11TH EDITION

Manual of Clinical Microbiology

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Manual of Clinical Microbiology

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Volume 1

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Acknowledgment of Previous Contributors

The *Manual of Clinical Microbiology* is by its nature a continuously revised work which refines and extends the contributions of previous editions. Since its first edition in 1970, many eminent scientists have contributed to this important reference work. The American Society for Microbiology and its Publications Board gratefully acknowledge the contributions of all of these generous authors over the life of this Manual.

PREFACE

The *Manual of Clinical Microbiology* (MCM) is the most authoritative reference text in the field of clinical microbiology. This edition of the *Manual* benefited from the talents of a team of 22 editors and almost 250 authors who were supported by a very capable production team at ASM Press. This, the 11th edition, is presented after the usual 4-year publication cycle following the 10th edition. All of the editorial team are proud members of the American Society for Microbiology and strong supporters of its book publishing arm, ASM Press. We have followed in the footsteps of previous authors and editors of the *Manual* and remain steadfastly committed to the utmost quality and timeliness that the MCM readership has come to expect. For the first time, we have had co-editors in chief of MCM. The length and scope of the *Manual* now require this division of labor to ensure thoroughness and timeliness of the editing process. We hope that readers of the *Manual* will recognize the commitment to excellence by everyone associated with its production.

We represent only the fifth and sixth editors in chief in the 45-year history of the *Manual*. We are grateful for the example set by our predecessors and by the sage advice offered by recent editors in chief Patrick Murray and James Versalovic. We offer our deep appreciation to Ken April, the production editor at the outset of this edition, and to Ellie Tupper, who succeeded him and completed the editorial production process.

This is only the second edition of the *Manual* to have a full-scale, searchable, Web-based HTML electronic edition. We hope that users of the *Manual* will find this electronic alternative to the print version of MCM to be convenient and user friendly. It is likely that future editions of MCM will rely more heavily on the electronic format for delivery of the vast content of the *Manual*.

This is a very dynamic era in clinical microbiology, with new technical tools (MALDI-TOF, ribosomal and total gene sequencing, and other molecular methods) that are profoundly influencing our approaches to organism detection and identification. The *Manual* continues to include classic microbiological techniques such as microscopy and culture as a foundation in addition to the newer methods cited above. Some organisms have become prominent causes of disease recently, e.g., Ebola, enterovirus D-68, and Gram-negative bacteria that produce carbapenemases. Every effort was made to include up-to-date information in the *Manual* on these recently emergent organisms. In addition, the studies of the human microbiome have informed our understanding of normal microbial communities and have posed the possibility of polymicrobial rather than single-agent infections.

In conclusion, we are profoundly grateful for the privilege of guiding the *Manual* through the publication of this 11th edition. We hope that the efforts of the editors and authors will prove useful to the clinical microbiology community until the next edition is available in about 4 more years.

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DIAGNOSTIC STRATEGIES AND GENERAL TOPICS

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Introduction to the 11th Edition of the *Manual of Clinical Microbiology*[*]

JAMES H. JORGENSEN AND MICHAEL A. PFALLER

1

The 11th edition of the *Manual of Clinical Microbiology* (MCM11) marks a significant change from prior editions in that there are co-editors in chief for the first time. There has been only one editor in chief for all prior editions since the first edition in 1970. However, as the *Manual* has grown in size and scope, it has become a Herculean effort for a single editor in chief. Since the 8th edition of the *Manual*, it has been published as two volumes. We have divided our duties primarily by volume: volume 1 (Jorgensen) and volume 2 (Pfaller). This edition of the *Manual* contains 151 chapters comprising almost 2,600 pages. It includes a comprehensive array of content on all aspects of microbiology contributed by world experts on each subject, with their chapters edited by a highly committed team of volume and section editors working within the usual 4-year publication cycle. This edition includes one new volume editor (Sandra Rich-ter) and three new section editors in addition to the new editors in chief. Four volume editors, Karen Carroll, Guido Funke, Marie Louise Landry, and David Warnock, served for the past edition of the *Manual*. Underscoring the international importance of the *Manual*, 20% and 37% of section editors and chapter authors, respectively, contributed content from countries outside the United States (up from 19 and 30% in the previous edition). There were 88 authors who contributed content to the *Manual* for the first time.

The overall organization of the *Manual* and the chapter formats are quite similar to those of the 10th edition. However, readers will note some new or expanded content in this edition. Since publication of the 10th edition, the application of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has rapidly been developed and embraced by clinical microbiology laboratories for identification of a vast array of bacteria and fungi. Most of the "organism" chapters now include comments on the utility of MALDI-TOF MS for the particular genera and species under discussion. Microbiologists have quickly joined this bandwagon due to the speed, accuracy of identifications, and low cost per test of this technique (despite the high initial instrument acquisition cost). The technology is still evolving, as the required extensive databases needed for all of the relevant organism groups are being painstakingly constructed. Assuming the adequacy of the databases, this technology may supplant much of the conventional phenotypic testing of the past and even the use of sequence-based identification approaches. Having said that, the cost and complexity of gene sequencing has continued to decrease rapidly, making it likely to be accessible to more clinical microbiology laboratories in the next few years. We have learned that no one genomic, proteomic, or phenotypic approach is perfect for the identification of all organisms, and different approaches may need to be used in concert to accurately identify some organisms.

Readers will note many new genus and species names in this edition. These result from initial descriptions of some species and reclassification of some genera and species from a previous taxon to a new or different one based upon 16S or 23S gene sequencing studies, sequencing of certain housekeeping genes, or DNA hybridization efforts that have demonstrated new phylogenetic associations. Some of these may seem bewildering at first due to the many new names that are unfamiliar to both microbiologists and practicing clinicians. Some of the newly designated organisms may not be recognized by conventional phenotypic testing and may not be found in the databases of FDA-cleared identification devices. They may require gene sequencing, use of microarrays, or perhaps mass spectrometry for accurate identifications. This will put pressure on clinical microbiology laboratories to either adopt newer technologies or place greater reliance on reference laboratories when it is important to know the contemporary identities of significant organisms. This offers an opportunity for dialogue between clinical microbiologists and the clinicians that they serve regarding the situations in which intensive identification efforts are justified.

The structure of volume 2 is largely the same as that found in MCM10. In Section IV (Virology), the chapter on Hendra and Nipah viruses from the previous edition has been folded into chapter 85 ("Parainfluenza and Mumps Viruses") and also includes other members of the *Paramyxoviridae*, and chapter 106 now highlights bocaviruses along with parvoviruses to recognize the emergence of the former as a human pathogen since its discovery in 2005. The Mycology and Parasitology sections remain largely unchanged, with the exception of the movement of the chapter on microsporidia from the Parasitology section to the Mycology section in recognition of the reclassification of these agents to the kingdom Fungi. As with the Bacteriology chapters,

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[*] This chapter contains some information presented in chapter 1 by James Versalovic in the 10th edition of the *Manual*.

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the chapters in volume 2 reflect the tremendous advances in the area of molecular taxonomy which have resulted in the reclassification of many different organisms, the recognition of several so-called "cryptic" species, and the discovery of several "new" pathogens as molecular and proteomic tools find their way from the research setting to the clinical laboratory.

Now entering its 6th decade, the *Manual* strives to continue to be the leading, most authoritative reference for the "real-world" practice of clinical microbiology. In order to create and assemble each edition, this publication builds on the content of past editions, and the process requires about 3 years of careful planning, design, writing, and review of chapters before the final phases of copyediting, composition, printing, and binding. In the intervening 1 to 2 years from the time of chapter acceptances until printing, new diagnostic trends, technologies, pathogens, and patterns of infectious diseases may emerge or change in ways that affect the timeliness and relevance of this comprehensive reference. This sobering reality simply "goes with the territory" of compiling any authoritative body of work. Hopefully the *Manual* continues to provide a highly respected benchmark and authoritative reference for the entire field of clinical microbiology. In the era of mass collaboration and rapid communication, our team at the *Manual* trusts that our readership, each of you, will contribute to the future of this field by pointing out errors, issues, and trends that serve to strengthen the *Manual* and its next edition. The work never stops, and the knowledge base keeps growing. So let us all continue to enhance the practice and contribute to the evolution of our cherished profession of clinical microbiology.

Microscopy

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2

The history of microscopy has been closely linked to the beginning of microbiology since 1665, when Hooke published his treatise *Micrographica*, which included illustrations of mold forms and the anatomy of the flea (1). Today, light microscopy is used not only in microbiology, pathology, and cell biology but also in metallurgy, materials science, computer chip design, and microsurgical applications. This chapter will attempt to describe the basic concepts of light microscopy as they are practiced in the microbiology laboratory.

TECHNICAL BACKGROUND AND DEFINITION OF TERMS

Aberration

Aberrations are unwanted artifacts in the microscopic image that are caused by elements in the optical path. Aberration can be caused by physical objects, such as dust or oils, on the optical surfaces, by alterations in the light path caused by improper alignment or aperture settings, and by lens system imperfections. Two main types of optical aberration, spherical aberration and chromatic aberration, can occur when white light passes through a convex lens. Spherical aberration is exhibited by images that appear to be in focus in the center of the field and out of focus at the periphery (2). Chromatic aberration occurs because shorter light wavelengths are refracted to a greater extent than longer wavelengths (2). This wavelength separation (also called dispersion) produces color fringes within the image field. Lenses that are not corrected for chromatic aberration can cause difficulties when interpreting Gram and other staining due to the presence of purple or green fringes around bacteria. This is especially problematic when the bacteria are very small.

Contrast

Contrast is a measure of the differences in image luminance that provide gray scale or color information. Contrast is expressed as the ratio of the difference in luminance between two points divided by the average luminance in the field (3). Under optimum conditions, the human eye can detect the presence of 2% contrast (3).

Depth of Field and Depth of Focus

Depth of field is a subjective measure of the vertical distance between the nearest and farthest objects in the specimen that appear to be in sharp focus. Depth of field decreases as the numerical aperture (NA) of the lens increases (4). Depth of focus is the area around the image plane where the image will appear to be sharply focused. The image plane is formed within the microscope tube at or near the level of the ocular lenses. Microscopes with greater depths of focus allow the user to employ ocular lenses with different working distances, magnification factors, and visual compensation systems without losing image sharpness. Like depth of field, depth of focus depends upon the numerical aperture of the objective. However, depth of focus increases as the numerical aperture increases (4).

Immersion Fluid (Immersion Oil)

Immersion fluid is a term used to describe any liquid that occupies the space between the object and microscope objective lens. Immersion fluids are usually required for objectives that have working distances of 3 mm or less (2). Many microscopy applications employ immersion fluids that possess the same refractive index as the glass slide (refractive index = 1.515) (2, 4). This procedure produces a homogeneous optical path which minimizes light refraction and maximizes the effective numerical aperture of the objective lens. Immersion fluids are also used between the condenser and the microscope slide in transmitted light fluorescence microscopy and in dark-field microscopy to minimize refraction, to increase the numerical aperture of the objective, and to improve optical resolution (2, 4).

Köhler Illumination

Köhler illumination was first introduced in 1893 by August Köhler of the Carl Zeiss Corporation as a method for providing the optimum specimen illumination (2). In this procedure, the collector lens projects an enlarged and focused image of the lamp filament onto the plane of the aperture diaphragm. Because the light source is not focused at the specimen, the specimen is bathed in a uniformly bright, glare-free light that is not seriously affected by dust and imperfections on the glass surfaces of the condenser. Köhler illumination is required to produce the maximum optical resolution and high-quality photomicrographs (2, 5).

Mechanical Tube Length

Mechanical tube length describes the light path distance within the microscope body tube. Tube length is measured

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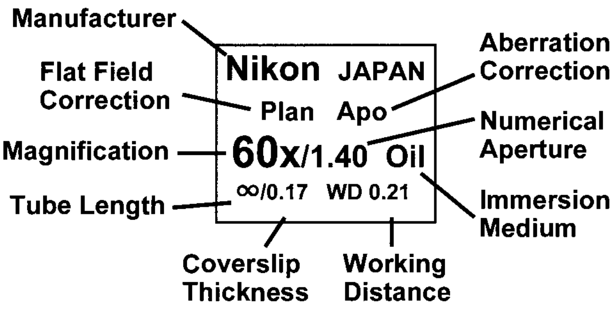

FIGURE 1 Objective lens labeling. Objective lenses are labeled with information about the manufacturer, correction factors, numerical aperture, tube length, coverslip thickness, working distance, and expected immersion medium. Objectives without a listed aberration correction are considered achromats. Objectives without a listed immersion medium (Oil, Oel, W, Gly) are considered dry objectives and are intended for use with air between the lens and the specimen.
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from the objective opening in the nosepiece to the top edge of the observation tube. Tube length is usually inscribed on the barrel of the objective as the length in millimeters (160, 170, 210 mm, etc.) for fixed lengths or the infinity symbol (∞) for infinity-corrected tube lengths (Fig. 1). Many of the newer objectives are infinity corrected, while older objectives are corrected for 160-mm (Nikon, Olympus, Zeiss) or 170-mm (Leica) tube lengths (2).

Numerical Aperture

NA is a measure of the light-gathering capability of a lens or condenser. Higher NA objectives have better resolving power and brighter images than lower NA objectives. Higher NA objectives also have a shallower depth of field. The equation for determining NA is given by NA = $n \times \sin(\theta)$, where n is the refractive index of the imaging medium between the objective and the specimen and θ is one-half the angular aperture of the objective (Fig. 2) (1, 2, 4).

Refractive Index (Index of Refraction)

Index of refraction is the ratio of the velocity of light in a vacuum to its velocity in a transparent or translucent medium (2, 4, 6). As the refractive index of a material increases, light beams entering or leaving a material are deflected (refracted) to a greater extent. The refractive index of a medium depends upon the wavelength of light passing through it. Light beams containing multiple wavelengths (e.g., white light) are dispersed when they move into a different medium because the wavelengths in the beam are refracted to slightly different degrees. Light dispersion causes chromatic aberration in microscope objectives (2). Refractive index is also an important variable in calculating numerical aperture (see "Numerical Aperture" above). Moving from a high dry microscope objective that uses air as the imaging medium (refractive index of air = 1.003) to an oil immersion objective of the same power (refractive index of immersion oil = 1.515) increases the maximum theoretical numerical aperture of a given lens from 1.0 to 1.5, producing a 50% increase in light-gathering capability (2, 4).

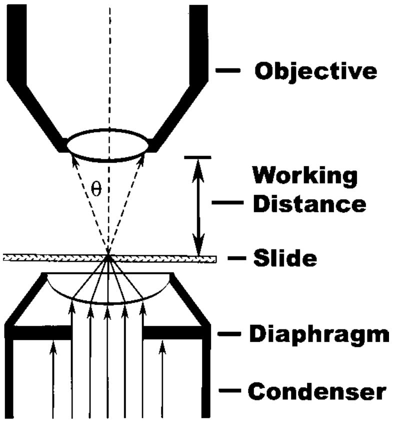

FIGURE 2 Typical configuration for bright-field microscopy. The column of light generated by the field lens and the field diaphragm enters the bottom of the condenser and is focused on the slide by the condenser lens. The condenser diaphragm controls the angle of the light, the numerical aperture of the condenser, and the amount of contrast in the image. The working distance is the vertical distance from the top of the specimen to the leading edge of the objective lens. The semiangle of the objective aperture (θ) is used to calculate numerical aperture. Modified from reference 4.
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Resolution (Resolving Power)

The resolution of an optical microscope is defined as the shortest distance between two points that can be distinguished as separate entities by the observer or camera system (4). The resolving power of a microscope is the most important feature of the optical system because it defines our ability to distinguish fine details in a specimen. The theoretical limit of resolution (r) for a given lens is defined mathematically as $r = \kappa/(2\text{NA})$, where κ is the imaging wavelength and NA is the numerical aperture of the lens (4). From this equation, it is obvious that only the light wavelength and NA directly affect the resolving power. Thus, a 40× oil objective with an NA of 1.30 can have the same resolving power as a 100× oil objective (Table 1). In the same manner, the resolving power of a 100× oil objective will be higher when ultraviolet (UV) wavelengths are used than when visible light is used (Table 1).

Working Distance

Working distance is the distance between the leading edge of the objective lens and the top of the cover glass when the specimen is in focus (Fig. 2). The working distance of an objective generally decreases as magnification increases (4). The working distance of an objective may not be inscribed on the barrels of older objectives, but newer objectives often contain the working distance in millimeters (Fig. 1). Longer working distance objectives are important when examining the inside surfaces of glass tubes (tube cultures) and cell culture flasks.

SIMPLE MICROSCOPE

Common objects, such as jeweler's loupes, photographic slide viewers, and simple magnifying or reading glasses, are all examples of simple microscopes. A simple microscope contains a single bi-convex magnifying lens which is

TABLE 1 Resolving the power of selected lenses with different numerical apertures.

Lens system	NA	Light color	Avg wavelength (nm)	Medium	Resolution (μm)
Eye		White	550	Air	700
Hand magnifier	0.03	White	550	Air	10
10× objective	0.30	White	550	Air	0.92
40× objective	0.75	White	550	Air	0.37
40× objective (oil)	1.30	White	550	Oil	0.21
100× objective	1.30	White	550	Oil	0.21
100× objective	1.30	UV	400	Oil	0.15

thicker in the center than at the periphery. In contrast, with compound microscopes, simple microscopes produce a magnified image that is in the same orientation as the original object. Because of their low NA, simple microscopes have limited resolution and magnifying power. Most commercial magnifiers are able to produce a ×2 to 30 magnification, and the better lenses will have a resolution of about 10 μm. Simple magnifiers are useful for dissection, examination of bacterial colonies, and interpretation of agglutination reactions.

COMPOUND MICROSCOPE

The first compound microscopes were constructed around 1590 by Dutch spectacle makers Zaccharias Janssen and Hans Janssen. The Janssen microscope consisted of an object lens (objective) that was placed close to the specimen and the eye, or an ocular lens that was placed close to the eye. The lenses were separated by a body tube. In this microscope, the objective lens projected a magnified image into the body tube and the eyepiece magnified the projected image, thereby producing a two-stage magnification. Modern compound microscopes still use this general design and have two separate lens systems mounted at opposite ends of a body tube.

The stereoscopic microscope combines two compound microscopes, which produce separate images for each eye. The three-dimensional stereoscopic effect is produced in the brain when two images are viewed with slightly offset viewing perspectives. Placing a camera on one of the eyepieces or on a dedicated camera port will not produce a three-dimensional photo image. Stereoscopic microscopes are used with reflected or transmitted illumination, but the absence of a substage condenser limits their NA and resolution. Stereomicroscopes are useful in examining the colonial morphology of bacteria, fungi, and cell cultures.

Optical Train

The modern light microscope is composed of optical and mechanical components that, together with the mounted specimen, make up the optical train. The optical train of a typical bright-field microscope consists of an illuminator (light source and collector lens), a substage condenser, a specimen, an objective, the eyepiece, and a detector. The detector can be a camera or the observer's eye.

Specimen illumination is one of the most critical elements in optical microscopy. Inadequate or improper sample illumination can reduce contrast in the specimen and significantly decrease the resolving power of any microscope (7). Fifty or 100-watt tungsten halogen lamp systems have been the most popular means of providing light for visible-light microscopy because they have a relatively low cost and provide a white light. Newer microscopy systems now use light-emitting diode (LED) light matrices. These systems have reduced energy requirements, do not heat the specimen or the lower optical train, and last for decades.

Light generated by the light source is passed through a collector and a field lens (Fig. 3) before being directed into the substage condenser and onto the specimen. Image-forming light rays are captured by the microscope objective and passed into the eyepieces or a camera port. Alignment of the optical components of a microscope is critical to producing a good image.

Field Diaphragm

The field diaphragm is located in the light path between the light source and the substage condenser (Fig. 3). This iris-like mechanism controls the width of the light beam that enters the substage condenser. The field diaphragm does not affect the optical resolution, numerical aperture, or intensity of illumination. However, the field diaphragm should be centered in the optical path and opened far enough that it just overfills the field of view. This adjustment is important for preventing glare and loss of contrast in the observed image. When the field diaphragm is opened

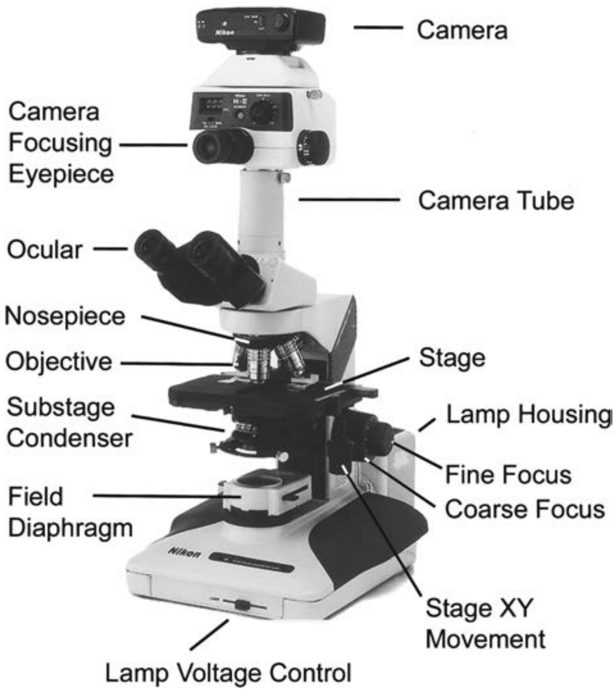

FIGURE 3 Anatomy of a typical clinical microscope with an integral camera. doi:10.1128/9781555817381.ch2.f3

too far, scattered light and reflections can degrade image quality (1).

Substage Condenser

The substage condenser is typically mounted beneath the microscope stage in a bracket that can be raised or lowered independently of the stage (Fig. 3). The substage condenser gathers light from the field diaphragm and concentrates it into a cone of light that illuminates the specimen with uniform intensity over the entire field of view. Adjustment of the substage condenser is probably the most critical element for achieving proper illumination, and it is the main source of image degradation and poor-quality photomicrography. The condenser light cone must be properly adjusted to optimize the intensity and angle of light entering the objective. Because each objective has different light-gathering capabilities (numerical aperture), the substage condenser should be adjusted to provide a light cone that matches the numerical aperture of the new objective. This is done by adjusting the aperture (or condenser) diaphragm control. Substage condensers on newer microscopes have a scale embossed on the condenser and an index mark on the aperture control that allows the user to quickly switch from one NA range to another. Many manufacturers are now synchronizing the NA gradations to correspond with the approximate numerical aperture of the objectives.

In clinical laboratory practice, the condenser aperture is often made smaller to improve the contrast of wet mounts and some stained preparations (1). This practice, while effective for some applications, will result in decreased resolution (3). It should be noted that the intensity of illumination should not be adjusted by opening and closing the condenser aperture diaphragm or by moving the condenser laterally in the light path. Illumination intensity should be controlled through the use of neutral density filters placed into the light path or by reducing voltage to the lamp. It should be noted that reducing the voltage will also alter the color of the light emanating from a tungsten halogen bulb (5). The lighting intensity provided by LED light systems can be reduced without altering the color of the light. The color of the incoming light will affect photomicroscopic color balance, and it can influence the interpretation of stained specimens.

Objectives

The objective lens is the most important single determinant of the quality of the image produced by a particular microscope (1). When choosing a microscope, the purchaser must select the magnification factor, the NA, and the level of correction for each objective. Lenses with higher NA values will have higher resolution and produce a brighter field of view. Choosing an appropriate level of optical correction will depend upon the ultimate use of the microscope. Achromatic (achromat) objectives are the least expensive objectives found on laboratory microscopes. Achromat objectives are corrected for axial chromatic aberration in two wavelengths (red and blue), and they are corrected for spherical aberration in one color (green) (2). The limited correction of achromatic objectives can cause a number of optical artifacts when specimens are examined and photographed in color (e.g., green images often have a reddish-magenta halo) (2). Achromat objectives produce the best results when the light passes through a green filter and when black-and-white photomicroscopy is performed. Flatness of field is also a problem when using straight achromat objectives because the center of the field is in focus while the edges are out of focus (2). In the past few years, most manufacturers have begun providing flat-field corrections for achromat objectives. These objectives are called plan-achromats.

The next-higher level of correction and cost is found in objectives called fluorites or semiapochromats. Fluorite objectives are produced from advanced glass formulations that allow for greatly improved correction of optical aberration. Like achromat objectives, fluorite objectives are corrected chromatically for red and blue light (2). Fluorites, like achromats, are corrected spherically for two or three colors instead of a single color (2). The superior correction of fluorite objectives compared to that of achromat objectives enables these lenses to be made with a higher numerical aperture. Fluorite lenses produce brighter images than achromats. Fluorite objectives also have better resolving power than achromats and provide a higher degree of contrast, making them better suited for color photomicrography in white light (2, 4).

Apochromats are the most highly corrected microscope lenses and the most costly. Apochromats are corrected chromatically for three colors (red, green, and blue), which almost eliminates chromatic aberration, and are corrected spherically for either two or three wavelengths (2). Apochromat objectives are the best choice for color photomicrography in white light. Because of their high level of correction, apochromat objectives usually have, for a given magnification, higher numerical apertures than do achromats or fluorites (2, 4).

Fluorescence objectives are designed with quartz and other special glasses that have high rates of transmission of UV, visible, and infrared light. These objectives are extremely low in auto-fluorescence and use specialized optical cements and antireflection coatings that protect the lens and allow it to operate with a wide variety of excitation wavelengths. Correction for optical aberration and numerical aperture values in UV fluor objectives usually approaches that of apochromats, which contributes to image brightness and enhanced image resolution (2, 8). The primary drawback of high-performance fluorescence objectives is that many are not corrected for field curvature and produce images that do not have uniform focus throughout the entire field of view. This is not a large problem when performing direct or indirect fluorescent antibody testing but it can be troublesome if you have to use the same objectives for bright-field or phase-contrast microscopy.

Microscope objectives that use air as the medium between the coverslip and the objective lens are considered dry objectives. The maximum working numerical aperture of a dry objective system is limited to 0.95, and greater values can be achieved only with optics designed for immersion media. Immersion media have the same refractive index and dispersion values as glass (refractive index = 1.51). The use of immersion media produces a homogeneous light path from the coverslip to the lens so that light is not refracted away from the objective. The use of immersion fluids and lenses significantly increases the numerical aperture and the optical resolution of the system. In addition to oil lenses, specially corrected objective lenses designed for glycerin and water immersion are available commercially. The proper immersion fluid type is always stamped on the side of the objective. The advantages of oil immersion objectives are severely compromised if the wrong immersion fluid is utilized. Microscope manufacturers produce immersion objectives with tight refractive index and dispersion tolerances (2). It is therefore advisable to use only the immersion fluid recommended by the objective manufacturer. Mixing of immersion fluids from different manufacturers should be avoided because mixing can produce unexpected crystallization artifacts or phase separations that compromise image quality.

Many high-power (NA ≥ 0.8) dry objectives are engineered to operate through 0.17-mm coverslips (designated number 1 1/2). In practice, however, the total thickness of the specimen/coverslip sandwich can be greater or less than 0.17 mm due to variations in coverslip and/or mounting fluid thickness (2, 4). Under these conditions, there will be noticeable spherical aberration in the microscopic image (2, 4). A 0.2-mm deviation in coverslip thickness will produce an 8% decrease in image intensity with a 0.79-NA objective and a 57% decrease with a 0.85-NA high dry objective (2). Therefore, some of the more advanced dry objectives are engineered with a coverslip correction collar that adjusts the objective lens elements to compensate for coverslip thickness variations. Objectives with a coverslip correction collar are labeled “Corr,” “w/Corr,” or “CR.” However, this labeling is usually unnecessary because the objective has a distinctive knurled ring and graduated scale on the side. The expected coverslip thickness for an objective is etched on the barrel of the objective (Fig. 1).

The eyepiece or ocular objective contains the final lens system in the optical train. The purpose of the ocular objective is to magnify and focus the projected image onto the eye of the viewer. Ocular lenses generally have a magnification factor of ×10 to ×20, and the total magnification of the microscope is the product of the objective magnification and the ocular magnification (1, 2, 6). Thus, a microscope with a 40× objective and a 10× ocular would have a magnification value of ×400. Many eyepieces have a shelf at the level of the fixed eyepiece diaphragm that allows for the insertion of ocular micrometers, pointers, or crosshairs. This shelf is located at the focal plane of the image projected by the objective lens so that the inserted element is in focus when the specimen image is in focus.

When upgrading microscope systems, the normal tendency is to save old objectives and oculars as backups. Check with your microscope manufacturer before doing this. Oculars, especially those from a different manufacturer, can have a different focal length than the one used in the new microscope. In addition, many manufacturers include optical corrections in the oculars that compensate for aberrations in their objective line. These corrections may cause unwanted chromatic aberrations when the old lens is used with the new microscope. Objectives with a standard tube length cannot be used on infinity-corrected optical systems.

DARK-FIELD MICROSCOPY

Dark-field microscopy is a specialized illumination technique used to detect thin organisms, such as spirochetes and *Leptospira* spp. High-resolution dark-field microscopy utilizes a specialized high-NA cardioid dark-field condenser that blocks the central light path light and produces a hollow cone of illumination that is directed away from the objective lens at an oblique angle (Fig. 4). Bacteria on the slide have a slightly different refractive index than the surrounding medium, and light rays passing through the organism are refracted into the objective lens, producing bright organism profiles against a dark background. Dark-field microscopy requires careful alignment of the condenser and placement of immersion oil between the slide and the substage condenser. Dark-field microscopy, when done correctly, increases the resolution of the microscope to 0.1 μm or less (3). The resolution of bright-field microscopy is 0.2 μm (1).

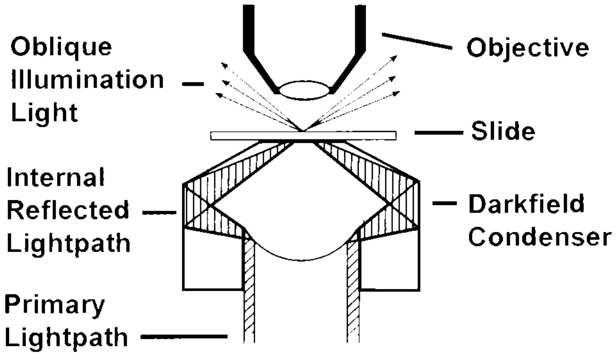

FIGURE 4 Dark-field illumination. The central light path interacts with the silvered dome located at the bottom of the condenser and is reflected away from the specimen. Peripheral light is reflected into the condenser, and it is reflected again by the internal condenser surfaces to produce a cone of light that is directed obliquely away from the objective.
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PHASE-CONTRAST MICROSCOPY

Many unstained biological specimens are virtually transparent when observed under bright-field illumination. To improve visibility in wet mounts and cell cultures, microscopists often reduce the opening size of the substage condenser iris diaphragm, but this maneuver is accompanied by a serious loss of resolution and the introduction of diffraction artifacts (2, 3). Phase-contrast microscopy significantly improves the contrast in these specimens without significant loss in resolution (3).

In phase-contrast microscopy, a ring annulus is placed directly under the lower lens of the condenser to produce a hollow cylinder of light. This light is essentially unchanged as it passes into the objective, and it arrives at the rear focal plane of the objective in the shape of a ring. Light that goes through the specimen is refracted and slowed slightly so that it is out of phase with the unchanged light by about 1/4 wavelength. This light is spread over the entire focal plane. Light passing through the rear focal plane of the objective interacts with a ring-shaped phase plate that alters the direct light path by 1/4 wavelength (3). When the direct light and the refracted light arrive at the image plane, they are out of phase by 1/2 wavelength. This out-of-phase light interacts destructively, so that specimen details appear as dark areas against a lighter background (3). Because the phase-shifting calculations are based upon on a 1/4 wavelength of green light, the phase image has the best resolution when a green filter is placed in the light path (3). Green filters also allow the microscopist to use less expensive achromat lenses that are spherically corrected for green light. Phase microscopy is an important tool for examining living and/or unstained material in wet mounts and cell cultures. However, phase-contrast microscopy has lower resolution than bright-field microscopy of stained specimens (3). In addition, viewed objects are often surrounded by halos that can obscure boundary details. Phase-contrast microscopy does not work well with thick specimens because the phase shift may be greater than the expected 1/4 wavelength.

FLUORESCENCE MICROSCOPY

The fluorescence microscope was developed in the early 1900s, and many of the initial microscopic studies involved identification and localization of compounds that autofluoresced when irradiated with UV light. In the 1930s, a number of investigators began using fluorescent compounds to identify specific tissue components and infectious agents that did not autofluoresce (8). Examples of this type of stain include acridine orange (intercalates into DNA and RNA), auramine-rhodamine (for mycolic acids), Calcofluor White (for fungal cell wall polysaccharides), Evans blue (for the cytoplasm of fixed cells), and Hoechst 33258 (for the minor groove of AT-rich double-stranded DNA).

The use of fluorochrome-antibody conjugates (immunofluorescence) was first described in the 1940s, when Coons et al. (9, 10) used fluorescein-labeled antibodies to detect pneumococcal polysaccharide antigens in tissue sections of infected mice. Fluorescent-antibody staining expanded significantly with the development of fluorescein isocyanate in 1950 (11) and the more stable fluorescein isothiocyanate (FITC) derivative in 1958 (12–16).

Quantum dots have recently emerged as a new class of fluorescent labels for biology and medicine (17). When conjugated to antibodies and other biological ligands, these tiny light-emitting particles can overcome many of the disadvantages of traditional fluorophores used in clinical pathology. Quantum dots have superior signal brightness, they are resistant to photobleaching, and multiple fluorescent colors can be excited by the same wavelengths of light. The last property makes multicolor fluorescence microscopy easy to perform and, when the wavelengths are tuned properly, provides quantitative information about ligand abundance (18). Quantum dots have been used for staining live cells (18), fixed cells (17), and tissues (17). Today, fluorescence microscopy is also used in conjunction with nucleic acid hybridization to visualize the locations of fluorescent *in situ* hybridization (FISH) and multicolor FISH probes (19, 20).

Fluorescence microscopy is dependent upon the ability of fluorescent substances to absorb near-UV energy and reemit that energy (light) at a lower wavelength (8). To work properly, the fluorescence microscope must irradiate the specimen with UV excitation light and separate the much weaker emitted light from the brighter excitation light so that only the emitted light reaches the eye. The resulting image consists of brightly shining areas against a dark background (8). Older fluorescence microscopes are configured for dark-field illumination or transmitted light fluorescence (8). These systems were cumbersome to use and lacked resolution. Most modern fluorescence microscopes use reflected light (epifluorescence). In these instruments, the excitation light is directed downward through the objective and onto the specimen. The emitted light and the reflected excitation light are collected by the objective, and they pass through a dichromatic mirror, which removes the excitation light and allows the longer-wavelength emitted light to form an image. With epifluorescence, the objective acts as a condenser and the alignment and oiling issues associated with a dark-field condenser are eliminated (8). The visual field is brighter with epifluorescence, the resolution is higher, and fluorescence quenching occurs only in the field of view (8).

Fluorescence microscopy requires high levels of illumination because the quantum yield of most traditional fluorochromes is low. The most common lamps used in fluorescence microscopy are mercury vapor (HBO) lamps ranging in wattage from 50 to 200 watts or Xenon vapor (XBO) lamps that range from 75 to 200 watts. It should be noted that lamp wattage is not necessarily a measure of usable brightness in a fluorescence lamp. The 100-watt HBO lamp is 4 times brighter than the 200-watt HBO lamp and 11 times brighter than the 150-watt XBO lamp (8). HBO and XBO lamps are under high pressure, and care must be taken to prevent the lamps from exploding. One should never touch these lamps with bare hands because oils on the fingers can etch or discolor the glass.

Fluorochromes must be excited by specific light wavelengths in order to generate the maximum amount of emitted light. Therefore, specific exciter and barrier filter combinations are used to maximize the quantum yield of the fluorophore. Exciter filters are used to select the required light wavelengths from the spectrum of light generated by the lamp (8). Excitation filters are provided in narrow, medium, and wide bandpass configurations that pass narrow, midsize, and wide ranges of light frequencies, respectively. Barrier filters block shorter light wavelengths and allow longer wavelengths to pass through the filter. Barrier filters are important because they remove the high-intensity excitation light, which can overwhelm the low-intensity emitted light. Barrier filters also prevent UV light from entering the eye, where it can cause cataracts and retinal damage. Wide-bandpass barrier filters generally produce brighter images, but care must be taken to prevent the introduction of background light, which can overwhelm the emitted light. Epifluorescence microscopes also have a dichromatic mirror (beam splitter) that reflects the incoming excitation light to the objective and allows the emitted light to pass to the barrier filter and on to the objectives (7). In most modern epifluorescence microscopes, the barrier filter, excitation mirror, and beam splitter are housed in removable optical blocks, and several of these blocks can be installed in the microscope at one time. This configuration allows the user to quickly change the excitation and barrier filters to accommodate different fluorochromes. Care must be exercised when selecting optical blocks. The excitation filter should match the excitation wavelength of the fluorophore (Table 2), and the emission barrier should allow the emitted light to

TABLE 2 Excitation and emission wavelengths of commonly used fluorochromes[a]

Fluorescent compound	Excitation wavelength (nm)	Emission wavelength (nm)
Acridine orange (single-stranded nucleic acid)	500	526
Acridine orange (double-stranded nucleic acid)	460	640
Auramine O	460	550
Calcofluor White	440	500–520
Ethidium bromide	545	605
Evans blue	550	610
Fluorescein isothiocyanate (FITC)	490	525
Hoechst 33258	352	461
Rhodamine B	540	625
Tetramethylrhodamine isothiocyanate (TRITC)	555	580

[a] Excitation and emission wavelengths can vary depending upon the solvent and the pH of the solution.

pass through. For example, direct fluorescent-antibody testing for viral antigens in cell smears typically employs FITC-labeled antibodies and an Evans blue counterstain. Choosing an optical block with a 450- to 490-nm excitation filter and a 515-nm wide-bandpass barrier filter will produce a bright field of view, and the counterstained cells will appear orange-red. By selecting a more restricted bandpass barrier filter (520 to 560 nm), the field of view will be darker and the red emitted light from the Evans blue counterstain will not be visible. The images produced by this optical block will have more contrast because the background is darker. Both filter combinations are appropriate for this task, but the final choice will depend upon user preference.

One of the major problems in the use and examination of fluorescent microscopic images is the tendency of traditional fluorophores to lose fluorescence when exposed to excitation light for several minutes. This loss of fluorescence is caused by two mechanisms, photobleaching and quenching. Photobleaching (fading) is a permanent loss of fluorescence that is caused by chemical damage to the fluorophore (8). Quenching is caused by the presence of free radicals, salts of heavy metals, or halogen compounds (8). Quenching can also be caused by transfer of emission light energy to other fluorescent molecules in close proximity to the fluorophore in a process called fluorescent resonance energy transfer (FRET). To lessen the effect of quenching, slides should be stored in the dark at 2 to 8°C. In addition, the user should block the excitation light path when not viewing or photographing the specimen. Most epifluorescence microscopes have a shutter in the light path for this purpose. Quenching can be a significant problem when photographing fluorescent images because the shutter may be open for a minute or more. Quenching can be reduced somewhat by adding free-radical scavengers, such as *p*-phenylenediamine (14), 1,4-diazabicyclo(2,2,2)-octane (DABCO) (21), or *n*-propyl gallate (22) to the mounting fluid. *p*-Phenylenediamine and *n*-propyl gallate can be used to reduce quenching in FITC and rhodamine. DABCO is slightly less effective than *p*-phenylenediamine for FITC fluorescence, but unlike *p*-phenylenediamine, DABCO does not darken when exposed to light and it is safer to use. Quench-resistant mounting media are also available from Vector Laboratories (Burlingame, CA), Molecular Probes Inc. (Carlsbad, CA), and Bio-Rad Laboratories (Hercules, CA). The major advantage of quantum dot fluorophores is their resistance to photobleaching.

LINEAR MEASUREMENTS (MICROMETRY)

The first reported micrometric procedures were credited to Antonie van Leeuwenhoek, who used fine grains of sand as a gauge to determine the sizes of human erythrocytes. Since then, a variety of methods have been used to determine the dimensions of microscopic organisms. The crudest method involves comparing the object size to the measured or calculated view field size. Another rough micrometric method is to compare the sizes of larger organisms to the size of a red blood cell (6 to 8 μm) in the image. Other micrometric techniques include the addition of polystyrene beads of known size into the specimen. Comparative measurements are then performed by utilizing a photomicrograph or digital image. The accuracy of these methods is variable and depends on the homogeneity of the comparison objects.

Microorganisms can be measured directly by placing them on a calibrated microscope slide or a counting chamber. The accuracy of this method depends upon the separation distance between ruled lines, but it averages between 10 and 50 μm.

The most common procedure used in the clinical laboratory utilizes a graduated scale (reticle) located within one of the eyepieces (23). Reticles must be calibrated against a stage micrometer for each objective (23). To avoid unnecessary recalibrations, the calibration information for each objective should be recorded and stored near the microscope workstation. The accuracy of reticle measurement is approximately 2 to 10 μm (3 to 5%), depending on magnification and the resolution of the stage micrometer (23).

PHOTOMICROSCOPY

Microscopists began capturing microscopic images on film shortly after the photographic process was invented (5). Micrographic images have long been used for investigations of morphology, in scientific publications and lectures, and in teaching. Modern film technologies have high resolution and clarity, but the use of photomicrographs in day-to-day microscopy has been hampered by long turnaround times associated with film development and printing. Reacquiring fluorescence images is a particular concern because the fluorescence can fade (8). The availability of high-quality digital cameras has significantly changed how photomicrographs are used in the microbiology laboratory. Today it is not unusual for digital photomicrographs to be shared with experts via the Internet. This process significantly extends the capabilities of the on-site microbiologist and can enhance patient care. Microscope-based digital cameras and video systems are also used to perform "plate rounds" in remote hospitals and clinics within a multihospital system. Newer Internet technologies involving robotic microscopes and high-resolution video systems now allow microbiologists to change the focus and change the slide positioning of a microscope located anywhere in the world and to view the resulting images on a monitor in their office. The availability of digital photomicroscopy has significantly enhanced the microbial identification process, and it has helped to standardize microbe identification.

A wide variety of microscopes can be purchased with integrated camera systems and sophisticated light metering and exposure controls. Accessory cameras are also available from a large number of aftermarket manufacturers. However, an expensive camera system does not automatically confer the ability to produce high-quality images. Publication-quality photomicrographs require proper specimen illumination and optical train alignment to achieve the microscope's ultimate potential (5). Color photography can be especially demanding because specimens may appear yellow or blue under tungsten halogen (3,200 K) light, depending upon whether the lamp voltage is above or below the recommended 9-volt setting. Newer camera systems have sophisticated exposure, lighting, and white balance controls that make image capture easier, but they cannot correct for poor technique.

Not all microbiologists can afford a microscope with an integrated camera system. Simple eyepiece cameras can be used to capture bright-field images for Internet consults, training manuals, and plate rounds. The simplest configuration for eyepiece photography involves the use of a point-and-shoot digital camera. A number of adapters that allow coupling a fixed-lens camera to a microscope eyepiece tube are now available. Photographs taken in this manner are often acceptable, but they may be dark and have some chromatic aberration (due to different lens correction factors) and vignetting (pipe view effect).

Another method for photomicroscopy is to use the camera port on microscopes fitted with a trinocular head. Olympus and Nikon have introduced adapters that allow their digital cameras to attach to the camera tube of their microscopes. In addition, camera tube and eyepiece adaptors for a number of digital cameras are available from Microscope Depot (Tracy, CA). Photography under these conditions is best done using a camera with through-the-lens exposure metering. These devices work well if the exposure is not longer than several seconds or shorter than 1/3 second (5). Many of these cameras have built-in flashes that should be turned off during photomicroscopy. Consumer-grade digital cameras may have problems with fluorescence microscopy due to the extreme contrast of fluorescent images and the tendency of metering systems to average exposure values over the entire field (5).

CARE AND USE OF THE MICROSCOPE

Proper care and maintenance of the microscope will prolong the usable life of the instrument and allow for more accurate interpretation of microbiological images. The microscope should be kept in a low-vibration, low-dust environment to facilitate viewing and to decrease damage to the optical systems. The optical elements should be kept completely free of dust, dirt, oil, solvents, and other contaminants (23). Ideally, the microscope should be covered and the lamp should be turned off when the microscope is not in use. Do not touch the optical surfaces with your fingers (23). Keep the lenses clean, and be sure to remove oil or mounting fluid from the objectives, condenser, and mechanical stage after each session. Immersion oils act as a slow-acting solvent that can weaken the optical mounting cement (7). Avoid dragging the high dry objective through oil or fluorescence mounting fluid. One way to avoid accidental contact with these fluids is to place the high dry objective and the oil immersion objective in the nosepiece on opposite sides of the low-power objective (5). Lenses should be dusted with a fine lens brush and a bulb syringe and cleaned with lens paper and a commercial lens cleaner that is approved by the microscope manufacturer (7). Compressed air is not recommended because it can leave a residue on glass surfaces (7). Commercial cleaners, such as Windex and Sparkle, should not be used on optical surfaces because they often contain acids or bases that can erode the antireflection coatings on the lens (7). Organic solvents, such as alcohols and acetone, should not be used on the lenses because these solvents may dissolve the optical mounting cement (7). Unused spaces in the nosepiece (Fig. 3) should be plugged, and the eyepieces should remain installed at all times to prevent introduction of dust into the body tube. The stage should be cleaned regularly, and any spilled immersion oil or mounting fluid must be removed, or slides will stick when they are moved across the stage. Oils and mounting fluids also collect dust and grit, which can damage the optical and mechanical parts. Microscopists should not attempt to remove or disassemble the objectives, as this increases the potential for damage (23). This is a job that is best left to professionals (23). Do not use lubricating oils on the gears or bearing surfaces of the microscope because this may cause the condenser and stage to sink from their own weight (23). Annual or semiannual cleaning and adjustment by a professional microscope repair person will also help to extend the usable life of the microscope.

ERGONOMICS

Peering into a microscope eyepiece for long periods is not an activity for which the body is well adapted. Microscope work requires the head and arms to be locked in a forward position and inclined toward the microscope with rounded shoulders. This unusual positioning is further exaggerated when the feet are placed on the ring-style footrests that are common on many laboratory stools. Poor posture and awkward positioning during microscopy can cause pain or injury to the neck, wrists, back, shoulders, and arms (24). In one regional survey of cytotechnologists, Kalavar and Hunting (25) found that 70.5% of respondents reported neck, shoulder, or upper back pain during microscopy and that 56% had an increased prevalence of hand/wrist symptoms. Eyestrain, leg discomfort, and foot discomfort have also been documented with long-term microscope use (26). With older microscopes, users often have their heads inclined up to 45 degrees from vertical and their upper backs may be inclined by as much as 30 degrees. Even 30-degree inclinations of the head can produce significant muscle contractions, fatigue, and pain (26). For this reason, microscopists should be taught to sit upright and hold their head in a neutral position (27).

During microscopy, the laboratorian should sit erect while maintaining the natural curve of the spine (27). The lower back and shoulder blades should be supported by the chair, and a lumbar support cushion should be used if necessary. The legs and feet should rest firmly on the floor or a footrest. The chair should have a pneumatic height adjustment (23), and the seat should have a sloping front edge to prevent undue pressure on the thighs. The backrest should be adjustable for both height and angle. The chair should have a five-pointed star base with caster wheels. Knee spaces, which are often used for laboratory storage, should be free from obstructions, and there should be a minimum of 2 inches of clearance between the thigh and the bottom of the desk or counter (25). Obstructions that prevent the microscopist from holding his or her shoulders perpendicular to the ocular axis of the microscope should be removed (24). The upper arms should be perpendicular to the floor with the elbows close to the body. The forearms should be parallel with the floor, and the wrists should be straight. The head should be upright, and the neck should bend as little as possible, preferably no more than 10 to 15 degrees. The eyepieces should be just below the eyes, and the eyes should look downward at a 30- to 45-degree angle. The use of tilting microscope heads can significantly improve the comfort of the microscopist (25, 28, 29). Repetitive motions of the hands and the contact stress of arms resting on (the edge of) a hard surface can cause pain and nerve injury, leading to repetitive stress injuries and/or carpal tunnel syndrome (24). The use of padded armrests can moderate some of these problems. In addition, microscopes should not be placed under an air vent in order to prevent stiffening of the muscles during microscopy.

Most laboratory microscopes are used by multiple individuals, and it is often a challenge to find conditions or microscope configurations that satisfy everyone. Some laboratories place microscopes on books or heavy blocks of wood to accommodate taller microscopists (23). This configuration creates a number of problems. If the microscope is raised to a sufficient height to prevent neck flexion, users may be forced to bend their wrists into an unnatural position. If the microscope is lowered to allow the forearms to remain parallel to the floor, the neck is forced to bend. Lowering the chair to its lowest position causes leg discomfort. Shorter individuals may have to raise the chair to a level where their feet no longer touch the floor. Footrests can ameliorate this problem, but some individuals may have insufficient space under the benchtop to accommodate their legs. In practice, most laboratories will elect to use a suboptimum, but workable, microscope configuration that all users can employ. Under these conditions, microscopists can reduce stress and fatigue by taking 1-min "microbreaks" every 10 to 15 min during which they can stand, stretch, and allow the eyes to focus at a distance.

Eye fatigue can be a major problem for microscope users, especially if they have poor vision. The diopter adjustment provided on most microscope eyepieces can be adjusted to compensate for minor near- and far-sightedness and allow the user to remove his/her glasses during microscope use. The diopter adjustments do not adjust for astigmatism, and users with moderate to severe astigmatism should wear glasses when using the microscope. Most microscope manufacturers now produce high-eyepoint eyepieces that move the visual observation point further from the eyepiece, thereby facilitating the use of glasses during microscopy. Ensuring that the microscope images are as bright, sharp, and crisp as possible will also help to reduce eye fatigue and associated headaches. The importance of proper alignment of the microscope and optical components cannot be overstressed. Proper optical alignment and the use of newer objectives with higher NA values will produce brighter images and better resolution, which eases the strain of searching for tiny specimen details. The use of a neutral blue (daylight) filter during bright-field microscopy can also help to lessen eye strain when examining microbiological specimens. In the future, many new microscopes will display the specimen image on a computer monitor. This innovation may alleviate many of the eyestrain problems that develop during extended microscope use (29).

Microscopes are as different as the people who use them, and the previous comments should not be construed as a prescription for alleviating strain or repetitive-motion injuries in every situation. When purchasing a microscope, every effort should be made to allow microscopists to evaluate the new microscope under their normal working conditions. Some microscopes will be comfortable for some users and uncomfortable for others. In the long run, the fit and feel of the microscope is just as important as the optical characteristics.

CONCLUSIONS

Advances in the design, resolution, and ergonomics of modern microscopes have greatly enhanced our ability to study and identify microorganisms. Microscopy still has a central role in the detection of infectious agents despite highly publicized advances in DNA and RNA detection systems. Microscopic examination of clinical specimens provides a rapid and inexpensive "first pass" in the detection and identification of infectious agents. Thus, clinical microscopy will continue to be a core competency in clinical microbiology laboratories for the foreseeable future.

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Laboratory Detection of Bacteremia and Fungemia

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3

The laboratory detection of bacteremia and fungemia remains one of the most important—and complex—roles of clinical microbiology laboratories. This is, in part, because the attributable mortality for bacteremia and fungemia remains as high as 12% (1) but also because rapid, accurate, and reliable identification of patients with bacteremia or fungemia is of critical importance in influencing treatment. Blood culture results guide antimicrobial therapy and also subsequent surgical procedures, removal of vascular access lines, and other clinical interventions. Moreover, diagnosis of bacteremia and fungemia requires more than just a single test. It requires more than one blood culture, drawn from different sites, identification of isolates recovered from blood specimens, antimicrobial susceptibility testing of isolates, and interpretation of results in conjunction with other tests and cultures.

The goal of this chapter is to provide a summary of the clinical importance of bacteremia and fungemia, the scientific and medical principles underlying current diagnostic methods, a summary of alternative diagnostic approaches, and a brief review of those tests that are emerging as potential additions to or replacements for traditional procedures.

ASSESSMENT OF METHODS FOR DETECTING MICROORGANISMS IN BLOOD

No method has been shown to be an ultimate standard for the detection of either bacteria or fungi from blood. It has been recognized for decades that even with the best blood culture systems, use of optimal methods for collecting blood specimens, and limiting collection of blood specimens from patients with a high pretest probability of bacteremia or fungemia, usually only 8 to 12% of blood cultures will yield microbial isolates, of which one-third to one-half will be contaminating skin flora. The reasons for this are not fully understood. The single biggest cause is that many blood cultures are obtained from patients who are at low or no risk for bacteremia or fungemia. A recent study, using a Bayesian prediction model of objective clinical and laboratory risk factors, showed the likelihood of true bacteremia to range from 0.4 to 18.4%, with a mean prevalence of 6.9% in a large cohort of patients (2). Other causes also play a role: (i) many patients are receiving empiric antimicrobial therapy at the time blood specimens are collected, thereby reducing the yield from blood cultures; (ii) patients may have temporarily cleared microorganisms from blood; (iii) specimens may be mishandled, leading to falsely negative results; (iv) some microorganisms cannot be recovered adequately using broth-based blood cultures; (v) the number of microorganisms in a given specimen may be too low to be recovered by current methods; and (vi) current use of 4- or 5-day incubation and testing cycles on automated systems will result in a small number of isolates being undetected.

It was long expected that molecular methods would provide a gold standard test for the detection of bacteremia or fungemia, but this has not been the case. As will be reviewed below, some molecular methods are not even as sensitive as cultures, some have been shown to detect nucleic acids in persons without bacteremia or fungemia, and some proteins in blood have been shown to act as inhibitors to some nucleic acid amplification assays.

One approach has been to use composite standards, such as the use of combined results from different assays. From a statistical standpoint, this is a flawed analysis because any given assay cannot be compared against itself (as part of the gold standard). Moreover, the entire point of developing a standard is to be able to use the standard in many different studies; these "combined" gold standard assays preclude that for the obvious reason that it quickly becomes impractical to use multiple assays as a standard in every evaluation. Assays also change over time, or are no longer marketed at all, which would eliminate their use as part of a combined gold standard.

For all these reasons, and despite their shortcomings, blood cultures remain the imperfect gold standard laboratory test for the diagnosis of bacteremia and fungemia.

DIAGNOSTIC IMPORTANCE

Determining which patients have bacteremia or fungemia and subsequent identification of pathogens and their antimicrobial susceptibility profiles are the most important objectives for using blood cultures as a diagnostic test. However, the identity of pathogens and the pattern of recovery from blood cultures provide important diagnostic clues as to the location and type of infection. There are strong associations between sites of infection and which pathogens are recovered from the bloodstream (3), observations that give critically important information to providers as to the nature of the infection. It should be remembered that up to 29% of blood culture isolates do not have an identifiable source of infection (1).

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PROGNOSTIC IMPORTANCE

The attributable mortality rate of about 12% is an overall rate for hospitalized adult patients with bacteremia or fungemia. When the site of infection, type of service, and other variables are used to further categorize patients, mortality rates have been shown to vary widely (1, 3). There is less known about the prognostic importance of blood culture isolates recovered from outpatients as a group, primarily due to a lack of controlled studies but also because most patients with bacteremia or fungemia are sick enough to be admitted to the hospital and therefore become inpatients. Moreover, the frequency of community-acquired occult bacteremia in children has been greatly reduced or eliminated where conjugate pneumococcal and *Haemophilus influenzae* type b vaccines are used. What data have been published indicate that, for bacteremic patients with uncomplicated pyelonephritis or other uncomplicated infections that do not require subsequent hospitalization, blood cultures generally are not needed because the results have minimal prognostic (and little or no diagnostic) value (4–7).

CRITICAL FACTORS

During the past 40 years, a number of studies established and clarified which factors were most important in the recovery of pathogens from patients with bacteremia or fungemia. Many of the findings are pertinent today, while others now largely have historic interest only. The factors that remain the most important are described in the following paragraphs, but it should be emphasized that optimal recovery of pathogens from blood requires that all of these factors be addressed together, not in isolation.

Volume of Blood Cultured

For adult patients and older children, the volume of blood cultured is the most important factor in recovery of pathogenic microorganisms from blood. It is still important in younger children, but there are practical limits to the volumes of blood that can be withdrawn for laboratory tests such as blood cultures (8). This importance stems from the observation that there is a direct relationship between the total volume of blood cultured and the likelihood of recovery of pathogens (8–12). The research that established this often was done in conjunction with controlled clinical evaluations of different blood culture bottles and systems, which guided development of many of the commercial products that are available today. The outcome of many studies was that, for adult patients, 8 to 10 ml of blood should be inoculated into each of two bottles per blood culture, for a total of 16 to 20 ml of blood. This observation, combined with the finding that 2 to 4 blood cultures are needed to detect bacteremia or fungemia during a septic episode, means that up to 80 ml (four blood cultures of 20 ml each) should be drawn to optimize microbial recovery. Although this is a large amount of blood, and the full 80 ml is not necessary for all patients, it may be necessary to establish whether or not a patient is bacteremic or fungemic depending on the clinical situation and causative organism. This volume of blood may be reduced by half to 40 ml with use of blood culture bottles containing certain additives. For patients with anemia or other reasons for concern about the overall volume of blood drawn for laboratory testing, it is of no benefit to decrease the volume of blood drawn per culture; to do so will only result in decreased recovery of pathogens. A better diagnostic alternative is to modify the number of blood cultures drawn (as discussed below) and to limit the overall number of blood draws to those that are essential for patient care.

For older children, the volume of blood that should be drawn for culture differs little or not at all from that specified for adults. There are no controlled clinical trials that provide guidance, but broadly speaking, children age 12 and older (except for very small children) should have blood cultures drawn according to the criteria used for adult patients. For younger children, the total volume of blood that should be drawn for culture is lower than that for adult patients. For neonates, infants, and very young children, the volume of blood drawn for culture needs to be reduced by two mechanisms: the volume drawn per culture as well as the number of cultures. The volume of blood drawn should not exceed 1% of the patient's blood volume, but this may need to be modified if the patient is anemic at the time of the blood draw (8). As discussed in the following section, the number of blood cultures drawn at one time is critical for proper interpretation of blood culture results. Therefore, no fewer than two blood cultures should be drawn from pediatric patients.

Number of Blood Cultures

Collecting the correct number of blood cultures has two benefits. First, it helps ensure that an adequate volume of blood is drawn for culture. Second, it allows providers to correctly interpret results of blood cultures. Single blood cultures yield information that is difficult to interpret, unless the isolate is one that rarely, if ever, is recovered as a contaminant (e.g., pathogenic fungi such as *Histoplasma capsulatum*). Aside from examples such as that, however, most isolates recovered from blood cultures require the isolation from more than one blood culture to be considered a cause of sepsis. Contaminants generally occur in only one blood culture of a series, whereas pathogens typically occur in more than one of the blood cultures in a series. For patients with intravascular foci of infection, such as infective endocarditis, all blood cultures in a series should yield the pathogen.

A common question concerns the diagnostic sensitivity and specificity of a blood culture. Because the clinical presentation of patients with bacteremia or fungemia is so varied, clinical signs and symptoms cannot be used as a gold standard against which blood cultures can be prepared. There also is no other laboratory test (or combination of tests) that can serve as a surrogate gold standard. In the absence of another gold standard test, then, only estimates can be made regarding diagnostic sensitivity and specificity. Four studies have addressed this question directly (9,13–15). Because these studies were conducted during the period from 1975 to 2007, only rough comparisons should be made of the results. This is because (i) the studies used different blood culture systems; (ii) different volumes of blood were cultured; (iii) the relative distribution of pathogenic species changed during this time; (iv) markedly different classes of antimicrobial agents were in use; (v) this time period was the one in which a large number of intravenous devices, implants, and prostheses were introduced and later used widely; (vi) new pathogens were discovered; and (vii) the study designs all were different. A summary of the results from these four studies is shown in Table 1.

The question, then, is whether the traditional recommendation of drawing two to three blood cultures for the detection of common pathogenic bacteria and yeasts remains valid. Clearly, a single blood culture is insufficient. Because two blood cultures will only recover 80 to 90% of pathogens, at least three blood cultures are necessary, as

TABLE 1 Cumulative percentage of recovery of pathogenic microorganisms by number of blood cultures

No. of cultures	% Recovery of microorganisms in study by:			
	Washington[a]	Weinstein et al.[b]	Cockerill et al.[c]	Lee et al.[d]
1	80	91.5	67.4	73.1
2	88	>99	81.8	85.7
3	99		95.7	98.2
4			100	99.8

[a] Data from reference 13. Of the four groups in this table, this is the only set that excluded patients with infective endocarditis.

[b] Data from reference 3.

[c] Data from reference 9.

[d] Data from reference 15.

that will recover 96 to 98% of pathogens. Although four blood cultures will recover close to 100% of pathogens, routinely drawing this number of blood cultures has several drawbacks: (i) it is not necessary for all patients, (ii) it adds substantially to costs if done on all patients, and (iii) it isn't practicable for some patients. One recommended approach is to draw two blood cultures in the first 24 h, followed by an additional two blood cultures during the subsequent 24 h (9). The main criticism of this approach is that it can delay detection of bacteremia for an additional 24 h. Because this approach also suffers from the aforementioned drawbacks, the available evidence supports drawing three blood cultures in the first 24 h.

Although adult patients vary substantially in both height and weight, in general the number of blood cultures drawn does not need to be adjusted by either parameter for previously healthy adult patients. For patients with anemia, for whom withdrawing blood for laboratory testing may worsen their anemia, the most important decision is whether blood cultures (or other laboratory tests) are necessary at all. If blood cultures are clinically indicated, it makes neither clinical nor laboratory sense to decrease the number of blood cultures or volume drawn; to do either is not in the patient's best interest.

Dilution of Blood

Dilution of blood was more important with older blood culture media, where the antimicrobial effects of blood per se, and antimicrobial agents in the blood, needed to be diluted by higher volumes of broth medium. Over time it became clear that, with modern blood culture systems using standardized blood culture media, anticoagulants, and other factors, a blood-to-broth ratio of between 1:5 and 1:10 was sufficient. With the addition of additives to the broth medium that bind or sequester antimicrobial agents, a blood-to-broth ratio of as little as 1:4 may be sufficient.

Anticoagulants

In the past, a number of different anticoagulants have been considered for blood culture bottles, since clotted blood diminishes yield (16). Almost all have now been abandoned based on bacterial inhibition studies with seeded blood cultures. Most commercial blood culture bottles today contain sodium polyanethol sulfonate (SPS) in a narrow range of concentrations; some also have sodium citrate alone or in combination with SPS. SPS, in addition to its anticoagulant property, inhibits complement activity, inactivates clinically achievable concentrations of some aminoglycoside antibiotics, inactivates lysozyme, and blocks phagocytosis. The relative effects these four properties have on recovery of pathogenic microorganisms from blood cultures is unknown; however, controlled clinical trials have shown improved recovery of Gram-negative rods and streptococci with adequate concentrations of SPS.

Agitation

Agitation of aerobic blood culture bottles, by any mechanism, has been shown to increase recovery of pathogens, and all automated systems include agitation of aerobic bottles and most anaerobic bottles as well.

Medium and Additives

Although at one time there were many types of broth media used for blood cultures, today most commercial blood culture bottles contain soybean casein digest broth, also known as Trypticase soy broth (BD). Other types of media have shown comparatively little advantage over soybean casein digest broth, even those that were designed to recover specific groups of pathogens.

In the past, a wide variety of additives were added to broth media, many of which are no longer available. Current additives, which were designed to improve recovery of pathogens primarily from patients receiving antimicrobial therapy at the time of culture, clearly are beneficial in terms of recovery of pathogens and have the added advantage of allowing for use of smaller volumes of blood without a concomitant decrease in microbial recovery. Use of some additives may also result in increased recovery of bacterial contaminants.

SPECIMEN COLLECTION

Skin Disinfection

Trials of different skin disinfectants have been published for half a century, with development of reasonable conclusions as to which disinfectants perform better. It cannot be overemphasized, however, that the margin of difference between disinfectants is small and that what is by far more important is the technique used to disinfect skin (17). As a general guide, the best skin disinfectant is chlorhexidine, followed by tincture of iodine, povidone-iodine, and then various alcohols. Specific procedures for disinfection of skin are provided with each product, and so will not be presented here, but should be followed closely by users. For infants less than 2 months of age, chlorhexidine should not be used but rather alcohol swabs should be used. Perhaps the single most important point to be made is that skin disinfection takes time: time to perform the procedure and adequate time for the disinfecting solution to work. As noted below, adequacy of disinfection can easily be audited by monitoring contamination rates.

For most adults and older children, blood should be drawn from veins in the antecubital fossae. Not only are these veins readily accessible by venipuncture, but also they are sufficiently large to allow for drawing blood by a needle and syringe (i.e., a "butterfly" apparatus is not needed) and the veins are less likely to collapse. Although the practice is common, as a best practice, blood cultures should not be drawn through indwelling vascular access lines, owing to higher contamination rates. For patients with suspected line-related infection, blood should be drawn through the line and from venipuncture sites and the results compared (8, 18), as described below.

Number and Timing of Cultures

As stated previously, three to four blood cultures should be drawn simultaneously to detect bacteremia or fungemia. A single blood culture lacks sufficient sensitivity to detect bacteremia, and for many isolates, the result cannot be interpreted (e.g., coagulase-negative staphylococci). There is no benefit to waiting an arbitrary amount of time between drawing blood cultures (19), even though that practice was widely used for decades.

For patients with suspected infective endocarditis, or other intravascular foci of infection, three microbiological factors are of importance: documenting bacteremia, determining the identity of the infecting pathogen, and characterizing the antimicrobial susceptibility profile. Documenting continuous bacteremia, while necessary in some cases, is probably of less relative importance for most patients. In cases where it is necessary because of a confusing clinical or microbiological presentation, drawing three to four blood cultures spaced 30 to 60 min apart is a reasonable approach. For patients with prosthetic valves or an implanted device, for whom infections are likely to be caused by coagulase-negative staphylococci, skin disinfection is of critical importance to minimize the risk of contamination. It is also important to not draw blood cultures through indwelling vascular catheters in these patients. Most cases of infective endocarditis are caused by common bacterial pathogens, and therefore, blood cultures do not require use of special media or prolonged incubation and monitoring. For patients who are more likely to have infection caused by *Abiotrophia* spp. or *Granulicatella adiacens*, although special media are not necessary, it is important to notify the laboratory so that the pattern of growth on agar plates can be monitored (e.g., lack of growth on sheep blood agar plates) (8). The duration of incubation does not need to be more than 5 days; two studies have documented that all isolates from patients with infective endocarditis are recovered within that time (9, 20).

INTERPRETATION OF BLOOD CULTURE RESULTS

Unlike straightforward laboratory tests that yield a binary test result of positive or negative, blood cultures yield a complex set of data that can only be interpreted in the context of the clinical scenario that prompted their collection. The information generated by blood cultures includes the identity of any bacteria or fungi isolated from the specimen (or combination of microorganisms), the number of positive and negative cultures, and the time for detection of microbial growth, all of which must be interpreted in light of the pretest probability for bacteremia or fungemia; the results of other clinical, laboratory, or radiographic findings; type(s) of isolates recovered from other body sites; response (or lack of response) to therapy; clinical signs and symptoms; and the clinical judgment of the ordering physician.

In routine clinical practice, interpretation should not rely solely on the identity of the microorganism(s) isolated. While some isolates almost never are contaminants (e.g., *Brucella* spp., *Francisella tularensis*, and *Histoplasma capsulatum*) and are easy to interpret, isolation of most bacteria and fungi requires further interpretation. Isolates such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, other members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Candida albicans* are usually pathogens but, in a minority of cases, can represent contaminants (3). In contrast, isolation of many other species (e.g., *Propionibacterium* spp., *Bacillus* spp. other than *Bacillus anthracis*, and most *Corynebacterium* spp.) almost always represents contaminating flora. Determining the clinical importance (if any) is often difficult for the coagulase-negative staphylococci, as the identity of isolates per se is of little or no help. This is particularly true for patients with indwelling catheters, implants, or prosthetic devices, for whom the coagulase-negative staphylococci are one of the most common causes of infection. Another good example is isolation of *Staphylococcus lugdunensis*, which can cause infections of foreign devices or of host tissue in a manner similar to that of *S. aureus*, but it also can be a contaminant (21). Isolation of this bacterium from blood cultures, as with other coagulase-negative staphylococci, requires additional information for proper clinical interpretation. Isolation from even a single culture can be clinically important.

Interpretation of blood culture results collected by venipuncture is more straightforward than that for blood cultures collected from indwelling venous lines. For blood cultures collected via lines, interpretation involves analysis of the additional factors of the type of line, where it is inserted in the body, how long it has been in place, and whether isolates are recovered from the line only, from an accompanying blood culture collected via venipuncture, or from both. A number of interpretive criteria have been proposed, but the multiplicity of studies with various methodologies makes interpretation and comparison difficult. Although complex by necessity, the current CLSI criteria (8) remain useful and are shown in Table 2.

Other criteria have been suggested for making the interpretation of blood culture results more accurate and reliable. The time required for an automated blood culture system to flag a bottle as positive (i.e., to detect a growth signal), or so-called "time to positivity," has been studied in a number of settings. Although there is a correlation between the earlier detection of pathogens compared with that of contaminants, as documented many years ago, there is so much overlap between the time needed to detect growth of both groups that the information is of minimal clinical use. Moreover, time to positivity obviously is not useful for slowly growing microorganisms and probably is not useful in patients who are receiving antimicrobial therapy, which can delay detection of microbial growth. One exception to this principle is that most isolates recovered after more than 72 h of incubation represent contaminants, with the obvious exceptions of some slowly growing pathogenic bacteria and yeasts.

Another commonly used criterion is that of considering the number of positive bottles, rather than the number of positive cultures. Although there are conflicting data regarding this issue, the most thorough study, by Mirrett and colleagues (22), showed that, for coagulase-negative staphylococci, there is no correlation between the number of bottles positive in blood culture sets and the clinical importance of the isolates. This observation held true for sets that consisted of two, three, or four blood culture bottles (22). Fewer data are available regarding other types of blood culture isolates, but because microorganisms in blood cultures show a Poisson distribution, there is no reason that the principle for coagulase-negative staphylococci should not hold true for other types of isolates.

Last, the issue of quantitative blood cultures still arises on occasion. Although methods for quantitation (or semiquantitation) have been devised and there are some data to

TABLE 2 Interpretive criteria for CRBSI[a, b]

- **Short-term peripheral[c] catheters**
  - *Method*
    - Obtain two sets of peripheral blood cultures via venipuncture
    - Remove catheter aseptically and culture using semiquantitative method of Maki
  - *Interpretation*
    - If one or more peripheral blood culture sets are positive AND the catheter segment culture is positive (≥15 colonies) for the same microorganism: suggestive of CRBSI
    - If one or more blood peripheral culture sets are positive AND the catheter segment culture is negative: inconclusive (except for *Staphylococcus aureus* or *Candida* spp.)
    - If both peripheral blood culture sets are negative AND the catheter segment culture is positive (any colony count): suggestive of catheter colonization
    - If both peripheral blood culture sets are negative AND the catheter-segment is negative: CRBSI is unlikely
- **Nontunneled and tunneled central venous catheters and VAP[d]**
  - *Method*
    - Obtain two sets of peripheral blood cultures with at least one set drawn via venipuncture; the other set should be drawn aseptically from the catheter hub or through the VAP septum
  - *Interpretation*
    - If both sets are positive for the same microorganism: suggestive of CRBSI
    - If both sets are positive for the same microorganism AND the set drawn through the catheter becomes positive ≥120 min earlier: suggestive of CRBSI
    - If both sets are positive for the same microorganism AND the set drawn through the catheter becomes positive ≤120 min earlier: CRBSI is still possible if both sets yield the same microorganism with an identical antimicrobial susceptibility profile
    - If both sets are positive AND the set drawn through the catheter has at least a 5-fold-greater colony count than the peripheral culture (for laboratories using a manual quantitative method such as lysis-centrifugation): suggestive of CRSBI
    - If only the blood culture set drawn from the catheter becomes positive: inconclusive for CRBSI and suggests either colonization of the catheter or contamination during specimen collection
    - If only the blood culture set drawn peripherally becomes positive: inconclusive for CRBSI except if *S. aureus* or *Candida* spp. is isolated; in those cases, documentation of CRBSI requires isolation of the same microorganism by culture of the catheter tip segment or additional positive catheter or peripheral blood cultures
  - *Alternative method*
    - Obtain two sets of peripheral blood cultures via venipuncture
    - Remove catheter aseptically and culture by semiquantitative method of Maki
  - *Interpretation*
    - If one or both of the peripheral blood culture sets AND the catheter segment culture are positive with the same microorganism: CRBSI is likely
    - If one or both of the peripheral blood culture sets are positive AND the catheter-segment culture is negative: this may represent CRBSI if positive for *S. aureus* or *Candida* spp.; in those cases, documentation of CRBSI requires further isolation of the same microorganism by additional peripheral blood cultures
    - If the peripheral blood culture sets are negative AND the catheter segment culture is positive: suggests colonization
    - If the peripheral blood culture sets and the catheter segment culture are negative: CRBSI is unlikely

[a] For all categories, interpretations are in the absence of any other identifiable source of infection. CRBSI, catheter-related bloodstream infections.

[b] Text modified from reference 8.

[c] The term "peripheral" in this table refers to blood specimens obtained via venipuncture of a peripheral vein.

[d] VAP, venous access ports.

support this approach, in reality, the approach is not practicable for routine use. Moreover, as newer technologies are developed with increasing analytical sensitivity, it is possible that methods will be devised to quantitate the number of pathogens in blood via surrogate markers.

LABORATORY DETECTION—CULTURE-BASED METHODS

Conventional detection of bacteremia or fungemia has been either direct, via recovery of pathogenic microorganisms by culture of blood specimens, or indirect, by identification of surrogate markers. The latter method was investigated widely during the 1970s and 1980s by attempts to identify methods such as detection of limulus endotoxin assays, or detection of bacterial antigens in blood, but these efforts are largely only of historic interest. As a result, culture remains the only widely used laboratory method for detection of bacteremia or fungemia.

Blood cultures were first described more than a century ago and, by the 1930s, had prompted a number of investigations that defined some of the methods still used today. By the 1960s, blood cultures were widely used, but methods varied considerably, a multiplicity of methods were in use (with varying degrees of success), and it became increasingly clear through time that the overall approach left much to be desired. During the 1970s, John Washington and other investigators began a series of experiments and controlled clinical trials to define the critical factors in the detection of bacteremia and fungemia, identify best practices for blood cultures, and establish scientific evidence upon which future blood culture systems could be developed and used on a widespread basis. Out of these studies emerged a growing body of knowledge regarding the critical factors for recovering pathogenic microorganisms from blood, knowledge that has resulted in more refined clinical approaches to blood cultures and to development of commercially available blood cultures systems that incorporate these critical factors into their design and use.

Manual Blood Culture Systems

Relatively few nonautomated blood culture systems are still manufactured and sold commercially in the United States. Those that are available commercially are well characterized as to their diagnostic strengths and limitations. These systems are adequate for the detection of common bacterial and fungal pathogens, are easy to use, require only a non-CO_2 incubator, and are inexpensive (12). The main drawback to manual systems is that they are labor-intensive, which precludes their use in laboratories processing even moderate numbers of blood cultures. Moreover, with the availability of smaller instrumented blood culture systems, even laboratories processing low numbers of blood cultures can opt for using an automated system.

The first manual blood culture systems consisted of little more than blood culture bottles containing various medium formulations, an anticoagulant, and occasionally additives or supplements. Detection of microbial growth was via two methods: subcultures after 24 or 48 h and at the end of the incubation period (so-called terminal subcultures) and visual observation of bottles for hemolysis (color change), gas production, or turbidity. This approach was not practicable for laboratories performing more than a small number of blood cultures. Because early instrumented/automated blood culture systems had their own issues with practicability, a number of alternative manual blood culture systems were developed and marketed. Of these, only two remain available in most markets: Septi-Chek (BD Biosciences, Sparks, MD) and Oxoid (Remel, Lenexa, KS).

Septi-Chek is a variation of the biphasic system originally developed by Castaneda to recover *Brucella* spp. from blood. These systems are called biphasic because there is the traditional liquid broth medium phase combined with a solid phase consisting of various agar media. In biphasic systems, blood is inoculated into the culture bottle and mixed with the broth medium. By one of several devices, the blood-broth mixture is then flooded over the solid media that acts as a direct subculture. The Septi-Chek variant of a biphasic system consists of a plastic paddle that is attached to the bottle after receipt in the laboratory. This obviously is an aerobic system, and for recovery of anaerobic bacteria, there is a separate bottle that does not have a corresponding paddle. Bottles are then incubated (with or without agitation) and inspected visually once or twice each day for evidence of microbial growth. The agar-coated paddle not only provides a second means of detecting microbial growth but also provides for early isolation of microbial colonies. Aerobic bottles are inverted following each inspection, providing further subcultures of the blood-broth mixture.

Current versions of Septi-Chek bottles contain one of several broth media with 0.05% SPS as an anticoagulant. Two bottle sizes are available: bottles containing 70 ml of broth, accommodating blood specimens of up to 10 ml (to maintain an adequate blood-to-broth ratio), and bottles containing 20 ml of broth accommodating blood specimens of up to 3 ml. Bottles are available that contain soybean-casein digest broth, soybean-casein digest/Columbia broth, soybean-casein digest/thioglycolate broth, or brain heart infusion broth. Agar paddles use chocolate, MacConkey, and malt agars.

In controlled clinical evaluations, Septi-Chek has been shown to perform well and to be an acceptable manual system for routine practice (23–27). It is not practicable for use in laboratories processing large numbers of blood cultures, but cost-effectiveness studies defining the practical upper limit of Septi-Chek bottles that can be handled before labor costs become prohibitive have not be performed.

In contrast to Septi-Chek, the manual blood culture system Oxoid Signal is a one-bottle system without an anaerobic version. Oxoid is another variant of the Castaneda bottle. With Oxoid Signal, bottles are inoculated with blood through a rubber septum in a lid. The Signal device consists of an empty plastic cylinder attached to a needle, which when inserted into the bottle extends to below the surface of the blood-broth mixture. During microbial growth, release of gases into the blood-broth mixture increases the concentrations of those gases, which reach equilibrium with those in the bottle headspace. Through time, this increases the atmospheric pressure within the bottle, eventually forcing some of the blood-broth mixture upward through the needle into the attached Signal device. Although this provides a second mechanism for detecting microbial growth, unlike Septi-Chek it does not provide for earlier isolation of microbial colonies. In controlled clinical evaluations, the Oxoid Signal system has not performed as well as other blood culture systems (28–31).

Lysis-Centrifugation

Only one commercial lysis-centrifugation blood culture system has been marketed, the Isolator blood culture system (Wampole Laboratories, Cranbury, NJ). With lysis-centrifugation, the blood specimen is inoculated into tubes containing a mixture of the lysing agent saponin, an anticoagulant, and a fluorocarbon-cushioning agent. The blood is then lysed by the saponin, and the mixture is centrifuged to separate the components. The supernatant is then removed and discarded, and the suspended pellet is used to inoculate whatever culture media are deemed necessary to recover specific pathogens. The system has been used for detection of routine bacterial pathogens, but because delayed processing has been reported to reduce recovery of anaerobic bacteria, some *Haemophilus* species, and *Streptococcus pneumoniae*, other commercial broth-based systems are likely better for routine use (32–36). Although at one time Isolator was a good method for recovery of pathogenic yeasts, dimorphic fungi, mycobacteria, and *Bartonella* spp., other systems (see below) are at least equal for recovering these pathogens. Detection of *Bartonella* spp. is best achieved by use of nucleic acid amplification; if not available, serologic testing can be used. These issues, combined with the manual nature of lysis-centrifugation, have made it less practicable for routine use.

Automated Blood Culture Systems

As the number of blood cultures performed began to increase during the 1960s and 1970s, with the resulting need for methods that were less labor-intensive, there developed a growing interest in development of automated blood culture systems. A number of these were developed and tested, but only a few have been commercially successful.

The first of these was the Bactec 460 radiometric system (Becton Dickinson, Sparks, MD), a derivative of the original Bactec 220 system that was developed in the late 1960s and first marketed in the early 1970s. During the next two decades, the Bactec 660, 730, and 860 systems in turn succeeded the 460 system. All of these systems had in common the detection of microbial growth by monitoring CO_2 production by growing microorganisms. The 220 and 460 systems used radiometric detection of ^{14}C-labeled CO_2, whereas the 660, 730, and 860 systems detected CO_2 production by infrared spectrophotometry. Because both radiometric and nonradiometric detection methods required removal of an aliquot of the atmosphere in the bottle headspace, monitoring more than once or twice per day was not possible. This is because an equal volume of sterile gas had to be added to bottles after sampling to maintain the appropriate pressure in each bottle. This, in turn, reduced slightly the CO_2 present in the atmosphere, thereby limiting the frequency of sampling. Because once or twice daily sampling did not allow for detection of microbial growth at the earliest possible time, a newer generation of blood culture technology was needed.

The next generation, conceived in the late 1980s and introduced in the 1990s, was that of the continuous-monitoring blood culture systems (CMBCS). These systems take their name from the fact that, unlike previous automated blood culture systems, which monitored bottles for CO_2 production only a few times each day, the newer systems monitor CO_2 production much more frequently, typically once every 10 to 15 min (12). Unlike older automated systems, which flagged potential microbial growth when the CO_2 level in a bottle exceeded an arbitrary threshold level, CMBCS use one of several computer algorithms to detect microbial growth. The first is use of a threshold level, second is a sustained linear increase in CO_2 production, and third is an increase in the rate of CO_2 production (although specific computer algorithms used by each manufacturer are proprietary). Because the latter two criteria depend upon actively growing microorganisms, delayed placement of bottles in instruments, which allows microorganisms to grow and thereby produce CO_2 prior to testing, can result in delayed (or lack of) detection of microorganisms in bottles. Because of the large amount of data to be analyzed for each bottle, and for the many bottles in each incubator, the success of CMBCS is as much due to the development of more powerful computer processors and memory as it is to anything else.

For CMBCS to monitor CO_2 levels on a frequent basis, two other barriers to testing had to be removed. The first was to eliminate the need for sampling and replenishing the atmosphere in the bottle headspace. This was achieved by the development of sensors that could be read through the wall of the bottles without need for invasive testing. The second was to eliminate the need for repeated manual loading and loading of bottles onto instruments for testing. Although this was achieved to some degree by the Bactec 860 system, each bottle still had to be moved to the testing apparatus. In contrast, CMBCS have a mechanism for monitoring the sensor of each bottle individually, thereby eliminating the need to ever move bottles during incubation and testing. Overall, the change to more frequent testing with CMBCS reduced the time to detect microbial growth by 1 to 1.5 days compared with Bactec radiometric and nonradiometric systems (12).

BacT/Alert

The first of the CMBCS was BacT/Alert, with prototypes under development in 1988 and the first limited clinical trials conducted by 1990 (37). BacT/Alert is a colorimetric system, detecting changes in CO_2 concentration in bottles via changes in color of a pH-sensitive device in the base of bottles. Since it was introduced, the system has undergone several changes in style and configuration, with the BacT/Alert 3D being the most current version of the instrument. Sequential medium formulations include standard aerobic and anaerobic media; aerobic, anaerobic, and pediatric (aerobic only) fastidious antimicrobial neutralization (FAN) media (which include activated charcoal and Fuller's earth [Ecosorb] designed to inactivate or bind antimicrobial agents); FAN aerobic (FA), FAN anaerobic (FN), and pediatric FAN (PF) bottles; FA Plus, FN Plus, and PF Plus media (which contain proprietary adsorbent polymeric beads); and the Mycobacteria Process (MP) bottle designed to recover mycobacteria. Since it was introduced more than 20 years ago, a number of clinical evaluations of BacT/Alert have been published that have established the performance characteristics of the system and different bottle types that have been produced. These findings are summarized in Table 3.

Broadly speaking, these characteristics hold true for all of the bottles available with CMBCS: bottles with additives outperform bottles without additives, only a 4- to 5-day incubation and testing cycle is necessary, blind and/or terminal subcultures are not necessary, and bottles containing additives increase recovery of contaminating microbial flora.

Bactec 9000 Series

In 1992, Becton Dickinson introduced the second of the CMBCS, the Bactec 9000 series. Initially there were two versions, the Bactec 9120 (holding 120 bottles per cabinet) and the Bactec 9240 (holding 240 bottles per cabinet). Several years later, the Bactec 9050 (holding 50 bottles) was introduced for laboratories needing an automated system with a smaller capacity (38). The Bactec technology is similar to that of BacT/Alert, the main difference being that Bactec CMBCS use a fluorescence-sensing mechanism to detect CO_2 production.

Bactec systems make use of several medium formulations, including standard aerobic/F and anaerobic/F bottles, Plus aerobic/F and Plus anaerobic/F bottles, which contain antibiotic-binding resins attached to tiny glass beads, lytic/10 anaerobic/F medium bottles, Peds Plus/F bottles, and Myco/F lytic bottles that are designed to increase recovery of fungi and mycobacteria (39). Each of these bottle types accepts up to 10 ml of blood, except for the Peds Plus/F bottles, which accept 1 to 3 ml of blood. A large number of clinical evaluations of Bactec Standard and Plus/F bottles used on the 9050, 9120, and 9240 systems have been published. The performance characteristics of these systems are summarized in Table 4.

In contrast to Standard/F and Plus/F bottles, Bactec Myco/F lytic bottles were developed to allow for recovery of mycobacteria and fungi as well as common bacterial pathogens. Myco/F lytic bottles differ from all other Bactec 9000 bottles in that the fluorescent sensor in the bottle detects decreasing oxygen concentration (i.e., detects oxygen consumption) as opposed to detecting increased concentrations of carbon dioxide. In a number of clinical trials, Myco/F lytic bottles have been shown to compare favorably with other systems for detection of mycobacteria and fungi. In an early study, the Myco/F lytic bottle detected fewer *H. capsulatum* isolates but more *Cryptococcus neoformans* isolates than the Isolator system (40). Myco/F bottles have been shown to be equivalent to ESP II bottles for recovery of mycobacteria overall, with significantly better recovery of *Mycobacterium avium* complex in Myco/F lytic bottles (40). More recent studies have shown Myco/F lytic bottles and BacT/Alert MB bottles to be equivalent to but appreciably faster than the Isolator 10 system for recovery of *Mycobacterium tuberculosis* and *Mycobacterium avium* complex clinical isolates (41, 42).

VersaTREK Blood Culture System

The third CMBCS introduced during the 1990s was the ESP system (Difco Laboratories), now marketed by Thermo

TABLE 3 Summary of performance characteristics of BacT/Alert blood culture system

BacT/Alert standard bottles are equivalent to other commercial bottles without additives for recovery of common pathogenic bacteria and yeasts (38, 66, 67)

BacT/Alert aerobic FAN bottles are superior to BacT/Alert aerobic standard bottles for recovery of common pathogenic bacteria and yeasts (68)

BacT/Alert anaerobic FAN bottles are superior to BacT/Alert anaerobic standard bottles for recovery of common pathogenic bacteria and yeasts with the exception of strict aerobes such as Gram-negative nonfermenters and yeasts (69)

BacT/Alert FA Plus and FN Plus bottles are superior to FA and FN bottles for recovery of common pathogenic bacteria and yeasts with earlier time to detection (70)

BacT/Alert PF Plus bottles are superior to PF bottles for recovery of common pathogenic microorganisms with earlier time to detection (71)

BacT/Alert FA Plus and FN Plus bottles are superior to BacT/Alert standard bottles for recovery of common pathogenic bacteria and yeasts with earlier time to detection for FA Plus bottles (70, 72)

BacT/Alert FAN bottles are equivalent to other commercial bottles with additives for recovery of common pathogenic bacteria and yeasts (73, 74)

BacT/Alert FAN bottles are equivalent to other commercial bottles for improving recovery of pathogens from patients who are receiving antimicrobial therapy when cultures are drawn

BacT/Alert FAN bottles are superior to BacT/Alert standard bottles for detection of episodes of bacteremia and fungemia (68, 69, 75)

BacT/Alert FAN bottles recover more contaminating bacteria than do BacT/Alert standard bottles

BacT/Alert bottles can be processed using a 5-day rather than a 7-day incubation period without a significant decrease in recovery of pathogenic bacteria and fungi (76, 77)

Terminal subcultures of bottles shown to be negative by the instrument do not increase recovery of pathogenic bacteria or yeasts

BacT/Alert aerobic standard bottle inoculated with 10 ml of blood detects more microorganisms than the same bottle inoculated with 5 ml of blood (78)

Scientific (Cleveland, OH) as VersaTREK. VersaTREK differs from the BacT/Alert and Bactec systems in several fundamental ways. First, VersaTREK uses a different system for detecting microbial growth. Once received in the laboratory, bottles are fitted with an adapter mechanism before being loaded into the instrument. The detector mechanism allows for the system to monitor pressure changes within the headspace of each bottle as oxygen, hydrogen, nitrogen, and carbon dioxide are consumed or produced by growing microorganisms. Second, the blood broth mixture within aerobic bottles is agitated by use of a stir bar contained within each bottle. Anaerobic bottles are not agitated. Third, the broth medium in VersaTREK Redox 1 aerobic bottles is soy casein peptone broth, whereas the broth medium in Redox 2 anaerobic bottles is proteose-peptone broth. Fourth, bottles are monitored for growth less frequently: at 12 min for aerobic bottles and 24 min for anaerobic bottles. Last, unlike BacT/Alert FAN and Bactec Plus bottles, both of which contain additives to minimize or negate antimicrobial activity in blood specimens, VersaTREK Redox 1 and Redox 2 bottles contain 80 ml of broth medium, the larger volumes providing greater dilution of blood and any antimicrobial agents contained therein. The smaller Redox 1 EZ Draw and Redox 2 EZ Draw bottles, which are marketed to allow for direct collection of blood specimens into the bottles, still contain 40 ml of broth medium for greater dilution of blood specimens compared with BacT/Alert and Bactec bottles. A number of clinical evaluations of VersaTREK have been published. Based on the results of these evaluations, the performance characteristics of this system are summarized in Table 5.

TABLE 4 Summary of performance characteristics of Bactec blood culture systems

Bactec standard bottles are equivalent to other commercial bottles without additives for recovery of common pathogenic bacteria and yeasts (79)

Bactec Plus/F bottles are superior to BacT/Alert standard bottles for recovery of common pathogenic bacteria and yeasts (80)

Bactec anaerobic Plus/F bottles are superior to Bactec standard anaerobic bottles for recovery of common pathogenic bacteria and yeasts (80)

Bactec Plus/F bottles are equivalent to other commercial bottles with additives for recovery of common pathogenic bacteria and yeasts (74, 81)

Bactec Plus/F bottles are equivalent to other commercial bottles with additives for improving recovery of pathogens from patients receiving antimicrobial therapy when cultures are drawn

Bactec Plus/F bottles are superior to bottles without additives for detection of episodes of bacteremia and fungemia (80)

Bactec Plus/F bottles recover more contaminating bacteria than do Bactec standard bottles

Pediatric Blood Culture Bottles

A number of blood culture bottles intended for use with pediatric patients have been developed and marketed. Because of the practical difficulties in conducting clinical trials involving large numbers of hospitalized children, these products have not been evaluated to the same extent as those for adult patients. While there is no drawback to using these bottles (other than cost differences), there appears to be little advantage to using them in regions where immunization with pneumococcal conjugate and *Haemophilus influenzae* type b vaccines are given widely.

LABORATORY DETECTION—NON-CULTURE-BASED METHODS

Surrogate Markers for Sepsis

A number of nonmicrobiological tests have been evaluated for their ability to detect bacteremia or fungemia. Although most of these evaluations state that assays were evaluated for the ability to detect sepsis, it should be remembered that sepsis is foremost a clinical diagnosis. What most of these

TABLE 5 Summary of performance characteristics of VersaTREK blood culture system

VersaTREK bottles are superior to Bactec standard bottles for recovery of common pathogenic bacteria and yeasts
A number of studies have shown VersaTREK bottles to be inferior for recovery of *Staphylococcus aureus* compared with bottles from other systems containing additives (33, 73, 82, 83), whereas one study found the opposite (84)
Several studies have reported decreased recovery of contaminants from VersaTREK bottles (11); another study found the opposite (85)
Several studies have reported increased recovery of streptococci and enterococci from VersaTREK bottles (82, 83, 86); another study found the opposite (84)
In some studies, VersaTREK bottles have been reported to detect significantly more pathogens in blood taken from patients receiving antimicrobial therapy at the time of blood culture (82, 83, 86)
VersaTREK bottles can be processed using a 4- or 5-day rather than a 7-day incubation period without a significant decrease in recovery of pathogenic bacteria and fungi (87, 88)

studies were evaluating, in fact, was the ability to detect patients with bacteremia or fungemia who subsequently developed the clinical signs and symptoms of sepsis.

Early assays, such as the limulus amoebocyte assay, showed promise but were never fully developed or marketed. Perhaps the most widely studied assays have been those that measure procalcitonin and serum lactate levels. For procalcitonin, the available evidence shows that the assay is not useful for the detection of bacteremia or fungemia, due to a lack of both sufficient sensitivity and specificity. The assay has been shown to be useful, however, when used to monitor patients with a diagnosis of bacteremia or fungemia as a guide to stopping antimicrobial therapy. The findings with serum lactate levels are similar. The assay lacks both sensitivity and specificity for the diagnosis of bacteremia or fungemia, but serial measurements may be of use both for prognosis and for modifying antimicrobial therapy.

Nucleic Acid Amplification Methods

A number of laboratory-developed assays designed for the direct detection of bacteremia or fungemia from blood specimens have been evaluated and reported (43). As presented elsewhere in this chapter, although laboratory-developed assays may serve a role as a proof of concept, they are of little use to the community at large because the assays cannot be marketed or distributed to other laboratories. Moreover, development and use of such assays is likely prohibitively expensive and beyond the expertise available in most clinical laboratories.

SeptiFast (Rotkreuz, Switzerland) is currently available in parts of the European Union but not in the United States. The system is a real-time PCR assay designed to detect, directly from blood, 10 species of Gram-negative bacilli, 6 species of Gram-positive cocci, 5 species of yeasts, and 1 filamentous fungus. The method uses the LightCycler system; although this technology is available for use in the United States, SeptiFast reagents are not.

Overall, the system has shown mixed results in clinical evaluations (44). Taken together, the data from these evaluations indicate that SeptiFast shows comparatively low sensitivity for detecting pathogens in blood. The number of studies, conflicting study designs and definition, and other factors make interpretation of the data difficult, but overall, the assay lacks sufficient sensitivity to be used as a standalone test. SeptiFast has been evaluated as an adjunct to blood cultures, in which a positive result from SeptiFast between days 3 and 7 following a positive blood culture result predicted an increased risk of patients developing complicated bloodstream infections (45). Whether the same risk stratification could be achieved using other laboratory tests has not been evaluated.

Another molecular assay, Xpert MTB/RIF (Cepheid, Sunnyvale, CA), has been evaluated for detection of *Mycobacterium tuberculosis* directly from blood specimens. In one small clinical evaluation, the calculated sensitivity of Xpert was only 21% (a figure notably similar to some of the data for SeptiFast, as described above), but in this group of patients, a positive Xpert test result was useful for stratifying patients into those who were or were not at increased risk for early death (46).

At this time, therefore, the data are clear that no molecular assay is available that can act as a substitute for blood cultures, but these tests may be useful as adjuncts to blood cultures.

RAPID IDENTIFICATION OF MICROBIAL ISOLATES

A number of rapid identification methods have been introduced over the years, with increasingly effective results. Early attempts were largely modifications of biochemical tests or early molecular methods (e.g., DNA probes) that did not have the desired effect. More-recent methods have yielded substantially better results for rapid identification of select groups of microbial pathogens. Moreover, there is evidence that earlier detection and identification of bloodstream pathogens, or antimicrobial resistance, improves outcomes (47) and facilitates antimicrobial stewardship (48, 49).

Peptide Nucleic Acid-Fluorescent *In Situ* Hybridization

The peptide nucleic acid-fluorescent *in situ* hybridization (PNA-FISH) method (AdvanDx, Woburn, MA), although limited to a few select pathogens, has been shown to reduce the time needed to identify microbial pathogens present in blood cultures. The principle of the method is simple, being based on the widely used principle of *in situ* hybridization. Unlike DNA and RNA probes, which have negatively charged sugar-phosphate backbone structures, the PNA backbone structure is composed of an electrically neutral polyamide (peptide) structure. The neutral electrical charge allows for more-rapid, tighter, and more-specific hybridization with nucleic acid targets. The probes are labeled with fluorescent dyes, which can be observed by using a fluorescent microscope. Once a blood culture bottle is flagged as positive, a smear is made of the blood-broth mixture and a Gram stain is performed. Based on those results, the appropriate PNA-FISH probe(s) can be selected for testing.

The method is easy to use, only modestly expensive, and requires little in the way of infrastructure beyond that already present in most clinical microbiology laboratories (other than a fluorescent microscope) (50). However, to take full advantage of the method, it is necessary to perform the test on an ad hoc basis for individual isolates, which is not practicable in many microbiology laboratories. The method does not provide any data regarding antimicrobial susceptibility or resistance.

Nucleic Acid Amplification Tests

Although nucleic acid amplification test technology would appear to lend itself to this application, surprisingly few commercial assays have been developed. In contrast, a large number of laboratory-developed assays have been reported, but their usefulness is limited by that of all user-developed assays: assays may not be adaptable for use in other laboratories, development and validation costs may be prohibitively expensive, and regulatory issues limit use of these assays in some countries (43). Their usefulness at this time is also limited by the relatively small number of pathogens that can be detected, preliminary evidence indicating that the assays are not as useful when more than one type of microorganism is in the blood-broth mixture (polymicrobial isolates), and the ability to test for only a small number of antimicrobial resistance determinants. Thus, the number of commercial assays available for this purpose is limited, as are clinical evaluations as to their effectiveness.

Multiplex Assays

Two commercial systems have been developed, the Verigene assay (Nanosphere, Northbrook, IL) and the Film Array (Biofire, Salt Lake City, UT). The Food and Drug Administration has cleared both for marketing in the United States. For Verigene, separate assays for detection of either Gram-positive or Gram-negative bacteria directly in blood cultures are FDA cleared; an assay for detection of common pathogenic yeasts is under development. The FilmArray product, called the blood culture identification panel, detects a combination of 24 Gram-positive bacteria, Gram-negative bacteria, and yeasts and three antimicrobial resistance genes in a single combined assay.

Verigene is a multiplex assay based on PCR amplification of nucleic acid targets with subsequent detection by hybridization with oligonucleotides bound to nanosphere particles, followed by signal amplification using what the manufacturer calls a silver staining process. FilmArray also is a multiplex assay, with an initial nucleic acid extraction and purification step followed by PCR-based amplification in two stages. The first is a multiplexed reaction; this is followed by individual PCR amplification reactions designed to detect specific products from the first-stage amplification step. Final detection is based on use of endpoint melting curves.

The Verigene Gram-positive blood culture assay, which is cleared for detection of 12 genera or species and three antimicrobial resistance genes, has been evaluated in several clinical trials. In the first, blood cultures processed on the VersaTREK blood culture system were used for the analysis. The evaluation of 203 total positive blood cultures showed 94% concordance for microbial identification and 97% concordance for detection of drug resistance for 178 monomicrobial isolates, with 92% and 96% concordance for 25 polymicrobial isolates (51). Results were available 24 to 48 h sooner than with conventional identification and susceptibility test methods. In the second study, blood cultures were processed using the BacT/Alert system (52). Results from this study of 186 blood cultures were similar to those of the first, in that for 176 monomicrobial cultures the concordance for identification was 96% for microbial identification and 99% for detecting drug resistance. Results were available 31 to 50 h sooner than with conventional methods. A third study showed overall concordance of 95% with conventional identification methods: 99% for monomicrobial isolates but only 33% for a small number of polymicrobial isolates (53). A fourth study was more specific and limited in scope, evaluating use of the assay as part of an antimicrobial stewardship program targeting patients with enterococcal bacteremia (49). In this study of 74 patients, compared with the preintervention use of conventional identification and antimicrobial susceptibility testing, the postintervention phase when Verigene was used showed shortening of the time required for appropriate antimicrobial therapy to be given, shortened length of hospitalization, and lower hospital costs. Finally, in an evaluation of the assay in a pediatric hospital, the findings reported were similar to those of the first three studies: the assay showed 95.8% concordance with conventional methods for identification of Gram-positive bacteria (54). The assay also showed 100% correlation for detection of methicillin-resistant *S. aureus* isolates, 100% correlation for detection of vancomycin-resistant *E. faecium* isolates, and 98% detection of methicillin-resistant *Staphylococcus epidermidis* isolates (54). As in earlier studies, time to detection was substantially shorter with the Gram-positive blood culture assay than with conventional methods (54).

The Verigene Gram-negative blood culture test, which is cleared to detect eight genera or species and six antimicrobial resistance genes, has been evaluated in only one published clinical trial (55). In this evaluation of 102 isolates, the Gram-negative blood culture test showed 97.9% concordance with conventional identification methods. The reported performance characteristics for detecting antimicrobial resistance or susceptibility were a positive predictive value of 95.8% and a negative predictive value of 100% (55).

Only one clinical evaluation of FilmArray blood culture identification has been published (56). In this study, FilmArray showed 91.6% concordance with conventional identification methods for samples with monomicrobial growth and 71% concordance for polymicrobial samples. The assay did detect additional microorganisms in 3.6% of samples for which isolates were not recovered by conventional culture methods.

Other Nucleic Acid Amplification Tests

The Gene Xpert (Cepheid, Sunnyvale, CA) has several assays that can be used in conjunction with blood cultures for identification of blood culture isolates or for detecting antimicrobial resistance. The two assays that would fit into this category are an assay for detecting the *mecA* gene in methicillin-resistant strains of *Staphylococcus epidermidis* and *Staphylococcus aureus* and one for detecting the *vanA* gene in vancomycin-resistant strains of enterococci. As with other methods for detecting pathogens or antimicrobial drug resistance in blood culture isolates, these assays do not replace blood cultures but are only an adjunct.

Antigen Detection

The BinaxNOW *Staphylococcus aureus* test (Alere Scarborough, Inc., Scarborough, ME) is an immunochromatographic assay that uses polyclonal antibodies to detect an *S. aureus*-specific protein, thereby allowing for differentiation between *S. aureus* and other Gram-positive cocci in blood culture bottles. Current FDA clearance is for use with BacT/Alert blood culture bottles. In one off-label study using VersaTREK blood culture bottles, the test showed 95.8% sensitivity and 99.6% specificity compared with culture and a direct tube coagulation test, both performed on aliquots of the blood-broth mixture (57). In a second off-label evaluation, the assay was compared with conventional methods using Bactec blood culture bottles (58). In this study, the BinaxNOW assay showed 97.6% sensitivity and 100% specificity.

MALDI-TOF (MS)

Matrix-assisted laser desorption ionization–time of flight (mass spectrometry) (MALDI-TOF [MS]) has been the most widely evaluated approach to rapid microbial identification of blood culture isolates. Currently two manufacturers of MALDI-TOF (MS) systems have instruments cleared by the FDA for marketing in the United States to identify microbial isolates from solid media (i.e., not directly from blood culture bottles): the Microflex LT Biotyper (Bruker Daltonics, Bremen, Germany) and the Vitek MS IVD (bioMérieux, Marcy l'Etoile, France). In an early study, 90% of bacterial isolates were identified directly from positive blood culture bottles (59). Other early studies of this technology also showed good results for identifying bacteria and fungi directly from blood culture broth specimens. One evaluation of the Biotyper evaluated the ability of MALDI-TOF (MS) to identify bacterial pathogens in 212 blood cultures (60). Of these, 42 (19.8%) showed insufficient numbers of bacteria in the blood-broth mixtures for MALDI-TOF (MS) to identify the bacteria (61). Of the other 170 blood cultures, MALDI-TOF (MS) showed 95.3% concordance in correctly identifying the bacteria compared with conventional identification methods (61). In another evaluation using the Biotyper (62), 330 positive blood culture bottles were analyzed, of which 318 showed growth on subcultures and 12 were considered to be false-positive signals by the blood culture instrument. Of the latter group, the MALDI-TOF (MS) results were fully concordant with culture results (62). For the 318 blood cultures that yielded growth on cultures, all were monomicrobial. When compared with results of conventional identification methods, MALDI-TOF (MS) results were concordant to the species level for 83.3% and to the genus level for 96.6% of blood cultures (62).

In the most comprehensive comparison of the two MALDI-TOF (MS) systems, a total of 202 positive blood culture bottles processed on the Bactec system were tested with both versions of MALDI-TOF (MS) (62). In this evaluation, there were 181 monomicrobial and 21 polymicrobial isolates. Biotyper correctly identified 177 of 181 (97.8%) monomicrobial isolates compared to 167 of 181 (92.3%) identified by the Vitek system (62). Neither system performed well for identification of polymicrobial isolates (62). Although time to identification was not evaluated in this study, two previous studies showed a reduction in the time to identification of between 26.5 and 34.3 h compared to conventional methods (63, 64). Despite this, the inability to perform antimicrobial susceptibility testing limits the usefulness of this method.

DIRECT RAPID ANTIMICROBIAL SUSCEPTIBILITY TESTING FROM BLOOD CULTURE BOTTLES

A large number of studies have attempted to answer whether direct testing of the blood-broth mixture (without the intervening step of subcultures) using conventional (not molecular) methods can be used to decrease the time needed for susceptibility test results. Theoretical obstacles to this approach are straightforward. The main obstacle is the inability to standardize the number of microorganisms in the blood-broth mixture to be used for testing. None of the CMBCS flag bottles as positive based on the number of microorganisms present. Depending on which system is used, the initial number of microorganisms present in the blood specimen, the metabolic characteristics of the microorganism (e.g., growth rate and gas production), and the number of microorganisms per milliliter when bottles are flagged as positive may vary. Second, testing aliquots of the blood mixture, with or without additives, introduces a complex liquid matrix (i.e., blood, broth, anticoagulant, and any additives) that was not intended for use in commercial antimicrobial susceptibility assays. Even with centrifugation, or other procedures, this "matrix effect" cannot be eliminated or mitigated fully. A third issue, although perhaps not as important, concerns the presence of antimicrobial agents present in blood. Because these agents are given in dosages to achieve blood concentrations at or above levels designed to inhibit bacterial growth, even with dilution by the broth medium, there may still be residual antimicrobial activity in the blood-broth mixture (65). Fourth, this is a moving target. Interpretive criteria for breakpoints may change over time, thereby requiring repeated validation of the method, which would not be practicable in most settings. A fifth obstacle is regulatory: most commercial antimicrobial susceptibility assays do not include direct testing of a blood-broth mixture in the package insert, making such use off-label and, in some cases, not reimbursable. Last, the published evidence on these approaches is not persuasive: some published studies have shown these approaches to work, but others have arrived at the opposite conclusion. In an era that emphasizes evidence-based laboratory medicine, objective analysis of the literature yields the conclusion that current evidence does not support this practice.

QUALITY AUDITS AND BENCHMARKS

The most commonly studied, documented, and reported quality metric regarding blood cultures is the contamination rate. There has been a long-standing recommendation that blood culture contamination rates be kept at or below 3% for hospitalized patients. This figure is not derived from anything more than the belief that it generally is not possible to maintain rates below 1% and that rates above 5% result in a confounding of the clinician's ability to distinguish between contaminants and pathogens. Because blood culture contaminants result in increased health care costs, contamination rates above 5% also are associated with increased costs. Whether the 3% figure is realistic for outpatient settings, particularly in emergency departments, is another unanswered question. For patient safety, quality, and costs, it makes sense to target the lowest possible contamination rates, but targeting specific rates should be done with the understanding that different contamination rates occur in different settings.

Another common assessment of quality is the number of blood cultures drawn per septic episode. As noted previously, interpretation of blood culture results depends heavily upon drawing both an adequate volume of blood and more than one culture. Determining the clinical importance of isolates recovered from single blood cultures can be impossible depending on the type of isolate recovered. At the same time, collecting more blood cultures than is necessary is wasteful, contributes to phlebotomy-caused anemia, and results only in recovery of more contaminants. For both reasons, laboratories should monitor the number of blood cultures collected per septic episode.

A third important measurement is the adequacy of fill of blood culture bottles. Weighing filled bottles and comparing weights against those of a known standard most readily achieve this goal. Bottles filled with inadequate volumes of blood diminish yield and should be reported to the provider, with a recommendation to recollect the blood culture. Adequacy of filling should be monitored through time and by site so that any patterns of underfilling (or overfilling) can be identified and the appropriate corrective action taken.

SUMMARY

Detection of bacteremia and fungemia remains one of the most important roles of clinical microbiology laboratories. Despite the development and introduction of a number of novel technologies, the blood culture using liquid-based media still remains the only practicable approach for routine patient care. Molecular detection methods show promise, but the available methods do not have the sensitivity of blood cultures (except for a select few pathogens), provide only limited information regarding antimicrobial susceptibility testing, if used alone would not allow for retention of isolates for epidemiologic investigations, at this time are not effective for detecting polymicrobial isolates, and are more expensive to use on a routine basis. Other methods such as MALDI-TOF (MS) also can achieve similar results for rapid identification of microorganisms isolated on solid media but currently are not cleared by the FDA for identification of pathogens directly from blood cultures. Experience has shown that novel technologies rarely replace older technologies but rather serve as adjunct methods to enhance older technologies. Because the isolation of pathogens from blood serves multiple clinical roles—prognosis, guiding therapy, monitoring response to therapy, and epidemiology—any approach to the laboratory detection of bacteremia and fungemia must be able to fulfill each of these roles. It is unlikely that any new technology will replace blood cultures entirely in the foreseeable future. What already is happening is that newer technologies are being integrated with blood cultures into an algorithmic approach that takes advantage of the benefits of each method.

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Systems for Identification of Bacteria and Fungi*

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4

Traditionally, the identification of bacteria and fungi has been based on conventional tube-based biochemical reactions, with results compared to historical charts of expected biochemical reactions. Due to the need for faster, simpler methods, manual biochemical-based testing kits and instrument-based semiautomated or automated methods were introduced. Automation in microbiology first occurred in the early 1970s with the introduction of semiautomated blood culture instruments (see chapter 3), followed by instrumented systems for identification and antimicrobial susceptibility testing of bacteria. More rapid semiautomated and automated systems for antimicrobial identification and susceptibility testing followed, relying on microorganisms' biochemical characteristics, fatty acid patterns, and/or other metabolic properties for their identification. Commercially available biochemical platforms may include decision support software integrating identification and susceptibility test results with surveillance strategies for antimicrobial resistance and guidelines for therapy. A recently introduced automated technique for microorganism identification relies on proteomic analysis of bacterial or fungal cells using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Nucleic acid-based approaches, typically used for bacterial and fungal identification when biochemical and/or proteomic strategies fail, include broad-range DNA target sequencing or array-based approaches and organism-specific amplification techniques. Whether a laboratory uses manual or automated, biochemical-, protein- or DNA-based methods, the scientific approach to identify microorganisms relies on the same fundamental principles. This chapter provides an overview of technologies used for the identification of bacteria and fungi recovered from clinical specimens. Discussions relevant to systems for automated blood cultures (chapter 3), antimicrobial susceptibility testing (chapter 72), immunoassays (chapter 7), molecular diagnostics (chapter 6), and detection of *Mycobacterium* species (chapter 30) are found elsewhere in this *Manual*.

ORGANISM IDENTIFICATION SYSTEMS

Overview of Methods and Mechanisms of Identification

Historically, microorganisms have been identified by what we now refer to as "conventional procedures," which include reactions in tubed media and observation of physical characteristics, such as colonial morphology and odor, coupled with results of Gram staining, agglutination tests, and antimicrobial susceptibility profiles. Over the years, identification methods miniaturized commonly used biochemical reactions into a more convenient format (http://www.asmusa.org/index.php/guidelines/sentinel-guidelines) and developed into a system-dependent approach that became the industry standard. The system-dependent method relies on a set of substrates that are carefully selected for their positive and negative reactions. These patterns create metabolic profiles that are compared with established databases.

Biochemical profiles are determined by reactions of individual organisms with each of the substrates in the system. The accuracy of the reactions is dependent upon users following the directions of the manufacturer regarding inoculum preparation, inoculum density, incubation conditions, and test interpretation. Most systems rely upon one or a combination of several indicators. These include (i) pH changes resulting from utilization of a substrate, (ii) enzymatic reactions that release a chromogenic or fluorogenic compound, (iii) tetrazolium-based indicators of metabolic activity in the presence of a variety of carbon sources, (iv) detection of volatile or nonvolatile compounds, and (v) recognition of visible growth (Table 1). Additional biochemical tests for microbial identification that use other means of detecting a positive response for a given substrate may also be included.

Although no formal definition of "rapid" exists for describing the time required for results to be generated, most microbiologists expect rapid systems to provide usable results within 4 h. Clearly, the generation times of microbes (typically 30 min or longer) will not allow growth-dependent methods to generate detectable biochemical responses within this time. To overcome the problem of generation times, manufacturers of rapid systems use novel substrates with which preformed enzymes, produced by the organisms to be tested, may react to elicit responses detectable within 1 to 4 h.

Proteomic analysis using MALDI-TOF MS, which enables identification of bacteria and fungi in a matter of

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*This chapter contains information presented by Caroline Mohr O'Hara, Melvin P. Weinstein, and J. Michael Miller in chapter 14 of the 8th edition of this *Manual*, by Melvin P. Weinstein and Karen C. Carroll in chapter 15 of the 9th edition of this *Manual*, and by Cathy A. Petti, Melvin P. Weinstein, and Karen C. Carroll in chapter 3 of the 10th edition of this *Manual*.

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TABLE 1 Technologies for microorganism identification

System reactivity	Need for growth	Analyte	Indicator(s) of positive result	Example(s) of system
pH-based reactions (mostly 15–24 h)	Yes	Carbohydrate utilization	Color change due to pH indicator; carbohydrate utilization = acid pH; protein utilization or release of nitrogen-containing products = alkaline pH	API panels, Crystal panels, Vitek cards, MicroScan conventional panels, Phoenix panels, Sensititre panels
Enzyme profile (mostly 2–4 h)	No	Preformed enzymes	Color change due to chromogen or fluorogen release when colorless complex is hydrolyzed by an appropriate enzyme	MicroScan rapid panels, IDS panels, Crystal panels, Vitek cards, Phoenix panels, Sensititre panels
Carbon source utilization	Yes	Organic products	Color change as a result of metabolic activity transferring electrons to colorless tetrazolium-labeled carbon sources and converting the dye to purple	Biolog
Volatile or nonvolatile compound detection	Yes	Cellular fatty acids	Chromatographic tracing based on detection of end products, which are then compared to a library of known patterns	MIDI
Visual detection of growth	Yes	Various substrates	Turbidity due to growth of organism in the presence of a substrate	API 20C AUX panels
DNA target sequencing	No	Nucleic acid	Electropherogram or raw sequence of nucleotide bases	Laboratory developed; MicroSeq, GenBank, RDP, RIDOM, SmartGene
PCR/ESI MS	No	Nucleic acid	Patterns of mass signals in a spectrum	IRIDICA (Abbott)
MALDI-TOF MS	Yes	Protein	Patterns of mass signals in a spectrum	Bruker, bioMérieux, Andromas

minutes, is rapidly being adopted into clinical microbiology laboratories (2). Molecular methods that amplify particular gene targets novel enough to distinguish among genera and species and automated sequencing technology are used for identification of difficult-to-identify microorganisms. Proteomic and molecular methods have expanded our knowledge of pathogenesis and have, in some cases, resolved erroneous taxonomic classifications.

System Construction

Microbial identification systems are either manual or automated. Manual methods use the analytical skills of the technologists for reading and interpreting the tests, whereas automated systems offer a hands-off approach, providing technologists more time for other duties and providing the laboratory with increased standardization. For all systems, the backbone of accuracy is the strength and utility of the database. Databases are constructed using known, clinically relevant strains as well as type strains of most taxa. In some cases, before an organism is added to the database, it is evaluated to confirm its relationship to other strains in the same taxon by using the likelihood fraction. This compares the characteristics of the new strain to those of a typical culture of the same species. Unusual microorganisms or common microorganisms with atypical properties often cannot be reliably identified by commercial systems unless they are well-represented in the system's database.

The number of species included in a database may vary from just a few for some manual assays to thousands for automated instruments. For most commercial systems, database maintenance is a continuous process and software upgrades incorporating major taxonomic changes are provided by the manufacturer at regular intervals. Some systems may allow users to make minor changes at the local workstation.

System identifications are supported by algorithm-based decision making that is generally available through a computer. Occasionally, these identifications are compiled into a preprinted index, which is used to manually convert the organism's profile number into identification. Bayes's theorem, or modifications of it, is often the basis of algorithm construction from data matrices.

Bayes's theorem is one of the statistical methods that manufacturers of biochemical identification systems use to arrive at an identification of a certain taxon based on the biochemical reaction profile produced by the unknown clinical isolate (3). Bayes's theorem considers two important issues in order to arrive at an accurate conclusion: (i) $P\ (t_i/R)$ is the probability that an organism exhibiting test pattern R belongs to taxon t_i, and (ii) $P\ (R/t_i)$ is the probability that members of taxon t_i will exhibit test pattern R. Before testing, we make the assumption that an unknown isolate has an equal chance of being any taxon and that each test used to identify the isolate is independent of all other tests. In this case, Bayes's theorem can be written as

$$P(t_i/R) = \frac{P(R/t_i)}{\sum_i P(R/t_i)}$$

By observing reference identification charts derived by conventional biochemical tests, we know the expected pattern of the population of taxon t_i (e.g., *Escherichia coli* is indole positive and citrate negative). R in the formula is the test pattern composed of R_1, R_2, . . . R_n, where R_1 is the result for test 1 and R_2 is the result for test 2, etc., for a given taxon. We can then incorporate the percentages (likelihoods that t_i will exhibit R_1, etc.) into Bayes's theorem to arrive at an accurate taxon.

Clinical microbiologists must not, however, become dependent upon these likelihoods and percentages when interpretive judgment would suggest an alternative taxonomic conclusion. Bacteria often tend to stretch the rules of nomenclature when isolated from clinical specimens, and they may not react as expected in a commercial system, even though a legitimate result is produced (e.g., lactose-positive *Salmonella* species or H_2S-positive *Escherichia coli*). The result from the most reliable systems can be misleading. In these cases, an alternative method of identification must be used. D'Amato et al. have described how the systems use the database profiles and probability matrices to arrive at an identification of an unknown taxon (4).

The manufacturers of commercial identification systems rely heavily on input from their customers. Laboratories are encouraged to communicate with product manufacturers about problems, such as unusual organism identifications that develop when a method or system is being used. Manufacturers depend on customer satisfaction, and most are willing to assist in problem solving or in projects that could add strength to their systems. These companies, like their users, are clearly interested in the highest quality of cost-effective patient care.

CRITERIA FOR SELECTING INSTRUMENTED SYSTEMS

When selecting a method for identification and antimicrobial susceptibility testing, the laboratorian must consider several important issues. Laboratory supervisors and managers should make such major decisions carefully and with expert consultation. The process begins by answering key questions about the needs for a new system in the context of laboratory versus patient benefits.

Once these questions are answered, the next step is to begin the search for the right instrument or system to meet the needs of the laboratory and the medical staff. As a general rule, it is best not to be the first to purchase a new system without having seen in the peer-reviewed literature the results of evaluations performed by reputable clinical laboratories. The manufacturer's representative can be asked to supply peer-reviewed articles about the ability of the system to correctly identify the range of isolates usually seen in the user's laboratory. This phase requires demonstrations and conversations regarding space requirements, technical applications, manufacturer issues such as interface capabilities and service contracts, and personnel-related concerns such as sample preparation and throughput.

It is often helpful to visit other laboratories similar to one's own that are using the system under consideration to ask if they like the system, whether they would buy it again, how much downtime they have experienced, whether the service from the manufacturer has been acceptable, and whether the system has been mechanically reliable.

The laboratory should select a system that has been fully evaluated and whose accuracy exceeds 90% in its overall ability to identify common and uncommon bacteria normally seen in that particular hospital or laboratory. The system should be able to identify commonly isolated organisms with at least 95% accuracy compared with conventional methods.

The accuracy of antimicrobial susceptibility testing for combination panels is as important as the accuracy of identification, perhaps more so. Chapter 72 of this *Manual* discusses the issues involved in instrument susceptibility test methods. Finally, with increasing use of automation in clinical microbiology laboratories, it is important to understand how automated identification systems fit in with other automated systems in the laboratory and with the laboratory information system (see chapter 5 of this *Manual*).

EVALUATING AN INSTRUMENT OR SYSTEM

Validating a New Instrument or Method

Several references provide useful information on the approach to evaluation, verification, and validation of kits, assays, and instruments in the clinical laboratory (5–9). When an identification system is added to the laboratory, laboratories must demonstrate that the system performs as described by the manufacturer (10, 11). Published reports by other laboratories that have evaluated the system in a sound, scientific manner provide the first level of evidence of acceptable performance (9). Next, the purchasing laboratory must provide evidence of acceptable performance of the new identification instrument by in-laboratory verification. Verification involves documentation of test accuracy in the laboratory where the instrument will be used (6). The Clinical Laboratory Improvement Amendments of 1988 (12) specify the conditions for systems placed into service.

Although smaller laboratories may have fewer resources than larger laboratories for verification of the accuracy of an identification system, laboratory size has no bearing on the need to ensure the accuracy of laboratory identification methods and of the work performed by a laboratory in support of patient care. The role of verification by the purchasing laboratory ensures that personnel can use the system at performance levels of accuracy already documented by the manufacturer and published in the literature. The laboratorian should expect a level of 95% agreement with the existing system or reference method and accept, in the final analysis, no less than 90% agreement. This takes into account the fact that the new system may be more accurate than the old one.

As of 1998, the Food and Drug Administration (FDA) no longer performs premarket [510(K)] evaluations to "clear" automated or manual phenotypic identification systems, nor does it receive or approve quality control protocols from these devices to meet the 1988 Clinical Laboratory Improvement Amendment requirements. Laboratorians must be aware that the identification component of the new or modified system that they are using is not cleared by the FDA because this approval is no longer required. This makes it even more important for laboratorians to search the literature for valid evaluations of their chosen instrument and to conduct their own in-house validation to make sure that the instrument meets the claims of the manufacturer regarding identification. Devices and methods incorporating probes, nucleic acid amplification and other genetic methods, MALDI-TOF MS, and the antimicrobial susceptibility test component of commercial instruments will continue to be reviewed by the FDA for clearance.

LIMITATIONS OF MICROORGANISM IDENTIFICATION SYSTEMS

The databases of microbial identification systems must be revised frequently to accommodate newly named species. For example, had *Cronobacter sakazakii* (the yellow-pigmented variant of *Enterobacter cloacae*) not been added to the databases of these instruments, the clinical correlation of *C. sakazakii* with neonatal meningitis would likely be obscured if only *E. cloacae* had been reported. Laboratorians must be aware that the accuracy of a system is limited to the claims of the manufacturer for the version of the database currently in the instrument and that the database may be outdated.

The laboratory procedure manual must stipulate the action to be taken when a result is questionable either because of the unusual biochemical profile of the organism or because of the appearance of an unexpected susceptibility profile. A backup method must be used to achieve an accurate identification profile. Otherwise the isolate should be sent to a reference laboratory for analysis.

Closely related species may be difficult or impossible to distinguish using certain systems; however, the inability to accurately identify all species within a genus does not always have a negative effect on patient outcome. For example, correct identification of all of the newly recognized *Citrobacter* species may not be possible for some of the systems. In this case, the effect on patient outcome because of the inability of a system to recognize *Citrobacter werkmanii* may be negligible, and a simple report of "*Citrobacter* species" may provide adequate data for patient management. Laboratorians should recognize when they might be handling a potential agent of bioterrorism; when such pathogens are suspected, laboratories should follow Laboratory Response Network protocols and not place these organisms on automated instruments (see chapter 14, "Biothreat Agents"). Users of automated systems should be aware of the limitations of commercial products with respect to their biopreparedness plans and substitute other tests for presumptive diagnosis per recommended guidelines (http://www.asmusa.org/index.php/guidelines/sentinel-guidelines). Microorganisms suspected of being biothreat pathogens should be referred to a public health or reference laboratory for definitive identification. Likewise, organisms that are potentially hazardous to laboratory personnel (e.g., *Brucella* species) should not be placed on automated systems unless this can be done in such a fashion as to eliminate any risk of disease acquisition to laboratorians.

As pathogens continue to evolve and taxonomic classifications are revised, laboratorians must pay attention to the manufacturer's communications about products, such as letters, notices, or test exclusions regarding the accuracy of their methods, as well as the published literature describing the potential problems encountered by others using these identification systems. Likewise, the user has a responsibility to report continued problems with a system or product where poor performance may lead to adverse patient outcomes.

PHENOTYPIC IDENTIFICATION SYSTEMS

Automated Instruments (Vitek, Phoenix, MicroScan, TREK)

The last five decades have witnessed an evolution of sophistication in automated organism identification and antimicrobial susceptibility testing systems. There are several systems available, and this section will highlight their features with respect to organism identification. Chapter 72 will discuss their antimicrobial susceptibility testing performance. The interested reader is encouraged to seek out the latest information provided by the manufacturer, as companies are constantly updating their products and, with the advent of MALDI-TOF MS, will be enhancing expert systems to link identification by proteomics with updated and expanded susceptibility testing panels.

The first automated identification system to become available for clinical laboratories more than 40 years ago was the Vitek system (bioMérieux, Inc., Durham, NC). This system was developed by NASA to test astronauts for unusual organisms acquired during the burgeoning space expeditions. The current versions, called the Vitek 2 systems, consist of a personal computer (PC), reader/incubator, and smart carrier station. The smart carrier station is a sample preparation module that standardizes the inoculum and identifies the specimen through a barcode label prior to loading the cassettes. There are several available systems depending upon the desired instrument footprint and volume of testing needed (Table 2). Depending upon the size, the instruments can accommodate 15, 30, 60, or 120 cards (Vitek 2XL). Two reader/incubator instruments can be connected to one computer. The PC has a bidirectional interface capability with the laboratory information system (www.biomerieux-usa.com).

The Vitek system has cards for the identification of anaerobes and coryneforms; yeast; *Neisseria* species, *Haemophilus* species, and other fastidious organisms; Gram-positive organisms; and Gram-negative pathogens (*Enterobacteriaceae*, non-*Enterobacteriaceae*, and highly pathogenic organisms such as *Brucella* and *Francisella* species) (Table 2).

The system also contains an advanced expert system that matches 2,000 organism phenotypes with 100 resistance mechanisms. Organisms for which the phenotype and MIC values match are flagged green and do not require user verification. Isolates that flag yellow (inconsistent results), red (unknown phenotype), or purple (phenotype is not in the database) require user review and problem resolution (www.biomerieux-usa.com).

The Vitek 2 system, as well as the other automated systems, continues to be reviewed in the literature as new features are added and software versions are updated. New publications revisit these systems in the context of comparisons to novel molecular array platforms and MALDI-TOF MS. A few recent papers have compared the performance of several platforms; this information is useful for laboratories seeking to purchase one of these systems (13–17).

Siemens Healthcare Diagnostics, Inc. (Deerfield, IL) manufactures the MicroScan WalkAway system. The available instruments include the WalkAway-40 *plus* and the WalkAway-96 *plus*, which accommodate 40 and 96 panels, respectively. The autoSCAN-4 system is a small instrument designed for low-volume laboratories; it accommodates and reads one panel at a time within several seconds. The instruments have front panel controls, and the systems include a PC keyboard, monitor, and LabPro software. The MicroScan uses system-wide bar code authentication of panels to minimize potential errors from manual labeling and/or keystroke entry when placing panels into the instrument (www.siemens.com/diagnostics).

There are a variety of MicroScan panels—conventional, rapid, specialty, and Synergies *plus*—which are available in over 60 configurations. The conventional panels contain traditional biochemicals for identification and broth microdilution MICs and can be read visually. The rapid panels provide organism identifications as early as 2 to 2.5 h and broth microdilution susceptibility results between 4.5 and 16 to 18 h. Results are finalized within 18 h. Synergies *plus* panels also provide identification within 2 to 2.5 h, and broth microdilution resistance results are flagged when ready. Specialty panels are available for identification of yeast, anaerobes, fastidious Gram-negative rods, and streptococci (www.siemens.com/diagnostics). Conventional panels contain modified biochemical and chromogenic tests for the identification of a broad range of species (Table 2) (18). Rapid panels utilize fluorogenic substrates or fluorogenic indicators to detect pH changes following substrate

TABLE 2 Features of automated systems for identification of bacteria and yeasts[a]

Manufacturer	Instruments	Principle(s)	Panels[b]	Organisms in database (no. of taxa)	No. of tests	Incubation (h)	Software/expert systems
bioMérieux	Vitek 2 XL Vitek 2 60 Vitek 2 Compact 60 Vitek 2 Compact 30 Vitek 2 Compact 15	Colorimetric carbon source utilization; enzymatic activity; resistance	GP	119	43	8	Advanced Expert System, Observa
			GN	143			
			NH	26			
			ANC	61			
			Yeast	52			
Siemens	MicroScan WalkAway *plus*	Overnight panels turbidimetric detection of carbon source utilization; enzymatic activity Rapid panels use fluorometric detection of preformed enzymes	GN Convent.	116	34	2.5	LabPro
			GN Rapid	139	36	16–18	
			GN Synergies	139			
			GP Convent.	51	27		
			GP Rapid	53	36		
			GP Synergies	53			
			Yeast	42	27		
			Anaerobe	54	25		
			NH	20	18		
BD Diagnostics	BD Phoenix	Colorimetric and fluorometric detection	GP	140	48	8–16	BDXpert, BD EpiCenter
			GN	161			
			Streptococcus	27			
			Yeast ID	64			
TREK Diagnostic Systems	ARIS	Fluorometric detection	GP	41	32	5–18	SWIN
			GN	137	31		
Biolog	OmniLog	Carbon source utilization detection by reduction of tetrazolium violet	GP GN	2,500	95	4–24[c]	GEN III
MIDI, Inc.	Sherlock microbial identification system	Cell wall fatty acid analysis using gas chromatography	GP GN Yeast	1,500	NA	24[c]	CLIN 50 database

[a] Derived from: http://www.bd.com/ds/productCenter/IS.asp; www.biomerieux-usa.com; www.siemens.com/diagnostics; www.biolog.com; http://www.midi-inc.com/; http://www.trekds.com/products/sensititre/c_standardid.asp.

[b] GP, Gram positive; GN, Gram negative; NH, *Neisseria*, *Haemophilus*; ANC, anaerobe; Convent., conventional; ID, identification; NA, not applicable.

[c] Overnight growth on particular media is required; assay takes about 2 h to perform.

utilization and the production of specific metabolic products (19). Similar to other systems, identification is based on detection of substrate utilization, pH changes, and growth in the presence of certain antimicrobial agents. Depending upon the panel used, results may be available from 2 to 42 h after incubation at 35°C (18, 19). Results can be read manually, which is useful for resolving aberrant reactions and for smaller facilities that do not have automation. Some of the panels can be stored for up to 1 year at room temperature.

The company offers a device called the PROMPT system for inoculating panels in place of having to create a 0.5 McFarland standard. The user touches three well-isolated colonies that are as large as the unique wand tip. The wand is then placed into a small bottle containing 30 ml of aqueous Pluronic-D solution. After mixing well by shaking, the suspension is poured into a seed tray for inoculation into a MicroScan panel. There is also the RENOK rehydrating inoculator that can be used to simultaneously inoculate all 96 wells of any available MicroScan panel from the seed tray created by the PROMPT device (www.siemens.com/diagnostics).

The LabPro with Alert$_{EX}$ contains a database of predefined rules based upon Clinical and Laboratory Standards Institute (CLSI) recommendations (20). The user can customize the rules as needed to match formularies and infection control alerts and for other uses. The software automatically notifies the user when an isolate requires attention. This may include the need for supplemental testing, a quality control issue, or another actionable item. The epidemiology management feature can provide information to infection control and pharmacy. Antibiogram tools are based on the CLSI M39 document (18) (www.siemens.com/diagnostics).

The BD Phoenix Automated Microbiology System (BD Diagnostics, Sparks, MD) became available in Europe in 2001 and in the United States in 2004. Currently, the complete system consists of the Phoenix instrument, numerous test panels, the AP instrument for panel inoculation (introduced in 2008), and the BD EpiCenter system for data management (http://www.bd.com/ds/productCenter/IS.asp). The Phoenix instrument can test up to 99 test panels and one control panel simultaneously. There are numerous panels available for Gram-positive and Gram-negative pathogen identification (ID) and/or antimicrobial susceptibility testing (AST); a yeast identification panel was introduced in 2011. The combined ID and AST panels consist of 136 wells divided into 51 wells on the ID side and 85 wells on the AST side. Organisms are identified on the basis of 48 individual biochemical tests using a combination of fluorometric and colorimetric detection of the various substrate reactions. Identification of Gram-negative and Gram-positive organisms requires 2 to 12 h, whereas yeast identification requires 4 to 15 h. Once results are available by the Phoenix instrument, the BDXpert system software manages the interpretation of the results. Users can finalize results directly at the Phoenix instrument, using the EpiCenter, or through the laboratory's information system.

The BD EpiCenter System is a data management software system. It has a bidirectional laboratory information system interface and tools that allow the user to create specific institutional rules for AST and other types of reporting. These tools or programs also allow the user to analyze epidemiological trends and generate reports from multiple BD instruments, not just the Phoenix instrument, including the new BD Bruker MALDI Biotyper.

One of the initial problems with the workflow using the Phoenix compared to other systems was the manual set up of the panels which resulted in a longer mean time of setup than for other instruments (e.g., 3 min for Phoenix compared to 1.5 min for Vitek 2) (21). In 2008, BD introduced the AP instrument to reduce the hands-on time required to set up Phoenix panels and to better standardize the inoculum preparation. The user places ID and AST broth for up to 5 isolates into a rack that gets loaded onto the AP system for automated standardization of the ID broth inoculation and subsequent inoculation of the AST broth and addition of the AST redox indicator. The user must manually transfer the rack to an inoculation station, scan the bar code labels, pour the ID and/or AST broths into the panel, seal them, and place them into the Phoenix instrument (http://www.bd.com/ds/productCenter/IS.asp). A study by Junkins et al. reports a reduction in hands-on time by 50% using the AP instrument (22).

Trek Diagnostic Systems (now part of Thermo Fisher Scientific) are best known for the extensive and customizable microbroth dilution panels for susceptibility testing (discussed in chapter 72). The company manufactures identification panels (Sensititre ID plates) that contain fewer substrates than the larger systems discussed above and are designed to identify the most commonly encountered aerobic Gram-negative and Gram-positive pathogens. The separate Gram-negative and Gram-positive plates contain 32 reaction wells containing fluorogenic substrates that allow for identification in as short a time as 5 h, and which can be extended to overnight incubation if needed. Each ID microtiter plate can test three separate organisms. ID panels can be read on the same instrumentation used to read the susceptibility MIC plates. In addition, laboratories can purchase an auto inoculator called the Sensititre AIM and a nephelometer to enhance inoculum standardization. The Sensititre ARIS 2X is a fully automated benchtop instrument that incubates and reads the bar-coded plates. The system has a 64-plate capacity and accommodates all TREK plates: MIC, breakpoint, and identification. Up to 192 tests per day can be run on a single instrument. The ARIS instrument is connected to a computer that contains the SWIN software, an expert system used for interpretation of the plates. In addition, the company has an epidemiology module on SWIN that allows the user to design five customizable reports for tracking workload, MIC values, and organisms of epidemiological significance. There are no recent publications evaluating the Sensititre Gram-negative identification panels. A publication by Staneck et al. comparing the Sensititre Gram-negative identification panel to API 20E and Rapid NFT using a large number of isolates demonstrated comparable performance for the more common genera of *Enterobacteriaceae* and nonenterics (23). A study from Mexico evaluated the Sensititre Gram-positive ID plate to assess its utility for identifying common staphylococci compared to API STAPH v4.1 (10). Discordant isolates were resolved by PCR and sequencing of partial sequences of the 16S rRNA, *sodA*, and *tuf* genes. The Sensititre plates correctly identified only 69% of the isolates compared to 90% by API STAPH, indicating limited utility for identification of this group of Gram-positive organisms (24).

The Biolog OmniLog ID System (Biolog, Hayward, CA), introduced in 1989, is a fully automated instrument based upon the ability of an organism to utilize or oxidize a panel of 95 carbon sources. The 96-well microtiter plate uses reduction of tetrazolium violet that is incorporated into each substrate as an indication of utilization of the carbon source (25). The "carbon fingerprint" is analyzed by the software of the instrument and compared to an extensive database (GEN III) of over 2,500 species of aerobic and anaerobic bacteria, yeasts, and fungi (26). Similarity indices are used to identify the test organism as follows: similarity index of <0.5, no identification; similarity index of 0.50 to 0.75, good identification; similarity index of >0.75, excellent identification (25). A variety of configurations, levels of automation, and identification databases are available (26). The fully automated GEN III OmniLog ID system has the capacity to incubate and monitor up to 50 Biolog MicroPlates (26). A requirement for testing is subculture of isolates to be tested to Biolog universal growth agar (25). The Biolog system has been evaluated in the literature over the last two decades (25, 27, 28). The most recent publication evaluated the accuracy of the system for identification of "atypical" clinical isolates, that is, isolates not routinely included in routine identification databases such as certain aerobic actinomycetes, *Bacillus* species, and fastidious Gram-negative rods (25). In this study of 159 bacterial isolates, the OmniLog system was compared to 16S rRNA gene sequencing and an extensive panel of biochemical assays; compared to conventional methods, the overall accuracy of the Biolog system was 68.3% (25). The best performance was seen with the aerobic actinomycetes (100 versus 74% accuracy with 16S rRNA gene sequencing), while the performance for fastidious Gram-negative rods was poor (20 versus 100%) (25). At least one study has also demonstrated variable performance of the Biolog system for identification of Gram-positive cocci (27).

The Sherlock microbial identification system (MIDI, Inc., Newark, DE) has been available since 1991 and spun out of a partnership between Hewlett-Packard Co. (now Agilent Technologies) and the University of Delaware's Plant Pathology Department (www.midi-inc.com). The system can identify a broad range of bacterial pathogens (approximately 1,500 species) using gas chromatographic analysis of cellular fatty acids. The traditional method of identification requires about 1.5 to 2 h perform. Organisms to be tested are subcultured to specific media and incubated for 24 h, and then a defined biomass is transferred to tubes for fatty acid extraction before gas chromatography is performed (29). The Sherlock software identifies organisms using qualitative and quantitative pattern matching of fatty acid methyl esters (29). A more recently developed rapid system for aerobes and anaerobes called Q-FAME requires less biomass and less time (approximately 24 min), but it is not FDA cleared. The Sherlock system is used as a reference method in food microbiology, by environmental laboratories, and in some clinical laboratories. Some users have expanded the manufacturer's library by incorporating their own strains over time, and this has been shown to enhance identification and differentiation of genera such as *Corynebacterium* (30). In 2005, MIDI received FDA clearance for its aerobic bioterrorism library (http://www.midi-inc.com/).

The BIOMIC V3 is a digital imaging system that automates the reading and interpretation of disk diffusion tests and the results of other commercial manual identification and AST kits or tests. This system requires off-line incubation after which the user manually places the plate or test onto the instrument. The BIOMIC is programmed to read API panels, RapID tests (Remel), and Crystal panels (Becton Dickinson). It also automates the reading of Etest MICs and broth microdilution panels (www.biomic.com).

Manual Kits, Assays, and Nonautomated Platforms

An array of kits, assays, and nonautomated platforms are available for identification of a variety of common and unusual pathogens. In many circumstances, these are used to supplement automated systems for identification of fastidious organisms that may fail to adequately grow in the panels of those systems or as a backup for unusual or failed results. These assays work on the principles of turbidity due to growth of organism in the presence of a substrate. Table 3 contains the list of many of the more commonly used assays stratified by the organism groups that they identify. It is recommended that the interested reader contact manufacturers for the most up to date information regarding availability and performance.

PHENOTYPE-INDEPENDENT METHODS FOR IDENTIFICATION

Phenotypic microbial identification can be limiting and is often not rapid. MALDI-TOF MS is being adopted into clinical microbiology laboratories as a rapid, cost-effective method for identification of a wide range of bacteria and fungi. For challenging organisms, nucleic acid-based methods may still be required, including broad-range DNA target sequencing or arrays and organism-specific detection techniques. Amplified DNA sequence-based methods do not necessarily require optimal growth or even a viable microorganism, enable data exchange between laboratories, and may help define taxonomic relationships between microorganisms.

PROTEOMIC IDENTIFICATION SYSTEMS: MALDI-TOF MS (BASED IN PART ON REFERENCE 31)

MALDI-TOF MS provides rapid, inexpensive identification of bacterial and fungal colonies (32). MALDI stands for *m*atrix which *a*ssists in *d*esorption and *i*onization of highly abundant bacterial and fungal proteins through energy from a *l*aser (2). Although protein extraction may be performed, the most user friendly approach is to test colonies directly by moving whole cells from a bacterial or fungal colony (using a plastic or wooden stick, loop, or pipette tip) to a "spot" on a MALDI-TOF MS target plate (a disposable or reusable plate with test spots) (Fig. 1). Spots are overlaid with matrix (or first with a formic acid solution, which is allowed to dry, and then with matrix) (33–36) and dried, and the target plate is placed into a mass spectrometer (Fig. 2). The matrix (e.g., α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile and 2.5% trifluoroacetic acid) isolates bacterial or fungal molecules from one another, protecting them from fragmentation and enabling their desorption by laser energy; the majority of the laser energy is absorbed by the matrix, converting it to an ionized state. As a result of random collision in the gas phase, charge is transferred from matrix to microbial molecules; ionized microbial molecules are then accelerated through a positively charged electrostatic field into a *t*ime *o*f *f*light, or TOF, tube. Inside the tube, which is under vacuum, the ions travel toward an ion detector, with small analytes traveling the fastest, followed by increasingly larger analytes; a mass spectrum is produced, representing the number of ions of a given mass impacting the detector over time. It is highly abundant (predominantly ribosomal) proteins which generate the mass spectrum. Although they are not individually characterized, together they provide a profile unique to individual types of microorganisms, with peaks specific to genera and species. Computer software compares the generated mass spectrum to a database of reference spectra, generating a list of the most closely related organisms with numeric rankings. Depending on how high the value (percent or score) of the top match is (and considering the next best matches), the organism is identified at the family, genus, or species level.

Commercial MALDI-TOF MS systems are available from bioMérieux, Inc. (Durham, NC) and Bruker Daltonics, Inc. (Billerica, MA). Another system, called Andromas (Paris, France), is primarily used in France and will not be further discussed. AnagnosTec (Zossen, Germany) marketed a microbial database called Spectral Archiving and Microbial Identification System (SARAMIS) used with Shimadzu's AXIMA Assurance mass spectrometer (Shimadzu, Columbia, MD), which bioMérieux acquired in 2010 and changed the name to VITEK MS RUO; bioMérieux then developed a new database, software, and algorithms called VITEK MS IVD (including a prerelease version of the VITEK MS v1.1 database, the v1 system/v1.1 database, the v2.0 system/v2.0 database and algorithms, and the v3.0 system/v3.0 database and algorithms). The FDA-cleared platform is called Vitek MS. VITEK MS Plus incorporates the VITEK MS and SARAMIS v4.0 databases. The Bruker Biotyper system includes a mass spectrometer, software, and library. The FDA-cleared platform is called the MALDI Biotyper CA System. At the time of this writing, there are more published studies using the Bruker than the bioMérieux system. The systems differ in databases, identification algorithms, and instrumentation. Numeric rankings, reported on different scales, are not directly comparable. Bruker's Microflex LT mass spectrometer is a desktop instrument, whereas bioMérieux's is a larger floor model.

Rapidly implemented progressive improvements in both systems render it difficult to compare studies because of diverse specimen preparations, organism test sets (i.e., enriched for unusual organisms or not), reference identification methods, mass spectrometers, software, interpretive guidelines, and databases. In general, the technology performs at least as well as, if not better than, automated biochemical systems for identification of common bacteria and yeast (Table 4) and better for many unusual organisms (37). Usually, organisms are either correctly identified or yield a low score/percent, indicating that identification has not been achieved; the latter typically implies no "match" in the database but can occur due to technically poor preparation. Misidentifications are unusual but occur with closely related organisms; *Escherichia coli* and *Shigella* species are notably not well-differentiated by MALDI-TOF MS.

Richter et al. performed a multicenter study comparing VITEK MS v2.0 to 16S rRNA gene sequencing (with supplemental phenotypic testing as needed) for identification

TABLE 3 Summary of nonautomated identification systems for bacterial identification available in 2013[a]

Assay	Manufacturer	Organisms targeted	Storage temp(°C)	No. oftests	Incubation
Systems for anaerobe identification					
API 20A	bioMérieux	Anaerobes	2–8	21	24–48 h, anaerobic
BBL Crystal Anaerobe	BD	Anaerobes	2–8	29	4 h, aerobic
RapID ANA II	Thermo Scientific (Remel)	Anaerobes	2–8	18	4–6 h, aerobic
Rapid ID 32A	bioMérieux	Anaerobes	2–8	29	4 h, aerobic
Systems for *Enterobacteriaceae* and other Gram-negative bacilli					
API 20E	bioMérieux	*Enterobacteriaceae* and nonfermenting Gram-negative bacteria	2–8	21	18–24 h, aerobic
API Rapid 20 E	bioMérieux	*Enterobacteriaceae*	2–8	20	4 h
API 20 NE	bioMérieux	Non-*Enterobacteriaceae*	2–8	20	24–48 h, aerobic
API NH	bioMérieux	*Neisseria* species, *Haemophilus* species, *Moraxella catarrhalis*	2–8	12	2 h, aerobic
BBL Crystal Enteric/ Nonfermenter ID	BD Diagnostics	*Enterobacteriaceae*, some Gram-negative nonfermenters	2–25	30	18–20 h
BBL Crystal Neisseria/ Haemophilus ID kit	BD Diagnostics	*Neisseria* species, *Haemophilus* species, other fastidious organisms	2–8	29	4 h
BBL Enterotube II	BD Diagnostics	*Enterobacteriaceae*, oxidase-negative Gram-negative rods	2–8	15	18–24 h
Microbact 12A, 12E[b]	Oxoid	*Enterobacteriaceae*, miscellaneous Gram-negative rods	2–8	12	18–48 h
Microbact 12B[b]	Oxoid	Supplements 12A for identification of other Gram-negative rods	2–8	12	24–48 h
Microbact 24E[b]	Oxoid	*Enterobacteriaceae* and other Gram-negative rods	2–8	24	24–48 h
RapID NF Plus	Thermo Scientific (Remel)	Nonfermenting and selected fermenting Gram-negative rods	2–8	17	4 h
RapID NH Plus	Thermo Scientific (Remel)	*Neisseria*, *Haemophilus*, *Moraxella*	2–8	13	4 h
RapID ONE	Thermo Scientific (Remel)	*Enterobacteriaceae*	2–8	19	4 h
Systems for identification of Gram-positive cocci					
API Staph	bioMérieux	Staphylococci and micrococci	2–8	20	18–24 h
RAPIDEC Staph	bioMérieux	Staphylococci	2–8	4	2 h, aerobic
API 20 Strep	bioMérieux	Streptococci and enterococci	2–8	20	4–24 h, aerobic
BBL Crystal Gram-positive ID kit	BD Diagnostics	Gram-positive organisms	2–8	29	18–24 h
BBL Crystal rapid Gram-positive ID kit	BD Diagnostics	Gram-positive organisms	2–8	29	4 h
RapID Staph Plus	Thermo Scientific (Remel)	Staphylococcal species	2–8	18	4 h
RapID STR	Thermo Scientific (Remel)	Streptococci and other Gram-positive cocci	2–8	14	4 h
Systems for identification of Gram-positive bacilli					
API Coryne	bioMérieux	*Corynebacterium* and corynebacteria-like organisms	2–8	20	24 h, aerobic
RapID CB Plus	Thermo Scientific (Remel)	Coryneform bacilli (40 taxa)	2–8	18	4 h

[a] Compiled from: http://vgdusa.com/bbl-crystal-identification.htm; http://www.oxoid.com; thermoscientific.com/oxoid; http://www.biomerieux-usa.com/.
[b] The 12A and 12B are in a strip format; the 12E and 24E have microplate formats.

of 965 *Enterobacteriaceae* isolates representing 17 genera and 40 species (38). MALDI-TOF MS results agreed with the reference methods for 96.7% of isolates, with 83.8% correctly identified to the species level, 12.8% limited to genus-level identification, and 1.7% yielding no identification. Seven isolates had wrong genus identification, including three *Pantoea agglomerans* isolates misidentified as *Enterobacter* species and single isolates of *Enterobacter cancerogenus*, *Escherichia hermannii*, *Hafnia alvei*, and *Raoultella ornithinolytica* misidentified as *Klebsiella oxytoca*, *Citrobacter koseri*, *Obesumbacterium proteus*, and *Enterobacter aerogenes*, respectively. Eight isolates were misidentified at the species level. In general, the *Enterobacter cloacae* complex is accurately identified using MALDI-TOF MS, although some species within the complex may not be discriminated from one another (39, 40). Mass spectra of *Raoultella planticola*, *Raoultella ornithinolytica*, and *Klebsiella oxytoca* may be similar (41). MALDI-TOF MS can identify *Campylobacter* species, *Arcobacter butzleri*, *Yersinia enterocolitica* (42), and *Aeromonas* species (43) and differentiate *Capnocytophaga canimorsus*

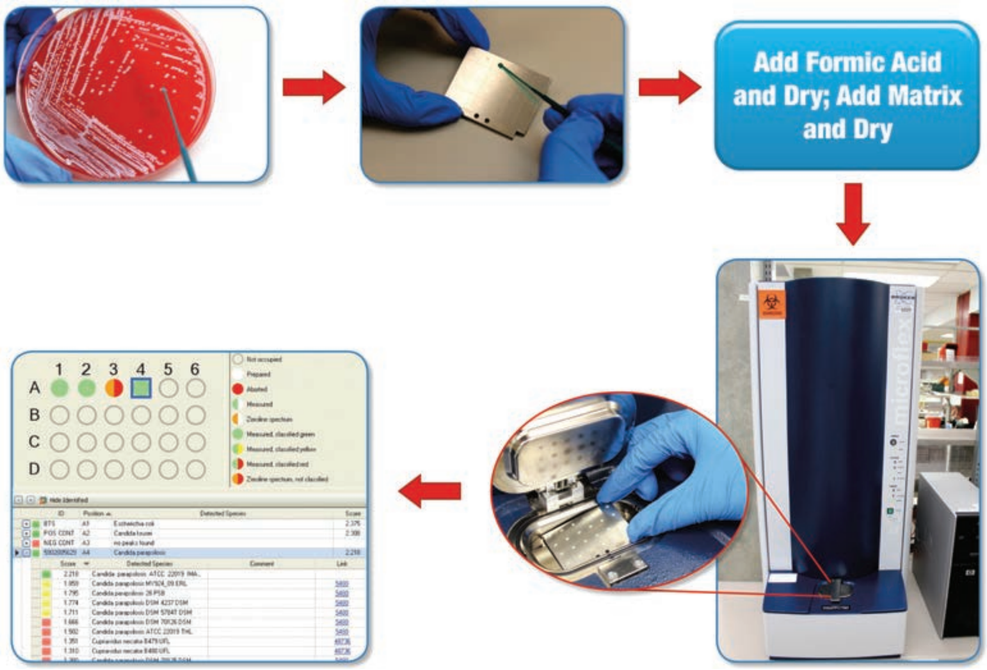

FIGURE 1 MALDI-TOF MS workflow (from reference 2). A colony from a culture plate is placed on a "spot" on a MALDI-TOF MS target plate (a reusable or disposable plate with a number of test spots). One or many isolates is tested at a time. In this example, the Bruker Biotyper system is shown. Cells are treated with formic acid on the target plate. Following drying, the matrix is added (34). After drying of the matrix, the plate is placed into the mass spectrometer for analysis (Fig. 2). A mass spectrum is generated and compared by the system's software against a database of mass spectra, resulting in identification of the organism (*Candida parapsilosis* in position A4 in the example). Reproduced by permission of Mayo Foundation for Medical Education and Research. All rights reserved. doi:10.1128/9781555817381.ch4.f1

from *Capnocytophaga cynodegmi* (44). *Salmonella enterica* subsp. *enterica* serovar Typhi may be distinguished from other *S. enterica* serovars, although typing within the genus *Salmonella* is generally not possible (45). It may be possible to identify *Burkholderia cepacia* complex members, including *Burkholderia cenocepacia*, *Burkholderia cepacia*, *Burkholderia stabilis*, and *Burkholderia vietnamiensis* (46). With database enhancement, HACEK organisms (*Haemophilus*, *Actinobacillus*/*Aggregatibacter*, *Cardiobacterium*, *Eikenella*, *Kingella*; 47) and *Legionella* species (48) can be identified. *Francisella tularensis* and *Brucella* species may be accurately identified; however, the Bruker Biotyper library does not contain and will not identify these organisms. Use of Bruker's "Security Relevant" database enables their identification (49).

Rychert et al. reported findings from a multicenter study evaluating the Vitek MS v2.0 system for the identification of 1,146 isolates of aerobic Gram-positive bacteria (50). For 92.8%, a single accurate, species-level identification was provided. With MALDI-TOF MS, overall identification of staphylococci (51), β-hemolytic streptococci (52), aerococci (53), and enterococci is excellent. Some α-hemolytic streptococci are problematic. *S. mitis* and *S. oralis* may be poorly differentiated from *S. pneumoniae*, at least with the Bruker system (54, 55); the VITEK MS system may overcome this limitation (56, 57). While MALDI-TOF MS reliably identifies viridans group streptococci to the species group level, it may not be able to discriminate some closely related species (58). Other streptococci, such as *S. canis*, *S. dysgalactiae*, and *S. pyogenes* and *S. infantarius*, *S. equinus*, and *S. lutetiensis* may not be well differentiated from one another (33). *Arcanobacterium haemolyticum* and *Rhodococcus equi* can be reliably identified, as can all but select closely related *Corynebacterium* species (using lower score cutoffs than recommended by the manufacturer) (59).

MALDI-TOF MS can be used to identify many clinically relevant anaerobic bacteria (60, 61). Jamal et al. reported species-level identification of 89 and 100% of 274 routinely isolated anaerobic bacteria (enriched in *Bacteroides fragilis*) using the Biotyper DB Update–V3.3 and the VITEK MS v1 system/v1.1 database, respectively (62). Identification of more esoteric anaerobes, including *Prevotella* species, has been successful in 83% of cases with user supplementation of Bruker's Reference Library 3.2.1.0 (63). Schmitt et al. evaluated a diverse collection of 253 clinical anaerobic isolates using the Bruker system and a user-supplemented database; 92 and 71% of isolates were correctly identified to the genus and species levels, respectively (64). Barreau et al. tested 1,325 anaerobic isolates using the Bruker system and correctly identified 92.5% to the species level (using lower score cutoffs than recommended by the manufacturer) (61). Garner et al. evaluated 651 anaerobic bacterial isolates using the VITEK MS v2.0 system and reported correct species-level identification in 91.2% (65).

A small number of studies have reported using MALDI-TOF MS for mycobacteria, an endeavor which requires special processing to kill tested bacteria (for safety), disrupt clumped cells, and break down cell envelopes. Current commercial libraries inadequately address *Mycobacterium*

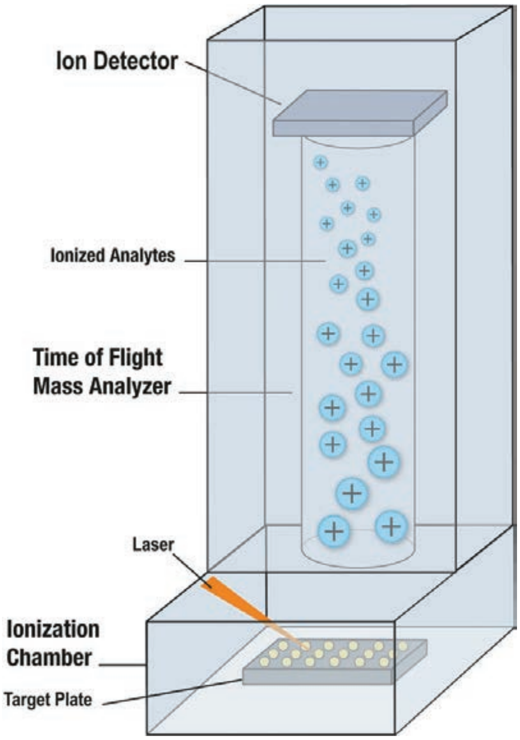

FIGURE 2 MALDI-TOF MS (from reference 2). The target plate is placed into the mass spectrometer. Spots to be analyzed are shot by a laser, desorbing microbial and matrix molecules from the target plate. Charge is transferred from matrix to microbial molecules, and the ionized molecules are accelerated through a positively charged electrostatic field into the mass analyzer, a tube under vacuum. The ions travel toward an ion detector with the smallest analytes traveling fastest, followed by progressively larger analytes. As ions emerge from the mass analyzer, they run into the ion detector, thereby generating a mass spectrum representing the number of ions hitting the detector over time. Although separation is by mass-to-charge ratio, since the charge is typically single for the described application, separation is by molecular weight. Reproduced by permission of Mayo Foundation for Medical Education and Research. All rights reserved.
doi:10.1128/9781555817381.ch4.f2

species, but with appropriate library construction, MALDI-TOF MS should be able to identify most clinically-relevant species, with a few caveats. Members of the *Mycobacterium tuberculosis* complex will likely be identifiable at the complex level only (66), and some species (e.g., *Mycobacterium abscessus* and *Mycobacterium massiliense*; *Mycobacterium mucogenicum* and *Mycobacterium phocaicum*; and *Mycobacterium chimaera* and *Mycobacterium intracellulare*) may not be well differentiated from one another (66). With enhanced databases, *Nocardia* species (67) may be identified, but as with *Mycobacterium* species, specific extraction procedures may be required.

MALDI-TOF MS can identify yeast (68), outperforming some conventional phenotypic systems, and distinguishing *Candida dubliniensis* and *Candida albicans*; *Candida rugosa* and *Candida pararugosa*; *Candida norvegensis*, *Candida krusei*, and *Candida inconspicua*; *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* (69); and (with database supplementation) *Cryptococcus neoformans* and *Cryptococcus gattii* (70–72). Dhiman et al. evaluated the Bruker system for identification of 138 common and 103 unusual yeast isolates and reported 96 and 85% accurate species-level identification, respectively (73). Although older studies used preparatory extraction for yeasts, we and others have used a direct colony testing strategy with formic acid overlay (Fig. 1) (34, 74). Westblade et al. performed a multicenter study assessing the Vitek MS system v2.0 for identification of 852 yeast isolates, including *Candida* species, *Cryptococcus neoformans*, and other clinically relevant yeasts, using direct application to a target plate followed by a formic acid overlay; 96.6% were identified to the genus level and 96.1% to the species level (74). MALDI-TOF MS may outperform current systems for esoteric species, such as *Candida famata* (69).

Filamentous fungi exhibit variable phenotypes and protein spectra may vary with growth conditions and with the zone of fungal mycelium analyzed; few are represented in current commercial databases. De Carolis et al. developed a library of *Aspergillus* species, *Fusarium* species, and *Mucorales* using the Biotyper system and identified 97% of 94 isolates to the species level (75). Using the VITEK MS v1 system/v1.1 database and direct on-plate testing, Iriart et al. identified 82% of 44 *Aspergillus* isolates (including all isolates with species in the database) (76). With appropriate database building, dermatophytes can be identified (77, 78). Preliminary studies indicate that *Pseudallescheria*/*Scedosporium* complex species are identifiable (79). Lau et al. used a special fungal extraction procedure and their own mass spectral database comprising 294 isolates representing 76 genera and 152 species to test 421 mold isolates; they achieved accurate species- and genus-level identifications with 88.9% and 4.3% of isolates, respectively (80).

Turnaround time for MALDI-TOF MS is 3 or fewer minutes per isolate. Compared to standard methods, turnaround time for bacterial and fungal identification is shorter by an average of 1.45 days (81), and since only a small amount of organism is required, testing can be performed on single colonies on primary culture plates without subculture. MALDI-TOF MS has a low reagent cost (81), and compared to conventional phenotypic identification and sequencing, reduces costs by 5- and 96-fold, respectively (37). An estimated 87% of isolates may be identified on the first day (compared with 9% with standard techniques), with final identifications several days earlier for biochemically inert, fastidious, or slow-growing organisms (81). DNA sequencing expenses can be avoided for some esoteric organisms, waste disposal decreased, and tests for screening for certain enteric pathogens (e.g., triple sugar iron agar for *Salmonella* species) and quality control and laboratory technologist training/labor for replaced/retired tests eliminated (82, 83).

MALDI-TOF MS has several limitations. Unlike publicly available sequence databases such as GenBank, MALDI-TOF MS databases are proprietary. Although low identification percentages for some organisms may be improved by user addition of mass spectral entries of underrepresented species or strains (to cover intraspecies variability), or even readdition of reference strain spectra to the database, doing so may be beyond the capability of some laboratories. Because of low scores/percentages, repeat testing may be required for ~10% of isolates (81). Growth on some media may be associated with low scores/percentages (84), and small or mucoid colonies may fail identification (40, 85). Refined criteria may be needed to distinguish closely related species and differentiate them from the next best taxon match (86). For certain species of organisms, genus- or

TABLE 4 Evaluations of MALDI-TOF MS for routine bacterial identification (2)

System	Isolates			% Identification to level:		Country	Comparator	Reference
	No.	Type	Period of isolate collection	Genus	Species			
Bruker Biotyper	1,013	Bacteria	2 mo	99	97	France	Phoenix, API, Biochemical	94
Bruker Biotyper	468	Bacteria	3 mo	97	92	Japan	MicroScan, API, Phoenix	95
Bruker Biotyper	2,781	Bacteria	1 mo	96	85	Australia	VITEK2, API, Biochemical	96
Vitek MS[a]	767	Bacteria	6 wk	95	87	France	VITEK2	97
Bruker Biotyper	986	Bacteria	3 mo	96	93	Belgium	Bruker Biotyper compared to Vitek MS	1
Vitek MS[b]				94	93			

[a] v1 system/v1.1 database.
[b] Prerelease version of v1.1 database.

species-specific (including lowered) cutoffs may be appropriate (51, 59, 87). Errors may occur, including colony inoculation in erroneous target plate locations, testing impure colonies, smearing between spots, failure to clean target plates, and wrong result entry into laboratory information systems. There is a learning curve to applying ideal colony amounts to target plates (40). Although results are generally reproducible, sources of variability include the mass spectrometer, matrix and solvent composition, technologists, culture conditions, and biological variability; quality control strategies are incompletely developed. Instrument (e.g., laser) and software failure may occur.

GENOTYPIC IDENTIFICATION SYSTEMS

DNA Target Sequencing

DNA target sequencing may be performed on organisms growing in pure culture. For a comprehensive review, refer to articles and guidelines that specifically address this topic (88, 89). The selection of DNA targets to identify bacteria and fungi relies on the concept that some genes have conserved segments flanking variable regions. Conserved regions of gene targets are locations where PCR and DNA sequencing primers anneal. Variable regions have unique nucleotide sequences, enabling sequence-based identification of a particular genus and species. The gene target most commonly used for bacterial identification is the 16S rRNA gene (16S ribosomal DNA), an ~1,500 bp gene that encodes a portion of the 30S ribosomal subunit. Partial (500 bp) 16S rRNA gene sequencing is commonly used for sequence-based identification of Gram-negative and Gram-positive bacteria, anaerobic bacteria, and mycobacteria (88). For genera with high conservation of the partially sequenced 16S rRNA gene, full-length 16S rRNA gene sequencing or sequencing of an alternative DNA target may be useful. Examples of alternative DNA targets for sequence-based bacterial identification include *rpoB* (the β-subunit of bacterial RNA polymerase), *tuf* (elongation factor Tu), *gyrA* or *gyrB* (gyrase A or B), *sodA* (manganese-dependent superoxide dismutase), and heat shock proteins. Although alternative targets may provide better discrimination between species than the 16S rRNA gene, because they are less conserved, genus- or group-specific PCR and/or DNA sequencing primers may be required, and there may be less available sequence data than exists for the 16S rRNA gene. Potential targets for identification of yeasts and medically relevant molds include the internal transcribed spacer regions ITS1 and ITS2, which are variable regions located between conserved genes encoding 18S, 5.8S, and 28S rRNAs, and the D1-D2 region of 28S rRNA.

To identify the microorganism, its DNA sequence is compared to reference sequences found in public (e.g., GenBank) and/or private (e.g., MicroSeq [Applied Biosystems, Calrsbad, CA], SmartGene [Lausanne, Switzerland]) databases. After comparing the query and reference sequences, the number of nucleotide mismatches between the query and reference sequences is used to determine relatedness, and the final result is reported as percent identity. The acceptable percent identity to identify a microorganism to the genus or species level is variable and depends on the DNA target and microorganism. 16S rRNA genes are multicopy targets in most bacteria, and variations in sequence amongst 16S rRNA genes in single organisms can result in difficultly interpreting sequence data. A computer program called RipSeq Mixed (iSentio, Bergen, Norway) can be used to computationally decatenate underlying sequences in such cases. Nucleotide databases must be carefully evaluated for accuracy, quality of sequence data, frequencies of database updates, software, cost, and breadth of nucleotide entries. The Clinical and Laboratory Standards Institute published a comprehensive consensus document for identifying microorganisms to the genus and species levels by DNA target sequencing; this document can serve as a useful guide for laboratorians who wish to pursue or have already implemented DNA target sequencing (88). In the future, whole-genome sequencing will become more routine for clinical laboratories, providing information about the diversity of species, their virulence properties, epidemiology, and antimicrobial resistance mechanisms, and disease causation.

PCR Electrospray Ionization Mass Spectrometry

Another technique to characterize bacteria is the application of electrospray ionization-MS to analyze PCR products (PCR/ESI-MS) (90–92). Rather than measuring the mass of bacterial proteins, masses of amplified DNA from broad-range (e.g., rRNA) and specific genes are measured. The masses of amplified DNA are measured with sufficient accuracy to enable unambiguous calculation of the nucleotide compositions of the amplified DNA. Typically, a number of PCR assays are performed and analyzed as a panel. By considering which PCR assays yield amplification products, along with their nucleotide compositions, the identity of the microorganism may be defined. This is conceptually similar to broad-range PCR followed by sequencing, except that here MS provides the base composition without knowing the order of the nucleotides. Analyzing multiple target sites on the microbial genome can compensate the lower information content of nucleotide composition over sequence. Simner et al. recently evaluated the PLEX-ID Broad Fungal assay (Abbott Laboratories, Abbott Park, IL) for identification of 91 characterized fungal isolates; 95.6% and 81.3% were identified to the genus and species levels, respectively (93). The advantage of PCR/ESI-MS over sequencing is that it is fast and has a high throughput; however, it is currently quite costly. PCR/ESI-MS is being developed as IRIDICA by Abbott (Abbott Park, IL).

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Automation and Design of the Clinical Microbiology Laboratory

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5

Clinical microbiology laboratories (CML) are central to the operations of any health care system and take many different forms depending on the type of system they serve. There is no one right way to design a laboratory because appropriate design will be dictated by the space available, staffing requirements, services provided, test volume, and many other factors. Despite institution-to-institution differences in laboratory design, a few elements should be common to all laboratories.

First, the laboratory should be a safe environment for employees and visitors. The microbiology laboratory can be a dangerous environment, as the diagnosis of infectious diseases often requires that pathogens be propagated to high concentrations and processed for identification. Laboratory design should be optimized to ensure that the diagnostic process can take place as efficiently and safely as possible. Guidance for safely operating and designing a laboratory can come from many resources. The Centers for Disease Control first published the *Biosafety in Microbiological and Biomedical Laboratories* in 1984. Although the information provided in this document is advisory in nature, legislation and regulations have in some cases made compliance with this document mandatory (1). At baseline, all CMLs must meet biosafety level 2 criteria and, depending on the services rendered and pathogens encountered, may need to meet biosafety level 3 criteria (1).

Second, laboratories should be designed to efficiently handle specimens from initial processing to final result without contaminating the specimen or culture. The efficiency with which a specimen can be processed is an increasing challenge as the centralized laboratory model becomes more common. In these models, a central laboratory provides testing for external institutions that ship specimens to a single location. This model helps to reduce redundancy of resources but at the possible expense of testing efficiency. The time and manner of transport within a hospital and within a system should be considered when designing a laboratory so as to optimize turnaround time (TAT) and reduce contamination.

Third, laboratories should be designed so that their configuration can be flexible and accommodate emerging technologies and changing demands.

The goal of this chapter is to provide information regarding the proper design of a laboratory, bearing in mind that each institution requires something different from its laboratory. Microbiology is rapidly changing and so too are the spatial and geographical demands placed on facilities. The chapter is presented in two sections. The first will focus on laboratory design and workflow. This section will focus on some key elements of laboratory design, including the geography of a laboratory (i.e., where testing is performed), preanalytical considerations, staffing strategies, workflow, process improvement, and clinical impact. The second will address a new and important subject for clinical microbiologists, laboratory automation. Some historical perspective will be provided along with a discussion of currently available options for laboratory automation and considerations for implementation.

GEOGRAPHY OF THE LABORATORY

Almost every clinical service is a customer of the laboratory. It is therefore critical that the laboratory be well connected, both electronically and physically, to the facilities for which it provides testing. A wide variety of laboratory information systems (LISs) and electronic medical records can be found among institutions. These systems are now a vital component of laboratory testing and will likely become even more important as digital microbiology and total laboratory automation (TLA) become more common. A review of these numerous systems is beyond the scope of this chapter. The Association for Pathology Informatics provides a toolkit which can be used to assess currently available LISs (http://www.pathologyinformatics.org/toolkit).

In any health care setting, but especially teaching hospitals, convenient access to the laboratory is an important factor that encourages physicians and trainees to interact with the hospital directly. It is difficult to quantify the clinical benefit of this interaction, but most microbiologists and infectious disease practitioners agree that being able to readily access the laboratory for personal consultation is of great benefit.

Clearly, there can be real advantages to locating a laboratory within the hospital it serves. However, there can be significant benefits to the centralized model as well. The primary advantage is one of scale. Centralizing a laboratory service, while cumbersome from the perspective of specimen transport, simplifies spatial, technological, and staffing needs. By centralizing services, laboratories can focus all of their resources in a single area, thus minimizing the

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significant costs of maintaining redundant laboratory infrastructure in external laboratories. Furthermore, in an age where more laboratorians are retiring than entering the work force, highly skilled expertise can be hard to find (2). Where expertise is sparse, centralization allows for many laboratories to take advantage of the skills of the very few. For example, in the United States, parasitic disease is rare. The diagnostic methodology of choice for most parasitic gastrointestinal disease (other than *Cryptosporidium* and *Giardia* spp.) is a microscopic ovum and parasite exam. This testing is time-consuming and difficult to maintain proficiency due to low prevalence. Many laboratories have realized they can no longer justify performing this testing in-house because they simply don't have the expertise. As a result, it is estimated that nearly 60% of laboratories in the United States currently send their ovum and parasite exams to a reference laboratory (3).

LOCATION OF TESTING

Traditional models of laboratory structure, where the analyte, rather than the test method, dictated the location of a given test, are now being challenged. There are many examples of infectious disease testing that now occur in the clinical chemistry lab or point-of-care tests that are performed in the Emergency Department rather than in the CML. As diagnostic methods are simplified, it is possible that home testing will become more common.

INFECTIOUS DISEASE TESTING IN THE CHEMISTRY AND CORE LABORATORIES

Technology continues to advance and simplify the performance of testing. Many tests that had previously existed in one section of the lab can now be moved to the location of greatest convenience. A good example of this phenomenon is the simplification of molecular testing. Instruments like the GeneXpert (Cepheid, Sunnydale, CA) and the FilmArray (Biofire Diagnostics, Inc., Salt Lake City, UT) have reduced molecular biology to simple specimen manipulation. It is no longer necessary to have skilled molecular biologists performing these tests. This allows institutions to consider relocating testing to a section of the laboratory that may not have previously been an option. Some microbiology laboratories are not staffed 24 h/day, 7 days/week (24/7), and in those scenarios, these "walk-away" molecular tests could be moved to a section of the laboratory that could handle them around the clock if necessary.

Deciding on the location of point-of-care testing (POCT) can be a contentious and controversial process. Traditionally, microbiologists have resisted requests to allow nonlaboratorians (such as Emergency Department providers) to perform POCT on the grounds that testing personnel will not adhere to the rigorous standards of protocol and quality control (QC) utilized by trained laboratory staff. In addition, it is sometimes the case that nonlaboratory departments do not fully understand the complexities of performing even a technically uncomplicated test. Tasks such as proper ordering, resulting, competency and proficiency testing, and inventory management must be considered when determining whether nonlaboratory departments should perform POCT. In cases where this is allowed, a rigorous system to monitor the quality of testing should be put in place. In addition to these practical matters, regulatory considerations may also dictate who can perform tests and where testing can be performed.

One advance that has greatly improved the ability to perform high-quality POCT outside the laboratory is instruments that autoverify results. In some cases, these instruments can be programmed for QC lockout, automated result interpretation, and autoverification of results. These instruments aid busy individuals in reading results at the appropriate time and automatically sending the information to the information management system. Lastly, the QC lockout is a function that forces proper QC testing by locking the instrument until QC is performed and passed.

PREANALYTICAL MICROBIOLOGY

A critical aspect of laboratory operations is the interaction with the client. Interactions include ordering, specimen tracking, and result reporting, all of which should be delivered electronically. The most obvious interaction with a laboratory involves specimen transport. There are three main forms of specimen transport utilized: manual, pneumatic tube, and robotic. Hospitals with internal laboratories commonly rely on a combination of all three transport modalities, although for most specimens, pneumatic tubes are often preferred. Transportation is a much bigger challenge for the centralized laboratory model. This applies both to commercial reference laboratories and to hospital systems which serve a network of external clients. Efficient transport of specimens is imperative to providing reliable and accurate results. With labile pathogens present in specimens that constitute less-than-ideal environments for maintaining organism viability, minimizing specimen transport time is critical. Although there is surprisingly little literature addressing the importance of specimen transport time, Table 2 in chapter 18 of this *Manual* provides guidance on the matter. Table 2 recommends that specimens such as cerebrospinal fluid, blood culture bottles, abscess material, and body fluids all be transported (collection to processing) in less than 2 h (4). In scenarios where specimens must be transported to distant laboratories, whether across town or across the country, it would seem unlikely that these recommendations could be met. In light of this fact, some laboratory systems have elected to inoculate and preincubate select, high-priority cultures (such as cerebrospinal fluid) so they can be transported at regular intervals rather than on a STAT basis. Organism viability is one reason that minimizing transport time is critical. A secondary reason is simply that results can be provided faster when specimens are transported efficiently.

STAFFING MODELS

The most important resources in a CML are the personnel that perform testing. It is well documented that there is a shortage of skilled medical technologists entering the field. It is estimated that 39% of United States laboratories have budgeted openings, with 57% of those openings being for medical technologists (MT) or clinical laboratory scientists and 14% for medical laboratory technicians (MLT) (2). It is also estimated that laboratories must hire approximately 12,000 new employees annually to keep up with test volume growth. However, training programs are disappearing and only about 5,000 medical technologists enter the field each year. As a result, institutions utilize a number of different staffing models to optimize the effectiveness of their workforce.

Most laboratories break down their workday into three 8-h shifts which generally include a daytime, early evening, and night shift. Another approach is the "7 on, 7 off" or

"8 on, 6 off" schedule in which employees work 7 or 8 straight days of 10-h shifts and then get 7 or 6 days off, respectively. It is not uncommon for laboratories to use a combination of 8- and 10-h shifts to cover the workload.

In many laboratories, the majority of staff are deployed to the first shift where most of the high-complexity testing is conducted. Second and third shift staff in this model may help to complete daytime work but will in many cases be responsible for performing only Gram stain interpretation and STAT laboratory functions.

An alternative approach to the traditional model is 24/7 staffing. In these laboratories, staffing is evenly distributed throughout the day and cultures are read continuously. The idea for this model was born out of lean/Six Sigma analyses which observed that some cultures were ready to be read during evening and night shifts. It followed that the TAT was unnecessarily delayed when these cultures weren't attended to until the following morning. In the 24-h staffing model, cultures are first read approximately 16 to 20 h after inoculation. Benches can then be subdivided by time of reading rather than by patient last name or by specimen source.

There is a paucity of peer-reviewed literature addressing the effectiveness of this approach. However, at least one lab has shared its experience with 24-h culture reads and shown that TATs are improved by almost 24 h for positive cultures (5). Some other benefits from this experience appear to be that work is more evenly distributed throughout the day. As a result, the morning shift staff do not get overwhelmed by cultures that have accumulated overnight. Lastly, there may also be some financial benefit in both staffing and supply expenses (J. Campos, personal communication).

There are some significant challenges to implementing 24/7 culture reading. It may be difficult to find experienced technologists to work on evening and night shifts. Employee turnover is typically higher on these shifts and may make it difficult to maintain trained personnel. Likewise, these shifts are generally unsupervised and without medical direction. Laboratories will want to ensure that they have the same resources for troubleshooting and guidance as are available during day shifts. Another consideration is whether the laboratory will be over-functioning by moving to 24/7 culture reading. Will results released during off-shifts be acted upon? Lastly, change management when moving to 24/7 microbiology will pose a significant challenge. Laboratories may experience resistance when moving from traditional to 24/7 microbiology.

STAFF TRAINING LEVEL

Laboratory staff can have various levels of education and experience which dictate the tasks they can perform. Becoming an MT (also referred to as a clinical laboratory scientist, biomedical scientist, or medical laboratory scientist) generally requires a bachelor's degree in medical technology or in a science such as biology or chemistry. MT programs are usually offered through a university and involve hospital setting internships, and some states may require that MTs pass an exam to be licensed. MTs are qualified to perform complex testing and analyses, including direct specimen microscopy, culture interpretation, organism identification, and susceptibility testing, to perform high-complexity molecular testing, and to supervise others. MTs are expected to understand the underlying scientific principles of laboratory testing as well as the causes of disease and the importance of test selection (6).

An MLT is defined as someone who has a working comprehension of technical and procedural aspects of laboratory tests. The technician correlates tests with disease processes, understands basic physiology, and recognizes abnormal test results (6). The tasks that can be performed by MLTs will vary by institution but generally include quality assurance monitoring, computer applications, and instrumentation troubleshooting and require an understanding of specimen collection and processing. The American Society of Clinical Pathologists states that a technician may make technical decisions related to testing but should be supervised by a technologist, supervisor, or laboratory director. MLTs usually have an associate degree from a community or junior college or a vocational school. MLTs may become MTs through additional education and experience.

Medical laboratory assistants are individuals who receive on-the-job training and may have specialized education, but that is not required. The primary task of the medical laboratory assistant is specimen processing and using preanalytical systems. These professionals must have in-depth knowledge of specimen acceptability.

WORKFLOW

Batch Versus Immediate Testing

The organization of workflow and the selection and extent of batching procedures depend on several factors. Specimens that have direct diagnostic or therapeutic consequences can be batched only to a limited extent. When analytical process automation is involved to a high degree, specimens can be combined in larger series. The effects on TAT must be determined and accordingly taken into consideration. It should also be determined when the findings should be made available to the clinician. When the above conditions are met, the ideal size of a batch can be determined on the basis of the number of specimens, specimen type, time allotted for transport, distribution of specimens after arrival at the laboratory, desired tests, and selected laboratory equipment. The effects of an order entry system or the various options of digital transmission of the findings must also be taken into account.

The use of matrix-assisted laser desorption–ionization time of flight (mass spectrometry) (MALDI-TOF [MS]) and molecular methods for organism identification can shorten the TAT (7). Identification using MALDI-TOF (MS) is possible with only a single colony from the primary culture, and fewer subcultures are needed to obtain a pure culture. It thus becomes possible to identify bacteria 1 to 2 workdays earlier than with current phenotypic methods (8, 9). The TAT can be further shortened by changing the reading intervals for the culture plates. Instead of reading the plates only at a fixed time, traditionally in the morning, they could also be read at regular intervals in the afternoon and/or evening. This is particularly true for specimens arriving at the laboratory in the afternoon or evening. Batching the specimens according to the above reading modalities and deploying staff as described above make it possible to maximize laboratory efficiency.

Introduction of digital image processing into diagnostic microbiology offers new opportunities for organizing workflows and shortening TAT. Reading plates digitally results in earlier recognition of colony growth. Consequently, subsequent identification and susceptibility testing can be accelerated. The pictures of the plates are taken at individually defined intervals and times for further processing. The computer can be supportive in preselection of culture-negative plates. The plates showing growth are sent through further diagnostic procedures. Imaging technology can detect even the smallest of colonies, which are difficult for the human eye to differentiate. Chromogenic media for diagnosing multidrug-resistant organisms such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and multidrug-resistant *Escherichia coli*, *Acinetobacter* spp., or *Salmonella* spp. are particularly suitable for imaging technology, since results of the cultures can be documented earlier. Adapting work hours to conform to the above procedures should be considered. Incubation and digital image processing can also be applied to manually inoculated plates. The benefits of automation can be put to full use by batching the specimens for the process described.

The rapid development of easy-to-use molecular diagnostic test systems for detecting pathogens like methicillin-resistant *S. aureus*, *Clostridium difficile*, enterovirus, and respiratory viruses has led to progressively smaller batch sizes. Batching specimens for such tests would thwart the advantages of these systems (e.g., time savings and easy handling).

Role of Process Improvement

Economic pressure and new approaches in the domain of laboratory automation, as described below, open up opportunities for reconsideration and possible revision of the entire laboratory process.

Potential areas for improvement range from analyzing and developing solutions for the current laboratory situation, concerning workflow, staff utilization, and certain selected methods, up to introducing entirely new laboratory concepts and equipment. Changes in these areas can lead to increased capacity and flexibility within the laboratory and enable it to deal with unforeseen or planned increased numbers of specimens for processing. Another reason for evaluating the entire laboratory organization is to compensate for a dwindling laboratory work force. For instance, in Germany, the number of graduates from schools for medical technologists decreased from 2,273 between 1994 and 1995 to 1,471 between 2009 and 2010 (10).

The following steps should be taken to achieve sustained optimization. First, a list of laboratory processes should be compiled. Then an implementation plan should be drawn up. The plan should describe the individual steps: the necessary resources, organization, productivity, target TAT, and ways to deal with additional workload. Sustainability of the new process is achieved by regularly and closely tracking the implemented changes and by monitoring them to determine whether or not objectives have been achieved.

The entire process should be preceded by a comprehensive informatory phase: visits to other laboratories and exchanges with colleagues. One can also seek the advice of external consulting firms. Manufacturers of medical equipment for diagnostic purposes also offer concepts. These firms provide a baseline evaluation and analysis of the laboratory's equipment in its entirety, including the work organization and the work processes, the stock of devices, and the computer systems and their usage. The proposed solutions generally lead to a greater degree of automation, which, along with potentially recommended middle software, provide better coordination of the devices and processes. Various methods for process analysis and optimization are being used in this context. The methods have been adopted from the automobile and shipping industries and are based on presentations from lean management and Six Sigma.

The lean management method originates from the automobile industry and was first used by Toyota. The aim was to identify all of the characteristics that enhance the value of the company and increase customer satisfaction. At the same time, superfluous activities and processes were to be discontinued. Lean management as it pertains to health care can be described in four components: (i) methods for analyzing processes, and thereby identifying and analyzing problems; (ii) methods for designing processes more effectively and efficiently; (iii) methods for better detecting errors, implementing solutions, and preventing damaging effects; and (iv) methods for managing these changes and problems and for finding solutions using a scientific approach (11).

A practical example is the implementation of lean management using the concept of the five Ss: exclusively, very necessary materials are allowed in the work area (**s**orting), the entire laboratory area is straightened up and each piece of working equipment is assigned its own designated place (**s**traightening), the area is cleaned systematically on a regular basis (**s**weeping), the processes in the laboratory are standardized (**s**tandardizing), and sustainability is achieved through regular evaluation and analysis of these processes (**s**ustaining).

The aim of the Six Sigma method is to reduce process variance and simultaneously reduce errors and deviations in the analysis to a minimum. This is achieved through implementation of control mechanisms that link workers, work processes, quality requirements, responsibilities, and costs. The method was developed in the 1970s in Japan for the shipbuilding industry and is used today in many branches of industry worldwide. Six Sigma is based on statistical methods for quality improvement aimed to increase organization success and customer satisfaction. The most frequently used Six Sigma method is the so-called DMAIC cycle (define, measure, analyze, improve, and control). Using DMAIC, processes become measured variables, which can be improved in a sustainable manner. The sigma value describes how often an error is expected to occur. The best sigma value in use is 6; this signifies that fewer than 3.4 defects or errors per million opportunities (DPMO: defects per million opportunities) are expected to occur. A sigma value of 1 for laboratory processes would signify that 691,462 defects are expected to occur per million analyses. A laboratory implementing Six Sigma was able to increase its sigma value from 3.9 (7,210 DPMO) to 4.5 (1,387 DPMO) (12).

The methods described above are used in various combinations to improve the laboratory organization as part of the optimization process and the implementation of high-grade automation. There are a number of publications on this subject that describe how this process can be applied successfully in many sectors of health care (11, 13–19). Rutledge et al. demonstrated in a clinical laboratory a decreased walk pattern for technologists by 70% and a mean TAT reduction of more than 50% for creatinine, complete blood count, urine analysis, and ionized calcium after implementing lean management and Six Sigma (13). Persoon et al. used the lean production system from Toyota to improve the preanalytic processes in a clinical chemistry laboratory (20). The median preanalytic processing time was reduced from 29 to 19 min. Overall, these process improvement systems promise to increase staff efficiency and reduce costs and errors with the ultimate goal of improving patient outcomes.

LABORATORY AUTOMATION: HISTORICAL PERSPECTIVES

The microbiology laboratory has changed very little over the past 30 years with respect to laboratory automation.

The field, though, is now poised to undergo a shift to automate what has traditionally been a very manual discipline. Despite the fact that clinical chemistry laboratories adopted TLA over 20 years ago, the concept has been slow to be accepted in microbiology (21). A number of factors have contributed to the delay in automating clinical microbiology laboratories.

First, the complexity of the microbiological specimen has hindered the development of TLA solutions. The clinical chemistry specimen is of relatively uniform volume and consistency and, perhaps most importantly, is collected in standardized containers. These factors are conducive to automation and are in sharp contrast to the specimen received in CMLs. The CML must be able to process and analyze an enormous variety of specimen types which are collected in highly variable containers.

Second, the nature of diagnosing an infectious disease is complicated by nonspecificity of clinical manifestations. The diagnostic process must therefore include testing for a large number of pathogens, and automated platforms must be able to accommodate this reality. Artificial intelligence programs may one day be able to replicate the complex interpretations required by microbiology technologists. Currently, though, culture interpretation requires human judgment and cannot be automated.

Third, the cost of automation is a significant barrier to entry into the CML. Relative to chemistry testing, microbiology specimen volumes are much smaller, thus reducing the need for automation and making for less appealing returns on investment. However, the high-volume, centralized laboratory model is becoming common and may be an attractive setting for automation. How these systems will fit into the smaller laboratory is yet to be determined.

CURRENT SYSTEMS FOR MICROBIOLOGY LAB AUTOMATION

Organism Identification and Susceptibility Testing

Microbiologists have a number of different options for performing organism identification and susceptibility testing. Susceptibility testing systems are reviewed in chapter 72 of this *Manual*. MALDI-TOF (MS) is a technology capable of providing identifications based on assessment of protein profiles and database comparison. In actuality, these systems are semiautomated, although they currently require significant manual manipulation prior to analysis. The two major platforms for MALDI-TOF (MS) organism identification are the Vitek MS (bioMérieux) and the Biotyper (Bruker Daltonic, Billerica, MA). These are discussed in more detail in chapter 4.

Molecular Automation

Traditional molecular biology is a tedious and labor-intensive process requiring experience and great care to prevent contamination. The future of molecular biology is going to look very different as manual molecular processes are being automated and consolidated into single systems. These systems are commonly referred to as being "sample-to-answer" or "walk-away." In addition to fully automated systems, a number of manufacturers offer automated nucleic acid extraction platforms. Discussing each product individually is outside the scope of this chapter.

Automated molecular testing platforms are reviewed in chapter 6 of this *Manual*.

Automated Specimen Processing

Automated inoculation of clinical specimen has been the center of research and development for several years (22). Just recently, these devices became available on the market. Automation of microbiological specimen processing promises to improve the quality of the streaking process, avoid cross-contamination, alleviate ergonomic issues, and reduce processing time and costs. It is expected that the automated streaking process will be reproducible and reliably yield isolated colonies. This will reduce the number of subcultures necessary for identification and susceptibility testing. Available devices have been scientifically evaluated in only a few cases (23–26). The systems usually process swabs in liquid transport media such as E-Swab (Copan Diagnostics, Murrieta, CA) or Sigma swab (Medical Wire, Corsham, United Kingdom). These swab systems consist either of an open-pore polyurethane foam tip and a modified Amies medium (Sigma swab) or a nylon flocked swab with Amies medium (E-Swab). These systems lead to a significantly improved transition of microorganisms from the swab to the transport medium and allow better evaluation of the microorganisms from the Gram stain (20, 25). Swabs in solid or half-solid transport media can only be processed in semiautomatic devices. The devices available are divided according to different inoculation techniques using the loop, comb applicator, or bead technique. In Table 1, the automated inoculation systems are compared regarding specimen type processed, inoculation technique, capacity (inoculated plates/hour), and additionally required disposables.

Innova

Innova (Becton Dickinson, Sparks, MD) has five specimen drawers that can accept a total of 200 specimens. A specimen drawer can only accept one type of specimen at a time. The specimens are decapped and recapped automatically and agitated. Up to 270 whole plates as well as biplates of up to six different types can be loaded simultaneously into the device. The streaking pattern can be defined according to the material and selected from a variety of streaking options. The inoculating loop is thermally sterilized. Each loop can be used for up to 15,000 inoculations. For inoculation volumes of 200 µl, a pipette is available. The inoculated plates are sorted according to the type of media into five different groups. The device is a closed system, containing air that is cleaned by a HEPA filter system.

InoqulA FA/MI

InoqulA (BD-Kiestra) FA/MI (full automation/manual interaction) can process liquid media in FA mode, and swabs or other types of specimen in MI mode. A barcode is attached to the side of the plates. In MI mode, the swab or the material to be inoculated is placed manually on the agar plate and streaking is performed with magnetic beads as in FA mode. Slides are prepared in MI mode. Processing can be carried out in MI mode or in FA mode but not in both simultaneously. In FA mode, the specimens are automatically agitated as well as decapped and recapped. A magnetic rolling bead (Fig. 1) is used for streaking, and a maximum of five whole plates or biplates can be inoculated at one time. The streaking pattern can be either selected from a variety of patterns or defined by the customer for each material. The inoculated plates can be presorted into four different cassettes for incubation. The system is equipped with a HEPA filter system.

TABLE 1 Automated inoculation systems

Instrument (manufacturer)	Specimen type	Inoculation technique	Capacity (no. of inoculated plates according to manufacturer)	Additionally required disposables
Innova (BD-Diagnostics, Sparks, MD)	Liquid-based specimen	Loop (1, 10, 30 µl)	180[a]	None; for volumes >200 µl, pipette
InoqulA FA, MI (BD-Kiestra B.V., Drachten, The Netherlands)	Liquid-based specimen (FA); swab specimen (MI)	Bead	400[a]	Pipette, bead
PREVI Isola, (bioMérieux, Marcy l'Etoile, France)	Liquid-based specimen	Comb applicator	180[b]	Pipette, comb
WASP (Copan Italia Spa, Brescia, Italy)	Liquid-based specimen	Loop (1, 10, 30 µl)	—[a]	None

[a] Capacity depends on the number of inoculated plates or biplates per specimen and chosen streaking pattern.
[b] —, capacity depends on the number of inoculated plates or biplates per specimen.

PREVI Isola

PREVI Isola (bioMérieux, Marcy l'Etoile, France) has five racks designed for various types of specimen. A rack can accept only one type of specimen container at a time. However, the device only processes liquid media. Whole plates and biplates can be loaded into five input cassettes, each of which can accept 30 plates. The specimens must be decapped and recapped manually. The type of streaking pattern is predefined by the comb applicator (Fig. 2). The inoculated plates are stored in three output cassettes (30 plates/cassette). A HEPA filter system is provided.

WASP

The WASP (walk-away specimen processor; Copan) processes various specimen types; the specimens are automatically decapped and recapped as well as agitated or centrifuged. Between 342 and 370 whole plates and biplates can be stored in up to nine-plate silos; only one plate type should be used per silo. The streaking pattern can be selected from a variety of options. The inoculating loop is thermally sterilized. The inoculum in the loop is documented per photo. An agar plate can be inoculated one-half each with two different specimens. The inoculated media are sorted according to the plate type. The plates are labeled on the side or on the base. Gram slide preparation, inoculation of enrichment broths, and an antibiotic disk dispenser for susceptibility testing are available. Cultured plates can be reloaded in the WASP for disc diffusion susceptibility testing. The WASP is equipped with a HEPA filter system.

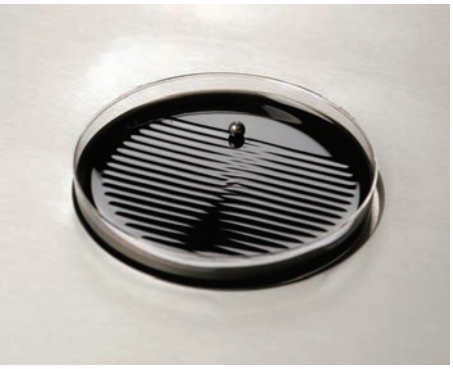

FIGURE 1 InoqulA magnetic bead.
doi:10.1128/9781555817381.ch5.f1

Total Laboratory Automation

TLA in microbiology aims to improve quality, reduce time to result, better manage an increasing number of specimens, compensate for reduction in skilled staff, and be more economically effective (27–30).

Three companies provide different solutions for TLA in microbiology: BD-Kiestra, bioMérieux, and Copan. The field is evolving quickly. More methods and devices are expected to be automated in the near future. These include automated colony picking for MALDI-TOF MS identification and preparation of dilutions for susceptibility testing.

BD-Kiestra TLA Concept

The BD-Kiestra TLA Concept is a conveyer-connected system (Fig. 3). This system comprises the following work steps: inoculation of liquid specimen as well as swabs and other nonliquid specimens using InoqulA, incubation in aerobic and CO_2 atmospheres, and digital imaging. Each incubator

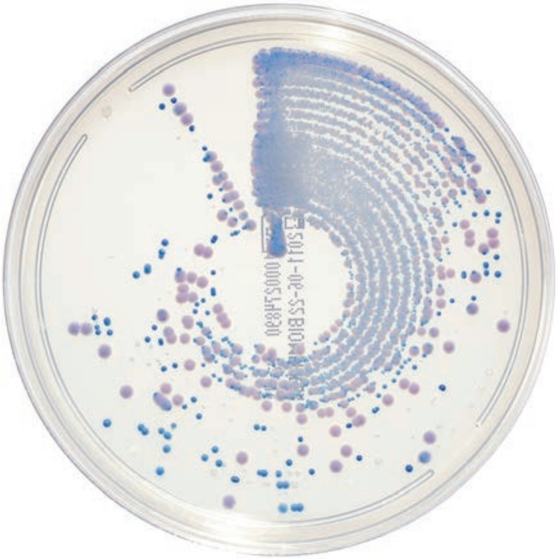

FIGURE 2 PREVI Isola-inoculated agar plate.
doi:10.1128/9781555817381.ch5.f2

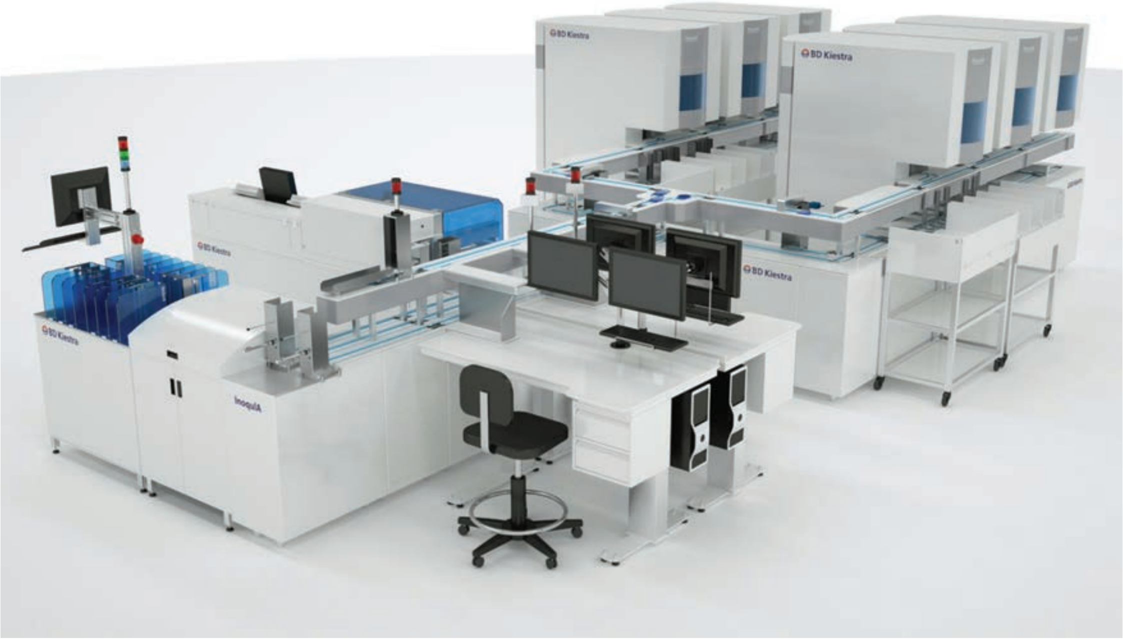

FIGURE 3 BD-Kiestra TLA with barcoding, inoculation (InoqulA) (depth, 93.5 cm; width, 417.2 cm; weight, 770 kg), and incubation (ReadA) (depth, 164.0 cm; width, 87 cm; weight, 430–730 kg). doi:10.1128/9781555817381.ch5.f3

has a capacity of 1,152 plates. The plates are stored individually in the incubator. Automated colony picking for identification with MALDI-TOF (MS), automated susceptibility tests, and expansion with molecular diagnostics equipment (BD-Max) are in the planning stage. Actual concepts are individualized with respect to the size and capacity of the installation. In this way, the laboratory can gradually approach full automation and remain open for future developments.

bioMérieux Concept FMLA

The bioMérieux Concept FMLA (full microbiology lab automation) (Fig. 4) is a modular design. The instruments are connected and regulated by the middle software Myla. This system automates the following processes: inoculation of fluid specimen on agar plates using PREVI Isola, incubation in aerobic and CO_2 atmospheres, and digital imaging in a smart incubator system. An incubator has a capacity of 1,000 plates with single-plate location. These instruments can be connected by a conveyor system. At present, measures are being taken to automate the colony picking from agar plates for identification using MALDI-TOF (MS) and susceptibility testing. Additional instruments can be connected to the system using the middle software Myla. These include blood culture systems, Gram-staining devices, Vitek 2, and others.

WASP Lab

The WASP Lab (Copan) (Fig. 5) is a modular construction and connects the individual machines through a conveyer-connected system. The system processes smears, sputum, stool, and liquid samples. It includes specimen processing with the WASP, incubation in aerobic and CO_2 atmospheres, and digital imaging. An incubator has a capacity of 800 to 1,760 plates with single-plate location. Preparation is being made to incorporate identification (using MALDI-

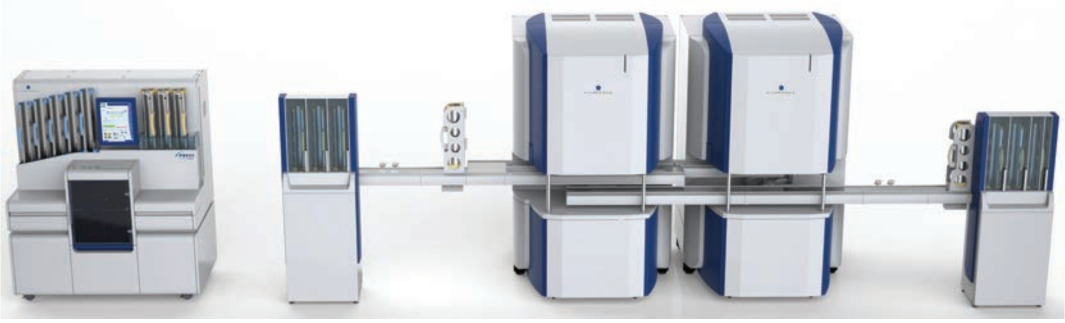

FIGURE 4 FMLA, bioMérieux, smart incubation unit (depth, 145.7 cm; width, 128.0 cm; weight, 850 to 950 kg); PREVI Isola (depth, 90.7 cm; width, 144.5 cm; weight, 298 kg). doi:10.1128/9781555817381.ch5.f4

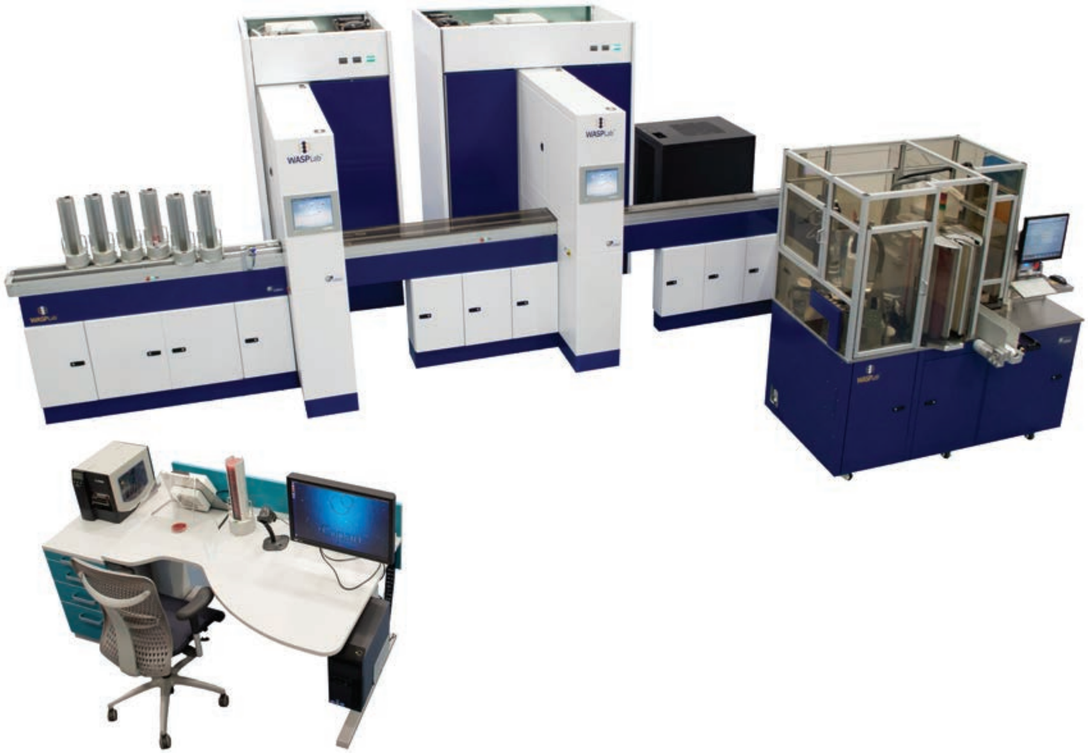

FIGURE 5 WASPLab, Copan: WASP (depth, 119.1 cm; width, 194.9 cm; weight, 700 kg); double incubator (depth, 83.0 cm; width, 174.0 cm; weight, 700 kg); imaging system (depth, 121.3 cm; width, 37.5 cm; weight, 250 kg). doi:10.1128/9781555817381.ch5.f5

TOF [MS]) and automated susceptibility testing into the WASP Lab system.

Digital Imaging

Digital imaging is one of the central components in TLA. The methods will be described collectively, since all of the manufacturers adhere to similar principles of image assessment. Digital imaging software is designed to simulate and improve the visual assessment of organism growth. Each system (BD-Kiestra, bioMérieux, and Copan) can take pictures of plates with several exposures and at various angles. With the incorporation of digital imaging and the automation of MALDI-TOF (MS) identifications, the percentage of plates needing manual processing can be greatly reduced. In each system, colonies of bacteria can be labeled for further processing on a screen. Digital processing facilitates early detection of organism growth and shortens the time of identification. Decision making can be automated for certain criteria, e.g., growth or no growth. In addition, all samples from one patient, e.g., urine, sputum, and multidrug-resistant organism screening, can be evaluated simultaneously with computer-assisted processing. Lastly, images can be archived and later used for training or for QC programs.

Limitations of the Systems

The strength of the automatic systems described lies in the processing of standardized and uncomplicated specimens. Those specimens needing special processing methods, such as organ or tissue biopsies, are not easily accommodated by such systems. However, the possibilities for automatic processing can be expanded considerably through the use of liquid-based specimen transport systems. This effectively converts a very high volume specimen, the swab, into a liquid specimen that can be easily managed with an automated specimen processor (25, 27, 31, 32).

A high level of flexibility and system compatibility is especially necessary for small laboratories. The modular nature of the automation systems described enables the laboratories to achieve various levels of automation. The combination of modular automation and process improvement, as explained, promises scalability of the current automation systems for laboratories independent of their size. For this reason, the costs of automation for small laboratories may not outweigh the benefits.

Criteria for Evaluation and Selection of an Automation System

Prior to choosing an automation system, the daily routine in the laboratory should be evaluated. Data regarding specimen volume, arrival time of specimen, and workflow must be determined to negotiate the future lab design with the manufacturers being considered. If possible, it is recommended that institutions conduct a thorough assessment of the systems installed in other institutions to determine whether or not each system is capable of fulfilling a laboratory's needs. All types of laboratory personnel should be involved in the project to incorporate their suggestions and ideas as well as to increase general support for the necessary changes. There are only a few publications addressing this issue (27, 29, 30, 33).

Factors to consider when selecting an automated system are outlined below.

Productivity

The productivity of the automation systems depends upon the number of specimens processed and the TAT for the entire analysis process. Productivity is affected by a number of factors. Specimen type and the number of plates per sample must be considered. Respiratory material, urine, wound swabs, genitourinary tract specimens, and screening swabs for multidrug-resistant organisms are processed with different types of media (34). The productivity is influenced by the choice of a streaking pattern, the number of pictures taken by digital imaging, and whether a whole plate or a biplate is inoculated.

Reliability, Stability, and Durability

Indicators showing possible malfunction of a system should be used. Contingency plans for possible failures of the system should be planned in advance. Allowance for a certain number of inspections and service procedures should be considered, allotting time for their duration. Staff to service the system should be available on weekends and during holidays.

Technical Aspects

In the beginning, do a survey of the buildings and determine the infrastructure of the rooms: calculate the required space and the weight of the machines; determine the power supply and, if necessary, the compressed-air outlets. At present, the dimensions of the devices and systems are continually decreasing in size. Future developments must also be taken into account. The possible integration of more devices, e.g., PCR equipment, should also be considered.

Software Applications

The instruments should be connected with a bidirectional interface to the LIS. It might be necessary to use middleware. Coordinating the middleware software and LIS is essential. If required, instruments from other manufacturers should be integrated.

Safety and Hygiene

The automated specimen processors should be closed and an appropriate air filter system, e.g., HEPA filter, applied. The risk of contamination must also be taken into account, and protocols for cleaning and disinfecting the incubators and conveyors should be established. Occupational health and safety regulations should be taken into account. There are no current regulations or guidelines addressing the issue of safety in laboratory automation. Of significant concern is the matter of containing exposures generated through errors in automatic incubation.

Quality Control and Scientific Aspects

The entire system should be integrated into the quality management program. The individual work steps must be monitored in compliance with the laboratory regulations: from reading the barcode on the samples and plates to inoculating and transporting the plates, digital image processing, and waste disposal. Inoculators should ensure that particles and air bubbles in the specimen are detected to avoid false-negative inoculations. Measures against transposition and cross-contamination of the samples should be ensured. Only one study evaluating cross-contamination by automated systems has been published (24). In this study, sterile and *E. coli*-inoculated Vacutainer tubes as well as E-Swab tubes were alternately loaded on the WASP. No colonies were observed from the sterile specimen.

Costs

Laboratories must take into consideration not only the amount invested in the purchase of the devices, but also the consumable supplies, e.g., pipettes, combs, or beads. It might be necessary to purchase agar plates and broths from different manufacturers. Changes in workflow can lead to alterations in the staffing requirements on different shifts and may necessitate installing a night shift if one does not currently exist. This must be taken into account when budgeting expenses. Service and repair charges, as well as the costs of the interfaces, must also be included. Possible structural changes in the laboratory should also be represented in the financial plan.

The Final Decision

The choice of the type and extent of lab automation is, of course, dependent upon the individual circumstances and the financial resources available. It has been a long time since diagnostic medical microbiology has experienced such an enormous innovative surge. It is therefore necessary to take into account future innovations in organism identification and susceptibility testing. New methods can influence procedures in the work process and drastically change the demands on capacity.

FUTURE PERSPECTIVES

The degree of automation as well as the variability of the procedures that can be automated will continue to increase. Continued development will make the systems more flexible and result in products that can better accommodate the conditions of each individual lab. Automated technology is evolving quickly. It will soon include additional testing, e.g., direct testing of blood culture systems and molecular diagnostics.

The primary objective of automating microbiology laboratories is to improve the quality and consistency of processes that suffer from high variability and are labor-intensive. The hope is that these technologies will allow the laboratory staff to concentrate on the processing of more technically demanding specimens. Software solutions for integrating POCT devices will further improve the analytic process.

Lastly, digital image processing as an integral part of bacteriological clinical diagnostics will promote further development in telemedicine. In many centralized laboratory models, sample processing and plate reading may not take place in the same location. In such situations, quality standards can still be maintained in small labs or in remote areas by offering access to experienced personnel. Telemedicine can help counter the lack of skilled personnel in these areas.

Further scientific evaluation of TLA could also facilitate an appraisal of its clinical relevance and its impact on patient care. It could reduce errors and improve the quality of diagnostic microbiology.

SUMMARY

New advances in technology as well as staffing shortages are causing microbiologists to rethink laboratory design. It may be that the traditional laboratory model as we know it today will cease to exist in the near future. TLA will certainly become commonplace in high-volume laboratories and may eventually be found in smaller laboratories. Automation is unlikely to replace medical technologists, but it will change requirements in two important ways. First of all, manual processing of the plates will be replaced by digital imaging. Second, the efficiency of automation may cause more laboratories to adopt the 24-h culture-reading strategy. Clinical microbiology is changing at a rapid pace, primarily due to a surge in technological advances. Laboratory designs will need to be more flexible to accommodate future developments.

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Molecular Microbiology*

FREDERICK S. NOLTE

6

Since the publication of the 10th edition of this *Manual*, significant changes have occurred in the practice of diagnostic molecular microbiology. Nucleic acid amplification techniques are now commonly used to diagnose and manage patients with infectious diseases. The growth in the number of Food and Drug Administration (FDA)-cleared/approved test kits and analyte-specific reagents (ASRs) has facilitated the use of this technology in the clinical laboratory. Technological advances in nucleic acid amplification techniques, automation, nucleic acid sequencing, and multiplex analysis have reinvigorated the field and created new opportunities for growth. Simple, sample-in, answer-out molecular test systems are now available that can be deployed in a variety of laboratory and clinical settings.

Molecular microbiology remains the leading area in molecular pathology in terms of both the numbers of tests performed and clinical relevance. Nucleic acid-based tests have reduced the dependency of the clinical microbiology laboratory on more traditional antigen detection and culture-based methods and created new opportunities for the laboratory to affect patient care. This chapter covers nucleic acid probes, signal and target amplification techniques, postamplification detection and analysis, clinical applications of these techniques, and the special challenges and opportunities that these techniques provide for the clinical laboratory. Applications of molecular methods used in epidemiological investigations, metagenomics, and new pathogen discovery are covered in chapters 10, 15, and 16, respectively.

NONAMPLIFIED NUCLEIC ACID PROBES

Nucleic acid probes are segments of DNA or RNA labeled with radioisotopes, enzymes, or chemiluminescent reporter molecules that can bind to complementary nucleic acid sequences with high degrees of specificity. Although probes can range from 15 to thousands of nucleotides in size, synthetic oligonucleotides of <50 nucleotides are most commonly incorporated into commercial kits. The probes can be designed to identify microorganisms at any taxonomic level. A number of commercially available DNA probes have been developed for direct detection of pathogens in clinical specimens and identification of pathogens after isolation by culture.

The commonly used formats for probe hybridization include liquid-phase, solid-phase, and *in situ* hybridization. The leading method used in clinical microbiology laboratories is a liquid-phase hybridization protection assay (Hologic Gen-Probe, Inc., San Diego, CA). In this method, a single-stranded DNA (ssDNA) probe labeled with an acridinium ester is incubated with the target nucleic acid. Alkaline hydrolysis follows the hybridization step, and probe binding is measured in a luminometer after the addition of peroxides. For a positive sample, the acridinium ester on the bound probe is protected from hydrolysis and, upon the addition of peroxides, emits light. The hybridization protection assay can be completed in several hours and does not require removal of unbound single-stranded probe or isolation of probe-bound double-stranded sequences (1).

In solid-phase hybridization, target nucleic acids are bound to nylon or nitrocellulose and are hybridized with a probe in solution (2). The unbound probe is washed away, and the bound probe is detected by means of fluorescence, luminescence, radioactivity, or color development. Although solid-phase hybridization is a powerful research tool, the length of time required and the complexity of the procedure limit its application in clinical practice.

In situ hybridization is another type of solid-phase hybridization in which the nucleic acid is contained in tissues or cells that are affixed to microscope slides and is governed by the same basic principles described previously (3). In most clinical applications, formalin-fixed, paraffin-embedded tissue sections are used. The sensitivity of *in situ* hybridization is often limited by the accessibility of the target nucleic acid in the cells.

In general, due to the poor analytical sensitivities of nonamplified-probe techniques, the application of these techniques to direct detection of pathogens in clinical specimens is limited to those situations in which the number of organisms is large. Such situations include cases of group A streptococcal pharyngitis and agents associated with vaginosis and vaginitis. These techniques are used most effectively in culture confirmation assays for mycobacteria and systemic dimorphic fungi. These culture confirmation tests have a positive effect on patient management by providing rapid and accurate detection of these slowly growing, often difficult-to-identify pathogens.

Nucleic acid probes for direct detection of group A streptococci, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae* are available from Hologic Gen-Probe. Probes for identification of *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma*

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*This chapter contains some information presented by Angela M. Caliendo in chapter 4 of the 10th edition of this *Manual*.

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capsulatum, campylobacters, enterococci, group A streptococci, group B streptococci, *Haemophilus influenzae*, *Listeria monocytogenes*, mycobacteria, *N. gonorrhoeae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* isolated in culture are also available from Hologic Gen-Probe.

A solid-phase nucleic acid probe test for detection and identification of *Gardnerella vaginalis*, *Trichomonas vaginalis*, and *Candida albicans* in vaginal fluid from patients with vaginosis or vaginitis is available from BD Diagnostic Systems (Sparks, MD). It uses two distinct probes for each organism, a capture probe and a color development probe, in an easy-to-use format.

Peptide nucleic acid (PNA) probes are DNA mimics in which the negatively charged sugar phosphate backbone of DNA is replaced with a noncharged polyamide or "peptide" backbone. PNA probes contain the same nucleotide bases as DNA and follow standard Watson-Crick base-pairing rules when hybridizing to complementary nucleic acid sequences (4). Because PNA probes are noncharged, they do not have to overcome the destabilizing electrostatic repulsion that occurs when two negatively charged DNA molecules hybridize. As a result, PNA probes bind more rapidly and tightly to nucleic acid targets. In addition, the relatively hydrophobic character of the PNA probes enables them to penetrate the hydrophobic cell membrane following preparation of a standard smear.

PNA fluorescent *in situ* hybridization probes targeting rRNA sequences for rapid, direct identification of *S. aureus*, coagulase-negative staphylococci, *Enterococcus faecalis*, selected other species of enterococci, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and several *Candida* spp. from positive blood culture bottles are available from AdvanDx (Woburn, MA) (5–7). Figure 1 shows an image of a mixture of *E. coli*, *K. pneumoniae*, and *P. aeruginosa* stained with PNA fluorescent *in situ* hybridization probes for each organism labeled with three different-colored fluorophores and examined by fluorescence microscopy.

AMPLIFIED NUCLEIC ACID TECHNIQUES

The development of the PCR by Saiki et al. (8) was a milestone in biotechnology and heralded the beginning of the modern era of molecular diagnostics. Although PCR is the most widely used nucleic acid amplification strategy, other strategies have been developed, and several have clinical utility. These strategies are based on signal, target, or probe amplification. Examples of each category are discussed in the sections that follow. These techniques have sensitivity unparalleled in laboratory medicine, have created new opportunities for the clinical laboratory to have an effect on patient care, and have become the new gold standards for laboratory diagnosis of many infectious diseases.

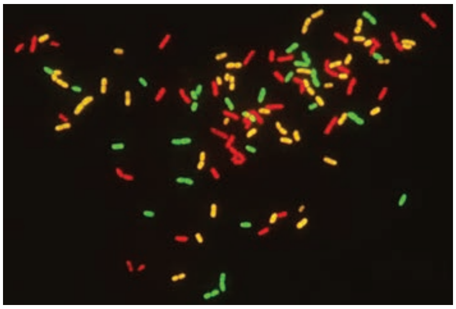

FIGURE 1 PNA probes for *E. coli* (green), *K. pneumoniae* (yellow), and *P. aeruginosa* (red). Reprinted with permission of AdvanDx from http://www.advandx.com/Technology/image-gallery. doi:10.1128/9781555817381.ch6.f1

SIGNAL AMPLIFICATION TECHNIQUES

In signal amplification methods, the concentration of the probe or target does not increase. The increased analytical sensitivity comes from increasing the concentration of labeled molecules attached to the target nucleic acid. Multiple enzymes, multiple probes, multiple layers of probes, and reduction of background noise have all been used to enhance target detection (9). Target amplification systems generally have greater analytical sensitivity than signal amplification methods, but technological developments, particularly in branched-DNA (bDNA) assays, have lowered the limits of detection to levels that rival those of some earlier target amplification assays (10).

Signal amplification assays have several advantages over target amplification assays. In signal amplification systems, the number of target molecules is not altered, and as a result, the signal is directly proportional to the amount of the target sequence present in the clinical specimen. This reduces concerns about false-positive results due to cross contamination and simplifies the development of quantitative assays. Since signal amplification systems are not dependent on enzymatic processes to amplify the target sequence, they are not affected by the presence of enzyme inhibitors in clinical specimens. Consequently, less cumbersome nucleic acid extraction methods may be used. Typically, signal amplification systems use either larger probes or more probes than target amplification systems and, consequently, are less susceptible to errors resulting from target sequence heterogeneity. Finally, RNA levels can be measured directly without the synthesis of a cDNA intermediate.

bDNA Assays

The bDNA signal amplification system is a solid-phase, sandwich hybridization assay incorporating multiple sets of synthetic oligonucleotide probes (11). The key to this technology is the amplifier molecule, a bDNA molecule with 15 identical branches, each of which can bind to three labeled probes.

The bDNA signal amplification system is illustrated in Fig. 2. Multiple target-specific probes are used to capture the target nucleic acid onto the surface of a microtiter well. A second set of target-specific probes also binds to the target. Preamplifier molecules bind to the second set of target probes and up to eight bDNA amplifiers. Three alkaline phosphatase-labeled probes hybridize to each branch of the amplifier. Detection of bound labeled probes is achieved by incubating the complex with dioxetane, an enzyme-triggerable substrate, and measuring the light emission in a luminometer. The resulting signal is directly proportional to the quantity of the target in the sample. The quantity of the target in the sample is determined from an external standard curve.

Nonspecific hybridization of any of the amplification probes and nontarget nucleic acids leads to amplification of the background signal. In order to reduce potential hybridization to nontarget nucleic acids, isocytidine (isoC) and isoguanosine (isoG) were incorporated into the preamplifier and labeled probes were used in the third-generation bDNA assays (12). IsoC and isoG form base pairs with each other but not with any of the four naturally occurring bases (13).

The use of isoC- and isoG-containing probes in bDNA assays increases target-specific signal amplification without

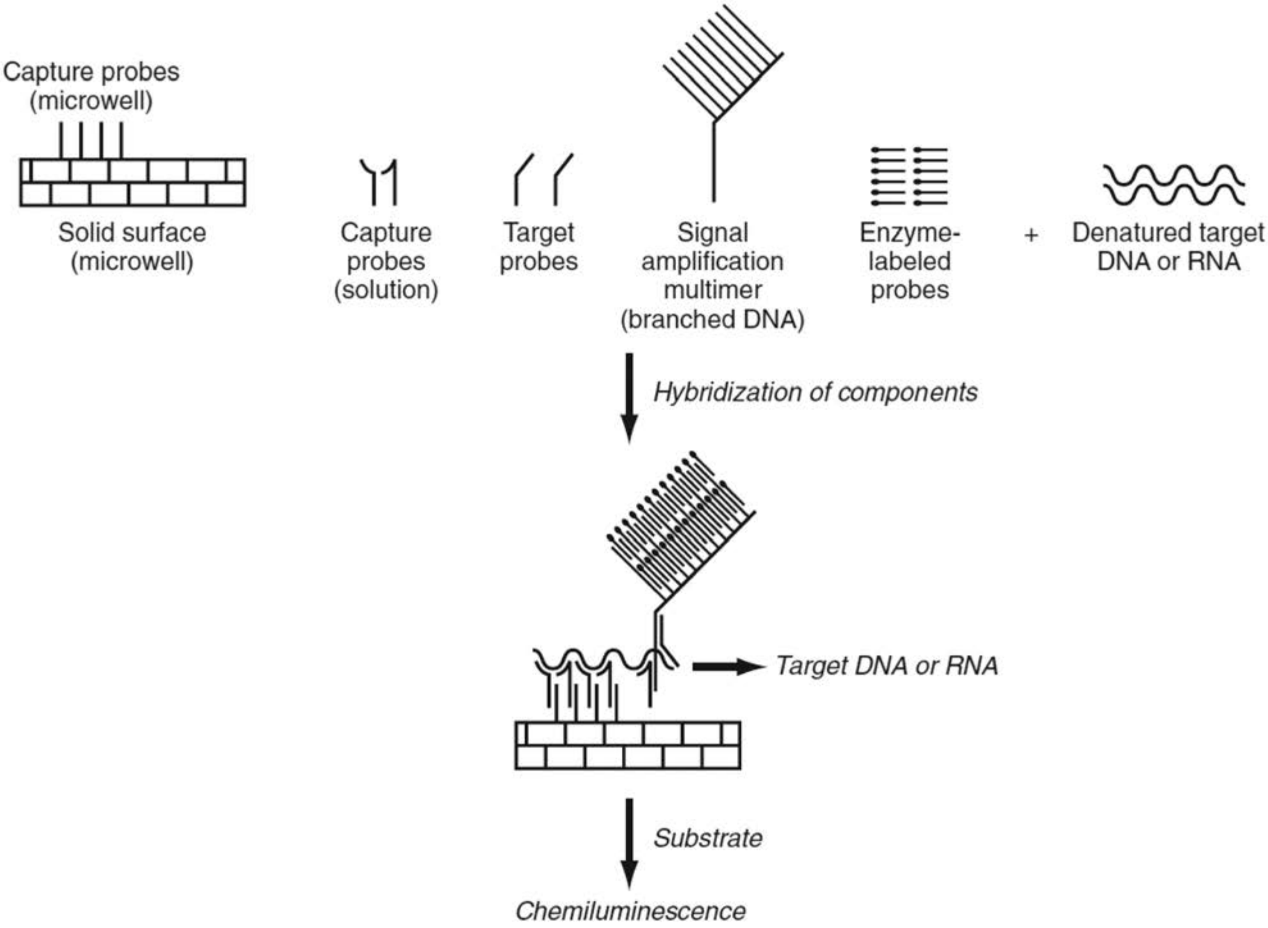

FIGURE 2 bDNA signal amplification. Reprinted with permission of Elsevier from reference 219. doi:10.1128/9781555817381.ch6.f2

a concomitant increase in the background signal, thereby greatly enhancing the detection limits without loss of specificity. The detection limit of the third-generation bDNA assay for HIV-1 RNA is 75 copies/ml. bDNA assays for the quantification of hepatitis B virus (HBV) DNA, hepatitis C virus (HCV) RNA, and HIV-1 RNA are commercially available (Siemens Healthcare Diagnostics, Deerfield, IL). The SiemensVersant 440 analyzer for bDNA assays automates the incubation, washing, reading, and data-processing steps.

Hybrid Capture Assays

The hybrid capture system is a solution hybridization-antibody capture method that uses chemiluminescence detection of the hybrid molecules (Fig. 3). The target DNA in the specimen is denatured and then hybridized with a specific RNA probe. The DNA-RNA hybrids are captured by antihybrid antibodies that are used to coat the surface of a tube. Alkaline phosphatase-conjugated antihybrid antibodies bind to the immobilized hybrids. The bound antibody conjugate is detected with a chemiluminescent substrate, and the light emitted is measured in a luminometer. Multiple alkaline phosphatase conjugates bind to each hybrid molecule, amplifying the signal. The intensity of the emitted light is proportional to the amount of target DNA in the specimen. Hybrid capture assays for detection of *N. gonorrhoeae*, *C. trachomatis*, and human papillomavirus (HPV) (14) in clinical specimens are available from Qiagen (Germantown, MD). There are manual and automated (Rapid Capture System) versions of these assays.

Cleavase-Invader Technology

Invader assays (Hologic, Bedford, MA) are based on a signal amplification method that relies on the specific recognition and cleavage of particular DNA structures by cleavase, a member of the FEN-1 family of DNA polymerases. These polymerases cleave the 5′ single-stranded flap of a branched, base-paired duplex. This enzymatic activity likely plays an essential role in the elimination of the complex nucleic acid structures that arise during DNA replication and repair. Since these structures may occur anywhere in a replicating genome, the enzyme recognizes the molecular structure of the substrate without regard to the sequence of the nucleic acids making up the DNA complex (15, 16).

In the invader assays, two probes are designed that hybridize to the target sequence in an overlapping fashion (Fig. 4). Under the proper annealing conditions, the probe oligonucleotide binds to the target sequence. The invader oligonucleotide probe is designed such that it hybridizes upstream of the probe with a region of overlap between the 3′ end of the invader and the 5′ end of the probe. Cleavase cleaves the 5′ end of the probe and releases it. It is in this way that the target sequence acts as a scaffold upon which the proper DNA structure can form. Since the DNA

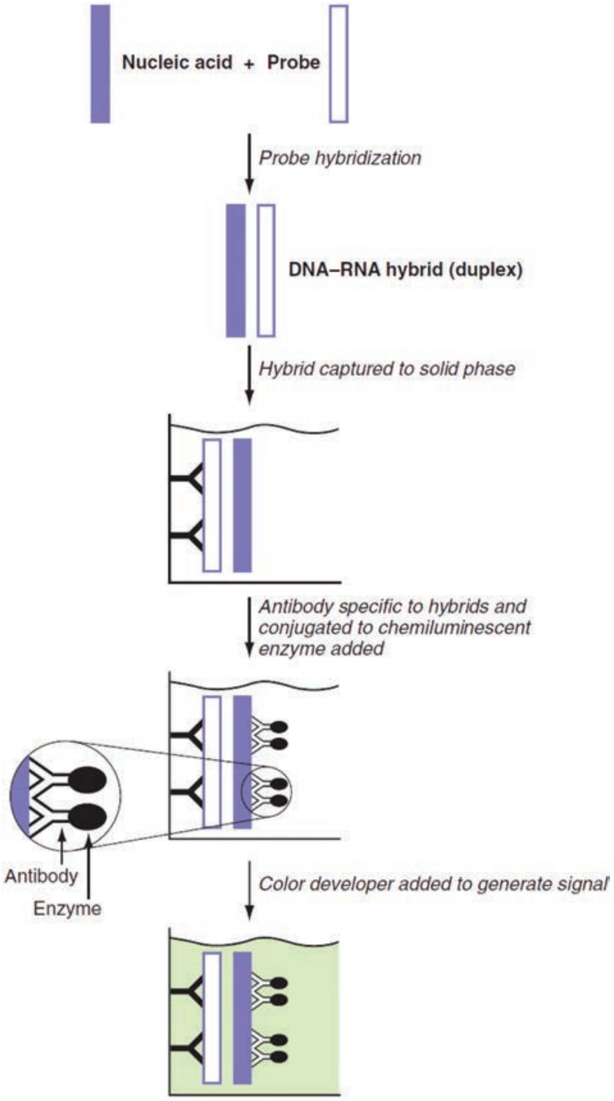
Nucleic acid + Probe
Probe hybridization
DNA–RNA hybrid (duplex)
Hybrid captured to solid phase
Antibody specific to hybrids and conjugated to chemiluminescent enzyme added
Antibody
Enzyme
Color developer added to generate signal

FIGURE 3 Hybrid capture signal amplification. Reprinted with permission of Elsevier from reference 219. doi:10.1128/9781555817381.ch6.f3

structure necessary to serve as a cleavase substrate occurs only in the presence of the target sequence, the generation of cleavage products indicates the presence of the target. Use of a thermostable cleavase enzyme allows reactions to be run at temperatures high enough for a primer exchange equilibrium to exist. This allows multiple cleavase products to form off of a single target molecule. Fluorescence resonance energy transfer (FRET) probes and a second invasive cleavage reaction are used to detect the target-specific products. FDA-cleared assays for detection of pools of high-risk genotypes and types 16 and 18 of HPV in cervical samples are available from Hologic (17, 18).

TARGET AMPLIFICATION TECHNIQUES

All of the target amplification systems share certain fundamental characteristics. They use enzyme-mediated processes, in which a single enzyme or multiple enzymes synthesize copies of target nucleic acid. In all of these techniques, the amplification products are detected by two oligonucleotide primers that bind to complementary sequences on opposite strands of double-stranded targets. All the techniques result in the production of millions to billions of copies of the targeted sequence in a matter of hours, and in each case, the amplification products can serve as templates for subsequent rounds of amplification. Because of this, all of the techniques are sensitive to contamination with product molecules that can lead to false-positive reactions. The potential for cross contamination is real and should be adequately addressed before any of these techniques are used in the clinical laboratory. However, the occurrence of false-positive reactions can be reduced through special laboratory design, practices, and workflow.

Polymerase Chain Reaction

PCR is a simple, *in vitro* chemical reaction that permits the synthesis of essentially limitless quantities of a targeted nucleic acid sequence. This is accomplished through the action of a DNA polymerase that, under the proper conditions, can copy a DNA strand (Fig. 5). At its simplest, a PCR consists of target DNA, a molar excess of two oligonucleotide primers, a heat-stable DNA polymerase, an equimolar mixture of deoxyribonucleoside triphosphates (dNTPs; dATP, dCTP, dGTP, and dTTP), $MgCl_2$, KCl, and a Tris-HCl buffer. The two primers flank the double-stranded DNA (dsDNA) sequence to be amplified, typically <100 to several hundred bases, and are complementary to opposite strands of the target.

To initiate a PCR, the reaction mixture is heated to separate the two strands of target DNA and is then cooled to permit the primers to anneal to the target DNA in a sequence-specific manner. The DNA polymerase then initiates extension of the primers at their 3′ ends toward one another. The primer extension products are dissociated from the target DNA by heating. Each extension product, as well as the original target, can serve as a template for subsequent rounds of primer annealing and extension.

At the end of each cycle, the PCR products are theoretically doubled. Thus, after n PCR cycles the target sequence can be amplified 2^n-fold. The whole procedure is carried out in a programmable thermal cycler that precisely controls the temperature at which the steps occur, the lengths of time that the reaction mixture is held at the different temperatures, and the number of cycles. Ideally, after 20 cycles of PCR a 10^6-fold amplification is achieved and after 30 cycles a 10^9-fold amplification occurs. In practice, the amplification may not be completely efficient due to failure to optimize the reaction conditions or the presence of inhibitors of the DNA polymerase. In such cases, the total amplification is best described by the expression $(1 + e)^n$, where e is the amplification efficiency $(0 \leq e \leq 1)$ and n is the total number of cycles.

Reverse Transcriptase PCR

As it was originally described, PCR was a technique for DNA amplification. Reverse transcriptase PCR (RT-PCR) was developed to amplify RNA targets. In this process, cDNA is first produced from RNA targets by reverse transcription and then the cDNA is amplified by PCR. As it was originally described, RT-PCR used two enzymes, a heat-labile RT, such as avian myeloblastosis virus RT, and a thermostable DNA polymerase. Because of the temperature requirements of the heat-labile enzyme, cDNA synthesis had to occur at temperatures below the optimal annealing temperatures of the primers. This presented problems in terms of both nonspecific primer annealing and inefficient

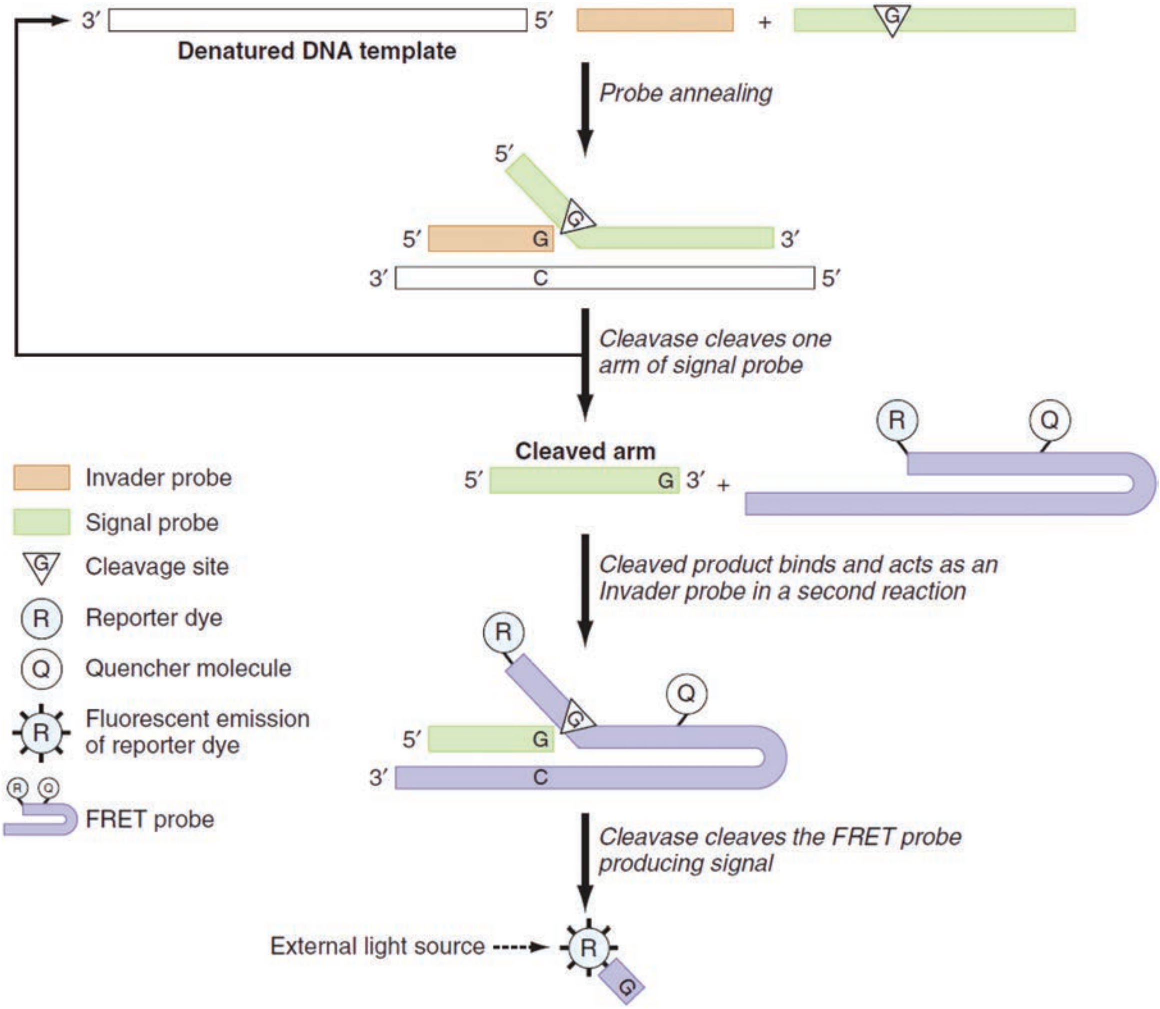
3′
5′
Denatured DNA template
+
G
Probe annealing
5′
5′
G
G
3′
3′
C
5′
Cleavase cleaves one arm of signal probe
Cleaved arm
5′
G
3′
+
R
Q
Invader probe
Signal probe
G
Cleavage site
R
Reporter dye
Q
Quencher molecule
R
Fluorescent emission of reporter dye
R
Q
FRET probe
Cleaved product binds and acts as an Invader probe in a second reaction
R
Q
G
5′
G
3′
C
Cleavase cleaves the FRET probe producing signal
External light source
R
G

FIGURE 4 Cleavase-invader probe-based amplification. Reprinted with permission of Elsevier from reference 219. doi:10.1128/9781555817381.ch6.f4

primer extension due to the formation of RNA secondary structures. These problems have largely been overcome by the development of a thermostable DNA polymerase derived from *Thermus thermophilus* that under the proper conditions can function efficiently as both an RT and a DNA polymerase (19). RT-PCRs with this enzyme are more specific and efficient than previous protocols with conventional, heat-labile RT enzymes.

Nested PCR

Nested PCR was developed to increase both the sensitivity and the specificity of PCR. It uses two pairs of amplification primers and two rounds of PCR. Typically, one primer pair is used in the first round of PCR for 15 to 30 cycles. The products of the first round of amplification are then subjected to a second round of amplification with the second set of primers, which anneal to a sequence internal to the sequence amplified by the first primer set. The increased sensitivity arises from the high total cycle number, and the increased specificity arises from the annealing of the second primer set to sequences found only in the first-round products, thus verifying the identity of the first-round product. The major disadvantage of nested PCR is the high rates of contamination that can occur during the transfer of first-round products to the second tube for the second round of amplification. This contamination can be avoided either by physically separating the first- and second-round amplification mixtures with a layer of wax or oil or by designing single-tube or completely contained amplification protocols. In practice, the enhanced sensitivity afforded by nested PCR protocols is rarely required in diagnostic applications, and the identity of an amplification product is usually confirmed by hybridization with a nucleic acid probe.

Multiplex PCR

In multiplex PCR, two or more primer sets designed for amplification of different targets are included in the same reaction mixture (20). By this technique, more than one target sequence in a clinical specimen can be coamplified in a single tube. The primers used in multiplexed reactions must be carefully selected so that they have similar annealing temperatures and lack complementarity. Multiplex PCRs have proved to be more complicated to develop and may be less sensitive than PCRs with single primer sets.

Many multiplex assays have been developed, especially for the detection of central nervous system (21, 22), respiratory (23–26), bloodstream (27–29), and gastrointestinal (30, 31) infections. The scale of the multiplexing can range from

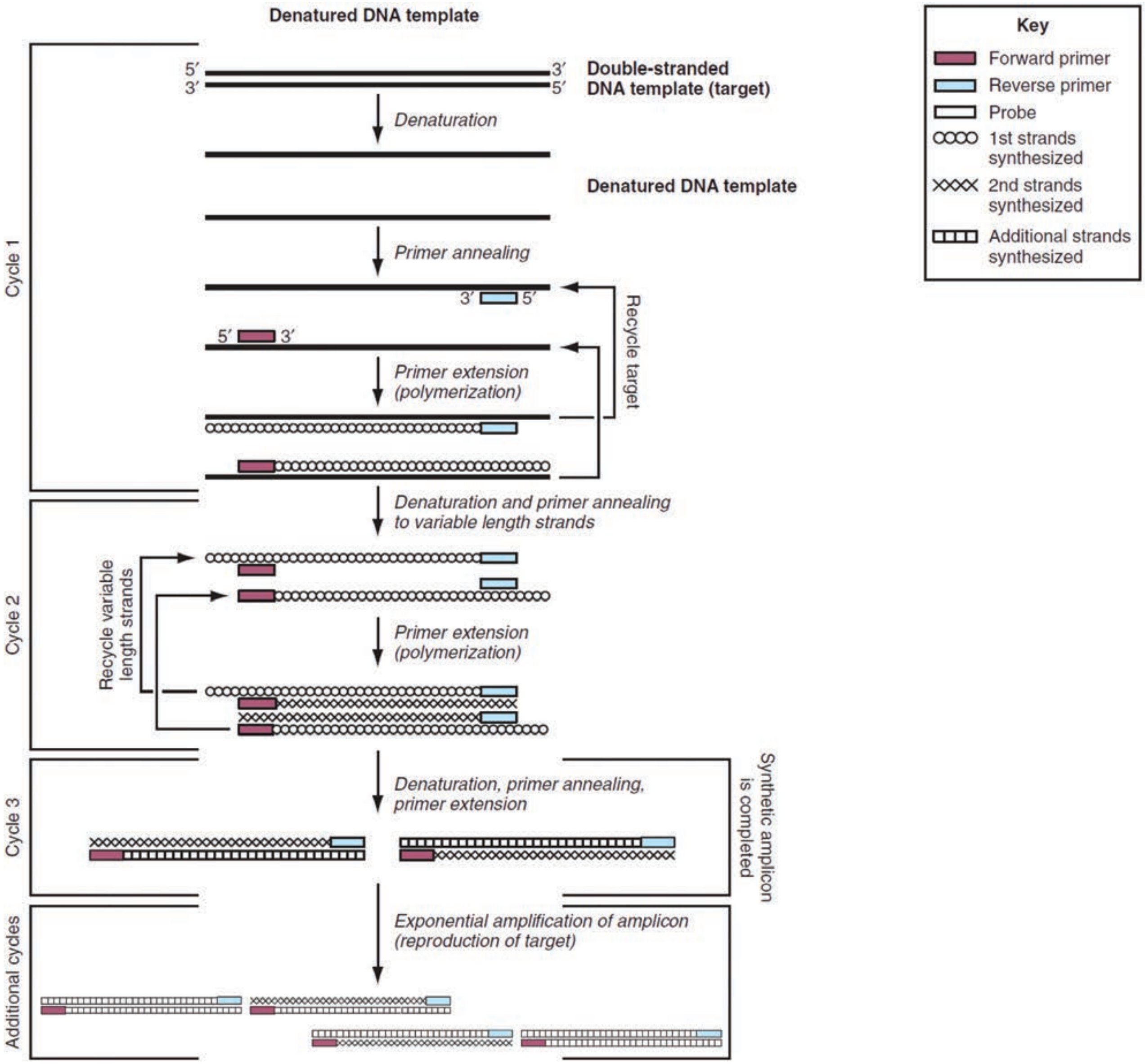
Denatured DNA template
5′
3′
3′
5′
Double-stranded DNA template (target)
Denaturation
Denatured DNA template
Primer annealing
3′ 5′
5′ 3′
Recycle target
Primer extension (polymerization)
Cycle 1
Denaturation and primer annealing to variable length strands
Recycle variable length strands
Primer extension (polymerization)
Cycle 2
Denaturation, primer annealing, primer extension
Synthetic amplicon is completed
Cycle 3
Exponential amplification of amplicon (reproduction of target)
Additional cycles
Key
Forward primer
Reverse primer
Probe
1st strands synthesized
2nd strands synthesized
Additional strands synthesized

FIGURE 5 PCR target amplification. Reprinted with permission of Elsevier from reference 219.
doi:10.1128/9781555817381.ch6.f5

several to 20 or more targets depending on the method used to identify the individual amplicons. Technological developments in multiplex nucleic acid amplification and detection have allowed an organ system-based approach to molecular microbiology that matches or exceeds the ability of culture methods to provide a comprehensive diagnostic result. One of the first platforms for high-order multiplex PCR analysis was the xMAP system (Luminex Corp., Austin, TX). The xMAP system incorporates a proprietary process to internally dye polystyrene microspheres with two spectrally distinct fluorochromes. By using precise ratios of these fluorochromes, an array is created consisting of 100 different microsphere sets with specific spectral addresses. Each microsphere set can possess a different reactant on its surface. For nucleic acid analysis, oligonucleotide probes would be covalently bound to the microsphere surface by carbodiimide coupling. Since each microsphere set can be distinguished by its spectral address, the sets can be combined, allowing up to 100 different analytes to be measured simultaneously in a single reaction vessel. A third fluorochrome coupled to a reporter molecule quantifies the biomolecular interaction that occurs at the microsphere surface.

Microspheres are interrogated individually in a rapidly flowing liquid stream as they pass by two separate lasers in the Luminex xMAP flow cytometer. High-speed digital signal processing classifies each microsphere based on its spectral address and quantifies the reaction on its surface. Thousands of microspheres are investigated per second, resulting in an analysis system capable of analyzing and reporting up to 100 different reactions in a single reaction vessel in a few seconds.

Multiplex assays run on the Luminex platform typically consist of three major steps: nucleic acid amplification by PCR, target-specific extension, and liquid bead array decoding. After PCR amplification, the amplicons are mixed with a second set of tagged primers specific for each target. If the target is present, the tagged primer will be extended through a process called target-specific extension. During this extension, a label is incorporated into the extension product. The color-coded beads are added to identify the tagged and labeled extension products. Attached to each different-colored bead is oligonucleotide complementary to the tag sequence for each target. Samples are then placed in the Luminex xMAP flow cytometer, where the beads are read by two color lasers. One laser identifies the color of the bead and the other laser detects the presence or absence of a labeled extension product on that bead. Luminex recently introduced a more streamlined protocol that reduces analysis time from 8 to 5 h with fewer hands-on steps, but this “fast” protocol may reduce sensitivity (32).

The technology has been adapted to a wide variety of applications in bacteriology (33), mycology (34), and virology (35, 36). Systems for the multiplex detection of respiratory viruses based on the Luminex xMAP system have been developed by Luminex Molecular Diagnostics, EraGen Biosciences (Madison, WI), and Qiagen (25, 37, 38). FDA-cleared assays for the Luminex xMAP system include those for respiratory viruses and gastrointestinal pathogens (32, 39).

Another promising technology for high-order multiplex PCR is the FilmArray, developed by BioFire Diagnostics (Salt Lake City, UT) (26). It is a completely automated, integrated, and self-contained lab-in-a-pouch system. The film portion of the pouch has stations for cell lysis, nucleic acid purification, reverse transcription to detect RNA targets, first-stage PCR, multiplex PCR, and an array of up to 120 second-stage nested PCRs. After extracting and purifying nucleic acids from the unprocessed sample, the FilmArray performs a nested multiplex PCR that is executed in two stages. During the first-stage PCR, the FilmArray performs a single, large-volume, massively multiplexed reaction. The products from first-stage PCR are then diluted and combined with a fresh, primer-free master mix. Aliquots of this second master mix solution are then distributed to each well of the array. Each well of the array is prespotted with a single set of primers. The second-stage, small-volume PCR is performed in singleplex fashion in each well of the array. At the end of the second-stage PCR analysis, melting-curve analysis is used identify each target. Though this assay uses nested PCR, the entire test is performed within a sealed pouch, thus eliminating concerns of carryover contamination. Using amplification and melting-curve data, the FilmArray software automatically generates a result for each target. FilmArray panels for detection of 20 different respiratory pathogens and identification of organisms in positive blood cultures are FDA cleared, and a gastrointestinal pathogen panel is in development.

Real-Time (Homogeneous, Kinetic) PCR

The term “real-time PCR” describes methods in which the target amplification and detection steps occur simultaneously in the same tube (homogeneous). These methods require special thermal cyclers with precision optics that can monitor the fluorescence emission from the sample wells. The computer software supporting the thermal cycler monitors the data throughout the PCR at every cycle and generates an amplification plot for each reaction (kinetic).

Figure 6 shows a representative amplification plot and defines the terms used in quantitative real-time PCR. The amplification plot shows the normalized fluorescence signal from the reporter at each cycle number. In the initial cycles of PCR, there is little change in the fluorescence signal. This initial signal level defines the baseline for the plot. An increase above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The cycle threshold (C_T) is defined as the cycle number at which the fluorescence passes the fixed threshold. A plot of the log of the initial target concentration versus C_T for a set of standards is a straight line (40). The amount of the target in an unknown sample is determined by measuring the sample C_T and using a standard curve to determine the starting copy number. Alternatively, the cycle number corresponding to the maximal change in fluorescence, the second derivative maximum, has a similar relationship to the initial target concentration.

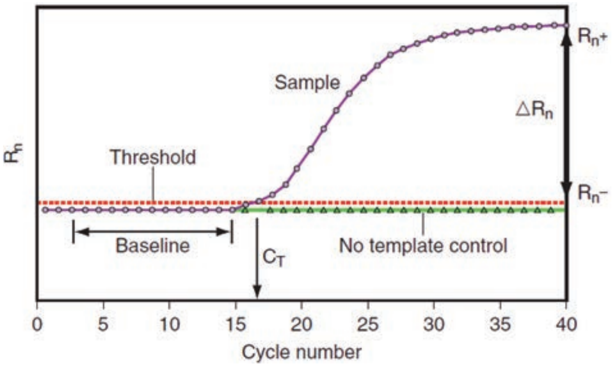

FIGURE 6 Real-time PCR amplification plot with commonly used terms and abbreviations. C_T, cycle threshold; R_n, normalized fluorescent signal from reporter dye. From *TaqMan Universal PCR Master Mix Protocol*, p 5-4 (Applied Biosystems, Foster City, CA, 2010). Reprinted with permission. doi:10.1128/9781555817381.ch6.f6

In its simplest format, the PCR product is detected as it is produced by using fluorescent dyes that preferentially bind to dsDNA. SYBR green I is one such dye that has been used in this application (41). In the dye’s unbound state, the fluorescence is relatively low, but when the dye is bound to dsDNA, the fluorescence is greatly enhanced. The dye binds to both specific and nonspecific PCR products. The specificity of the detection can be improved through melting-curve analysis. As the temperature is slowly raised, the two strands of the amplicon melt apart and the amount of fluorescence decreases. The data are transformed and analyzed by plotting the first derivative of the fluorescence on the *y* axis and the temperature on the *x* axis. The specific amplified product will have a characteristic melting peak at its predicted melting temperature (T_m), whereas the primer dimers and other nonspecific products should have different T_ms or give broader peaks (42).

The specificity of real-time PCR can also be increased by including FRET probes in the reaction mixture. These probes are labeled with fluorescent dyes or with combinations of fluorescent and quencher dyes. In 5′ exonuclease PCR (TaqMan) assays, the 5′-to-3′ exonuclease activity of *Taq* DNA polymerase is used to cleave a nonextendable hybridization probe during the primer extension phase of PCR (43). This approach uses dual-labeled fluorogenic hybridization probes and is illustrated in Fig. 7. One fluorescent dye serves as a reporter, and its emission spectrum is quenched by the second fluorescent dye. The nuclease degradation of the hybridization probe releases the reporter dye, resulting in an increase in the peak fluorescent emission.

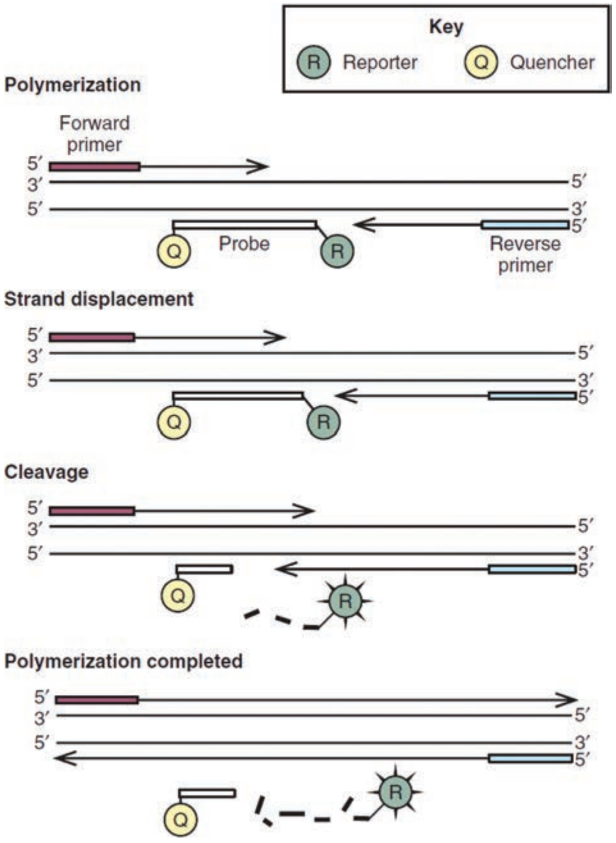

FIGURE 7 5′ Exonuclease chemistry for real-time PCR applications. Modified with permission of Elsevier from reference 219. doi:10.1128/9781555817381.ch6.f7

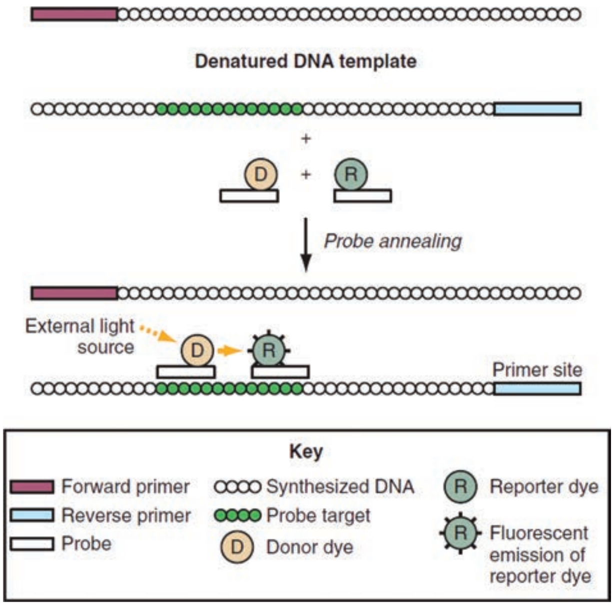

FIGURE 8 Dual hybridization probes for real-time PCR applications. Reprinted with permission of Elsevier from reference 219. doi:10.1128/9781555817381.ch6.f8

The increase in fluorescent emission indicates that specific PCR product has been made, and the intensity of fluorescence is related to the amount of the product (44). The specificity is increased because a signal is generated only when the primer and probe are bound to the same template strand.

The use of dual hybridization probes is another approach to real-time PCR (45). This method uses two specially designed sequence-specific oligonucleotide probes (Fig. 8). These hybridization probes are designed to hybridize within 1 to 5 nucleotides apart on the product molecule. The 3′ end of the anchor probe is labeled with a donor dye, and the 5′ end of the reporter probe is labeled with an acceptor dye. The 3′ end of the reporter probe is phosphorylated to prevent extension during PCR. The donor dye is excited by an external light source, and instead of emitting light, it transfers its energy to the acceptor dye by FRET. The excited acceptor dye emits light at a longer wavelength than the unbound donor dye, and the intensity of the acceptor dye light emission is proportional to the amount of PCR product.

Real-time detection and quantification of amplification products can also be accomplished with molecular beacons (46). Molecular beacons are hairpin-shaped oligonucleotide probes with an internally quenched fluorophore whose fluorescence is restored when the probes bind to a target nucleic acid (Fig. 9). The probes are designed in such a way that the loop portion of each probe molecule is complementary to the target sequence. The stem is formed by the annealing of complementary arm sequences on the ends of the probe. A fluorescent dye is attached to one end of one arm, and a quenching molecule is attached to the end of the other arm. The stem keeps the fluorophore and quencher in close proximity such that no light emission occurs. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than the stem and undergoes a conformational change that forces the stem apart, causing the fluorophore and the quencher to move away from each other, restoring the fluorescence.

Scorpion probes combine a PCR primer with a molecular beacon (47, 48). Intramolecular hybridization of the loop structure to a downstream portion of the amplification product separates the reporter and quencher dyes. The hybridization kinetics of Scorpion probes are generally faster than those of molecular beacons because the primer and probe are located on the same molecule (Fig. 10).

Dark quencher probes are also used in real-time PCR applications (ELITech Group, Princeton, NJ). Dark quencher probes contain a fluorophore on the 5′ end and a nonfluorescent quencher molecule on the 3′ end (49). The fluorescence is quenched when the probe is a random coil and emitted when the probe anneals to the target sequence. Unlike fluorogenic 5′ nuclease probes, these probes are not degraded by the DNA polymerase during target amplification. Since the dark quencher is not fluorescent, it does not contribute to the background signal. This trait has the advantage of improving the signal-to-noise ratio for the detection system, which may improve sensitivity. These probes also incorporate a hybridization-stabilizing compound, known as a minor groove binder. It is a small, crescent-shaped molecule that is covalently linked to the 3′ end of the probe that spans about 3 or 4 nucleotides and snugly fits into the minor groove of DNA, where it forms hydrogen bonds with the template. Minor groove binders increase the T_m of the probe. The minor groove binder allows for the use of shorter probes because of the increased T_ms and enables

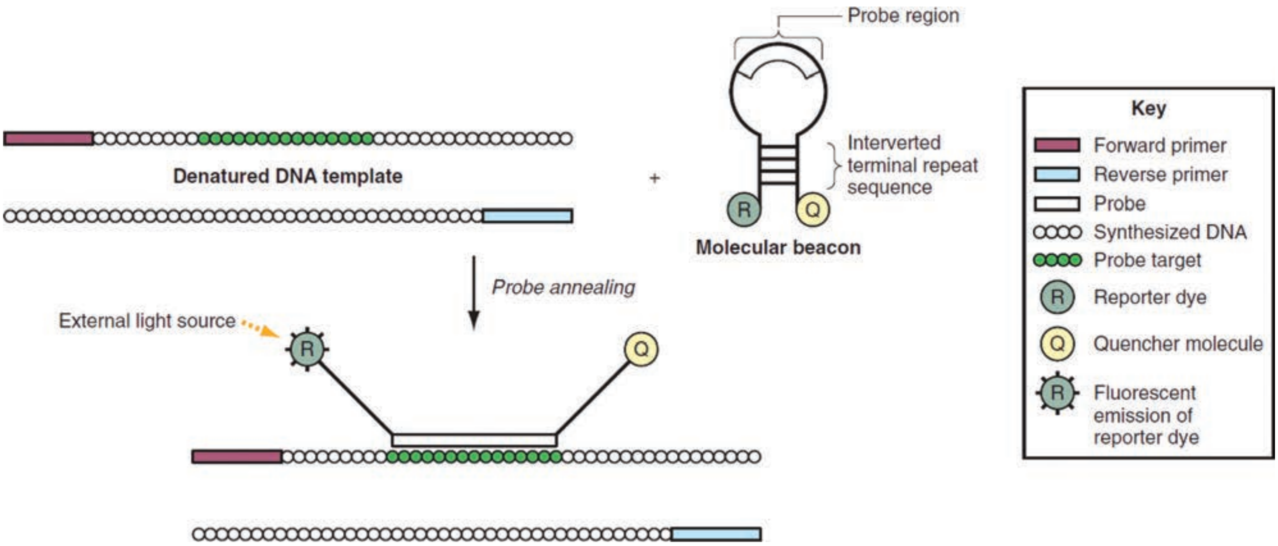

FIGURE 9 Molecular beacon probes for real-time amplification applications. Modified with permission of Elsevier from reference 219. doi:10.1128/9781555817381.ch6.f9

improved T_m leveling, which increases the specificity of the detection reaction.

Another approach to detection, characterization, and quantification of real-time PCR amplicons involves the use of a nonstandard DNA base pair constructed from isoG and isoC (50–52). These synthetic bases pair with each other, but not with the natural bases guanine and cytosine, and can be covalently coupled to a wide variety of reporter groups. In the MultiCode-RTx assays (Luminex Corp.), the target is amplified using a forward primer with a single isoC nucleotide with fluorescent label at the 5′ end and an unlabeled standard base reverse primer. Amplification is performed in the presence of isoG coupled to a fluorescence quencher molecule, and site-specific incorporation by the DNA polymerase places the quencher in close proximity to the fluorophore, resulting in a decrease of fluorescence with every PCR cycle. The number of cycles in which the fluorescence change can be detected is dependent on the initial number of target molecules in the reaction. The decrease in fluorescence is easily monitored by a number of different standard real-time PCR instruments. Postreaction amplicon melting-curve analysis can be performed to confirm the identity of the amplicon and to detect sequence variants. MultiCode-RTx ASRs for detection and/or quantification of a variety of bacteria, viruses, fungi, and parasites are available from Luminex.

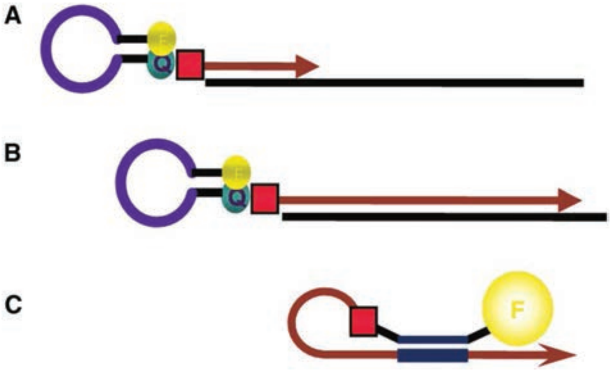

FIGURE 10 Scorpion probes. (A) The primer element binds to the DNA target. The probe element is in the closed, nonfluorescent configuration. (B) The primer is extended and incorporates a probe-binding site into the new strand. The probe element remains in the closed, nonfluorescent configuration. (C) After a cycle of denaturation and reannealing, the probe flips forward to bind its target site on the same molecule. The fluorophore and quencher are now separated, and the fluorescence increases. Reprinted with permission from reference 220. doi:10.1128/9781555817381.ch6.f10

A novel class of asymmetric, partially double-stranded, linear probes can be used in real-time PCR assays (53). A schematic representation of their design and principles is shown in Fig. 11. The partially double-stranded probe is composed of two complementary oligonucleotides of very different lengths. The long, target-specific strand is 5′ labeled with a fluorophore and is blocked on the 3′ end to prevent extension. The shorter strand is complementary to the 5′ end of the long strand and has quencher dye attached to its 3′ end. In the absence of the target, the quencher oligonucleotide hybridizes to the target-specific oligonucleotide and the duplex does not fluoresce due to the close proximity of the reporter and quencher dyes. When the target is present, the long strand binds preferentially to the target, resulting in increased fluorescence due to separation of the reporter and quencher dyes. Partially double-stranded probes are better able to detect targets with a high level of genetic heterogeneity (e.g., HIV-1 and HCV) than are molecular beacons and TaqMan probes due primarily to their increased length and less stringent hybridization conditions (54). Partially doubled-stranded probes are used in HIV-1 and HCV viral load assays manufactured by Abbott (Abbott Park, IL).

Real-time PCR methods decrease the time required to perform nucleic acid assays because there are no post-PCR processing steps. Also, since amplification and detection occur in the same closed tube, these methods eliminate the postamplification manipulations that can lead to laboratory contamination with the amplicon. In addition, real-time

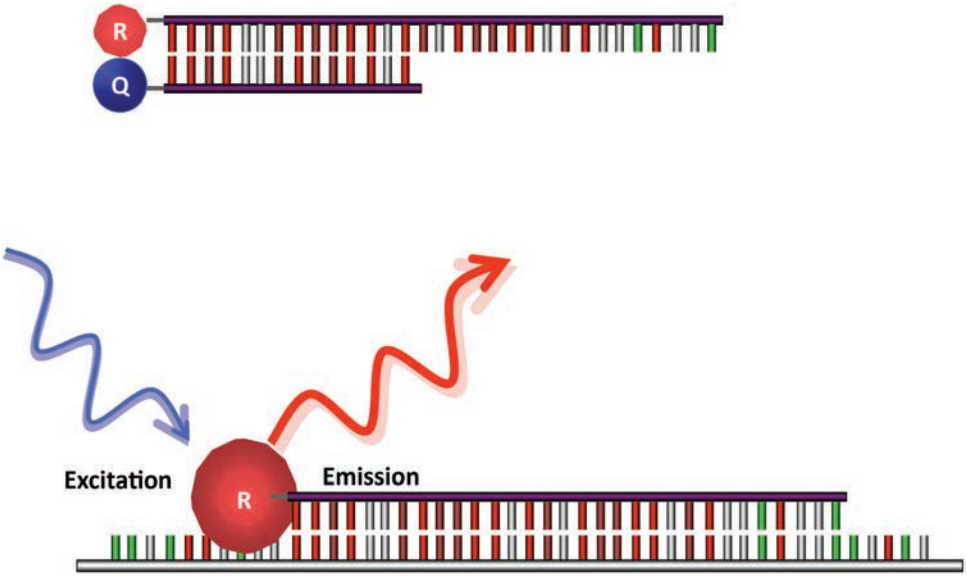

FIGURE 11 Principles of partially double-stranded linear probes. A double-stranded linear probe contains two complementary oligonucleotides of very different lengths. The longer, positive strand is 5′ labeled with a fluorophore reporter, while the shorter, negative strand carries a quencher moiety at its 3′ end. When not bound to a target, the probe is nonfluorescent due to the close proximity of the fluorophore and the quencher. In the presence of a specific target, the positive strand preferentially hybridizes to the target, resulting in increased fluorescence signal generation. Modified from reference 53. doi:10.1128/9781555817381.ch6.f11

PCR methods lend themselves well to quantitative applications because analysis is performed early in the log phase of product accumulation, and as a result, they are less prone to error resulting from differences in sample-to-sample amplification efficiency. However, the multiplexing capabilities of these methods are limited due to the overlapping absorption and emission spectra of available fluorophores, thus restricting the number of multiplexed targets to four or five (55).

Digital PCR

PCR exponentially amplifies nucleic acids and the number of amplification cycles, and the amount of amplicon allows the computation of the starting quantity of targeted nucleic acid. However, many factors complicate this calculation, often creating uncertainties and inaccuracies, particularly when the starting concentration is low. Digital PCR attempts to overcome these difficulties by transforming the exponential data from conventional PCR to digital signals that simply indicate whether or not amplification occurred (56–59). An additional benefit of digital PCR is that it can provide absolute quantification of target nucleic acid without reference standard curves.

Digital PCR is accomplished by capturing or isolating each individual nucleic acid molecule present in a sample within many chambers, zones, or regions that are able to localize and concentrate the amplification product to detectable levels. After PCR amplification, a count of the areas containing PCR product is a direct measure of the absolute quantity of nucleic acid in the sample. The capture or isolation of individual nucleic acid molecules may be done in capillaries, microemulsions, arrays of miniaturized chambers, or on surfaces that bind nucleic acids (Fig. 12). Digital PCR has many applications, including detection and quantification of low levels of pathogen sequences, rare genetic sequences, gene expression in single cells, and clonal amplification of nucleic acids for sequencing mixed nucleic acid samples. Clonal amplification enabled by digital PCR is a key element of many of the "next-generation" sequencing methods described later in this chapter. Digital PCR systems are available from RainDance Technologies (Billerica, MA), Bio-Rad Laboratories (Hercules, CA), Life Technologies (Carlsbad, CA), and Fluidigm Corp. (South San Francisco, CA).

Transcription-Based Amplification Methods

Nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification (TMA) are both isothermal RNA amplification methods modeled after retroviral replication (60–62). The methods are similar in that the RNA target is reverse transcribed into cDNA and then RNA copies are synthesized with an RNA polymerase. NASBA uses avian myeloblastosis virus RT, RNase H, and T7 bacteriophage RNA polymerase, whereas TMA uses an RT enzyme with endogenous RNase H activity and T7 RNA polymerase.

Amplification involves the synthesis of cDNA from the RNA target with a primer containing the T7 RNA polymerase promoter sequence (Fig. 13). The RNase H then degrades the initial strand of target RNA in the RNA-cDNA hybrid. The second primer then binds to the cDNA and is extended by the DNA polymerase activity of the RT, resulting in the formation of dsDNA containing the T7 RNA polymerase promoter. The RNA polymerase then generates multiple copies of single-stranded, antisense RNA. These RNA product molecules reenter the cycle, with subsequent formation of more double-stranded cDNA molecules that can serve as templates for more RNA synthesis. A 10^9-fold amplification of the target RNA can be achieved in <2 h by this method.

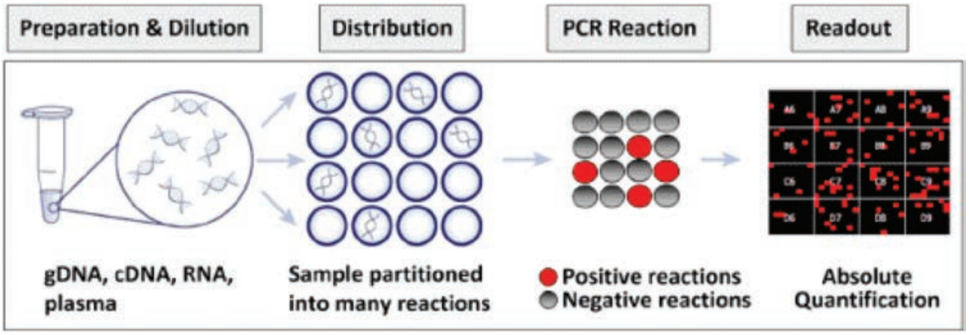

FIGURE 12 Digital PCR works by partitioning a sample into many nanoliter-scale individual PCR reactions, some of which contain a target molecule (red) while others do not (gray). Following PCR, the ratio of positive to negative reactions is used to calculate the starting number of target molecules. gDNA, genomic DNA. Reprinted with permission from Applied Biosystems QuantStudio OpenArray Digital PCR Application Note. doi:10.1128/9781555817381.ch6.f12

The single-stranded RNA products of TMA in the Gen-Probe tests are detected by modification of the hybridization protection assay. Oligonucleotide probes are labeled with modified acridinium esters with either fast or slow chemiluminescence kinetics so that signals from two hybridization reactions can be analyzed simultaneously in the same tube. The NASBA products in the bioMérieux (Durham, NC) tests are detected by hybridization with probes labeled with tris(2,2′-bispyridine)ruthenium and electrochemiluminescence. NASBA has also been used with molecular beacons to create a homogeneous, kinetic amplification system similar to real-time PCR (63).

Transcription-based amplification systems have several strengths, including no requirement for a thermal cycler, rapid kinetics, and a single-stranded RNA product that does not require denaturation prior to detection. Also, single-tube clinical assays and a labile RNA product may help minimize contamination risks. Limitations include the poor performance with DNA targets and concerns about the stability of complex multienzyme systems. Gen-Probe has developed FDA-cleared, TMA-based assays for detection of *Mycobacterium tuberculosis*, *C. trachomatis*, *N. gonorrhoeae*, HPV, and *T. vaginalis*. NASBA-based kits (bioMérieux) for the detection and quantification of HIV-1 RNA and detection of enterovirus and methicillin-resistant *S. aureus* are commercially available. A basic NASBA kit is also available for the development of other applications defined by the user. In its latest iteration, NucliSENS EasyQ, NASBA is coupled with molecular beacons for real-time amplification and detection of target nucleic acids (64).

Strand Displacement Amplification

Strand displacement amplification (SDA) is an isothermal template amplification technique that can be used to detect trace amounts of DNA or RNA of a particular sequence. SDA, as it was first described, was a conceptually straightforward amplification process with some technical limitations (65). Since its initial description, however, it has evolved into a highly versatile tool that is technically simple to perform but conceptually complex. SDA is the intellectual property of BD Diagnostics.

In its current iteration, SDA occurs in two discrete phases, target generation and exponential target amplification (66). Both are illustrated in Fig. 14. In the target generation phase, a dsDNA target is denatured and hybridized to two different primer pairs, designated as bumper and amplification primers. The amplification primers include the single-stranded restriction endonuclease enzyme sequence for BsoB1 located at the 5′ end of the target-binding sequence. The bumper primers are shorter and anneal to the target DNA just upstream of the region to be amplified. In the presence of BsoB1, an exonuclease-free DNA polymerase, and a dNTP mixture consisting of dUTP, dATP, dGTP, and thiolated dCTP (C_s), simultaneous extension products of both the bumper and amplification primers are generated. This process displaces the amplification primer products, which are available for hybridization with the opposite-strand bumper and amplification primers.

The simultaneous extension of opposite-strand primers produces strands complementary to the product formed by extension of the first amplification primer, with C_s incorporated into the BsoB1 cleavage site. This product enters the exponential target amplification phase of the reaction. The BsoB1 enzyme recognizes the double-stranded site, but because one strand contains C_s, it is nicked rather than cleaved by the enzyme. The DNA polymerase then binds to the nicked site and begins synthesis of a new strand while simultaneously displacing the downstream strand. This step recreates the double-stranded species with the hemimodified restriction endonuclease recognition sequence, and the iterative nicking and displacement process repeats. The displaced strands are capable of binding to opposite-strand primers, which produces exponential amplification of the target sequences.

These single-stranded products also bind to detector probes for real-time detection. The detector probes are ssDNA molecules with fluorescein and rhodamine labels. The region between the labels includes a stem-loop structure. The loop contains the recognition site for the BsoB1 enzyme. The target-specific sequences are located 3′ of the rhodamine label. In the absence of a specific target, the stem-loop structure is maintained with the fluorescein and rhodamine labels in close proximity. The net effect is that very little emission for the fluorescein is detected after excitation. After SDA, the probe is converted to a double-stranded species, which is cleaved by BsoB1. The cleavage causes physical separation of the fluorescein and rhodamine labels, which results in an increase in emission from the fluorescein label.

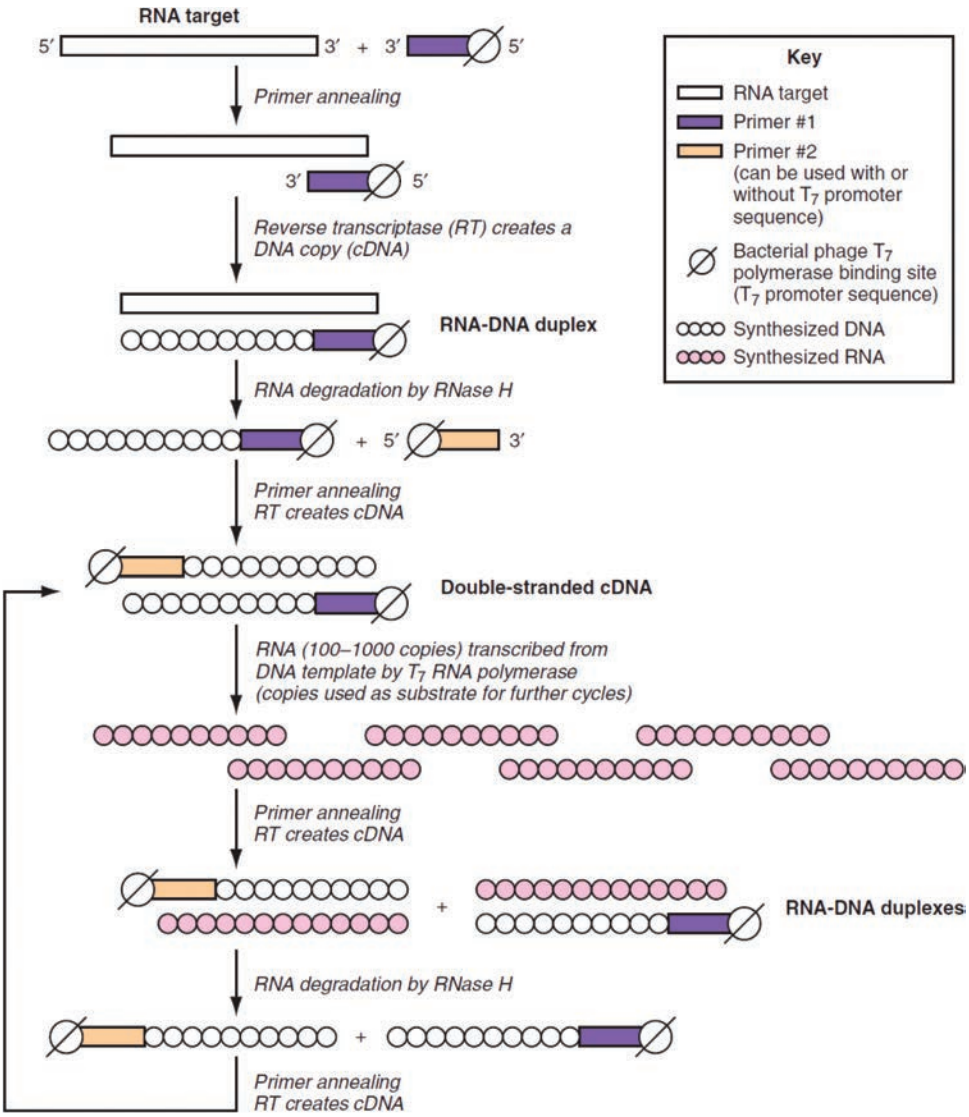
RNA target
5′
3′
+
3′
5′
Primer annealing
3′
5′
Reverse transcriptase (RT) creates a DNA copy (cDNA)
RNA-DNA duplex
RNA degradation by RNase H
+
5′
3′
Primer annealing
RT creates cDNA
Double-stranded cDNA
RNA (100–1000 copies) transcribed from DNA template by T$_7$ RNA polymerase (copies used as substrate for further cycles)
Primer annealing
RT creates cDNA
+
RNA-DNA duplexes
RNA degradation by RNase H
+
Primer annealing
RT creates cDNA
Key
RNA target
Primer #1
Primer #2 (can be used with or without T$_7$ promoter sequence)
Bacterial phage T$_7$ polymerase binding site (T$_7$ promoter sequence)
Synthesized DNA
Synthesized RNA

FIGURE 13 Transcription-based target amplification. NASBA and TMA are examples of transcription-based amplification systems. Reprinted with permission of Elsevier from reference 219.
doi:10.1128/9781555817381.ch6.f13

SDA has a reported sensitivity high enough to detect as few as 10 to 50 copies of a target molecule (65). By using a primer set designed to amplify a repetitive sequence with 10 copies in the *M. tuberculosis* genome, the assay is sensitive enough to detect 1 to 5 genome copies from the bacterium. SDA has also been adapted to quantify RNA by adding an RT step (RT-SDA). In this case, a primer hybridizes to the target RNA and an RT synthesizes a cDNA molecule. This cDNA can then serve as a template for primer incorporation and strand displacement. The products of this strand displacement then feed into the amplification scheme described above. RT-SDA has been used for the determination of HIV viral load (67). FDA-cleared tests using SDA for the direct detection of *C. trachomatis*, *N. gonorrhoeae*, and herpes simplex virus (HSV) 1 and 2 in urogenital specimens are available from BD Diagnostics. These assays can be run on either a semiautomated (ProbeTec) or fully automated (Viper) system.

The main advantage of SDA is that it is an isothermal process that, unlike PCR, can be performed at a single temperature after initial target denaturation. This eliminates the need for expensive thermal cyclers. Furthermore, samples can be subjected to SDA in a single tube, with amplification times varying from 30 min to 2 h. The main disadvantage of SDA lies in the fact that, unlike those at which PCR is performed, the relatively low temperature at which SDA is carried out (52.5°C) can result in nonspecific primer hybridization to sequences found in complex mixtures such as genomic DNA. Hence, when the target is in low abundance compared to background DNA, nonspecific amplification products can swamp the system, decreasing the sensitivity of the technique. However, the use of organic solvents to increase stringency at low temperatures and the recent introduction of more thermostable polymerases capable of strand displacement have alleviated much of this problem.

Loop-Mediated Amplification

Loop-mediated amplification (LAMP) is an isothermal method that relies on autocycling strand displacement DNA

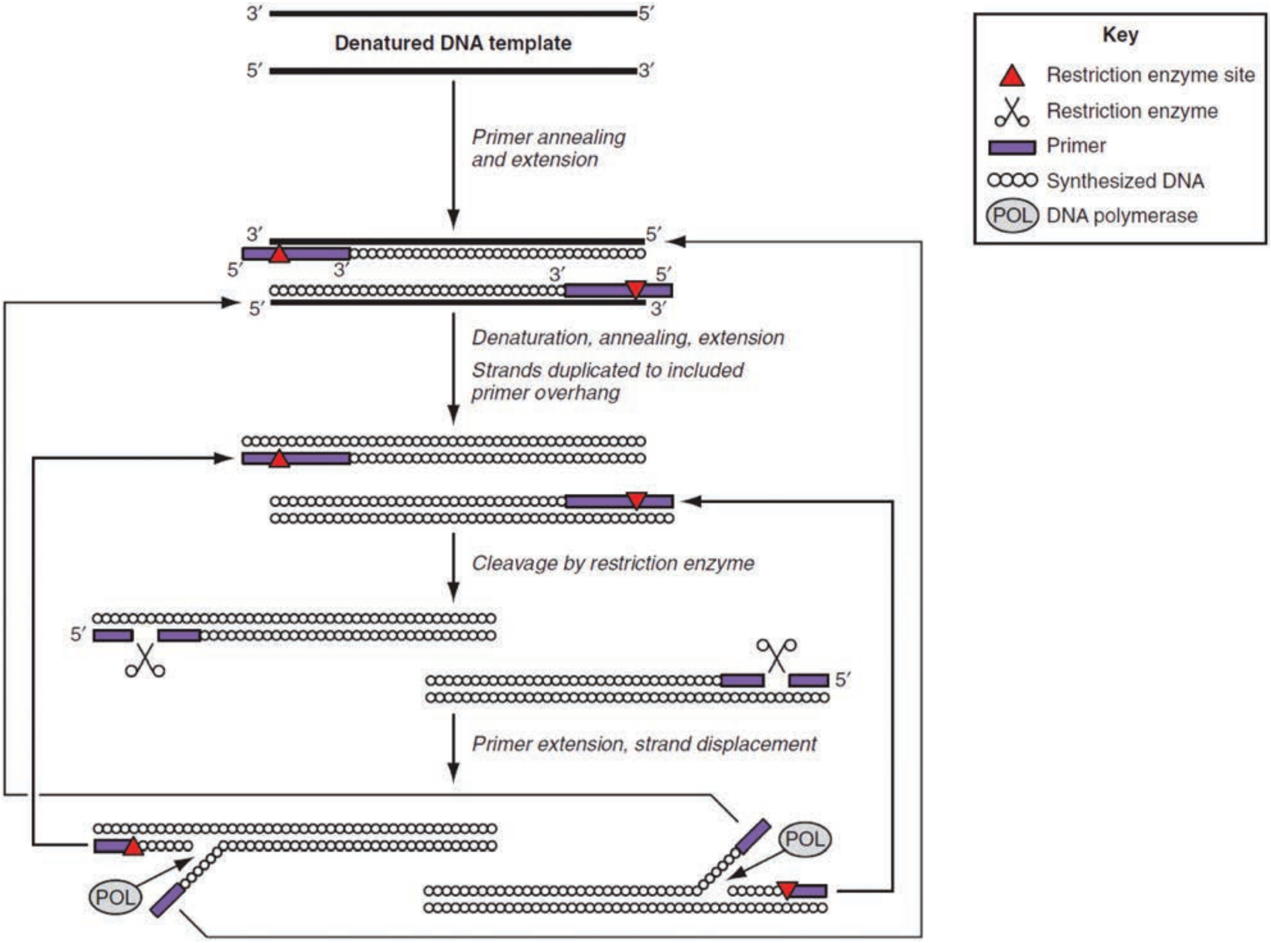
Denatured DNA template
3′
5′
Primer annealing and extension
Denaturation, annealing, extension
Strands duplicated to included primer overhang
Cleavage by restriction enzyme
Primer extension, strand displacement
POL
Key
Restriction enzyme site
Restriction enzyme
Primer
Synthesized DNA
DNA polymerase

FIGURE 14 Strand displacement target amplification. Modified from reference 219.
doi:10.1128/9781555817381.ch6.f14

synthesis by *Bst* DNA polymerase and a set of four to six primers (68). Two inner and two outer primers define the target sequence, and an additional set of loop primers is added to increase the sensitivity of the reaction. The final products of the LAMP reaction are DNA molecules with a cauliflower-like structure of multiple loops consisting of repeats of the target sequence (Fig. 15) (69). The products can be analyzed in real time by monitoring the turbidity in the reaction tube resulting from production of magnesium pyrophosphate precipitate during the DNA amplification. Amplification products can also be visualized in agarose gels after electrophoresis and staining with ethidium bromide or SYBR green.

LAMP has been used successfully in a number of laboratory-developed assays to detect DNA and RNA viruses (70–73) and diagnose mycobacterial infections (74). Since LAMP is an isothermal process and positive reactions can be detected by simple turbidity measurements or visualized directly with the naked eye, it requires no expensive equipment. These attributes make it an attractive technology for resource-poor settings and field use (75). However, primer design for LAMP is more complex than for PCR, with specialized training and software required for their design. Meridian Bioscience, Inc. (Cincinnati, OH) has licensed LAMP technology from Eiken Chemical Company, Ltd. (Tokyo, Japan) for the development of infectious-disease diagnostics in the United States. Meridian currently has FDA-cleared tests for detection of *Clostridium difficile*, *Mycoplasma pneumoniae*, and group A and group B beta-hemolytic streptococci (76).

Helicase-Dependent Amplification

Helicase-dependent amplification (HDA) is an isothermal process developed by BioHelix (Beverly, MA) that uses helicase to separate dsDNA and generate single-stranded templates for primer hybridization and subsequent extension by a DNA polymerase (77). As the helicase unwinds dsDNA enzymatically, the initial heat denaturation and subsequent thermocycling steps required by PCR can all be omitted. In HDA, strands of dsDNA are separated by the DNA helicase and the ssDNA-coated ssDNA-binding proteins. Two sequence-specific primers hybridize to each border of the target sequence, and a DNA polymerase extends the primers annealed to the target sequence to produce dsDNA. The two newly synthesized products are used as substrates by the helicase in the next round of amplification. Thus, a simultaneous chain reaction proceeds, resulting in exponential amplification of the selected target sequence (Fig. 16).

HDA is compatible with multiple detection technologies, including qualitative and quantitative fluorescence technologies, and with instruments designed for real-time

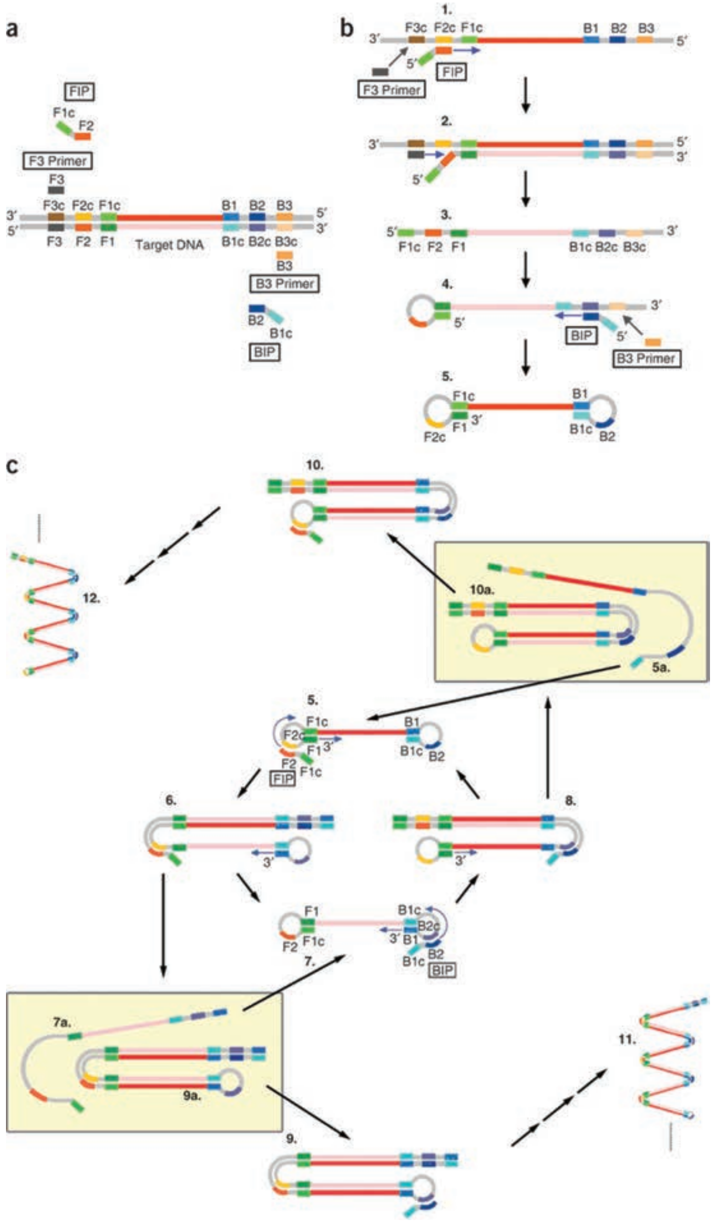

FIGURE 15 (a) Primer design of the LAMP reaction. For ease of explanation, six distinct regions are designated on the target DNA, labeled F3, F2, F1, B1c, B2c, and B3 from the 5′ end. As c represents a complementary sequence, the F1c sequence is complementary to the F1 sequence. Two inner primers (FIP and BIP) and outer primers (F3 and B3) are used in the LAMP method. FIP (BIP) is a hybrid primer consisting of the F1c (B1c) sequence and the F2 (B2) sequence. (b) Starting structure-producing step. DNA synthesis initiated from FIP proceeds as follows. The F2 region anneals to the F2c region on the target DNA and initiates the elongation. DNA amplification proceeds with BIP in a similar manner. The F3 primer anneals to the F3c region on the target DNA, and strand displacement DNA synthesis takes place. The DNA strand elongated from FIP is replaced and released. The released single strand forms a loop structure at its 3′ end (structure 3). DNA synthesis proceeds with the ssDNA as the template and BIP and B3 primer, in the same manner as described earlier, to generate structure 5, which possesses the loop structure at both ends (dumbbell-like structure). (c) Cycling amplification step. Using self-structure as the template, self-primed DNA synthesis is initiated from the 3′-end F1 region, and the elongation starts from FIP, annealing to the single strand of the F2c region in the loop structure. Passing through several steps, structure 7 is generated, which is complementary to structure 5, and structure 5 is produced from structure 8 in a reaction similar to that which led from structures 5 to 7. Structures 9 and 10 are produced from structures 6 and 8, respectively, and more elongated structures (11, 12) are also produced. Reprinted with permission of Nature Publishing Group from reference 69. doi:10.1128/9781555817381.ch6.f15

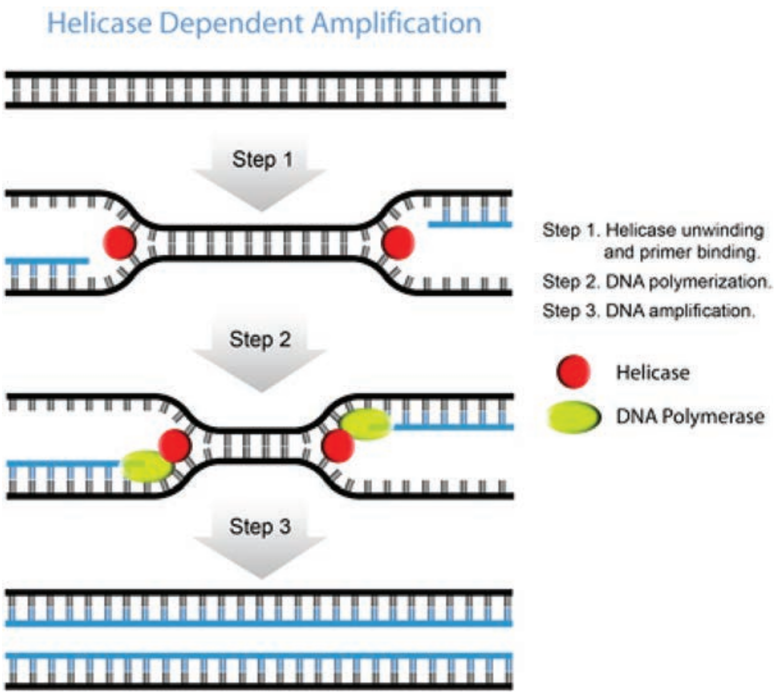
Helicase Dependent Amplification
Step 1
Step 2
Step 3
Step 1. Helicase unwinding and primer binding.
Step 2. DNA polymerization.
Step 3. DNA amplification.
Helicase
DNA Polymerase

FIGURE 16 HDA amplifies target sequences using two sequence-specific primers flanking the fragment to be amplified and a mixture of enzymes for DNA strand separation and polymerization. In the first step of the HDA reaction, the helicase enzyme loads onto the template and traverses along the target DNA, disrupting the hydrogen bonds linking the two strands. Exposure of the single-stranded target region by helicase allows primers to anneal. The DNA polymerase then extends the 3′ ends of each primer using free deoxynucleotides (dNTPs) to produce two DNA replicates. The two replicated DNAs independently enter the next cycle of HDA, resulting in exponential amplification of the target sequence. Reprinted with permission of BioHelix from http://www.biohelix.com/HDA_mechanism.asp. doi:10.1128/9781555817381.ch6.f16

PCR (78). Furthermore, HDA has shown potential for the development of simple, portable DNA diagnostic devices to be used in the field or at the point of care (79–81). FDA-cleared tests for detection of HSV 1 and 2 DNA in oral and genital lesions are available from BioHelix and for detection of *C. difficile* from Quidel (San Diego, CA).

POSTAMPLIFICATION DETECTION AND ANALYSIS

Gel Analysis

Visualization of amplification products in agarose gels after electrophoresis and ethidium bromide staining was the earliest detection method. After gel electrophoresis, DNA is often transferred onto a nitrocellulose or nylon membrane and hybridized to a specific probe to increase both the sensitivity and the specificity of detection. Membranes with bound radiolabeled probes are placed in proximity to X-ray film, and the hybrids are visualized as dark bands. Enzyme-labeled probes can be visualized through either light or color production after the addition of the appropriate chemiluminescent or chromogenic substrates. Many of these nonisotopic approaches are at least as sensitive as isotopic methods and are faster. In addition, the enzyme-labeled probes are more stable. Although gel electrophoresis and blotting remain important research tools, these techniques are being replaced by faster and simpler methods in the clinical laboratory.

Single-strand conformation polymorphism (SSCP) analysis and restriction fragment length polymorphism (RFLP) analysis have been used to ascertain information about the base compositions of the amplification products visualized in a gel. In SSCP analysis, the PCR product is denatured and then subjected to electrophoresis in a nondenaturing gel (82). Variations in the physical conformations of the PCR products are related to the base compositions and are detected by differential gel migration. This technique has successfully been used to detect mutations causing rifampin resistance in *M. tuberculosis* (83).

RFLP analysis uses restriction endonucleases to cleave amplification products at specific recognition sites. The fragments are separated by electrophoresis, and the resulting banding pattern provides information about the nucleic acid sequence. When coupled with a hybridization reaction, RFLP analysis can also provide information about the location and number of loci homologous to the probe. Both SSCP analysis and RFLP analysis of short products have largely been replaced by direct DNA sequencing as this technology has improved and the costs have decreased.

Capillary Electrophoresis

Capillary electrophoresis allows for accurate size discrimination of fluorescently labeled nucleic acids from 50 to 1,000 bases with single-base precision. PCR and capillary electrophoresis have been functionally integrated by PrimeraDx (Mansfield, MA) to produce highly multiplexed assays that can simultaneously detect up to 60 targets whose identities are defined by the specific size of the corresponding amplicons (84, 85).

PrimeraDx developed a multiplexed assay for the simultaneous quantification of cytomegalovirus (CMV), HSV, BK virus, human herpesvirus 6 (HHV-6), and HHV-7 viral loads that integrates PCR and capillary electrophoresis. In this assay, amplification of the nucleic acid targets is monitored by sampling the PCR during sequential cycles and separating and quantifying the PCR products by capillary electrophoresis. These data are used to construct amplification curves. Similar to real-time PCR amplification, a cycle threshold is determined from the amplification curve for each of the targets in the exponential phase of amplification. Unlike real-time PCR, where standards in a separate reaction are used, the PrimeraDx assay uses multiple internal standards in each reaction to generate calibration curves for each individual assay in the multiplex reaction. Qualitative assays for detection of fungal pathogens and respiratory viruses have also been developed. PrimeraDx's ICEPlex system is an automated, high-throughput instrument that performs the multiplex real-time PCR and capillary electrophoresis.

Seegene, Inc. (Seoul, South Korea) has developed a wide variety of multiplexed infectious-disease assays, including sexually transmitted disease, HPV genotyping, mycobacterial, and respiratory pathogen panels (23, 86). The targets of these multiplexed assays are designed to be discriminated by size and are compatible with several different microfluidic and capillary electrophoresis systems, including the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and Applied Biosystems (Foster City, CA) sequencers.

Colorimetric Microtiter Plate Systems

Colorimetric microtiter plate (CMP) systems are convenient alternatives to traditional gel and blotting techniques for detection of amplified products. In these systems, the amplified product is captured in microtiter plate wells by specific oligonucleotide probes coating the plastic surface. Bound product is detected by a color change that takes place after addition of an enzyme conjugate and the appropriate substrate. These systems resemble enzyme immunoassays and use microtiter plate washers and readers commonly found in clinical laboratories. CMP systems are more practical and faster than the traditional membrane hybridization techniques described above.

Several variations of CMP systems are commercially available. In one popular approach, biotinylated primers are used to amplify the target, and the biotin-containing PCR product is denatured and added to the microtiter well. After hybridization with a capture probe, the bound product is detected with a streptavidin-enzyme conjugate and a chromogenic substrate (87). Enzyme-conjugated antibodies directed against dsDNA have also been used to detect PCR products in CMP systems (88). Another approach uses digoxigenin-dUTP to label the PCR product and enzyme-conjugated antidigoxigenin antibodies to detect the captured product (221). With the development and widespread adoption of real-time amplification and detection systems, CMP systems are now used infrequently in clinical laboratory settings.

Allele-Specific Hybridization

Line probe assays are manufactured by Innogenetics (Ghent, Belgium) for genotyping of HCV, HPV, and HBV; identification of mycobacteria; and analysis for drug resistance mutations in HBV and M. *tuberculosis* (89–94). The HCV line probe assays are distributed by Siemens. In these assays, a series of probes with poly(T) tails are attached to nitrocellulose strips. Biotin-labeled PCR product is then hybridized to the immobilized probes on the strip. The labeled PCR product hybridizes only to the probes that give a perfect sequence match under the stringent hybridization conditions used. After hybridization, streptavidin labeled with alkaline phosphatase is added and binds to the biotinylated hybrids. Incubation with a chromogen results in a purple precipitate. The pattern of hybridization provides information about the nucleic acid sequence of the amplicon. This method is capable of detecting single-nucleotide polymorphisms.

A line probe for identification of 37 HPV genotypes is available from Roche Diagnostics (Indianapolis, IN) and is registered for use in the European Union (95). The method employs multiplex PCR with biotinylated primers targeted to the L1 region of the HPV genome and a linear array of L1 sequence-specific probes fixed to a nitrocellulose strip. The pattern of hybridization provides the genotype and is determined as described above.

Nucleic Acid Sequencing

The combination of PCR and Sanger dideoxynucleotide chain termination methods can be used to determine DNA sequences in clinical samples (96). The application of capillary electrophoresis techniques to the separation of PCR and dideoxy chain termination products has streamlined the sequencing process by eliminating some of the labor-intensive steps, which makes the technology a better fit for diagnostic applications (97). The current detection method for automated Sanger sequencing involves size-dependent separation of fluorescently labeled DNA fragments by capillary electrophoresis. The different fluorescent tags on the fragments are interrogated by a laser as the fragments pass in single file in the gel-filled capillary tube. The information is collected by a computer, which generates chromatograms using a different color for each of the four bases, from which the template DNA sequence can be determined. Typically automated Sanger sequencing is accurate for sequences up to 800 bp in length. To obtain a full sequence of longer regions of DNA, it is necessary to break it into smaller pieces and sequence them individually. This is typically at random (shotgun sequencing) or in an ordered stepwise fashion (primer walking).

CLIP, a coupled amplification and sequencing method, uses oligonucleotide primers labeled with different fluorescent dyes, standard dideoxynucleotide termination reagents, and PCR to produce extension products that end with a chain-terminating nucleotide (98). The nucleic acid sequence is deduced from the electrophoretic mobilities of the different extension products from a set of four reactions, each product containing a different chain-terminating nucleotide. A unique feature of CLIP sequencing is that one reaction produces sequence information for both nucleic acid strands. CLIP sequencing serves as the basis for commercially available assays for HIV-1 drug resistance (Siemens Medical Solutions, Malvern, PA).

The ViroSeq HIV-1 genotyping assays (Celera Diagnostics, Alameda, CA; distributed by Abbott Molecular, Des Plaines, IL) also use dideoxy chain-terminating sequencing, but each dideoxynucleotide is labeled with a different fluorescent dye. Each reaction mixture contains one primer but all four uniquely labeled dideoxynucleotides. Separation of the terminated PCR products is done by capillary electrophoresis.

Although direct sequencing of PCR products by capillary electrophoresis is a powerful research tool, its routine use in the clinical laboratory depends on the development of high-throughput systems with integrated databases and data analysis software. Such systems are available for HIV-1 and HCV genotyping and for identification of bacteria and fungi by rRNA gene sequence analysis.

Pyrosequencing (Qiagen) represents an alternative approach to conventional sequencing and is useful for genotyping and short-read-length sequencing (99). Pyrosequencing is based on the luminometric detection of pyrophosphate that is generated during DNA synthesis.

A sequencing primer is hybridized to a single-stranded PCR amplicon and incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase, and apyrase and the substrates adenosine 5′-phosphosulfate and luciferin. The first of four dNTPs is added to the reaction mixture. DNA polymerase catalyzes the incorporation of the dNTP into the DNA strand. Each incorporation event is accompanied by release of pyrophosphate (PP_i) in a quantity equimolar to the amount of incorporated nucleotide. The ATP sulfurylase quantitatively converts PP_i to ATP in the presence of adenosine 5′-phosphosulfate. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin, which generates light in amounts that are proportional to the amount of ATP. The light produced in the reaction is detected by a charge-coupled device camera. A program is produced in which the height of each peak is proportional to the number of nucleotides incorporated. Apyrase, a nucleotide-degrading enzyme, continuously degrades ATP and unincorporated dNTPs. This degradation switches off the light and regenerates the reaction solution. The next dNTP is added, and the process is repeated.

Pyrosequencing has been used in microbiology to detect drug resistance mutations and to identify and type bacteria, viruses, and fungi (100–103). Unlike conventional sequencing strategies, pyrosequencing provides reliable data for sequences adjacent to the sequencing primer termini. Pyrosequencing provides a simple-to-use and robust platform for short-read-length sequencing.

Multiple new sequencing technology platforms have emerged since 2005 and have greatly surpassed conventional dideoxynucleotide chain termination methods in terms of increased total sequence production and decreased cost. Collectively, these new sequencing methods are referred to as next-generation or massively parallel sequencing, and they have considerable potential for clinical diagnostics (104–106). The major next-generation sequencing platforms as of this writing are the Roche 454 GS-FLX (454, Branford, CT), the Illumina HiSeq and MiSeq (Illumina, San Diego, CA), the ABI SOLiD (Applied Biosystems), and the Ion Torrent (Life Technologies). The sequencing methods, read lengths, depths of coverage, run times, total bases per run, sequence accuracies, usability, and costs vary for each of these platforms.

The Roche 454 platform, using the pyrosequencing technology described earlier to carry out hundreds of thousands of sequencing reactions simultaneously on independent beads, works as follows. Target DNA is first randomly sheared into fragments and then ligated to adapters. Single-stranded template DNA is isolated, mixed with beads, and then subjected to emulsion PCR to clonally amplify the template on each bead. The beads are then distributed into a "picotiter plate" that contains millions of tiny wells. Within each well that receives an individual bead, an isolated environment is created for the sequencing of each template, resulting in massively parallel sequencing of different templates simultaneously.

DNA templates sequenced with the Illumina systems are ligated to adapter sequences that incorporate a sequence complementary to anchor oligonucleotides, which are covalently linked to the surface of a flow cell. After annealing to the anchor oligonucleotides, the template DNA molecules are clonally amplified in a modified isothermal PCR termed a "bridge PCR," in which the DNA molecules are free to flex and form a bridge with an adjacent anchor oligonucleotide. Bridge amplification generates clusters of amplified template on the solid surface, where each cluster represents a different template. Next, the clusters are denatured to provide a single-stranded template, and a sequencing primer is hybridized to the strand. It uses a unique sequencing chemistry that incorporates fluorescently labeled, reversible terminator nucleotides. These nucleotides are labeled with different-colored fluorophores so that all four nucleotides can be added to the reactions simultaneously. Only one terminator nucleotide can be incorporated into each sequence during one sequencing cycle, and the color of the fluorescent label incorporated into the sequences of each cluster is recorded. Removal of the terminator group on the nucleotide just added enables incorporation of the next complementary nucleotide, and the cycle is repeated.

The ABI SOLiD system chemistry starts with emulsion PCR of adapter-modified ssDNA molecules. After PCR, the templates are denatured and bead enrichment is performed to select beads with extended templates. The template on the selected beads undergoes a 3′ modification to allow covalent binding to a glass slide. The modified beads are deposited randomly on the slide. The sequencing occurs by ligation. Primers hybridize to the adapter sequence within the library template. A set of four fluorescently labeled di-base probes compete for ligation to the sequencing primer. Specificity of the di-base probe is achieved by interrogating every first and second base in each ligation reaction. Multiple cycles of ligation, detection, and cleavage are performed, with the number of cycles determining the eventual read length. Following a series of cycles, the extension product is removed and the template is reset with a primer complementary to the $n - 1$ position for a second round of ligation cycles. Five rounds of primer resets are completed for each sequencing tag. Consequently, each base is interrogated in two independent ligation reactions by two different primers. This dual interrogation provides highly accurate sequences.

The Ion Torrent system employs an integrated circuit to directly perform nonoptical DNA sequencing in a massively parallel fashion (107). Genomic DNA is fragmented and ligated to adapters, and adapter-ligated libraries are clonally amplified onto 2-μm acrylamide beads. Template-bearing beads are enriched through a magnetic bead process. Sequencing primers and DNA polymerase are then bound to the templates and pipetted into the ion chip's loading port. The chip contains millions of sensor wells. Individual beads are loaded into the individual sensor wells by spinning the chip in a centrifuge. The well depth allows only a single bead to occupy a well.

In ion sequencing, all four nucleotides are provided in a stepwise fashion during an automated run. When the nucleotide in the flow is complementary to the template base directly downstream from the sequencing primer, the nucleotide is incorporated into the nascent strand by the bound polymerase. This increases the length of the sequencing primer by one base, or more if a homopolymer stretch is directly downstream from the primer, and results in hydrolysis of the incoming nucleotide triphosphate, which causes the liberation of a single proton for each nucleotide incorporated during that flow. The release of the proton produces a shift in the pH of the surrounding solution proportional to the number of nucleotides incorporated in the flow. This is detected by the sensor on the bottom of each well, converted to a voltage, and digitized by off-chip electronics. After the flow of each nucleotide, a wash is used to ensure that nucleotides do not remain in the well. To change raw voltages into base calls, signal processing software converts the raw data into measurements of incorporation in each well for each successive nucleotide flow using a physical model. The use of most widely used technology for constructing integrated circuits allows the manufacturer to produce a more scalable and lower-cost alternative to many of the currently available massively parallel sequencing systems.

Next-generation sequencing will have a major impact on genomics research. In the field of medical microbiology, applications are evolving in the areas of metagenomics, microbial identification, and detection of rare mutations. Ultradeep sequencing can detect rare viral variants consisting of as little as 1% of the population, levels far deeper than those achievable with traditional Sanger sequencing methods, and the detection of these low-abundance drug resistance mutations may significantly affect treatment outcomes in HIV-1 and HCV infections (108–110). The CLSI has recently developed guidelines for nucleic acid sequencing in clinical laboratories (106).

Hybridization Arrays

High-Density Arrays

High-density DNA hybridization arrays are produced by attaching or synthesizing hundreds or thousands of oligonucleotides on a solid support in precise patterns. A labeled amplification product is hybridized to the probes, and hybridization signals are mapped to various positions within the array. If the number of probes is sufficiently large, the sequence of the PCR product can be deduced from the pattern of hybridization (resequencing arrays). A number of manufacturers have developed high-density DNA microarrays and the instrumentation required to acquire and analyze the data, including Affymetrix (Santa Clara, CA), Roche NimbleGen (Madison, WI), and Agilent Technologies. Hybridization arrays have a number of applications in microbiology, including microbial and host gene expression profiling, pathogen identification, antimicrobial resistance detection, viral discovery, molecular surveillance, strain typing, and diagnostic sequencing (111). The CLSI has published a guideline for the use of diagnostic nucleic acid microarrays (112).

One of the most developed approaches brings together advances in synthetic nucleic acid chemistry with photolithography, a process used in the manufacture of semiconductors for the computer industry (Affymetrix). This approach uses light to direct the synthesis of short oligonucleotides (20 to 25 bp) on a silica wafer (113). On a 15-mm-square chip, thousands of individual sites or features can be established. At each feature, specific oligonucleotides are assembled one nucleotide at a time by light-activated chemistry.

NimbleGen's approach to *in situ* synthesis of oligonucleotide probes on silica wafers is similar to that of Affymetrix, but photolithographic masks are replaced by virtual or digital masks. The array synthesizer uses an array of programmable micromirrors to create digital masks that reflect the desired pattern of UV light to deprotect the features where the next nucleotide will be coupled with probe size up to 85 bp. Each array contains $>10^6$ features.

Agilent microarrays use glass slides and ink-jet printing, which eliminates the need for either lithographic or digital masks. The *in situ* synthesis of 60-mer oligonucleotides is achieved using five-"ink" (four bases plus catalyst) printing of nucleotide precursors combined with coupling and deprotection steps (111). Agilent microarrays are available in a number of formats with up to 244,000 features.

Due to the complex nature of the chemical synthesis and the expense involved in production of *in situ*-synthesized arrays, this is limited to commercial manufacturers. Consequently, they are not conducive to user-defined or laboratory-developed applications.

Another method of producing DNA hybridization arrays involves the precise micropipetting of premade dsDNA probes (typically 200 to 2,000 bp in length) onto glass slides with a robotic device (114, 115). These arrays are not suitable for mutation detection due to the size and density of the arrayed DNA probes but have facilitated gene expression profiling. DNA arrays of this type can be used to determine the activation states (mRNA levels) of thousands of genes simultaneously. Gene expression profiling of pathogens by use of arrays may provide new insights into pathogenic mechanisms and help identify new therapeutic and vaccine targets.

High-density microarrays coupled with sequence-independent PCR have also been used in the discovery and characterization of pathogens and have the potential to provide rapid, unbiased, differential diagnosis of infectious diseases. Wang et al. described the first microarray designed to detect large numbers of viruses (116). The microarray consisted of 1,600 70-mer oligonucleotides derived from 140 different virus species, with an average of 10 oligonucleotides per virus species. They demonstrated that a wide variety of viruses could be detected by the microarray with sensitivities and specificities similar to those of individual virus-specific PCR assays (117). In addition, this approach has facilitated the discovery of a number of novel viruses from humans and animals, including the severe acute respiratory syndrome coronavirus (118). The current version of the panviral microarray contains 60,000 probes representing all viral species in GenBank. The field of diagnostic microarrays is rapidly developing, with multiple broad-range microarrays described (119–122).

There are a variety of sample preparation methods for the different array types, but all share a few fundamental characteristics. All methods start with extraction of total RNA, poly(A), or genomic DNA that is then converted to either cDNA or cRNA by enzymatic methods that modestly amplify the sample with tagging or incorporating biotinylated or fluoresceinated nucleotides. In expression applications, the amplification must maintain the relative abundance levels of the different transcripts present, whereas for resequencing applications, the relative abundance of information is rarely important. The DNA chip is hybridized in a flow cell with the sample for 2 to 12 h. After hybridization, a scanning laser confocal microscope evaluates the surface fluorescence intensity of the chip. Automated scanning by the microscope takes only a few minutes to acquire an image of the entire surface of the chip, and computer software analyzes the fluorescent image and determines the nucleic acid sequence or gene expression profile of the sample.

High-density microarrays hold much promise for molecular diagnostics. However, the complexity of fabricating the arrays, limited commercial availability, and high test costs are obstacles to their routine use in clinical laboratories.

Low- to Moderate-Density Arrays

Recent developments of new detection techniques and simplified methodologies have facilitated the transition from expensive high-density arrays to cost-effective low- to medium-density systems for clinical diagnostics. The three microarray systems described in the following paragraphs all are FDA-cleared platforms.

The INFINITI analyzer (AutoGenomics, Carlsbad, CA) is a fully automated, multiplexing platform that uses novel BioFilmChip microarrays for a wide range of molecular diagnostic applications. Fluorescent-labeled PCR amplicons are hybridized to probes immobilized on a BioFilmChip microarray. The microarray is film-based microarray, which consists of multiple layers of thin hydrogel matrices on a polyester solid support. Each spot on the array is scanned with a built-in confocal microscope. The system has integrated controls for all steps and automatically processes and analyzes data. Infectious-disease applications available as research-use-only (RUO) assays include microarrays for detection of drug resistance in *M. tuberculosis*, respiratory viruses, sexually transmitted disease agents, and nontuberculous mycobacteria (123).

The Verigene system (Nanosphere, Inc., Northbrook, IL) uses gold nanoparticle-labeled probes to detect target nucleic acid hybridized to capture oligonucleotides arrayed on a glass slide. Silver signal amplification is then performed on the gold nanoparticle probes that are hybridized to the captured DNA targets of interest. The Verigene Reader optically scans the slide for silver signal, processes the data, and produces a qualitative result. Tests for the detection of influenza A, influenza B, respiratory syncytial virus, and *C. difficile* and for the identification of Gram-positive bacteria and selected antimicrobial resistance genes from positive blood culture bottles have been cleared by the FDA for the Verigene system (124, 125). A Gram-negative bacteria panel for positive blood cultures, an enteric pathogen panel, and an expanded respiratory pathogen panel are in the development pipeline.

The eSensor system (GenMark Dx, Carlsbad, CA) uses electrochemical-detection-based DNA microarrays (126). These microarrays are composed of a printed circuit board consisting of an array of 76 gold-plated electrodes. Each electrode is modified with a multicomponent, self-assembled monolayer that includes presynthesized oligonucleotide capture probes. Nucleic acid detection is based on a sandwich assay principle. Signal and capture probes are designed with sequences complementary to immediately adjacent regions on the corresponding target DNA sequence. A three-member complex is formed between capture probe, target sequence, and signal probe based on sequence-specific hybridization. This process brings the 5′ end of the signal probe containing electrochemically active ferrocene labels into close proximity to the electrode surface.

The ferrous ion in each ferrocene group undergoes cyclic oxidation and reduction, leading to loss or gain of an electron, which is measured as current at the electrode surface using alternating-current voltammetry. Higher-order harmonic signal analysis also facilitates discrimination of ferrocene-dependent faradic current from background capacitive current.

The eSensor cartridge consists of a printed circuit board, a cover, and a microfluidic component. The microfluidic component includes a diaphragm pump and check valves in line with a serpentine channel that forms the hybridization channel above the array of electrodes. The eSensor instrument consists of a base module and up to three cartridge-processing towers, each with eight slots for cartridges. The cartridge slots operate independently of each other. The throughput of a three-tower system can reach 300 tests in 8 h. A respiratory pathogen panel for the eSensor system that detects 14 different types and subtypes of respiratory viruses is FDA cleared (127), and an HCV genotyping test is in the development pipeline.

Mass Spectrometry

One of the most exciting developments in clinical microbiology is the application of mass spectrometry (MS) to identification and characterization of pathogens (128, 129). All MS applications are based on direct measurement of two intrinsic properties of the analyte, molecular mass and charge. The mass spectrometer consists of three functional units. The first unit, the ion source, is used to ionize the analyte and transfer it to the gas phase. The second unit is the mass analyzer, which serves to separate the ions by their mass-to-charge ratio, which defines their time of flight (TOF). The analyzer can use a vacuum chamber and a static or dynamic magnetic/electric field to separate the ions. The third unit uses an electron multiplier or fast oscilloscope to detect the ions. A wide variety of ionization methods can be used, but matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) are used for analysis of proteins and nucleic acids that are too fragile to be ionized by older conventional methods.

MALDI-TOF MS of whole bacterial cells has greatly reduced the time needed to identify bacteria and fungi grown in culture and is increasingly being deployed in clinical laboratories. Two commercially available systems, the MALDI Biotyper (Bruker Daltronics, Billerica, MA) and VITEK MS (bioMérieux), are FDA cleared and are reviewed in chapter 4. MALDI-TOF and ESI MS can also be used to analyze PCR amplicons. Two fully integrated systems for infectious-disease applications are available from Ibis Biosciences/Abbott Molecular (Carlsbad, CA) and Sequenom (San Diego, CA) and are described below.

The T5000 Universal Biosensor/PLEX-ID (Ibis/Abbott Molecular) is a commercially available system capable of identification and characterization of a broad range of pathogens (130). In this system, all nucleic acids present in a clinical sample are extracted and aliquoted into wells of a microtiter plate that each contain one or more pairs of broad-range primers for PCR. The primers are designed to amplify a product from a selected group of microorganisms, for example, all bacteria, specific species, or individual strains. The PCRs produce a mixture of products reflecting the complexity of the original mixture of microorganisms present in the clinical sample.

The PCR products are desalted and sequentially electrosprayed into a mass spectrometer for TOF analysis. The spectral signals are processed to determine the masses of each of the PCR products present with sufficient accuracy that the base composition of each amplicon can be unambiguously deduced. Using the combined base compositions from multiple PCRs, the identities of the pathogens and their relative concentrations in the sample can be determined. Although it is not immediately intuitive, nucleic acid composition (i.e., the numbers of A's, G's, C's, and T's) in specific regions of the genome is equally as informative as the nucleic acid sequence. MS is remarkably sensitive and can measure the weight and determine the base composition from small quantities of nucleic acids in complex mixtures essentially instantaneously.

A key element of the Ibis system is a curated database of genomics that associates base counts with primer pairs for thousands of organisms. Broad-range PCRs are capable of producing products from groups of organisms rather than single species. That, coupled with the ability of the mass spectrometer to rapidly and accurately derive base compositions from PCR amplicons, provides high information content and obviates the need to anticipate which pathogen is present in the sample. The Ibis system has been used for the rapid identification and strain typing of a variety of bacteria, viruses, fungi, and protozoa (131). Although the PCR ESI MS system is no longer commercially available, Abbott plans to launch a new platform in 2014.

Sequenom developed comparative sequencing by base-specific cleavage and MALDI-TOF MS for automated, high-throughput microbial DNA sequence analysis in its iPlex MassArray system (132). In this innovative genotyping method, PCR-amplified signature sequences are subjected to *in vitro* transcription and base-specific RNA cleavage by RNase A. Mass signal patterns of the resulting cleavage products, a mixture of RNA fragments known as compomers, are acquired and provide a fingerprint of the microorganism. Each RNA compomer is defined by its nucleotide composition with the cleavage base terminating its 3′ end and thus by its mass in the resulting mass spectrum.

The list of detected experimental compomer masses is compared with a calculated list of molecular weights derived from an *in silico* digest of a set of reference sequences in the system database. The simulated patterns of the reference set are used to identify the microorganism by its best match to a reference sequence. Small differences between the best-matching reference and sample sequence show up as a difference between the *in silico* and detected sample spectra. They can be used to identify and localize sequence differences down to a single base change and identify novel sequences. Depending on the gene target, MALDI-TOF MS can provide high-level discrimination of individual microbial taxa or be used to identify lineages within a species (133–137).

QUANTITATIVE METHODS

Many of the methods discussed above can be used to quantify the amount of RNA or DNA in a clinical sample. The most commonly used methods include PCR and RT-PCR, transcription-based amplification, and bDNA assays. The principle of quantitative molecular methods is that there is a linear relationship between the quantity of the input template and the amount of the product or signal generated. Competitive PCR (cPCR) is a reliable and robust method that was the basis of the first generation of viral load assays for HIV-1 and HCV (Amplicor Monitor System; Roche Diagnostics) that were commonly used in clinical laboratories. These assays, based on conventional standard PCR, are still in use by clinical laboratories but are rapidly being replaced by real-time amplification methods. The basic concept behind cPCR is the coamplification in the same reaction tube of target and calibrator templates with equal or similar lengths and with the same primer-binding sequences (138). Since both templates are amplified with the same primer pair, identical thermodynamics and amplification efficiencies are ensured. The amount of the calibrator must be known, and after amplification, products from both templates must be distinguishable from each other. Different types of calibrators have been used in cPCR, but in general those calibrators similar in size and base composition to the target work most effectively. RNA competitors should be used in quantitative RT-PCRs to address the problem of variable RT efficiency. This competitive amplification approach has also been used effectively with transcription-based amplification methods using RNA targets and RNA calibrators.

For cPCR, the concentration of the target template in the clinical sample can be determined by a simple calculation. The yield of the PCR product is described by the equation $Y = I(1 + e)^n$, where Y is the quantity of the PCR product, I is the quantity of the template at the beginning of the reaction, e is the efficiency of the reaction, and n is the number of cycles. In cPCR, this equation is written for both templates, as follows: competitor, $Y_c = I_c(1 + e)^n$; target, $Y_t = I_t(1 + e)^n$. Since e and n are the same for both the competitor and the target, the relative product ratio, Y_c/Y_t, directly depends on the initial concentration ratio, I_c/I_t, and the function $Y_c/Y_t = I_c/I_t$ is linear.

Real-time amplification and detection methods are particularly well suited for quantification of nucleic acid because the amount of the fluorescent signal generated is proportional to the concentration of the target DNA or RNA in the original sample. Real-time PCR and transcription-based amplification methods are the most commonly used quantitative methods. For real-time PCR, the fluorescent signal is measured during the exponential phase of amplification, which is where the amplification plot crosses the threshold (Fig. 6). This is in contrast to standard PCR methods that measure the endpoint signal. There are advantages to measuring the fluorescent signal during the exponential phase of amplification; the reaction components are not limiting, and the assay is less sensitive to the effects of inhibitors. As a result, real-time PCR assays are more reproducible than standard PCR assays. Both internal and external calibrators can be used with real-time assays, but the improved precision of real-time assays allows more reliable results to be obtained with an external calibration curve than would be obtained with standard PCR. When external calibrators are used, a calibration curve is generated by plotting the $\log_{10}$ concentration of the external calibrator versus the C_T, and this plot is used to calculate the concentration of nucleic acid in the sample. The concentration of nucleic acid in the sample is inversely related to the C_T: the higher the concentration of the nucleic acid, the lower the C_T (40). In general, quantitative real-time PCR assays are not more sensitive than standard PCR assays; however, they have a much broader linear range, typically 6 to 7 orders of magnitude.

Digital PCR is the next advance in nucleic acid quantification. It can provide a lower limit of detection than real-time PCR methods with better precision at the very low concentrations. As opposed to relative quantification, digital PCR provides absolute quantification with no need for reference standards. Currently, digital PCR is used as a research tool, but it may find applications in clinical laboratories to resolve ambiguous results obtained with quantitative real-time PCR assays or for creating accurate viral reference standards as the technology becomes less costly (139–141). The CLSI has published guidelines for quantitative molecular methods for infectious diseases that address the development and application of quantitative PCR assays and other nucleic acid amplification methods (142).

AUTOMATION AND INSTRUMENTATION

Molecular assays consist of three major steps: specimen processing, nucleic acid amplification, and product detection. Sample processing is usually the most labor-intensive step and has represented the biggest challenge for manufacturers of automated test systems. However, in the past several years there have been considerable advances in this area with the availability of both semiautomated and fully automated systems. Automation of the nucleic acid extraction process offers laboratories several advantages, including ease of use, limited handling of the sample, improved reproducibility, reduced opportunity for cross contamination, and, for some systems, postelution functions such as adding samples into the master mix. These advantages need to be weighed against the costs of automated systems, the inflexibility of batch size, and the large sizes of many of the automated instruments. The systems vary in the types of nucleic acid extraction methods that they provide and include total nucleic acid, DNA-only, and RNA-only protocols. Other features of automated extraction systems to consider are the availability of protocols for various specimen types and volumes, variable elution volumes, the availability of target-specific and/or generic target extraction methods, and specimen throughput. The available automated systems range from fully automated high-throughput systems such as the MagNA Pure and COBAS AmpliPrep systems (Roche), *m*2000sp (Abbott), and QIAsymphony (Qiagen) to those designed for a small number of specimens such as BioRobot EZ1 (Qiagen) and NucliSENS (bioMérieux).

There are a wide variety of instruments commercially available for real-time PCR testing. These instruments vary as to speed, capacity of samples per test run, reaction volume, optics, and support for different fluorescence probe types. The time required for analysis depends to a great extent on the time required for thermocycling, and the speed of thermocycling depends on how quickly the instrument can change temperature over time. For example, some instruments can change the temperature at 20°C per s, permitting instrument analysis of up to 32 samples in as little as 30 min. Capacity may offset thermocycling speed. Although a higher-capacity instrument may have a longer thermocycling time than a lower-capacity instrument, potentially more samples may be analyzed by the high-capacity instrument in a specific time period than by the low-capacity instrument.

The reaction mixture volume assayed may also vary from one system to another. If target nucleic acid is present in extremely small amounts in a sample, an instrument that permits higher-volume analysis may be preferred.

Real-time PCR instruments utilize a variety of optics for fluorescence detection. A tungsten source lamp for excitation and selectable filters for excitation and emission wavelength detection are used in a number of instruments. Light-emitting diodes or laser excitation devices coupled with emission wavelength detection may also be used. The new real-time PCR instruments allow up to six different fluorogenic dyes to be used simultaneously in one reaction. The Prism series of sequence detection systems (Applied Biosystems), LightCycler (Roche), SmartCycler (Cepheid, Sunnyvale, CA), and Rotor-Gene (Qiagen) are examples of instruments designed initially for research applications that now find widespread use in molecular diagnostics laboratories. The COBAS TaqMan analyzer (Roche) and the *m*2000rt system (Abbott) were the first real-time instruments designed specifically for use in clinical laboratories (143).

Many manufacturers are coupling automated nucleic acid extraction instruments with amplification and detection systems to create high-throughput, fully automated nucleic acid analyzers. The TIGRIS and Panther systems (Hologic Gen-Probe), the COBAS AmpliPrep/COBAS TaqMan and COBAS 4800 systems (Roche), the *m*2000 system (Abbott), and the Viper and BD MAX systems (BD Diagnostics) are examples of fully automated and integrated systems designed to perform sample processing, nucleic acid amplification, and product detection.

The GeneXpert system (Cepheid) represents the other end of the automation spectrum, in which a single sample is added to a disposable fluidic cartridge that fully automates and integrates sample preparation, amplification, and real-time detection. The instrument has a random-access design, amenable to on-demand molecular diagnostic testing. Other examples of simple, sample-in, answer-out systems designed to be performed on demand include FilmArray (BioFire) and AmpliVue (Quidel/BioHelix).

A number of criteria need to be considered before selecting and implementing a new molecular microbiology instrument platform (144). These include vendor support by way of test menu, technical support, pricing, and kit configuration. The functionality of the instrument platform should be also be assessed by workflow, reliability, flexibility, and ease of use. Finally, the specifications for the instrument in terms of environmental conditions (e.g., temperature, humidity, and airflow) and utility requirements should be carefully reviewed prior to the final selection.

CURRENT APPLICATIONS

Molecular methods have created new opportunities for the clinical microbiology laboratory to affect patient care in the areas of initial diagnosis, disease prognosis, and monitoring of response to therapy. Over time the methods have become more automated, the cost of testing has decreased, and clinical utility has been proven for the diagnosis and management of a variety of infectious diseases. As a result, molecular testing is now routinely performed in many clinical microbiology laboratories, and clinical applications will continue to expand in the future. A number of near-patient or point-of-care nucleic acid amplification tests for a variety of infectious diseases are commercially available or close to market. These point-of-care tests could extend molecular microbiology into resource-poor settings and potentially drive innovation in global public health programs for control of HIV and M. *tuberculosis* infections.

Initial Diagnosis

With the development of molecular methods, the clinical microbiology laboratory is no longer reliant solely on the traditional culture methods for detection of pathogens in clinical specimens. Culture-based methods have long been the gold standard for infectious-disease diagnosis, but for several diseases, nucleic acid-based tests have replaced culture as the gold standard. HCV infection, enteroviral meningitis, pertussis, HSV encephalitis, and genital infections due to *C. trachomatis* and *N. gonorrhoeae* are some examples of infectious diseases in which nucleic acid-based tests are the new gold standards for diagnosis. In other diseases such as tuberculosis, nucleic acid amplification tests are useful adjuncts to culture that can provide more rapid initial diagnosis and permit timelier public health interventions. This technology has been used to best advantage in situations in which traditional methods are slow, insensitive, expensive, or not available. These techniques work particularly well with fragile or fastidious microorganisms that may die in transit or be overgrown by contaminating biota when cultured. *N. gonorrhoeae* is an example for which the nucleic acid can be detected under circumstances in which the organism cannot be cultured. The use of improper collection media, inappropriate transport conditions, or delays in transport can reduce the viability of the pathogen but may leave the nucleic acid still detectable. It is beyond the scope of this chapter to review all of the possible applications or to provide a compendium of methods for detection of various pathogens. The reader is directed to another excellent resource for this information (145).

Opportunities to actually replace culture for bacterial pathogens in routine practice are limited by the need to isolate the organisms for antibiotic susceptibility testing. In those applications in which culture has actually been replaced by nucleic acid testing, the pathogens are of predictable susceptibilities and, consequently, routine susceptibility testing is not performed, or the genetics of resistance are well defined and simple to detect, such as methicillin resistance in *S. aureus*. Since the last edition of this book, most clinical microbiology laboratories have replaced antigen detection with nucleic acid amplification methods for diagnosis of *C. difficile*-associated disease because the nucleic acid amplification tests have performance characteristics similar to toxigenic culture but with a much shorter analysis time (146).

Molecular methods have had the biggest impact in clinical virology, in which the molecular approaches are often faster, more sensitive, and more cost-effective than the traditional methods. The diagnoses of enteroviral meningitis, HSV encephalitis, and CMV infections in immunocompromised patients are examples of clinically relevant and cost-effective applications of nucleic acid-based tests. There are greater opportunities to replace the conventional methods in virology than in bacteriology because the culture-based methods are costly and antiviral susceptibility testing is not routinely performed. In those situations in which antiviral susceptibility testing is required, such as identification of ganciclovir-resistant CMV, molecular methods (i.e., sequencing) are the method of choice for rapid identification of mutations. The diagnostic role of molecular tests has been further expanded with a new HIV diagnostic testing algorithm proposed by the Centers for Disease Control and Prevention (CDC) (147). In this new algorithm, HIV-1 RNA testing with either the Aptima HIV-1 RNA qualitative assay (Hologic Gen-Probe; FDA approved for HIV diagnosis) or an HIV-1 viral load assay can be used to facilitate prompt diagnosis of acute HIV-1 infection when faced with discordant fourth-generation screening (antigen and antibody combination assays) and supplemental antibody test results to differentiate acute HIV-1 infection from false-positive immunoassay results. Similarly, HCV RNA testing has replaced the recombinant immunoblot assay as a supplemental test for HCV infection (148). Clinical laboratories will be performing more HCV RNA tests with the adoption of the CDC's amended testing recommendations for one-time HCV testing for all persons born from 1945 through 1965 regardless of risk factors (149).

Until recently a major limitation of molecular tests for infectious diseases was the clinical need for simultaneous identification of multiple pathogens associated with defined syndromes such as respiratory tract infections, sepsis, gastroenteritis, and meningoencephalitis (150). This syndromic approach to molecular microbiology was facilitated by the technological developments of multiparametric or multiplex analysis described earlier in this chapter. At the time of this writing, there are FDA-cleared highly multiplexed panels for the detection of respiratory, gastrointestinal, and bloodstream infections. It is likely that this trend in development and deployment of syndromic molecular microbiology tests on multiparametric platforms will increase in the future.

Perhaps the greatest impact of molecular methods has been in the discovery of previously unrecognized or uncultivable pathogens. During the past 25 years, a number of infectious agents were first identified directly from clinical material by using molecular methods. HCV, the principal etiologic agent of what was once known as non-A, non-B hepatitis, was discovered in 1989 through the application of molecular cloning techniques by investigators from the CDC and the Chiron Corporation (151). Cloning and analysis of the HCV genome led to production of viral antigens that now serve as the basis of the specific serologic tests used to screen the blood supply and to diagnose hepatitis C. To date, HCV has resisted all attempts at sustained *in vitro* propagation. As a result, RT-PCR is used to detect, quantify, and genotype HCV in infected individuals.

Tropheryma whipplei, the causative agent of Whipple's disease, is another example of a microorganism that was initially identified by molecular methods (152). It was discovered by the use of broad-range PCR, in which primers are directed against conserved sequences in the bacterial 16S rRNA gene. Sequence analysis of the PCR product and comparison with known 16S rRNA gene sequences were used to characterize the organism and establish its disease association. This approach provides a new paradigm for discovery of unrecognized pathogens that is of value in other diseases with features that suggest an infectious etiology.

Molecular methods are very powerful tools for the identification of emerging pathogens and are covered in detail in chapter 16. RT-PCR with consensus primers and a panviral DNA microarray was used to rapidly identify the etiologic agent of severe acute respiratory syndrome as a coronavirus (153, 154). Within a few months of the recognized outbreak, the virus was identified and sequenced and the molecular assays were developed that played an essential role in diagnosing the infection and defining the epidemiology of the infection.

Similarly, high-throughput shotgun sequencing offers important new opportunities for discovery of microbial pathogens. It was used to identify a novel polyomavirus, WU virus, from a nasopharyngeal aspirate from a 3-year-old with pneumonia (155). Using a specifically designed real-time PCR assay, this virus has been shown to be present in 0.7 to 3.0% of patients with acute respiratory infections; the majority of patients were coinfected with other respiratory viruses (156). This approach has also been used to detect previously known and unknown viruses in feces of children with gastroenteritis and a novel Old World arenavirus that caused fatal disease in three recipients of organs from a single donor (157).

Identification of Bacteria and Fungi by Nucleic Acid Sequencing

Nucleotide sequence analysis of the 16S bacterial rRNA gene has expanded our knowledge of the phylogenetic relationships among bacteria and is the new standard for bacterial identification. rRNA contains several functionally different regions, with some regions having highly conserved and others having highly varied nucleic acid sequences (158). The sequence of the 16S rRNA gene is a stable genotypic signature that can be used to identify an organism at a genus or species level. The 16S gene sequence can be determined rapidly and provides objective results independent of phenotypic characteristics. As discussed in the preceding section, it can also be used to characterize previously unrecognized species. A similar approach that targets the nuclear large subunit of the rRNA gene can be used for the identification of fungi (159). This gene is universally found in all fungi and contains sufficient variation to identify most fungi accurately to the species level.

The DNA sequencing approach to microbial identification involves extraction of the nucleic acids, amplification of the target sequence by PCR, sequence determination, and a computer software-aided search of an appropriate sequence database. The major limitations of this approach to microbial identification include the high cost of automated nucleic acid sequencers, the lack of appropriate analysis software, and limited databases.

Applied Biosystems has developed ribosomal gene sequencing kits for bacteria and fungi. A sequence from an unknown bacterium is compared with either full or partial 16S rRNA sequences from >1,000 type strains by using the MicroSeq analysis software (160). The software analysis provides percent base pair differences between the unknown bacterium and the 20 most closely related bacteria, alignment tools to show differences between the related sequences, and phylogenetic tree tools to verify that the unknown bacterium actually clusters with the 20 closest bacteria in the database. The MicroSeq fungal identification system is similar to the bacterial identification system but targets D2 large-subunit rRNA (161, 162). Continued improvements in automation, refinements of analysis software, and decreases in cost should lead to more widespread use of nucleic acid sequence-based approaches to microbial identification.

More recently, pyrosequencing, or sequencing by synthesis, has been used for the identification of infectious pathogens. Since the length of high-quality sequence generated is limited to 50 to 100 bp, it is very useful for single-nucleotide polymorphism analysis, but it has also been applied to taxonomic categorization of microorganisms. This approach requires identifying a variable region that contains a unique sequence for the different microorganisms within the group. Pyrosequencing has been successfully used to classify mycobacteria and nocardiae into clinically important groups and to identify yeast and filamentous fungi (163, 164). CLSI has published a guidance document focused on interpreting and reporting results for microbial identification by DNA sequencing (165).

DNA-based microbiome studies frequently fall into two categories (166). Targeted amplicon studies focus on one or a few marker genes and use these markers to reveal the composition and diversity of the microbiota. Others use a shotgun metagenomic approach in which genomic sequences are randomly obtained. The gene used most often in targeted amplicon studies is the 16S rRNA gene because of the availability of large databases of reference sequences and taxonomies of bacteria. A variety of sequencing technologies, including massively parallel sequencing, have been used for taxonomic profiling. See chapter 15 for a more complete discussion of the experimental and analytical tools used for studying the human microbiome.

Disease Prognosis

Molecular techniques have created opportunities for the laboratory to provide important information that may predict disease progression. Probably the best example is HIV-1 viral load as a predictor of progression to AIDS and death in infected individuals. This predictive value was first demonstrated in 1996 as part of a multicenter AIDS cohort study (167). The investigators showed that the risk of progression to AIDS and death was directly related to the magnitude of the viral load in plasma at study entry. The viral load in plasma was a better predictor of disease progression than the number of CD4$^+$ lymphocytes. Subsequent studies have confirmed that baseline viral load critically influences disease progression.

Subtyping of certain viruses by molecular methods may also have prognostic value. Subtyping of respiratory syncytial viruses may provide information about the severity of infection in hospitalized infants, with those infected with group A viruses having poorer outcomes (168). HPV causes dysplasia, intraepithelial neoplasia, and carcinoma of the cervix in women. HPV types 16 and 18 are associated with a high risk of progression to neoplasia, and types 6 and 11 are associated with a low risk of progression (169). The clinical utility of molecular testing for high-risk HPV DNA has been established for managing women with the cervical cytologic diagnosis of atypical squamous cells of undetermined significance. Women with this condition can be referred for colposcopy based on the detection of high-risk HPV DNA (170). HPV DNA testing is approved by the FDA for use as an adjunct to cytology for cervical cancer screening in women aged 30 years or more (171). The most recent consensus screening guidelines for the prevention and early detection of cervical cancer emphasize the importance of HPV testing in women 30 to 65 years of age even in the absence of cervical cytological abnormalities (172).

CMV viral load testing is useful for deciding when to initiate preemptive therapy in organ transplant recipients and distinguishing active disease from asymptomatic infection. The level of CMV DNA can predict the development of active CMV disease (173, 174), with higher viral load values increasing the risk of symptomatic disease. Quantitative assays are also useful in distinguishing disease from infection with other herpesviruses such as Epstein-Barr virus (EBV) and HHV-6.

Duration of and Response to Therapy

Molecular methods have been developed to detect the genes responsible for resistance to single antibiotics or classes of antibiotics in bacteria and in many cases are superior to the phenotypic, growth-based methods. The detection of methicillin resistance in staphylococci, vancomycin resistance in enterococci, carbapenem resistance in *Enterobacteriaceae*, and rifampin resistance in *M. tuberculosis* provides examples of where molecular methods are used to supplement the growth-based methods (175, 176). However, it is difficult to imagine, given our current state of knowledge of the molecular genetics of antimicrobial resistance and the technological limitations, that a genotypic approach to routine antimicrobial susceptibility testing of bacteria could rival the phenotypic methods in terms of information content and cost in the near future.

Molecular techniques are playing an increasing role in predicting and monitoring patient response to antiviral therapy. The laboratory may have a role in predicting response to therapy by detecting specific drug resistance mutations, determining viral load, and genotyping. Both viral load and genotype are independent predictors of response to combination therapy with pegylated interferon and ribavirin in chronic HCV infections, although genotype is the main predictor of response (177–180). Those patients with high pretreatment viral load values (≥2 million copies/ml or 600,000 IU/ml) or genotype 1 infections have lower sustained response rates compared with those with genotype 2 and 3 infections (177–179). Genotype is also used to determine the duration of therapy, with genotype 1 infection requiring a longer course of therapy than genotype 2 or 3 infections (180, 181). Recent studies have more closely defined duration of therapy based on the extent of the viral response. Patients who do not reach a ≥2-$\log_{10}$ drop in viral load at 12 weeks after initiating therapy are very unlikely to respond to pegylated interferon and ribavirin. Moreover, patients with a rapid virologic response (HCV RNA level of <50 IU/ml 4 weeks after initiating therapy) may require a shorter duration of therapy, provided they have a low baseline HCV RNA level (≤400,000 IU/ml) and minimal hepatic fibrosis (180).

The FDA approval of the first direct-acting antiviral agents for treatment of chronic HCV genotype 1 infections in 2011 changed the paradigm for treatment and monitoring the response to treatment (182). Both drugs, boceprevir and telaprevir, are serine protease inhibitors to be used in conjunction with pegylated interferon and ribavirin. A number of other compounds encompassing at least five distinct drug classes are currently under development or in clinical trials (183). In patients treated with boceprevir, HCV viral load is determined at baseline and at weeks 8, 12, and 24 of therapy. If the HCV viral load is undetectable at weeks 8 and 24, then patients should be treated for a total of 24 weeks. If HCV viral load is >100 IU/ml at week 12 or detectable at week 24, then therapy should be discontinued. The algorithm is similar for patients receiving triple therapy with telaprevir. HCV viral load is determined at weeks 4, 12, and 24. If HCV RNA is undetectable at weeks 4 and 12, then patients should be treated for a total of 24 weeks. If the HCV viral load is >1,000 IU/ml at week 4 or 12 and/or detectable at week 24, then therapy should be discontinued. Currently, there is no clinical indication for viral resistance testing, but that may change as different classes of direct-acting antiviral agents are used for treatment.

Quantitative tests for HIV-1 RNA are the standard of practice for guiding clinicians in initiating, monitoring, and changing antiretroviral therapy. Several commercially available HIV-1 viral load assays have been FDA approved, and guidelines for their use in clinical practice have been published (184). Viral load assays have also been used in monitoring response to therapy in patients chronically infected with HBV (185) and in predicting the risk for developing BK virus-associated nephropathy in renal transplant recipients (186). In organ transplant recipients, the persistence of CMV viral load after several weeks of antiviral therapy is associated with the development of resistance (187).

LABORATORY PRACTICE

The unparalleled analytical sensitivity of nucleic acid amplification techniques coupled with their susceptibility to cross contamination presents unique challenges to the routine application of these techniques in the clinical laboratory. There are special concerns in the areas of specimen processing, workflow, quality assurance, and interpretation of test results. Additional information can be found in the CLSI documents MM3-A2, *Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline—2nd Edition* (188); MM6-A2, *Quantitative Molecular Methods for Infectious Diseases; Approved Guideline—2nd Edition* (142); MM13-A, *Collection, Transport, Preparation, and Storage of Specimens and Samples for Molecular Methods; Approved Guideline* (189); and MM19-A, *Establishing Molecular Testing in Clinical Laboratory Environments; Approved Guideline* (190).

Specimen Collection, Transport, and Processing

Proper collection, transport, and processing of clinical specimens are essential to ensure reliable results from molecular assays. Nucleic acid integrity must be maintained throughout these processes. Important issues to consider in specimen collection are the timing of specimen collection in relationship to disease state and the proper specimen type. Other factors that come into play include the use of the proper anticoagulant, transport and storage temperatures, and time to processing of the specimen. HIV-1 viral load testing is an example in which the proper conditions for specimen collection, transport, and processing have been well described and has provided insight into the importance of these factors. For HIV-1 viral load testing, the plasma needs to be separated from the cells within 6 h of collection to minimize degradation of RNA. Once the plasma has been separated, it can be stored at 4°C for several days, but −70°C is recommended for long-term storage (191). Most types of specimens are best stored at −20 to −70°C prior to processing.

Molecular methods have several advantages over conventional culture with regard to specimen collection. It may be easier to maintain the integrity of nucleic acid than the viability of an organism. Molecular tests for the detection of *C. trachomatis* and *N. gonorrhoeae* are an example in which DNA is stable on dry cervical swabs for a week at room temperature or refrigeration temperatures, which is in stark contrast to the conditions required to maintain organism viability for culture. Nucleic acid persists in specimens after initiation of treatment (192, 193), thus allowing detection of a pathogen even though the organism can no longer be cultured. Also, due to the increased sensitivity of molecular assays, it may be possible to test a smaller volume of specimen or use a specimen that is collected using a less invasive method.

The major goals of specimen processing are to release nucleic acid from the organism, maintain the integrity of the nucleic acid, render the sample noninfectious, remove inhibiting substances, and in some instances concentrate the specimen. These processes need to be balanced with minimizing manipulation of the specimen. Complex specimen processing methods are time-consuming and may lead to the loss of target nucleic acid or result in contamination between specimens. Care must be taken to avoid carrying over inhibitory substances, such as phenol or alcohol, from the nucleic acid isolation step to the amplification reaction.

There are several general methods for nucleic acid extraction. Different methods may be used depending on whether the desire is to purify RNA or DNA or both. Another factor to consider when deciding on a nucleic acid extraction method is the type of pathogen sought. Some pathogens, such as viruses, can be very easy to lyse, while mycobacteria, Gram-positive bacteria, and fungi can be very difficult to lyse. Enzyme digestion, harsh lysis conditions, or mechanical disruption may be required to disrupt the cell walls of these organisms.

DNA isolation methods often use detergents to solubilize the cell wall or membranes, a proteolytic enzyme (such as proteinase K) to digest proteins, and EDTA to chelate divalent cations needed for nuclease activity (194, 195). The lysate can be used directly in amplification assays, or additional steps may follow to purify the nucleic acid. These additional steps remove proteins and traces of organic solvents and concentrate the specimen. In order to successfully use a crude lysate, the target DNA must be present in a relatively high concentration and there must be minimal inhibitors of amplification in the sample. If these criteria are not met, additional purification steps should be used.

Another commonly used method of nucleic acid isolation involves disruption of cells or organisms with the chaotropic agent guanidinium thiocyanate and a detergent (196). After a short incubation, the nucleic acid can be precipitated with isopropanol. Guanidinium thiocyanate denatures proteins and is also a strong inhibitor of ribonucleases, making it a very useful tool for RNA isolation, although it is also used for purification of DNA. The Boom extraction method is also based on the lysing and nuclease-inactivating properties of guanidinium thiocyanate but utilizes the acid-binding properties of silica or glass particles to purify nucleic acid (197).

Detection of target organisms that are present in small numbers in a large-volume clinical sample requires that target organisms be concentrated to a detectable level. One way to accomplish this is to isolate the particular nucleic acid of interest by binding it to a solid phase, which allows the support, with the target bound to it, to be separated from the original sample. These techniques are referred to as target capture. Target capture techniques immobilize nucleic acids on magnetic beads by the use of a capture probe that attaches to the bead and to the target nucleic acid. A magnetic separation device is used to concentrate the target by drawing the magnetic beads to the sides of the sample tube, while the remainder of the sample is washed away and removed. Target capture techniques also remove materials in the sample that might otherwise interfere with amplification.

Over the past several years, various manufacturers have developed commercially available reagents using one of these basic methods or a modification of these methods. Many of these methods rely on the use of spin column technology, are easy to use, and provide a rapid, reproducible method for purification of nucleic acid from a wide variety of clinical specimens. In recent years, further advances have been made with the introduction of magnetic silica particles that are coupled with instruments providing various degrees of automation, thus further simplifying nucleic acid extraction and purification. These reagents tend to be expensive, but the additional cost can be offset by labor savings. Laboratories are increasingly using automated systems for nucleic acid extraction, as they require less hands-on time, may reduce the risk of cross contamination between specimens, and provide more consistent yields. There are now many automated systems available for use in clinical laboratories; they should be thoroughly evaluated because not all isolate nucleic acids with the same efficiency and purity. The quality of the nucleic acid can have a significant impact on the performance of a molecular test.

Tissue samples need to be disrupted prior to the nucleic acid extraction process. This can be accomplished by cutting the tissue into small pieces or mechanically homogenizing the tissue prior to proceeding with one of the above-described extraction methods. Preserved tissue specimens require removal of the paraffin with solvents and slicing into fine sections prior to processing.

Removing inhibitors of amplification is a key function of the nucleic acid extraction process. Simple methods of nucleic acid extraction that involve boiling of the specimen have been used for relatively acellular specimens such as cerebrospinal fluid (CSF). Though the boiling method is fast and easy, there are problems with inhibitors of amplification in CSF that are not inactivated by boiling (198). The inhibition rate can be reduced to <1% by using a silica-based extraction method. Similarly, crude lysates of urine and cervical swab specimens are commonly used for the detection of *C. trachomatis* and *N. gonorrhoeae*. Specimens containing amplification inhibitors have been reported to range from 1 to 5% for urine to as much as 20% for cervical swabs (199). Common inhibitory substances include hemoglobin, crystals, β-human chorionic gonadotropin, and nitrates. Blood samples are used commonly for detection and/or quantification of a variety of viral pathogens, including HIV-1, HCV, and CMV. HIV-1 viral load testing is an example in which the effects of different anticoagulants have been well studied. HIV-1 viral RNA is most stable when collected in EDTA, and heparin has been shown to be inhibitory to amplification and should be avoided (200). In addition, very small volumes of whole blood (1%) can be inhibitory to *Taq* DNA polymerase (201). Other compounds such as acidic polysaccharides, which are components of glycoproteins present in sputum and cervical specimens and bile salts found in stool, can also inhibit polymerase (202). Human DNA, when present in the sample in high quantities, for example, tissue or blood, may also interfere with the detection of a low concentration of pathogen nucleic acid. With the recognition of such a wide array of inhibitors of amplification and the availability of simple, reliable, semiautomated, and automated nucleic acid extraction methods, the use of crude lysates for testing becomes more difficult to justify. Regardless of the nucleic acid extraction method employed, the laboratory should monitor inhibition rates for different specimen types and nucleic acid extraction methods (see "Quality Control and Assurance" below).

Contamination Control

Several types of contamination can occur with molecular testing: cross contamination of specimens during the nucleic acid extraction step, contamination of specimens with positive control material, and carryover contamination of amplified products. Contamination with amplified products can occur with DNA or RNA target amplification and with probe amplification methods. It does not occur with signal amplification assays since nucleic acid molecules are not synthesized with these methods. Cross contamination that occurs during specimen processing or handling of positive control material can occur with all amplification methods. The approach to the control of contamination due to amplified products has changed dramatically with the widespread use of real-time amplification and detection methods. Since the reaction tube is not opened after amplification, there is minimal risk of contamination from the amplified product. Many laboratories using real-time methods continue to use a variety of good laboratory practices to control for contamination, but the focus is on minimizing cross contamination between specimens rather than contamination from the amplified product. Refer to CLSI document MM3-A2, *Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline—2nd Edition* (188), and *Molecular Microbiology: Diagnostic Principles and Practice*, 2nd ed. (144), for detailed descriptions of good laboratory practices to minimize contamination.

Clinical microbiologists have long been concerned about minimizing contamination between samples with microorganisms during specimen processing. Molecular methods have raised the level of concern considerably, and for good reason, as current methods can detect a few molecules. The previously undetected low levels of contamination that occurred in processing specimens for routine culture can lead to false-positive results in molecular assays. Prevention of contamination due to target DNA or RNA is best done by careful handling of specimens to avoid splashing, opening only one specimen tube at a time, pulse-spinning tubes prior to opening, using screw-top tubes rather than snap-cap tubes to minimize aerosolization, bleaching work surfaces, and using plugged pipette tips. Some of these approaches can be difficult for high-volume laboratories, which is why automated extraction systems can be very useful. Care must be taken with these systems to ensure that there is no cross contamination during the automated process. This is often done by alternating negative and high-titer specimens in a checkerboard arrangement and monitoring for carryover of sample into the negative specimens. These experiments should be designed with an understanding of the concentration of the organism in the clinical specimen. For example, the concentration of HSV in CSF from patients with meningitis is quite low compared with the concentration of BK virus in the urine of a patient with nephropathy.

Preventing contamination of the laboratory with DNA from a clinical specimen or positive control material is very important, because eliminating contamination with target DNA once it occurs can be very difficult. This is why care should be taken to use a positive control at the lowest concentration that consistently amplifies. The enzymatic and photochemical inactivation methods used to control carryover contamination of amplified products are not effective in preventing contamination with target DNA.

Enzymatic inactivation of amplified product can be accomplished with uracil-*N*-glycosylase (UNG), a DNA repair enzyme found in a variety of bacterial species. During the PCR, dTTP is replaced with dUTP so that dUTP is incorporated into the newly synthesized DNA products. This allows for a distinction between starting template DNA and amplified products; only newly synthesized PCR products will contain deoxyuracil. If UTP-containing amplification products are present as contaminants, the addition of UNG to the reaction mixture will result in the cleavage of deoxyuracil residues, thus destroying the contaminating DNA (203). The use of UNG increases the amount of carryover DNA needed to contaminate the reaction mixture by several orders of magnitude (204). When UNG is used, it is important to keep the annealing temperature above 55°C so that the UNG remains inactive, thus avoiding degradation of newly synthesized product. For the same reason, after completion of amplification, the reaction mixture should be held at 72°C (205). UNG can be inactivated at 94°C, but prolonged inactivation at 94°C may also affect the activity of the polymerase enzyme. UNG will not remove uracil from RNA molecules and is therefore ineffective in controlling contamination in RNA amplification assays, such as TMA and NASBA.

When UTP and UNG are used, the PCR reaction conditions should be reoptimized, as the magnesium requirement may increase. The efficiency of amplification may be reduced when UTP is substituted for TTP. This can be overcome by adding a mixture of dUTP and dTTP into the master mix. The efficiency of inactivation using UNG depends on the size of the amplified product and its G+C content. Inactivation may not be effective with amplified products of <100 bp, as maximum UNG efficiency requires the DNA molecule to be 150 bp (206).

Contamination of laboratory work surfaces, equipment, reagents, and clothing of laboratory personnel with previously amplified nucleic acid products is of particular concern for clinical laboratories, since these products can accumulate over time with routine testing and can be inadvertently transferred to subsequent assay reactions, resulting in false-positive test results. To minimize the potential for such amplicon contamination and false-positive results, laboratories performing molecular tests with target amplification methods were designed traditionally to have physical separation of preamplification (i.e., reagent preparation and sample processing), amplification-detection, and postamplification (i.e., DNA sequencing) areas with separate ventilation systems. In addition to the use of dedicated rooms, biological cabinets, and dead-air boxes for various processes involved in specimen testing, laboratories have also typically employed a unidirectional workflow for the movement of specimens, supplies, and personnel from preamplification to postamplification areas through each phase of testing. The physical separation of pre- and postamplification activities and a unidirectional workflow are particularly important for those laboratories performing postamplification analyses in which the reaction vessel is opened and the amplicon transferred to another vessel or device (e.g., sequencing or liquid bead microarrays). The strict separation of pre- and postamplification areas is less important for laboratories using real-time amplification methods, particularly those using fully automated systems that perform nucleic acid extraction, amplification, and detection.

Quality Control and Assurance

"Verification" and "validation" are terms that are often used interchangeably, but it is important to remember that they are different processes (207). Verification is the process by which assay performance is determined; parameters such as sensitivity, specificity, positive and negative predictive values, and accuracy are established. The verification of an assay is completed before the assay is used for patient testing. Validation is the ongoing process of proving that the assay is performing as expected and achieves the intended result or intended use.

The analytical verification of an assay provides information on the performance characteristics of the assay, including the limit of detection, linear and measuring ranges (quantitative tests), trueness, precision, and specificity, while the clinical verification determines the clinical utility of the assay. The analytical performance characteristics of a test should be well understood prior to determining the clinical utility of a test, and any analytical limitations need to be considered when determining clinical uses. For example, a qualitative HSV DNA test may have adequate sensitivity to detect cases of HSV encephalitis but have inadequate sensitivity to detect the lower levels of DNA found in cases of HSV meningitis.

Determining the clinical utility of a molecular assay can be difficult when the molecular assay is more sensitive than the gold standard. This situation was seen with the commercial assays designed to detect *C. trachomatis* in genital specimens. Molecular assays proved to be much more sensitive than the gold standard method of culture. An insensitive gold standard can make a molecular assay appear to have a falsely low specificity. In this situation, an expanded gold standard can be used. For *C. trachomatis*, this included direct fluorescent-antibody testing and/or another molecular method (192, 193, 208). There are additional challenges in determining the clinical utility of molecular assays that detect rare pathogens. These assays are usually laboratory-developed tests (LDTs), and any given medical center may see very few cases of the disease, making clinical verification difficult. Moreover, standards and control material can be difficult to obtain for rare pathogens. Several companies now provide control material for the more common molecular assays, such as those for *C. trachomatis*, *N. gonorrhoeae*, HIV-1, HCV, and CMV. A complete list of reference materials and international standards for molecular microbiology tests is maintained by the Genetic Testing Reference Materials Coordination Program, CDC, and can be accessed at http://www.cdc.gov/clia/Resources/GetRM/

default.aspx. The availability of calibrators that are made based on a consensus standard (such as the World Health Organization international standards for HIV-1, HCV, CMV, and EBV) is very important in establishing the clinical utility of viral load tests. The lack of such calibrators makes standardization of LDTs very difficult. This has been particularly problematic for CMV and EBV, for which the presence of latent infection and asymptomatic reactivation leading to low levels of viral replication underscores the need to establish clinical cutoffs for initiating therapy or reducing immunosuppression. With the lack of an international standard, there is poor agreement of viral load values between LDTs (209), so clinically important cutoffs to predict the development of disease need to be determined by the individual laboratory. These standards are now available and should improve the agreement of viral load values between LDTs, which will facilitate the establishment of clinically relevant cutoffs.

A positive control is designed to ensure that the test can consistently detect a concentration of target nucleic acid at or near the limit of detection of the assay. The positive control should be at the lowest concentration that can be reproducibly amplified. A positive control that is significantly greater than the cutoff of the assay may not detect small decreases in amplification efficiency. In addition, use of large amounts of target DNA can increase problems with contamination in the laboratory. For a quantitative test, two levels of positive control are required, a low positive control near the lower limit of quantification and a high positive control near the upper limit of quantification. For real-time methods that have an upper limit of quantification of 10^7 or 10^8 copies/ml, it may not be possible to find adequate amounts of control material, so a sample in the range of 10^5 copies/ml is often used. Depending on the availability of material, the positive control may be purified nucleic acid or lysed or intact organisms. An extraction control tests the ability of the nucleic acid extraction or purification method to successfully release nucleic acid from the organism. The extraction control, which should be intact organisms, can also serve as a positive control if it is used at the appropriate concentration.

Monitoring for the presence of inhibitors in a specimen is important, particularly for complex specimens such as blood or sputum. Several methods can be used to control for inhibition. One method is to amplify two aliquots of a clinical specimen, one directly and the second spiked with an aliquot of positive control DNA. For a specimen to be considered negative for the target analyte, testing results for the direct specimen must be negative and those for the spiked specimen must be positive. If an inhibitor of amplification was present, the spiked specimen would be negative. The concentration of positive control used for the spike must be near the limit of detection of the assay to ensure that low-level inhibition of amplification is detected.

Another approach to monitoring for inhibition of amplification is adding an internal control to the clinical specimen prior to nucleic acid extraction. As discussed in "Quantitative Methods" above, the internal control molecule may be designed with the same primer-binding sites as the target molecule but modified in some manner so as to allow detection separate from the target based on size or sequence. An internal control may be designed that does not share the same primer-binding sites as the target molecule; in this situation a separate set of primers is needed for amplification. An internal control is an effective way to monitor for inhibition, but it may decrease the sensitivity of the assay due to competition for assay components. Amplification of a human housekeeping gene such as the β-globin gene may also be used as an internal control, but the gene should not be present in vast excess of the target molecule or inhibition of amplification of the target molecule can occur without evidence of inhibition of the housekeeping gene. Inhibition controls should be included in assays that use a new specimen extraction method or specimen type. However, a cost-effective approach is to discontinue these controls once the inhibition rate is determined to be <1%. However, discontinuing the use of an internal control limits the ability to detect inhibition due to preanalytical factors, such as collection of the specimen in a tube containing heparin rather than EDTA.

Under certain conditions, there may be a need to determine if there is adequate nucleic acid in a specimen, for example, when using paraffin-embedded tissue or when evaluating the quality of a specimen. In these situations, amplification of housekeeping genes can be used to determine if the specimen contains human DNA. The absence of amplifiable human DNA from the specimen raises concern about whether the specimen quality is adequate.

Negative controls should be included in all assays and processed in a manner similar to the processing of the clinical specimens. The negative control should be taken through all steps of the assay, including the nucleic acid extraction process. However, lack of target amplification in the negative control does not ensure that there is not contamination in the run, as contamination is often low level and sporadic. Including multiple negative controls in the run may provide additional assurance that there is no contamination, but this approach may be cost prohibitive. Ideally, the negative control should be a clinical specimen that does not contain the analyte of interest. These types of controls may be difficult to obtain, so water or buffer is often substituted.

Currently, the College of American Pathologists (CAP) is the only Centers for Medicare and Medicaid Services-approved proficiency program for molecular testing for infectious diseases. The CAP provides proficiency testing for many common pathogens for which routine tests are done in the clinical laboratory. The Quality Control for Molecular Diagnostics proficiency program, which is jointly sponsored by the European Society for Clinical Virology and the European Society for Clinical Microbiology and Infectious Diseases (Glasgow, Scotland, United Kingdom), also provides testing for a variety of pathogens. When formal external proficiency testing programs are not available, laboratories may split samples with other laboratories, split samples between a new method and an established laboratory-developed method, or clinically validate the test result by clinical diagnosis. When exchanging specimens between laboratories for proficiency testing, it is important that both laboratories use the same method, particularly for quantitative methods, as viral load values may differ substantially among the various assays.

Reporting and Interpretation of Results

The interpretation of molecular assays requires a basic understanding of the strengths and limitations of these technologies. There are unique problems in interpreting molecular testing results that are not routinely encountered with traditional microbiological assays, such as culture and serology. Some of the problems that may occur in interpreting molecular assays include recognizing false-positive results, distinguishing viable from nonviable organisms, and correlating nucleic acid detection with the presence of disease.

For interpretation of a positive test result, the issues that need to be considered are assay specificity and contamination. The specificities of most molecular assays are established by the primers and probes used during amplification and detection steps; if they cross-react with other pathogens, then false-positive results are possible. For example, primers designed to detect *M. tuberculosis* from respiratory specimens must not cross-react with organisms that are part of normal oral biota or other common respiratory pathogens, such as *S. pneumoniae*. Although uncommon, problems with primer specificity do occur. Rhinoviruses and enteroviruses are closely related, and finding a conserved gene target that will detect all rhinoviruses and all enteroviruses without some cross-reaction is difficult. This would not be a problem for testing of CSF specimens for enteroviruses since rhinoviruses do not cause meningitis, but it can complicate the interpretation of tests for rhinoviruses in respiratory specimens since enteroviruses can be found in the oropharynx. Problems with primer specificity have also been reported for a commercially available PCR assay designed to detect *N. gonorrhoeae*. The primers used in this assay cross-react with *Neisseria subflava*, a nonpathogenic organism found in the oropharynx (210). False-positive results can also be due to contamination, which may occur during specimen processing or as a result of carryover contamination of previously amplified products.

The interpretation of a negative result requires consideration of assay sensitivity, specimen quality, nucleic acid extraction efficacy, and amplification efficiency. Problems with any of these factors can lead to a false-negative result, which is why measures to control for each of these parameters should be included in assays whenever feasible. Another source of false-negative results is sequence variation, which may prevent binding of either primers or probes. To minimize this problem, one should perform a thorough search of known sequences before designing the assay and occasionally reexamine the available databases after the assay is put into clinical use. False-negative results may also occur when the specimen type is not optimal (throat swab versus nasopharyngeal aspirate for the detection of respiratory pathogens) or when the specimen is collected at an inappropriate time in the disease course.

Molecular assays detect pathogen nucleic acid but cannot determine whether that nucleic acid is found in a viable or nonviable organism. Pathogen nucleic acid can be detected for long periods of time after appropriate treatment is initiated. For example, *C. trachomatis* DNA can be found in the urine of patients for up to 3 weeks after completion of a course of therapy (192). Similar results have been reported for the detection of HSV DNA in the CSF of patients with encephalitis. DNA can persist for 2 weeks or longer after the initiation of acyclovir therapy (211). Due to the persistence of pathogen DNA after initiation of therapy, qualitative molecular assays should not be used to monitor response to therapy. One notable exception is the use of an HCV RNA RT-PCR assay to monitor the response to therapy. In this instance, the absence of detectable viral RNA from plasma is used to define treatment response (178, 179).

The detection of pathogen nucleic acid does not ensure that the organism is the cause of disease. The organism may be present as part of the normal biota, as a colonizer of a particular area, or as a cause of infection. Distinguishing between colonization and infection may be more difficult when molecular techniques that are more sensitive than culture are used. Organisms present in very low concentrations, which may have gone undetected by routine culture methods, may be detected by using molecular techniques.

Distinguishing colonization from infection is easier when testing a specimen from a normally sterile site such as CSF or blood; however, this factor alone does not ensure that the organism is a true pathogen. This distinction is a concern with the detection of herpesviruses, which cause lifelong latent infections. An important example of distinguishing these two states is monitoring transplant recipients for CMV disease using molecular methods. Initial studies used very sensitive qualitative PCR assays (212, 213), and it was clear that CMV DNA could be detected in the blood of patients who never went on to develop symptomatic disease. Quantitative molecular tests have been useful is stratifying the risk of active disease; the higher the viral load result, the higher the risk of active CMV disease (173, 174). Similar data are emerging for *S. pneumoniae* infections; early studies showed that qualitative tests of respiratory specimens could not distinguish pneumonia from asymptomatic colonization of the respiratory tract. The clinical utility of quantitative tests to differentiate infection from colonization is under investigation (214).

Reporting the results of a qualitative molecular assay is usually straightforward; results are often reported as DNA detected or not detected. Several key parameters that may also be reported are the limit of detection of the assay, data pertaining to the rate of inhibition for a given sample type, the gene target, and the amplification method used for testing. Reporting results from quantitative assays is more complex and requires consideration of several parameters, including measuring range, units, and precision. Results of quantitative assays can be expressed as copies, weight (nanograms or picograms), or international units of the target nucleic acid in a defined volume, such as milliliters of plasma or blood, grams of tissue, or number of leukocytes. Viral load values are reported as integers, in scientific notation, or as $\log_{10}$ transformed data. Ideally, $\log_{10}$ transformed results should be reported, as this better reflects biologically relevant changes in load of microorganisms that usually replicate exponentially, and because most assays exponentially amplify the target. Moreover, clinicians may be less likely to overinterpret insignificant changes in viral load values when results are reported as $\log_{10}$ transformed values. Since patients and clinicians may not be as familiar with $\log_{10}$ transformed results, laboratories may choose to report both integers and $\log_{10}$ transformed numbers. For quantitative tests, the number of significant digits reported for viral load values should be limited due to the precision of the test. Integers should be rounded to 10s or 100s (734 copies/ml to 730 copies/ml; 52,321 copies/ml to 52,300 copies/ml), and $\log_{10}$ transformed values should be limited to two decimal places.

When the results of quantitative assays are reported, the precision of the assays needs to be considered. For the currently available HIV-1 viral load assays, the intra-assay and biological variability are approximately 0.5 $\log_{10}$ (191). Therefore, changes in viral load must exceed 0.5 $\log_{10}$ (3-fold) in order to represent a biologically significant change in viral replication. For HCV, the intra-assay variability is about 0.1 to 0.2 $\log_{10}$ copies/ml, while the biological variation is 0.75 $\log_{10}$ copies/ml (215). So, a biologically important change in viral load value is closer to 1.0 $\log_{10}$ copies/ml. Quantitative assays have a defined linear or measurement range. Values below the lower limit of quantification should be reported as less than the lower limit of the linear range, rather than as negative. Values above the upper limit of quantification should be reported as greater than the upper limit of the linear range. For values above the limit of detection but below the limit of quantification, results may be reported as detectable, less than the lower limit of the linear range. For example, if the lower limit of quantification of an HIV-1 viral load assay is 40 copies/ml, a value of 25 copies/ml could be reported as "detectable, <40 copies/ml." Inclusion of the amplification method and specimen type in the report is particularly important for quantitative assays, as values from different assay types are not always comparable.

Regulatory and Reimbursement Issues

The medical needs for new molecular microbiology tests have exceeded the capacity of the diagnostic industry to provide FDA-cleared test kits to fill these needs. A current list of FDA-cleared/approved nucleic acid-based tests for infectious diseases can be found at http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm330711.htm.

Notably absent from the list are many tests that have become a standard of care in a variety of infections, including *Bordetella pertussis*, EBV, varicella-zoster virus, and BK polyomavirus. Many laboratories have developed tests to fill these unmet needs. These LDTs must be appropriately verified and validated as specified in the Centers for Medicare and Medicaid Services final rule for laboratory requirements, 42 CFR part 493 (216). Such tests are eligible for reimbursement by Medicare and other payers if they are determined to be part of a standard of care or to be of proven clinical benefit.

LDTs often utilize a combination of reagents from different manufacturers, some of which are ASRs. ASRs are chemical substances, for example, antibodies or nucleic acid sequences, that are used in diagnostic tests to detect another specific substance in a specimen and are purchased from manufacturers under this label. The value of ASRs is that they ensure the quality of reagents used in LDTs. ASRs do not include a protocol for use or information on analytical performance or clinical indication. The FDA requires a disclaimer on reports for LDTs using ASRs, and it reads as follows: "This test result was developed and its performance characteristics determined by [laboratory name]. It has not been cleared or approved by the U.S. Food and Drug Administration." This disclaimer was not intended to cover LDTs not using ASRs or the off-label uses of FDA-cleared products.

A laboratory may want to include clarifying statements in the reports of results from LDTs employing ASRs. These statements may point out that FDA clearance is not necessary for these tests and that they are used for clinical purposes. Additional information may include that the laboratory is certified under the Clinical Laboratory Improvement Amendments of 1988 to perform high-complexity testing and that, pursuant to the requirements of the amendments, the laboratory has established and verified the test accuracy and precision.

While the FDA assumes authority for regulating LDTs and is currently exercising enforcement discretion, it has indicated that it will be increasing oversight of these tests in the near future. It is not clear when the FDA will release its guidance on LDTs, but the FDA has stated that the framework for oversight is complete and under review.

The FDA released a draft guidance that clarified the regulatory requirements applicable to *in vitro* diagnostic products intended for RUO and investigational use only (IUO). In addition, it emphasizes that products so labeled should not be used in clinical diagnosis and patient management (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm253307.htm). RUO and IUO reagents, instruments, and systems have been in use for many years as components of LDTs, and limiting their use would disrupt clinical laboratory testing and as a result negatively affect patient care. The CAP and other professional organizations believe that the FDA should adopt an enforcement discretion policy for RUO/IUO reagents since it is expected that LDTs, which incorporate these components, will soon be under enhanced FDA oversight.

Correct Current Procedural Terminology coding of molecular microbiology tests is essential to coverage and reimbursement by payers. In 1998, many analyte-specific codes for tests using direct probes, amplified probes, and amplified probes with quantification were established in the microbiology section of the Current Procedural Terminology coding manual, and this list of available codes continues to expand (217). The introduction of analyte-specific codes has simplified the coding process and in many cases increased the reimbursement for molecular microbiology procedures, although there continues to be considerable regional variation in reimbursement rates for the codes.

Credentials

Staffing a molecular diagnostics laboratory with individuals who have an appropriate knowledge base and skill set remains a challenge. Until recently, molecular diagnostics was not part of the core curriculum in medical technology programs. However, the situation is changing, and the acquisition of credentials in this area is now available for medical technologists and technicians from the American Association of Bioanalysts, the National Credentialing Agency for Laboratory Personnel, and the American Society for Clinical Pathology. The National Credentialing Agency and the American Society for Clinical Pathology merged their credentialing activities in July 2009. Laboratory directors may receive credentials in molecular diagnostics through the American Association of Bioanalysts (physicians and clinical laboratory scientists), the American Board of Clinical Chemistry (physicians and clinical laboratory scientists), and jointly through the American Boards of Pathology and Medical Genetics (physicians only).

FUTURE DIRECTIONS

Nucleic acid testing will continue to be one of the leading growth areas in laboratory medicine. The number of applications of this technology in diagnostic microbiology will continue to increase, and the technology will increasingly be incorporated into routine clinical microbiology laboratories as it becomes less technically complex and more accessible. However, now more than ever clinical and financial outcome data will be needed to justify the use of this expensive technology in an era of declining reimbursement and increased cost consciousness.

The clinical utility of molecular testing is now well established, and the gap between the availability of FDA-cleared/approved tests and clinical need is improving. However, the pending enhanced oversight of LDTs and restriction of the use of RUO and IUO reagents and systems by the FDA could limit the ability of laboratories to develop tests to meet clinical needs not met by *in vitro* diagnostic products. Although there has been considerable progress in recent years, there are other important unmet needs, including the availability of international standards and traceable and commutable calibrators that can be used for assay verification and validation. These materials, when widely available, should improve agreement of the results between different tests and aid in the establishment of the clinical utility of molecular tests. Another need is for the continued development of effective proficiency testing programs that will help ensure that the results of molecular tests are reliable and reproducible among laboratories.

To a great extent, the future of molecular microbiology depends on automation. Many of the available tests are labor-intensive, with much of the labor devoted to tedious sample processing methods. Several fully automated systems for molecular diagnostics have been developed for high- and mid-volume laboratories; however, most suffer from a limited test menu. To increase access to molecular tests, simple, affordable, fully automated, random-access platforms with broad test menus are needed, particularly for laboratories that perform low- to mid-volume testing. Nucleic acid testing for infectious diseases at the point of care is beginning to enter clinical practice in developed and developing countries, particularly for applications that require short turnaround times and in settings where a centralized laboratory approach is not feasible (218).

The use of multiplex nucleic acid-based assays to screen at-risk patients for panels of probable pathogens remains a goal for molecular microbiology. Several such tests are currently available, but success to date has been limited by the technical complexity of some systems. The development of simpler, multiparametric technologies is a key to providing molecular tests with the same broad diagnostic range provided by culture and other conventional methods for diagnosis of syndromic infections.

Metagenomic studies have provided new insights into the human microbiome, and alterations in these communities of microorganisms have been linked to a number of disease states. With the continued decrease in the cost of massively parallel nucleic acid sequencing and the increasing availability of the necessary bioinformatics tools, it is likely that our understanding of the human microbiome will result in novel microbiome-related diagnostics and clinical interventions.

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Immunoassays for Diagnosis of Infectious Diseases*

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7

By definition, immunoassays are biochemical assays that detect the presence of an analyte, either antibody (Ab) or antigen (Ag), using labeled antibodies as the analytical reagent (1–6). Immunoassays can be adapted for detection of analytes across laboratory disciplines and are often more cost-effective than other diagnostic methods. In the clinical microbiology laboratory, immunoassays often serve as confirmatory tests, and therefore, the results are typically not intended to be used as the sole basis on which a diagnosis is made. However, for certain infectious diseases, including Lyme disease, cryptococcal meningitis, and syphilis, antibody/antigen detection by immunoassays is the primary means by which the infection is established. Due to their ease of use, rapid turnaround time, and generally high specificity, immunoassays are increasingly becoming available for point-of-care testing.

This chapter summarizes the common immunologic testing methods currently used in clinical microbiology laboratories and their application for the diagnosis of infectious diseases. The following discussion emphasizes general assay design, with important caveats relevant to test development and interpretation. While some examples relating to clinical testing are included, for an in-depth, pathogen-focused discussion, the reader is directed to the designated chapters in this *Manual*.

HISTORIC PERSPECTIVE ON IMMUNOASSAY DEVELOPMENT

The first immunoassays were developed in the early 1940s and were dependent on the visualization of antigen-antibody precipitation bands in various agar preparations. These types of assays, referred to as immunodiffusion assays, were able to detect milligram to microgram quantities of analyte (Table 1) (7–9). Interpretations of immunodiffusion results are notoriously subjective, requiring extensive technician expertise, and assays are labor-intensive to set up and perform. These limitations, along with concurrent improvement in antibody/antigen purification techniques and the introduction of new detection systems, brought about the development of increasingly more sensitive and objective immunoassay methods (1–3, 5, 6, 10).

The first significant improvement of the immunoassay occurred in the clinical chemistry arena in 1959. Solomon Berson and Rosalyn S. Yalow developed a novel radioimmunoassay (RIA) method for detection of human antibodies to insulin and received the Nobel Prize in Medicine in 1977 (11). Using purified antibodies linked to one of a variety of radioisotypes (e.g., ^{125}I, ^{3}H, or ^{14}C), results from RIAs could be objectively evaluated using a scintillation counter or photographic paper. The RIA dramatically improved sensitivity and greatly expanded the repertoire of analytes available for testing (Table 1). This was also the method after which all future enzyme immunoassays were modeled. The discovery of monoclonal antibodies and the development of antibody-producing hybridomas by Kohler and Milstein in 1975 enabled the production of RIAs with improved specificity and further expanded the repertoire of analytes available for measurement (12). While among the most sensitive of immunoassay techniques, RIAs presented laboratorians with a number of significant challenges, including the needs for safe disposal of hazardous waste and well-trained, attentive laboratory technicians. Due to these concerns, the RIA detection method has subsequently been replaced by avidin-biotin detection assays, chemiluminescence immunoassays (CLAs), and other enzyme-based detection systems.

In recent years, laboratory automation has expanded into the immunoassay arena, with many tests requiring only limited technologist time. As the basic concept of the immunoassay has evolved, there has been increased utilization of different solid-phase matrices for adherence of either antigens or antibodies. While polypropylene test tubes were used as the solid-phase matrix initially, these have largely been replaced by microtiter plates. More recently, with the influx of large, complex automated systems, smaller solid-phase matrices, such as microdisks or -spheres, have become increasingly popular. Collectively, immunoassay technology has significantly advanced in both the level of sensitivity of the assays and the breadth of their utilization to the point that immunoassays are now some of the most popular and widely used of all laboratory tests.

DEFINITION OF TERMS

The array of terms used for immunoassays can be a confusing alphabet soup. This chapter discusses some commonly used conventions in terminology; however, the reader may find some references in which the terms are used differently. Overall, most assays utilize the term "immuno-" coupled with a second term, which describes the type of assay or label

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TABLE 1 Approximate sensitivities of various immunoassays[a]

Technique	Approx sensitivity (per ml)
Tube precipitation	100 mg
Immunodiffusion	1–3 mg
Agglutination	1 μg
Complement fixation	1 μg
Hemagglutination	50 ng
Particle immunoassay	30–50 ng
EIA	~1 ng
Radioimmunoassay	1 pg
Chemiluminescence immunoassay	10^{-18} to 10^{-21} mol

[a] Data are from references 9 and 28.

used. For example, immunoprecipitation is an immunoassay utilizing a precipitation reaction. RIA is an immunoassay that utilizes radioactivity as the label. The term enzyme immunoassay (EIA) is a more general term that can be applied to any immunoassay which uses an enzyme label, although EIA is often used to refer to reagent-limited, competitive-type assays. The term enzyme-linked immunosorbent assay (ELISA) can also be used as a general term for any assay utilizing an enzyme label. However, it is most often used to refer to assays in which the antigen or antibody is adhered to a solid-phase matrix and a second, enzyme-labeled antibody is used for detection; this entire method is also referred to as the "sandwich" assay. Finally, "immunometric" is a term generally referring to any reagent used in excess. For the purposes of this chapter, the term EIA is used to refer to any assay using an enzyme, while ELISA refers only to solid-phase sandwich-type assay formats.

GENERAL CONCEPTS OF ASSAY DESIGN

Categorization of Serologic Assays

There are a number of ways to characterize immunoassays. One useful classification scheme stratifies assays based on the amount of available label and reagent (13). There are three major groups of immunoassays: assays that are label free, reagent excess, and reagent limited. Label-free assays rely upon the ability of antigen and antibody to bind and form a detectable agglutination or precipitation reaction. There are many classic agglutination assays used in the diagnosis of infectious disease, including the Widal test for typhoid fever and the Weil-Felix OX-K reaction for scrub typhus. Reagent excess assay formats use solid-phase, adhered antigen or antibody incubated with the sample. Subsequently, excess labeled secondary antibody is added to detect the analyte of interest. These are the most commonly employed immunoassays in clinical microbiology laboratories today. Finally, reagent-limited assays are competitive tests in which sample antigen or antibody competes for binding sites to a solid-phase reagent with an identical enzyme-labeled analyte. The level of enzyme activity is inversely proportional to the level of analyte in the sample. These competitive assays may be performed using classic RIA and EIA formats but are less often used in diagnosis of infectious disease.

Alternatively, immunoassays can be categorized as either heterogeneous (solid-phase assays) or homogeneous (free-solution assays) (1, 3). Heterogeneous assays require the separation of bound from free components, whereas homogeneous assays do not require this separation step. Therefore, heterogeneous assays involve some type of solid phase (e.g., microtiter plate or polyacrylamide bead) to which the immunoreactants are attached. This is in contrast to homogeneous reactions, which occur free in solution and are most often applied for detection of drugs or hormone levels in clinical chemistry laboratories. While the aforementioned classification schemes are useful and can be applied to commonly used immunoassays, the reader should be aware that not all immunoassays strictly fit into either category.

Determination of Assay Performance Characteristics

When choosing an assay for use in the clinical laboratory, it is critical to understand the concepts of sensitivity, specificity, and predictive values (6). The sensitivity of an assay is defined as the proportion of individuals with the disease that are correctly identified by the particular test of interest; these are the true positives (TP) (Table 2). Conversely, false negatives (FN) are the patients with the disease whose condition is not detected by the test. The formula to determine assay sensitivity is as follows: sensitivity = [TP/(TP + FN)] × 100. The specificity of an assay is defined as the proportion of individuals without the disease that are correctly classified; these are the true negatives (TN). False positives (FP) are the proportion of patients without disease who test positive. The formula to determine the specificity of an assay is as follows: specificity = TN/(TN + FP)] × 100. Clinical tests that show high sensitivity ensure that the majority of individuals with the disease will be detected, and thus the number of false-negative results is very low. In contrast, a highly specific test indicates that the majority of individuals without disease will test negative, so the number of false-positive results will be low. When an assay is developed, the diagnostic cutoffs for a positive or negative result can be modified to alter both the sensitivity and the specificity. For example, if one lowers the cutoff, assay sensitivity will increase, with a corresponding decrease in specificity. The gain in sensitivity at the cost of specificity (or vice versa) must be weighed for each individual assay, as optimization of these components will depend on many factors, including (i) disease prevalence in the test population and (ii) whether the test is a screening or diagnostic assay. Finally, it is important to note that when we discuss sensitivity and specificity of an assay compared to a "gold standard" laboratory method, we refer to analytical sensitivity and specificity, which is not the same as clinical sensitivity and specificity. Determination of the clinical performance characteristics requires comparison of assay results to a patient's clinical diagnosis. Unfortunately, this is not always feasible for the laboratory to determine, and close collaboration with health care providers may be necessary.

The probability of having the disease, given the results of a test, is called the predictive value of the test. The positive predictive value (PPV) determines the percentage of patients with positive results who have the disease (PPV = [TP/(TP + FP)] × 100), while the negative predictive value

TABLE 2 Assay performance characteristics

Result by new method	Result by gold standard method[a]	
	Positive	Negative
Positive	TP	FP
Negative	FN	TN

[a] TP, true positive; FP, false positive; FN, false negative; TN, true negative.

(NPV) calculates the percentage of patients with negative test results who lack the disease (NPV = [TN/(TN + FN)] × 100) (Table 2). The predictive value of a test incorporates the prevalence of disease in a particular population with the sensitivity and specificity of the assay of interest. Positive and negative predictive values are important because they assess the ability of a test to predict the presence or absence of disease in a patient from a particular population. In this context, disease prevalence, defined as the proportion of the population with the disease in question, is a critical component of assay evaluation. If a disease has a low prevalence in the target population, a positive result will most likely be a false-positive result, whereas the opposite is true in a high-prevalence population. The potential use of an assay with a high negative predictive value is that a negative test may be used to exclude disease. In addition to the values discussed above, a variety of other statistical methods can be used to evaluate laboratory tests, such as odds ratios, receiver-operator curve (ROC) analysis, and likelihood ratios. It is beyond the scope of this chapter to discuss these statistical methods, and the reader is referred to other sources for a more complete discussion (14, 15).

Screening versus Diagnostic Assays

An important component of assay design is based upon the ultimate use of the test (16, 17). In other words, will the test be used as a screening tool or for diagnostic purposes? Screening tests are designed to detect infection in asymptomatic individuals, whereas diagnostic tests are intended for patients with specific indications of possible disease. The ideal screening test should be both highly specific and sensitive; however, this is often difficult to achieve, and therefore, assays with high sensitivity are preferred as screening assays. Furthermore, for a screening test to be effective, the prevalence of the disease in the test population should be high, since a highly sensitive assay in a low-prevalence population will lead to an increased number of false-positive results and potentially unnecessary additional testing or treatment. Though a highly sensitive screening assay is optimal, assay specificity is also an important consideration, since a significant number of people without the disease may screen falsely positive. Due to the interplay of sensitivity and specificity, the screening procedure itself should not be used solely to diagnose the illness, but rather, those individuals with a positive screening test should be evaluated by other methods to confirm the result. This is often accomplished by supplementary, reflex testing with a confirmatory or diagnostic assay. Implementation of screening assays should also include prior consideration of assay cost versus the impact of early disease detection. Overall, screening tests should be utilized in populations with high disease prevalence, they should be inexpensive and easy to perform, and perhaps most importantly, early detection of the disease should have a measurable positive impact on patient outcome.

GENERAL CONCEPTS OF SEROLOGIC ASSAYS

Basic Principles of Immunology

In order to facilitate understanding of antibody titers, a brief review of basic immunologic reactions is required (18–21). Antibody production is a function of the humoral branch of the immune system and, more specifically, of B lymphocytes. These cells utilize membrane-bound immunoglobulin (Ig) molecules, or antibodies, to recognize a wide array of antigens, which in the case of microbial agents are often expressed on the pathogen surface. Based on their electrophoretic mobility patterns, five different immunoglobulin classes have been identified (IgA, IgD, IgE, IgG, and IgM), though the infectious disease serology field focuses primarily on detection of IgG-, IgM-, and, in some cases, IgA-class antibodies.

The section of the antigen bound or recognized by the antibody is referred to as the "epitope," and the strength of the antibody-epitope interaction is termed "affinity"; the higher the affinity, the stronger the antibody-epitope bond. Following primary exposure to a novel antigen, immature B lymphocytes are stimulated and differentiate into either plasma cells, which secrete soluble antibody, or resting memory B cells. Due to their prior priming, upon subsequent exposure to the same antigen, resting memory B cells rapidly transition to plasma cells and produce specific antibody much sooner than during the initial exposure. Furthermore, repeat exposure to an antigen leads to an increase in strength of the antibody-epitope bond, a phenomenon called "affinity maturation." The strength of the antibody-antigen bond is also dependent on the number of antigen binding sites per immunoglobulin (i.e., in IgG, 2 sites; in polymerized IgA, 4 sites; and in IgM, 10 sites), and therefore, the total strength of the complex is much greater than the affinity of a single interaction. This is called "avidity." Just as with affinity maturation, there is an increase in avidity with additional exposure to an antigen. For example, upon initial interaction with an antigen, IgG avidity is low; however, upon secondary exposure, IgG avidity increases. Measurement of the avidity index can therefore be used as an aid to determine the stage of infection.

There are four phases of antibody response following initial exposure to an infectious agent (i.e., the primary antibody response): (i) an initial lag phase, when there is no antibody detected; (ii) a log phase, when the antibody titer increases in a logarithmic fashion; (iii) a plateau phase, in which the amount of antibody stabilizes; and (iv) a decline phase, during which time the antibody is cleared or catabolized. The actual time course and ultimate maximum antibody titer depend on the antigen and the individual host. The period after initial exposure but before antibody is produced or is at sufficient levels to be detected is called the "window period," which can vary from as short as 7 days to as long as 6 months, depending on the infectious agent and the patient's immune status. During a primary exposure, the initial antibody response begins with the production of IgM, which typically appears 7 to 10 days postinfection. IgG antibody production begins approximately 10 days after exposure; however, detectable levels are often much lower than those of the IgM response. Typically, as the IgM antibody level decreases, the IgG level will increase so that by the end of the second to third month, only IgG-class antibody is detectable. If there is a repeat exposure with the same infectious agent, the Ig response kinetics is markedly different: the lag phase typically lasts only 1 to 3 days, and IgG-class antibodies are the primary isotype produced. In the months following antigen exposure, the IgG levels will plateau, at which point the antibody may remain detectable for life, even in the absence of subsequent exposure to the antigen.

Although there is exquisite specificity in the antigen-antibody reaction, a spectrum of antibodies with various affinities and avidities to multiple different antigens can be produced. It is these lower-specificity antibodies that are primarily responsible for the cross-reactions seen between assays aimed at detecting Ig against closely related microorganisms. Recognition of cross-reactivity is critical in infectious disease testing, as organisms within the same genus, yet causing different disease manifestations, may share multiple antigenic determinants that can potentially lead to nonspecific results. Although current assays are designed to help obviate these problems, laboratorians and clinicians should remain cognizant of the potential for cross-reactivity.

Specimen Requirements

Traditionally, the term "serologic assay" has been applied to the use of serum or plasma samples for the detection of antibodies to various infectious disease antigens. This concept has broadened over time and currently refers to the detection of antibody or antigen in a variety of patient sample types, including cerebrospinal fluid (CSF), urine, and other body fluids. While molecular techniques and mass spectrometry (and in the future, microbiome and genomics applications) have dramatically altered the diagnostic field, there remain multiple clinical scenarios in which serological assessment is not only advantageous but also the diagnostic method of choice. Serologic assays are particularly useful for the identification of microbial agents that cannot readily be cultured or when antimicrobials have been administered prior to sampling, which can potentially delay or inhibit organism growth. In these scenarios, determination of antibody titers or detection of antigen can often provide a rapid diagnosis. Furthermore, many of these assays serve as the diagnostic tool, without the requirement of confirmatory testing or microorganism isolation, particularly if an antigen test is positive. A common example is the diagnosis of group A beta-hemolytic streptococcal pharyngitis. If the rapid immunoassay for the detection of the group A streptococcal (GAS) antigen is positive, treatment can be instituted immediately. Only if the rapid antigen test is negative is it necessary to perform a culture, though this is recommended only for children and adolescents (22).

Use of Serologic Analysis as a Measure of Protective Immunity

In general, a positive IgG titer is indicative only of exposure to a particular infectious agent at some time in the past. While the following discussion will use the term "immune," this does not necessarily mean that the level of antibody is protective against reinfection. The actual amount of antigen-specific antibody present in the serum of a particular individual is host determined and is controlled by immune response genes, which are products of the human histocompatibility system. For each infectious agent, the positive/negative cutoff for the serologic assay is usually determined such that a positive result is the minimum amount of IgG antibody present to consider the individual immune. However, this level may not always correlate with the actual level required for protective immunity in each individual.

Use of Serologic Analysis as a Measure of Recent versus Remote Infection

A single IgM or IgG serum result should not be used to determine the stage of infection (i.e., acute versus past). While it may be tempting to consider a high IgG titer as indicative of recent infection, antibody levels are largely host specific, and some antigens are more effective than others in stimulating the immune system. Furthermore, the ability of the immune system to respond to antigens can also be affected by a variety of other host factors, such as age, genetic disposition, and comorbidities (18, 19). Ultimately, single-time-point antibody testing typically indicates only that the individual is a high responder to that particular antigen and confirms prior exposure to the infectious agent. There are, however, several ways to differentiate between recent and remote infection using serologic methods.

IgM-Class Antibodies

Perhaps the most frequently used method is measurement of IgM-class antibodies specific to the infectious agent. While a positive IgM result to a particular organism is considered evidence of an active (i.e., recently acquired) infection, cautious interpretation is required for certain pathogens, as IgM levels may persist beyond the acute stage (i.e., for months to years following infection). This phenomenon has been described for various organisms, including cytomegalovirus, *Mycoplasma pneumoniae*, hepatitis A virus, *Toxoplasma gondii*, and *Borrelia burgdorferi* (17, 23–25). Conversely, a negative IgM titer does not immediately exclude a recent exposure. It may take 1 to 2 weeks for IgM-class antibodies to develop, and therefore, if a sample is drawn too early in the clinical course, a second specimen may be needed.

Significant Change in Antibody Titers

A second technique used to establish the presence of primary infection is to determine acute- and convalescent-phase titers. This requires drawing two serum samples: the first being collected soon after exposure to the infectious agent and the second being collected 2 to 3 weeks later. Evidence of an acute infection is confirmed if there is at least a 4-fold increase in antibody titer between the first and second collections. Drawbacks to this practice include the requirement for multiple return visits and a significant delay between onset of symptoms and confirmation of disease, thus obviating its use for early clinical management. Furthermore, false-negative results are not uncommon in serum samples collected early in disease. Therefore, the diagnostic criteria may include seroconversion (conversion from negative to positive) between acute- and convalescent-phase serum samples. Based on these caveats, serologic testing should not be relied upon for a primary diagnosis in the acute phase of most infectious diseases. Molecular techniques, such as polymerase chain reaction (PCR) and culture-based detection with identification by methods such as mass spectrometry, are the preferred diagnostic methods for early clinical management. The identification of certain microorganisms, however, including those which are not readily detected by molecular methods or easily cultured (e.g., *Leptospira* species, *Treponema pallidum*, *B. burgdorferi*), relies heavily on serologic evaluation.

Avidity Testing

Duration of infection can also be determined by measuring the avidity level of IgG-class antibodies for certain diseases (24, 26, 27). Generally, IgG antibodies produced early in infection display low avidity, which subsequently increases with prolonged exposure to the agent. Use of an avidity assay, in conjunction with assessment for the presence of IgG and IgM antibodies, can provide a clearer indication of infection state. Avidity tests are performed by using a standard EIA (defined in detail below); however, samples are tested in duplicate with and without the addition of an IgG dissociation agent (usually high concentrations of urea). Low-avidity antibodies readily dissociate from the antigen in the presence of urea and are removed during subsequent wash steps, whereas high-avidity antibodies remain bound to the antigen despite the presence of urea. Avidity is subsequently estimated by comparing the levels of IgG in treated versus untreated samples. Determination of IgG avidity can be particularly useful during evaluation of pregnant women for exposure to pathogens associated with pre- and perinatal infections, including toxoplasmosis, rubella, and cytomegalovirus (24, 26). For example, if a pregnant woman presents with symptoms consistent with toxoplasmosis and initial IgG screening results are positive, IgG avidity testing may be recommended. If IgG avidity is high, an acute infection may be ruled out. Alternatively, if the IgG avidity is low and subsequent IgM testing results are positive, acute *T. gondii* infection can be suspected. Despite the potential utility of avidity testing, certain caveats remain. Importantly, low avidity does not guarantee recent infection, as low-avidity antibodies may persist for months to years depending on the infectious agent and the host immune system. Additionally, few avidity assays are currently FDA approved, and variability in testing may occur due to differences in the applied dissociation agents and the types of reagents used.

Quantification of EIAs

At present, many immunoassays report results as absorbance values, optical densities, or international units, making it problematic to apply the concept of a 4-fold increase in titer as discussed above. While it is possible to develop an approximate equivalency between titers and absorbance values, this has to be determined individually for each assay. One method involves collecting multiple pairs of acute- and convalescent-phase reference sera, the titers of which are determined using traditional methods (e.g., immunofluorescence assays). These are subsequently tested by the EIA for the agent in question, and the previously determined titers are compared to the reported EIA value. Assuming that the EIA is linear over the reportable range and that it is able to provide distinct absorbance values between acute- and convalescent-phase sera, this technique can theoretically provide an adequate method to evaluate acute- and convalescent-phase titers.

However, this approach is not commonly performed in clinical laboratories for a number of reasons. First, establishment of the EIA reference range has to be performed separately for each individual assay, which can be both cost-prohibitive and time-consuming. Second, this approach requires access to multiple sets of positive acute- and convalescent-phase paired sera for each organism tested, which may be quite difficult to obtain. Finally, depending on the EIA used and the range of the standard curve, titers may not easily be converted into equivalent and meaningful absorbance values. Therefore, considering the challenges associated with this type of analysis, conversion of titer results to EIA values is generally not recommended. Instead, evaluation for the presence of acute infection should be performed by testing for IgM-class antibodies, by demonstrating seroconversion of IgG-class antibodies, by determining IgG avidity, or, preferably, by direct detection of the organism through molecular and/or culture-based methods.

SPECIFIC IMMUNOASSAYS

The spectrum of immunologic assays is discussed in detail in the following sections. Table 1 lists selected assays in order of relative sensitivities and provides approximate levels of detection.

Precipitation Reactions

The interaction of soluble antigen and antibodies is best described by a precipitin curve (Fig. 1). When the concentrations of antigen and antibodies are equimolar (i.e., in the zone of equivalence), they bind and form an insoluble antigen-antibody lattice, which can be visualized as a precipitate by a number of different techniques (3, 4). The formation of stable antigen-antibody lattices is less efficient on either side of the zone of equivalence when either antibodies (i.e., prozone) or antigen (i.e., postzone) is in excess. Passive immunodiffusion is the simplest of the precipitation assays and involves placing immune reactants (i.e., antibody and antigen) together in an inert semisolid medium (i.e., agarose gel) and viewing it for the formation of a precipitin band. Antigen and antibody will passively diffuse at a rate dependent on their size, room temperature/humidity, and gel viscosity; this often requires overnight incubation, but the turnaround time can be reduced by the application of an electric current (i.e., immunoelectrophoresis).

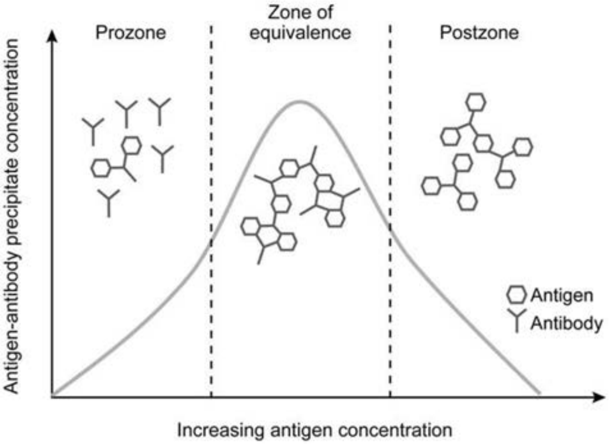
Prozone
Zone of equivalence
Postzone
Antigen-antibody precipitate concentration
Antigen
Antibody
Increasing antigen concentration

FIGURE 1 Precipitin curve showing the zone of equivalence, prozone phenomenon, and postzone phenomenon. doi:1128/9781555817381.ch7.f1

There are several variants of the immunodiffusion technique. The Oudin immunodiffusion assay is a single-dimension test in which antibodies are added to liquid agarose, which is then allowed to solidify in a test tube. Antigen is subsequently added to the top of the gel and allowed to diffuse until a precipitin band is formed. Radial immunodiffusion is a modification of the Oudin assay and designed to provide antigen quantification. The antibody-agarose mixture is allowed to solidify in a petri dish instead of a test tube, and antigen is added to a center well and diffuses out radially. The diameter of the resulting precipitin band is compared to the diameters from known antigen concentrations, and the final amount of antigen is quantified. Among laboratories that maintain immunodiffusion protocols, double immunodiffusion, or Ouchterlony analysis, is most common. In this method, immune reactants, typically patient serum and pathogen-specific antigen, are placed in separate wells in an agarose gel and both are allowed to diffuse. At the zone of equivalence, the precipitation line becomes visible, an example of which is shown in Fig. 2.

Overall, immunodiffusion reactions have excellent specificity (some approach 100%), are inexpensive and simple to perform, and can be adapted to a variety of health care settings. Currently, however, these methods are maintained primarily at large hospitals and public health and reference laboratories due to the expertise required to accurately interpret the results. At these facilities, immunodiffusion is most commonly used for detection of antifungal antibodies (e.g., to *Coccidioides immitis*/*Coccidioides posadasii*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Aspergillus* spp.). There

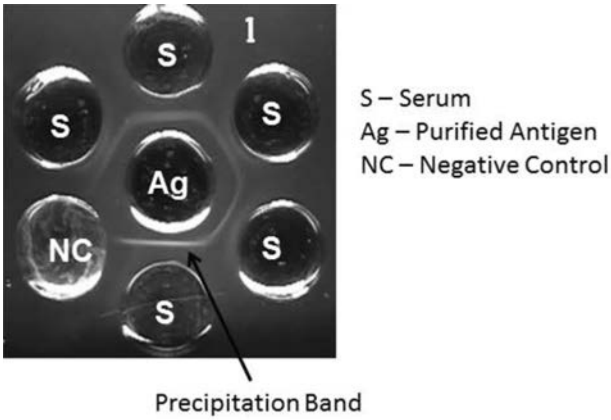

FIGURE 2 Image of an immunodiffusion reaction. doi:1128/9781555817381.ch7.f2

are, however, important limitations to immunodiffusion. First, immunodiffusion generally demonstrates low sensitivity, as microgram quantities of antibody are required for a positive reaction. To overcome this challenge, immunodiffusion assays are performed alongside complement fixation tests (see below). Second, immunodiffusion methods require large amounts of purified antigen, which, depending on the pathogen, may be difficult to acquire from commercial vendors. Third, the interpretation of results can be subjective, and the turnaround time varies from 24 to 48 h. Due to these challenges, smaller clinical laboratories are commonly converting from immunodiffusion assays to rapid, automated enzyme immunoassays.

Agglutination Reactions

Agglutination reactions utilize a particulate, insoluble antigen, in contrast to precipitation reactions, which are based on the interaction of soluble antigen and antibodies. Binding of the particulate antigen and the corresponding antibody results in visible clumping and a positive agglutination reaction (3–5, 28). Many varieties of agglutination assays exist and can be classified based on the type of particle to which the antigen or antibody binds. Direct agglutination reactions involve antigens found on naturally occurring particulates (e.g., whole bacterial organisms and red blood cells [RBCs]). This class of agglutination assay continues to be performed for rare pathogens, including *Francisella tularensis* and *Brucella* species, by utilizing an inactivated, whole organism mixed with patient sera. Alternatively, indirect or passive agglutination assays utilize antigen coupled to particles or beads composed of an inert material (e.g., latex, colloidal gold, gelatin, or silicates). Indirect agglutination assays are often preferred over direct agglutination reactions due to the enhanced visibility of positive reactions. Finally, soluble antigen can be detected in a patient sample by incubation with antibody-coated particles; this is termed reverse passive agglutination.

IgM-class antibodies are several hundred times more efficient at agglutination than IgG antibodies due to their large pentameric structure. Therefore, if the immune response involves primarily IgG-class antibodies, the reaction may require some type of chemical enhancement or an antiglobulin reagent to bind to the Fc portion of IgG antibodies and enhance their precipitation (Coombs' test) (29).

Agglutination assays that employ RBCs are termed hemagglutination assays and can be designed in a direct or an indirect format. Direct agglutination of RBCs is commonly used in blood banking for ABO blood group typing. For infectious disease testing, one of the most frequently ordered direct hemagglutination assays is the monospot test. This test detects the presence of heterophile antibody, produced during the early stages of infectious mononucleosis caused by Epstein-Barr virus (EBV). More recently, multiple assays have become available for detection of heterophile antibodies to EBV using indirect hemagglutination assays in which antigen is adsorbed to latex or polystyrene beads. The indirect hemagglutination reaction is a popular assay format due to its improved sensitivity and ease of performance without the need for sophisticated equipment. For these reasons, these assays have been used in many developing countries for testing of a variety of infectious diseases, including human immunodeficiency virus (HIV), hepatitis viruses (A, B, and C), and *T. pallidum*.

Viral serology uses a unique type of hemagglutination assay, termed hemagglutination inhibition (HI), to detect and quantify anti-influenza virus antibodies. Certain viruses, including influenza virus, have surface proteins (hemagglutinins) that agglutinate RBCs. Taking advantage of this reaction, the HI assay can be used to detect antibodies to influenza virus by incubating RBCs with virus and serum. The presence of anti-influenza virus antibodies will inhibit RBC agglutination, indicative of a positive reaction. The viral antibody titer can also be reported as the last dilution of patient serum still able to inhibit agglutination.

Classic agglutination assays are both straightforward and cost-effective to perform and, therefore, continue to be utilized in a variety of clinical settings, including point-of-care settings. They can be performed either on a card, in a test tube, or in microtiter plates. While typically designed to provide only qualitative results, an antibody or antigen concentration can be obtained through serial sample dilutions. The major limitation to direct agglutination assays is their limited sensitivity, as they detect only microgram to milligram quantities of an analyte. Improved sensitivity can be achieved by variation of the direct agglutination method. For example, microtiter passive hemagglutination assays for infectious disease agents can achieve a sensitivity equivalent to that of a conventional EIA, with a lower limit of detection of approximately 15 to 30 μg/ml. These assays can likewise be reported as endpoint titers, with the caveat that the value has an expected variation of ±1 dilution. Therefore, a reciprocal endpoint titer of 16 is not considered significantly different from endpoint titers of 8 or 32. Finally, agglutination sensitivity may be affected by the prozone phenomenon (Fig. 1). The prozone effect refers to a lack of agglutination due to an excessive amount of antibodies in the patient sample and can readily be overcome by testing serial dilutions of the specimen (1, 3, 4).

Agglutination assays can also display limited specificity, particularly in the presence of rheumatoid factor (RF) (1, 3, 4, 30, 31). This is most commonly observed with assays using IgG-coated latex beads and has been reported as an important limitation of the latex agglutination test for cryptococcal antigen (3, 32). If the patient is known to have RF (of which the laboratory is rarely made aware), there are several measures that can be taken to overcome or identify potentially false-positive reactions. First, if a false-positive reaction is suspected, the sample result can be compared to results from a reaction using control particles coated with normal IgG. If the control reaction is also positive, indicative of a false-positive reaction, then the sample can be treated with a reducing agent, such as 2-mercaptoethanol or pronase, to inhibit RF. Alternatively, the sample can be pretreated with aggregated IgG to remove the RF. This, however, can result in loss of antigen or specific antibodies and lead to falsely negative results. An alternate test method may be necessary to overcome challenging samples known to be positive for RF.

Complement Fixation

The complement fixation (CF) assay is based on the interaction of immune complexes with components of the classical complement pathway (4). This classic technique is a two-step process used to detect antibodies and determine an endpoint titer. Briefly, patient serum is heated (56°C for 30 min) to inactivate native complement and subsequently incubated with pathogen-specific antigen. During the incubation, Ag-Ab complexes will form if specific antibody is present in the sample. Purified complement is then added, components of which will be bound ("fixed") by any Ag-Ab complexes in the solution. Hemolysin-sensitized RBCs are added next and act as the final indicator system. If complement was fixed by Ag-Ab complexes, the RBCs are protected from lysis and will pellet to the bottom of the reaction well following centrifugation (i.e., a positive result for the presence of antigen-specific antibodies). If, however, Ag-Ab complexes have not been formed, complement will remain active and lyse the RBCs, resulting in no cell pellet following centrifugation (i.e., a negative result).

CF assays are significantly more sensitive than agglutination, precipitation, and immunodiffusion assays and are relatively inexpensive to perform. However, they are technically demanding and have a long turnaround time, and the required reagents may be difficult to acquire. Therefore, many of these assays have been converted to EIA-based formats. However, a number of laboratories continue to use CF as a confirmatory method for a variety of infectious agents, including *C. immitis*/*C. posadasii*, *H. capsulatum*, adenovirus, herpesvirus, influenza virus, *M. pneumoniae*, and some rickettsial organisms.

Neutralization Assays

Another classic serologic technique is the neutralization assay, which can be used to detect the presence of antibody able to neutralize viral infectivity (4). This method involves mixing a patient serum sample with live virus and inoculating the solution onto a cell line or a preparation of peripheral blood mononuclear cells. The readout involves evaluation for viral replication by either observation for viral cytopathic effect or performance of an immunoassay for the increased presence of viral protein. Evidence of decreased viral replication confirms the presence of neutralizing antibody in the patient sample. Neutralization assays are often used by reference and public health laboratories to confirm presumptively positive viral serologic results. Additionally, these tests are commonly utilized to assess viral vaccine efficacy, particularly in HIV vaccine studies, in order to demonstrate that the vaccine candidate appropriately induces host antibody production (33). While relatively simple to perform, these assays have a prolonged turnaround time and require cultivation of live virus, and depending on the readout method, the interpretations of the results can be subjective. Furthermore, they can be difficult to standardize, both within and between different laboratories.

Immunofluorescence Assays

Immunofluorescence assays (IFA) use fluorescently labeled antibodies (e.g., labeled with fluorescein or phycoerythrin dyes) to detect either antigen or antibody in serum and other clinical specimens (3–5). There are two main classes of IFA: direct and indirect (Fig. 3).

Direct IFAs use fluorescently tagged monoclonal antibodies to directly detect the analyte of interest in tissue or body fluids. For example, detection of influenza virus in a nasal wash specimen begins by applying the sample to a slide, and then a fluorescently tagged anti-influenza virus antibody is laid over it. The slide is then reviewed using a fluorescence microscope and viewed at the appropriate excitation wavelength for the fluorescent tag. If the tagged antibody has bound influenza antigen present in the sample, fluorescent light will be emitted and observed by the technologist. In contrast, indirect IFAs involve a two-step process. An unlabeled target antigen, antibody, or whole-cell organism is adhered to a microscope slide. Next, the patient sample is added and allowed to incubate. Antigen-antibody complexes will form if the target analyte is present in the sample, and any unbound sample will be washed away. Fluorescently labeled secondary antibody is added, and the slide is observed using a fluorescence microscope.

In general, IFAs are inexpensive tests that are relatively easy to perform. In addition, IFA allows for localization of the analyte to a specific location in the tissue or cell monolayer, which is of particular value for histopathology studies. IFAs also allow for the determination of an endpoint titer, which can be used in some situations to determine the stage of disease or response to therapy. However, IFAs can be tedious and require the purchase of expensive fluorescence microscopy equipment. In recent years, commercially available, automated analyzers that perform IFA slide processing have been developed (34). Additionally, performance of IFAs requires well-trained, experienced laboratory personnel who are able to differentiate between background reactivity and specific fluorescence. Interpreting IFA results is

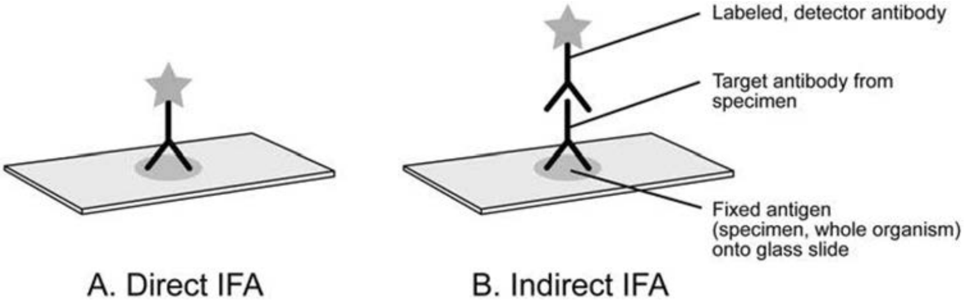
Labeled, detector antibody
Target antibody from specimen
Fixed antigen (specimen, whole organism) onto glass slide
A. Direct IFA
B. Indirect IFA

FIGURE 3 Diagram of a direct and an indirect immunofluorescence assay (IFA). doi:1128/9781555817381.ch7.f3

subjective, and the assays can demonstrate both inter- and intralaboratory variation in results. As with agglutination reactions, acceptable variability between endpoint titers is generally considered ±1 dilution; therefore, seroconversion or at least a 4-fold difference in endpoint titers is required for the change to be considered significant.

Enzyme Immunoassays

EIAs are becoming increasingly used in clinical laboratories and continue to be developed for detection of various analytes by many commercial manufacturers (1–3, 5, 6, 35, 36). The RIA has largely been replaced by the EIA in nearly all clinical laboratories, as EIAs offer comparable results without the challenges of radioactive waste disposal. The enzyme label used in EIA can be either fluorogenic, luminescent, or chromogenic in nature, and because a single activated enzyme can catalyze many molecules of the substrate, the detected signal from a single reaction is amplified, leading to sensitivities in the nanogram range. EIAs are also increasingly preferred over IFAs and agglutination reactions, as they often provide more-objective results, are readily adaptable to automation, and have a higher throughput with less technician hands-on time.

Competitive EIAs

EIAs can be designed as either competitive or noncompetitive assays (35). Competitive assays are commonly used to measure serum antigen levels and typically utilize unlabeled antibody bound to a solid phase. Briefly, both the patient sample containing the antigen of interest and purified, labeled antigen are added, thereby competing for the adhered antibody bound to the solid phase (Fig. 4). As a negative control, a similar reaction is set up using enzyme-labeled antigen and buffer alone. Following a washing step, the enzyme substrate is added, and the resulting color reaction is assessed. If the patient sample contains the target antigen, it will compete with the enzyme-labeled antigen for binding to the solid-phase antibody and reduce or prevent the color change from occurring. This reaction is then compared to the negative control (buffer plus enzyme-labeled antigen) to determine the specificity of the color change. For competitive assays, the amount of labeled immunoreactant detected is inversely proportional to the amount of antigen present in the sample.

Compared to noncompetitive assays, competitive EIAs often provide greater specificity, though at the cost of lower sensitivity. Competitive assays are ideal for measuring relatively small levels of analyte that can be obtained in large-enough quantities at high purity to be labeled with an enzyme. These assays generally require small amounts of antibody and, therefore, are ideal for use in systems which have a limited sample volume or antibody available.

Noncompetitive EIAs

Noncompetitive EIAs have become the mainstay of immunologic testing in the clinical microbiology laboratory, primarily due to their improved sensitivity compared to those of competitive assays. A variety of solid-phase supports can be used and include microtiter plates, nitrocellulose strips, and polystyrene beads. Reaction wells coated with purified antigen bind to specific antibodies in the sample, which will subsequently be detected with an enzyme-labeled secondary antibody (Fig. 5). To identify the specific antibody isotype (e.g., IgM, IgG), isotype-specific, enzyme-labeled anti-Ig antibodies can be used as the secondary antibody. This is perhaps the most commonly used approach for the measurement of immune status with regard to infectious agents and for autoantibody assessment. There are numerous variations of this type of assay, as the captured analyte can be an immunoglobulin, a viral protein, or any antigen with at least two epitopes. This format is often referred to as a "capture" or "sandwich" assay as the sample analyte is "sandwiched" between two antibodies.

EIA Technical Challenges

As with any other laboratory test, there are always factors that can affect assay performance. Immunoassays are certainly no exception and have been shown to be adversely affected more often than other routine clinical chemistry and microbiology assays (2, 6, 31, 36–40). There are various

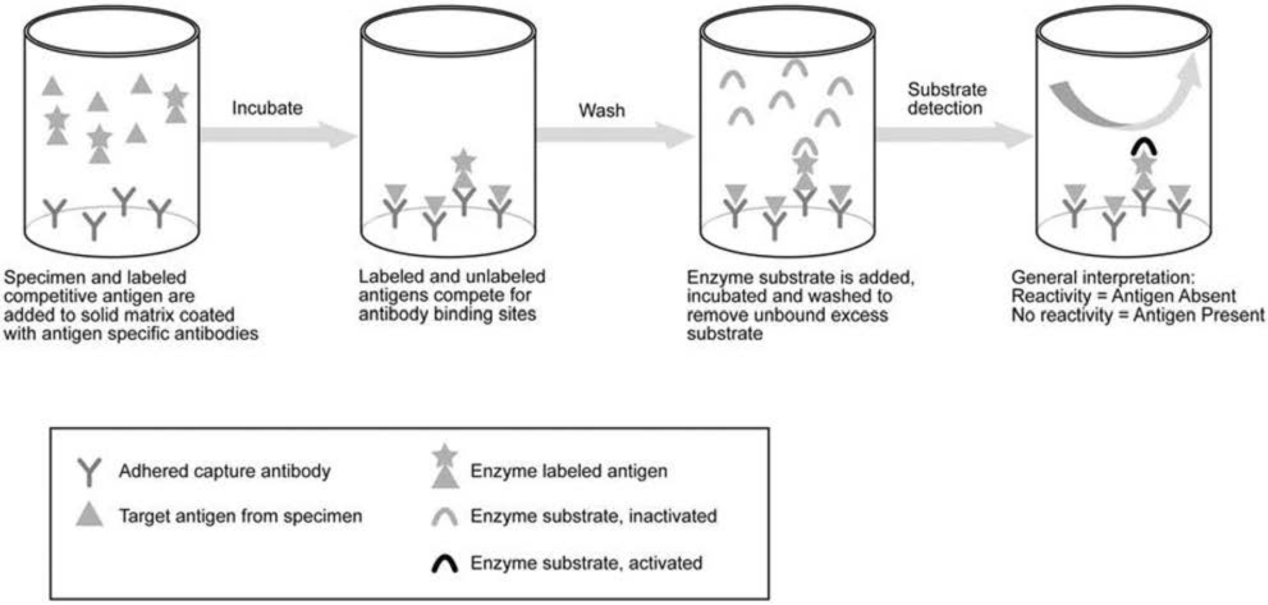
Incubate
Wash
Substrate detection
Specimen and labeled competitive antigen are added to solid matrix coated with antigen specific antibodies
Labeled and unlabeled antigens compete for antibody binding sites
Enzyme substrate is added, incubated and washed to remove unbound excess substrate
General interpretation:
Reactivity = Antigen Absent
No reactivity = Antigen Present
Adhered capture antibody
Target antigen from specimen
Enzyme labeled antigen
Enzyme substrate, inactivated
Enzyme substrate, activated

FIGURE 4 Diagram of a competitive EIA. doi:1128/9781555817381.ch7.f4

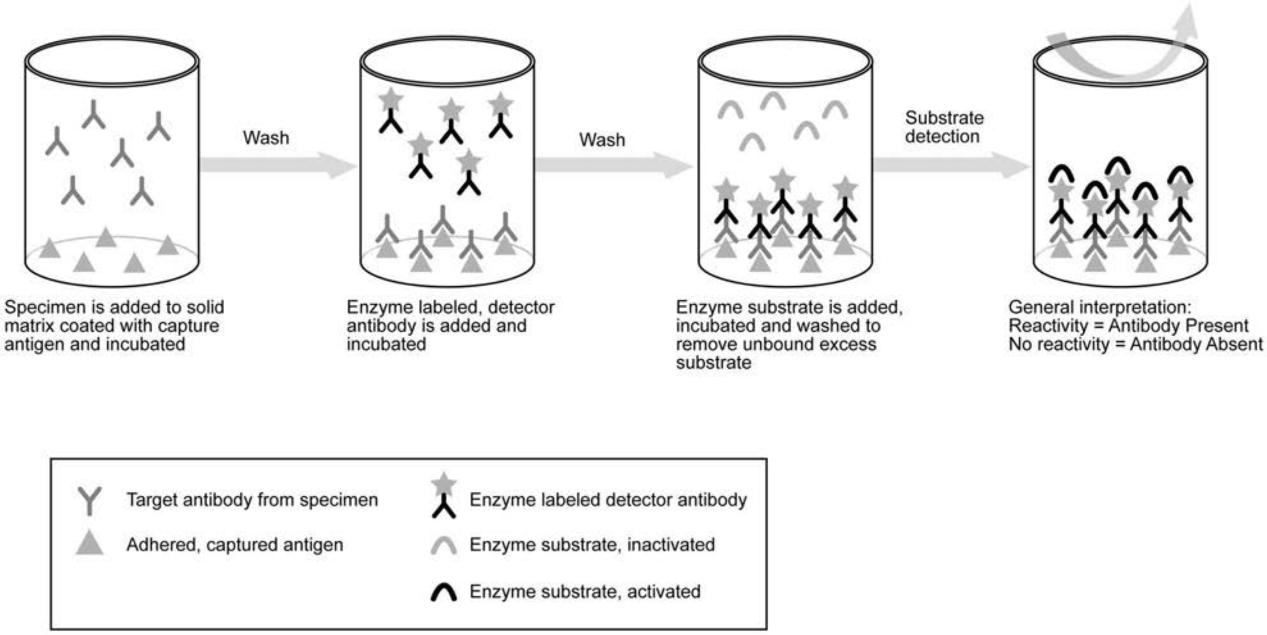
Wash
Wash
Substrate detection
Specimen is added to solid matrix coated with capture antigen and incubated
Enzyme labeled, detector antibody is added and incubated
Enzyme substrate is added, incubated and washed to remove unbound excess substrate
General interpretation:
Reactivity = Antibody Present
No reactivity = Antibody Absent
Target antibody from specimen
Adhered, captured antigen
Enzyme labeled detector antibody
Enzyme substrate, inactivated
Enzyme substrate, activated

FIGURE 5 Diagram of a noncompetitive EIA. doi:1128/9781555817381.ch7.f5

clues that should alert the laboratory to the possibility of an erroneous EIA result, including (i) feedback from a health care provider that results do not correlate with a patient's clinical presentation, (ii) results for a particular patient that have changed dramatically in a short period of time, (iii) a significant shift in positivity or negativity rates for the test, and (iv) an increase in run failures. These issues should prompt an investigation for potential technical problems associated with the EIA, some of which are discussed below.

Plate Variability

There are several issues to consider when performing sandwich EIAs using solid-phase microtiter plates (6, 35, 36). First, there can be variability between readings in adjacent wells of a microtiter plate that are inoculated with the same sample. This variability may be due to the lack of consistency during coating of the wells with capture antigen/antibody. This variability can be expressed as the well coefficient of variation (CV), which manufacturers strive to maintain between 3 and 5% for wells in the same plate. A second consideration is the "edge effect," which refers to the variability between readings in the outer wells and those in the inner wells for the same sample in a microtiter plate. While plate manufacturing variability may be a possible cause, this occurs primarily due to temperature differences between outer and interior wells, affecting both antigen-antibody and enzyme-substrate reactions. There are several ways to minimize the impact of the edge effect, including the use of single-column microtiter strips and close monitoring of the temperature and light exposure of samples during testing.

Hook Effect

The hook effect refers to an unexpected fall in the amount of an analyte at the high end of the dose-response curve, resulting in a gross underestimation of the amount of an analyte. This is equivalent to the prozone effect of agglutination reactions (2, 6, 31, 36–40). The hook effect is particularly problematic in sandwich immunoassays with patient samples that contain an extremely high level of analyte. During testing, the patient sample may give a low to moderately high result when a low dilution is used; however, upon further sample dilution, the result may unexpectedly continue to increase or go beyond the reportable range. Therefore, if laboratories run samples at a single routine dilution, they should be aware of the hook effect and potentially follow up on results that are considered by health care providers to be falsely negative.

Although the explanation for this phenomenon is not completely established, many investigators feel that the hook effect is due to excess antigen, which binds up the majority, if not all, of the antigen binding sites on both bound and unbound, enzyme-labeled detector antibodies. The free-floating antigen-labeled antibody complexes are removed by washing, preventing completion of the sandwich and signal detection. Alternatively, some feel that the hook effect may arise from a combination of the presence of low-affinity antibodies unable to form the sandwich complex, inadequate washing, or suboptimal levels of labeled antibody.

Numerous suggestions regarding ways that laboratories can overcome the hook effect have been made. One obvious strategy is to run all patient samples using 2 dilutions to screen for this phenomenon. If the sample is nonreactive or minimally reactive at the lower dilution but reactive or out of range at the higher dilution, this would alert the technician to the possibility of the hook effect. Although this is an effective approach, many laboratories are concerned about the time, cost, and labor involved with running 2 dilutions for every sample. Alternative strategies to reduce the risk of the hook effect include ensuring that adequate washing is performed between all steps of the EIA, especially following the addition and incubation with each antibody. Automatic plate washers are relatively inexpensive and can simplify this task and minimize potential ergonomic consequences. Additionally, close communication with the kit manufacturers, some of whom have identified a level at which the hook effect may occur, may lessen the frequency of this problem. Also, during new-kit evaluations, it is crucial that the laboratory test specimens with excess antibody levels, as the frequency of the hook effect may vary between different kits. Lastly, open dialogue with the clinician is invaluable, and laboratories are encouraged to discuss the limitations of EIAs with the direct end users, particularly in light of potential false-negative scenarios.

Antibody Interference

There are a number of endogenous antibodies in a patient's serum that may interfere with the performance of an immunoassay (2, 6, 31, 36–40). There are multiple types of antibody interferences, which can be caused by antibodies binding to the actual analyte (e.g., antipathogen antibodies), binding to components of the detection system (e.g., anti-alkaline phosphatase), or binding to reagent antibodies (e.g., anti-immunoglobulin antibodies). The last category is the most common and involves three types of antibodies. Heterophile antibodies are the first class of these cross-reacting immunoglobulins and are characterized by their weak affinity for immunoglobulins from multiple species, without a known or obviously identifiable immunogen. Second, RF, often found in patients with connective tissue disorders, can have a deleterious effect on a variety of immunoassays, often leading to falsely positive reactions. Finally, there are various types of anti-animal antibodies, the most common of which are the human anti-mouse antibodies (HAMA), which can react with the kit-associated, mouse-derived immunoglobulins (41). A number of techniques have been developed to decrease the interference associated with these cross-reacting antibodies, including heating samples to 70°C to inactivate all antibody in the specimen or precipitation with polyethylene glycol, protein A, or protein G (42). These methods are useful only if the EIA is designed to detect circulating antigen. Close communication with the kit manufacturer is also strongly advised for troubleshooting assistance.

Measurement of IgM

Detection and quantitation of IgM-class antibodies can pose unique technical challenges (3, 6, 26, 35). The most common issue regarding detection of IgM-class antibodies is the development of falsely positive reactions, most often due to the presence of RF in the patient sample. However, false-negative results can also occur due to competitive inhibition of IgM binding in the presence of high levels of specific IgG. Initially, EIAs designed to detect IgM were developed using a standard, indirect solid-phase EIA with an immobilized antigen and an IgM-specific secondary antibody. However, these assays were fraught with both low sensitivity and low specificity.

To obviate these problems, the IgM capture assay was developed (Fig. 6). In this procedure, a polyclonal anti-IgM antibody is bound to the solid phase. Upon incubation with the patient sample, IgM is captured on the plate. The test antigen is then added, binding any specific IgM present on the plate. An enzyme-labeled secondary antibody is subsequently added, and it will bind any immobilized antigen. This assay also obviates the problems with false-negative results due to competitive inhibition, as IgG in the patient sample is removed following the first wash step. False-positive reactions may still occur, however, due to either RF reacting with the IgG conjugate or binding any antigen-specific IgG in the sample. One method to avoid this problem is to use $F(ab')_2$-conjugated capture antibodies. These molecules lack the Fc portion of natural antibodies and, therefore, have minimal reaction with RF. Alternatively, the assay can be modified to a direct technique by employing enzyme-labeled antigen in the second step, thus eliminating any Ig which could bind RF. Even with these modifications, problems can still occur with borderline and slightly positive IgM results. For this reason, all IgM-specific antibody results should be evaluated with caution. As mentioned above, in certain clinical scenarios, performing an IgG avidity assay can help with the interpretation of positive IgM results.

Chemiluminescence Immunoassays

The chemiluminescence immunoassay (CLA) is a popular technique widely utilized in a variety of formats (1, 3). Chemiluminescence is defined as the emission of light

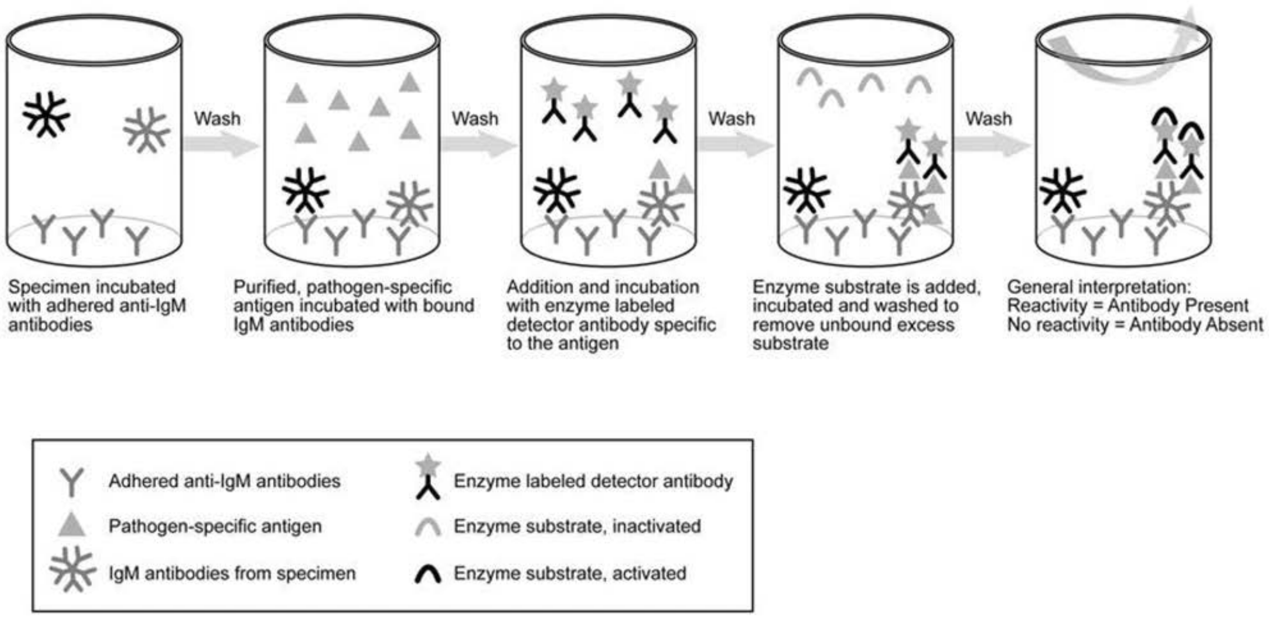
Wash
Wash
Wash
Wash
Specimen incubated with adhered anti-IgM antibodies
Purified, pathogen-specific antigen incubated with bound IgM antibodies
Addition and incubation with enzyme labeled detector antibody specific to the antigen
Enzyme substrate is added, incubated and washed to remove unbound excess substrate
General interpretation: Reactivity = Antibody Present No reactivity = Antibody Absent
Adhered anti-IgM antibodies
Pathogen-specific antigen
IgM antibodies from specimen
Enzyme labeled detector antibody
Enzyme substrate, inactivated
Enzyme substrate, activated

FIGURE 6 Diagram of an IgM capture EIA. doi:1128/9781555817381.ch7.f6

when a substrate decays from an excited state to a ground state. In contrast to fluorescence, chemiluminescence derives energy from the chemical reaction itself, most often an oxidation reaction. CLAs display the highest analytical sensitivity among immunoassays, with a lower limit of detection in the attomole (10^{-18}) to the zeptomole (10^{-21}) level. The antigen or antibody can be tagged directly with the chemiluminescent labels, or the label can be used as the substrate for an enzyme-labeled immunoreactant. Acridinium ester is the most commonly used CLA label due to its high sensitivity. Oxidation of these compounds, typically through the addition of sodium hydroxide or hydrogen peroxide, results in a burst of light that is detectable by a luminometer or by capture on photographic film. Advantages of CLA include label stability, sensitivity, and low cost. Limitations of this method include the requirement for specialized instrumentation and the potential for false-negative results if quenching agents are present in the specimen.

Western Blotting and Immunoblotting

Western blotting is a classic solid-phase assay in which pathogen-specific proteins (either a crude lysate or purified derivatives) are separated by denaturing gel electrophoresis and transferred (i.e., blotted) to nitrocellulose filter paper (Fig. 7). The filter paper is subsequently incubated with patient sera, and the presence or absence of antibodies specific to the separated proteins is determined using labeled secondary anti-human antibodies. Notably, most clinical laboratories forgo the initial protein separation step and purchase preblotted nitrocellulose membranes for testing. In addition, numerous platforms enabling the entire blotting procedure (e.g., washing, incubating, and developing) to be entirely automated have been developed, minimizing the amount of technician hands-on time required.

The difference between an immunoblot and a Western blot is that the protein bands on immunoblots are applied directly to the membrane, using purified or recombinant protein, and not transferred from a gel. Immunoblots offer increased specificity and clarity for interpretation, as the potential cross-reactions and background noise due to nonspecific binding are substantially reduced. Regardless of the method, blotting patterns can be read visually, using radiolabeled isotopes or using a CLA substrate, which is then developed on X-ray or photographic film. The presence or absence of a band, however, can be subject to multiple interpretations and may lead to over- or underinterpretation of results. Advances in optical scanning instrumentation which allow for a more objective interpretation of blot results have recently been made.

Western blotting and immunoblotting are most commonly used for confirmatory purposes following an initial screening assay and are typically interpreted by identifying a specific antibody banding pattern. As an example, Western blotting or immunoblotting is currently recommended as a second-tier, confirmatory assay for the presence of antibodies to *B. burgdorferi*, the causative agent of Lyme disease.

Rapid Immunoassays

Immunodiagnostic testing has been revolutionized with the development of rapid immunoassays for numerous analytes that were previously performed only at large reference laboratories. CLIA regulations for many of these assays are waived, and they can now be performed as point-of-care tests in as little as 15 to 30 min (4, 16, 43–49). These assays have been designed in a variety of formats and are able to detect antigen, antibody, or even products from nucleic acid amplification tests.

Among the most popular formats is the immunochromatographic method, more commonly referred to as the

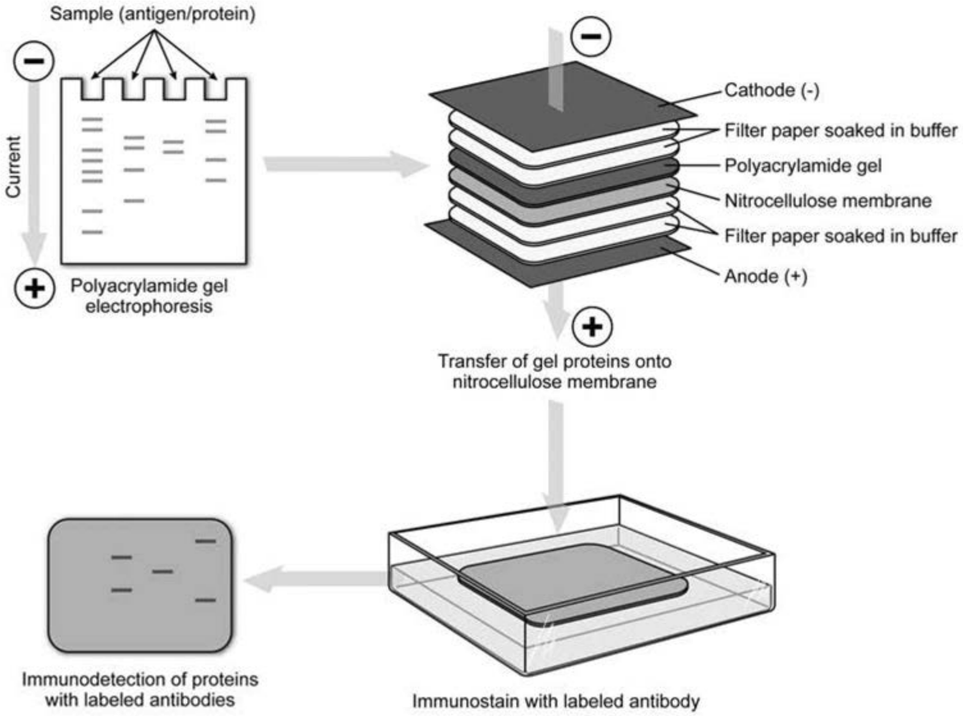
Sample (antigen/protein)
−
Current
+
Polyacrylamide gel electrophoresis
−
Cathode (-)
Filter paper soaked in buffer
Polyacrylamide gel
Nitrocellulose membrane
Filter paper soaked in buffer
Anode (+)
+
Transfer of gel proteins onto nitrocellulose membrane
Immunodetection of proteins with labeled antibodies
Immunostain with labeled antibody

FIGURE 7 Diagram of the Western blot procedure. doi:1128/9781555817381.ch7.f7

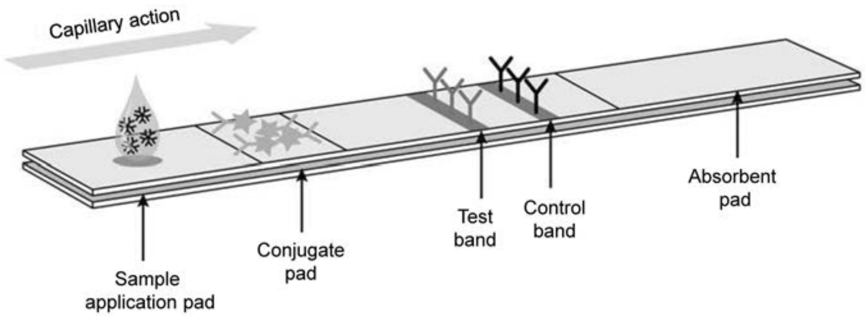

FIGURE 8 Diagram of a lateral-flow immunoassay (LFA). doi:1128/9781555817381.ch7.f8

lateral-flow immunoassay (LFA). This one-step assay uses a chromatographic pad with three zones: a sample application area, a conjugate pad, and the reaction area (Fig. 8). The sample (e.g., serum, CSF, and urine) is applied to the sample pad and flows laterally by means of capillary action. On the conjugate pad is lyophilized antibody or antigen (depending on the target analyte) tagged with a specific label, which generates a visible signal on the reaction region. Commonly used conjugates include colloidal-gold particles or latex beads. If present, the target analyte will bind to the conjugated detector analyte, and the resulting immune complex will migrate to the reaction pad by capillary action. The reaction area is impregnated with a secondary antibody or antigen specific to the target analyte and a control antibody specific to the conjugated analyte at two distinct locations. Capture of the immunocomplex leads to concentration of the detector particles and the development of a colored band at both locations, indicative of a positive reaction. The presence of a control band indicates a negative reaction, whereas the absence of a control band invalidates the test.

As variations to this assay format, systems that do not require separate venipuncture or specimen collection procedures have been designed. Often a finger stick device is combined with the one-step lateral-flow assay. Rapid assay formats have been especially useful for HIV testing and have been used extensively in testing for HIV in underdeveloped countries. More recently, LFAs have been developed for detection of bacterial, fungal, and parasitic antigens from various specimen types: *Legionella pneumophila* serogroup 1 (urine), *Streptococcus pneumoniae* (urine or CSF) or *Cryptococcus* (serum or CSF) antigen, and *Plasmodium* spp. (serum) (50).

Overall, rapid assays are straightforward to perform, are inexpensive, and do not require specialized instrumentation. While LFAs are considered to have lower sensitivity than conventional EIAs, some rapid assays display sensitivities approaching those of traditional assays (44). Limitations of rapid assays include the inability for automation and a subjective interpretation.

Automated Technologies

With the continuous expansion of commercially available EIAs, the development and market for automated EIA analyzers have followed suit. It is beyond the scope of this chapter to provide a detailed review of the available instrumentation; however, the reader is referred to the College of American Pathologists' website (http://www.cap.org), which provides a comprehensive, annual review of each individual, commercially available immunoassay analyzer (51). These instruments are designed as either semi- or fully automated platforms able to process samples in batches or by random access. Automated systems have been adapted to capture the analyte of interest (antigen or antibody) using a variety of solid-phase matrices, including microtiter plates, dyed polyacrylamide or polystyrene beads, magnetic beads, or solid-phase receptacles. Analyte detection can likewise vary depending on the EIA kit, which has necessitated the design of automated systems with the ability to detect colorimetric, chemiluminescent, and fluorescent end products. Currently, these instruments can run a broad range of serological assays to detect both antigens and antibodies for, e.g., HIV, hepatitis (A, B, and C), *T. gondii*, cytomegalovirus, rubella virus, and *Chlamydia trachomatis*, provided that an EIA-based kit is available. An increasing number of automated analyzers are also designed to directly interface with the laboratory information system, allowing easier result reporting while minimizing the potential error associated with manual result entry.

An exciting new area of automated immunoassay technology has been the development of multiplex immunoassay systems (52, 53). Many of these instruments combine several technologies, including chemiluminescence, fluorescence immunoassays, flow cytometry, and molecular diagnostics, to allow for rapid and simultaneous analysis of multiple analytes in a single sample. An example of this class of analyzers is the multiplex flow immunoassay, which uses microspheres as the solid-phase matrix, each impregnated with a different fluorescent indicator. The spheres are coated with a unique capture molecule (antigen or antibody) and, depending on the target analytes of interest, can be added in almost any combination to a patient specimen. Following incubation, a secondary, fluorescently labeled antibody specific to the target analyte is added and the microspheres are analyzed by flow cytometry. Each bead, and therefore the associated coating antigen, is identified through its unique dye fluorescence, and the presence of the target analyte is determined based on the presence or absence of fluorescence from the secondary antibody. Using this technology, a recent report described the multiplex detection of IgG-class antibodies to the measles, mumps, rubella, and varicella-zoster viruses in a single reaction (54). Detection of other analytes using this multiplex technology has also been reported (55–57).

A second example of a highly automated system with the potential for multiplexing capabilities is the immuno-PCR (IPCR) assay. This is a novel technique that combines the traditional EIA format with PCR technology (3, 10, 38, 58–65). It follows a standard EIA protocol in which the secondary antibody is directly linked to a nucleic acid tag rather than an enzyme label. The linker DNA is subsequently amplified by PCR, and the amount of PCR product is directly proportional to the amount of target analyte present in the sample (66). This is an ultrasensitive technique, in some cases with a limit of detection in the attogram (10^{-18}-g) range, and has already been applied toward the detection of multiple viral and bacterial agents (e.g., HIV, rotavirus, *Staphylococcus aureus* protein A, norovirus, and Shiga toxin) (66, 67). IPCR also gained recognition for its ability to detect prion proteins, which are difficult to identify with currently available methods (39, 64). Despite the potential benefits of IPCR, many challenges with this technology must be overcome prior to its broad implementation in clinical laboratories. These challenges include issues with high background reactivity due to nonspecific interactions, the general multistep, complicated assay protocol, which may lead to performance errors, instrumentation cost, and, finally, the potential for cross-sample contamination with amplified PCR products. Development is ongoing to resolve these potential pitfalls.

SUMMARY

The role of immunodiagnostics in clinical microbiology is a rapidly advancing and evolving area. There has been significant progress made in the performance of serologic tests for infectious diseases, and automated platforms now allow testing laboratories to increase sample throughput. In addition, automated instruments reduce hands-on time, provide better standardization, and increase efficiency while decreasing turnaround time to result reporting. Furthermore, in the current era of cost reduction, automated instruments are more commonly being designed to process and test samples across clinical laboratory disciplines. Despite these advantages, automated instrumentation can present clinical laboratories with a number of challenges, including a high upfront purchase cost, which may be difficult to overcome in smaller laboratory settings. Additionally, some of these platforms have large footprints, and instrument errors can occur. Finally, as automated platforms continue to have more-expansive test menus, the potential for both improper test utilization and incorrect interpretation of results will likely increase. This results in an even greater need for clinical laboratories to work closely with health care providers to guide proper test utilization and provide accurate result interpretation for the diagnosis of infectious diseases.

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Prevention of Health Care-Associated Infections

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8

Health care-associated (or nosocomial) infections (HAIs) represent one of the most common complications of health care delivery, affecting an estimated 1.7 million persons admitted to acute-care hospitals in the United States each year (1). The World Health Organization reports that millions more worldwide suffer each year from infections acquired in the health care setting, estimating the pooled HAI prevalence among hospitalized patients worldwide to be 10.1% (2). Every health care facility should therefore have an infection prevention program (IPP) charged with monitoring, preventing, and controlling the spread of infections in the health care environment. Because infection prevention requires the ability to detect infections when they occur, the clinical microbiology laboratory (CML) is inextricably linked to any comprehensive IPP. In this chapter, we discuss the impact of HAIs, outline the organization of the hospital IPP, and describe the important roles of the CML in the prevention of HAIs.

HEALTH CARE-ASSOCIATED (OR NOSOCOMIAL) INFECTION

Definition

An HAI is one that is acquired in a hospital or health care facility (i.e., the infection was not present or incubating at the time of contact with the health care environment). For most bacterial infections, an onset of symptoms more than 48 h after admission is evidence for health care acquisition. To determine whether some infections, such as legionellosis, are health care associated, one must consider the usual incubation periods and determine whether the patient was exposed to the health care environment during that time period. Because hospital stays are getting shorter and more patients are being treated in the outpatient setting, many HAIs are not recognized during hospitalization or may be associated with health care environments other than acute-care hospitals (e.g., ambulatory surgery centers, dialysis, and long-term care). IPPs must therefore devise strategies for effective outpatient surveillance in order to accurately monitor HAI rates (3).

Infection Rates, Infection Sites, and Predominant Pathogens

At least 5 to 10% of patients admitted to acute-care hospitals acquire an infection during hospitalization (2, 4, 5). The urinary tract is the most commonly involved site, comprising 30 to 40% of all HAIs. Surgical wound and lower respiratory tract infections are the next most frequent, with each accounting for about 15 to 20% of HAIs, followed by bloodstream (5 to 15%) infections. The vast majority of HAIs are associated with medical devices (e.g., urinary tract catheters, endotracheal tubes in ventilated patients, and central venous catheters). For this reason, and as a way to adjust for risk when comparing rates over time or between similar units in different facilities, the Centers for Disease Control and Prevention (CDC) recommends calculating HAI rates in the intensive care unit (ICU) using device days as the denominator (see Table 1 for definitions of common epidemiology terms) (6).

Table 2 lists the five most common bacterial pathogens isolated from various infection sites in U.S. hospitals (7). However, the spectrum of organisms causing HAIs is continuously changing, and organisms may vary from region to region or even from hospital to hospital (8). From the 1970s through 2000, the spectrum of nosocomial pathogens shifted from Gram-negative to Gram-positive organisms, and *Candida* spp. emerged as a major problem (9). The incidence of nosocomial infections caused by staphylococci and enterococci increased as these organisms became increasingly resistant to antimicrobial agents (10, 11). More recently, multidrug-resistant Gram-negative rods (MDR-GNRs; defined by *in vitro* resistance to at least three of the antimicrobial classes used for treatment) have become increasingly prevalent in many hospitals (12–14). For example, 2009–2010 data from the National Healthcare Safety Network (NHSN) reveals that multidrug resistance (including carbapenem resistance) was reported in more than 60% of *Acinetobacter* species among most HAI types (7), and a recent CDC report documented a 4-fold increase in the proportion of carbapenem resistance among *Enterobacteriaceae* (CRE) in U.S. hospitals, from 1.2% in 2001 to 4.2% in 2011 (15).

Morbidity, Mortality, and Cost

HAIs cause or contribute to thousands of deaths annually (1, 2, 4). Because patients with the most severe underlying illness are also those most vulnerable to HAI, it is very difficult to estimate the proportion of crude or overall mortality that is directly attributable to HAIs. Studies that attempt to address this by carefully controlling for many potentially confounding variables are called attributable mortality studies. Estimates of the attributable mortality associated with health care-associated bloodstream infections have ranged from

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TABLE 1 Commonly used terms in health care epidemiology[a]

Term	Definition or summary
Epidemiology	Study of the occurrence, distribution, and determinants of health and disease in a population. Hospital or health care epidemiology is the study of disease occurrence and distribution in the hospital or health care system.
Nosocomial infection	Infection acquired in a hospital or other health care facility.
Endemic infections	Infections occurring as part of the background or usual rate of infection in a specified population.
Epidemic infections	Infections occurring as part of an outbreak (or epidemic) of an infection, defined as a significant increase in the usual rate of that infection in the specified population.
Incidence rate	Ratio of the number of new cases of infection in a specified population at risk during a defined time period to the overall number of people in the population at risk (the denominator).
Device-associated incidence rate	Ratio of the number of new cases of device-related infection in a specified population at risk during a defined time period to the number of days of device utilization in the population at risk.
Prevalence rate	Total number of cases of infection in the defined population at risk at one point in time (point prevalence) or in a given time period (period prevalence).
Observational or descriptive study	Study of the natural course of events, without an intervention in the process.
Case-control study	Study frequently done as part of an outbreak investigation in which a group of patients with the outcome of interest (e.g. cases of nosocomial infection) is compared to a control group of patients without the outcome. A comparison of specific factors between groups (i.e., exposures of interest) may suggest why infection occurred.
Crude or overall mortality rate	Ratio of the number of patients who die to the overall number of patients in a specified population.
Attributable mortality	Ratio of the number of patients who die as a direct result of the disease of interest to the number of the overall population with the disease.

[a]Data are adapted from reference 6.

14% for infections caused by coagulase-negative staphylococci (16), 31% and 37% for infections caused by vancomycin-susceptible and vancomycin-resistant enterococci (VRE) (17, 18), respectively, and 38 to 49% for infections caused by *Candida* spp. (9, 19, 20) (Table 3).

HAIs also increase hospital costs and lengths of stay (LOS); a recent CDC report estimated the annual direct medical costs of HAIs in U.S. hospitals at between \$28.4 and \$33.8 billion (21). At the University of Iowa, the median excess LOS for nosocomial bloodstream infections caused by coagulase-negative staphylococci and *Candida* spp. were 8 and 30 days, respectively (16, 20). Health care-associated bloodstream infections in the ICU were associated with an excess LOS of 24 days and excess hospital costs of \$40,000 per survivor (22). Kirkland found that surgical-site infections increased LOS by more than 6 days and increased hospital costs by over \$3,000 per infection (23). Surgical-site infections after major orthopedic surgery (e.g., hip replacement) are associated with an even greater increase in length of stay and costs (24).

The premise upon which IPPs operate is that many of these life-threatening and costly HAIs are preventable. The landmark Study of the Efficacy of Nosocomial Infection Control (SENIC) indicated that the presence of an active IPP was associated with a 32% decrease in HAI rate, while the absence of such a program was associated with an 18% increase in HAI rate (25). During the 1990s, the CDC National Nosocomial Infections Surveillance (NNIS) system of hospitals reported a reduction in risk-adjusted infection rates in ICUs for all monitored infection sites (urinary tract, bloodstream, lung). The elements that were critical for reducing rates included targeted surveillance in high-risk populations (using standard definitions), adequate numbers of trained infection preventionists (IPs), who inform health care providers of their infection rates, and prevention efforts designed to address issues identified during evaluation of infection rates (26). More recently, hospitals using combinations ("bundles") of evidence-based prevention strategies have demonstrated greater reductions in device-associated HAIs than previously thought possible (27, 28). For example, a collaborative study involving over 100 ICUs in Michigan demonstrated a two-thirds reduction in rates of central-line-associated bloodstream infection (CLABSI), with a reduction of the median CLABSI rate to zero among participating ICUs (27). Thus, although it is not known what proportion of all HAIs are truly preventable, an effective IPP clearly improves patient care, saves lives, and decreases health care costs.

HOSPITAL INFECTION PREVENTION PROGRAM

Structure, Personnel, and Responsibility

The responsibilities of the hospital IPP include surveillance and prevention of HAIs, continuing education of medical

TABLE 2 Distribution of the five most common nosocomial pathogens isolated from major infection sites reported to the NHSN from 2009 to 2010[a]

Infection site and pathogens	% of total at each infection site
Central line-associated bloodstream infection	
CoNS	20.5
Enterococcus spp.	18.1
Candida spp.	14.6
Staphylococcus aureus	12.3
Klebsiella pneumoniae/Klebsiella oxytoca	7.9
Ventilator-associated pneumonia	
Staphylococcus aureus	24.1
Pseudomonas aeruginosa	16.6
Klebsiella pneumoniae/Klebsiella oxytoca	10.1
Enterobacter spp.	8.6
Acinetobacter baumannii	6.6
Catheter-associated urinary tract infection	
Escherichia coli	26.8
Enterococcus spp.	15.1
Candida spp.	12.7
Pseudomonas aeruginosa	11.3
Klebsiella pneumoniae/Klebsiella oxytoca	11.2
Surgical-site infection	
Staphylococcus aureus	30.4
CoNS	11.7
Enterococcus spp.	11.6
Escherichia coli	9.4
Pseudomonas aeruginosa	5.5

[a] NHSN, National Healthcare Safety Network; CoNS, coagulase-negative staphylococci. Data are from reference 7.

staff, control of infectious disease outbreaks, protection of employees and visitors from infection, and advice on new products and procedures. The program is generally directed by a physician/epidemiologist and enforced by the infection prevention committee. Every hospital must also have a working infection prevention staff, comprising one or more infection preventionists (IPs). The IPs coordinate surveillance and prevention of HAIs, among many other related activities, and provide their data to the infection prevention committee.

Infection Prevention Committee

The infection prevention committee is responsible for reporting and evaluating HAI data and for drafting and implementing policies, procedures, and guidelines pertinent to the practice of infection prevention. The committee should be multidisciplinary, with representatives from all departments, including clinical microbiology, and should meet every 1 to 3 months to review hospital-specific HAI data and to formulate policy. The members bring the needs and perspectives of their departments to the committee and, in turn, take back important information about infection prevention initiatives and policies. Other responsibilities of the committee include reviewing technical information about new products, devices, or procedures pertinent to infection prevention and instituting all necessary control measures in the event of an outbreak or other infection-related emergency.

TABLE 3 Attributable mortality of nosocomial bloodstream infection due to selected pathogens

Organism(s)[a]	Mortality among cases (%)	Mortality among matched controls (%)	Attributable mortality (%)	Reference(s)
CoNS	31	17	14	16
Enterococcus spp.	43	12	31	18
VRE	67	30	37	17
Candida spp.	57–61	12–19	38–49	19, 20

[a] CoNS, coagulase-negative *Staphylococcus* spp.; VRE, vancomycin-resistant *Enterococcus* spp.

The clinical microbiologist, or the microbiology supervisor in an institution that does not have a doctoral-level microbiologist, is an integral component of the infection prevention team and thus must be an active member of the infection prevention committee. Because the infection prevention committee frequently bases its decisions on the results of microbiological tests, the clinical microbiologist must instruct members of the committee how to integrate culture results and which microbiological approaches can be used to solve specific infection prevention problems. The microbiology laboratory can benefit if the infection prevention staff understands the routine processes in microbiology, e.g., timelines for the processing of blood, wound, or urine cultures and related techniques (29). Specimen processing timelines enable infection prevention staff to understand various expectations of turnaround times (TATs) for specific results and time constraints of microbiology test services and can educate them as to when they can expect follow-up information for routine cultures, thus minimizing premature phone calls to the laboratory requesting culture information. Conversely, while serving on the committee, the microbiologist will learn about the problems confronting infection prevention personnel and thus will be better able to organize the laboratory's response to such problems.

In addition, the microbiologist should inform the committee about changes in methods, reagents, or instrumentation that may substantially affect the laboratory's ability to detect and characterize HAI pathogens. These include changes in the sensitivities and specificities of diagnostic methods and changes in antimicrobial susceptibility testing (AST) interpretive criteria, as well as taxonomic changes that may create confusion. An example of changes in testing and reporting criteria that directly affect infection prevention efforts is the recent change in the interpretive breakpoints for cephalosporins and carbapenems against the *Enterobacteriaceae* enacted by both the Clinical and Laboratory Standards Institute (CLSI) (30) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (31). The new (lower) breakpoints are intended to obviate the need for extended-spectrum β-lactamase (ESBL) confirmatory testing or modified Hodge testing (confirmation of carbapenemase) for clinical use. The result of this change in testing and interpretive criteria has been the loss of considerable epidemiologic data available from routine testing, as isolates with ESBL production may no longer be confirmed or flagged as such (32), and in some instances, this has led to an increase in the number of isolates characterized as resistant to these agents and therefore potentially MDR, with major implications for infection prevention (33–36). One institution recently reported that this change resulted in a 35% increase in the number of MDR-GNRs identified and a concomitant increase in the hospital's use of contact precautions (33).

Members of the infection prevention team must communicate with each other to accomplish their goals. Communication can be enhanced if the infection prevention staff members regularly make rounds in the laboratory to ask questions, review microbiological and molecular testing results, and discuss current problems and views. Likewise, the microbiology staff should attend conferences at which infection prevention personnel discuss epidemiological principles and contemporary topics. Unfortunately, several ongoing trends challenge these valuable personal interventions between microbiology and infection prevention personnel (34). Consolidation of CML services, off-site relocation of microbiology laboratories, and total reliance on electronic medical records to the exclusion of first-hand observation (e.g., review of plates or Gram stains) too often keep clinicians and infection prevention personnel out of the microbiology laboratory and keep microbiologists confined to the laboratory.

HAI Surveillance

Active HAI surveillance programs are associated with reduced rates of infection and their consequent morbidity and mortality (25, 37); national and state accrediting agencies require hospitals to do HAI surveillance, and public reporting of certain HAI rates is now a requirement for U.S. hospitals that participate in the Medicare program (38). Thus, systematic surveillance of HAIs is the IPP's most important activity. Surveillance is also the IPP's most costly and time-consuming activity. Surveillance allows the IPP to monitor the frequency and types of HAIs, detect outbreaks, evaluate compliance with infection prevention guidelines, provide data for policy development, and monitor the effect of infection prevention interventions on HAI rates. To accomplish the overall goal of decreasing infection rates, the IPP must provide surveillance data to clinic units as soon as possible, accompanied by suggestions for improvement and reminders of existing infection prevention practices. IPPs can compare their hospital rates with national benchmarks compiled and reported by the CDC NHSN system (39). Figure 1 is a sample format for comparing infection rates in an ICU with national benchmark data. The IPP should provide infection rates, recommendations for reducing rates, and assistance in implementing interventions to unit personnel, such as medical directors, nurse managers, and clinicians.

The IPP should design a surveillance system that is compliant with state and federal mandates, appropriate for the specific needs of the hospital, and feasibly based on their budget. Because surveillance consumes more resources than any other infection prevention activity (40), IPPs must design the most efficient surveillance system possible. The most complete and accurate surveillance program would require an IP to review charts of all hospitalized patients daily, but this approach obviously is not practical in any but the smallest of hospitals. IPPs should focus limited resources in the highest-risk areas (e.g., intensive care, hematology/oncology, burn, and organ transplant wards) and use various screening techniques to increase surveillance efficiency. IPs can use a variety of data sources (e.g., microbiology reports, nursing care plans, antibiotic orders, radiology reports, vital signs, and discharge diagnoses) to determine which charts should be further reviewed.

Review of microbiology data is probably the most common single method for case finding, and it compares favorably in some circumstances with more-comprehensive ward-based surveillance (41, 42). For example, Yokoe and colleagues reported that review of microbiology data alone was both more resource efficient than and as effective as applying the CDC's definition of health care-associated bloodstream infection (43). Such laboratory-based surveillance allows the IP to efficiently review a large amount of data. Moreover, medical information systems can enhance laboratory-based surveillance further by linking laboratory data with data from many other sources (44, 45), including pharmacy (antimicrobial use), radiology, billing (diagnostic codes), patient census data, and nursing notes (vital signs, care plans).

Although reviewing microbiology reports is an essential part of surveillance, these data alone may not detect all HAIs or all outbreaks. The sensitivity and specificity of laboratory-based surveillance depend upon both the

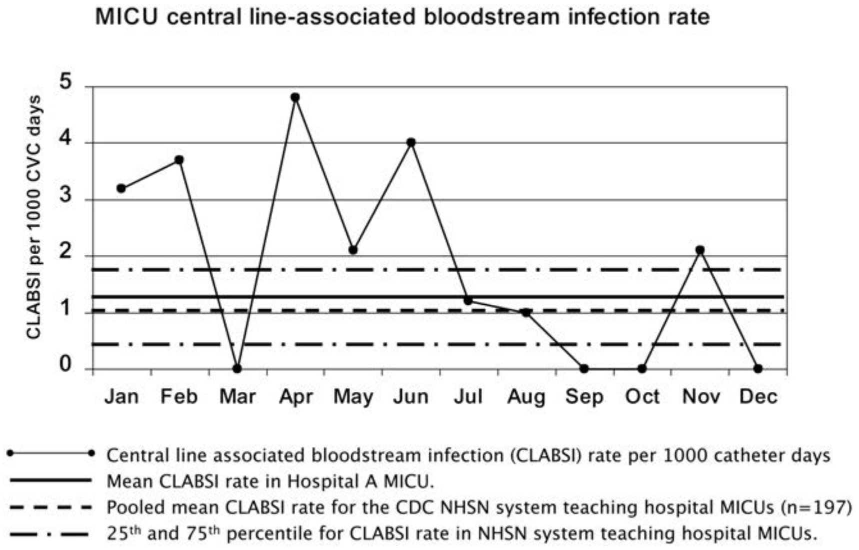

FIGURE 1 Rates of central venous catheter (CVC)-associated infection in a medical ICU (MICU). Sample chart format for reporting nosocomial infection rates in an ICU compared with CDC NHSN benchmarks. doi:1128/9781555817381.ch8.f1

frequency with which clinicians obtain cultures and the quality of the culture specimens received by the lab. In addition, while laboratory-based surveillance may quickly detect outbreaks due to unusual pathogens, or infections at unusual sites, outbreaks or clusters due to common pathogens at common sites (e.g., *Escherichia coli* urinary tract infection) may go undetected for longer periods of time. An optimal surveillance program will include data from multiple sources to help IPs determine which charts deserve further review. The University of Iowa previously validated a surveillance strategy using primarily microbiology reports and nursing care plans and found the sensitivity and specificity to be 81% and 98%, respectively (46). More recently, we introduced and validated a computer-based screening algorithm that provides for each IP each day a list of all patients in their units that had specific abnormalities in vital signs or white blood cell counts, positive microbiology findings, or different combinations of tests ordered within a 24-h period (e.g., chest radiograph and culture of respiratory secretions or cultures from two or more body sites). After reviewing this list, the IPs review the medical records of a smaller percentage of patients, thereby reducing the amount of time required for surveillance.

The overall approach to surveillance in each health care facility should be monitored and adjusted by the infection prevention committee based on the types of patients treated, the procedures performed, the resources available for surveillance, prevailing infection rates, state and federal mandates, and other factors. The surveillance approach should be reevaluated each year during an annual risk assessment performed by the IPP and approved by the infection prevention committee. In addition, the infection prevention committee, in consultation with CML personnel, should decide the mechanism by which the CML provides specific information needed for surveillance (e.g., all positive results and selected or sorted reports).

Increasing public disclosure of HAI rates (38, 47–50) and increasing pressure for hospitals to demonstrate reductions in HAIs have important implications for surveillance. If interhospital comparisons of HAI rates are to have any meaning, all hospitals must use the same methods for surveillance and risk adjustment (47, 49) and HAI definitions must be as objective as possible so that they are not subject to “gaming” (38). Unfortunately, methods for HAI surveillance vary widely among hospitals, and validated methods for risk-adjusting rates are not available (51). So although public disclosure of HAI rates is a laudable goal and is now reality in the United States, much work needs to be done to ensure that such reporting improves, rather than hinders, efforts to prevent HAIs (48, 52).

Process Surveillance

Several recent studies clearly demonstrate that implementing evidence-based infection prevention practices, such as good hand hygiene (53, 54), and the guidelines for placement and care of central venous catheters (27, 55) can dramatically reduce HAI rates (27, 54). The obvious implication of these studies is that health care workers do not routinely adhere to the safest processes of care. For this reason, IPs must perform surveillance not only for important outcomes (infections) but also for important processes (e.g., rates of hand hygiene performance, use of maximal sterile barriers during central venous catheter placement, and elevation of the head of the bed to 30 degrees for ventilated patients). Process measures can help infection prevention personnel understand some of the variation in HAI rates. In addition, reporting adherence rates to personnel may improve practice and reduce HAI rates.

Antimicrobial Stewardship

Every hospital in the United States must now have an antimicrobial stewardship program (ASP), guidelines for which have been published by the Infections Disease Society of America (IDSA) and the Society for Healthcare Epidemiology of America (SHEA) (56). Antimicrobial stewardship efforts are directly dependent on reports from the CML, so good communication between the laboratory, pharmacy, infection prevention personnel, and a stewardship team is essential. For guiding empirical antimicrobial therapy, unit-specific and tailored antibiograms should be updated on a regular basis and provided to clinicians for use at the bedside. Such antibiogram data can also be used for evaluation of trends in important antimicrobial resistance rates and for education of clinicians regarding optimal antimicrobial use. Directed antimicrobial therapy requires patient-specific culture and susceptibility data. This allows for a prospective audit of antimicrobial use with feedback to the prescriber.

A major challenge to effective stewardship is the ability to obtain antimicrobial susceptibility data from the laboratory in a timely and efficient manner. Efforts to reduce the analytical turnaround time are helpful; however, the data must then be incorporated rapidly into antimicrobial management. The task of the ASP is made considerably more difficult if laborious chart reviews are required for each patient on antimicrobial therapy. The use of a computer decision support system can streamline this process by automatically analyzing patient data from multiple sources (pharmacy, laboratory, electronic medical record, unit census) and generating a variety of alerts for the ASP (e.g., bug-drug mismatch, allergy, drug interactions, duplicative coverage, and the opportunity to convert from intravenous to oral drug administration) (57, 58). One ASP projected that a computer decision support system could save the institution over $600,000 annually compared to the ASP without the decision support system (58).

ROLE OF THE CML IN INFECTION PREVENTION

With this overview of the structure and activities of the hospital IPP in mind, we will now focus on the most important specific roles played by the CML in the day-to-day practice of infection prevention.

Specimen Collection and Transport

Many HAI pathogens (e.g., coagulase-negative staphylococci) also commonly colonize patients’ skin or mucous membranes and can easily contaminate cultures if specimens are not collected or handled properly. If contaminants are mistakenly considered to be infecting organisms, IPs may inadvertently count these as HAIs, thereby inflating the infection rates (29, 59, 60). Consequently, the laboratory must provide guidance for acceptable collection and transport of clinical specimens (see chapter 18). Enforcement of rejection criteria and a quality assurance program with monitors for blood culture contamination rates and specimen transport time help to optimize the preanalytical phase of testing.

Accurate Identification and Susceptibility Testing of HAI Pathogens

Commercial identification and susceptibility testing systems allow most laboratories to identify microorganisms to the species level and to perform antimicrobial susceptibility testing (AST) (see chapters 4 and 72). However, the expanding spectrum of organisms that colonize and infect seriously ill patients challenges the ability of the CML to identify and characterize HAI pathogens accurately. Consequently, the laboratory frequently must update the methods used to identify and characterize HAI pathogens. For example, the introduction of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) as an identification method provides the opportunity for more precise (and more rapid) characterization of some previously difficult to identify HAI pathogens (see chapter 4).

The degree to which organism identification is routinely conducted can be important to HAI control efforts. Infection prevention involves a constant search for evidence that a common organism has spread from patient to patient (34, 61). The ability to detect such events is enhanced by identifying the organism to the species level. Laboratories that identify HAI pathogens to the species level may find outbreaks that would otherwise have gone undetected because clusters of unusual organisms or unusual clusters of organisms may be clues to outbreaks. Furthermore, incomplete or incorrect identification of organisms may obscure real problems and make retrospective epidemiologic investigation impossible. For example, a report of the "*Klebsiella/Enterobacter* group" fails to distinguish between two genera (*Klebsiella* and *Enterobacter*) that may have different epidemiologic patterns of infection within the hospital.

Unusual microorganisms, or common microorganisms with atypical phenotypic properties, often cannot be reliably identified by commercial systems and may require the use of molecular or proteomic methods to obtain an accurate identification. As pathogens continue to evolve and taxonomic classifications are revised, microbiologists must pay attention to the manufacturers' communications about products, such as letters, notices, or test exclusions regarding the accuracy of the test methods, as well as the published literature describing the potential problems encountered by others using these identification systems. Prompt notification of infection prevention personnel of such issues will avoid confusion.

New antimicrobial resistances continue to emerge, and existing resistances are increasing in frequency. To guard against significant AST errors for some organism-antimicrobial combinations, laboratories must supplement automated systems with other methods (see chapters 70 to 73). AST errors are most likely for organisms that display heteroresistance, inducible resistance mechanisms, or mechanisms for newly emerging resistance. For example, automated systems did not perform well in the detection of vancomycin resistance in staphylococci or of carbapenem resistance among *Klebsiella* species when these phenotypes first emerged (62, 63), necessitating the use of additional or confirmatory test methods. If the laboratory uses methods that do not accurately identify organisms or particular resistance patterns, the infection prevention program may not identify serious problems or even outbreaks. Conversely, infection prevention personnel may investigate spurious problems, thereby diverting and wasting precious resources. The CLSI M100 document provides an important annual update to guidance regarding susceptibility testing methods and evaluation of unexpected susceptibility results (30).

Laboratories that recognize problems with commercial automated AST or identification systems should bring them to the manufacturers' attention so that they can improve the instrumentation, panels, or software programs to improve accuracy. This process of ongoing independent evaluation of automated systems and feedback to responsive industry representatives is extremely important. Unfortunately, in an era of shrinking laboratory resources, fewer laboratories have the ability to perform rigorous internal evaluations of new technology. The most important resistances emerging in nosocomial pathogens include those to ESBLs (most notably the carbapenemases) among *Enterobacteriaceae* (15, 64), to vancomycin among enterococci (10) and *Staphylococcus aureus* (65–67), to methicillin in *S. aureus* (68), and to multiple drugs among nonfermenters, such as *Pseudomonas aeruginosa*, *Acinetobacter* spp., and *Stenotrophomonas maltophilia* (69).

The infection prevention program must implement measures to prevent the spread of important multidrug-resistant organisms (MDROs), and the CDC has published a comprehensive guideline to assist in this effort (70). However, the success of any program to control MDROs depends upon the ability of the laboratory to detect these organisms. Laboratory directors must read current literature regarding automated systems' ability to detect emerging resistances and implement if necessary additional methods to detect or confirm particular resistance patterns. The CDC website provides fact sheets summarizing current recommendations for detecting these resistances (http://www.cdc.gov/HAI/settings/lab/lab_settings.html). They are also reviewed in chapters 73 and 77 of this *Manual*.

Laboratory Information Systems

An information system that can perform prospective data mining and interface with other parts of the computerized patient record could help IPs perform surveillance, monitor patient-to-patient spread of pathogens, and detect outbreaks early (44, 45, 71, 72). Thus, persons choosing a laboratory information system (LIS) must consult with both laboratory and infection prevention personnel before purchasing the best system for the hospital.

Rapid Diagnostic Testing for Infection Prevention and Antimicrobial Stewardship

During the past decade, numerous rapid diagnostic tests that use molecular or immunologic methods have been developed. For example, a variety of methods are now available for rapid detection of respiratory viruses (73), *Clostridium difficile* (74), *Mycobacterium tuberculosis* (75), and *Legionella pneumophila* serogroup 1 (76). Rapid methods for detecting important antimicrobial resistances have also been developed, with most of the current focus being on rapid detection of methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE), with increasing need for rapid and accurate detection of the MDR-GNRs (77, 78). A positive result from any of these tests may allow for early intervention to improve infection prevention (e.g., earlier implementation of appropriate isolation precautions to prevent the spread of MDROs or faster investigation and mitigation of health care facility water sources to curb *Legionella* transmission).

Of course, if clinicians order the tests indiscriminately or the laboratory has poor quality control, rapid diagnostic tests can lead to errors, including falsely positive tests that lead to inappropriate treatment and isolation of the patients. Erroneous results may also cause the infection prevention program to waste time investigating a pseudo-outbreak (79).

As mentioned previously, more-rapid detection of pathogens (and their susceptibility patterns) is also critically important to good antimicrobial stewardship (80). Specific approaches to more-rapid detection of organisms and their resistance determinants are discussed in chapters 4 and 70 to 77 of this *Manual*.

Reporting Laboratory Data to the Infection Prevention Program

Culture and AST results are an important data source for infection prevention and are usually reviewed daily by IPs. Thus, routine microbiology laboratory results should be readily accessible to IPs. In addition to having microbiology data available in real time, both clinicians and IPs benefit from periodic summaries of selected microbiology results, such as an antibiogram specifically for HAI pathogens. These results can be presented in a table that includes the antibiograms of the most common HAI pathogens by anatomical site and hospital service and also includes antimicrobial cost information. This information will help clinicians choose empirical antimicrobial therapy for patients with health care-associated infection. The CLSI has developed guidelines for antibiogram preparation (81).

Laboratory personnel should notify the IP directly, either by phone or electronically, to report some results to ensure that appropriate control measures are implemented. Examples of organisms requiring immediate notification of the infection prevention program include *Neisseria meningitidis*, *Legionella*, acid-fast bacilli, enteric pathogens (such as *Salmonella* or *Shigella*), and MDROs, such as MRSA, vancomycin-resistant *S. aureus* (VRSA), VRE, and ESBL-producing or carbapenem-resistant *Enterobacteriaceae*. In addition, new or unusual pathogens or potential agents of bioterrorism (e.g., *Bacillus anthracis*, *Yersinia pestis*, orthopox viruses) should be reported immediately. The method of notification for each of these pathogens should be discussed with the IPP (for example, isolation of MRSA or VRE might be indicated electronically, while phone or pager contact is necessary for acid-fast bacilli, VRSA, CRE, or potential agents of bioterrorism).

In addition to providing printed and verbal reports, laboratory staff should meet regularly with infection prevention staff to ensure that their communication is direct and clear. They can discuss areas of mutual concern, such as the status of epidemiological and microbiological investigations of clusters or outbreaks. Together they can determine whether supplementary studies, such as molecular typing or environmental cultures, will be necessary. If these studies are necessary, they can determine exactly what needs to be done, who will do these procedures, and when they will be done.

Outbreak Recognition and Investigation: Epidemic versus Endemic Infections

Most nosocomial infections are not associated with outbreaks; they are endemic rather than epidemic infections. If HAI rates are consistently defined by prospective surveillance, infection prevention personnel may occasionally identify HAI outbreaks—increases in rates of infection beyond the expected during a defined time period—by reviewing these rates. However, more often, infection prevention personnel learn about potential outbreaks while interacting with personnel on the ward, in clinics, or in the laboratory.

When the infection prevention team detects a cluster or outbreak of health care-associated infection, they must act promptly to identify the etiologic agent if it is not known, define the extent of the outbreak, learn the mode of transmission for the pathogen, and institute appropriate control measures. The CML must provide appropriate laboratory support during this time. Table 4 outlines recommended steps in an outbreak investigation and points out the important role of the CML at each step.

Because the demands on the laboratory may be great during outbreaks, the laboratory staff should prepare in advance. Laboratory personnel should ask IPs what types of outbreaks have occurred in the past or can be anticipated in the future and what laboratory resources would be required should similar outbreaks occur. Laboratory staff should also anticipate the extra costs associated with outbreak investigations so that they can work with hospital administrators to include funds for these efforts in annual budgets. Costs should not be borne by the laboratory or charged to individual patients involved in the outbreak.

Some problems and potential pitfalls of outbreak investigation are pertinent to the CML and bear specific mention. Foremost among these is the problem of determining when to proceed with an outbreak investigation in the first place. The number of cases necessary to constitute an outbreak depends upon the organism, the patient population, and the institution involved. For example, while numerous cases of *Escherichia coli* urinary tract infection in a long-term-care facility may not constitute an outbreak, even a single nosocomial case of group A streptococcal surgical-wound infection or VRSA infection merits an outbreak investigation. Laboratories should consider implementing computer decision support systems that recognize clusters of pathogens within the hospital (82, 83). Organisms that appear to be part of a cluster could be further characterized to evaluate whether they are genetically related, which would suggest patient-to-patient spread or exposure to a contaminated common source. Investigators at the hospital of Northwestern University implemented such a system and noted that their rates of HAIs decreased in temporal association with this intervention (82).

A second important problem is that of a pseudo-outbreak. A pseudo-outbreak has occurred when an apparent outbreak turns out not to be an outbreak after all. The usual cause of a pseudo-outbreak is either misdiagnosis (e.g., infection has not actually occurred) or misinterpretation of epidemiologic data (e.g., infections have occurred but clustering or epidemic transmission has not). The microbiology laboratory can be the source of pseudo-outbreaks (84–90). Problems in the laboratory that lead to pseudo-outbreaks include contamination of reagents for stains (85), false antimicrobial susceptibility test results (84), and contamination of culture specimens (often from construction or renovation projects [87] or cross-contamination during specimen processing [90]). Careful attention to quality control, sterile techniques in specimen processing, and preventive measures during construction and renovation projects can decrease the likelihood of pseudo-outbreaks that originate in the laboratory.

Molecular Typing to Support Infection Control Activities

Outbreaks of nosocomial infection often result when hospitalized patients are exposed to a common source or a reservoir of a pathogenic agent (e.g., water from a hot water tank colonized with *Legionella* spp.). The organisms causing such outbreaks usually derive from a single strain (i.e., they are clonally related). The infection control program may, therefore, request that the microbiology laboratory characterize isolates that may be associated with outbreaks to determine whether they are genetically related. In the appropriate clinical setting, species-level identification and AST results (antibiogram) may provide strong evidence for an epidemiological link. However, more-sensitive methods

TABLE 4 Steps in nosocomial outbreak investigation and the role of the laboratory at each step[a]

Investigative step(s)	Role(s) of the clinical microbiology laboratory
Recognize problem	Serve as a surveillance and early warning system, ideally as part of the Laboratory Information System. Notify infection control personnel of infection clusters, unusual resistance patterns, and possible patient-to-patient transmission.
Establish case definition	Assist and advise regarding inclusion of laboratory diagnosis in case definition.
Confirm cases	Perform laboratory confirmation of diagnosis.
Complete case finding	Characterize isolates with accuracy. Store all sterile-site isolates and epidemiologically important isolates. Search laboratory database for new cases.
Establish background rate of disease; compare to attack rate during suspected outbreak	Provide data for use in ongoing surveillance, including baseline rates for selected units and infection sites. Search laboratory database for all prior cases of the entity if the baseline rate is not prospectively monitored.
Characterize outbreak (descriptive epidemiology)	Perform typing of involved strains and compare types to those of previously isolated endemic strains to determine if the outbreak involves a single strain (see chapter 10). This can be done only if selected pathogens are routinely stored (see above).
Generate hypotheses about causation: reservoir, mode of spread, vector	Perform supplementary studies or cultures as needed but only if justified by an epidemiologic link to transmission by personnel, patients, or the environment.
Perform case-control study or cohort study	Perform supplementary studies as needed.
Institute control measures	Adjust laboratory procedures as necessary.
Maintain surveillance to document efficacy of control measures	Maintain surveillance and early warning function of the laboratory.

[a]Adapted from reference 6.

of strain delineation are often necessary. In this setting, genotypic or DNA-based typing methods have replaced phenotypic typing methods (e.g., AST, biochemical profiles, bacteriophage susceptibility patterns), which discriminate poorly among isolates (91, 92).

Genotypic typing methods provide meaningful data and are cost-effective only when they are used for well-defined epidemiologic objectives. These objectives include (i) determining the source and extent of an outbreak, (ii) determining the mode of transmission of HAI pathogens, (iii) evaluating the efficacy of preventive measures, and (iv) monitoring transmission of pathogens in high-risk areas (e.g., intensive care units), where cross-infection is a recognized hazard.

The ideal genotypic typing system should be standardized, reproducible, stable, sensitive, broadly applicable, readily available, and inexpensive. The typing method should also have proven value in previous epidemiologic investigations. Further discussion of the relative advantages and disadvantages of the many available typing systems is beyond the scope of this chapter, and the discussion has been summarized in published reviews (91, 92) and in chapter 10 of this *Manual*.

Organism Banking and Storage

Of course, the CML cannot provide the infection prevention program with supplemental testing, such as molecular typing, if the appropriate isolates have not been saved. The laboratory should plan ahead and be sure to save all epidemiologically important isolates (see chapter 11). Laboratory and infection prevention personnel should decide which isolates should be banked and how long they should be stored based upon their epidemiological importance and the available resources. We recommend that all isolates from normally sterile sites (e.g., blood, cerebrospinal fluid [CSF]), important MDROs (e.g., MRSA, VRE, ESBL producers, CRE) from any site, and other epidemiologically important pathogens (e.g., *Mycobacterium tuberculosis*) be saved for a period of 3 to 5 years.

Surveillance Cultures from Patients, Hospital Personnel, and the Environment

The clinical microbiology laboratory is often called upon to detect potential pathogens that may be colonizers of patients, health care workers, and the hospital environment.

For example, several U.S. states (as well as the VA health care system) have mandated the routine use of "active surveillance and isolation" (using culture or molecular methods to identify patients that are MRSA colonized but not clinically infected, in order to place them in contact isolation and/or attempt decolonization), a process that remains controversial (93). As more-extensive experience with MRSA active surveillance accumulates, there is also interest in how best to apply active surveillance to prevent the transmission of other MDROs, including VRE and MDR-GNRs.

A major reason for careful consideration before launching into large-scale active surveillance efforts is the fact that such measures are complex and resource intensive. Indeed, the prospect of performing active surveillance cultures for a large number of diverse MDROs (which requires that culture samples be obtained from several different anatomic sites of each patient) threatens to overwhelm and divert the resources of CMLs, IPPs, and hospital units. In addition, recent data suggest that the "horizontal" infection prevention approaches (those approaches that target all infection types and are applied to an entire population, such as hand hygiene) are preferred over a focus on "vertical" approaches (those that target a specific pathogen, such as active surveillance of MRSA) (94). For example, a recent cluster-randomized trial demonstrated that universal decolonization of all ICU patients was more effective than active surveillance in reducing rates of bloodstream infection and positive MRSA clinical cultures (95). In our view, active surveillance culture for MDROs should be limited to (i) new introduction of problematic pathogens, (ii) continued transmission of an MDRO despite implementation of standard and enhanced IP practices, and (iii) outbreaks. In these settings, active surveillance should always be seen as an adjunct to, not a replacement for, those well-established horizontal IP practices (e.g., hand hygiene and bundled practices for prevention of device-associated infection).

Health care workers are also occasionally screened for carriage of epidemiologically significant organisms. The most common organisms for which screening is performed are the MDROs, most often MRSA, usually as one aspect of an outbreak response. Screening for other organisms (e.g., group A streptococci) may also be performed as part of an infection or outbreak investigation. Finally, hand cultures may be performed as part of educational efforts in support of a hand hygiene campaign or to confirm the mechanism of cross-infection during an outbreak investigation (96).

For some organisms (e.g., MRSA), screening methods are standardized and well established, while for others (e.g., MDR-GNRs), such methods are evolving and will continue to evolve as more-complex resistance phenotypes emerge (78). Table 5 outlines current approaches to screening patients and health care workers for organisms of epidemiologic significance (97–100).

Laboratory and infection prevention personnel should weigh two important factors before deciding to culture specimens from hospital personnel during an outbreak investigation. Finding the outbreak strain on the hands or in the nares of a health care worker does not establish the direction of transmission or definitively implicate a health care worker as the source or reservoir for the outbreak, and culturing hospital personnel indiscriminately can lead to confusing results and can generate ill will toward the infection prevention program. In general, only health care workers epidemiologically linked to cases should be cultured. We recommend that infection prevention programs obtain cultures of hospital personnel only after consulting with a hospital epidemiologist experienced in outbreak investigation.

At one time, the hospital environment was felt to be the major source of HAI pathogens. Since then, it has been recognized that patients most often acquire infection from

TABLE 5 Screening patients and health care workers for asymptomatic carriage of organisms of epidemiologic significance[a]

Organism(s)	Diagnostic procedure(s)	TAT (h)	Optimum specimen(s)
Staphylococcus aureus, including MRSA	Routine aerobic culture and AST	48–96[b]	Nares swab[c] Throat, perirectal, skin, wounds
	Chromogenic agar medium	18–48[d]	Nares swab Throat, perirectal, skin, wounds
	Real-time PCR (MRSA)	1–4	Nares swab[e]
Vancomycin-resistant enterococci (VRE)	Routine aerobic culture and AST	48–72	Perirectal or stool swab
	Real-time PCR	1–4	Perirectal or stool swab
Multidrug-resistant Gram-negative rods (*P. aeruginosa*, *Acinetobacter* spp., *S. maltophilia*, ESBL- and carbapenemase-producing organisms)	Routine aerobic culture and AST[f]	48–72	Perirectal or stool swab, endotracheal or sputum sample, sites of prior infection or colonization[g]
Group A streptococcus	Routine aerobic culture	24–48	Rectal, vaginal, skin, and throat swabs
Various organisms on hands	Routine aerobic cultures: Contact agar plates Broth-based culture (glove juice technique)	48–96	Hand cultures Direct imprint on agar plate Culture of broth after 1 min of hand immersion with agitation of broth

[a] Such cultures should be done only for the following reasons: (i) as part of an outbreak investigation to seek carriage of an organism among patients or health care workers who are epidemiologically linked to cases, (ii) to seek carriers of MDROs as part of enhanced MDRO control strategies, or (iii) to identify *S. aureus* carriers in order to proceed with a decolonization strategy to decrease the risk for acquisition of *S. aureus* infection during a period of vulnerability (e.g., the perioperative period).

[b] The "gold standard" method includes overnight broth enrichment and confirmation of species identification and antimicrobial susceptibility, which can increase the TAT to 96 h. Most conventional agar-based screens (e.g., mannitol salt agar with or without oxacillin), without broth enrichment, provide TATs of approximately 48 h.

[c] The nares provides the best sensitivity and specificity of any single site for detection of *S. aureus* (including MRSA) detection (97). However, several studies have demonstrated that sampling of additional sites, including the oropharynx and perirectal sites, may increase yield by 10 to 40% (98–100).

[d] Positive results for chromogenic agar medium can be reported at 18 to 24 h, but negative results are reported at 48 h.

[e] Currently available real-time PCR assays are FDA approved only for nares samples but have been used in some studies for oropharyngeal, skin, and perirectal samples.

[f] Several modifications of culture methods may enhance recovery by increasing medium selectivity for MDROs (e.g., addition of ceftazidime for ESBL-producing *Enterobacteriaceae*, levofloxacin for fluoroquinolone-resistant *E. coli*, imipenem or meropenem for carbapenem-resistant GNRs, etc.). Chromogenic agar is now commercially available, and the CDC has guidance for detection of carbapenem-resistant *Enterobacteriaceae* (http://www.cdc.gov/hai/pdfs/labsettings/klebsiella_or_ecoli.pdf). Laboratory-developed molecular methods for more-rapid detection of specific resistance determinants (e.g., bla_{KPC}) are in use in some centers, and multiplex assays to detect multiple resistance determinants are now commercially available.

[g] The sample site(s) should be guided by likely reservoirs, with gastrointestinal (e.g., *Enterobacteriaceae*) and respiratory (e.g., *Acinetobacter*) being most common.

their own colonizing biota (101, 102). Nonetheless, the hospital environment can serve as a source of this colonizing biota, which is transmitted to patients on the clothing and hands of health care personnel. Thus, there are specific circumstances in which environmental sampling for quality assurance or for detection of potential pathogens is necessary. Routine sampling for quality assurance should be limited to biologic monitoring of sterilization processes and monthly cultures of water and dialysate for hemodialysis. Rarely, it may be useful to perform a short-term evaluation of the effectiveness of hospital cleaning and disinfection (for example, sampling surfaces for VRE or *Clostridium difficile* after terminal room cleaning to evaluate the effectiveness of cleaning practices). Likewise, sampling the hospital potable water for *Legionella* spp. is indicated after diagnosis of a nosocomial legionellosis case or as part of a comprehensive program to decrease the risk of nosocomial legionellosis (101, 103, 104). Air sampling for mould spores can also be an important step in identifying the source of fungal disease transmission in highly immunocompromised patients. Rarely, sampling of other inanimate objects or surfaces may be indicated, if such objects or surfaces are implicated in pathogen transmission. Table 6 outlines current approaches to screening the hospital environment for organisms of epidemiologic significance.

As a general rule, routine, undirected cultures of health care workers or the hospital environment should not be performed; such cultures are labor-intensive, nonstandardized, and difficult to interpret, and they rarely provide useful information (101). Except as previously outlined, such sampling should be done only as part of an epidemiologic investigation in consultation with the hospital epidemiologist. When such an epidemiologic investigation reveals a common organism in patient and/or environmental samples, the laboratory should also provide access to epidemiologic typing methods, as previously discussed.

Emerging Issues and Trends in Health Care Infection Prevention

The landscape of health care infection prevention is ever changing. At the time of this writing, the emerging issues of greatest import (at least in the U.S. health care system) include (i) the growing perception that every HAI is preventable and therefore that the only acceptable HAI rate is zero and (ii) the starkly increased stakes for health care facilities to demonstrate HAI elimination, given the impact of greater transparency and increased financial consequences for HAIs (38). These trends provide both challenges and opportunities for the CML.

While many HAIs are preventable by use of current best practices, there are still gaps in our knowledge that result in HAI risks for severely immunocompromised patients who require invasive devices that breach normal host defenses (38, 105). However, improved prevention approaches have resulted in HAI reductions that are greater than previously thought possible (27). As HAI rates get lower, each HAI is scrutinized more carefully than ever; in some institutions, HAIs may be subject to adjudication by consensus panels or individual clinicians (106). For HAIs that include a microbiology laboratory test result as part of the definition,

TABLE 6 Screening environmental sources (air, water, and surfaces) for organisms of epidemiologic significance[a]

Source(s) and agent(s)	Diagnostic procedure(s)	TAT	Optimum specimen(s)
Air			
Fungi	Fungal cultures	48 h–7 days	Air processed with large-volume air sampler[b]
Bacteria[c]	Routine aerobic cultures	48–72 h	Air processed with large-volume air sampler Settle plates
Water			
Legionella species	Culture on selective media[d]	5–10 days	500–ml to 1-liter water samples[e] Swabs of internal surfaces of faucets, showerheads, and aerators[e]
Fungi[f]	Fungal cultures	48–96 h	500-ml to 1-liter water samples[e] Swabs of internal surfaces of faucets, showerheads, and aerators[e]
Bacteria	Routine aerobic cultures	48–72 h	Water and dialysate samples as outlined by AAMI[g]
Surfaces			
Aerobic bacteria (including MDROs)	Routine aerobic cultures	48–72 h	Surface swab or sponge,[h] contact agar plate (Rodac)
Clostridium difficile	Anaerobic cultures	48–72 h	Surface swab or sponge,[h] contact agar plate (Rodac)[i]

[a] With the exception of water and dialysate cultures for monitoring of hemodialysis and potable water cultures for *Legionella* spp., environmental cultures should be performed only when an epidemiological investigation suggests that the environment may be involved in pathogen transmission.

[b] Large-volume air samplers are preferred for air sampling for mould spores; settle plates should not be used for this purpose.

[c] There are no standards for acceptable levels of bacteria in air samples, nor is there any evidence correlating bacterial burden to infection risk. Air sampling for bacteria should be performed only rarely, as part of either an outbreak investigation or a research protocol.

[d] *Legionella* spp. do not grow on routine aerobic culture media. Buffered charcoal yeast extract (BCYE) agar is the most common medium used for *Legionella* isolation.

[e] A large volume (1 liter) is preferred. If the water source is chlorinated, 0.5 ml of 0.1 N sodium thiosulfate should be added to each liter of sample to neutralize the chlorine. Water samples are filter concentrated. Swabs should be immersed in 3 to 5 ml of water taken during sampling of the same site to prevent drying.

[f] The role of waterborne fungi in infection transmission in the hospital environment remains poorly described, but cultures may be indicated as part of a search for environmental sources during an outbreak of invasive fungal infections in an immunocompromised patient population.

[g] Standards of the Association for the Advancement of Medical Instrumentation (AAMI) govern microbiological monitoring of hemodialysis.

[h] The sterile swab or sponge should be moistened (e.g., with nutrient broth, sterile saline, etc.) prior to sample collection.

[i] For C. *difficile*, the contact agar plate should be optimized for anaerobic recovery (selective, prereduced media, promptly placed in an anaerobic environment).

this scrutiny places increased pressure on those diagnostic tests to provide unrealistic levels of accuracy. For example, the more successful an institution is at reducing its rate of health care-associated C. *difficile*, the greater the likelihood that a positive result from a highly sensitive PCR-based assay will be falsely positive (107). This increases the importance of enforcing strict criteria for testing submitted samples (e.g., no testing of nondiarrheal stool, no repeat testing, no "test of cure") and also makes clear that no institution will demonstrate "elimination" of health care-associated C. *difficile*, if only because some falsely positive results will undoubtedly result in misclassification. The same concepts hold for any HAI definition that relies heavily on microbiology results. As the stakes rise for each HAI, labs will undoubtedly be called upon to defend the accuracy of their diagnostic approach, if not to become involved in adjudicating individual HAIs. The Healthcare Infection Control Practices Advisory Committee (HICPAC) of the CDC has recently published guidance for the use of surveillance data in this new, high-stakes environment (38).

This high-stakes environment, resulting from financial consequences and public disclosure about HAI rates, also provides an opportunity for the CML to play a more active role in HAI prevention. Once health care system leaders realize that the CML is an integral component of any effective IPP, they will understand how shortsighted it is to cut resources for CMLs. While such cuts may appear to save money in the short term, they will undoubtedly increase long-term costs and, more importantly, morbidity and mortality related to elevated HAI rates and uncontrolled outbreaks due to insufficient CML support of IP activities.

CONCLUSION

The CML is an essential element of the infection prevention program in every health care facility, playing critical roles in surveillance, outbreak detection and management, antimicrobial stewardship, risk assessment and planning, and education. The development and application of new technologies in the clinical laboratory can greatly enhance infection prevention efforts. Close collaboration between CML and infection prevention personnel is required to ensure optimal HAI prevention, which saves money and lives.

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Investigation of Disease Outbreaks

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9

INTRODUCTION/DEFINITIONS

A disease outbreak (or cluster) is the occurrence of a disease in excess of what would otherwise be expected. Outbreaks can have a major impact on health care expenditure whether they occur in a restricted area or result in cases over a wide geographic spread. The time period may be limited or may last for years. To classify an outbreak, it is vital to know the epidemiology of the implicated infectious agent. A single case in an unrecognized area or community, as in the case of smallpox, can be considered an outbreak and should be investigated. Whether the cases are few in number or enter into the hundreds or thousands, outbreaks can be fear and anxiety provoking for large populations and divert a large amount of public resources (1).

Recognizing an increase in the number of cases relative to the ongoing background of diseases is difficult. Increasing the specificity of the case definition by describing outbreaks in terms of time, place, and person can minimize this challenge. This involves collecting detailed information on the similarities of clustered cases as well as identifying the important outliers. Geography was traditionally a major clue as to the source of the outbreak. However, as a result of the globalization of the world economies as well as increases in travel and trade, pathogens have expanded beyond their typical geographical ranges. Both people and foodstuff now regularly travel far distances and challenge prior demarcations for infectious agents. Public health officials must have the knowledge and flexibility to be prepared for presentations of disease in uncommon locations and the ability to trace back a source across continents. It is easy to see in today's world how an outbreak can become a pandemic, an epidemic spreading to several countries, even with a limited number of cases. The spread of severe acute respiratory syndrome (SARS) in 2003 is a good example of this. Originating as a small cluster of cases from the Guangdong Province in southern China, the outbreak lasted approximately 6 months and spread to 29 countries and regions (http://www.who.int/csr/sars/country/table2004_04_21/en/).

Outbreaks are usually triggered when individuals travel into an area of endemicity or when a new infectious disease is introduced into a susceptible population. Thus, host factors in addition to behavior play a key role. In the 21st century, the population of persons vulnerable to an outbreak is growing due to the number of people living with chronic, immunologically debilitating conditions, such as malnutrition, cancer, and HIV.

In addition to host immunity, new or unusual behavioral, sexual, cultural, or political changes can make populations particularly at risk (2). An example of this is the migration of refugees during wartime. In 2012, there were around 6.5 million newly displaced persons, largely associated with large-scale population movements due to armed conflict (3). This is the highest global figure ever reported. There are also voluntary mass movements, such as international sporting events or pilgrimages to religious places. In 2012, nearly 700,000 people traveled to the United Kingdom for the Olympics, 66,000 of those from the United States (http://www.ons.gov.uk/ons/rel/ott/travel-trends/2012/sty-visits-to-the-uk.html). In the same year, over 3 million people performed the Hajj pilgrimage, with 14,567 visas issued for persons in the United States (http://www.saudiembassy.net/latest_news/news10271201.aspx).

The modern response to outbreaks can be vastly different than in years past due to advances in the understanding of infectious disease pathogenesis. Many outbreaks are identified and investigated retrospectively. In these cases, the cause may be difficult to identify, as the investigation is remote from the event and the source of the outbreak may no longer be present. The laboratory, which has always been integral to an outbreak investigation, has become even more central now that advancements in microbial identification, even in cases of novel pathogens, are more likely to be identified at the time of the outbreak.

This chapter will review concepts associated with infectious disease outbreaks, including disease surveillance, mechanisms used to detect outbreaks, and the epidemiological steps used to investigate an outbreak. Since this *Manual* is largely for laboratorians, this chapter focuses (i) on routine and novel laboratory detection that can be used in outbreak settings and (ii) on examples of situations in which laboratory testing played a key role in the investigation of an outbreak. In past editions of this *Manual*, the focus was largely foodborne outbreaks; this chapter expands the focus to include many different types of pathogens and settings for outbreaks.

DISEASE SURVEILLANCE

Outbreak (or epidemic) intelligence is the process used to detect, verify, analyze, assess, and investigate signals that may represent a threat to public health (4). Around the world, this effort is coordinated by international health organizations that receive information from national public

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health agencies, which in turn rely on local authorities. Improved coordination has been shown to be highly effective and necessary in large-scale epidemics. Comparing the largest influenza pandemic of the last century with the first pandemic of the 21st century, the average numbers of secondary cases generated by contact with one infected individual, "R," were similar (5, 6). However, while the 1918 pandemic affected almost a half a billion persons and killed 3% of the world's population, the SARS epidemic was limited to fewer than 10,000 persons thanks to the coordinated responses of public health agencies worldwide (http://www.who.int/csr/sars/country/table2004_04_21/en/).

At every level, multidisciplinary teams that are comprised of epidemiologists, laboratory personnel, environmentalists, and other technical staff are involved. Table 1 illustrates the vast types and levels of organizations that work hand in hand to address infectious disease outbreaks in the United States. Most of the investigations and most of the legal authority to conduct those investigations are at the state or local level. Basic laboratory testing and interviews of a select number of individuals may provide sufficient information to identify the source and take appropriate measures. This is well within the means of the local authorities and can usually be performed with public cooperation without a public health mandate. Outbreak investigations provide a unique opportunity for enhanced education and awareness, making local authorities that have insight into their area particularly suited.

Federal agencies usually become involved only when outbreaks involve multiple states, coordination across multiple agencies is needed, or specific expertise is requested. In the United States, there are a variety of federal agencies that assist in setting standards and coordinating efforts, such as the Centers for Disease Prevention and Control (CDC), Federal Drug Administration (FDA), U.S. Department of Agriculture (USDA), and U.S. Department of Health and Human Services (HHS). The CDC, for example, regularly collects surveillance data from health departments in the 50 states, five territories, New York City, and the District of Columbia. Important for laboratories to note is that in addition to information regarding the pathogens, patient data are collected. Reporting is typically done through the National Electronic Disease Surveillance System (NEDSS), and a list of the diseases involved in mandatory reporting is included in Table 2 (7). This consistent data source allows for agencies to easily identify spikes in case numbers. For example, in the United States in 2011, an increase to 222 measles cases and 17 outbreaks marked a significant change from a median of 60 cases and 4 outbreaks reported annually during 2001 to 2010 (8). Even though the cases were reported from 31 states, the increase could be identified through mandatory reporting of a predetermined laboratory and clinical case definition.

Internationally, the World Health Organization (WHO) has a 24-h global alert and response system for epidemics and health emergencies and supports member states in their preparedness for epidemics by pooling the resources of existing entities with particular expertise. The 2005 International Health Regulations published by the WHO provides a framework for the climate under which member nations develop and maintain surveillance capabilities. Any nation with knowledge of a disease outbreak of international concern is obligated to report it to the WHO within 24 h (http://www.who.int/ihr/global_alert/en/). It is then the role of larger, international agencies to collect and disseminate information to local agencies and keep them current on the global perspective.

OUTBREAK DETECTION

The investigation of an outbreak is initiated for many reasons, but in general, potential outbreaks come to the attention of health authorities through one of three ways: direct reporting, active pathogen surveillance, and syndromic surveillance (http://www.cifor.us/documents/CIFORGuidelinesforFoodborneDiseaseOutbreakResponse.pdf).

Direct reporting is when the public notifies health authorities of cases of which they have an immediate knowledge through a notification or complaint system. Reporting via notification has been important to the identification of outbreaks since the inception of public health agencies. Typically, an individual will call in to report an illness, perhaps after a group function or event. A physician caring for that individual may also report one or more cases. Reporting via this route enables identification of cases caused by any organism, not just those under active monitoring, such as norovirus, and is usually timely. However, notification-based systems require that the public and clinicians be aware of them and rely on a high degree of resources to appropriately triage and investigate these reports. As described earlier, each state and local area defines a list of specific infectious diseases that are considered reportable. This usually

TABLE 1 Key agencies involved in outbreak response

Level of response required	Example(s) of agencies involved[a]
Local: events contained to one city or county	Health care providers and local laboratories from public and private agencies, including city and county Departments of Health
State: events across several cities/counties	State Health Departments and state-level private organizations
Federal: large numbers of people or outbreaks that are particularly widespread	National private organizations or public national agencies, such as the Centers for Disease Control, Federal Drug Administration, United States Department of Agriculture, or U.S. Department of Health and Human Services
International: global outbreak preparation and response	World Health Organization

[a] More information can be obtained at http://www.cdc.gov, http://www.fda.gov, http://www.usda.gov/wps/portal/usda/usdahome, http://www.hhs.gov, and http://www.who.int/en/.

TABLE 2 Infectious diseases designated as notifiable at the national level[a]

- Anthrax
- Arboviral diseases, neuroinvasive and nonneuroinvasive (California serogroup, eastern equine encephalitis, Powassan, St. Louis encephalitis, West Nile, and western equine encephalitis viruses)
- Babesiosis
- Botulism (foodborne, infant, and other)
- Brucellosis
- Chancroid
- *Chlamydia trachomatis* infection
- Cholera
- Coccidioidomycosis
- Cryptosporidiosis
- Cyclosporiasis
- Dengue virus infections:
  - Dengue fever
  - Dengue hemorrhagic fever
  - Dengue shock syndrome
- Diphtheria
- Ehrlichiosis/anaplasmosis
- Giardiasis
- Gonorrhea
- *Haemophilus influenzae*, invasive disease
- Hansen disease (leprosy)
- Hantavirus pulmonary syndrome
- Hemolytic uremic syndrome, postdiarrheal
- Hepatitis, viral
  - Hepatitis A, acute
  - Hepatitis B, acute, chronic, or perinatal infection
  - Hepatitis C, acute, past or present
- Human immunodeficiency virus (HIV) infection diagnosis
- Influenza-associated pediatric mortality
- Legionellosis
- Listeriosis
- Lyme disease
- Malaria
- Measles
- Meningococcal disease
- Mumps
- New World arenaviruses (Guanarito, Machupo, Junin, and Sabia viruses)
- Novel influenza A virus infections
- Pertussis
- Plague
- Poliomyelitis, paralytic
- Poliovirus infection, nonparalytic
- Psittacosis
- Q fever
  - Acute
  - Chronic
- Rabies, animal and human
- Rubella
- Rubella, congenital syndrome
- Salmonellosis
- Severe acute respiratory syndrome-associated coronavirus (SARS-CoV) disease
- Shiga toxin-producing *Escherichia coli* (STEC)
- Shigellosis
- Smallpox
- Spotted fever rickettsiosis
- Streptococcal toxic shock syndrome
- *Streptococcus pneumoniae*, invasive disease
- Syphilis
- Syphilis, congenital
- Tetanus
- Toxic shock syndrome (other than streptococcal)
- Trichinellosis
- Tuberculosis
- Tularemia
- Typhoid fever
- Vancomycin-intermediate *Staphylococcus aureus* (VISA) infection
- Vancomycin-resistant *Staphylococcus aureus* (VRSA) infection
- Varicella (morbidity and mortality)
- Vibriosis
- Viral hemorrhagic fever (Crimean-Congo hemorrhagic fever, Ebola, Lassa, Lujo, and Marburg viruses)
- Yellow fever

[a] Adapted from the work of Adams et al. (7).

includes language that allows for reporting any "unusual clusters" or "pathogens that may take on some unexpected significance." In addition, since reporting from the public and clinicians is passive, most states and local areas have a dual system whereby laboratories report similar lists of pathogens/conditions in parallel. Lab-based reporting can be electronic and is likely to be more reliable but will not have the specifics of a clinical presentation. Thus, these forms of reporting are complementary.

Second, authorities regularly conduct pathogen-specific surveillance to look for clusters cases or numbers previously not seen in certain geographical areas using predetermined case definitions. In order to identify an increase in the number of illnesses that define an outbreak, public health officials must routinely collect data to serve as a predetermined case definition. By collecting routine data, there are often predetermined thresholds that are set for an alert and a response based on known epidemiology.

Sometimes outbreaks come to the attention of health authorities as a combination of direct reporting and surveillance. For example, at the end of June 2011, the French Institute for Public Health Surveillance was informed of eight cases of bloody diarrhea or hemolytic uremic syndrome (HUS) in adults living in southwest France (9), but authorities were already on high alert because of known cases of HUS in Germany in that year that resulted in 3,816 identified Shiga toxin-producing *Escherichia coli* infections and 54 deaths (10). The cause was identified as sprouts in both outbreaks.

Finally, public health authorities actively engage in syndromic surveillance by looking at nonspecific health indicators. An example of this is the CDC's practice of looking at diagnosis codes in all emergency departments around the time of notable events, such as the Olympics. This allows epidemiologists to look for cases of intentional food poisoning or biological terrorism (11). It is important to note that evidence collected by any of these methods may include false positives, and case definition becomes vital in the development of a reporting threshold at which action is taken.

EPIDEMIOLOGICAL APPROACHES TO AN OUTBREAK INVESTIGATION

Once an outbreak is suspected, the decision to perform a full investigation can be initiated. The purposes of an outbreak investigation can be multiple, including, but not limited to, identifying the etiologic agent responsible, finding the source of the infection, determining the specific attack rate, and formulating recommendations to prevent further transmission. Speed and accuracy are two key components that must be utilized, and a systematic framework is vital to ensure that neither is sacrificed for the other. The basic steps of an outbreak are listed in Table 3 (http://www.cdc.gov/excite/classroom/outbreak/steps.htm) (12). These steps serve as a guide to stay organized, work systematically, and allow for planning. In the urgency of an outbreak investigation, many of these steps can occur concurrently or be rearranged but must not be overlooked and should serve as a mental checklist. The extent and scope of the investigation may change based on resources, timing, the clinical or public health significance of the outbreak, and whether the outbreak is ongoing.

Depending on the nature of the outbreak, a team of experts knowledgeable in the area should be brought together. In order to verify that an outbreak is occurring, the observed number of cases must exceed the expected number. Unrelated cases of clinically similar illnesses but different diseases and sporadic and unrelated cases of the same disease may confound the investigation and must be ruled out. The diagnosis must be confirmed by testing or through the collection of laboratory specimens to be analyzed at a later time point (in cases where the pathogen is unknown). An initial case definition should be established. This involves classifying cases as (i) confirmed, i.e., laboratory test positive, (ii) probable, i.e., displaying clinical features without lab confirmation, and (iii) suspect, i.e., displaying only some clinical features. This allows for an accurate case count and allows for some evolution in case definition and diagnosis.

The next step in an outbreak investigation is to perform some simple epidemiologic analyses. Creating a line listing of cases, some early demographic factors, and potential exposures will start to identify patterns in those affected and possible sources or exposures that might be causing the outbreak or at least be related to the source. Creating a histogram at this stage is key to representing the number of cases that occurred over time in order to determine how they were propagated relative to the exposure. A point source outbreak is a cluster of cases around the time of the exposure, while person-to-person spread results in cases that cluster around the exposure (the primary cases) and then in secondary cases (household contacts, school contacts, etc.) that occur when the epidemic is propagated (Fig. 1).

It is important that laboratory data, including any additional specimens not originally collected when propagation was unknown, continue to be collected as the cases are identified. It is important to develop a hypothesis of the source agent early on but to revise the theories as information becomes available. Included in this hypothesis should be the mode of transmission and the likely mechanism of exposure.

Once a hypothesis is developed, control measures should be implemented to contain the outbreak. These measures may be revised and should become more-permanent existing measures as needed to protect vulnerable populations or prevent the occurrence of a repeat or similar outbreak. The investigation does not end with the outbreak. Ongoing surveillance must be conducted to ensure that there are no

TABLE 3 Steps in an outbreak investigation

1. Prepare to investigate
   Identify team, review literature, notify appropriate entities, determine if immediate control measures are needed
2. Verify the diagnosis
   Confirm that cases are “real”
   Exclude pseudo-outbreaks
   Compare preepidemic and epidemic rates
3. Establish a case definition
   Establish a set of criteria for selecting ill persons
4. Find cases
   Find cases and define the scope
5. Describe the epidemiology
   Describe and orient the data in terms of time, place, and person
   Create a line list and epidemic curve
6. Develop a hypothesis
   Determine the how and why of the outbreak
7. Evaluate the hypothesis
   Possibly perform a cohort or case-control study to compare risk factors
8. Refine the hypothesis and carry out additional studies
   Perform culture surveys of the environment Type isolates
9. Implement control and prevention measures
   Develop strategies for future prevention
10. Communicate findings
   Educate public health staff as well as the community at large

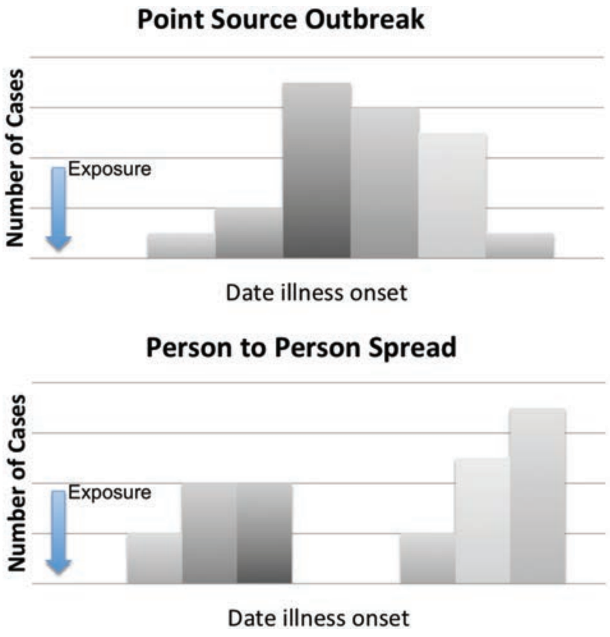

FIGURE 1 Epidemic curve examples.
doi:10.1128/9781555817381.ch9.f1

new cases that are linked or that exceed a normal threshold for that particular disease. Public compliance is key to the establishment of new control measures; health officials can effectively educate the public and ensure compliance to new measures. The WHO provides guidelines for communicating with the public during an outbreak, but most communications should be handled by local health departments, which have experience in outreach (http://www.who.int/csr/resources/publications/WHO_CDS_2005_28en.pdf). Monitoring after an outbreak may need to be enhanced, and more-permanent active surveillance measures may need to be put in place.

In some outbreaks, additional, more sophisticated, epidemiological, comparative, or other studies may be considered (e.g., case-control studies, cohort studies, and practice observations). Situations where this may be appropriate include when resources are available, when the outbreak is associated with high mortality or severe disease, with new or unusual pathogens or methods of transmission, and/or with outbreaks which continue despite implementation of control measures. Discussion of these more advanced studies is beyond the scope of this chapter but can be found in *Epidemiology* by Gordis (13).

ROLE OF THE LABORATORY IN OUTBREAKS

Routine

Although outbreaks may last decades (as in the case of AIDS), some last mere hours (bacterial food poisoning). Laboratory investigation and specimen collection are key at every juncture, and the team should be prepared with the tools and the materials needed to collect, process, and transport specimens at the time of an outbreak investigation. After the suspected pathogens are determined, the appropriate clinical specimens can be determined, and laboratories with the capability of handling specimens should be contacted and informed. The logistics required for the collection, processing, and storage of each specimen should be considered prior to collection.

Specimens obtained in the acute phase of the disease, preferably prior to the administration of any treatments (i.e., antimicrobials) are much more likely to yield the infective pathogen. The specimens should be collected in sufficient quantity and should not be contaminated by other specimens or sites. Protective clothing must be worn if indicated, and specimen collection sites should be cleaned and decontaminated as necessary. All specimens should be correctly labeled with a patient identifier, the location of the specimen, and the time of collection. Additionally, in cases in which bioterrorism is suspected, chain of custody may need to be maintained. The CDC and WHO have guidelines for general as well as disease-specific specimen collection; they are summarized in Table 4 (http://www.who.int/csr/resources/publications/surveillance/whocdscsredc2004.pdf.). Culture is the mainstay of outbreak investigation, and although newer techniques have emerged, it remains the gold standard for testing therapeutics (i.e., drug susceptibilities).

In outbreak cases in which fatalities have occurred, it is important to save tissue specimens for current examination and also for possible later reference analysis, including, but not limited to, skin biopsy specimens, liver biopsy specimens, and brain, cornea, and/or neck biopsy specimens if indicated. Once the disease is known, specimen collection can be limited to only those required to confirm the diagnosis of the disease.

Current and Novel Lab Detection Methods (Illustrative Examples)

It is an exciting time in which the rate at which pathogens are discovered through the use of increasingly powerful technology is rapidly growing. These modern techniques for identifying pathogens have become integral to both surveillance activities and sporadic case investigations and have advanced the speed at which outbreak investigations can be conducted. Once the etiology of an outbreak is known, the focus can shift from pathogen detection to intervention. Resources can then be directed toward employing treatments, such as antimicrobials, therapeutic antibodies, and vaccines. Thus, a method for rapid and accurate organism detection is highly desirable. Table 5 discusses some examples of the use of the laboratory in selected outbreaks in this modern era of molecular microbiology (14–21). Molecular techniques are discussed in detail in chapter 10, but select examples of their use in outbreak investigations are discussed below.

Genomic sequencing is a technology that involves identifying sequential nucleotides from a template strand. It is often employed to identify new pathogens in which the etiology is unknown. This was the case in the identification of West Nile virus, which caused an outbreak of human encephalitis in New York City in 1999, along with the death of birds. Necropsy samples from the birds were inoculated into embryonated chicken eggs for viral isolation, and the entire viral genome was sequenced and subsequently identified through phylogenetic analysis (22). Sequencing (either in whole or in part) is becoming more available due to declining costs and the increases in taxonomy databases (23–25). The total time in which an organism, even a novel one, is sequenced has vastly decreased. In fact, the challenge is how to best analyze the output. Another complication surrounding sequencing and the availability of the genetic code revolves around the potential to weaponize pathogens. Although there have been no recent examples of bioterrorism, the accessibility of techniques to synthesize whole synthetic genomes may allow for the mass production of current or modified pathogens and explains why outbreak surveillance for eradicated diseases, such as smallpox, is ongoing.

Once the nucleotide sequence is known, target amplification, usually by PCR technology, can also be used as a detection method. This was seen in the 2012 novel coronavirus (CoV) outbreak in which the CDC developed a novel PCR assay to qualitatively detect Middle East respiratory syndrome CoV (MERS-CoV) RNA in respiratory specimens in patients that met the clinical criteria. For the detection of pathogens with known sequences, PCR is typically highly sensitive and specific but is dependent on adequately obtained specimens. In some investigations, these are difficult to obtain. In the case of MERS-CoV, specimens of the lower respiratory tract are required (26).

In order to test for multiple pathogens simultaneously, multiplex PCR can be used (27). It is best employed in an outbreak to narrow the differential when a patient presents with a common complaint (e.g., respiratory panel for common respiratory pathogens, gastroenteritis panel, etc.). The nosocomial implications of this technology are particularly important, and these methods can be used to monitor baseline levels and outbreak clusters within health care institutions, particularly when platforms with more-specific identifications (i.e., Rous sarcoma virus type A [RSV A] versus

TABLE 4 Laboratory studies for specific outbreak syndromes[a]

Syndrome	Select example(s) of disease(s)/ pathogen(s)	Suggested laboratory test(s)
Acute diarrhea syndrome	Amebic dysentery	Microscopic ova and parasite examination with wet mount and trichrome stain, *Entamoeba histolytica* ELISA, NAAT (stool)
	Cholera	Bacterial culture, rapid lipopolysaccharide dipstick test (stool)
	Cryptosporidiosis	Microscopic examination with modified acid-fast stain or DFA, ELISA, NAAT (stool)
	Escherichia coli	Bacterial culture, Shiga toxin immunoassay, NAAT for Shiga toxin genes (stool), DFA
	Giardiasis	Microscopic ova and parasite examination, enzyme immunoassay, DFA (stool)
	Salmonellosis	Bacterial culture (stool)
	Shigellosis	Bacterial culture (stool)
	Viral gastroenteritis	NAAT, enzyme immunoassay for rotavirus, viral culture (stool)
Acute hemorrhagic fever	Ebola and hemorrhagic fevers, hantaviruses, Lassa fever, arenaviruses, flaviviruses, or yellow fever	ELISA, NAAT, or viral culture (serum, biopsy, or postmortem tissue), IHC (biopsy or postmortem tissue)
Acute jaundice syndrome	Hepatitis A, B, and E	Antigen and antibody testing (serum), NAAT (plasma)
	Yellow fever	Antibody testing (serum) NAAT, IHC, or viral culture (biopsy or postmortem tissue)
Acute neurological syndrome	Enteroviral meningitis	NAAT (CSF), viral culture (pharynx, stool)
	Japanese encephalitis	ELISA (CSF or serum), NAAT (CSF)
	Leptospirosis	Bacterial culture (CSF, serum), *Leptospira* antibody, microscopic agglutination test (serum)
	Malaria	Blood smear, immunochromatographic card assay, NAAT (blood)
	Meningococcal meningitis	Bacterial culture (CSF, blood)
	Poliomyelitis	Viral culture (pharynx, stool, CSF), serology (serum)
	Rabies	NAAT (saliva), DFA (nuchal skin biopsy, brain biopsy, postmortem brain tissue), serology (serum, CSF)
	Tick-borne encephalitis viruses	Serology (serum), NAAT (serum, CSF)
Acute respiratory syndrome	Anthrax	Bacterial culture (blood, pleural fluid, CSF), NAAT (pleural fluid, CSF), IHC (bronchial biopsy specimens, postmortem tissue), anthrax lethal toxin (serum), antibody (serum)
	Influenza	NAAT, DFA, viral culture (nasopharyngeal aspirate or lower respiratory tract specimen), serology (serum)
	Legionellosis	Antigen test (urine), bacterial culture, NAAT (sputum, bronchoscopy specimen)
	Pertussis	*Bordetella* culture or NAAT (nasopharyngeal swab)
	Pneumonic plague	Bacterial culture (blood, lymph node aspirate, sputum, lower respiratory tract specimen), NAAT (postmortem)
	Respiratory syncytial virus	NAAT, DFA, rapid antigen detection test, or viral culture (nasopharyngeal aspirate/swab, or lower respiratory tract specimen)
Acute dermatological syndrome	Chickenpox	DFA, viral culture, NAAT (vesicular fluid or scraping), serology (serum)
	Cutaneous anthrax	Bacterial culture, NAAT (scraping of vesicular fluid, eschars, or ulcers) histopathology, IHC (full-thickness punch biopsy), anthrax lethal toxin, antibody (serum)
	Measles	Serology (serum, CSF), viral culture, NAAT (pharynx, urine, blood, CSF)
	Rubella	Serology (serum), NAAT (CSF)
Acute "systemic" syndrome	Brucellosis	Bacterial culture (blood, bone marrow), serology (serum), NAAT (blood or body tissue)
	Dengue fever	Serology or NAAT (serum or CSF)

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[a] Data are adapted from the WHO (http://www.who.int/csr/resources/publications/surveillance/whocdscsredc2004.pdf). Note that not all tests are commercially available or FDA approved. Abbreviations: ELISA, enzyme-linked immunosorbent assay; NAAT, nucleic acid amplification test; DFA, direct fluorescent antibody test; IHC, immunohistochemistry; CSF, cerebrospinal fluid.

TABLE 5 Examples of the laboratory techniques and control interventions used in significant outbreaks by pathogen type

Organism type and reference(s)	Pathogen	Location(s), no. of cases	Source	Lab technique(s) used	Control measures
Bacterial					
14[a]	*Vibrio cholerae* O1	Haiti, 684,085 cases and rising	Contaminated drinking water	Culture, susceptibility testing, PFGE, serotyping, and whole-genome sequence analysis	Improving sanitation, educating health workers on rehydration
15, 16[b]	*Neisseria meningitidis* group A	West Africa (primarily Nigeria and Niger) 88,199 suspected cases	Person-to-person transmission	CSF culture and susceptibility testing, PCR, serogrouping, and multilocus sequence typing for strain identification	Isolation wards, postexposure prophylaxis, and vaccination campaigns, including introduction of a new group A conjugate vaccine
Viral					
17, 18[c]	Hepatitis B	Gujarat, India, 456 cases	Contaminated injection devices	Serology and hepatitis B virus genotyping	Improved education of health care workers on safe injection practices
19	Dengue	Punjab, Pakistan, over 1,000 cases	*Aedes* mosquito	PCR for serotyping	Spraying campaign
20, 21[d]	Mumps	Several states (primarily Midwestern U.S. colleges), 6,584 cases	Person-to-person transmission	Viral culture and reverse transcription-PCR	Infection control efforts, vaccination campaign
Parasitic[e]					
	Cyclospora cayetanensis	United States, 631 cases	Packaged salad mix	Stool examination with staining and PCR	Temporary suspension of production of salad mix from Taylor Farms de Mexico

[a] http://mspp.gouv.ht/newsite/.
[b] http://www.who.int/mediacentre/factsheets/fs141/en/.
[c] http://www.cdc.gov/hepatitis/Outbreaks/2013/A1b-03-31/index.html.
[d] http://www.cdc.gov/mumps/outbreaks.html.
[e] http://www.cdc.gov/parasites/cyclosporiasis/outbreaks/investigation-2013.html.

RSV B) are used. These more commercial platforms usually rely on the fact that the organism and its sequence are both known and suspected; however, they may also be used to employ degenerate, genus-wide primers to discover novel pathogens (28).

In another promising technology, DNA microarray, millions of genetic probes are bound to glass or silicon wafers and tested for their capacity to bind complementary sequences (29). The binding is detected by measuring tagged fluorescent molecules. This adds increased specificity and has the potential to allow technicians to survey multiple microbes simultaneously and even identify antibiotic resistance markers. It has shown some promising results, but sensitivity is currently low and processing is cumbersome, so use in outbreak investigations is still limited (30, 31).

With any of these laboratory methods, it is key that appropriate controls are established and that methodology is validated. Remember that the laboratory can be the cause of a pseudo-outbreak or the presumption of an outbreak when there is none. It must be considered that an uptick in the rate of infection may be a lab artifact rather than clinical reality.

SELECT EXAMPLES OF OUTBREAK SCENARIOS: HEALTH CARE AND FACILITIES, CONTAMINATED PRODUCTS, AND FOODBORNE ILLNESSES

Outbreaks in health care facilities are discussed in detail in chapter 8. As in other locations, mandatory reporting in health care facilities exists in order to aid in ongoing baseline surveillance. The increased number of reportable health care-associated infections places a particular burden on the lab to constantly monitor and report certain infections. In addition, some facilities require ongoing environmental surveillance to be performed by their laboratory, depending on that location's infection control needs. It is important to let the epidemiology guide the investigation, as sporadic environmental cultures may either overestimate or underrepresent the pathogens present and infection may still occur (32, 33).

Another area in which outbreak investigations are becoming increasingly important is in the preparation, handling, and distribution of medications. In one case, an outbreak of *Serratia marcescens* and *Enterobacter cloacae* bacteremia in a surgical intensive care unit was traced to

TABLE 6 Steps of an outbreak investigation using fungal meningitis cases linked to contaminated steroid injections from a 2012 multistate outbreak[a]

Step in the outbreak	Specific illustrative finding(s)
1. Prepare to investigate	The Tennessee Department of Health was aware of baseline data, including the uncommon nature of fungal CNS infections and the known association of infection with epidural injections.
2. Verify the diagnosis	The index case was confirmed to be *Aspergillus fumigatus* by CSF culture, and a Gram stain of the L4 L5 space on autopsy yielded branching hyphal elements consistent with *A. fumigatus*.
3. Establish a case definition	In response to a single reported case of *Aspergillus* meningitis in a patient who had a recent epidural injection, active surveillance for additional cases was performed. Cases were defined as patients with fungal meningitis, posterior circulation stroke, spinal osteomyelitis, or epidural abscess that developed after epidural or paraspinal glucocorticoid injections that were administered after 21 May 2012 in Tennessee.
4. Find cases	Additional cases were identified on active surveillance. Initially, cases from the same clinic were investigated, including those that received methylprednisolone injections. After the index case, 21 additional patients had laboratory confirmation of infection, all with *Exserohilum rostratum*.
5. Describe the epidemiology	Detailed chart reviews captured patient data and other factors including common products used in the procedures.
6. Develop a hypothesis	Based on the finding of an association with single-dose vials of methylprednisolone injections, the Massachusetts Department of Health contacted the supplier, the New England Compounding Center (NECC).
7. Evaluate the hypothesis	Cerebrospinal fluid, isolates, and tissue obtained from clinical specimens were sent to the CDC for identification of the pathogen with the use of PCR amplification of fungal DNA and genomic sequencing. The FDA also analyzed unopened vials of methylprednisolone that had been collected from NECC and were later found to be positive. Patients who received injections of other medications at the same clinic did not manifest disease.
8. Refine the hypothesis and carry out additional studies	Based on the distribution of the methylprednisolone vials from the manufacturer, 2 additional clinics were quickly included in the investigation in Tennessee, and over 65 patients were identified.
9. Implement control and prevention measures	In October 2012, NECC recalled all products compounded at and distributed by its facility in Framingham, MA.
10. Communicate findings	The CDC regularly received reports of and communicated rates of fungal infections due to NECC products. The FDA advised follow-up with patients who were administered products from NECC and advised withholding any products still in circulation.

[a] See references 26 and 27.

extrinsic contamination of the parenteral narcotic fentanyl by likely tampering by a health care worker. The laboratory was key in analyzing patient, environmental, and drug samples, in ruling out environmental causes, and in showing similarities between the fentanyl and blood isolates (34).

In another case involving a multistate outbreak of fungal meningitis, the laboratory was key in confirming cases that met the case definition and in investigating the contaminated product, particularly in the identification of which lots were infected. Table 6 reviews this investigation and how the laboratory played a role at the key steps in the outbreak investigation. Using Table 3 as a template, we show how a multistate outbreak of fungal meningitis was recently linked to cases from Tennessee and extrinsic contamination from a compounding pharmacy (35, 36). By adding clear associations between products and infection, the laboratory provides evidence to warrant change either at the level of an individual institution (as in the removal of the implicated health care worker in the outbreak of *Serratia* bacteremia or in the move to revise legislation involving the regulation of compounding pharmacies, as with the cases of fungal meningitis).

Due to the critical supply of our food network and distribution chain, monitoring of foodborne diseases is one of the most active, coordinated surveillance systems in the United States, involving local and reference laboratories. FoodNet is a sentinel surveillance system undertaken at 10 participating sites in the United States in collaboration with the CDC, USDA, and FDA. It concentrates on laboratory testing and documented diseases and gives a baseline incidence of foodborne and diarrheal diseases and their distribution in the United States. Together, these sites survey about 15% of the U.S. population (37).

Foodborne illnesses can cause outbreaks at any time and are monitored regularly. It is estimated that there are 9.4 million domestically acquired foodborne illnesses each year caused by over 30 pathogens. This results in an estimated 55,961 hospitalizations and 1,351 deaths annually (38). In recent years, changes in the American diet to include undercooked foods, imported foods, and mass-produced foods add to the challenge of investigating foodborne outbreaks. It is not uncommon in today's market to find food that is processed in a variety of locations distributed over multiple states or multiple countries.

In addition to FoodNet, PulseNet is a national subtyping network of over 80 state and local public health laboratories and federal food regulatory laboratories that perform molecular surveillance of foodborne infections. They analyze DNA fingerprints through testing with pulse-field gel electrophoresis (PFGE) and routinely detect and report on subtypes of eight bacteria, including *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* serotypes. Isolate subtypes are constantly being uploaded, and in 2012, participating laboratories reported over 60,000 isolates to PulseNet, providing baseline data on infections down to the molecular level (http://www.cdc.gov/pulsenet/).

One example of PulseNet at work is its handling of the *Listeria monocytogenes* multistate outbreak in 2011. On 2 September 2011, the Colorado Department of Public Health and Environment (CDPHE) reported to the CDC seven cases of listeriosis since 28 August 2011, whereas on average they would report two cases annually in August. All patients were interviewed, and three reported eating cantaloupe marketed as "Rocky Ford." Outbreak strains were analyzed with PFGE, and PulseNet picked up an additional PFGE pattern that emerged as a multistate cluster. A sample of cantaloupes collected from the implicated farm also had that PFGE pattern. Although two serotypes and five widely differing PFGE pattern combinations were found, both ongoing surveillance and outbreak investigations were able to identify cases that spanned 28 states. The accuracy of detection also meant that by 9 September 2011, the CPDHE could inform the public that cantaloupes were the likely cause of the illness and instruct consumers not to eat them, and by the next day, distribution was stopped and grocery stores were instructed to remove the product. Despite the rapid action taken by public health authorities, the case number, at 147 infected persons and 33 deaths, was the largest listeriosis outbreak since the CDC has been keeping record (http://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/index.html) (39).

RESOURCES TO STAY INFORMED AND CONNECTED IN GLOBAL OUTBREAKS

With the rise of Internet-based surveillance and other social media tools, the way that we identify, communicate during, and react during an outbreak is being revolutionized. In the last few decades, the WHO has often responded to outbreak investigations that have been triggered by what had previously been considered nontraditional sources, such as media surveillance systems (40). Even with national and transnational public health organizations engaging in continual information gathering, often the most vulnerable countries have no infrastructure for reporting or alerting populations (41). Internet or mobile resources can support high-risk areas that may be the most susceptible to disease but the most lacking in epidemic infrastructure. A sample of some widely used and cited resources is listed here. Many of these systems track not only human but also zoonotic infections, as many outbreaks originate in domestic animals or wildlife and animals are critical to comprehensive surveillance.

GeoSentinel (http://www.istm.org/geosentinel/main.html) is a worldwide communication and data collection network for the surveillance of travel-related morbidity. The International Society of Travel Medicine (ISTM) and the CDC initiated it in 1995 as a network of ISTM member travel/tropical medicine clinics. Its members are connected through e-mail, which provides a linked network that can quickly communicate with public health officials. They can informally provide leads when they encounter any unusual diagnosis or exposure and be surveyed to assess rates of background disease (42, 43).

ProMED-mail (http://www.promedmail.org), established in 1994 by Joshua Lederberg after a meeting of the Federation of American Scientists and the WHO, is a free e-mail alert system about new or evolving outbreaks (44). A panel of experts in each field selects and reviews ongoing member submissions. They then provide expert commentary and supply literature references before disseminating reports to a >60,000-member subscriber base in over 180 countries. It served as an early warning system in the SARS outbreak and has served as a communication network among laboratories. The Global Public Health Intelligence Network (GPHIN) is a fee-based subscription service run by the Public Health Agency of Canada that scans news services in real time and posts to its members in six languages (45). Posts are not verified or tied to references, and more than 60% of the initial reports come from informal sources, but the WHO has incorporated the GPHIN into its Global Outbreak Alert Response Network and works to verify the epidemic reports (http://www.who.int/csr/alertresponse/epidemicintelligence/en/). Google Flu Trends uses Google search queries to track and map influenza globally (46) and was found to perform well during the 2009 H1N1 pandemic (47).

HealthMap (http://healthmap.org), developed by Boston Children's Hospital, is a multistream surveillance program that integrates Google News, ProMED-mail, Eurosurveillance, and official documents from the WHO, among other sources, into a user-friendly map that is updated in real time (48). Users can be updated on a mobile app or can report outbreaks using their smart phone. InSTEDD (Innovative Support to Emergencies, Diseases, and Disasters) is a growing number or nonprofits that work to develop open-source technology that specifically serves vulnerable populations. It currently develops local media tools and hopes to eventually aggregate medical services data to promote awareness in areas of need and create sustainable approaches to health problems (http://instedd.org).

For those investigating health care-associated outbreaks, the Outbreak Database (http://www.outbreak-database.com) can be used. It is an open-access resource maintained by physicians at the Charité University Hospital

(Berlin, Germany) and contains updated articles related to worldwide nosocomial outbreaks. It can provide relevant literature pertaining to an acute outbreak and literature to address science-oriented questions or to aid in the development of successful infection prevention policies. It can be particularly valuable for laboratory personnel by identifying molecular diagnostics that were used in similar outbreak settings and to locate reference laboratories that may be used in an ongoing investigation (49).

CONCLUSIONS

This chapter has reviewed concepts associated with outbreaks, including how to approach an epidemiological investigation, routine and novel laboratory methods, and infectious disease reporting. Examples of routine and novel laboratory testing that have been used in outbreak settings and examples where laboratory testing played a key role in the investigation of an outbreak have been reviewed.

The rise in globalization highlights the importance of thinking about the world health community when responding to an outbreak. Local clinicians, laboratorians, and public health practitioners remain the stalwart members of the outbreak team. However, they must interface with state, national, and international authorities and professional organizations in order to act fast, precisely define, and stem the tide of an outbreak. Pathogen identification is becoming faster and more sensitive, and reference laboratories should be employed in order to obtain the most accurate diagnosis in a timely fashion. The laboratory will continue to play a crucial and likely expanding role in the investigation of infectious disease outbreaks in the coming years.

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Molecular Epidemiology

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10

Our knowledge and understanding of infectious diseases have increased dramatically in recent decades primarily from observations drawn from the interconnected fields of clinical microbiology and epidemiology. In the context of infectious diseases, epidemiology is the study of the transmission of human pathogens, including their risk factors for and control of infectious disease in human populations (1). The epidemiology of infectious diseases has, since the discovery of microbial pathogens, been closely linked to the laboratory sciences. Until the introduction of molecular techniques in the 1970s, infectious disease epidemiology was driven mostly by discoveries in classical microbiology, with the development of culture and phenotypic identification methods, subtyping techniques, like bio-, sero-, and phage typing, and antimicrobial susceptibility testing. These methods were used to discover pathogens and study their reservoirs, transmission routes, geographical distribution, infection dynamics (including outbreak detection and investigations), vaccine efficacy, and other disease prevention measures.

Since the 1970s, molecular methods have been introduced and, with increased frequency, continue to replace phenotypic methods in the microbiological laboratories. The emergence of these new methods led to the creation of a new discipline: molecular epidemiology. In its simplest form, molecular epidemiology can be defined as the application of molecular (i.e., nucleic acid- or protein-based) tools to answer epidemiological questions. With the introduction of molecular epidemiology, the study of the epidemiology of infectious diseases has reached new heights. Molecular epidemiology is no longer a science for the elite; molecular biological devices and reagents are widely available, and almost any clinical or research laboratory has the expertise and the equipment required for performing molecular studies. We are now obtaining deeper recognition of the molecular mechanisms that form the basis of the virulence of microbial pathogens, and as time passes, the subtyping methods used to trace them are becoming faster, more discriminatory, and therefore more powerful. The etiologies of many infectious diseases may now be diagnosed without culture in minutes or hours, thereby enabling a fast specific therapeutic response and in some instances also a rapid public health response.

Molecular epidemiology is often confused with another related but distinct microbiology discipline: molecular taxonomy. Taxonomy is the discipline that studies the classification, identification, and naming of microorganisms. A subdiscipline of taxonomy is phylogeny, which is the study of the evolutionary relationships of microorganisms. Before the introduction of molecular methods, phylogeny was based solely on phenotypic traits and, as a result, was imprecise and often yielded erroneous information. The use of nucleic acid hybridization techniques and the analysis of housekeeping gene sequences have greatly improved our understanding of microbial evolution. Compared to taxonomy and phylogeny, molecular epidemiology focuses on the study of more recent population dynamics. In addition, taxonomy and phylogeny describe interactions between the organisms themselves, whereas epidemiology describes interactions between the organisms, their hosts, and the surrounding environment. The same molecular methods may be used in both disciplines; it is their application that determines if they are used for taxonomy or epidemiology.

In molecular epidemiology, molecular methods are used for detection, identification, virulence characterization, and subtyping to generate isolate-specific molecular markers (or fingerprints) for assessment of epidemiological relatedness (1). This chapter is an introduction to molecular epidemiology and important concepts associated with this discipline. Some terms commonly used in molecular epidemiology are provided in Table 1. A nonexhaustive list of subtyping methods that are commonly used now or are under development and are anticipated to supplement or replace the currently used ones is provided. Key characteristics and applications of these methods are summarized in Table 2. Strategies for the development of molecular subtyping tools, including validation and quality control, will be discussed. Criteria for the selection of methods to be used in the different contexts of epidemiology and how the choice of the method influences the interpretation of data will also be addressed.

SUBTYPING METHODS

Subtyping Method Characteristics

Typeability

An ideal typing method should be able to produce data that can lead to the establishment of subtypes for the majority, if not all, of the strains of the pathogen being studied. The degree with which a method achieves this can be expressed, in mathematical terms, as the percentage of typeable isolates among the total number of isolates being subtyped. All molecular subtyping methods (except plasmid profiling with some organisms) show very high typeability with the organisms that they target.

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TABLE 1 Definitions commonly used in molecular epidemiology[a]

Term	Definition
Isolate	A population of microbial cells from a pure culture derived from a single colony on an isolation plate.
Strain	An isolate or group of isolates exhibiting phenotypic and/or genotypic traits which are distinctive from those of other isolates of the same species.
Clone	A group of isolates descending from a common ancestor as part of a direct chain of replication and transmission from host to host or from the environment to host. The term "outbreak strain" is often used with this meaning in the context of epidemiologic subtyping.
Subtype	A specific pattern or set of markers displayed by a strain when a particular typing system is used.
Typeability	The proportion of strains for which a subtype may be generated by a given subtyping method.
Reproducibility	Same as repeatability. The ability of a subtyping method to produce the same results upon repeated testing. Usually stated as the proportion of strains in a given population that displays the same subtype upon repeated testing.
Stability	The ability of a subtyping method to assign the same subtype to epidemiologically related strains, e.g., as part of the same single-strain outbreak or originating from the same patient or from serial passage *in vitro* or *in vivo*. Usually stated as the proportion of epidemiologically linked strains showing the same subtype.
Discriminatory power	The ability of a subtyping method to differentiate between epidemiologically unrelated strains.
Epidemiological concordance	The ability of a subtyping method to link epidemiologically related strains.
Comparative subtyping	Subtyping results generated in the same or in a very few experiments in the same laboratory may be compared due to poor interexperiment reproducibility.
Definitive subtyping	Subtyping results generated in different laboratories and/or at different times may be compared and stored in a reference library.
Library subtyping	Subtyping results generated by definitive subtyping are stored in a database (library). Sometimes used synonymously with definitive subtyping.
Cluster	The occurrence of clinical isolations of microbes with a particular subtype greater than would otherwise be expected in a particular time and place with no further supporting epidemiological information.
Outbreak	Same as an epidemic. The occurrence of disease greater than would otherwise be expected in a particular time and place.
Sporadic	Antonym to outbreak. Occurring with no clear relation to an outbreak.
Endemicity	Constant presence of a disease at a significant frequency; typically restricted to, or peculiar to, a locality or region.

[a] In part adopted from Van Belkum et al. (1) and Struelens (242).

Reproducibility and Stability

Reproducibility refers to the ability of a method to assign the same type to an isolate tested on independent occasions separated in time and/or place. It may be calculated as the percentage of strains that, upon repeated testing using the same parameters, yield the same result. This term is sometimes also referred to as the repeatability. Typically, there is a direct correlation between the reproducibility and robustness of a method and the quality of the data being generated. Reproducibility may be influenced by many steps in the procedure, such as preparation of materials (growth conditions, DNA extraction), different batches of reagents, different types of instruments, and, finally, bias in observing, analyzing, and interpreting results. Some methods have such a poor reproducibility that it is not possible to compare results generated in different experiments. Such methods are said to be comparative. Other methods are so reproducible that it is possible to recognize the same subtypes even though they have been generated at separate times and/or in separate places. Such methods are considered definitive subtyping methods.

Reproducibility has both intralaboratory and interlaboratory dimensions. As will be discussed later, some subtyping methods, e.g., amplified fragment length polymorphism (AFLP) analysis, show excellent reproducibility when performed on the same instrument in one laboratory (i.e., they have good intralaboratory reproducibility) but poor or suboptimal reproducibility when testing is performed in different laboratories (i.e., they may have a poor interlaboratory reproducibility).

Reproducibility is also indirectly affected by the stability of the genetic markers being targeted by the method. The assessed markers should remain stable during outbreaks and among multiple individual patient isolates not varying to a

TABLE 2 Characteristics and application of a number of subtyping methods

	Quality of or applicability to:												
Characteristic	Plasmid profiling	PFGE	Whole-genome mapping	RAPD	rep-PCR	PCR-ribotyping	AFLP	MLST	MLVA	Gene sequencing	SNP detection with DNA microarrays	Whole-genome sequencing	Mass spectrometry
Reproducibility	Good	Good	Good	Poor	Poor	Good	Good	Good	Good	Good	Good	Good	Good
Stability	Variable	Good	Good	Poor	Moderate	Good	Good	Moderate to good	Moderate to good	Moderate to good	Good	Moderate to good	Good
Discriminatory power	Variable	Excellent	Excellent	Good	Good	Good	Excellent	Low to moderate	Excellent	Excellent	Good to excellent	Excellent	Poor
Universal applicability	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	Yes	No
Applicable for library subtyping[a]	Yes	Yes	Yes	No	No/yes (in local libraries)	Yes	Yes (in local libraries)	Yes	Yes	Yes	Yes	Yes	Yes
Complexity of data	Simple	Complex	Complex	Complex	Complex	Simple	Complex	Simple	Simple	Simple	Simple	Very complex	Simple
Ease of use[a]	Simple	Moderately labor-intensive	Moderately labor-intensive	Simple	Simple/simple	Simple	Moderate	Simple to moderately labor-intensive	Simple	Simple to moderately labor-intensive	Simple to moderately labor-intensive	Labor-intensive	Simple
Cost[a]	Low	Moderate	High	Low	Low to moderate	Low	Moderate	Moderate	Moderate	Moderate	Moderate	High	Low
Suggested use of the method	Supplement to other methods	Outbreak surveillance, large-scale libraries	To aid in the assembly of next-generation short sequence reads	To answer specific limited epidemiological questions/ small-scale outbreak investigations	As RAPD	First-line subtyping of *C. difficile*	Local outbreak surveillance, suitable for local library subtyping	Phylogenetic studies, attribution of *Campylobacter*, potential forensic use	Outbreak surveillance, large-scale library subtyping if standardized, potentially good for forensic and attribution purposes	Outbreak surveillance, large-scale library subtyping, phylogenetic studies, forensic microbiology, attribution	Outbreak surveillance, large-scale library subtyping, phylogenetic studies, forensic microbiology, attribution	Outbreak investigation, (large-scale) library subtyping, phylogenetic studies, forensic microbiology, attribution	Subspecies level (serotype, pathotype) typing
Comments		Gold standard for highly discriminatory bacterial subtyping			Semiautomated method may possibly be used for local surveillance								

[a]Manual/automated version.

degree that confuses the epidemiological picture. The genetic fingerprints generated by a method should also not be affected by *in vitro* manipulations, such as freeze-thaw cycles and serial passages.

Discriminatory Power

Discriminatory power is defined as the ability of a method to distinguish between unrelated strains. An objective measure of discriminatory power can be obtained by calculating Simpson's index of diversity (DI) (2), which is an estimate of the probability that two epidemiologically unrelated strains will display different subtypes. The formula reads

$$\mathrm{DI} = 1 - \frac{1}{N(N-1)} \Sigma_{j=1}^{N}$$

where *N* is the number of unrelated strains tested and *aj* is the number of strains with a subtype that is indistinguishable from the *j*th strain. The DI value of 1.0 indicates that all strains can be differentiated from each other. A value above 0.95 is a desirable cutoff for subtyping methods to be used for outbreak investigations.

The DI is a function of both the number of subtypes in the strain population (richness) and the proportion of strains representing each subtype, i.e., the evenness of the distribution of the isolates among the different subtypes. It is not possible to determine the contribution of the richness or the evenness of a subtyping method to the DI from the number *per se*. This should also be taken into consideration when comparing different subtyping methods. Several other diversity indices have been devised for this purpose, among which Shannon's index (H) (3) is the most commonly cited. However, the maximal size of this index is a direct function of the number of subtypes generated within a given strain population, and it is therefore difficult to interpret differences in the sizes of the index unless the populations studied generate the same number of subtypes. An easier and more comprehensible way to judge the richness of a subtyping method is to create a histogram showing the distribution of different subtypes. The results of a hypothetical experiment of subtyping the same population of 100 strains by two different methods are shown in Fig. 1A and B. Method A has a slightly higher DI than method B. However, the difference between the methods becomes obvious when one observes the histograms: method A has a higher richness than method B. The 100 strains are differentiated into 74 subtypes, with all containing 5 or fewer strains by method A, whereas the 6 most common subtypes by method B contain half of the strains, and overall, only 43 subtypes are generated by this method.

An ideal method should have such a high discriminatory power that it is capable of discriminating all epidemiologically unrelated isolates from each other (i.e., it has a high specificity). However, the method should also be able to group together isolates that are associated with the same source (i.e., it has a high sensitivity). In other words, a method with high sensitivity and specificity generates epidemiologically relevant data. The ability of a method to group together epidemiologically related isolates is sometimes also referred to as the epidemiological concordance.

Convenience Parameters

There are many convenience parameters to be considered, including speed of analysis, cost, technical demands, accessibility of the method, and ease of data analysis. The

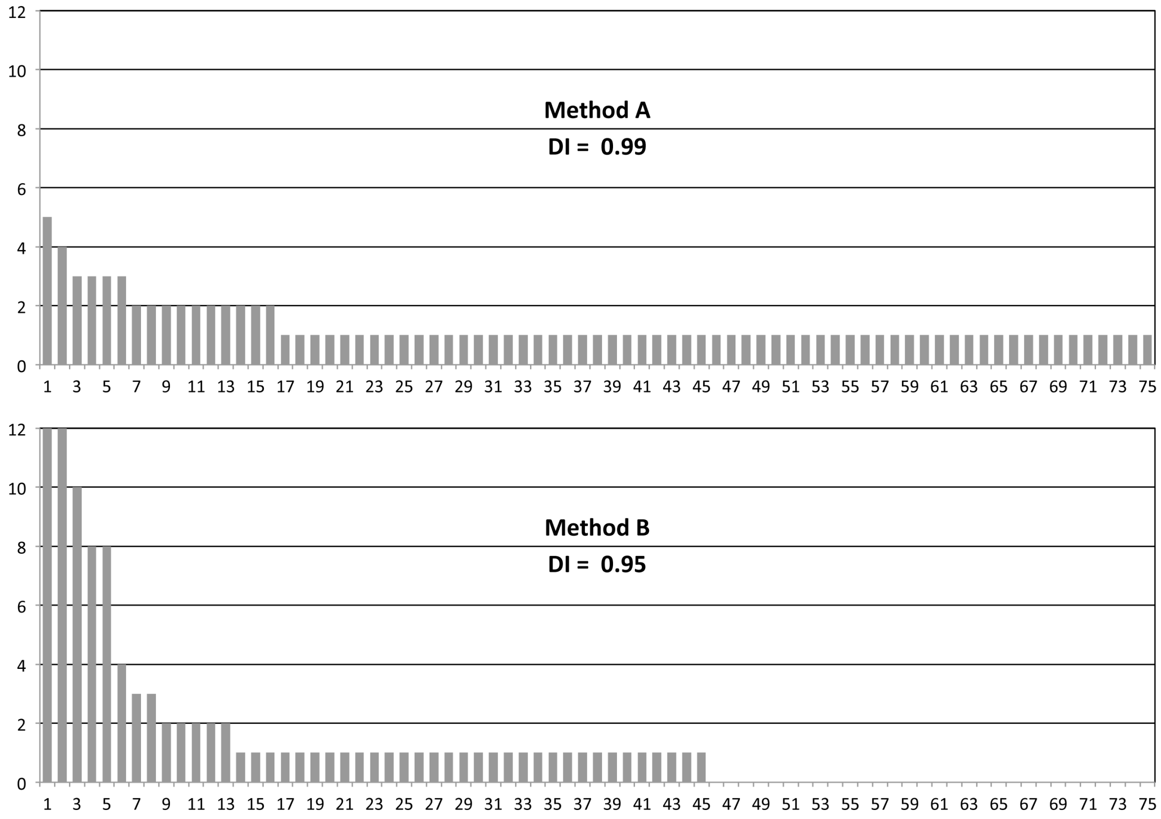

FIGURE 1 Hypothetical example of the subtype distribution of 100 epidemiologically unrelated microbial strains generated by two subtyping methods. doi:10.1128/9781555817381.ch10.f1

ability of a method to generate data rapidly is affected by the throughput of the method, the number of steps involved, the amount of hands-on technical time required to perform the method, and whether the method is amenable for automation. Ideally, typing results should be available within a single working day. The cost of performing a method depends on numerous factors, such as the initial investment in equipment and infrastructure, the price of reagents and consumables, and the number and skill level of staff needed. Cost is usually also the most critical factor affecting the accessibility of the method to general microbiology laboratories. Ease of use encompasses technical simplicity, high throughput, and ease of scoring the results. For easy analysis, data should be objective, amenable for computerized analysis, and easily disseminated between laboratories.

Another convenience parameter rarely considered is the universal applicability of the method; can the same method be used to subtype a broad range of organisms while maintaining a universally high typeability, reproducibility, and discriminatory power? The broader the range of microbial species that can be studied, the more central the position of the method in the general typing laboratory will be.

Non-Target-Specific Methods

A short description of the principle of some non-target-specific methods is shown in Fig. 2.

Plasmid Profiling

Plasmids are circular extrachromosomal autonomous self-replicating genetic elements that are found in bacteria and some eukaryotes. Their sizes range from approximately 1 kb to >1 Mb. Besides carrying genes regulating their own replication and transmission, plasmids may carry genes that confer specific properties in the host organism, e.g., antimicrobial resistance or toxins. Plasmid profiling was the first widely used nucleic acid-based bacterial subtyping method dating back to the 1970s. The rationale for using plasmid profiling for subtyping is that bacterial strains typically differ in the numbers and sizes of plasmids that they carry. The isolates are lysed using a method that disrupts chromosomal DNA while retaining the integrity of the plasmid, e.g., alkaline lysis (4), followed by separation of the plasmids by agarose gel electrophoresis (5), staining using a fluorescent dye, e.g., ethidium bromide, and visualization of the plasmids under UV light.

The advantages of this method are that it is universal (i.e., it may be used to characterize any organism that contains plasmids by using the same basic procedure) and that it is inexpensive, rapid, and simple, with no requirements for special equipment. The typeability of the method is variable, the discriminatory power varies between organisms, and the reproducibility is in general good, but since plasmids may be lost during strain propagation *in vivo* and *in vitro*, the stability is suboptimal. Additionally, plasmids can be unintentionally nicked, causing changes in their conformational structure (from supercoiled to a relaxed or linear form), impacting their migrating properties and resulting in an incorrect profile. These changes may happen if the purification process is performed too harshly. Two or all three conformations may be present at the same time in significant amounts and be visible as two or three bands in the gel, and since they have different migration properties in agarose, interpretation of the profiles may be difficult. In addition, large plasmids, those >100 kb in size, are poorly

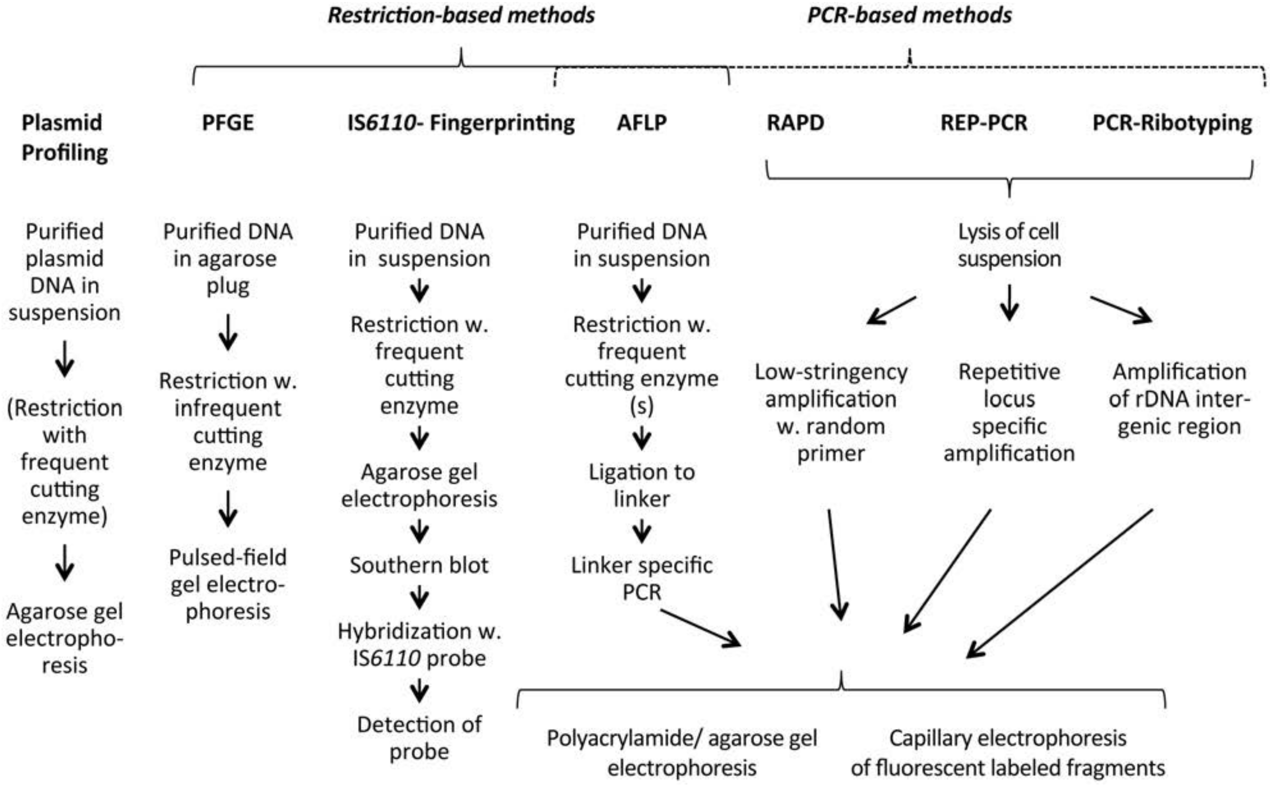
Restriction-based methods
PCR-based methods
Plasmid Profiling
PFGE
IS6110- Fingerprinting
AFLP
RAPD
REP-PCR
PCR-Ribotyping
Purified plasmid DNA in suspension
(Restriction with frequent cutting enzyme)
Agarose gel electrophoresis
Purified DNA in agarose plug
Restriction w. infrequent cutting enzyme
Pulsed-field gel electrophoresis
Purified DNA in suspension
Restriction w. frequent cutting enzyme
Agarose gel electrophoresis
Southern blot
Hybridization w. IS6110 probe
Detection of probe
Purified DNA in suspension
Restriction w. frequent cutting enzyme (s)
Ligation to linker
Linker specific PCR
Lysis of cell suspension
Low-stringency amplification w. random primer
Repetitive locus specific amplification
Amplification of rDNA inter-genic region
Polyacrylamide/ agarose gel electrophoresis
Capillary electrophoresis of fluorescent labeled fragments

FIGURE 2 Procedural principles of some commonly used non-target-specific subtyping methods. doi:10.1128/9781555817381.ch10.f2

separated by ordinary agarose gel electrophoresis. Unrelated plasmids with similar sizes may not be differentiated in an agarose gel. However, if they are digested with a restriction enzyme before electrophoresis, it will often be possible to differentiate between them.

Plasmid profiling has been used in numerous epidemiological investigations of a wide range of community-acquired or nosocomially acquired infections (6, 7) and is still used as an adjunct in investigations of outbreaks of foodborne infections in some countries (8). Plasmids may spread between different organisms and cause outbreaks of, for example, antimicrobial resistance. Plasmid profiling is the first step in the investigation of such outbreaks (9, 10).

Restriction Fragment Length Polymorphisms

A restriction endonuclease is an enzyme that recognizes a short DNA sequence and cuts it at a specific location within the restriction site. Genomic DNA from a single microorganism will always be cut into fragments of the same size and number if it is digested by the same restriction enzyme. The restriction fragments may be separated according to their size by electrophoresis using agarose or a similar medium as a matrix, followed by staining and visualization under UV light to reveal the DNA fingerprint or restriction profile of that organism. Since different strains have different genomic contents, even strains of the same species that are epidemiologically unrelated to each other will usually show different restriction profiles. Subtyping methods that explore the polymorphisms of restriction profiles are called restriction fragment length polymorphism (RFLP) methods, and the process is called restriction endonuclease analysis (REA). The method was introduced in the late 1970s and the 1980s for subtyping of viruses, parasites, fungi, and bacteria (11–14) using high-frequency cutting enzymes, which resulted in DNA fingerprints containing up to 500 restriction fragments ranging in size from <1 kb to 30 kb. Although the method had universal applicability, was simple, and required few resources, the resulting fragments were often difficult to resolve and the DNA fingerprints too complex to analyze accurately, especially for organisms with large genomes, e.g., bacteria, fungi, and parasites. For this reason, ways to simplify the RFLP DNA fingerprints while maintaining the discriminatory power of the method were sought. This was achieved by reducing the number of restriction fragments in the fingerprints by (i) reducing the number of fragments generated during the restriction reaction or (ii) reducing the number of fragments being visualized. Pulsed-field gel electrophoresis (PFGE) falls within the former category. There are numerous examples of the latter technique, among which IS*6110* fingerprinting will be mentioned herein. Finally, optical mapping or whole-genome mapping (WGM), which does not involve electrophoresis but visualizes high-frequency RFLP fingerprints on a glass surface with the restriction fragments in the order that they occur in the chromosome, will be described.

Pulsed-Field Gel Electrophoresis

In pulsed-field gel electrophoresis (PFGE), the DNA fingerprint is simplified by using rarely cutting restriction enzymes with the goal of reducing the number of restriction fragments to be analyzed (macrorestriction). The resulting fragments usually range between ~20 kb and >1 Mb in size. Because organisms differ in the guanine and cytosine (GC) contents of their DNA, the optimal restriction enzymes for PFGE vary between organisms (15). The optimal restriction enzyme(s) generates between approximately 8 and 25 DNA fragments that are well separated and evenly distributed throughout the gel from each strain tested.

Large DNA fragments cannot efficiently be purified in a liquid suspension because this will cause random shearing. To avoid that, the genomic DNA is released and purified from cells that have been embedded in a solid agarose plug. The plug stabilizes the DNA against breaking or shearing as the cells are lysed chemically. The intact genomic DNA is then digested with an infrequently cutting restriction enzyme. Large restriction fragments cannot be resolved with conventional agarose gel electrophoresis, which works best for separation of fragments smaller than approximately 30 kb. This limitation is overcome by subjecting the plug with the macrorestricted DNA to electrophoresis in an alternating or pulsing electric field using an agarose gel as the separation matrix. Since its introduction in 1984 (16), several different PFGE platforms have been developed (17–19). In all formats, the electric field alternates in direction in a predefined manner throughout the course of the electrophoresis. During PFGE, smaller DNA fragments reorient (i.e., make a directional change) in the electrical field faster than larger fragments and therefore move more rapidly through the gel, resulting in separation of the fragments in a size-dependent manner. In some platforms, the interval of time that the electrical current is applied in one direction before it is switched to another direction may be changed. Usually the switch times are set to be short in the beginning of the electrophoresis and are then increased or ramped up during the course of the run.

PFGE has been performed since the 1980s and still remains the gold standard for bacterial molecular subtyping due to its universal applicability, virtual 100% typeability, high discriminatory power, good reproducibility, and stability. By nature, all electrophoresis-based methods are comparative; i.e., only isolates investigated in the same experiment may be compared. However, this problem may be overcome by rigorous standardization of the procedure, with the choice of restriction enzyme, type and brand of agarose, and electrophoresis running conditions being critical. Since 1996, PulseNet USA, the national molecular subtyping network for foodborne disease surveillance, has used PFGE as the preferred method for molecular surveillance, thereby proving its utility as a library subtyping method (20). The drawbacks of the method are that special electrophoresis equipment is required and that the procedure is rather labor-intensive. Up to 20 isolates may be subtyped within 2 working days by one person.

IS*6110* Fingerprinting

IS*6110* fingerprinting is an RFLP fingerprinting method used for subtyping of strains of the *Mycobacterium tuberculosis* complex. It was the first RFLP method to become standardized internationally (21), and it remains the gold standard for the molecular surveillance of tuberculosis (TB). The principle of the method includes whole-genomic-DNA restriction by a high-frequency cutting endonuclease, e.g., PvuII, followed by standard agarose gel electrophoresis, transfer of the DNA fragments onto a nitrocellulose or nylon membrane by Southern blotting (22), hybridization of the labeled probes containing IS*6110* insertion sequences to membrane-bound fragments, and visualization of the hybridized fragments (23). Only strains of M. *tuberculosis* and *Mycobacterium bovis* contain this insertion sequence (24), with M. *tuberculosis* strains containing between 10 and 12 copies and M. *bovis* only between 1 and 3 copies. Because of the low number of copies of IS*6110* in M. *bovis*, the method is not optimal for subtyping this species.

The strength of the method is that it has enabled the monitoring of the global spread of *M. tuberculosis* and tracing outbreaks to their sources because of its high typeability, reproducibility, and discriminatory power. The drawbacks of the method are that it may be used only for surveillance of tuberculosis and that it is complicated and labor-intensive.

Whole-Genome Mapping

Whole-genome mapping (WGM) or, as it was initially called, optical mapping is a technique where single DNA molecules are stretched out on a glass surface, after which they are subjected to restriction endonuclease analysis; the resulting fragments are visualized by fluorescence microscopy in the order that they occur in the genome (25, 26). The generated restriction maps of complete genomes can be compared using specialized software. The technology is a powerful tool for subtyping that can facilitate genome assembly from whole-genome shotgun sequences and that can perform comparison of the sizes and contents of genomes from related organisms, e.g., virulence genes and phages (25, 27–31). However, the technology is complex and costly. It has been automated and commercialized by the company OpGen (Gaithersburg, MD), but the cost is still prohibitive for large-scale use for public health purposes, particularly when the current cost of whole-genome sequencing (WGS) is equal to or, with some sequencing platforms, less expensive than that of whole-genome mapping.

PCR-Based Subtyping Methods

Numerous subtyping methods employing PCR alone or in combination with restriction analysis have been described previously. These techniques vary in amplification approach and/or in the genomic regions that they interrogate. Following amplification and restriction, if this is part of the method, the resulting fragments are separated by electrophoresis and visualized following staining with ethidium bromide. Since the fragments generated by PCR usually are small, capillary electrophoresis using a DNA sequencer may also be used to visualize the amplicons if the primers are fluorescently labeled.

Random Amplification of Polymorphic DNA/Arbitrarily Primed PCR

In random amplification of polymorphic DNA/arbitrarily primed PCR (RAPD/AP-PCR), small genomic fragments are amplified using short primers (less than approximately 14 bp) with a random sequence under low-stringency conditions (32, 33). Because of the low-stringency PCR conditions, the primers will bind to genomic regions with perfectly matching sequences as well as regions with a few mismatches. The result is a number of unevenly amplified fragments of different sizes yielding a profile that, theoretically, should be specific to each strain. The method is universally applicable, and it has a typeability of virtually 100%, a discriminatory power that is good but typically less than that of PFGE, and a reproducibility that is difficult to control because of the low-stringency amplification (34). The method has been used for subtyping of a broad range of prokaryotes and eukaryotes and is best suited for the comparison of a limited number of isolates subtyped under highly standardized conditions in one laboratory over a short period of time. Because of the poor reproducibility of the method, it is virtually impossible to create meaningful reference libraries of RAPD/AP-PCR fingerprints.

Repetitive Element PCR

Repetitive element PCR (rep-PCR) is the common denominator for PCR-based subtyping methods in which the region between interspersed repetitive DNA elements is amplified. Repetitive elements are present in many copies of the genomes of most organisms and conserved among phylogenetically related genera. If they are located close to each other in the genome, the sequence in between them may be amplified by PCR. Usually, several amplicons will be generated, and their numbers and sizes will often vary between strains. Many different repetitive elements have been identified. The most commonly used are the repetitive extragenic palindromic (REP) elements, which are 33 to 40 bp in length (35, 36), the enterobacterial repetitive intergenic consensus (ERIC) elements, which are 124 to 127 bp in length (36, 37), and the BOX element, which is a 154-bp repetitive sequence composed of three subunits, BoxA, BoxB, and BoxC, that may be present in different combinations (38). Theoretically, it is possible to perform highly reproducible and specific rep-PCRs due to the lengths of the primers used. However, in practice, rep-PCR suffers from the same reproducibility problems as RAPD, and when performed manually, results generated in different laboratories or with different PCR equipment and analysis platforms cannot be readily compared (39, 40).

A commercial semiautomated platform for rep-PCR, the DiversiLab system (bioMérieux, Marcy l'Etoile, France), is now available. In this system, quality-controlled reagents are provided in a kit format with Internet-based computer-assisted analysis, reporting, and data storage (41). This way, the reproducibility problems encountered with manual rep-PCR seem to have been alleviated.

Thus, rep-PCR, like RAPD, is a universal and simple subtyping method for most pathogens with a high typeability and a discriminatory power that in general is good but typically less than that of PFGE. Because of the problems with reproducibility, it is comparative and best suited for subtyping of a limited number of isolates during a short time span, but some laboratories have succeeded in creating small libraries of rep-PCR patterns. The reproducibility of the DiversiLab system is likely better, and it is used as a universal subtyping tool in some reference and hospital laboratories (42–44). A drawback of the DiversiLab system is that the software and database used to analyze the data are located on a server with the manufacturer and that the data cannot easily be transferred to a local database and analyzed using other commercially available image analysis software. Manual rep-PCR is inexpensive, whereas the cost of the semiautomatic system is a little higher than that of PFGE.

PCR Ribotyping

In PCR ribotyping, the region between the genes encoding 16S and 23S rRNA is amplified. The method takes advantage of the fact that the genomes of most organisms contain more than one rRNA gene operon and that the size of the amplified region between the 16S and the 23S rRNA gene sequences often varies both within the same strain and between different strains (45). The method is rapid, simple, inexpensive, and in general universally applicable; the typeability is virtually 100%, the reproducibility is good, but the discriminatory power is moderate. Although PCR ribotyping protocols have been described for numerous organisms, the method has gained general acceptance for the subtyping of mainly one organism, *Clostridium difficile* (46, 47).

Combined RFLP and PCR-Based Methods

Amplified Fragment Length Polymorphism

AFLP is a patented fingerprinting technique that is based on the selective amplification of a subset of DNA fragments generated by restriction enzyme digestion (48). The method involves three steps. First, the genomic DNA is restricted with one or two restriction enzymes, followed by ligation of oligonucleotide adapters to the ends of the restriction fragments. Then a subset of the fragments are amplified by PCR using primers that are complementary to the adapters extending 1 to 3 bases into the restriction fragments, thereby reducing the number of fragments that are amplified by selection of those that are complementary to the extra (selective) bases. Finally, the amplicons are separated by polyacrylamide electrophoresis or, if the primers are fluorescently labeled, in an automated DNA sequencer using capillary electrophoresis (49). Typically, 50 to 100 fragments are amplified, thereby ensuring the high discriminatory power of the method. The method is universally applicable and has been used to study numerous organisms. Its typeability is very high, the discriminatory power is in general of the same magnitude as that of PFGE, and the reproducibility is good if the procedure is standardized (e.g., the same equipment is used in all experiments). However, even if the protocol is standardized, AFLP profiles may not be comparable if different detection platforms or different models of the same brand of DNA sequencer are used (50). Therefore, the intralaboratory reproducibility of the method may be excellent but the interlaboratory reproducibility rarely is, rendering this approach unsuitable for the creation of large-scale surveillance libraries involving multiple laboratories. Finally, the method is fairly complicated, as is the analysis of data generated by it.

Target-Specific Methods

A short description of the principles of some of the target-specific methods is shown in Fig. 3.

PCR-RFLP

In PCR-RFLP, a short DNA fragment containing the whole or part of a gene or an operon is amplified, followed by digestion of the amplicon with a suitable restriction enzyme. The resulting fragments are then separated and visualized in an agarose gel. In this way, information about the content of the amplified fragment may be generated without sequencing it. These methods with a few exceptions do not provide much discrimination beyond the species level but may assist in the grouping of isolates at a higher level and in the identification of virulence determinants. For example, several protocols have been described for subtyping of Shiga toxin type 2 (Stx2) in Shiga toxin-producing *Escherichia coli* (STEC) (51). Toxin subtype information is useful, since there seems to be a correlation between the Stx2 subtype and severity of disease (52). A recently described PCR-RFLP method for further subtyping STEC organisms by targeting the region located upstream from *stx* genes on the *stx* phage appears promising, though the resolution was somewhat less than that of PFGE (53).

Another PCR-RFLP application is amplified ribosomal DNA restriction analysis (ARDRA). In this method, the 16S rRNA gene in prokaryotes and the 18S rRNA gene in eukaryotes is the amplification target. Isolates of closely related but different bacterial and fungal species will usually be differentiated, whereas the variation of the restriction patterns is low below the species level. For this reason, this method is an easy way to identify bacterial species that are difficult to differentiate phenotypically, e.g., *Acinetobacter* genomospecies (54) or mycobacteria (55).

Finally, PCR-RFLP has been used to assist in the rapid identification of *Salmonella* and STEC O157 flagellar antigens by targeting the *fljB* and/or the *fliC* genes (56, 57).

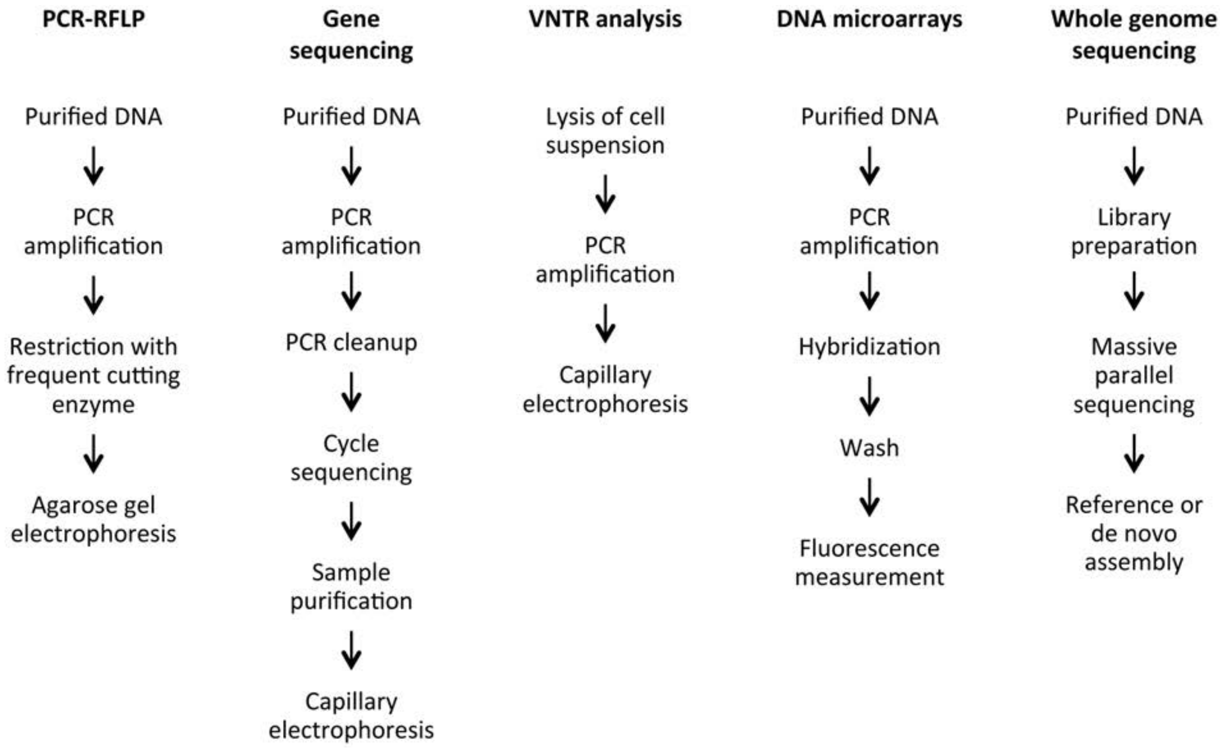
PCR-RFLP
Gene sequencing
VNTR analysis
DNA microarrays
Whole genome sequencing
Purified DNA
PCR amplification
Restriction with frequent cutting enzyme
Agarose gel electrophoresis
Purified DNA
PCR amplification
PCR cleanup
Cycle sequencing
Sample purification
Capillary electrophoresis
Lysis of cell suspension
PCR amplification
Capillary electrophoresis
Purified DNA
PCR amplification
Hybridization
Wash
Fluorescence measurement
Purified DNA
Library preparation
Massive parallel sequencing
Reference or de novo assembly

FIGURE 3 Procedural principles of some commonly used target-specific subtyping methods. doi:10.1128/9781555817381.ch10.f3

Similarly, a PCR-RFLP protocol for subtyping thermotolerant *Campylobacter* spp. has been developed. In this protocol, the flagellar gene *fla*A is targeted (58). The discriminatory power of the method is good albeit less than that of PFGE (59, 60), but due to its simplicity, it is a useful screening tool and a supplement to other methods.

Gene Sequencing

Multilocus Sequence Typing/Multivirulence Locus Sequence Typing

Multilocus sequence typing (MLST) was one of the first DNA sequence-based subtyping methods. It was originally introduced by Maiden et al. (61) for *Neisseria meningitidis* as a sequencing-based counterpart to the phenotypic method multilocus enzyme electrophoresis. MLST detects variation due to mutations or recombination by direct nucleotide sequencing of 350- to 600-bp fragments typically in 5 to 10 housekeeping genes. Innovative technologies, such as electrospray ionization-mass spectrometry (MS), can also be used to determine the base composition instead of direct sequencing (62).When a gene has a base pair change, the new gene sequence is considered a separate allele type. The allele types for each of the gene sequences are combined to define the multilocus sequence type (ST) to characterize the genetic relatedness of the strains. Additionally, MLST results can be analyzed using clustering techniques, such as the eBURST algorithm, which is able to divide isolates based on their STs into clonal complexes (CCs). With eBURST clustering, isolates with a high level of genetic similarity (such as those having six of seven identical alleles) are combined as members of a CC (63). Thus, isolates in different CCs are less closely related to one another than those within the CC.

Since housekeeping genes are under little selective pressure, the accumulation of changes occurring is typically slow. Because the original MLST approach often had a limited discriminatory power in many organisms for it to be an effective epidemiological tool, other similar systems targeting more rapidly evolving genes, such as virulence-associated genes or genes encoding outer membrane proteins, have been developed. This use of virulence factors in an MLST scheme has been referred to as multivirulence locus sequence typing (MVLST). The experience so far has been mixed. Noller et al. (64) used a combination of seven housekeeping genes (*arcA*, *aroE*, *dnaE*, *mdh*, *gnd*, *gapA*, *pgm*) and two membrane protein genes (*espA*, *ompA*) to subtype a collection of STEC O157:H7/H– strains with highly diverse PFGE patterns. Overall, they found a lack of diversity in all housekeeping genes, as well as in *espA*, and only minimal diversity in *ompA*. In contrast, an MLST scheme developed by Zhang et al. (65) for subtyping *Listeria monocytogenes* exclusively targeted virulence genes (*prfA*, *inlB*, *inlC*) and virulence-associated genes (*dal*, *lisR*, *clpP*) and was able to discriminate between sporadic isolates that were indistinguishable by PFGE. An MLST assay targeting only two genes, the *por* gene, encoding the outer membrane protein porin, and the *tbpB* gene, encoding the transferring binding protein subunit B, has been proven to be a highly discriminatory method for subtyping of *Neisseria gonorrhoeae* while still being able to accurately identify sexual contact pairs (66). While MLST can be used for epidemiological surveillance for some organisms with plastic genomes, such as *Neisseria* species, the key application for it in general is studying the phylogeny and evolution of population lineages (67). MLST may also be used to study differences in host preference or virulence potential in human infection between clonal complexes (68).

One key advantage of MLST over gel-based techniques, such as PFGE, is that the sequence data are nonambiguous, regardless of the type of the instrument used to generate it, and can readily be compared between laboratories through the curated MLST websites, such as http://pubmlst.org, http://www.mlst.net, and http://www.pasteur.fr/mlst. MLST also has a 100% typeability and a high reproducibility. However, sequencing both directions of the DNA strands in multiple loci is rather labor-intensive and expensive compared to some other technologies, such as fragment analysis.

Other Gene Sequencing Applications

The Lancefield M protein is an important virulence factor in group A streptococci (GAS) that is also used for serotype classification. The protein is encoded by the *emm* gene, and the M serotype may be predicted from its sequence (69). The complete set of *emm* sequences and the sequencing protocol are available at http://www.cdc.gov/streplab/M-ProteinGene-typing.html. *emm* typing has made M serotyping available to the broader scientific community while retaining the historical and pathogenic information generated in a relatively few reference laboratories in the course of many years of research using the traditional method. Since there is more variation in *emm* sequences than is apparent from the M type, *emm* typing provides additional discrimination of GAS compared to the classical M serotyping method (70).

Similarly, the capsular type is an important virulence factor for pneumococci. This capsule is encoded by the *cps* gene, and the *cps* sequences of all the pneumococcal serotypes are available at http://www.sanger.ac.uk/Projects/S_pneumoniae/CPS/. A recent study described a single PCR sequencing strategy based on a primer pair spanning the regulatory gene *cpsB* that could putatively amplify 84 out of the 92 serotypes studied and differentiate 46 of them (71). A protocol using sequential nested PCRs has been developed to identify the 29 most common pneumococcal serotypes without sequencing the whole gene (72).

Methicillin-resistant *Staphylococcus aureus* (MRSA) contains a large heterogenous mobile genetic element, the staphylococcal chromosomal cassette *mec*, or SCC*mec*, carrying the *mecA* gene, the central element of methicillin resistance, and the *ccr* locus, which encodes recombinases (*ccrAB* or *ccrC*) involved in SCC*mec* mobility. SCC*mec* types are defined by combining the information on the genetic organization of the *mec* complex with the *ccr* type and with subtypes defined by variations in the junkyard (J) regions. So far 11 major types (I to XI) have been identified (73). The up-to-date nomenclature and guidelines for reporting novel SCC elements are available on the SCC*mec* website curated by the International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (http://www.sccmec.org). Determining the SCC*mec* type is epidemiologically particularly relevant, since different SCC*mec* types are associated with hospital and community MRSA infections (74, 75).

Molecular epidemiologic approaches have been used to study the transmission patterns of numerous viruses. The most common approach is to perform sequence analysis on a defined region of the viral genome that contains sufficient genetic diversity to make valid conclusions based on sequence comparison and phylogenetic analyses. The lengths of these sequence "windows" vary with each viral species. Typical windows include hypervariable regions or genes which are often flanked by relatively conserved regions that make convenient PCR targets. For the purpose of molecular epidemiology, viruses are usually assigned to clades, genotypes, or clusters represented by established reference strains. In particular, RNA viruses have high mutation rates, which increase the amount of sequence variation in the genome (76, 77).

Variable-Number Tandem-Repeat Analysis

Many microbial genes and intergenic regions contain loci of repetitive DNA which may be varied among strains with respect to the number of repeat units present or their individual structure. These so-called variable-number tandem-repeat (VNTR) regions have been identified in essentially all pro- and eukaryotic species and have been successfully used for subtyping purposes (78, 79). Slipped-strand mispairing (SSM) is the molecular model that best explains the variability of short sequence repeats (80). Stretches of relatively short arrays of repeat units, when being copied by DNA polymerase, may engage in illegitimate base pairing. This forces the polymerase to introduce or delete individual repeat units. The evolutionary clock speeds of different repeat loci are varied; some loci evolve very slowly and others so fast that they are too unstable to be considered epidemiological markers. Therefore, some loci are better suited for epidemiological tracing than others (81). In the simplest form, VNTR analysis may include only a single locus (82). When multiple loci are targeted, the technology is often referred to as multiple-locus VNTR analysis, or MLVA (83).

VNTR loci can be identified from a partial or complete reference genome sequence by using free-of-charge software available on the Web, such as Tandem Repeats Finder (http://tandem.bu.edu/trf/trf.html) or TRedD (http://tandem.sci.brooklyn.cuny.edu/). Tandem-repeat databases (http://minisatellites.u-psud.fr; http://tandem.bu.edu/cgi-bin/trdb/trdb.exe) have also been developed to assist in identifying VNTRs from published sequences using various parameters and query tools (84). A typical MLVA protocol involves a multiplex PCR amplification of VNTR targets, followed by fragment sizing using high-resolution capillary electrophoresis (85). The use of multiple fluorescent dyes to label the forward primers facilitates multiplexing targets with overlapping fragment sizes. The observed fragment size for each VNTR defines the allele type (either a copy number or an arbitrary number), and the string of allele types constitutes the MLVA type.

The key application of MLVA is in the field of epidemiological surveillance used either alone or in combination with other techniques. Since MLVA targets fast-evolving repeat sequences, the discriminatory power of the method can be quite high for many highly clonal organisms that show low diversity with other methods. For example, a MLVA scheme targeting eight VNTRs was able to differentiate laboratory contaminants and non-outbreak-related strains during the 2001 anthrax outbreak in the United States (86). Other organisms that show relatively high diversity with other commonly used methods, such as PFGE, may have clonal subpopulations that can be differentiated using MLVA. This is the case for strains belonging to the *Salmonella enterica* serotype Typhimurium definitive type 104 (DT 104) complex that, until MLVA was introduced, could not be differentiated efficiently by any subtyping method (87, 88). The downside of targeting fast-evolving genetic elements, such as VNTRs, is that patterns can evolve during an outbreak, complicating data analysis and compromising epidemiological concordance. For example, single- and even double-locus variants have been detected during STEC O157 and *S. enterica* serotype Typhimurium outbreaks (89, 90).

The advantages of MLVA are its high throughput, low labor intensity, and relatively low per-sample cost. As with many other PCR-based techniques, MLVA is vulnerable to PCR and fragment analysis artifacts, such as minus-A, stutter peaks, and fluorescence carryover, which can interfere with the data analysis. Robustness and reproducibility of the method can be affected by instrument- and reagent-related issues, which in turn can have an impact on the portability of the method and interlaboratory data sharing. For example, significant sizing discrepancies are known to occur between different capillary electrophoresis platforms (91). However, all these issues can be overcome by the proper assay design and optimization, strict adherence to standard operating procedures, and staff training. When different instrument platforms are employed, interlaboratory data sharing can be facilitated by a calibration strain set that is used to normalize the allele types to be comparable to the actual sequenced copy number (92). The overall main weakness of MLVA is that the assays are typically specific to the organism or even the serotype for which they were developed. Only a few published assays have attempted to cover multiple serotypes or species (93–95). On the other hand, the specificity may also be an advantage, since virtually all strains are typeable and a fingerprint can also be generated without a pure culture from a primary enrichment sample (unpublished PulseNet USA data).

Clustered Regularly Interspaced Short Palindromic Repeat Analysis

Clustered regularly interspaced short palindromic repeats (CRISPRs) represent the most widely distributed family of DNA repeats in prokaryotes. They were initially discovered in *E. coli* (96) and have recently been identified in approximately 90% of archaeal and 40% of bacterial genomes (97). Most CRISPR-containing prokaryotes possess multiple CRISPR clusters (from 2 to 20 loci), each of which is organized as a tandem array of up to 100 short (21- to 47-bp) and highly conserved direct repeats (DRs) regularly separated by stretches of variable sequences called spacers. Many CRISPR loci are flanked by an AT-rich leader sequence and CRISPR-associated (*cas*) genes. Spacers are derived from foreign nucleic acids, such as phages or plasmids, and can bestow on the host bacteria protection from subsequent infection by homologous phages or plasmids (98, 99). As a bacterial immune system, CRISPRs evolve rapidly in response to changing phage pools. CRISPRs evolve by either deletion or acquisition of units (a DR and a spacer). In the majority of cases, new units are added at one end of the CRISPR adjacent to the leader, whereas motif deletions can occur randomly (100).

CRISPR loci can be identified from partial or complete genome sequences and their structures characterized using the CRISPRfinder program available at http://crispr.u-psud.fr/Server/CRISPRfinder.php (101). The extracted CRISPRs are stored in a public database (CRISPRdb), which is also automatically updated from published genomes. The first CRISPR application developed was spoligotyping of *M. tuberculosis*, which consisted of detection of the presence or absence of a range of spacers after PCR by hybridization on a membrane (102). Nowadays, a typical CRISPR protocol involves PCR amplification of CRISPR loci followed by either Sanger sequencing of the PCR products or detection of the presence or absence of spacers using oligonucleotide probes coupled on a DNA microarray (103, 104). The free-of-charge Web resource CRISPRcompar (http://crispr.u-psud.fr/CRISPRcompar/index.php) can be used to identify the spacers from the sequenced alleles, to annotate them, and to compare them to previously known spacers (97).

CRISPR analysis can be used for epidemiological surveillance either alone or in combination with other targets. CRISPR analysis and MLST of two virulence genes have been successfully used to subtype *Salmonella* strains, with particularly promising results for serotype Enteritidis, which is highly clonal by other methods (105, 106). It remains to be seen how effective CRISPR analysis is as an attribution tool, but given that different phages are known to be unique to specific niches, the spacer composition may give clues about the source of a particular strain (107).

WGS

The introduction of high-throughput, next-generation sequencing (NGS) technologies resulted in a dramatic decrease in the per-base cost of genomic sequencing over the course of the past decade, with a corresponding increase in the volume of data output. The earliest NGS platforms were based on massively parallel pyrosequencing (108, 109), and subsequent generations of the technology have either refined this approach or applied new advances, such as ligation chemistry (110), semiconductor-based detection (111), single-molecule sequencing (112), and engineered protein nanopores (113). There are currently four main commercial platforms for NGS, namely, 454 GS FLX+ (Roche Diagnostics, Branford, CT), Ion Torrent PGM and Proton (Life Technologies, Guilford, CT), MiSeq and HiSeq (Illumina, San Diego, CA), and PacBio RS/RS II (Pacific Biosciences, Menlo Park, CA). With the exception of PacBio RS/RS II, which can perform single-molecule, long-read sequencing of libraries >10 kb, all of these systems generate between several hundred thousand and several billion short-read sequences between 100 and 800 bp long. Despite this similarity, there are nonetheless important differences in read length, sequence output, turnaround time, accuracy, error model, and cost (114–116). Each of these factors must be considered when selecting an appropriate sequencing strategy for specific objectives and applications. To further complicate matters, as NGS technologies continue to mature, the hardware, software, chemistries, and consumables are all subject to intense development and innovation, with improvements to sample throughput and data quality, and continual decreases in per-isolate sequencing costs.

WGS represents the ultimate tool in molecular epidemiology, as it allows the identification of single genomic changes between two isolates. The technology has not yet been widely used for real-time surveillance and outbreak investigations at the community level, though it has proven its power in a few recent investigations in health care settings (117–119). WGS has been widely used to develop new diagnostic tests for known and emerging pathogens (120), and it has become an important tool in pathogen discovery and characterization and in studying pathogen evolution and transmission. A recent example is the analysis of the complete genome of the swine origin H1N1 strain that emerged in 2009, which showed that the virus had been circulating in humans for an extended period as the result of a single introduction. The new strain had genome segments derived from swine, human, and avian strains (121). Similarly, in the 2011 German outbreak of *E. coli*, WGS played a key role in understanding the determinants and modeling the evolutionary events that led to a hypervirulent strain (122, 123).

As the cost and complexity of sequencing continues to decline, the management and analysis of the massive volumes of output data have emerged as a significant challenge to the routine use of NGS for infectious disease surveillance, outbreak detection, and response. In order to be useful for public health intervention, raw whole-genome sequence data from suspected pathogens must be analyzed, assembled, interpreted, and compared in a timely fashion, so that action may be taken to prevent or minimize disease-associated morbidity and mortality. In the case of a small outbreak investigation, these data may represent tens of gigabytes of raw sequence, but for large molecular surveillance efforts, such as the PulseNet network for foodborne disease surveillance (124), the expected raw sequence output could easily surpass a hundred terabytes of raw genomic sequence data each year. The challenge of genomic "big data" will require the development of a dedicated informatics infrastructure to support NGS and other high-throughput laboratory technologies and the incorporation of genomics, bioinformatics, and data science skill sets by the public health workforce. Since most end users of the technology will be microbiologists in clinical and public health laboratories with limited bioinformatics skills, it is critical that user-friendly and intuitive bioinformatics tools be developed for most sequencing-related surveillance activities. Equally important for global health is the development of clear and concise international consensus standards on sequence quality; data compression, storage, and transmission; sequence-associated metadata; and analytical methods for outbreak detection and response. Work to address these issues has already been initiated through the Global Microbial Identifier (GMI) network (http://www.globalmicrobialidentifier.org/), which consists of approximately 200 experts from at least 30 countries, including clinical, food, and public health microbiologists and virologists, bioinformaticians, epidemiologists, representatives from funding agencies, data hosting systems, and policymakers from academia, public health, industry, and governments.

While most viral sequences can be directly compared from WGS data using multiple-sequence alignment, the analysis of most bacterial, fungal, and parasitic genomes is complicated by their larger relative sizes. While some WGS-based subtyping strategies for these organisms consider the entire genomic sequence, most focus on sets of specific markers or polymorphic regions within the genomic sequence. Several of the most common strategies are described below. A glossary of terms commonly used in bioinformatics is provided in Table 3.

Whole-Genome SNP Typing

Whole-genome single nucleotide polymorphism (SNP) typing involves mapping sequence reads from each query (or test) isolate against a reference genome to identify and compare positions of single nucleotide variations. The reference sequence is typically selected from among closely related, finished genomes from public repositories or from a high-quality *de novo* assembly of an epidemiologically important internal reference isolate (e.g., an index case or putative source) in cases where an external reference sequence is either not available or not appropriate for the investigation. An example of the latter case is illustrated in Fig. 4.

The initial pool of candidate SNPs can vary greatly in number between organisms according to their genome sizes, their complexity and plasticity, their population diversity, and the quality and coverage of mapped sequence reads. Candidate SNPs identified from each mapping are typically

TABLE 3 Definitions commonly used in bioinformatics

Term	Definition
Annotation	The identification and functional determination of genes and genetic elements within a sequence.
Genome assembly	Process by which many short DNA sequence fragments, such as those generated by next-generation sequencers, are reassembled into a representation of the original genomic sequence.
Contig	Contiguous consensus sequences derived from the assembly of many short, overlapping DNA fragments.
Coverage (read depth)	Avg number of reads representing a given nucleotide in the reconstructed sequence.
De novo assembly	Genomic assembly without an *a priori* reference sequence to inform the genomic structure.
High-confidence SNP	Single nucleotide polymorphism that has been verified using specific criteria, such as sequence coverage, sequence quality, and population and allelic frequency. While these parameters will vary depending on the context and application of the SNP calling, the typical minimum criteria are 10 to 20× coverage, >Q20 (see below for definition), and 100% frequency.
Indel	*In*sertion or *del*etion of nucleotides, which results in a net change of the total number of nucleotides.
k-mer	Oligonucleotide of length "k."
N50 statistic	Commonly used as a rough assessment of genomic assemblies. It represents the contig length for which all contigs of that length or greater include at least half of the total base pair length of the sequence set.
Open reading frame	Uninterrupted reading frame in a given sequence that may be used for gene prediction.
Per-base sequence quality	The sequence quality score for each individual base position in a sequence. Typically, Phred scores are used, where $Q = -10\log(\text{error probability})$. A Q30, for example, means a 1-in-1,000 likelihood of an incorrect base call at that position.
Read	A unit of continuous DNA sequence derived from target DNA.
Reference-mapped assembly	Assembly of a genome by aligning new sequence reads against a preexisting and similar reference sequence.
Suffix array	An array of all substring suffixes of a longer string. For example, the sequence ATGCATGC, represented as an array (A[i]), includes suffixes of ATGCATGC, ATGCATG, ATGCAT, ATGCA, ATGC, ATC, AT, and A.

filtered according to sequence quality; the position, genomic distribution, and functional impact of variant alleles; and population-level considerations, such as allelic frequency, genetic convergence, homoplasy, and linkage disequilibrium. With sufficient bioinformatics expertise and computational resources, the distillation from reference mappings to a minimum set of high-confidence, parsimoniously informative loci can be completed relatively quickly. Once validated, sets of high-confidence canonical SNPs can be used as targets for the analysis and subtyping of subsequent isolates or as the basis for high-throughput surveillance assays using real-time PCR or other lower-cost molecular technologies (125, 126).

There are several circumstances in which SNP-based strain typing may perform suboptimally, primarily due to its dependence on consistent reference mapping. For some organisms, there are no circularized reference genomes necessitating the use of *de novo* assemblies as references. Even when a closed reference genome exists, genomic plasticity in the population may be sufficiently high in species such as *Neisseria gonorrhoeae* or *Burkholderia* that mapping to historical reference strains may be inefficient or result in

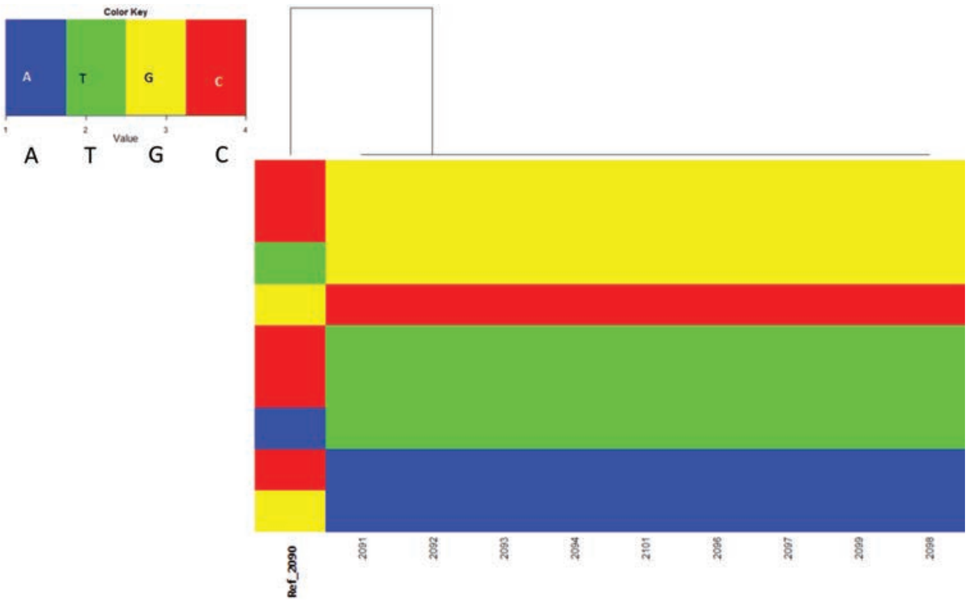

FIGURE 4 Heat map showing the SNPs among 10 STEC O157 isolates belonging to the same outbreak. In order to elucidate the genetic relationships among the outbreak isolates, high-quality SNPs were called against the outbreak reference strain (index case) that appears at the far left. A total of nine SNPs were detected among the outbreak isolates. doi:10.1128/9781555817381.ch10.f4

important gaps in genomic coverage. Similarly, in organisms where critical virulence and diversity factors are carried by plasmids, transposons, or other mobile genetic elements, information about important discriminatory loci may be absent from the available reference sequences. One strategy is to construct composite reference sequences from the microbial chromosome, with major plasmids or transposons of interest appended as a pseudoassembly. While this approach allows for the consideration of these extrachromosomal sequences, the limited stability of many mobile genetic elements and important differences in selective pressure between chromosomal and extrachromosomal alleles may present important problems. Some organisms also contain mutational hot spots (clustered mutations) from which SNP calls need to be assessed carefully, with the natural error rate of *Taq* polymerase kept in mind. A final consideration relates primarily to fungi and parasites, where the ploidy (number of sets of chromosomes) of the organism and the presence of complex or heterogenous alleles may impact the number and quality of high-confidence, discriminatory SNPs that are useful for subtyping and analysis.

WGS, MLST, and Binary Typing

As the sequencing, assembly, and annotation of large numbers of microbial genomes become increasingly cost-effective and feasible, it is possible to define and query large-scale MLST or binary typing schemes, which include dozens or even hundreds of different genes or sequences of interest. This approach can be applied to both microbial subtyping and characterization, as important differences in gene complement may be associated with the mobilization of plasmids, transposons, bacteriophages, or horizontal gene transfer from other organisms and may signal phenotypic differences in levels of virulence or antimicrobial susceptibility. Publicly available database schemas and software, such as the Bacterial Isolate Genome Sequence Database (BIGSdb) (http://pubmlst.org/software/database/bigsdb/), allow bacterial genomes to be catalogued and systematically compared across many different MLST targets (127). Other measures, such as BLAST (Basic Local Alignment Search Tool) score ratios (BSR), provide a more general assessment of the functional similarity of two genomes, based on the pairwise comparison of all open reading frames (ORFs) (128).

Although gene-based comparisons are emerging as an important strategy for strain typing and characterizing bacteria and other pathogens using WGS data, current limitations with short-read next-generation sequencing and bioinformatic analysis may impact their usefulness, particularly for large-scale comparisons. The reliance of these techniques on high-quality genomic assemblies and the consistent prediction or annotation of open reading frames present challenges in terms of the technical complexity and throughput of the analysis. Draft bacterial genomes may include hundreds of contigs, and the interpretation of complex MLST schemes will invariably be affected by breaks, duplications, or misassemblies of important sequences or reading frames. Differences in assembly or gene prediction algorithms may also impact the throughput of the assay and interpretation of results, and the development of standardized bioinformatic methods and workflows is critical, particularly as sequence databases scale to hundreds or thousands of genomes with many different alleles.

Binary typing data for the presence or absence of genes or genetic variants may also be used to provide useful information for microbial characterization, particularly when used in conjunction with other strain typing methods. Interrogating sets of known resistance and virulence markers can provide important information during an outbreak investigation or response, and these features can often be used to support strain type identification or to correlate with transmission or patient outcomes.

k-mer Analysis

Recently, a number of different k-mer-based approaches have emerged to analyze and compare large genomic data sets in a computationally efficient manner. In general, these algorithms deconstruct genomic sequences or unassembled raw reads into large sets of k-mers, or oligonucleotides of length “k,” for comparison by means of suffix arrays. The k-mers are then sorted, deduplicated, and compared to other k-mer sequence sets to identify variations (SNPs and indels) that can be used for clustering and comparison (129).

Unlike traditional SNP-mapping approaches, which take into consideration the genomic context of the variant position and the underlying sequence quality of the reads that support its identification, k-mer-based SNP analysis focuses on the putative SNP position and the 10 to 12 bases of surrounding sequence, without consideration of quality scores. These are important limitations, which may restrict the ability of k-mer-based methods to reliably detect closely spaced SNPs or indels and result in limited resilience against low sequence quality or sequencing errors. Even so, k-mer methods have significant advantages in terms of speed and implementation, since they do not require assembly or alignment of sequences and can be equally applied to whole-genome sequence sets and unassembled sets of short reads.

k-mer-based algorithms continue to improve, and recent implementations for strain typing can assess horizontal gene transfer, annotate SNP positions based on a known reference, and accommodate missing alleles and evolutionary convergence, and they are easily adapted to new applications, such as the metagenomic analysis of complex samples (129). During outbreak investigations in particular, k-mer-based SNP analysis is an excellent tool for initial genomic comparison and clustering, since it provides actionable strain typing information quickly and can be used to guide the application of more-resource-intensive downstream bioinformatics analyses, which may take several days to complete.

DNA Microarrays

A DNA microarray is a molecular platform in which a few hundred to thousands of specific DNA oligonucleotides or short sequences (capture molecules) are bound to a matrix. The array is used to detect specific DNA sequences (target sequences) in a test sample of fluorescently labeled DNA, with or without amplification, by hybridization followed by detection of the array-bound test DNA. Two types of arrays, planar and liquid, are used.

Analysis with planar microarrays is typically performed by deposition of DNA probes complementary to the genome targets of interest on a glass slide (solid matrix). Probes are typically synthetically produced oligonucleotides (9 to 100 bp) or PCR products (100 to 1,000 bp). They can target ORFs amplified from the sequenced reference isolate or short intergenic oligonucleotides based on the available sequence (130). Alternatively, PCR probes may be attached to microspheres that are internally dyed with two spectrally distinct fluorophores and combined to create a suspension array (liquid matrix), such as those seen with the Luminex platform (131). Overall, platforms developed may differ in a number of aspects, such as probe content, density, deposition technology, labeling, and hybridization of probes, as well as sample detection and analysis. An ORF-based DNA array can detect losses of entire ORFs but does not detect minor deletions, point mutations, deletions restricted to intergenic spaces, genetic rearrangements, deletions of homologous repetitive elements, or gene insertions. Short oligonucleotide arrays are more precise at detecting shorter nucleotide polymorphisms but require a larger number of probes at high cost. Such oligonucleotide arrays have been developed by companies such as Affymetrix (Santa Clara, CA), Operon (Huntsville, AL), Agilent Technologies (Santa Clara, CA), and NimbleGen (Madison, WI). Insertions of genes compared with the sequenced reference strain cannot be detected in comparative genome hybridization DNA array analysis. This problem can be alleviated by adding nonredundant amplified sequences from several closely related organisms to the array (132).

DNA microarrays represent an attractive platform for use in microbial identification and subtyping, since they allow for rapid and accurate analysis of large numbers of different DNA molecules. Besides its remarkable resolution, DNA array analysis unveils the genetic region responsible for diversity, often allowing phenotypic predictions at the same time. In addition, due to the basic design of microarrays (i.e., detection of signal associated with sample DNA bound to fixed DNA probes), the positional variation associated with gel-based methods, such as PFGE, is eliminated and the analysis of results is easier to automate and standardize. Limitations of DNA array technology include the initial high cost for the synthesis and spotting of target-specific probes and for the fluorophores used to label the reactions. Ambiguities in the interpretation of the ratios of hybridization and cross-hybridization to analogous targets are also important limitations of the technique (133). The interlaboratory reproducibility of DNA array-based methods remains unclear, since multilaboratory validation studies are largely missing (134). Even though planar arrays allow for the analysis of hundreds of thousands of targets at the same time, sample throughput is an obvious bottleneck of the technology. If the total number of probes can be limited to a few hundred, suspension arrays offer a higher sample throughput platform with lower cost. Currently, the Luminex xMAP system (Luminex Corp., Austin, TX) allows for up to 100 different probes to be analyzed simultaneously in a single well of a 96-well plate.

The main application for DNA arrays is pathogen identification. One approach is to amplify more-universal genes (e.g., 16S rRNA, 18S rRNA, 23S rRNA) and to screen for pathogen-specific polymorphisms (135, 136). However, universal PCR can be challenging, since commercial PCR reagents are frequently contaminated with trace amounts of bacterial DNA. Additionally, since the detection is based on pathogen-specific point mutations in these genes, naturally occurring variants may not all be represented on the array, resulting in false-negative detection events. An alternative strategy for coupling PCR and DNA array detection is to use multiplex PCR to amplify a number of discrete, pathogen-specific genetic markers. The ability to construct high-density microarrays with multiple probe sequences makes it relatively trivial to include multiple markers for specific pathogens and thus permit greater validity of a positive detection event (137). The disadvantage of this approach is that there is a practical limit to the number of primer sets that can be included in the PCR. Instead of coupling PCR amplification and DNA array detection, applying direct detection to extracted DNA or RNA is achievable when the amount of target is abundant (138). The primary goal in this case is to avoid amplification biases associated with PCR.

Several microarrays have been developed for rapid identification of viruses, including the ViroChip and the GreeneChip (139–141). These arrays have the capacity to detect a large number of viral pathogens and can quickly identify the etiologic agent in unknown source outbreaks (142, 143). In some cases, the arrays are designed to detect specific groups of viruses, such as respiratory pathogens (144–146). The most comprehensive pathogen detection arrays reported to date were designed by a team at the Lawrence Livermore National Laboratory. The Lawrence Livermore microbial detection array (LLMDA) can test over 6,000 viruses and 15,000 bacteria as well as fungi and protozoa (147, 148). The array contains both "discovery" probes, which match conserved genome regions that are unique to a taxonomic family or a subfamily but shared by species within that family, facilitating detection of novel species within a family, and "census" probes, which target highly variable regions unique to an individual species or a strain and can hence be used for forensic purposes.

Microarrays have also been used to genotype viral and bacterial agents based on SNPs occurring on specific sequence regions. In many cases, though, whole-genome sequence analysis may be the more cost-effective means of obtaining a viral or bacterial genotype. Nevertheless, there are numerous examples of the use of microarrays for viral genotyping, including human rotavirus (149), human and avian influenza viruses (150, 151), hepatitis B virus (152), HIV (153), varicella-zoster virus (154), measles virus (155), human papillomavirus (156, 157), polioviruses (158), and noroviruses (142, 143). Similarly, microarrays have been utilized for bacterial subtyping by molecular serotyping (159), SNP analysis (160, 161), and binary typing (162) and for detection of the presence or absence of antimicrobial resistance genes (163, 164) or specific mutations conferring antimicrobial resistance (165, 166).

Microarrays, besides being used for pathogen identification and genotyping, have been used for resequencing the complete genomes of viral agents (167, 168) and partial bacterial genomes (169, 170); however, the development of next-generation sequencing methods has made array-based sequencing impractical.

Mass Spectrometry

In recent years, matrix-associated laser desorption ionization–time of flight (MALDI-TOF) MS systems have become increasingly common in clinical and public health microbiology laboratories throughout the world. In contrast to earlier generations of mass spectrometry equipment, which were complex, costly, and difficult to maintain, these newer benchtop MALDI-TOF systems offer simple and standardized workflows for many genus- and species-level microbial identification tasks, with high throughput, accurate and fast results, and a low overall per-test cost (171). While a few proof-of-concept studies have demonstrated the feasibility of MALDI-TOF for strain- and subspecies-level identification of select organisms (172–175), standardized or generalizable methods have yet to be developed, and in most cases, the current level of resolution of MALTI-TOF, when used alone, remains at the genus or species level. Attempts have been made by some commercial companies to combine mass spectrometry with other technologies and typing approaches. The PLEX-ID (Abbott Laboratories, Abbott Park, IL) system uses PCR and reverse transcription-PCR (RT-PCR) electrospray ionization mass spectrometry to identify and/or type viruses, bacteria, and fungi. For most bacterial species, the level of resolution is generally equivalent to that of MLST, with the inclusion of other important multiplexed targets for further confirmation or characterization (176). Another system, the Agilent MassCode PCR system (Agilent Technologies), uses highly multiplexed PCR panels and identifies/subtypes microorganisms through the detection and measurement of cleavable mass tags using atmospheric-pressure, chemical-ionization, quadrupole mass spectrometry (177). Other platforms, such as the Sequenom MassArray (Sequenom Inc., San Diego, CA), use PCR MALDI-TOF to interrogate thousands of SNPs through a single primer base extension and can be used for high-resolution SNP-based strain typing using standardized sets of canonical targets (178). However, these applications remain under active development and in most cases to do not currently provide the discriminatory power needed for epidemiologically relevant high-resolution strain typing.

APPLICATIONS

Molecular Surveillance

Molecular Serotyping

Serotyping is a subtyping method in which specific antigens on the surfaces of microorganisms are detected by antigen-antibody reactions using diagnostic antisera. The method is definitive but only moderately discriminatory. It has nevertheless proven to be extremely useful for epidemiologic classification of a number of pathogens and has for this reason been a mainstay in the surveillance of different infectious diseases for more than 70 years. It is a cumbersome method that requires the availability of high-quality antisera and substantial expertise to perform the assay and interpret the results. More convenient, rapid, and reliable molecular alternatives to serotyping are being developed. These molecular serotyping methods are designed to generate data that are in close or complete agreement with conventional serotyping in order not to lose the connection to the historical data. In the previous sections, molecular flagellin typing of *Campylobacter* (*flaA* typing), M serotyping (*emm* typing) of GAS, and capsular (*cps*) typing of pneumococci have been mentioned.

In the genus *Salmonella*, more than 2,500 serotypes are currently recognized (179). Two types of molecular serotyping approaches have been used; in one, a molecular subtyping method is correlated with the serotype, and in the second, the genes acrually encoding the serotype are targeted directly. In this latter case, for O antigens the genes are within the *rfb* gene cluster, and for the flagellar antigens the *fliC* and *fljB* genes encode phases 1 and 2, respectively. Some examples of subtyping methods that correlate with serotyping are MLST, PFGE, ribotyping, and rep-PCR (180); the correlation between subtyping methods is never perfect, and ideally, serotypes determined this way should be confirmed by traditional serotyping or a molecular serotyping method targeting the serotype-encoding genes. Regarding the latter, the U.S. Centers for Disease Control and Prevention (CDC) has developed an assay that reliably identifies the vast majority of the serotypes involved in human infections using a combination of PCR amplification of the *rfb* genes, *fliC*, and *fljB*, with detection of the amplicons using a liquid microarray (159, 181). This assay was recently commercialized by Luminex. With the advances in sequencing technology, it is also becoming increasingly cost-efficient to determine the serotype directly from the sequences of the serotype-encoding genes (182). The methods targeting *rfb*, *fliC*, and *fljB* are directly compatible with the Kauffman-White scheme, which contains the phenotypic descriptions of all *Salmonella* serotypes (179), and the results will not need to be confirmed by traditional serotyping.

Cluster Detection/Outbreak Investigations

Subtyping methods that are used for community-wide routine surveillance to detect disease case clusters and to support outbreak investigations need to be rapid, highly dis-

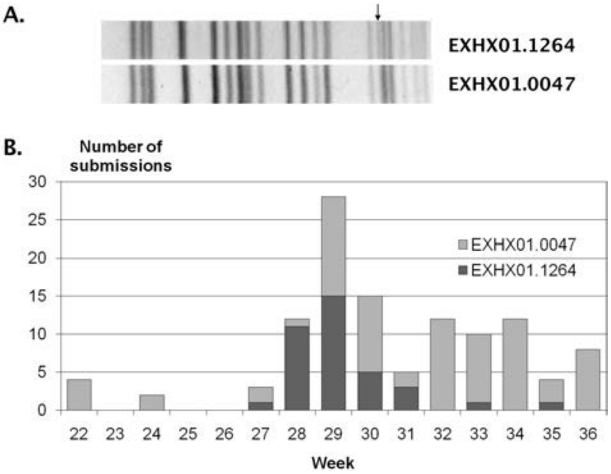

FIGURE 5 PFGE profiles of two STEC O157 strains, namely, the most common sporadic pattern, EXHX01.0047, and the outbreak-associated pattern, EXHX01.1264, and their distribution during the 2002 outbreak period.
doi:10.1128/9781555817381.ch10.f5

criminatory, reproducible, and amenable to library subtyping so that trends in the occurrence of the organisms that they target may be followed easily. Often subtyping results are the only information available to determine whether an outbreak investigation should be initiated. In this situation, the case definition needs to be very specific; i.e., the discriminatory power of the subtyping method needs to be high in order not to include patients that are not associated with the outbreak as cases, which would confuse the epidemiological investigation. The molecular methods that are most suitable for this purpose are PFGE, MLVA, AFLP (when used in one laboratory), and highly discriminatory DNA array and gene-sequencing methods. These methods may be used in conjunction with less discriminatory traditional and molecular methods, e.g., biotyping, serotyping, phage typing, antibiograms, and/or plasmid profiling.

PulseNet, the national molecular subtyping network for foodborne disease surveillance, is a prime example of a network performing real-time surveillance of infectious diseases at the community level (124). In a subtyping network, the choice of method is less important than that all participants use the same method, as long as the preferred method has sufficient performance characteristics. In PulseNet, the preferred subtyping method is PFGE, and only isolates with indistinguishable patterns are considered when clusters are being detected. An outbreak of STEC O157:H7 that occurred in the summer of 2002 was caused by a strain with a pattern that differed from the most common pattern in the PulseNet database by just one band (Fig. 5A). Forty-four case patients with the outbreak pattern were identified, but during the outbreak period, 41 isolates with the highly similar most common pattern were also detected (Fig. 5B); in case-control or other interview studies, the inclusion of these 41 patients infected with isolates displaying the common pattern as cases would have completely obscured the association with the vehicle, ground beef from one particular plant (183). As more supporting epidemiological information is gathered, it may be possible to amend the microbiological case definition. In the above-mentioned outbreak, two cases with epidemiological links to the outbreak were added to the list of case patients, even though they were infected with a strain displaying the most common variant pattern.

The requirements for methods used for surveillance and investigation of outbreaks in hospitals and other institutions may be different from the ones used in the community. The setting is much smaller in scale and usually attended by a single laboratory, and the outbreaks are often caused by pathogens transmitted from person to person and/or with pathogens that persist in the environment. Strains that are involved in outbreaks transmitted between patients or persistently from the environment often evolve during the outbreak, and variant subtypes may be present at the same time. This was the basis for the much-cited “Tenover criteria” for interpretation of PFGE results (184); they state that strains may be related if they differ from each other by up to six restriction bands, corresponding to two genetic events. Obviously, these criteria do not apply to point source outbreaks. The methods that are used for community-wide surveillance may also be used for these nosocomial outbreaks, and methods that do not show good interlaboratory reproducibility may be used as long as the intralaboratory reproducibility is sufficient. AFLP has excellent intralaboratory reproducibility, and even rep-PCR may be used for short-term surveillance and to delineate outbreaks that have been detected by other means, e.g., by phenotypic methods (185–187).

The combination of molecular epidemiologic techniques and standard case classification and reporting provides a very sensitive means of describing the transmission pathways of many viruses. In particular, analysis of the sequence data can help to confirm the source of a virus or suggest a source for cases in which the source was unknown. Molecular data can be used to establish epidemiologic links, or lack thereof, between various cases and outbreaks. Molecular techniques have been particularly useful for the study of outbreaks of foodborne viruses, such as norovirus (188). In these cases, sequence data were used to identify the source and to trace the transport and distribution of contaminated food (189, 190).

For highly infectious viruses, such as measles virus, transmission can occur anywhere. Molecular techniques have been used to identify the source of the infections when standard case investigations have failed to identify a source (191).

Source Attribution

Source attribution is the epidemiological science that studies the relative contributions of different sources to the burden of infectious diseases. Strictly speaking, outbreak investigations are source attribution analyses, but the term is more commonly used in a broader sense encompassing both outbreak-related and sporadic disease. Molecular methods may be used to study the geographic distribution or sources and spread of microbial strains, and this is discussed in a section below.

Source attribution using microbiological methods is gaining increasing attention, especially in the study of foodborne disease, to estimate where in the food production chain the largest public health problems originate. The decision makers use these estimates to prioritize where the control efforts can be spent most efficiently. A subtyping method that is used for source attribution creates subtypes that are distributed unevenly between the different possible sources of human infection. When this knowledge is combined with information about the distribution of the same subtypes in sick humans and, if available, the human rates of exposure to each source, it is possible to estimate the relative contribution of each source to the burden of human disease (192). This approach was introduced in the United Kingdom to attribute the sources of *Campylobacter jejuni* and *Campylobacter coli* to predominantly chicken meat, with ruminants being another important source (193), and has since been used in many countries to attribute the sources of campylobacteriosis and to guide and to demonstrate the effect of intervention measures (194, 195).

The limitation of the model is that it will attribute only to sources for which there are data available; i.e., if no isolates are included from a potential major source, no estimates for this source will be generated. Subtyping methods used for source attribution do not need to be as discriminatory or reproducible as those used for outbreak investigations, as long as they clearly differentiate isolates from different sources. Choosing a subtyping method with an unnecessarily high discriminatory power will increase the complexity of the analysis of the data set. In regard to *Campylobacter*, PFGE is the gold standard for outbreak investigations, whereas the less discriminatory method MLST works well for attribution. Theoretically, subtyping methods that provide phylogenetic information are more useful for source attribution than methods that do not provide this information, since the data collected from each source are likely not to be all-inclusive. Even if some subtyping information is missing, the data may still be useful, since strains that are related phylogenetically to each other are more likely to originate from the same source.

Dynamics of Infectious Disease

Pathogen Evolution

Molecular techniques have made enormous contributions to our understanding of the evolution of pathogens, but a detailed description of these studies is beyond the scope of this chapter. Molecular techniques have been used to monitor the distribution of circulating strains of various pathogens, to monitor the stability of antigenic sites that are important for diagnostics and as vaccine targets, and to assess susceptibility to antimicrobial drugs.

Improvements in computational molecular biology resources and new analytical methods such as BEAST (Bayesian Evolutionary Analysis and Sampling Trees) (196) incorporate sample date and allow the evolutionary dynamics of a population to be inferred from sequence data. These approaches are called phylodynamics (197). With these approaches, sequence information from infectious agents can be used to infer the transmission patterns of that agent over time. Analysis of hepatitis C virus sequences from Egypt suggested an exponential increase in the number of cases in 1930 and 1955, when unsterile needles were used to distribute an antischistosomal therapy (198). Similar studies documented the episodic transmission of HIV into London during the late 1990s (199). More recently, these methods were used to investigate the origins of the swine origin influenza A H1N1 (2009) viruses that were associated with the influenza pandemic in 2009 and 2010 (200).

Geographic Spread

The ability of a molecular technique to identify the source of an infectious agent can be applied on a global scale, and the information can be used to monitor the spread of the pathogen. The sensitivity of this approach depends on the level of surveillance activity and the availability of a global database of genetic information. Sequence analysis was used to identify the source of the West Nile virus that was introduced into the United States in 1999 and to track the spread of this lineage of virus across the entire country (201, 202), as well as to track the global spread of various genetic variants of HIV (203).

Sustained Transmission in Areas That Are Destined for Elimination

Molecular surveillance for viral diseases that are prevented by vaccination is especially beneficial when it is possible to observe the change in viral genotypes over time in a particular country or region. This information, when analyzed in conjunction with standard epidemiologic data, has helped to document the interruption of transmission of endemic viruses and provides a means to measure the success of vaccination programs.

Molecular characterization of measles viruses has provided a valuable tool for measuring the effectiveness of measles control programs. In general, three patterns of measles genotype distribution have been described. In countries that still have endemic transmission of measles, the majority of cases are caused by several endemic genotypes that are distributed geographically. In these cases, multiple cocirculating lineages within the endemic genotype or genotypes are present. In countries that have eliminated measles, the small numbers of cases are caused by a number of different genotypes that reflect various sources of imported virus and suggest the lack of sustained transmission of an endemic genotype or genotypes. The third pattern occurs in countries or regions that have had very good measles control but are experiencing an increase in the numbers of susceptible individuals because of failure to maintain high rates of vaccination coverage. In this situation, reintroduction of measles usually results in a large outbreak associated with a single genotype of virus with nearly identical sequences (191, 204–206).

Pathogen Discovery/Identification

Molecular methods are sometimes crucial for the discovery of new pathogens, especially viral pathogens, and to determine if a pathogen is truly new or has arisen from known pathogens through recombination or other genetic events. For example, coronaviruses have the ability to recombine with other coronaviruses as well as to incorporate cellular genes into their genomes (207). The initial analysis of the complete genome sequence of the coronavirus associated with severe acute respiratory syndrome (SARS-CoV) confirmed that the virus was a novel coronavirus and not a recombinant between previously described coronaviruses (208, 209). In 2012, rapid WGS of a novel coronavirus causing acute respiratory syndrome in humans (HCoV EMC or MERS-CoV) showed that the new virus was a new variant of *Betacoronavirus* that was closely related to a bat coronavirus and more distantly related to SARS-CoV (210–212).

Vaccination Issues

Live attenuated vaccines are used to control a number of viral diseases. In some cases, these vaccines can cause symptoms that are similar to those caused by infection with the wild-type virus. When the risk of exposure to disease is very low, these vaccine reactions can be identified by temporal association with vaccination. However, when vaccination is used as part of an outbreak response, it may be difficult to distinguish vaccine reactions from symptoms caused by the wild-type virus. Since serologic techniques usually lack the sensitivity to distinguish between antibodies directed at the vaccine or the wild-type strain, genetic characterization of the viruses is usually the only means available to clearly classify the case as vaccine associated or due to a wild-type infection. Of course, this approach requires that appropriate specimens for virologic detection be collected from the suspected cases and that sequence information be available for the vaccine strains.

The availability of molecular methods that can clearly identify vaccine reactions, as described above, has contributed to our knowledge about the safety of live attenuated vaccines. In addition, molecular techniques now play an important role in the postlicensure evaluation of live attenuated viral vaccines. Here, genetic characterization is used to monitor the stability of vaccines in the field and to clearly identify vaccine reactions. In one example, PCR and RFLP analysis were used to monitor the safety of varicella-zoster vaccine by classifying vesicular rash or zoster cases as being associated with wild-type varicella-zoster virus of the Oka vaccine strain (213). Additionally, some viruses have the capacity to recombine or reassort their genomes, and in these cases, WGS is an important component of molecular epidemiologic studies. The live attenuated strains used in the WHO polio eradication program replicate in the human gut and can be excreted for several weeks after immunization. If the attenuating mutations in the vaccine strains revert, the vaccine may cause vaccine-associated paralytic poliomyelitis in vaccinees or result in transmissible, neurovirulent, circulating vaccine-derived poliovirus strains which have been associated with outbreaks of poliomyelitis (214, 215). The vaccine-derived polioviruses often contain mosaic genomes that result from recombination between the vaccine strains and other lineages of poliovirus or other enteroviruses (215, 216), and these variants can be detected only by full-genome sequencing.

In some cases, data from molecular surveillance of viral and bacterial pathogens are used to decide on the most appropriate formulation for vaccines. The geographic distribution of strains may affect the efficacy of the vaccines if the genetic changes are accompanied by changes in antigenicity. Therefore, for agents with variable antigenic properties (e.g., rotavirus and influenza virus), careful monitoring of the strains associated with cases in vaccinated populations is necessary (217–219).

Forensic Microbiology

Sometimes infectious disease incidents are criminally investigated if there is a suspicion that they may be the result of deliberate actions or criminal gross negligence. HIV-infected individuals have been imprisoned for spreading the virus intentionally (220) or through negligence (221), and anthrax has been spread through the mail (86, 222). Numerous outbreaks of foodborne infections occur each year, and some have been the result of intentional contamination of the food supply (223). Forensic molecular epidemiology comes into play when regulators and laymen, judges, and lawyers have to decide if an infection (or infections) is a result of criminal action. In principle, criminal microbiological investigations are not different from outbreak investigations, except that in criminal investigations, the link between the infection(s) and its (their) source(s) needs to be proven beyond reasonable doubt. In outbreak investigations, the precautionary principle is relatively often used when deciding on public health actions, since the aim of an outbreak investigation is to stop the outbreak from spreading as soon as possible. This is in contrast to criminal investigations, where the precautionary principle is not used since an offender is presumed innocent until proven otherwise. Subtyping methods that may quantify the probability that two microbial strains are related are therefore preferred over methods that do not yield direct quantifiable information. Non-target-specific methods are therefore not ideal for criminal investigations because DNA fragments of the same size may have completely different sequence contents; e.g., it is not possible from the PFGE pattern to tell if two isolates are identical, hence the use of the term “indistinguishable” for PFGE patterns that cannot be differentiated from each other. In contrast, differences observed with a target-specific method may be quantifiable if the prevalence of the detected alleles is known in the relevant microbial populations.

SUBTYPING METHOD SELECTION, VALIDATION, AND DATA INTERPRETATION

Method Selection

All too often, it appears that the selection of subtyping methods is guided only by convenience criteria, in particular, the accessibility of a method to the laboratory. This has led to numerous studies in which an inappropriate method was used to study an organism (1). When selecting a subtyping method, one should first of all consider the epidemiological context in which it is going to be used; i.e., what is the question that the typing data should answer? A method that is appropriate for long-term surveillance is most likely not appropriate for a short-term investigation of a nosocomial outbreak. Careful consideration should also be given for the genetic makeup of the target organisms, including the clonality, mode of transmission, and outbreak potential.

Factors That Impact the “Clonality” of a Given Population

The clonal relatedness of isolates is manifested by their display of a significantly higher level of similarity in their genotype and/or phenotype than can be expected from randomly sampled and epidemiologically unrelated isolates of the same species (1). The simplest explanation for a genetically monomorphic pathogen is that the population size of the ancestors of all extant organisms was so strongly reduced during a recent bottleneck that genetic diversity was abolished. One possibility that can result in such a bottleneck is a crucial genetic event that happened only once, such as a change in ecological niche due to the acquisition of two plasmids by the progenitor of *Yersinia pestis* (224). In the case of STEC O157, it has been suggested that clonality can be explained by source-sink evolution dynamics (225–227). According to this theory, mutations in microbes that reside mainly in reservoirs (the source, e.g., cattle) in which they are not pathogens confer on a small subset a phenotype that results in injury to an accidental host (the sink, e.g., humans). Alternatively, it has been argued that a strong selective advantage conferred by a mutation enabled certain strains of STEC O157 to flourish in cattle, making them more available for spillover into humans (227). On the other hand, some host-restricted pathogens, such as *S. enterica* serotype Typhi, are under relatively little selection pressure from their host or environment and therefore do not diversify through point mutations, recombination, or acquisition of new sequences (228). Finally, a sampling bias may also contribute to the appearance of population clonality. Sampling from one part of the phylogenetic tree will overlook much of the variation present in the population and collapse all isolates outside the studied population into a single type (229). In order to be able to adequately discriminate clonal organisms, subtyping methods targeting fast-evolving genetic elements, such as VNTRs or SNPs, are usually preferred (230, 231). With some organisms, optimal results may be obtained by using multiple techniques, e.g., PFGE and VNTR analysis (232).

Method Validation

Once a proper method or approach has been selected, it must be subjected to the highest scrutiny possible to ensure that it meets the criteria discussed at the beginning of this chapter. This includes validation using a population of strains and isolates that originate from the epidemiological context to which the method is going to be applied. Techniques that will be applied in local investigations need to be validated on a strain collection from the same locality, whereas methods that will be used for community-wide surveillance need to be validated on a collection of strains reflecting the diversity in the whole community. When a subtyping method that has been validated for use in one given epidemiological context is to be used in a different one, it may be necessary to validate it in the new context before it is implemented. This supplementing validation may not need to be as thorough as the original validation, depending on how similar the two contexts are.

Unlike methods developed in isolation (for use by one laboratory), methods to be used in multiple laboratories for the generation of data archived in reference libraries must be particularly carefully tested, evaluated, and validated. All methods need to go through four phases of validation: initial development, internal validation, external validation, and, finally, postimplementation evaluation. The first two phases often overlap. Additionally, before the new method is implemented, a quality assurance and quality control (QA/QC) program needs to be established.

The goal of the initial development is to identify the optimal conditions or parameters to ensure that the protocol is robust and reproducible and generates highly discriminatory and epidemiologically concordant data on all strains. Ten to 50 isolates that represent the diversity in the study population at large is typically used in this phase.

During the internal validation, the method is tested by individuals not involved in the method development in order to ascertain the robustness of the protocol in the hands of laboratorians with no prior experience with it. The panel of test isolates is expanded to include the full genetic diversity of the study population and should contain both sporadic and outbreak-related isolates in order to test the true discriminatory power and the epidemiological concordance of the method. Duplicate isolates of the same strain and multiple isolates from single-source outbreaks need to be included to evaluate the reproducibility and stability of the method. The test panel will usually contain 250 to 500 isolates. The isolates need to be selected from a collection of strains with a known subtype if a gold standard subtyping method exists for the organism in order to be able to evaluate the performance of the new method against this gold standard.

During the external validation, the robustness and portability of the method is further tested typically by 5 to 10 external partner laboratories, ideally with different levels of subtyping expertise and access to different types or brands of equipment and reagents. The assay is evaluated using 10 to 50 isolates selected by the laboratory that developed the method. The interlaboratory reproducibility of the method is also assessed during this phase. Sometimes the external validation is further expanded to include a prospective or retrospective testing of up to 50 isolates from each laboratory's own collection.

Following the successful completion of these phases, the method may be implemented. However, even after its implementation, the performance of the assay needs to be assessed on a regular basis to detect problems not identified during the initial validation and to assess emerging situations, such as the impact of introduction of new brands of reagents that may have become commercially available.

Quality Assurance/Quality Control

A quality assurance program needs to be in place before any molecular subtyping technique is implemented to ensure consistently high quality and reproducibility of the data generated. At a minimum, subtyping should be performed only by personnel trained in working with the procedure; a written standard operating procedure should be in place, and a strain with a well-established stable subtype should be included in all experiments in order to detect procedural failures. For library subtyping systems involving multiple laboratories, participation in an external quality assessment (EQA) program that includes an initial certification and annual proficiency testing is mandatory.

Data Interpretation

When interpreting subtyping results, one must consider the epidemiological context and all other available data, such as associated demographic and other epidemiological information, and other subtyping information, e.g., biochemical reaction profiles, serotype, phage type, antimicrobial susceptibility profiles, and the presence of virulence factors; reliance on a single parameter for characterization should be avoided whenever possible. Knowledge of the subtyping method, including the quality of the data, the diversity of the organism, and the history of the subtypes encountered, should also be considered (233).

Quality of the Data

Even when a carefully standardized procedure is followed, artifacts may occur, which may lead to erroneous conclusions about relationships between profiles. It is therefore important to know the nature of these artifacts in order to recognize and correct them. In this, the role of the database curator is extremely important. In PFGE, the artifacts include, among other things, ghost bands caused by incomplete restriction and subtle differences in band resolution (one thick band versus two thinner bands) (233). In PCR-based methods, such as RAPD and rep-PCR, differences in band intensities are a huge problem, and in MLVA, PCR, and fragment analysis artifacts, such as minus-A and stutter peaks and fluorescence carryover, can confuse data interpretation (234). Also, Sanger sequence trace files should be routinely checked for quality either manually or by using software (e.g., Phred/Phrap, http://www.phrap.org). Most major sequence databases require the submission of the raw sequence trace files from the laboratory when a new allele type is proposed (235). The quality of the next-generation WGS data can be assessed using various quality metrics that are based on the raw reads (e.g., per-base sequence quality, sequence length distribution, sequence duplication level, and sequence coverage) or assemblies (e.g., N50 and the number of contigs) and can be generated by using either the software built into the sequencing system or external software packages. It is important to recognize that the quality of the genome assemblies reflects the quality of the sequencing technology used but also of the analysis software employed for assembly and annotation (236).

Diversity of the Organism

Optimal interpretation of the differences (or lack thereof) between subtypes of two isolates depends largely on the variability of the organism being typed. Large databases should provide sufficient data to make reasonable determinations of diversity. If an organism displays little diversity, one should be cautious in assuming that closely related patterns, or even indistinguishable patterns, indicate a high likelihood that they originate from a common source. In this case, additional data from other typing methods and other available information should be considered. If the organism shows substantial diversity, one must still consider whether there are clonal subpopulations within a nonclonal organism. On the other hand, when an organism demonstrates extreme variability, any pattern matches may be significant.

Epidemiological Context

If the epidemiological setting from which the isolates are derived appears to be a point source outbreak without continued transmission, only very minor differences are likely to be observed, because the outbreak strain has very little time to undergo genetic changes. In contrast, when there is ongoing transmission, such as prolonged hospital or community outbreaks, with strains being passed from person to person, more variability should be expected. Additionally, the amount of variation seen during an outbreak will depend on the stability of the genetic markers targeted by the typing method. Fast-evolving genetic markers, such as VNTRs, tend to change slightly even during shorter outbreaks (90). During investigations of known outbreaks, it is fairly easy and often helpful to designate patterns that differ slightly from the primary outbreak pattern as subpatterns or variants. However, when performing surveillance for cluster/outbreak detection, accepting such variations may mislead the epidemiological investigation (183), especially if one of the variants represents a common pattern.

It is also important to consider how the laboratory results fit together with the epidemiologic and environmental investigations. The fact that common subtypes exist for many organisms demonstrates that indistinguishable subtypes alone do not unequivocally prove an epidemiologic connection. It is always possible that two individuals were infected with the same strain from different sources. On the other hand, clearly differing subtypes do not prove that isolates are epidemiologically unrelated, since multiple strains can be associated with the same outbreak (237) and some strains contain genetic elements, e.g., prophages, which are incorporated into the genome in an unstable manner and therefore may affect the subtype of different colonies picked from the same pure culture of the organism (238).

LIBRARIES FOR MOLECULAR EPIDEMIOLOGY

International travel, migration, and the food trade are the main factors that have contributed to the worldwide spread of microbes. Therefore, the need for international databases with standardized type nomenclature and information on epidemiologically relevant strains has emerged. Building such databases relies upon standardization of typing methods and on regular ring trials for all participating laboratories to guarantee consistently comparable data.

Strain Catalogues

Strain catalogue databases are mainly public access websites with limited access components in some of them. Their main function is to standardize subtype nomenclature and facilitate easy data sharing. Most of them are curated but typically contain minimal epidemiological or demographic information about the strains and are therefore not very useful for real-time epidemiological surveillance. The laboratories that contribute data to strain catalogues use protocols that vary in their levels of standardization from none (MLVA) to medium (*spa* typing).

The best-known examples of strain libraries are the three MLST databases that are hosted on Web servers located at Imperial College London (http://www.mlst.net) (235), Oxford University (http://pubmlst.org) (239), and the Institut Pasteur (http://www.pasteur.fr/mlst). On http://www.mlst.net and the Institut Pasteur websites, species-specific information, including limited epidemiological data, can be accessed on each of the species websites, whereas http://pubmlst.org software enables multiple client databases to query a single species-specific profile database, so that the information on isolates can be kept private for security or confidentiality reasons. Each species-specific database holds the sequences of all known alleles at each of the MLST loci and through the curator assigns new allele numbers and sequence types. The websites also provide clustering tools to explore the relationship of the query strain with other strains in the database.

SeqNet (http://www.seqnet.org/), coordinated by the Münster University Hospital and Robert Koch Institute in Wernigerode, Germany, is an example of a non-MLST-based strain catalogue. SeqNet is an initiative of 60 national reference laboratories and university laboratories from 29 European countries to establish a network of sequence-based typing of microbial pathogens (240). SeqNet currently has only a database for *S. aureus spa* types through the curated *spa* server. Unlike the three MLST databases, SeqNet has a QA/QC aspect built in it in the form of a one-time certification and annual ring trials.

The *ccrB* typing tool (http://www.ccrbtyping.net) is a public online resource for storage and automatic analysis of MRSA *ccrB* sequences (241). The user's sequence is assigned to an allele based on 100% homology with an existing allele or to a new allele if a homology between 90% and 100% is found to any of the available alleles. In the case of a new allele, the most similar allele is indicated. Based on the allele assignment, a prediction of the *ccrAB* allotype and SCC*mec* type is also provided.

International databases for MLVA data have also been recently established. MLVAbank (http://mlva.u-psud.fr), hosted by the University of Orsay, France, has both public and private databases available for a few organisms (94). The public databases have been derived from published data, sometimes by merging publications from different groups. Since the VNTR markers used by different groups are not always the same because of the current lack of standardization, different data sets may be available for different sets of isolates. Since different sets of primers are sometimes used for the same marker by different groups and since there are sizing discrepancies between different capillary electrophoresis platforms, the data are not always directly comparable even though they are uploaded to MLVAbank as repeat copy numbers.

The TB database (http://www.tbdb.org) is an integrated database for tuberculosis (TB) research that houses annotated genome sequence data for several *M. tuberculosis* strains, microarray and RT-PCR expression data from *in vitro* experiments, and TB-infected tissues (243). Experimental data may be deposited into the database by any TB researcher prior to publication, providing prepublication access to tools for the analysis, annotation, visualization, and sharing of data. The data are then made public at the author's request or following publication. The database curators also actively search the literature for publications containing relevant TB or host microarray data and then obtain the raw data from the researchers and load them into the database.

Surveillance Databases

Surveillance databases are restricted-access curated databases for real-time sharing of subtyping data and detailed demographic information associated with the strains. The main function of surveillance databases is to rapidly detect and define clusters of disease in order to initiate and support epidemiological investigations that are aimed at tracing the source and limiting the scope of outbreaks. Laboratories contributing data to surveillance databases typically are required to follow highly standardized protocols and comply with an extensive QA/QC program to ensure the reproducibility and the high quality of data.

PulseNet, the national and international surveillance network for foodborne disease, is the most successful example of a surveillance database. PulseNet USA was established in 1996 (20), and similar networks later followed in Canada, the Asia Pacific region, Latin America, the Middle East, and Europe, and the latest is in Africa (244) (www.pulsenetinternational.org). The participating laboratories perform PFGE on all foodborne organisms that they receive from clinical and food specimens in real time. The generated patterns are analyzed locally using highly customized software and uploaded to the central databases of each region via the Internet. The database managers confirm the quality of the patterns, name them according to a standardized scheme, and compare them against the patterns submitted to the database within the previous 60 days (120 days for *Listeria*). The epidemiologists are alerted if a new cluster of indistinguishable isolates is detected. Until now, much of the work in PulseNet International has focused on establishing the infrastructure of the network, but eventually each regional network will be able to log on to the server of any other international network, query the databases for matches to pathogen subtypes of interest, and, if matches are found, access the epidemiologic information on matching isolate patterns (244). Such collaboration is already taking place between PulseNet USA and PulseNet Canada.

Other PulseNet-like surveillance databases include the CaliciNet network in the United States (245) and the Foodborne Viruses in Europe (FBVE) network (246), dealing mainly with norovirus. The virological networks employ primarily DNA sequence analysis. A number of virus-specific, curated databases have been established. These can be identified by an Internet search, though many may have restricted access.

CONCLUSIONS AND FUTURE TRENDS

Molecular methods have improved our understanding of the epidemiology of infectious diseases and the well-known emerging or reemerging pathogens causing them during the past 4 decades. Molecular methods have been used not only to subtype and otherwise characterize the pathogens following their culture but also to identify and detect nonculturable or slowly growing organisms (247–249). The emergence and increased use of a plethora of culture-independent diagnostic tests (250, 251) in clinical laboratories in the last few years has resulted in decreased availability of cultures for further characterization in public health laboratories. This trend may place culture-dependent surveillance networks such as PulseNet in serious jeopardy. However, with the rapid evolution of next-generation sequencing technologies, including metagenomics (252), it is to be expected that these methods will be used to an increasing extent for rapid nonculture diagnostics of infectious diseases directly from a clinical sample, providing detection, virulence characterization, and subtyping of the organisms at the same time, thereby creating the potential for detection of public health events, e.g., outbreaks, in real time, with no delays caused by isolating and growing the organisms as pure cultures. Before this can be accomplished, some formidable challenges need to be tackled, such as building comprehensive sequence reference libraries for metagenomics containing a representative diversity not only of pathogenic microbes but also of the human microbiome (253). Additionally, a significant investment in public health informatics infrastructure is needed to ensure that the users of the data are alerted to and know how to use the growing information flowing from clinical laboratories.

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Procedures for the Storage of Microorganisms*

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Long- and short-term preservation of microorganisms for future study has a long tradition in microbiology. As early as the 1880s, it was observed that bacteria survived well in ice and that freeze-thaw cycles damaged the cell wall (1). By the 1900s, researchers had optimized methods to preserve tissues, viruses, and bacteria for later use by freezing and drying. It had become apparent to early researchers that preserving microorganisms allowed them to be studied at a later time and to remain viable when transported to others (2). Indeed, culture collections of microorganisms are valuable resources for scientific research in microbial diversity and evolution, patient care management, epidemiological investigations, and educational purposes. Preserved individual strains of microorganisms serve as permanent records of microorganisms' unique phenotypic profiles and provide the material for further genotypic characterizations. Such reference collections can encompass rare infectious agents unique to an individual or catalog the history of disease caused by common pathogens such as those responsible for community outbreaks.

There are multiple methods for microbial preservation. Effective storage is defined by the ability to maintain an organism in a viable state free of contamination and without changes in its genotypic or phenotypic characteristics. Secondly, the organism must be easily restored to its condition prior to preservation. Microbial preservation methods have been evaluated extensively over the past 60 years, and often, optimal methods for preservation depend on a microorganism's taxonomic classification. Review articles, monographs, and books have been published that provide detailed information about the storage of various types of microorganisms (3–7). For clinical microbiology laboratories, simple and broadly applied methods are necessary to maintain organisms for short- and long-term recovery. This chapter presents methods that can be used for the storage of bacteria, protozoa, fungi, and viruses.

OVERVIEW OF PRESERVATION METHODS

Short-Term Preservation Methods

Direct Transfer to Subculture

The simplest method for maintaining the short-term viability of microorganisms, most often used for bacteria, is periodic subculture to fresh medium. Although simple, if microorganisms are saved for >1 week, this method is potentially labor-intensive, requires extensive laboratory space, and may compromise a microorganism's phenotypic profile. Each transfer to a new subculture increases the likelihood of mutation with undesirable changes in a microorganism's characteristics. Furthermore, plasmids may be lost with subculturing.

The interval between transfers varies among organisms. Additionally, the rate of mutation is quite variable. Some organisms appear stable indefinitely with repeated transfer, and others may change phenotypic traits after as few as two or three passages. The actual rate of mutation, however, has not been studied until recently using sequencing technology (8, 9). Issues that must be addressed with direct transfer include the medium to be used, the storage conditions, and the frequency of transfer.

Maintenance Medium

The medium should support the survival of the microorganism but minimize its metabolic processes and slow its rate of growth. Extreme environments should be avoided because microorganisms have the unique ability to adapt through mutation events in order to survive in suboptimal surroundings. A medium with too high a nutrient content will induce rapid replication that requires more frequent transfers. The optimal medium for maintaining microorganisms has not been clearly defined and most likely varies from one species to another and may even depend on the individual strain. Media that have been used include distilled water, tryptic soy broth, and nutrient broths, all of which may be used with or without cryopreservatives.

Storage Conditions

Many laboratories store organisms, most often bacteria, for short periods on routine agar media at the workbench. Cultures kept in this fashion are subject to drying. A better method is to transfer organisms into an agar slant tube with a screw top and to store them in an organized location away from light and significant temperature changes. To prevent drying, caps can include rubber liners, or film can be wrapped over the top of the tube before or after the cap is screwed on. Storage at lower temperatures (5 to 8°C) slows metabolic processes and maintains viability for longer periods.

Frequency of Transfer

There is no set protocol for the frequency of transfer since storage conditions, media used, and types of microorganisms

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vary among laboratories. Individual laboratories may conduct studies for each category of microorganism to determine optimal intervals between transfers under their conditions used for storage. Such studies would involve performing subcultures at scheduled times until the laboratory identifies an acceptable interval between transfers at which a microorganism can reliably and reproducibly be recovered. (When transfers are performed, 5 to 10 representative colonies should be used to avoid the possibility of introducing an altered genotypic or phenotypic characteristic.) For the passage and storage of quality control organisms for antimicrobial susceptibility testing, published standards should be followed (10).

Quality Control Procedures

Although it is not necessary with each transfer, the status of the specimen should be assessed periodically. Ongoing viability, stability of phenotype, microorganism identity, and the rate of contamination of specimens should be determined and noted in a log.

Immersion in Oil

An alternative to capping tubes is to add a layer of mineral oil to the top of the specimen. Many bacteria and fungi can be stored for periods of up to 2 to 3 years by this method, and transfers are not needed as frequently. Microorganisms are still metabolically active in this environment, and mutations can still occur. Mineral oil should be medicinal-grade oil with a specific gravity of 0.865 to 0.890. Contamination of the specimen can occur if the mineral oil is not adequately sterilized. For sterilization, it should be heated to 170°C for 1 to 2 h in an oven (4). Autoclaving is not considered acceptable. Sterile mineral oil is also commercially available.

To prepare the specimen, an inoculum of 5 to 10 colonies of the microorganism should be placed on an agar slant or in tubed broth media. Once growth is identified, a layer of mineral oil at least 1 to 2 cm deep is added, and the agar must not be exposed to air. As with the simple transfer method, tests for viability should be performed to determine the optimal transfer schedule that will ensure microorganism recovery. Transfers will be less frequent than those of microorganisms stored without oil; however, oil is more difficult to add to vials and to clean up in the event of spills.

Freezing at −20°C

Refrigeration or freezing in ordinary freezers at −20°C may be used to preserve microorganisms for periods longer than those that can be accomplished by repeated transfers. Viability may be maintained for as long as 1 to 2 years for specific microorganisms, but overall, damage caused by ice crystal formation (6) and electrolyte fluctuations (4) results in poor long-term survival. The medium used for storage appears to be important, since preservation times vary from a few months to 2 years depending upon which medium is used (6, 11, 12). Modern self-defrosting freezers with freeze-thaw cycles must be avoided because cyclic temperature fluctuation will destroy the microorganism.

Drying

Although most microorganisms do not survive drying, molds and some spore-forming bacteria may be dried and stored for prolonged periods. Soil has been described as a storage medium if it is autoclaved and air dried, but it is not a standardized, defined, and consistent product for use over long periods (4). Instead, commercial silica gel can be used in small cotton-plugged tubes after being heated in an oven to 175°C for 1.5 to 2 h (6), with moderately successful recovery of fungi. Alternatively, a suspension of 10^8 microorganisms can be inoculated onto sterile filter paper strips or disks. The paper is dried in air or under a vacuum and is placed in sterile vials. These vials can be stored in the refrigerator for up to 4 years, and then single strips or disks can be removed as needed (4). This method is commonly used for quality control organisms.

Storage in Distilled Water

Most organisms do poorly in distilled water, but some survive for prolonged periods. Many fungi and *Pseudomonas* spp. survive for several years in distilled water at room temperature (6, 13). McGinnis et al. found that with the exception of fungi that do not easily sporulate, 93% of yeasts, molds, and aerobic actinomycetes can be easily and inexpensively preserved this way (14).

Long-Term Preservation Methods

Whereas the methods described above may be used to store microorganisms for periods of up to a few years, ultralow-temperature freezing and freeze-drying (lyophilization) are recommended for long-term storage. Although the initial investment in ultralow-temperature freezers and lyophilization may be costly, these methods are less labor-intensive over time, require less laboratory space (e.g., a cryovial versus broth or agar media), and reduce the chances of mutation events. Of course, mutations and loss of mobile genetic elements can still occur, and this phenomenon was observed in *Staphylococcus aureus* strains that lost the *mecA* gene during long-term preservation at −80°C (15). Similar to those with other preservation methods, survival rates after freeze-drying vary with species. Evaluating microorganisms over a 10-year period, Miyamoto-Shinohara et al. found that survival rates after freeze-drying for *Brevibacterium* spp. and *Corynebacterium* spp. approached 80%, whereas those for *Streptococcus mutans* decreased to 20% after 10 years (13).

Ultralow-Temperature Freezing

Microorganisms can be maintained at temperatures of −70°C or lower for prolonged periods. Systems for achieving these temperatures include ultralow-temperature electric freezers and liquid nitrogen storage units. With either system, unwanted heating can occur due to the loss of electrical power or liquid nitrogen. Close observation of the system and an adequate alarm mechanism are essential, since any increase in temperature will reduce viability. In the event that the temperature does rise, restoring power and returning to the target storage temperature as quickly as possible are essential. The presence of a cryopreservative such as glycerol may reduce the risk to microorganisms upon short exposure to higher temperatures (16). If thawing does occur, there are no guidelines for rapid restoration of the storage condition. Refreezing of the sealed vials as described below may be considered.

For long-term storage, temperatures below −130°C are recommended for fastidious cells, such as fungal hyphae and protozoa. Cellular activity and chemical reactions cease at these low temperatures, but at −70°C they may still continue to a limited extent. Hence, for long-term cryopreservation of certain organisms, storage in liquid nitrogen (−196°C) or liquid nitrogen vapor (−150°C) is recommended (3).

Storage Vials

Storage vials must be able to withstand very low temperatures and maintain a seal for their contents. Plastic (polypropylene) or glass (borosilicate) tubes may be used. Plastic vials with screw tops and silicone washers are much easier to use than glass vials that must be sealed with a flame and then scored and broken open. Several commercial suppliers stock acceptable vials, e.g., Fisher Scientific Products (Pittsburgh, PA), VWR Scientific (Radnor, PA), Wheaton Science Products (Millville, NJ), and Becton Dickinson and Co. (Franklin Lakes, NJ). Vials come in a variety of sizes. Half-dram vials are available from several suppliers and can be conveniently packaged in a 12-by-12 grid so that 144 vials are stored in one box or layer.

Cryoprotective Agents

To protect microorganisms from damage during the freezing process, during storage, and during thawing, cryoprotective agents are often added to the culture suspension. Whereas most bacteria, fungi, and viruses survive better with such additives, studies have shown that cryoprotective agents significantly damage others. The reader is referred to detailed references for specifics (Table 1) (3, 6). Rapid freezing without additives may still be acceptable for the long-term survival of protozoa, although freeze-drying may be preferred.

There are two types of cryoprotective agents: those that enter the cell and protect the intracellular environment and others that protect the external milieu of the organism.

TABLE 1 Common procedures for preservation of microorganisms

Organism group	Storage method	Cryopreservative	Storage temp (°C)	Storage duration (yr)
Streptococci	Freezing	Skim milk	−20	0.2
	Ultralow-temp freezing	Skim milk	−70 to −196	0.2–1
	Lyophilization	Skim milk	4	0.5–30
Mycobacteria	Freezing	Skim milk	−20	3–5
	Ultralow-temp freezing	Skim milk	−70 to −196	3–5
	Lyophilization	Skim milk	4	16–30
Spore-forming bacteria	Transfer	None	Room temp	0.2–1
	Immersion in mineral oil	None	4	1
	Drying	None	Room temp	1–2
	Freezing	Glucose	−20	1–2
	Ultralow-temp freezing	Skim milk, glycerol	−70 to −196	2–30
	Lyophilization	Skim milk, lactose	4	30
Other Gram-positive bacteria	Transfer	None	Room temp	0.2–0.3
	Immersion in mineral oil	None	4	0.6–2
	Freezing	Sucrose, glycerol	−20	1–3
	Ultralow-temp freezing	Skim milk, sucrose, glycerol	−70 to −196	1–30
	Lyophilization	Skim milk, sucrose	4	30
Gram-negative bacteria	Transfer	None	Room temp	0.1–0.3
	Immersion in mineral oil	None	4	1–2
	Freezing	Sucrose, lactose	−20	1–2
	Ultralow-temp freezing	Sucrose, lactose, glycerol	−70 to −196	2–30
	Lyophilization	Skim milk, sucrose, lactose	4	30
Filamentous fungi	Transfer	None	4 to 25	2–10
	Immersion in mineral oil	None	Room temp	1–40
	Storage in distilled water	None	Room temp	1–10
	Drying	Soil, silica gel	Room temp	1–4
	Ultralow-temp freezing	Glycerol, DMSO	−70 to −196	2–30
	Lyophilization (sporeformers)	Glycerol, sucrose, DMSO, skim milk	4	2–30
Yeasts	Storage in distilled water	None	Room temp	1–2
	Drying	Nutrient medium	Room temp	1–2
	Ultralow-temp freezing	Glycerol, DMSO, skim milk	−70 to −196	2–30
	Lyophilization	Nutrient medium	4	2+
Protozoa	Freezing	Blood, nutrient broth with DMSO or sucrose	−20 to −40	
	Ultralow-temp freezing	Blood, nutrient medium with DMSO or glycerol	−70 to −196	
Viruses	Transfer	Nutrient medium	4	0.5
	Ultralow-temp freezing	SPGA	−70 to −196	1–30
	Lyophilization	SPGA	4	6–10

Glycerol and dimethyl sulfoxide (DMSO) are most often used for the former; sucrose, lactose, glucose, mannitol, sorbitol, dextran, polyvinylpyrrolidone, polyglycol, and skim milk are used for the latter. Combinations of agents as well as detergents (e.g., Tween 80 and Triton WR 1339), other carbohydrates (e.g., honey), and calcium lactobionate have also been used. The most universal cryoprotectant is DMSO; however, the optimal cryoprotectant often varies with the microorganism. For example, glycerol appears to be best suited for the preservation of bacteria. A current and comprehensive review of protectant additives used in the cryopreservation of microorganisms is provided by Hubalek (17).

Glycerol is typically added at a concentration of 10% (vol/vol), and DMSO is added at 5% (vol/vol). Prior to use, glycerol is sterilized by autoclaving. Once prepared, it can be stocked at room temperature for months. DMSO must be filter sterilized and can be stored in open containers for only 1 month prior to use.

Of the external products, skim milk is the most often used. Dehydrated skim milk is purchased from medical product suppliers, e.g., Becton Dickinson and Co. and Hardy Diagnostics (Santa Maria, CA). It is autoclaved and used in a final concentration of 20% (wt/vol) in distilled water (3). This is double the concentration suggested by the manufacturers if the intent is to make a reconstituted equivalent of regular milk.

Preparation of Microorganisms for Freezing

Microorganisms are inoculated into a medium that adequately supports maximal growth. Cultures are allowed to mature to the late growth or stationary phase before being harvested. Broth specimens are centrifuged to create a pellet of microorganisms. The pellet is withdrawn and resuspended in 2 to 5 ml of broth with the appropriate concentration of cryoprotectant additive. For agar specimens, broth containing the cryoprotectant is placed on the surface of the agar. The surface is scraped with a pipette or sterile loop to suspend microorganisms, and then the broth mixture is pipetted directly into freezer vials. Alternatively, the agar surface can be scraped with a sterile loop. The microorganisms can then be transferred directly into the vial of cryoprotectant and emulsified into a final dense suspension. The volume of the aliquots to be frozen is typically 0.2 to 0.5 ml.

Freezing Method

The American Type Culture Collection (ATCC) recommends slow, controlled-rate freezing at a rate of 1°C per min until the vials cool to a temperature of at least −30°C, followed by more rapid cooling until the final storage temperature is achieved (3). This protocol is best achieved using a controlled-rate freezer. As an alternative, the ATCC suggests the use of an insulated freezing chamber, such as one commercially available or simply an appropriate polystyrene box packed with insulating materials. Studies in the 1970s showed that uncontrolled-rate freezing may be acceptable for most organisms and is much less expensive or labor-intensive (6). When organisms are stored in liquid nitrogen, however, it is still recommended that vials be placed initially in a −60°C freezer for 1 h and then transferred into the liquid nitrogen. When organisms are stored permanently at −60 to −70°C, the vials can be placed directly into the freezer.

Vials with small glass beads or plastic beads can also be used for freezing microorganisms, e.g., CryoBank (Copan Diagnostics, Murrieta, CA) or Microbank (Pro-Lab Diagnostics, Round Rock, TX) systems. The culture suspension will coat the beads, and then the excess culture suspension is aspirated from the vial. Individual beads can then be removed from storage for reconstitution without thawing the entire sample (18).

Thawing

Damage to microorganisms occurs as they are warmed from the frozen state. Critical temperatures appear to be between −40 and −5°C. Studies suggest that rapid warming through these temperatures improves recovery rates. For optimal results, stored culture vials should be warmed rapidly in a 35°C water bath until all ice has disappeared (3, 6). Once a vial is thawed, it should be opened and the organism should be transferred to an appropriate growth medium immediately. Great care must be exercised during the thawing phase, since rapid temperature changes and resulting air pressure changes inside vials can cause the vials to explode. Protective clothing and eyewear must be worn during this process.

For most practical purposes in the clinical laboratory, however, thorough thawing of stored bacteria or yeast in a water bath is not practical or necessary. The frozen vial can be thawed at room temperature and plated with good results for most routine organisms. If the organism vial must be saved for reuse at a later date, one may scrape off a small portion of the frozen contents with a sterile loop or pipette tip and then inoculate the appropriate media. The vial may be returned to frozen storage immediately without thawing and may be reused at a later date with limited damage to the organism.

Specialized Storage Systems

Computerized technology can facilitate the organization and database management of large storage collections. Some laboratories have developed their own tracking system, while other products are commercially available, e.g., Freezerworks (Dataworks Development, Inc., Mountlake Terrace, WA) and FreezerPro (Ruro Inc., Frederick, MD). These kinds of software allow the user to enter data regarding each sample in a repository, and the data are stored in a highly organized, searchable fashion. Radio-frequency identification (RFID) labeling may also be used for compact storage of information associated with each sample, e.g., ColdTrack vials (BioTillion LLC, Skillman, NJ) or FreezerPro RFID kit.

Freeze-Drying (Lyophilization)

Freeze-drying is considered to be the most effective way to provide long-term storage of most bacteria, yeasts, sporulating molds, and viruses. Better preservation occurs with freeze-drying than with other methods because freeze-drying reduces the risk of intracellular ice crystallization, which compromises viability. Removal of water from the specimen effectively prevents this damage. Among bacteria, the relative viability with lyophilization is greatest with Gram-positive bacteria (sporeformers in particular) and decreases with Gram-negative bacteria (6, 19), but overall, the viability of bacteria can be maintained for as long as 30 years. In addition, large numbers of vials of dried microorganisms can be stored with limited space, and organisms can be easily transported long distances at room temperature.

The process combines freezing and dehydration. Organisms are initially frozen and then dried by lowering the atmospheric pressure with a vacuum apparatus. Freeze-drying has been extensively reviewed in the past (5), and the required equipment includes a vacuum pump connected in line to a condenser and to the specimens. Specimens can be connected individually to the condenser (manifold method) or can be placed in a chamber where they are dehydrated in one larger air space (chamber or batch method). Alexander et al. and Heckly have both published detailed descriptions of equipment options (3, 5).

Storage Vials

Glass vials are used for all freeze-dried specimens. When freeze-drying is performed in a chamber, double glass vials are used. In the chamber method, an outer soft-glass vial is added for protection and preservation of the dehydrated specimen. Silica gel granules are placed in the bottom of the outer vial before the inner vial is inserted and cushioned with cotton. For the manifold method, a single glass vial is used. For both methods, the vial containing the actual specimen is lightly plugged with absorbent cotton. The storage vial in the manifold method or the outer vial in the chamber method must be sealed to maintain the vacuum and the dry atmospheric condition. All vials are sterilized prior to use by heating in a hot-air oven.

Cryoprotective Agents

Research concerning cryoprotective agents has been extensively reviewed (5). In general, the two most commonly used agents are skim milk and sucrose. Skim milk is used most often for chamber lyophilization, and sucrose is used most often for manifold lyophilization. Skim milk is prepared by making a 20% (vol/vol) solution of skim milk in distilled water. The solution is divided into 5-ml aliquots and autoclaved at 116°C, with care taken to prevent overheating and caramelization of the solution. The preparation is then used in smaller volumes as described above for freezing. Sucrose is prepared in an initial mixture of 24% (vol/vol) sucrose in water and added in equal volumes to the microorganism suspension in growth medium to make a final concentration of 12% (vol/vol).

Preparation of Microorganisms for Lyophilization

As with simple freezing, maximum recovery of organisms is achieved by using microorganisms in the late growth or stationary phase from the growth of an inoculum in an appropriate growth medium. High concentrations of microorganisms are considered to be important. The ATCC recommends a concentration of at least 10^8 CFU/ml (3), and Heckly suggests a concentration of 10^{10} CFU/ml or higher (6).

Freeze-Drying Methods

In the chamber method, inner vials with the microorganism suspension are placed in a single layer inside a stainless steel container. This container is placed in a low-temperature freezer at −60°C for 1 h. The container is then transferred to a chamber containing a low temperature bath, e.g., dry ice in ethyl Cellosolve or other solvent, and covered with a sealable vacuum top, which is connected in sequence to a condenser reservoir also filled with the dry ice-solvent mixture and to a vacuum pump. The vacuum is maintained at a minimum of 30 μm Hg for 18 h. At the same time, the outer vials are prepared by being heated in an oven overnight, filled with silica gel granules and cotton, and placed in a dry cabinet (with <10% relative humidity). The freeze-dried inner vials are inserted into the outer vials, and the outer vials are heat sealed. Multiple different strains or species should probably not be processed in the same batch. Cross contamination rates vary from 0.8 to 3.3% when two different microorganisms are placed on opposite sides of the same container and are as high as 8.3 to 13.3% when microorganisms are intermingled (20).

In the manifold method, a rack of individual vials is used rather than a single container. The rack is placed in a dry ice bath. After the freezing process, the vials are connected by individual rubber tubes in sequence to the condenser container filled with the dry ice-solvent mixture and to the vacuum pump. As in the method described above, the vacuum is maintained at 30 μm Hg for 18 h and then the individual vials are sealed.

Storage

Individual vials need to be appropriately labeled and sorted. Storage at room temperature does not maintain viability and is not recommended. Storage at 4°C in an ordinary refrigerator is acceptable, but survival rates may be improved at temperatures of −30 to −60°C (3, 5).

Reconstitution

Care must be taken when opening vials for reconstitution because of the vacuum inside the vial. Safety glasses should always be worn, and vials should be covered with gauze to prevent injury if the vial explodes when air rushes in. Reconstitution should also be conducted in a closed hood to avoid dispersal of microorganisms. The surface of the vial should be wiped with 70% alcohol, and then the top of the glass vial can be scored and broken off or punctured with a hot needle. A small amount (0.1 to 0.4 ml) of growth medium is injected into the vial with a needle and syringe or a Pasteur pipette, the contents are stirred until the specimen is dissolved, and then the entire contents are transferred with the same syringe or a pipette to appropriate broth or agar media. A purity check must be done on each specimen because of the possibility of either cross contamination or mutation during the preservation process.

Newer Technologies

The long-term preservation methods previously described are specifically designed for recovery of microorganisms for further cultivation. Culture-independent tests based on antigen or nucleic acid technologies are in widespread use and do not require viable microorganisms. In this regard, storage of microorganisms to preserve their antigens or nucleic acids is also important for clinical laboratories. The use of Whatman Flinders Technology Associates (FTA) matrix cards (Whatman International Ltd., Maidstone, United Kingdom) or other filter paper-based products is a novel approach for long-term storage of microbial DNA that is safe (microorganisms are inactivated), inexpensive, and fast (21, 22). Bacterial and/or fungal cell suspensions are applied directly to dry FTA paper. The FTA cards are impregnated with buffers, free radical trap and protein denaturants that lyse cell membranes on contact, entrap DNA, and protect DNA from degradation. This technology has been successfully applied to a variety of bacteria and fungi and serves as a reusable DNA archiving system. Although beyond the scope of this chapter, direct specimens such as blood can be preserved using a dry blood spot on filter paper or with a non-paper-based matrix for future antibody or nucleic acid testing to detect HIV (23–25), hepatitis B virus (23), hepatitis C virus (23, 25), *Rickettsia typhi*, and *Orientia tsutsugamushi* (26).

Procedures for Specific Organisms

Procedures for specific organisms are described below and summarized in Table 1.

Bacteria

All of the material presented in this chapter applies primarily to the preservation of bacteria. Simple transfer, storage under mineral oil, drying, or freezing at −20°C can maintain bacteria for short periods; freezing in ultralow-temperature electric freezers at −70°C or in liquid nitrogen at −196°C or freeze-drying can provide long-term preservation. A summary of the studies of bacterial preservation has been published (6). In general, serial transfer will preserve bacteria for up to a few months, storage under mineral oil or with drying will last 1 to 2 years, freezing at −20°C will preserve bacteria for 1 to 3 years, freezing at −70°C will preserve bacteria for 1 to 10 years, and freezing in liquid nitrogen and freeze-drying will preserve bacteria for up to 30 years (4). For fastidious bacteria such as *Streptococcus pneumoniae*, *Neisseria* spp., and *Haemophilus* spp., the optimal methods are lyophilization and freezing at −70°C by using Trypticase soy broth with glycerol as a preservation medium (27–29). Stock cultures of quality control microorganisms can be maintained in a cryopreservative suspension for up to 1 year at −20°C or indefinitely at −70°C.

Protozoa

Information concerning the preservation of protozoa is limited, in keeping with the infrequent need for such a process in clinical microbiology laboratories. Variable methods for individual genera are described. In general, freezing appears to be preferred to freeze-drying. Storage in liquid nitrogen is recommended for many of the protozoa below, although cryopreservation up to several months may be successful at −70°C (1). Cryopreservation of other parasites such as helminths is rarely done in clinical laboratories, and methods for storing and preserving viability for various parasites are reviewed elsewhere (30). All of the following procedures are as described by the ATCC (3).

Acanthamoeba spp., *Leishmania* spp., *Naegleria* spp., *Trichomonas* spp., and *Trypanosoma* spp. can be handled as described above for ultralow-temperature freezing with 5% (vol/vol) DMSO as the cryoprotective agent. These organisms should be stored in liquid nitrogen.

Acanthamoeba spp. and *Naegleria* spp. can also be dried at room temperature onto filter paper. Aliquots of a microorganism suspension (0.3 ml) are pipetted onto the paper in a shell vial and dried in air for 14 days at room temperature and then in a vacuum desiccator for an additional week. The vials are sealed and stored in liquid nitrogen.

Entamoeba spp. are stored frozen at −40°C. Specimens should be suspended in a mixture of growth medium containing 12% (vol/vol) DMSO and 6% (vol/vol) sucrose.

Leishmania spp. may also be prepared by inoculation of the organism into an animal host. At the peak of infection, the spleen is harvested and homogenized in half the final volume of ATCC medium 811 salt solution. Freezing is completed with 10% glycerol as the cryoprotectant.

Plasmodium spp. can be stored from infected blood samples. At the height of parasitemia, blood is obtained and anticoagulated with the following preparation: 1.33 g of sodium citrate, 0.47 g of citric acid, 3.00 g of dextrose, 200 mg of heparin (sodium), and 100 ml of distilled water. The final concentration of anticoagulant added to blood is 10%. To this anticoagulated blood, 30% glycerol in 0.0667 M phosphate buffer is added to a final concentration of 10% (vol/vol) glycerol. Freezing should occur in liquid nitrogen.

Trypanosoma spp. must be harvested from an animal host. At the peak of parasitemia, blood is withdrawn into heparinized tubes and diluted 1:1 in Tyrode's solution (8.0 g of NaCl per liter, 0.02 g of KCl per liter, 0.2 g of $CaCl_2$ per liter, 0.1 g of $MgCl_2$ per liter, 0.05 g of NaH_2PO_4 per liter, 1.0 g of $NaHCO_3$ per liter, and 1.0 g of glucose per liter) with 1 to 5% phenol red added. Then 5% DMSO is added as the cryoprotectant, and the specimen is stored in liquid nitrogen.

Yeasts and Filamentous Fungi

All of the techniques described above have been applied to the storage of yeasts and fungi (4, 6, 7, 31). The individual method employed depends on the species to be preserved and whether or not it sporulates.

Subculturing. Subculturing is the simplest method of maintaining living fungi and involves serial transfer to fresh solid or liquid media. Storage is accomplished usually at room or refrigerator temperature. Fungi may be maintained by subculturing for a number of years. Care must be taken to avoid aerosolization and contamination of the laboratory or other specimens.

Storage under oil. Whereas species of *Aspergillus* and *Penicillium* have remained viable under oil for 40 years (7), many species have shown deterioration after 1 to 2 years and must be transferred periodically. Taddei et al. also reported the successful storage and recovery of actinomycetes stored under paraffin oil for 10 to 30 years (32).

Water storage. Many fungi can be stored successfully for prolonged periods in distilled water (13, 33). A simple method is to pipette 6 to 7 ml of sterile distilled water onto 2-week-old culture slants in screw-cap tubes. The spores and fragments of hyphae are dislodged by scraping with the pipette, and the suspension is transferred to a sterile 1-g vial, which is tightly capped and stored at 25°C. Fungi are revived by subculturing 0.2 to 0.3 ml of the suspension to appropriate media (34).

An alternative method is to cut agar blocks from the growing edge of a fungal colony and place them in sterile distilled water in bottles with screw-cap lids (35). The cultures are stored at 20 to 25°C. The fungi are retrieved by removing a block and placing it mycelium side down on growth medium appropriate for that species (7). Contamination (22.8%) is a significant problem with this method (35).

Drying. Drying as described above has been used for fungi. Only 6 of 16 genera of fungi stored in this fashion survived for 4 years (36). The greatest success is reported for sporulating fungi stored in silica gel or in soil (7).

Freezing. Fungi have been successfully preserved by storage in liquid nitrogen by using glycerol or DMSO as a cryopreservative. Yeasts may also be stored at ultralow temperatures in skim milk. Broth cultures containing nonpathogenic fungi are disrupted in a Waring blender and suspended in equal parts of DMSO or glycerol to achieve final concentrations of 5 or 10%, respectively. Pathogens should not be disrupted in a mechanical blender because of the potential biohazard associated with aerosolization. *Histoplasma*, *Paracoccidioides*, and *Blastomyces* species should be frozen in the yeast phase, and *Coccidioides* species should be frozen in the early mycelial phase to minimize exposure of laboratory personnel. Otherwise, procedures for freezing are as described above.

Freeze-drying. Most spore-forming fungi can be preserved by freeze-drying. Cultures to be stored by freeze-drying should be grown on agar or broth media to the point of maximum sporulation (3) and processed as described above. Survival in storage for many years has been demonstrated (37, 38), but this is true only for sporulating organisms. Young vegetative hyphae of fungi do not survive freeze-drying (7).

Viruses

Ultralow-temperature freezing is effective in a number of situations. In addition to cryoprotectants described above, sucrose-phosphate-glutamate containing 1% bovine albumin (SPGA) (6, 39) and hypertonic sucrose are particularly effective, the latter for storing labile viruses such as respiratory syncytial virus (40). If ultralow-temperature freezing is employed, the rate of freezing should be as high as possible, using small-volume suspensions (0.1 to 0.5 ml). In addition to freezing of pure isolates, stool specimens known to contain viral enteric pathogens have been maintained at −70 to −85°C for 6 to 10 years with reasonable recovery and no change in the morphological characteristics of astroviruses, small round structured viruses, enteric adenoviruses, rotaviruses, and caliciviruses (41).

Gallo et al. evaluated five types of media for storage of HIV-infected peripheral blood lymphocytes and concluded that freezing peripheral blood lymphocytes in RPMI 1640 containing 10% fetal bovine serum and 10% DMSO and storing them at −60°C is acceptable for HIV isolation (42).

Freeze-drying is probably the optimum method for preserving viruses for extended periods. A detailed review of acceptable procedures has been published (43). Virus suspensions freeze-dried in medium supported with SPGA appear to survive better (6, 44). Lyophilization of polioviruses and other enteroviruses works best when electrolytes are removed by dialysis or ultrafiltration (6).

Select Agents

In response to the Public Health Security and Bioterrorism Preparedness and Response Act of 2002, federal regulations require laboratories that store select agents to register and comply with the standards established by the act (45). A current and complete list of microorganisms considered to be select agents can be found at http://www.selectagents.gov. Regardless of the method for long-term preservation, laboratories must register with the Federal Select Agent Program if they wish to use, possess, or transfer select agents or toxins. In order to minimize risk to public health and safety, select agents must be stored in a highly secured area with restricted access and appropriate safeguards. Only registered individuals who have completed training for handling select agents can access and retrieve these microorganisms from storage. An accurate and current inventory of select agents held in long-term storage must be maintained. Further guidance on storing select agents is available from the Centers for Disease Control and Prevention (http://www.selectagents.gov/resources/Long_Term_Storage_version_3.pdf).

DISASTER PREPAREDNESS

Especially in light of recent natural disasters like Hurricanes Katrina and Sandy (22), laboratories should consider the value of their organism collection and have a written emergency plan for the biorepository. For valuable or large collections of microorganisms in long-term storage, the International Society for Biological and Environmental Repositories provides guidance on emergency preparedness (http://www.isber.org). It is good practice to maintain backup copies of written or electronic data linked to the stored organisms. A laboratory may consider dividing irretrievable collections of microorganisms between containers or laboratory locations if these options are feasible. Liquid nitrogen storage containers do not require constant electrical power for operation. They may be filled up prior to an anticipated evacuation in order to maximize their temperature stability. For electric-powered freezers, laboratories should consider connection to a backup energy source such as an uninterruptable power supply or motor generator.

FUTURE DIRECTIONS

The field of microbiology is rapidly evolving, especially in the era of genomics and proteomics. Scientific research in human genetics, health care epidemiology, global health, antimicrobial resistance, microbial taxonomy, and infectious diseases relies heavily on carefully curated repositories of microorganisms. Clinical laboratories have various levels of resources for the preservation of microorganisms and their long-term storage, but anyone can contribute to national or international culture collections. Additionally, scientific advancements of pathogen discovery, genomics, and proteomics are supported by collecting and storing biological specimens such as human blood and urine, particularly from patients with noncultivable pathogens or ill-defined clinical syndromes. Scientific protocols have been developed to preserve the integrity of these biological specimens for long-term storage, up to 30 years (47, 48). All should partner with the scientific community (e.g., regional research centers of excellence or government-sponsored projects) by contributing to both specimen and microorganism repositories. The answers to many future questions lie in our clinical laboratories of today.

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Prevention of Laboratory-Acquired Infections

MICHAEL A. NOBLE

12

Laboratory biosafety is the active, assertive process based on evidence-based principles to ensure safety from microbial contamination/infection or toxic reaction for workers, the public, and the environment as a result of the active manipulations of live microorganisms or their products during academic, research, industrial, and clinical investigations. Laboratory accidents, injuries, and infections not only impact laboratory workers but can have consequences that impact laboratory coworkers, the institution, the community (including workers' families and friends), and the environment. Active laboratory biosafety addresses not only the prevention of laboratory-acquired infections in workers but also the potential accidental release of live agents, which could endanger humans, animals, and plants.

EPIDEMIOLOGY

The characterization of a person's infection as laboratory acquired is usually retrospective and is based on the assumption that the only likely exposure occurred while the person was in a laboratory. A trivial laboratory event may be considered the possible exposure because no other circumstance outside the laboratory could account for infection (1).

It is, however, important to appreciate that the total laboratory testing cycle begins well before the sample actually reaches the laboratory (the preanalytical phase of laboratory testing) and that exposures during the collection and transport of the sample should also be considered. In the past, infections acquired during the collection of some samples were included if it could be ascertained that the collection was solely for the purpose of a laboratory investigation. Infections experienced by phlebotomists as a result of needle stick injuries are now routinely included as laboratory-acquired infections (2). In contrast, phlebotomist infections, such as influenza and chicken pox acquired while collecting samples in patients' rooms, are not included (3).

Although difficult to date precisely, the first microbiology laboratories of Pasteur and Koch were active by 1840 to 1860. The first report of a laboratory-acquired infection, Mediterranean fever, was in 1899 (4).

Various compilations of laboratory-acquired infection have been published over the past 60 years. In 1953, the first survey published was of 5,000 American laboratories by Sulkin and Pike. These authors provided additional updates in 1961, 1965, and 1976. The authors cited 3,921 laboratory infections dating between 1930 and 1974, with a mortality of 4.1%. Of note, 2,307 (58.8%) of the infections were reported from research facilities, 677 (17.3%) were from diagnostic facilities, 134 (3.4%) were from the generation of biological products (industry), and 106 (2.7%) were from teaching facilities. The sources of the remaining 697 (17.8%) were unspecified.

Four series performed in the United Kingdom between 1971 and 1991 revealed that within clinical facilities, the majority of infections were reported from workers in the microbiology laboratory, followed by the autopsy service. Over the 20-year period, the number of infections reported dropped over 80%, from 104 to only 17.

Compiled information tends to be limited to events that are reported in the literature or specific databases. Thus, while uncommon community infections, such as those associated with *Brucella* species, are reported commonly as laboratory acquired, very common infections with *Staphylococcus aureus* (including methicillin-resistant *S. aureus* [MRSA]) are rarely identified (5) as being acquired in the laboratory. It is assumed that even complete listings reflect an immeasurable minority of infections that actually occur (6).

More recently, Internet-based discussion groups have worked to create information-gathering approaches (7, 8). While these newer surveys have challenges similar to those of former retrospective compilations, they demonstrate their potential for rapidly gathering important information on laboratory safety and infection.

Formal reporting programs have been implemented as part of patient and worker safety initiatives (9).

Two hundred cases of laboratory-acquired infections with parasites resulting from laboratory accidents from 1929 through 1999 have been previously published (1, 10). While the distribution of cases changed from decade to decade, the number of cases identified in each decade (19 to 28) remained relatively constant. Sharps-related injuries (e.g., needles and glass) were common factors and were often associated with the manipulation of research animals and the production of blood smears for malaria.

LABORATORY BIOSAFETY PROGRAM

Laboratory safety is an integral component of active programs that address the convergence of quality, safety, and risk (11). It involves all aspects of laboratory activity starting from before microorganisms arrive in the facility and progressing through the training of personnel, the establishment and monitoring of safe working practices, the proper use of reagents, materials, and equipment, the safe storage

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and transport of agents, and ultimately the terminal sterilization and destruction of microorganisms.

The International Organization for Standardization's Technical Committee 212 has developed an integrated suite of documents to improve the quality of medical laboratories, including ISO 15189:2012 (*Medical Laboratories—Requirements for Quality and Competence* [12]), ISO 15190:2003 (*Medical Laboratories: Requirements for Safety* [13]), ISO 22870:2006 [*Point-of-Care Testing (POCT)—Requirements for Quality and Competence* (14)], and ISO/TS 22367:2008 (*Medical Laboratories—Reduction of Error through Risk Management and Continual Improvement* [15]).

In those countries where laboratories are certified or accredited to ISO requirements, these documents are considered essential standards. ISO 15190:2003 addresses a range of topics relevant to medical laboratory safety, including management responsibility, the role of the safety manager, the need for a safety manual, implementation of a safety program, education, training, and competence, and performance of audits and review. It states that laboratory management is responsible for the safety of all employees and visitors to the laboratory and that ultimate responsibility rests with the laboratory director. The laboratory must identify an appropriately trained and experienced laboratory safety officer to assist the laboratory director and managers with safety issues. The laboratory safety officer must have the authority to stop activities that are deemed unsafe. The laboratory safety officer is responsible for designing and maintaining the laboratory safety program and is responsible for monitoring its effectiveness.

The elements of a laboratory safety program include development of the laboratory safety manual, safety audits, inspections (see Table 1 for audits required by ISO 15190:2003), risk assessments, and the maintenance of records. For further details, ISO 15190:2003 is available through the International Organization for Standardization website (www.iso.ch) or the Clinical and Laboratory Standards Institute (www.clsi.org).

LABORATORY SAFETY AND RISK MANAGEMENT

Laboratory biosafety is a working model of risk management where judgments on policies and procedures are implemented to avoid potentially negative outcomes, with the understanding that evidence and information may contain uncertainty (16). Biosafety is best viewed as a dynamic rather than a static program where a variety of factors, such as microbial pathogenicity, volume of sample, routes of exposure, workload, host health (immunosuppression or pregnancy), knowledge, and experience, can influence outcome. Using tools such as failure mode effects analysis (FMEA) and an internal laboratory audit can help identify weak points in the process and unappreciated hazards. Probability-severity tables can assess information, such as the likelihood and the severity of potential outcomes, and help determine the impact of different steps that can be implemented to mitigate risk. As described in ISO/TS 22367:2008 (*Medical Laboratories—Reduction of Error through Risk Management and Continual Improvement* [15]), the interventions required to address a low-hazard inconvenience that occurs commonly should be different from those required to address a high-hazard effect even if it occurs only rarely.

By taking a more dynamic view, levels of safety can be augmented through appropriate and selective use of enhanced practices, such as the combination of biosafety level 3 practices being used in biosafety level 2 containment facilities to address situations of increased risk associated with potential aerosolization of particular pathogens.

TABLE 1 Laboratory safety audits required by ISO 15190:2003[a]

Health and safety policy
Presence of written procedures that include safe work practices
Safety-oriented education and training of staff
Safety-oriented supervision
Use and maintenance of hazardous materials and substances
Health surveillance
First aid equipment and services
Accident and illness investigations
Health and safety committee review
Maintenance of records and statistics on accidents and near misses
Review of safety program
Regular site safety inspections

[a] See reference 13.

LABORATORY SAFETY AND PERSONAL ATTRIBUTES

A small but still-relevant matched case-control study of 33 laboratory workers who experienced a laboratory-associated injury over a 2-year period was previously described (17). Accident-involved persons were significantly more likely to have had a laboratory accident or laboratory infection prior to the 2-year study period and were significantly more likely to have a low opinion of laboratory safety programs. When the conditions surrounding the accidents were examined, 36% occurred when the employee was working too quickly, either just before lunch or at the end of the day. In 30% of accidents, the employee acknowledged a breech in safety regulations. This is consistent with the observation that organizational human error is most commonly the consequence of actions by well-meaning and usually well-informed people caused by slips and mistakes (18). One recent questionnaire-based study suggested that hospital laboratory workers may have less regard for an organizational safety culture and commitment than other hospital worker groups (19).

These observations continue to have relevance today. Laboratories continue to evolve with increasing samples and complexity. At the same time, the workforce is shrinking, and those that remain are older; this combination of circumstance is likely to result in increasing stress and time pressures, two factors that contribute to medical laboratory error (20, 21).

In a study in which laboratory practices were directly observed and then related to laboratory environmental contamination with hepatitis B virus (HBV), contamination was strongly related to a flawed technique and high workload. Unsafe work practices were also related, but to a lesser degree (22).

Over time, laboratorians have learned that even conventionally accepted practices can result in serious infection. Mouth pipetting, marking of blood spots, transport of samples to the laboratory in corked or sheathed sharps, recapping of needles, eating, and smoking were all at one time commonly practiced in properly run medical laboratories.

All of these practices are now appreciated as risky and are prohibited.

Injuries with sharp objects continue to be identified as an area of concern. Examination of bacterial culture plates with an eyeglass and sniffing plates to help identify organisms are now controversial (23). Laboratory safety requires diligent review and ongoing critique of current conventional practice, as well as openness to change when new risks are identified.

RISK-BASED CLASSIFICATION OF MICROORGANISMS

As a foundation for determining environmental requirements and best laboratory practices, the international community has developed a common risk classification of microorganisms. Group 1 biological agents are unlikely to cause human disease. Group 2 biological agents can cause human disease and might be a hazard to workers but are unlikely to spread to the community; there is usually effective prophylaxis or treatment available. Group 3 biological agents cause severe human disease and present a serious hazard to workers; they may present a risk of spreading to the community, but there is usually effective prophylaxis or treatment available. Group 4 biological agents cause severe human disease and are a serious hazard to workers; they may present a high risk of spreading to the community, and there is usually no effective prophylaxis or treatment available. A partial list of microorganisms by category is shown in Table 2.

While there is general consistency in classification of organisms by different countries, there are some examples where the same organism can be classified differently. This can have implications with respect to certification of the laboratory or to regulations surrounding transportation of these agents. Laboratories need to be aware of both domestic and international requirements prior to transport.

Increasingly, the emphasis has been to rely less on the classification of organisms and to focus more on the levels of safety with respect to containment and practices. For example, this approach allows for recommendations to contain organisms using the requirements of biosafety level 2 but to increase the caution by using a higher level of safety practices.

BIOSAFETY AND CLINICAL LABORATORY DESIGN

Knowledge of the classification of microorganisms flowing into a laboratory aids in the design of containment equipment and facilities. For research laboratories where the microorganism load is known, the process of matching risk and containment is straightforward. In the clinical laboratory, the content of samples is more likely to be unknown and may in certain situations contain microorganisms across the spectrum of classification. That being said, most isolates recovered from clinical samples can be categorized within biosafety level 1 or 2; thus, most facilities require containment level 2 (Table 3). The design elements required for containment level 2 laboratories are described in Table 4.

Laboratories that process viral cultures or investigate mycobacterial cultures should be designed to accommodate biosafety level 3. For many clinical laboratories, biosafety level 3 is considered beyond the scope of their practice.

In the wake of the 2001 terrorist attacks in New York and the anthrax mail scares that happened shortly afterwards, the interest in laboratory biosafety measures as a component of bioterrorism defense was brought to the fore. Specific funding has been allocated for construction of new national and regional biocontainment laboratories. Laboratories with increased levels of containment and security not only are of benefit for bioterrorism and biosecurity but also provide facilities to address potential epidemics, such as severe acute respiratory syndrome (SARS) and potential

TABLE 2 Risk-based classification of microorganisms[a]

Group	Bacteria	Viruses	Fungi	Parasites
1				
2	*Bacillus* species (not *B. anthracis*), *Clostridium* species, *Corynebacterium diphtheriae*, *Escherichia coli*, *Enterobacteriaceae*, mycobacteria (other than M. *tuberculosis*), *Staphylococcus* species, *Streptococcus* species	Adenovirus, calicivirus, coronavirus (not CoV)[b], herpesvirus, influenza virus	*Cryptococcus* species, *Candida* species, all dermatophytes, *Aspergillus* species	All clinical parasites
3	*Bacillus anthracis*, *Brucella species*, *Coxiella burneti*, *Francisella tularensis*, *Mycobacterium tuberculosis*, *Mycobacterium avium*	Lymphocytic choriomeningitis virus, Hantaan virus, St. Louis encephalitis virus, Japanese encephalitis virus, Western equine encephalitis virus, West Nile encephalitis virus, SARS coronavirus, prions	*Coccidioides immitis*, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Paracoccidioides braziliensis*	
4		Lassa virus, Marburg virus, Ebola virus, herpesvirus simiae		

[a] Based upon *Laboratory Biosafety Guidelines* (146). Note that this table is presented as a guide only. It should not be considered either as complete or as consistent with regulations in all jurisdictions. Specific national requirements may differ from this table as presented.

[b] not CoV, coronaviruses other than SARS coronavirus.

TABLE 3 Internationally accepted classification of microbial pathogens by risk group[a]

Risk group	Attributes
1	No or low individual and community risk. The microorganism is unlikely to cause human or animal disease.
2	Moderate individual risk and low community risk. The pathogen can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock, or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventive measures are available, and the risk of spread of infection is limited.
3	High individual risk but low community risk. The pathogen usually causes serious human or animal disease but does not ordinarily spread from one infected individual to another. Effective treatment and preventive measures are available.
4	High individual and community risk. The pathogen usually causes serious human or animal disease and can be readily transmitted from one individual to another, directly or indirectly. Effective treatment and preventive measures are not usually available.

[a] See reference 138.

influenza pandemics. In order to protect workers, the public, and the environment, laboratories need to ensure that access to their facilities is controlled, that procedures for specimen receiving and disposal are controlled, that incident reporting and emergency response plans are in place, that stored agents are under high security, and, for research facilities, that animal tracking and trace back systems are accurate (24–26). Caution needs to be taken to ensure that the needs for confidentiality and information containment do not interfere with the open communications necessary for laboratory safety and public health (27).

Handling of exotic pathogens of risk group 4 requires high containment level 4 facilities. Discussion of these facilities is beyond the scope of this chapter.

The essential practice standards for microbiology laboratories are cited in Table 5.

TABLE 4 Laboratory design requirements for biosafety level 2 laboratories[a]

Laboratory design requirement
1. The laboratory should be separated from public areas by lockable doors.
2. Laboratory doors should have appropriate signage.
3. Door openings should be of sufficient size to allow safe passage of equipment.
4. Work surfaces should be nonabsorptive and scratch, stain, chemical, and heat resistant.
5. The means of waste treatment should be readily available prior to disposal.
6. Windows should be designed to prevent ingress of flying insects.
7. There should be separate spaces for street and laboratory clothing.
8. Hand-washing sinks should be readily accessible.
9. There should be emergency access to showers and eye wash stations.
10. Biosafety cabinets and other primary containment equipment are recommended.
11. Hands-free operation of hand-washing sinks is recommended.
12. The air supply should be 100% outside air with no recirculation (recommended).

[a] Adapted from *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed (30), and *Laboratory Biosafety Guidelines*, 3rd ed. (146).

SAFETY EQUIPMENT AND THE CLINICAL LABORATORY

Splashguards

Splashguards made of clear glass or plastic represent the minimum level of equipment for protecting workers. They are more likely to protect workers from gross splashes than from aerosols. They can be an appropriate alternative to biosafety cabinets for opening vacuum blood tubes. They should be of sufficient size and be cleanable to remove the occasional blood splatter. The effectiveness of splashguards depends on appropriate placement with respect to both workflow and worker height. It is inappropriate to obscure vision by using splashguards as a convenient location for taped memos and procedures.

Biosafety Cabinets

Biosafety cabinets can protect the laboratory worker and the laboratory environment from splashes and aerosols and also reduce the risk of sample contamination (28–30). Biosafety cabinets are enclosed units with variable degrees of openness and access and which control exhausted air. Class 1 cabinets have an open front but work under negative pressure and exhaust their air through a HEPA filter. Exhausted air usually is returned to the work area. Class 2 cabinets increase the level of safety by including a HEPA-filtered downward-flow air curtain designed to increase the degree of separation between room air and interior cabinet air. Class 2 cabinets may exhaust air back into the room (class 2A) or outside the building environment through an exhaust system (class 2B). Class 2B cabinets can be further subclassified based on additional features. Class 3 cabinets are completely enclosed, providing gas-tight containment. They are accessible only through front-end glove ports. Class 3 cabinets provide the most suitable containment for working with exotic pathogens. Class 3 cabinets must be vented through ductwork supplemented with double HEPA protection and incineration. Because class 1 and class 2A units exhaust to the laboratory air, they are unacceptable for use with volatile chemicals and reagents. Biological safety cabinets cannot be used as alternatives to chemical fume hoods.

Many institutions require that safety cabinets be tested and certified to the National Sanitation Foundation standard NSF/ANSI 49:2009 (*Biosafety Cabinetry: Design, Construction, Performance, and Field Certification* [42]) by a qualified person on a regular basis to ensure that they maintain their required face velocity and negative pressure.

Chemical Fume Protection

A fume hood is a mechanically ventilated, partially enclosed workspace where harmful volatile chemicals and reagents can be handled safely. The primary function of a fume hood is to contain and remove gases and vapors.

TABLE 5 Standard microbiology practices for all laboratories of biosafety level 2 and greater[a]

Standard practice
1. A documented safety manual must be available for all staff.
2. Personnel must receive training on the potential hazards associated with the work involved and the necessary precautions to prevent exposure to infectious agents.
3. Eating, drinking, smoking, storage of either food or personal belongings, and application of cosmetics are not permitted.
4. Mouth pipetting is prohibited.
5. Long hair is to be tied back or restrained.
6. Access to laboratory and support areas is limited to authorized personnel.
7. Doors to working areas in laboratories must not be left open.
8. Personnel's open wounds and cuts should be covered with waterproof dressings.
9. Laboratories are to be kept clean and tidy.
10. Protective laboratory clothing, properly fastened, must be worn by all personnel, including visitors, trainees, and others working in the laboratory. Suitable footwear requires closed toes and heels.
11. Eye and face protection must be used where there is a known or potential risk of exposure to splashes or flying objects.
12. Gloves must be worn for all procedures that might involve direct skin contact with biohazardous material. Gloves are to be removed at the completion of the laboratory task and before leaving the laboratory.
13. Protective laboratory clothing must not be worn in nonlaboratory areas.
14. If a known or suspected exposure occurs, contaminated clothing must be decontaminated before laundering.
15. The use of needles, syringes, and other sharp objects should be strictly limited. Needles should not be bent, sheared, recapped, or removed from the syringe; they should be promptly placed in a puncture-resistant sharps container for disposal.
16. Hands must be washed with an appropriate disinfectant after gloves have been removed before leaving the laboratory and at any time after materials known or suspected of being contaminated are handled.
17. Work surfaces must be cleaned and decontaminated with a suitable disinfectant at the end of the day and after any spill of potentially biohazardous material.
18. Contaminated materials and equipment leaving the laboratory for servicing or disposal must be appropriately decontaminated.
19. Autoclaves used for decontamination should be regularly monitored with biological indicators.
20. All contaminated materials must be decontaminated before disposal or reuse.
21. Leakproof containers are to be used for the transport of infectious materials.
22. Spills, accidents, or exposures to infectious materials and losses of containment must be reported immediately to the laboratory supervisor.
23. An effective rodent and insect control program must be maintained.

[a] Adapted from *Biosafety in Microbiological and Biomedical Laboratories*, 5th ed (30), and *The Laboratory Biosafety Guidelines*, 3rd ed. (146).

Most fume hoods use ducts and a fan to ensure that heat and airborne contaminants are captured, transported out of the work area, and eventually discharged into the atmosphere outside the building. Chemical fume hoods differ from biosafety cabinets in that they are usually all ducted. They must be constructed of noncombustible materials, and they must also be explosion-proof.

Nonducted or recirculating fume hoods are of limited use in the laboratory and should not be considered acceptable substitutes for ducted fume hoods for containment of volatile chemicals (31). Special fume hoods are designed to protect workers from specific highly corrosive reagents or chemicals, such as perchloric acid and radioisotopes.

Installation of Biological Safety Cabinets and Chemical Fume Hoods

These large pieces of safety equipment protect users and the environment through the use of negative air pressure drawing air away from the user and through the equipment. This requires air to move at sufficient volume and velocity without developing turbulence. This can be measured using the ASHRAE 110 tracer gas test. Airflow can be negatively affected by overcrowding the cabinet or stacking of equipment against either the front or the back grill. This will disrupt the airflow, resulting in backwash out the front of the unit, and impair dexterity (32, 33).

Cabinets that are improperly positioned in relationship to each other or in relationship to room space and ventilation outlets can result in airflow imbalance and reversal (34), thereby increasing rather than decreasing the risk to the user. When airflow is being measured, the tester should not only test individual pieces of equipment but also ensure that airflow is not hampered when more than one cabinet is being used at the same time. Installation of equipment must take into consideration the proximity of equipment as well as timing of use.

Centrifuges

Although the safety centrifuge was first described in 1975 (35), accidental contamination of the laboratory and personnel is regularly reported (36, 37). While other equipment may result in greater aerosol dispersal, the commonness of centrifuge use increases its potential impact on laboratory risk (38). Accidents occur because of lack of tight seals on containers and rotors. Centrifuges can be susceptible to contamination because prolonged use without regular inspection can lead to worn O-ring container seals (39). Plastic centrifuge tubes can crack or distort and result in increased risk (37). Accidents can be avoided when centrifuges are included in preventive maintenance and are used safely. Lids must be closed at all times during operation. The centrifuge should not be left until full operating speed is attained and the machine is running safely without vibration. If vibration does occur, the centrifuge should be stopped immediately and load balances checked. Swing-out buckets should be checked for clearance and support. It is recommended that rotors and cups be cleaned and disinfected after each use with noncorrosive cleaning solution because of unrecognized leaks (40). (Cleaning and disinfection after each use may be a difficult regimen for all laboratories. A less frequent but regular locally established routine may be an acceptable alternative.) All spills and breakage should be reported to the laboratory safety officer and should be cleaned immediately, after first vacating the laboratory to give time for aerosols to settle. Depending on the materials involved in the spill and the air handling in the facility, aerosols may remain suspended for over 35 min (38). In the context of quality control, a log should be maintained and include the rotor serial number, speed in revolutions per minute, the duration of spin, times of use, and the operator's name.

Sharps Protection

Scalpels, needles, and broken glass, as well as other sharps, are commonly associated with wound injuries and laboratory-acquired infections (41). To the extent possible, the use of sharps should be eliminated or safety barriers implemented (5, 43). Sharps may be contaminated with infectious or cytotoxic agents or both. All sharps should be considered potentially infectious and be securely discarded in an appropriate sharps container.

Sharps containers used in medical laboratories should be specifically designed for the containment and disposal of needles, syringes with needles, scalpel blades, clinical glass, or other items capable of causing cuts or punctures (43). If containers are not resistant to penetration or compression, they pose a health risk to those involved in their handling and disposal.

The characteristics of well-designed containers are that they are leakproof and puncture resistant, do not degrade in autoclaves, either require no assembly or are easy to assemble, are appropriately labeled, and are available in a variety of sizes. Within this framework, manufacturers can implement a variety of designs. Sharps containers should be stable enough to resist toppling over and durable enough to withstand being dropped onto a hard surface (44). They should be constructed in a manner that ensures sufficient puncture resistance. International standards recommend a thickness able to resist a puncture pressure of 15 to 20 N (45, 46).

Using locally available tin cans or other containers in lieu of designed containers is inappropriate, as they do not address important aspects of containment.

Sharps containers must have a prominently displayed universal biohazard symbol. In addition, sharps containers intended for sharps contaminated with cytotoxic agents must display the cytotoxic hazard symbol. The international color code is yellow for biohazardous medical sharps and red for cytotoxic medical sharps.

Containers should not be filled to more than three-quarters of their maximum capacity to avoid accidents from overfilling, and sharps should never be forced into the container. Properly designed containers have a designated fill line.

Most sharps containers are designed for single use only. Once filled, they are securely and irreversibly closed for containment. Following autoclave treatment, the containers should be disposed of in accordance with local requirements. Containers with cytotoxic sharps or probable prion proteins should not be autoclaved but rather should be incinerated.

To reduce the challenges associated with disposal of single-use containers, reuse container services can provide collection and transport to an offsite location for safe, secure reopening, emptying, and decontamination prior to redistribution for reuse.

Medical Waste as an Infection Hazard

Medical facilities generate large volumes of waste that can be hazardous to workers and the community (47).

Liquid medical laboratory waste, such as urine, in many jurisdictions can be disposed of directly through a drain leading to the sanitary sewer system, although some jurisdictions require prior decontamination using a product such as 10% bleach. The remainder of medical waste requires sterilization.

In a recent survey, most university hospitals in the United States continue to use autoclaves to sterilize medical waste. Autoclaves effectively sterilize biological waste at a temperature of 121°C and with pressure at 100 kPa for 15 min. The more densely packed the autoclave is loaded, the longer it can take to reach these conditions in the middle of a load. Many organizations do not monitor autoclave effectiveness appropriately with biological indicators (48). Contractual arrangement with biomedical waste management organizations may be an economic or environmental alternative. Alternatives, including microwave inactivation, can be considered if the necessity of required rigorous conditions is recognized (49). In some areas where economic or environmental issues are of extreme concern, solar disinfection (50) of biomedical waste may be a viable consideration. In rare circumstances when zoonotic outbreaks are addressed and, more commonly, in resource-limited regions, incineration of medical waste continues to be performed, even though it has been demonstrated to be harmful to health and the environment (51).

SAFETY AS A QUALITY MANAGEMENT INITIATIVE

Safety Preparedness and the Material Safety Data Sheet

Guidelines for the protection of laboratory workers describe the preparations required in case of accidents. In the event of an accident, a prompt and appropriate response requires preparedness, rapid access to critical information, and equipment. Every laboratory should have a written and prepared plan in case of emergency. Routes for evacuation in case of fire or spill should be planned and practiced on a regular basis. Personnel trained in first aid should be readily available. Up-to-date material safety data sheets (MSDS) for all chemicals and microorganisms should be available. The locations of emergency equipment, including showers and eyewash stations, should be known to all and should be tested on a regular basis to ensure functionality at the time of need. Equipment and materials for containment of spills, including personal protective equipment, absorbent, and disinfectant (bleach or accelerated hydrogen peroxide), should be readily available and their locations known. Accident preparedness should be the subject of a regular internal safety audit program.

MSDS for chemicals should be obtained from the supplier or may be available from a variety of commercial and free Internet sites. A list of appropriately formatted MSDS for microorganisms is available through the Public Health Agency of Canada website (http://www.phac-aspc.gc.ca/msds-ftss/).

Every incident must be reported to appropriate management and the required authority. Every incident should be internally investigated for details of the accident, root causes, and the steps necessary to prevent similar events. Recommendations for required changes should be the subject of safety audits to ensure that they have been acted upon in a timely manner.

Hand Washing and the Use of Personal Protective Equipment

Hand washing is the single most useful technique to stop the transmission of microorganisms and acquisition of infection in medical laboratories (52). Hands can be contaminated during sample collection, handling of sample containers, handling of contaminated equipment, and touching of sample storage units.

While contamination can be reduced by the use of gloves, gloves alone are not completely effective. Hand hygiene can be performed with running water and either plain or antimicrobial soaps. Nonmedicated detergent-based soap products and water alone do not disrupt normal skin microbiota but can have some effect on the reduction of transient hand microbiota, including both bacteria and viruses (52, 53). The efficacy is directly related to the duration of hand washing. Plain soaps, like other products, can be associated with detrimental effects, including skin drying and irritation.

It is less clear whether soaps containing antimicrobial products are essential for the vast majority of hand-washing situations, even in the microbiology laboratory. For example, hand washing with plain soap is as efficacious as washing with antiseptic soap for removing *Clostridium difficile* (54), nonenveloped viruses, and *Bacillus anthracis* (55). The two product types are equally efficacious for the removal of common bacterial pathogens (53, 56).

Most laboratories continue to regularly use antiseptic soaps. Selection of an appropriate product for the laboratory depends upon both the types of organisms in specimens processed by the laboratory and issues such as fragrance, consistency, and potential for irritation and skin drying. Commonly used products contain chlorhexidine, iodophors, triclosan, or related compounds. Other ingredients, such as tea tree oil, are acceptable alternatives (57).

Waterless alcohol-based (60 to 62%) products can be a rapid and convenient alternative to conventional hand washing, especially when a sink with running water is not immediately accessible (53, 58). Used correctly, alcohol-based hand gels are as active as traditional 70% alcohol for removing MRSA, *Serratia marcescens*, and *Candida albicans* from contaminated hands (59). Alcohol-based products have reduced efficacy when hands are contaminated with spore-forming organisms, including *C. difficile* or *B. anthracis* (55) and nonenveloped viruses (53). In addition, alcohol-based products should not be relied upon when hands are visibly soiled, contaminated with proteinaceous materials, or contaminated with materials that have a high microbial load.

It is common in many laboratories to place alcohol-containing bottles by hand wash sinks. Others have noted that health care workers are more compliant with hand care when agents are close to the site of contamination (60). Accordingly, laboratories might consider having containers of alcohol-containing hand gel closer to workstations.

Gloves

Gloves can provide an important barrier within the laboratory, provided that they are used appropriately. Clearly, gloves are essential to prevent damage when hands are exposed to heat, cold, and toxic materials. Insulated gloves are essential when taking materials out of −70°C freezers or autoclaves or when hands are exposed to liquid nitrogen. In the microbiology laboratory, gloves create a protective barrier to reduce the risk of hand exposure to pathogens and their products.

General-purpose utility gloves ("kitchen" or "rubber" gloves) provide ample protection for clearing up biological spills, for cleaning, and for decontamination. Utility gloves can be cleaned and reused, although they should regularly be examined for cracks, tears, and peeling. Damaged utility gloves should be discarded. Utility gloves may be inappropriate when handling chemical solvents and should not be used. Chemical-resistant gloves should be available in all laboratories that handle chemical solvents and other toxic chemicals and dyes.

The degree of protection that gloves can provide depends upon many factors, including composition, size, fit, grip, and thickness, all of which can affect user dexterity. Latex-containing gloves may provide superior fine finger dexterity, which might be associated with fewer spills or accidents (61).

Disposable gloves of latex, vinyl, or nitrile can provide an effective barrier, especially when handling blood, body fluids, and excrement. This is especially true since open abrasions on hands may often go unnoticed (62). In specific settings, gloves of increased length (to the elbow or shoulder) may be appropriate. Gloves are easily torn. In-use durability studies indicate that vinyl gloves may tear as often as 40% of the time, depending upon the presence of powder and the length of the user's fingernails (62). Latex gloves are more durable but may be associated with atopic reactions.

Disposable gloves do not provide significant protection against needle stick injury. If sharps exposure and the risk of injury are possible, double gloving can provide some protection. Cotton undergloves provide more protection than a second vinyl or latex glove. In the morgue, use of chain mail gloves may be appropriate.

Gloves provide an important protective barrier; however, they may also be a source of harm. Using vinyl, latex, or cotton undergloves can reduce the contact irritation noted with rubber gloves. Excessive glove use can result in moisture damage to skin (63–65). Surveys of dentists wearing gloves for 6 h daily indicated that many, especially young women with preexisting eczema, suffered from glove intolerance (66). In addition, contaminated gloves often result in contamination of equipment (67) and can also contribute to transmission of serious infection (68).

While it has become common for phlebotomists to wear gloves during specimen collection, this may not be essential if the risk of exposure to blood and body fluids is considered sufficiently low and gloves of an appropriate size or material are not readily available.

A common concern in the microbiology laboratory is the appropriateness of regular glove use while working on the bench in a routine laboratory. In the accessioning area, where original sample containers are handled, gloves are appropriate because it is not uncommon to have sample on the outside of the container.

There is little evidence to support or reject glove use while handling culture plates. On the one hand, gloves may provide a layer of protection in case a plate is dropped or mishandled. On the other hand, poorly fitting gloves can reduce dexterity and increase risk. Glove use may be a topic of personal preference or local policy.

Wearing gloves is appropriate when protecting an existing hand injury; however, prolonged glove use can induce further moisture-associated skin injury. If gloves are worn, it is critical for the user to remove them at the end of the task or when the task is interrupted and whenever he or she leaves the bench in order to reduce the risk of laboratory contamination.

Regardless of the types of gloves and their compositions, it is essential that the user always wash his/her hands as soon as gloves are removed, either with running water and soap or, in many settings, with alcohol-containing hand hygiene products.

Respirator Masks

For health care workers with direct exposure to patients, including phlebotomists, it is a common recommendation to require wearing an N95 mask while performing certain tasks. Many institutions require that people wearing an N95 mask pass a qualitative-fit test or quantitative testing (69, 70), which can be time-consuming, costly, and subjective.

An alternative protection may be provided by use of a surgical mask. Surgical masks are not fit tested, are less uncomfortable (71), and provide an equal level of protection (71–73). While caution and reasonable practices have been demonstrated to contain outbreaks, effective influenza immunization in conjunction with personal protective equipment is required for optimal protection.

Ultimately the choice of usage will be governed by regulatory requirements.

Immunization

Immunization provides protection against some laboratory-acquired infectious diseases but should be considered secondary to mental alertness and good laboratory practices. Immunization may not prevent infection but can protect against serious illness. All adults, including pregnant women, should have a complete primary immunization with tetanus and diphtheria toxoids and should receive a booster every 10 years (74).

Laboratory workers, especially those with direct patient exposure, should receive annual influenza immunization (75). Similarly, those with direct patient exposure, especially to children and infants, should consider an immunization booster for pertussis (75). All staff with possible occupational exposure to human blood and body fluids should receive hepatitis B vaccine (75). The value of meningococcal immunization for laboratory workers has been discussed (76–78). Cases of meningococcal illness possibly linked to laboratory exposure have been published, and it has been recommended that microbiologists routinely exposed to meningococci, especially if aerosolized, should consider meningococcal immunization. Laboratorians working with specific pathogens and in specific situations should consider additional immunizations, for example, human diploid cell rabies vaccine, typhoid vaccine, and vaccinia vaccine (79, 80).

In the past, *Mycobacterium bovis* BCG vaccination has been considered of value for health care workers. However, it is no longer recommended as a primary tuberculosis (TB) control strategy because the protective efficacy of the vaccine in health care workers is uncertain and immunization with BCG may cause difficulty in the interpretation of tuberculin skin test responses caused by true infections with *Mycobacterium tuberculosis*. In laboratory and other health care workers with positive tuberculin skin tests, the new interferon gamma release assays can help differentiate likely TB exposure (positive) from BCG vaccination (negative).

Notable Laboratory-Acquired Infections

While the variety of microorganisms associated with laboratory-acquired infection is broad, certain ones have stood out over time as being of increased concern.

(i) *Brucella* Species

Brucellosis is a frequent cause of laboratory-acquired infection, especially in laboratories in regions of endemicity (81, 82) or in research facilities. In the clinical laboratory, infrequency and lack of familiarity with *Brucella* species is a risk factor, especially if relevant clinical information does not accompany the sample. *Brucella* species are pathogenic, even with very low inocula. The route of laboratory-acquired infection is often speculative, but direct exposure to hands and procedures that increase aerosols are thought important (83–85).

Often, small Gram-negative bacilli recovered from blood, joint fluid, cerebrospinal fluid (CSF), or pleural fluid are not appreciated as possible *Brucella* species (86), and work may continue on an open workbench, increasing the risk of exposure. Early action may be impeded as a result of biochemical misidentification as *Moraxella* species (87) or *Ochrobactrum anthropi* (88, 89).

Once the possibility of a *Brucella* isolate present on an open bench is raised, laboratories should transfer all work to a more secure laboratory environment with a properly working biological safety cabinet. In the event of a suspected or documented accident leading to possible exposure to *Brucella* species, laboratory workers can be monitored for seroconversion or clinical illness. In high-risk situations, prophylaxis with doxycycline, rifampin (90), or trimethoprim-sulfamethoxazole with or without rifampin (87) should be considered. Immunization with a *Brucella* vaccine is currently unavailable, although much progress is under way (91).

(ii) *Francisella tularensis*

Francisella tularensis is a Gram-negative coccobacillus associated with zoonosis-associated pneumonia (30, 92–95) and potentially with bioterror events (96). Because of its infrequency, delayed identification may occur among laboratory workers (94). This reinforces the value of taking a cautionary approach toward the isolation of small Gram-negative bacteria. Potential exposure to *Francisella tularensis* should raise the consideration of doxycycline (97) for prophylaxis in laboratory workers.

(iii) *Burkholderia pseudomallei*

Melioidosis is a significant cause of fatal sepsis (98). It is caused by *Burkholderia pseudomallei*, which is commonly found as an environmental saprophyte in Southeast Asia (30, 98, 99) and northern Australia but is also found in the Caribbean (100) and in Central America (101). Laboratory exposures can potentially result from many routine practices and result in serious infection. While definitive human data are lacking, animal studies strongly suggest that prophylaxis with co-trimoxazole, started as soon as possible after exposure, can be highly effective in preventing clinical infection (101–103).

(iv) *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is internationally accepted as a risk group 3 bacterium (Table 3). Although there are no statistics readily available, it is likely the most common risk group 3 microorganism to which clinical laboratory workers are exposed. Tuberculosis is one of the 10 most commonly reported laboratory-acquired infections (104). Because of the silent and delayed nature of conversion and frequency of tuberculosis in the community, it can be difficult to trace skin test conversion to laboratory exposure. The organism is most commonly in sputum or urine samples, which may or may not specify or request examination for possible *M. tuberculosis* infection. Handling samples, especially sputum, under a biological safety cabinet can reduce exposure to aerosols (104). It is most commonly transmitted through aerosol production. Clinical laboratory worker exposure may be difficult to trace (104), although exposure to aerosols and autopsy (105, 106) are recognized. Laboratory workers with frequent direct patient exposure are also at risk (107). Laboratory workers should have annual skin tests.

(v) *Coccidioides* Species

Coccidioidomycosis is a lung infection associated with *Coccidioides immitis* or *Coccidioides posadasii*, commonly regionally located (30). In 60 to 65% of cases, this infection is asymptomatic (108). Laboratory workers are potentially at risk due to culture exposure (109) or exposure during autopsy (110). *Coccidioides immitis* has been previously identified as a potential agent for bioterror (109), although it was removed from the list of select agents and toxins in 2012. While respiratory exposure to *Coccidioides* species by laboratory workers would be concerning, most infections are asymptomatic or self-limited. Immunoincompetent individuals may be at greater risk (111). The value of prophylaxis with antifungal agents is unproven but may be recommended (112, 113).

(vi) Laboratory-Acquired Viral Infections, Including HIV, Hepatitis B, and Hepatitis C

A broad range of surveys and case reports describe viral infections in laboratory workers (104, 114–120). Over the last 75 years, the variety and frequency of laboratory-acquired viral infections have progressively increased, perhaps reflecting greater awareness and improvements in diagnosis. Most are reported from research and/or veterinary laboratories. When reported, the most probable routes of infection include percutaneous infection (sharps), aerosol (fomite) inhalation, and oral ingestion, indicating the importance of laboratory safety practices. The incidence of new cases of human immunodeficiency virus (HIV) in the United States has stabilized to between 40,000 and 50,000 new cases per year (121). There are no recent reported cases in laboratory workers in the United States, but that may not be true for all countries (122–124). Following a deep tissue exposure injury, such as with a needle or scalpel, it is recommended that workers consider postexposure prophylaxis (PEP), even though PEP does not always prevent HIV infection following an exposure (125).

Laboratory workers are at risk of blood-borne infections, such as hepatitis C virus (HCV), HBV, and HIV. However, the safeguards introduced into medical laboratories have decreased the risk to workers.

There is little information on the prevalence of hepatitis C in health care workers, although it is estimated by the National Center for Infectious Diseases (126) that after needle stick or sharps exposure to HCV-positive blood, about 2 (1.8%) health care workers out of 100 will get infected with HCV (range, 0% to 10%). A recent review of a 14-year prospective study did not find health care workers at a heightened risk of HCV as a consequence of sharps injuries (127). Following the introduction of hepatitis B vaccine in 1982, the incidence of hepatitis B infection was reduced by over 95% (5).

While blood-borne infection information may be thought to be well established, recent knowledge surveys of health care workers have demonstrated that education sessions to update existing staff and inform new staff are regularly required (128).

(vii) Influenza and Other Severe Viral Respiratory Infections

Influenza and other severe outbreaks are a common event in many communities. Study of influenza outbreaks (129–131) has pointed out potential gaps in biosafety protection for laboratory workers. Although laboratory workers may be at risk (132), the level of risk is low because in-laboratory containment is easily addressed (133, 134). Laboratory workers are among those with the lowest incidence of illness or serological conversion (132). That being said, phlebotomists and microbiology and accessioning laboratory workers receiving respiratory samples, especially via vacuum tube delivery systems, were deemed to be at increased risk. Laboratories equipped and designed to biosafety level 2 should use practices more consistent with biosafety level 3, which require all samples to be opened and handled only under a biosafety cabinet (30, 133). In addition, centrifugation of samples should be performed only with sealed safety buckets that are opened within a cabinet. Frequent hand washing with either soap and running water or alcohol-based hand gels should be required (131).

(viii) Laboratory-Acquired Infections and External Quality Assessment

Infections acquired in the clinical laboratory are not always associated with clinical samples. Documented clusters of bacterial infections have been associated with samples sent to laboratories for proficiency testing (135). Contamination of other samples by quality control organisms has also been documented (136), although no in-laboratory infections resulted. Regardless of source, all viable microorganisms processed within a clinical laboratory must be handled with full awareness of appropriate biosafety practices.

(ix) Prions

Samples from patients with Creutzfeldt-Jakob disease (CJD) may be submitted to a laboratory for investigation. To date, there are no known cases of laboratory-acquired CJD, and there is no evidence that laboratorians are at increased risk of developing CJD. That being said, the progressive deteriorating nature of CJD raises concerns for those handling samples from patients with CJD, especially those of neurological origin (137). This represents a special problem in medical laboratories because of the difficulty of inactivating the underlying prions.

Current precautions while handling tissue samples (brain, spinal cord, dura mater, pituitary, and eye samples) require full use of personal protection equipment to protect the worker and the workspace (138, 139). All tissue samples must be discarded as medical waste. No special precautions are required for disposal of body fluids.

Equipment that has been exposed to CJD patient tissues should either be discarded or, if tolerant to heat, be autoclaved at 134°C for 18 min (prevacuum sterilizer) or 121°C to 132°C for 1 h (gravity displacement sterilizer). Autoclaving in water may be more effective than autoclaving in its absence (140). Equipment may also be soaked in 1 N NaOH for 1 h (140, 141). Work surfaces should be cleaned with a 1:10 dilution of sodium hypochlorite.

Milder chemical treatments based upon combinations of enzymes, including proteinase K and pronase, in conjunction with detergents, such as alkaline cleaners or SDS, have been shown to experimentally reduce PrPSc material to levels below detection and to prevent infectivity. The advantages of these methods are described as being inexpensive, noncorrosive, and nonhazardous to staff (140, 141). *In vitro* studies suggest that maintaining equipment in a moist environment reduces the adhesion of PrPSc materials to surgical stainless steel and can increase the efficacy of decontamination (142).

Safety and Point of Care

Medical laboratories are responsible for all aspects of laboratory testing throughout the total testing cycle, even when the testing is performed outside the laboratory itself. Increasingly, medical laboratories are responsible for point-of-care testing (POCT), such as blood glucose monitoring, coagulation, and oxygenation. Despite improvements in equipment and hygiene protocols, cases of hepatitis B and C continue to be associated with point-of-care devices. Prevention of transmission requires strict adherence to infection control protocols for equipment cleaning (143).

Safety Considerations Associated with MALDI-TOF Mass Spectrometry

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis is a new process that provides rapid identification of microorganisms through mass spectrometry of microbial proteins. The procedure includes application of the microbial target onto the sample plate, where it can be exposed to laser light. When addressing organisms, particularly those of risk group 3, in order to reduce potential biohazardous exposure, the sample can be rapidly (30 min) inactivated by exposure to trifluoroacetic acid (TFA), with minimal risk of altering the protein mass spectrometry peak fingerprint (89). Regardless of prior treatment, upon completion, all samples should be considered biohazardous waste and be treated accordingly.

Transportation of Samples

Laboratories have a responsibility to prevent exposure to infectious agents in laboratory samples to the highest extent possible. This responsibility includes during the transport of samples to and from the laboratory. For local transport within a clinic or hospital, the laboratory should ensure that leakproof containers are available and that the sample is transported in secure outer packaging, such as a sealable plastic bag, preferably emblazoned with the international biohazard label. For transport outside the facility, especially by road, rail, ship, or air, local and federal regulations apply. Even for short distances, it is appropriate that samples be transported in secure, firm outer containers with sufficient absorbent materials inside in case of a spill. In most jurisdictions, transport of samples with infectious agents by postal services is prohibited.

Air transport is under the authority of federal regulations directed by the requirements of the International Civil Aviation Organization (ICAO) as adopted by the International Air Transport Association (IATA). ICAO specifies the packaging and labeling requirements, including the proper shipping name and appropriate UN number, for samples known to contain infectious agents based on the source of the sample and the likely pathogens contained. Every laboratory that transports samples is required to have at least one person certified as knowledgeable with respect to packaging and transport requirements, including the completion of shipping documents.

For additional information, direct reference can be made to the International Civil Aviation Organization technical instructions for the safe transport of dangerous goods by air or to federal requirements (144).

Postexposure Management for Accidents Involving Infectious Agents

It is beyond the scope of this chapter to address the specifics of medical management following accidents that involve infectious agents. However, it is important that the following steps always be undertaken or considered. Every accident or injury, including those that are seemingly trivial (1), should be reported to the appropriate safety officer or supervisor. Scratches and puncture wounds should be cleaned immediately. For some injuries, especially those involving blood exposures, time may be a critical factor (145). If it is deemed appropriate to seek medical attention, it is important to indicate that the accident was laboratory acquired. If the likely or probable agent is known, bringing the microorganism MSDS can be helpful and save time. In research or animal facilities, preparation of information sheets on specific organisms and the availability of standardized investigation and treatment protocols in the event of an accident can be invaluable.

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Decontamination, Disinfection, and Sterilization

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Decontamination, disinfection, and sterilization are basic components of any infection control program. Patients expect that any reusable instrument or device used for diagnosis or treatment has undergone a process to eliminate any risks for cross-infection. However, the infection control literature documents many reprocessing failures, including numerous reports of transmission of nosocomial pathogens from contaminated endoscopes (1–5). Before 1990, it was very difficult to prove a causal relationship between a contaminated device and a subsequent nosocomial infection. Today, state-of-the-art clinical epidemiology supported by molecular typing tools such as pulsed-field gel electrophoresis, PCR, and genome sequencing can enable the hospital epidemiologist to prove a causal relationship between the use of a contaminated device and a consequent infection. Molecular epidemiology has thus provided scientific tools that can identify the limitations of the available disinfection and sterilization methods and can provide the impetus to improve reprocessing technologies. Despite those advances, little research has been done that will lead to major breakthroughs in disinfection and sterilization in the near future. Thus, we believe the key issues instead will be to standardize and optimize our applications of current knowledge.

Clearly, more research is needed in this field, but resources for such work have been limited. In fact, most disinfectants were introduced to the market more than 30 years ago, and little is known about their modes of action and the mechanisms of resistance. Excellent reviews on this topic have been published by Block (6) and by Russell and coworkers (7, 8). In addition, few basic procedures in decontamination, disinfection, and sterilization have been tested in randomized clinical trials. In this chapter, we have tried to cite the highest level of evidence available. However, given the dearth of studies, we have often had to cite results of observational studies, animal models, *in vitro* tests, and expert opinion because higher levels of evidence were unavailable.

Despite the lack of resources, reprocessing techniques, disinfectants, and general infection control practice have garnered more attention recently than in the past. This is due in part to the increasing frequency of multiresistant bacterial pathogens at a time when pharmaceutical companies have shifted from developing antimicrobial agents to designing drugs for chronic diseases (9). Moreover, new pathogens, such as the viruses causing severe acute respiratory syndrome (SARS), avian influenza (10), and swine flu (11) or the prions causing Creutzfeldt-Jakob disease (CJD) and variant CJD (vCJD), are emerging, for which there are few if any treatments. Consequently, the medical community needs better knowledge on disinfection and sterilization to prevent the spread of these pathogens.

PRINCIPLES, TERMINOLOGY, AND BACKGROUND

There is no uniform terminology for antiseptics, disinfection, and sterilization, and many problems arise as a result. Many terms are ill defined even within the United States or Europe. In addition, the testing procedures for disinfectants are not as far advanced and well defined as MIC testing based on the recommendations of the Clinical and Laboratory Standards Institute (CLSI). However, there currently are efforts to standardize and harmonize the terminology on an international level. For example, the International Organization for Standardization (ISO) norms for sterilization were published in 2004 and included in the new guidelines published in 2010 (12). In addition, manufacturers now must provide specific data on how to reprocess their medical devices. In the past, such information was frequently missing in the user's manuals.

For this chapter, we will use the term antiseptics for chemical compounds that are used to disinfect living tissue. The term disinfection will be used for compounds or methods that reduce microorganisms to a level at which the treated product cannot cause infection. In contrast to disinfection, sterilization will be used for procedures that will lead to a product that is free of microorganisms.

ANTISEPTICS

Compounds that disinfect living tissue are called antiseptic agents. They must meet many more requirements than compounds used to disinfect inanimate surfaces because they have to come in direct contact with the skin or mucous membranes. In addition, some of the agents are considered drugs and, thus, are regulated by the U.S. Food and Drug Administration (FDA). The antimicrobial spectra of commercially available agents are summarized in Table 1. The choice of the agent should be based not only on the desired effect but also, like antimicrobial agents, on side effects. Antiseptics rarely cause serious side effects, and most agents on the market have excellent safety profiles. Nevertheless, health care workers (HCWs) must remember that these

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TABLE 1 Overview of common antiseptic compounds[a]

Compound(s)	Antiseptic effect on:					Rapidity of action	Residual activity	Typical concn (%)	Affected by organic matter	Safety for humans
	Gram-positive bacteria	Gram-negative bacteria	Viruses	Fungi	*M. tuberculosis*					
Alcohols	+++	+++	++[b]	+++	+++	15–30 s	None	70–95	?[d]	Drying, flammable
Chlorhexidine	+++	++	++	+	++	Minutes	+++	4, 2, 0.5 in alcohol	Minimally	Ototoxicity, keratitis
Iodophors	+++	++	++	++	++	Minutes	+	10, 7.5, 2, 0.5	Yes	Skin irritation
Octenidine[c]	+++	+++	++	++	++	Minutes	+++	0.1	Minimally	Limited data
PCMX	++	+	+	+	+	Minutes	++	0.5–3.75	Minimally	Appears to be safe
Triclosan	++	++	++	±	+	Minutes	+++	0.3–1.0	Minimally	Appears to be safe

[a] Data from references 30, 69, and 334. Symbols: ±, poor; +, fair; ++, good; +++, excellent.

[b] Ethanol at >95% is highly effective against viruses; isopropanol has limited effectiveness against small or nonlipid viruses.

[c] Not available in the United States.

[d] ?, conflicting data.

agents can cause side effects such as anaphylactic shock in patients who have had contact with chlorhexidine, mainly patients originating from the Far East (13, 14).

Common Antiseptic Compounds

Alcoholic Compounds

Alcohol is the most important skin antiseptic. For centuries, alcohols have been appreciated for their antimicrobial properties. Alcohol is defined by the FDA as having one of the following active ingredients: ethyl alcohol, 60% to 95% by volume in an aqueous solution, or isopropyl alcohol, 50% to 91.3% by volume in an aqueous solution. Ethyl alcohol (ethanol) and isopropyl alcohol (isopropanol) are the alcoholic solutions most often used as surface disinfectants and antiseptic agents in health care institutions because they possess many qualities that make them suitable both for disinfection of equipment and for antisepsis of skin. They are fast acting, minimally toxic to the skin, nonstaining, and nonallergenic. Alcohols evaporate readily, which is advantageous for most disinfection and antisepsis procedures. The uptake of alcohol by intact skin and the lungs when alcohol is used topically is below toxic levels for humans (15). Some studies indicated dermal and pulmonary absorption by inhalation of vapors of isopropyl alcohol from a commercial hand rub may occur and could interfere with religious beliefs of HCWs (15, 16). However, the World Health Organization (WHO) resolved this issue in their most recent guidelines published in 2009, and Muslims, for example, are allowed to use alcoholic compounds for hand hygiene.

Alcohols have better wetting properties than water due to their lower surface tensions, which along with their cleansing and degreasing actions make alcohols effective skin antiseptics. Alcoholic formulations used to prepare the skin before invasive procedures should be filtered to ensure that they are free of spores, or 0.5% hydrogen peroxide should be added (17). Although the risk of infection is minimal, the low additional cost for a spore-free product is justified.

Alcohols have some disadvantages. If alcoholic antiseptics are used repeatedly, they may dry and irritate the skin. Therefore, preparations for hand disinfection should contain emollients.

The exact mechanism by which alcohols destroy microorganisms is not fully understood. The most plausible explanation for the antimicrobial action is that alcohols coagulate (denature) proteins (e.g., enzymatic proteins), impairing specific cellular functions (18). Ethyl and isopropyl alcohols at appropriate concentrations have broad spectra of antimicrobial activity that include vegetative bacteria, fungi, and viruses. In fact, their antimicrobial efficacies are enhanced in the presence of water, with optimal alcohol concentrations being 60 to 90% by volume.

Alcohols (i.e., 70 to 80% ethyl alcohol) rapidly (i.e., within 10 to 90 s) kill vegetative bacteria, such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterobacteriaceae*, and *Pseudomonas aeruginosa* in suspension tests (17). Isopropyl alcohol is slightly more bactericidal than ethyl alcohol (18) and is highly effective against vancomycin-resistant enterococci (19). It also has excellent activity against fungi, such as *Candida* spp., *Cryptococcus neoformans*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Aspergillus niger*, and dermatophytes, and mycobacteria, including *Mycobacterium tuberculosis*. However, alcohols generally do not destroy bacterial spores. In fact, spores of *Clostridium difficile* are stored in ethanol in the microbiology laboratory. In addition, fatal infections due to *Clostridium* spp. occurred when alcohol was used to sterilize surgical instruments. Both ethyl and isopropyl alcohols inactivate most viruses with a lipid envelope (e.g., influenza virus, herpes simplex virus, and adenovirus). However, several investigators found that isopropyl alcohol had lower virucidal activity against naked, nonenveloped viruses (20). In the experiments by Klein and DeForest, 2-propanol, even at 95%, could not inactivate the nonenveloped poliovirus type 1 and coxsackievirus type B in 10 min (21). In contrast, 70% ethanol inactivated these enteroviruses (21). Neither 70% ethanol nor 45% 2-propanol killed hepatitis A virus (HAV) when their activities were assessed on stainless steel disks contaminated with fecally suspended virus. Among 20 disinfectants tested, only 3 reduced the titer of HAV by greater than 99.9% in 1 min

(2% glutaraldehyde, sodium hypochlorite with >5,000 ppm free chlorine, and a quaternary ammonium formulation containing 23% HCl) (22). Bond et al. (23) and Kobayashi et al. (24) demonstrated that 2-propanol (70% for 10 min) or ethanol (80% for 2 min) made human plasma contaminated with hepatitis B virus (HBV) at a high titer noninfectious for susceptible chimpanzees (24). Both 15% ethyl alcohol and 35% isopropyl alcohol (25) readily inactivate human immunodeficiency virus (HIV), and 70% ethanol rapidly inactivates high titers of HIV in suspension, independent of the protein load. However, the rate of inactivation decreased when virus was dried onto a glass surface and high levels of protein were present (26). In a suspension test, 40% propanol reduced the rotavirus titer by at least 4 log in 1 min (27) and both 70% propanol and 70% ethanol reduced the release of rotavirus from contaminated fingertips by 2.7 log units. In comparison, the mean reductions obtained with liquid soap and an aqueous solution of chlorhexidine gluconate were 0.9 and 0.7 log units, respectively (28).

Chlorhexidine

Chlorhexidine gluconate, a cationic bisbiguanide, has been recognized as an effective and safe antiseptic for nearly 40 years (29, 30). Chlorhexidine formulations are extensively used for surgical and hygienic hand disinfection (see previous discussion). Other applications include preoperative showers (or whole-body disinfection), antisepsis in obstetrics and gynecology, management of burns, wound antisepsis, and prevention and treatment of oral disease (plaque control, pre- and postoperative mouthwash, and oral hygiene) (29, 30). When chlorhexidine is used orally, its bitter taste must be masked and it can stain the teeth. Intravenous catheters coated with chlorhexidine and silver sulfadiazine are used to prevent catheter-associated bloodstream infections (31). In fact, an infection control program to prevent catheter-associated bloodstream infections included hand hygiene, chlorhexidine site care, and full-barrier precautions in a large clinical study in intensive care units: these interventions led to an infection rate close to zero (32). Today, chlorhexidine compounds are considered the gold standard for catheter site care (33). Chlorhexidine is most commonly formulated as a 4% aqueous solution in a detergent base. However, alcoholic preparations have been demonstrated in numerous studies to have better antimicrobial activity than detergent-based formulations (34). Bactericidal concentrations destroy the bacterial cell membrane, causing cellular constituents to leak out of the cell and cell contents to coagulate (29). The bactericidal activity of chlorhexidine gluconate against vegetative Gram-positive and Gram-negative bacteria develops within minutes. In addition, it provides a persistent antimicrobial action that prevents the regrowth of microorganisms for up to 6 h. This effect is desirable when a sustained reduction in the microbiota reduces infection risk (e.g., during surgical procedures). Chlorhexidine has little activity against bacterial and fungal spores except at high temperatures. Mycobacteria are inhibited but are not killed by aqueous solutions. Yeasts and dermatophytes are usually susceptible, although the fungicidal action varies with the species (35). Chlorhexidine is effective against lipophilic viruses (e.g., HIV, influenza virus, and herpes simplex virus types 1 and 2), but viruses such as poliovirus, coxsackievirus, and rotavirus are not inactivated (29). Unlike what occurs with povidone iodine, blood and other organic materials do not affect the antimicrobial activity of chlorhexidine significantly (36). However, inorganic anions and organic anions such as soaps are incompatible with chlorhexidine, and its activity is reduced at extreme acidic or alkaline pH and in the presence of anion- and non-ion-based moisturizers and detergents. Microorganisms can contaminate chlorhexidine solutions (37), and resistant isolates have been identified. For example, Stickler found chlorhexidine-resistant *Proteus mirabilis* after chlorhexidine was used extensively over a long period to prepare patients for bladder catheterization (38). The chlorhexidine resistance among vegetative bacteria was thought to be limited to certain Gram-negative bacilli (such as *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Proteus mirabilis*, and *Serratia marcescens*) (39). However, genes conferring resistance to various organic cations, including chlorhexidine, have been identified in *S. aureus* clinical isolates (40, 41). Chlorhexidine has several other limitations. When absorbed onto cotton and other fabrics, it usually resists removal by washing. If a hypochlorite (bleach) is used during the washing procedure, a brown stain may develop (29). Long-term experience with the use of chlorhexidine has demonstrated that the incidence of hypersensitivity and skin irritation is low. However, severe allergic reactions including anaphylaxis have been reported (13, 42). Although cytotoxicity has been observed in exposed fibroblasts, no deleterious effects on wound healing have been demonstrated *in vivo*. There is no evidence that chlorhexidine gluconate is toxic if it is absorbed through the skin, but ototoxicity can occur when chlorhexidine is instilled into the middle ear during operative procedures. High concentrations of chlorhexidine and preparations containing other compounds (e.g., alcohols and surfactants) may damage eyes (43). In the report "Strategies to Prevent Central Line-Associated Bloodstream Infections in Acute Care Hospitals," it has been acknowledged that no recommendations with regards to chlorhexidine gluconate antisepsis can be made for infants less than 2 months of age due to incomplete safety data in this population (44). In fact, 51% of participants in a survey who used chlorhexidine in their neonatal intensive care unit reported adverse reactions. All were skin reactions and included erythema (32%), erosions (7%), or burns (61%). Of the reported skin burns, 13 of 17 (76%) were reported to have occurred in neonates with a birth weight of less than 1,500 g (45).

Iodophors

Iodophors essentially have replaced aqueous iodine and tincture as antiseptics. Iodophors are chemical complexes of iodine bound to a carrier such as polyvinylpyrrolidone (PVP) iodine (also known as povidone iodine) or ethoxylated nonionic detergents (poloxamers). These complexes gradually release small amounts of free microbicidal iodine. The most commonly used iodophor is PVP iodine. Its preparations generally contain 1 to 10% PVP iodine, which is equivalent to 0.1 to 1.0% available iodine. The active component appears to be free molecular iodine (I_2). A paradoxical effect of dilution on the activity of PVP iodine has been observed. As the dilution increases, bactericidal activity increases up to a maximum and then falls (46). Commercial PVP iodine solutions at dilutions of 1:2 to 1:100 kill *S. aureus* and *Mycobacterium chelonae* more rapidly than do stock solutions (47). *S. aureus* can survive a 2-min exposure to full-strength PVP iodine solution but cannot survive a 15-s exposure to a 1:100 dilution of the iodophor (47). Thus, iodophors must be used at the dilution stated by the manufacturer. The exact mechanism by which iodine destroys microorganisms is not known. Iodine may react with microorganisms' amino acids and fatty acids, destroying cell structures and enzymes (46). Depending on the concentration of free iodine and other factors, iodophors exhibit a broad range of microbicidal activity. Commercial preparations are bactericidal, mycobactericidal, fungicidal, and virucidal but not sporicidal at the dilutions recommended for use. Prolonged contact times are required to inactivate certain fungi and bacterial spores (20). Despite their bactericidal activity, PVP iodine and poloxamer-iodine solutions can become contaminated with *B. cepacia* or *P. aeruginosa*, and contaminated solutions have caused outbreaks of pseudobacteremia and peritonitis (48, 49). In fact, *B. cepacia* was found to survive for up to 68 weeks in a PVP iodine antiseptic solution (50). The most likely explanation for the prolonged survival of these microorganisms in iodophor solutions is that organic or inorganic material and biofilm may mechanically protect the microorganisms. Iodophors are widely used for antisepsis of skin, mucous membranes, and wounds. A 2.5% ophthalmic solution of PVP iodine is more effective and less toxic than silver nitrate or erythromycin ointment when used as prophylaxis against neonatal conjunctivitis (ophthalmia neonatorum) (51). In some countries, PVP iodine alcoholic solutions are used extensively for skin antisepsis before invasive procedures (52). Iodophors containing higher concentrations of free iodine may be used to disinfect medical equipment. Solutions designed for use on the skin should not be used to disinfect hard surfaces because the concentrations of the antiseptic solutions are usually too low for this purpose (20). The risk of side effects, such as staining, tissue irritation, and resorption, is lower for iodophors than for aqueous iodine. Iodophors do not corrode metal surfaces (46). However, a body surface treated with an iodine or iodophor solution may absorb free iodine. Consequently, increased serum iodine levels (and serum iodide levels) have been found in patients, especially when large areas were treated for a long period (46). For this reason, hyperthyroidism and other disorders of thyroid functions are contraindications for the use of iodine-containing preparations. Likewise, iodophors should not be applied to pregnant and nursing women or to newborns and infants (53). Because severe local and systemic allergic reactions have been observed, iodophors and iodine should not be used in patients with allergies to these preparations (54). Iodophors have little if any residual effect. However, for a limited time, they may have residual bactericidal activity on the skin surface because free iodine diffuses into deep regions but also back to the skin surface (46). The antimicrobial efficacy of iodophors is reduced in the presence of organic material such as blood.

Triclosan and PCMX

Triclosan (Irgasan DP-300 or Irgacare MP) has been used for more than 30 years in a wide array of skin care products, including hand washes, surgical scrubs, and consumer products. A review of its effectiveness and safety in health care settings has been published (55). A concentration of 1% has good activity against Gram-positive bacteria, including antibiotic-resistant strains, but less activity against Gram-negative organisms, mycobacteria, and fungi. Limited data suggest that triclosan has a relatively broad antiviral spectrum, with high-level activity against enveloped viruses, such as HIV type 1, influenza A virus, and herpes simplex virus type 1. However, the nonenveloped viruses proved more difficult to inactivate. Clinical strains of *S. aureus* with low-level resistance to triclosan have been identified, but the clinical significance of this remains unknown (56). Triclosan is added to various soaps, lotions, deodorants, toothpastes, mouth rinses, commonly used household fabrics, plastics, and medical devices. Moreover, the mechanisms of triclosan resistance may be similar to those involved in antimicrobial resistance (57), and some of these mechanisms may account for the observed cross-resistance of laboratory isolates to antimicrobial agents (58). Consequently, concerns have been raised that widespread use of triclosan formulations in non-health-care settings and products may select for biocide resistance and even cross-resistance to antibiotics. However, environmental surveys have not demonstrated an association between triclosan usage and antibiotic resistance (59). Triclosan solutions produce a sustained residual effect against resident and transient microbiotas, which is minimally affected by organic matter. Numerous studies have not identified toxic, allergenic, mutagenic, or carcinogenic potential. Triclosan formulations may help control outbreaks of methicillin-resistant *S. aureus* (MRSA) when used for hand hygiene and as a bathing cleanser for patients (55). However, some MRSA isolates have reduced triclosan susceptibility. Triclosan formulations are less effective than 2 to 4% chlorhexidine gluconate when used for surgical scrub solutions; properly formulated triclosan solutions may be used for hygienic hand washing. PCMX (chloroxylenol) is an antimicrobial used in hand-washing products. It is available at concentrations of 0.5 to 3.75%. Its properties are similar to those of triclosan. Nonionic surfactants may neutralize PCMX.

Octenidine

Octenidine dihydrochloride is a novel bispyridine compound which is an effective and safe antiseptic agent. The 0.1% commercial formulation favorably compared to other antiseptics with respect to antimicrobial activity and toxicological properties. *In vitro* and *in vivo* it rapidly killed both Gram-positive and Gram-negative bacteria as well as fungi (60, 61). Octenidine is virucidal against HIV, HBV, and herpes simplex virus. Similar to chlorhexidine, it has a marked residual effect. No toxicological problems have been found when the 0.1% formulation was applied according to the manufacturer's recommendations. The colorless solution is a useful antiseptic for mucous membranes of the female and male genitals and the oral cavity (62), but its bad taste limits its use orally. In a recent observational study, the 0.1% formulation was highly effective and well tolerated for the care of central venous catheter insertion sites (63). The results of this study have also been supported by a randomized controlled clinical trial (64). Octenidine is not registered for use in the United States.

Hygienic Hand Washing and Hand Disinfection

Hand hygiene is the single most important infection control measure (65). However, it remains difficult to motivate HCWs to perform this simple procedure faithfully (66). The Centers for Disease Control and Prevention (CDC) has published detailed guidelines on hand hygiene (65), and in 2006, the WHO launched a global effort to improve hand hygiene in health care facilities with a reference book published in 2009 (http://whqlibdoc.who.int/hq/2009/WHO_IER_PSP_2009.07_eng.pdf). In-depth reviews have been published by several authors (67–69). Microorganisms on the hands can be classified into three groups (70): (i) the transient biota, which consists of contaminants taken up from the environment; (ii) the resident biota, which consists of permanent microorganisms on the skin (69); and (iii) the infectious biota. Resident bacteria, most of which are on the uppermost level of the stratum corneum, have low pathogenicity and infectivity, and persons with normal immune systems who do not have implants or foreign bodies rarely acquire infections with these organisms. The density of resident bacteria on the skin ranges between 10^2 and 10^3

CFU/cm^2, and these resident bacteria limit colonization with more pathogenic microorganisms (i.e., colonization resistance). During their daily work, HCWs can contaminate their hands with pathogens. If they do not practice good hand hygiene, they can transmit these organisms to susceptible patients. Several studies indicated that pathogens such as *S. aureus* (71), *Klebsiella pneumoniae* (72), *Acinetobacter* spp., *Enterobacter* spp., or *Candida* spp. can be found on the hands of >20% of HCWs. Moreover, numerous epidemics have been traced to HCWs' contaminated hands (73–77). The goal of hand hygiene outside the operating room is to eliminate the transient biota without altering the resident biota. Hand washing for 15 and 30 s kills 0.6 to 1.1 and 1.8 to 2.8 log units, respectively (78). However, HCWs are very busy and frequently wash their hands for less than 10 s, which is insufficient to kill the transient biota (68, 69). One major advantage of the alcohol-based hand rub is that performance with these products takes about 25% of the time required for hand washing (68, 69). Moreover, compliance with hand-washing procedures does not exceed 40% even under controlled study conditions (65). However, recent studies have shown that compliance with using the alcohol-based hand rubs exceeds that of hand washing (79). Furthermore, other studies have demonstrated that rubbing one's hands with an alcohol-based hand rub kills bacteria and most viruses more effectively than hand washing with a medicated soap (80, 81). Of note, investigators have not determined whether the level of killing is associated with the efficacy of preventing nosocomial infections. Alcohol-based hand rubs have several other practical advantages for hand hygiene over washing with soap and water. Compared with sinks, dispensers for the alcohol-based products are inexpensive, and they can be installed at locations that are more convenient for HCWs. Furthermore, unlike sinks (82), the dispensers have not been associated with outbreaks. Given the numerous advantages of these products, CDC's current hand hygiene guidelines recommend that health care facilities consider introducing alcohol-based hand rubs as the primary mode of hand hygiene (65). Most U.S. institutions promote hand hygiene using an alcoholic hand rub as standard of care, driven by the guidelines issued by WHO in a draft form in 2004 and finalized in 2009.

The advantages of alcohols also apply in the laboratory setting. Alcohol-based hand rubs can be provided in locations near the benches. They offer personnel protection and prevent cross-contamination of laboratory specimens (83).

Because alcohols are flammable, health care facilities or laboratories in the United States should consult with the fire marshall before installing dispensers. Many states have laws that prohibit placing multiple containers in emergency exits and halls. However, there are only few published reports of fires caused by these products (84, 85).

Surgical Hand Washing (Scrub) or Surgical Hand Disinfection (Rub-In)

In contrast to hand hygiene outside the operating room, the surgical hand scrub aims to eliminate both transient biota and resident biota so that if the surgeon's gloves are punctured or torn, bacteria from his or her hands do not contaminate the surgical site. Tiny holes are observed in ≥30% of surgeons' gloves after operations, even when high-quality gloves are used. Cruse and Foord found that the incidence of surgical site infection was three times higher if the surgeon's gloves were punctured than if they were intact after the procedure (5.7 and 1.7%, respectively) (86). An experimental study demonstrated that the level of bacterial leakage through pinholes ranged between 10^3 and 10^4 CFU (87). Recently, a clinical trial clearly demonstrated that the presence of holes in a surgical glove without adequate antimicrobial prophylaxis increases the risk of postoperative surgical site infections fourfold (88). Moreover, a persistent antimicrobial effect is required after washing or disinfection to limit bacterial regrowth underneath the gloves (89). Thus, antiseptic preparations intended for use as surgical hand preparation are evaluated for their ability to reduce the number of bacteria released from hands (i) immediately after scrubbing, (ii) after wearing surgical gloves for 6 h (persistent activity), and (iii) after numerous applications over 5 days (cumulative activity). Immediate and persistent activities are considered the most important. Guidelines in the United States recommend that agents used for surgical hand preparation should significantly reduce microorganisms on intact skin, contain a nonirritating antimicrobial preparation, have broad-spectrum activity, and be fast acting and persistent. Agents, such as chlorhexidine, that have a prolonged postexposure effect are preferred because of this theoretical advantage, but there are no data from controlled clinical trials proving that the incidence of surgical site infections is lower when this agent is used. The WHO has issued a guideline for surgical hand antisepsis (www.who.int) with an executive summary published (90, 91).

Alcohol-based surgical rubs have several advantages over traditional surgical scrubs. Alcoholic preparations are more effective than any medicated soap for the surgical scrub, and they do not alter the skin as much as chlorhexidine washes do. Moreover, the water supply in an operating room could harbor *Pseudomonas* spp. that might contaminate the hands of surgical personnel after they perform their surgical scrub (92). Brushes, which are used during a surgical scrub, may do more harm than good, and they should be used only to clean the fingernails, not to clean the skin. Given the advantages of the alcohol-based preparations, the presurgical scrub has been replaced in many European countries by the alcohol-based surgical rubs (68), and the WHO guidelines recommend the surgical hand rubs. Alcoholic gels are frequently promoted, but most of them are significantly less effective than liquids and should not be used in the operating room (93). A very rapid protocol (1.5 min) for the surgical hand rub has been proposed and was rapidly accepted by surgeons at the author's institution (94). The results of this investigational study were confirmed in a clinical trial (91, 95). However, few commercially available products have been successfully tested, and quite a few failed. Therefore, a 1.5-min surgical hand antisepsis is only acceptable if the product is cleared for such a short exposure to the hands.

Of note, both routine hand hygiene and surgical hand preparations must balance removing unwanted bacteria from HCWs' hands and maintaining the integrity of the HCWs' skin because damaged skin is more likely than normal skin to become colonized with pathogenic organisms. Therefore, either hand hygiene products should contain emollients or health care facilities should provide moisturizing hand lotions that do not damage latex for their staff so that their skin does not become dry, cracked, and irritated.

Presurgical Skin Disinfection

The aim of skin disinfection is to remove and kill the skin biota at the site of a planned surgical incision rapidly. However, currently available antiseptics do not eliminate all microorganisms at the incision site. In fact, coagulase-negative staphylococci can be frequently isolated even after three applications of agents such as iodine-alcohol to the skin (96). The FDA defines a skin disinfectant as a "fast-acting, broad-spectrum, and persistent antiseptic-containing preparation that significantly reduces the number of microorganisms on intact skin" (97). Spore-free alcohols are well suited for this purpose, but they lack persistent activity. Iodine is frequently added for this purpose (98). PVP iodine continuously releases free iodine, which results in a limited prolonged antimicrobial effect. Chlorhexidine, with its profound prolonged effect after application, seems to be favorable compared to PVP iodine; however, PVP excelled against chlorhexidine according to a clinical trial (99). The WHO guideline on "safe surgery" recommends PVP iodine or chlorhexidine as a reasonable choice for preoperative skin preparation (http://www.who.int/patientsafety/safesurgery/en). A recent randomized controlled clinical trial indicates a preference for chlorhexidine-alcohol over iodophors (100), but the addition of alcohol may be the reason for the favorable effect rather than chlorhexidine, as shown in a similar trial favoring iodophor in alcohol (99). A recent study also showed that microorganisms causing surgical site infection (SSI) did not at all correlate with remaining bacteria after standardized three-step disinfection with povidone iodine-alcohol (101).

A Cochrane review found some evidence that preoperative skin preparation with 0.5% chlorhexidine in alcohol was associated with lower rates of SSIs following clean surgery than alcohol-based povidone iodine paint. However, the authors stated that further studies are needed that evaluate the iodine-containing and chlorhexidine-containing solutions relevant to current practice as well as the type of solution used (alcohol versus aqueous) (102).

Before a patient's skin is prepared for a surgical procedure, the skin should be free of gross contamination (i.e., dirt, soil, or any other debris) (103). Although preoperative showering has not been shown to reduce the incidence of surgical site infections (104), this practice may decrease bacterial counts and ensure that the skin is clean (105). The antiseptics used to prepare the skin should be applied using sterile supplies and gloves or by a no-touch technique, moving from the incision area to the periphery (103). The person preparing the skin should use pressure because friction increases the antibacterial effect of the antiseptic. For example, alcohol applied without friction reduces bacterial counts by 1.0 to 1.2 log CFU compared with 1.9 to 3.0 log CFU when friction is used. In comparison, alcoholic sprays have little antimicrobial effect and produce potentially explosive vapors (106).

Decolonization

The term "decolonization" when used in the context of MRSA carriers refers to measures primarily aimed at reducing the number of MRSA colonies present on the skin and mucous membranes or even at eradicating (i.e., eliminating) the microorganism completely. In contrast to medical treatment, decolonization of MRSA can also be indicated in the absence of infection. In most situations, complete MRSA eradication will be attempted; however, in many circumstances, the decreased risk of infection or decreased rates of transmission may be considered a desirable outcome.

Antiseptics have long been used on MRSA-colonized intact skin for decolonization. However, until now, no randomized controlled clinical studies on the efficacy of skin antiseptics used as monotherapy have been published, and the efficacy of several antiseptic agents has been demonstrated in experimental laboratory studies only (107–109).

Chlorhexidine is the most extensively investigated antiseptic agent. It has been used for many years all over the world to decolonize the MRSA-colonized skin. As early as 1977, Davies et al. (110) noted that chlorhexidine produced a significant decrease of the local skin flora in comparison with soap baths and other antiseptic agents (PVP iodine and hexachlorophane) (111, 112). This observation was confirmed by several other studies (112, 113). In the 1980s, chlorhexidine added to shower or bath water preoperatively with the aim of preventing SSIs was investigated with inconclusive results (105, 111, 112–115). Subsequently, chlorhexidine was repeatedly used for decolonization of MRSA patients (116–118); however, these investigations conducted in the setting of an outbreak were essentially observational in nature and did not include control groups. In two randomized placebo-controlled trials of whole-body washing with chlorhexidine in addition to nasal mupirocin, the superiority of chlorhexidine could not be demonstrated (112, 119). Although MRSA may not be completely eradicated by chlorhexidine, Sandri et al. (120) observed in a 5-year prospective study that the incidence of nosocomial MRSA infections was diminished following the administration of chlorhexidine plus nasal mupirocin ointment for all patients in an intensive care ward. Furthermore, in a multicenter study, it was demonstrated that universal decolonization of all patients in intensive care units was more effective than targeted decolonization or screening and isolation in reducing rates of MRSA clinical isolates and bloodstream infection from any pathogen (121).

Due to the limitations of chlorhexidine, other antiseptics have been used as part of decolonization bundles.

Various case reports, but no controlled studies, are available for octenidine dihydrochloride. A significant reduction in numbers of bacteria on the skin was demonstrated for several body sites (122, 123), but side effects that led to discontinuation of treatment in 14% of patients were described (123). Further antiseptics that have been proposed for eradication of MRSA skin carriage include povidone iodine (PVP iodine) (124), polyhexanide, triclosan (125), hexachlorophane (126, 127), undecylenamidopropyltrimonium methosulfate 4%, a quaternary surfactant, and phenoxyethanol 2% (128). However, none of these drugs has been studied in controlled trials. Povidone iodine (PVP iodine) is not recommended for application to extensive body surface areas due to systemic absorption (129, 130). Triclosan 0.3% added to hand or bathing soap was able to stop a MRSA outbreak affecting a neonatal unit (125), but it has been shown that MRSA strains can develop resistance to triclosan (131–133). Results on efficacy of hexachlorophane are inconclusive, and they are derived from an outbreak setting alone (126).

Since antiseptic agents may be applied to extensive surfaces of the body, depending on the indication, it is advisable to select antiseptic products with low absorption to limit the risk of adverse effects.

Although MRSA carriage in the throat is not uncommon (116, 134, 135), no studies have been reported that focused on eradication of MRSA from this location. Antiseptic agents potentially suitable for oropharyngeal MRSA decolonization include octenidine, triclosan, and chlorhexidine (136–140). The main difficulty consists of achieving a sufficiently long contact time between the throat and the antiseptic agent. Mouth rinses and gargling do not ensure adequate contact times, and administration of lozenges does not lead to sufficient distribution of the antiseptic. Hence, sprays may be more suitable for oropharyngeal administration (140). However, eradication of MRSA from the throat remains a largely unresolved issue and has been treated by tonsillectomy in extreme cases (141). Difficulties of oropharyngeal MRSA eradication may arise from the colonization pattern of *S. aureus* in tonsillar tissue. Brook et al. (135) demonstrated a higher recovery rate for *S. aureus* from tonsillar cores than from tonsillar surfaces.

CLEANING AND DECONTAMINATION

In Europe, decontamination basically means cleaning an item to remove organic material, protein, and fat. In the United States, the term describes a cleaning step and any additional step required to eliminate any risk of infection to HCWs while they handle a device without protective attire. The CDC guideline defines cleaning as the removal of visible soil (e.g., organic and inorganic material) from objects and surfaces by using water with detergents or enzymatic products. Thorough cleaning is essential before high-level disinfection and sterilization because inorganic and organic materials that remain on the surfaces of instruments interfere with the effectiveness of these processes. Decontamination removes pathogenic microorganisms from objects so they are safe to handle, use, or discard (142). The FDA defines the cleaning process as including all steps necessary to remove, inactivate, or contain contamination, beginning immediately after an item has been used for clinical purposes, continuing with the steps to decontaminate, clean, and package a device up to the first step of the sterilization process and ending with quality control tests.

Regardless of regulations, cleaning is always the initial step of the decontamination process on both continents. In this chapter, we use the term "decontamination" to describe the removal of debris, blood, proteins, and most microorganisms. This process usually, but not necessarily, renders the device "safe to handle" by HCWs who are not wearing protective attire. Basic definitions are outlined in Table 2.

The first step in reprocessing used medical devices is for HCWs to prevent debris from drying on the item. Research on prion diseases demonstrated that removal of debris is seriously impaired if the debris is allowed to dry on a medical device (143). Therefore, the reprocessing cycle should start as soon as possible: the item should be kept wet if delays in reprocessing are anticipated (144, 145). Cleaning can be done physically or chemically; it can also be done manually, by sonication, or with washers. In the United States, cleaning is

TABLE 2 Definitions and terms

Term	Standard	Technical-microbiological log CFU reduction	Comment
Sterilization	A (closely monitored) validated process used to render a product free of all forms of viable microorganisms, including all bacterial endospores[a]	≥6 log CFU reduction of the most resistant spores for the sterilization process studied, achieved at the half-time of the regular cycle (ISO 14937)	Prions require an adapted definition because of their high resistance to any form of sterilization
Disinfection	Elimination of most if not all pathogenic microorganisms, excluding spores	There is not a clear-cut defined reduction level; a minimum estimate is ≥3 log CFU reduction of microorganisms, excluding spores; common reduction is 4 to 5 log for devices; these are estimates, because there is no international standardization	Some high-level disinfectants achieve microbial reduction, including spores, similar to sterilization, if long incubation times and/or temperatures of >25°C are applied; this is called liquid sterilization by sterilants
Decontamination	Reduction of pathogenic microorganisms to a level where items are "safe to handle" without protective attire	Elimination of debris and proteins by cleaning and/or disinfection/sterilization process; in Europe, it is restricted to cleaning only, which achieves a minimum of ≥1 log CFU; most cleaning processes achieve 3 to 5 log CFU reduction; these are estimates, because there is no international standardization	Manual and/or mechanical cleaning (with water and detergents or enzymes), a prerequisite before disinfection or sterilization; in Europe, this term is used for cleaning the items; in the United States, it defines an item to be "safe to handle"; it may include a cleaning process but also a disinfection or even a sterilization process; the U.S. term "decontamination" refers to the HCWs' safety; in Europe, the term is used for the item only
Antisepsis			
Patient related	Disinfection of living tissue or skin	Preoperative skin preparation with an alcohol-based iodine compound	Antiseptic agents are handled as drugs by the FDA
HCW related	Reduction or removal of transient microbiota	Hand washing (scrub): reduction of ≥1 log CFU Hand disinfection (rub-in): reduction of ≥2.5 log CFU	

[a] Examples of standards for sterilization methods: ethylene oxide, ISO 11135 (industrial facility use) and ANSI/AAMI ST 41 (health care facility use); moist heat, ISO 11134 (industrial facility use) and ANSI/AAMI ST 46 (health care facility use).

frequently performed manually with water and a detergent. In Europe, many countries rely primarily on washer-disinfectors that rinse items with cold water and then with warm water plus a detergent. The cycle is completed with hot water at ≥90°C. Items such as bedpans and urinals can be cleaned and disinfected by putting the items into a machine, pushing a button, and removing them after a 2- to 5-min procedure. All sterilization techniques other than steaming have been shown to fail in 1 to 40% of sterilization cycles if residual proteins and/or salts are not removed by a proper cleaning process (146). Even steam sterilization at 134°C for 18 min, recommended by the WHO to inactivate prions, can fail to prevent cross-transmission if the device does not undergo a cleaning process beforehand (143–145).

For floors, surfaces, and noncritical items, cleaning with a detergent is sufficient in most situations, and a disinfection process adds little if any additional effect (147). In addition, disinfectants may interfere and even lose activity with residual proteins and debris that escape the cleaning process (147). Routine disinfection of environmental surfaces in patient care areas is recommended in the United States to add a step of additional safety in cases of unrecognized body fluids, but it is restricted to intensive care units and emergency rooms in Europe (148).

DISINFECTION

Disinfection is the second critical step in reprocessing medical devices. To be effective, disinfection must be preceded by thorough cleaning and must be done properly. Staff members must check the disinfectant's concentration regularly if it is diluted at the place of use, even if it is diluted with an electronically monitored dilution device. Failures of the valve or other critical parts of the device can result in an insufficient final concentration, which usually cannot be detected by checking either the appearance or the odor of the disinfectant. Many manufacturers provide test strips to check for the appropriate concentration. Of note, numerous outbreaks have occurred when staff members have not followed appropriate protocols (149). For example, *Klebsiella oxytoca* caused an outbreak after an infection control committee allowed staff members to decrease the concentration of a glutaraldehyde-based surface disinfectant because they didn't like the odor. The outbreak stopped after staff resumed using the disinfectant at the recommended concentration (150). An outbreak of 58 cases of *Mycobacterium xenopi* infection occurred when instruments used for discovertebral operations were rinsed with tap water after they were disinfected (151).

Definitions and Terms (Adapted from FDA and Environmental Protection Agency [EPA] Definitions)

Since the FDA regulates the most critical part of disinfection and sterilization, FDA definitions are used throughout the chapter unless stated otherwise. The most important definitions are given in Table 3.

Principles and Antimicrobial Activities of Compounds

The antimicrobial spectrum of disinfectants is tested differently than that of antimicrobial agents. Microbiology laboratories that test disinfectants must know the special methods needed to accurately assess their activity. In fact, MICs are of little help because the goal of disinfection is to kill rather than inhibit the growth of microorganisms. In contrast to sterilization, but similar to antimicrobial agents, killing curves for disinfectants are not linear and the rate of log killing decreases as the inoculum concentrations decrease (i.e., as the number of CFU per milliliter decreases). Therefore, a 3-log-unit killing is more easily achieved with disinfectants if the inoculum is large, e.g., 10^8 CFU, than if the inoculum is 10^4 CFU. Most disinfectants must be inactivated before they are incubated in media or plated because bacteria do not grow in the presence of very low concentrations of a disinfectant (inhibitory effect). However, if the compound is inactivated, bacterial growth can be demonstrated. Like antimicrobial agents, some disinfectants display a postexposure effect on bacterial growth. The postexposure effect has been quantified for a variety of disinfectants. Alcohols lack a postexposure effect, but chlorhexidine, octenidine, polyhexanide, and chloramine delay regrowth after exposure for several hours (23).

Low-level disinfectants destroy lipid-enveloped viruses, such as HIV, and most vegetative bacteria (Fig. 1) (23), but many disinfectants, including alcohol, are ineffective against non-lipid-enveloped or small viruses such as poliovirus. For example, isopropyl alcohol has little activity against poliovirus but >90% ethanol is very active (152). The FDA requires that the microbicidal efficacy of liquid chemical sterilants and high-level disinfectants be assessed in three different types of tests before they can be legally marketed in the United States.

TABLE 3 FDA and EPA definitions of important terms

Term	Definition
Cleaning (or precleaning)	Removal of foreign material (e.g., organic or inorganic contaminants) from medical devices as part of a decontamination process
Germicide	Agent that destroys microorganisms; the prefixes of terms with the suffix "-cide" (e.g., virucide, fungicide, bactericide, sporicide, and tuberculocide) indicate which microorganisms the germicide kills
High-level disinfectant	Germicide that when used according to the labeling kills all microbial pathogens except large numbers of bacterial endospores
Intermediate-level disinfectant	Germicide that when used according to the labeling kills all microbial pathogens except bacterial endospores
Low-level disinfectant	Germicide that when used according to the labeling kills most vegetative bacteria and lipid-enveloped or medium-size viruses; such disinfectants are regulated by the EPA
Minimum effective concentration	Lowest effective concentration of a liquid chemical germicide that achieves the microbicidal activity claimed by the manufacturer
Sterilant (chemical)	Chemical germicide that achieves sterilization

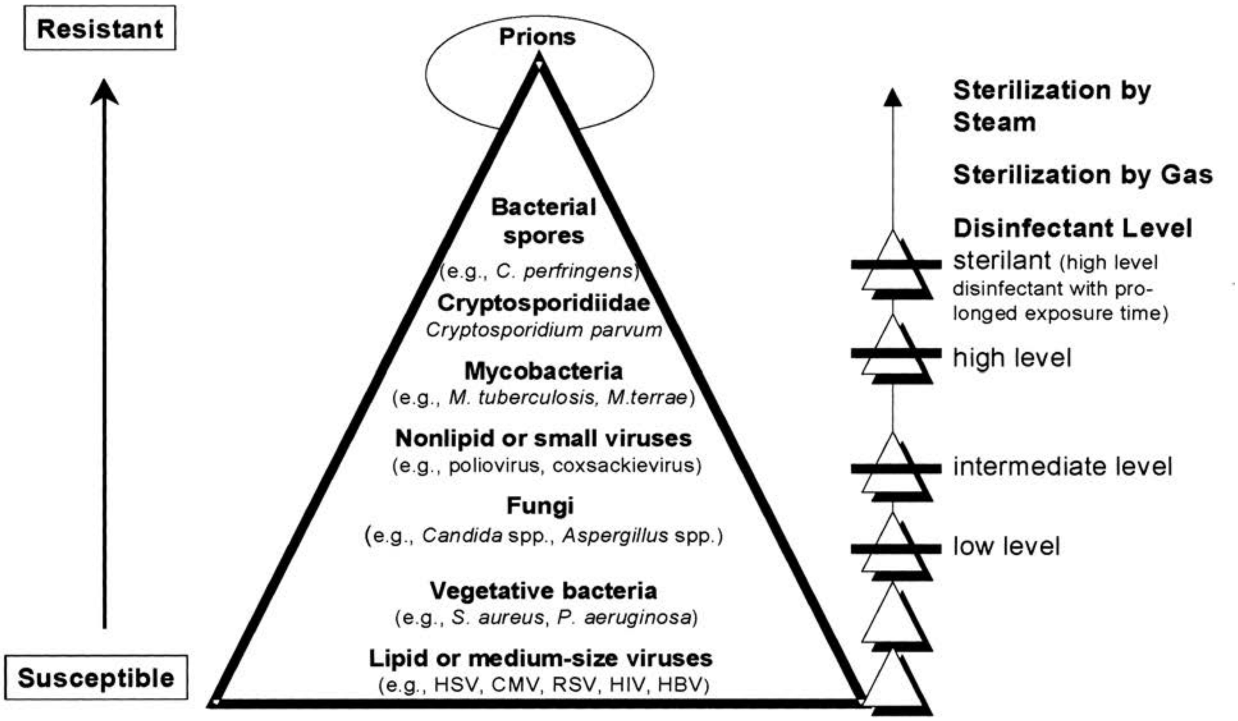
Resistant
Susceptible
Prions
Bacterial spores
(e.g., *C. perfringens*)
Cryptosporidiidae
Cryptosporidium parvum
Mycobacteria
(e.g., *M. tuberculosis, M.terrae*)
Nonlipid or small viruses
(e.g., poliovirus, coxsackievirus)
Fungi
(e.g., *Candida* spp., *Aspergillus* spp.)
Vegetative bacteria
(e.g., *S. aureus, P. aeruginosa*)
Lipid or medium-size viruses
(e.g., HSV, CMV, RSV, HIV, HBV)
Sterilization by Steam
Sterilization by Gas
Disinfectant Level
sterilant (high level disinfectant with prolonged exposure time)
high level
intermediate level
low level

FIGURE 1 Microorganisms' resistance to disinfectants. HSV, herpes simplex virus; CMV, cytomegalovirus; RSV, respiratory syncytial virus. doi:10.1128/9781555817381.ch13.f1

1. Potency testing incorporates the EPA test requirements for registration of germicides, such as the Association of Official Analytical Chemists' (AOAC) sporicidal test, tuberculocidal test, and use-dilution tests for *S. aureus* ATCC 6538, *Salmonella enterica* serovar Choleraesuis ATCC 10708, and *P. aeruginosa*; EPA virucidal tests for viruses, including poliovirus type 2 and herpes simplex virus; and FDA-recommended tests, such as total killing or endpoint analysis and comparing survivor and predicted curves. (Note: in Europe, disinfectants should have been tested by the methods defined by European Norms [EN] such as EN 1040 [bactericidal activity] and EN 1275 [fungicidal activity]).

2. Simulated-use testing involves testing the disinfectant under artificially created worst-case scenarios to determine how long instruments need to be in contact with the disinfectant if cleaning failed and the instruments are still contaminated with substantial organic matter and microbes. The instruments are contaminated with an organic load and appropriate test microorganisms (the organism depends on the level of disinfection being claimed), and the conditions of the artificially contaminated devices represent worst-case postcleaning conditions prior to exposure to the germicide.

3. "In-use" testing involves cleaning medical devices used for clinical purposes according to a facility's operating procedures.

As noted above, the FDA includes a tuberculocidal test in its testing procedures. This test does not account for the effect of cleaning before devices are disinfected. Devices are treated with 2% horse serum (proteinaceous load) and with 10^5 to 10^6 CFU of *Mycobacterium terrae* or equivalent nontuberculous mycobacteria. Under these conditions, a device would need to be immersed in a disinfectant (e.g., 2.4% alkaline glutaraldehyde) for ≥45 min at ≥25°C for complete tuberculocidal killing. However, Rutala and Weber demonstrated that proper cleaning eradicates at least 4 log units of microorganisms (153), and Hanson et al. showed that cleaning bronchoscopes before disinfection removed all detectable contaminants, with up to an 8-log-unit reduction in the viral load (154). Therefore, Rutala and Weber recommended that the FDA accept a standardized cleaning protocol followed by a 20-min immersion at 20°C with an FDA-approved disinfectant as adequate to kill mycobacteria (153). An updated list of low-level and intermediate-level disinfectants registered by EPA or high-level disinfectants and sterilants approved by the FDA is provided on their websites: http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/ReprocessingofSingle-UseDevices/ucm133514.htm and http://www.epa.gov/oppad001/chemregindex.htm.

Overview of Commonly Used Disinfectants for Devices

Glutaraldehyde

Among aldehydes that exhibit biocidal activity, including glyoxal, ortho-phthalaldehyde (OPA), succinaldehyde, and benzaldehydes, glutaraldehyde and formaldehyde are the most extensively studied aldehydes. In-depth reviews may be found elsewhere (20, 155, 156).

In commercially available products, glutaraldehyde is the predominant aldehyde. Because it has potent and broad-spectrum microbicidal activity and is compatible with many materials (including metal, rubber, and plastic), glutaraldehyde is often regarded as the high-level disinfectant and chemical sterilant of choice in many health care facilities. Glutaraldehyde-based formulations are most commonly used for high-level disinfection of medical equipment such as endoscopes, transducers, dialysis systems, and anesthesia and respiratory therapy equipment (156). The mechanism of action is complex and is related to alkylation of sulfhydryl, hydroxyl, carboxy, and amino groups in the cell wall, cell membrane, nucleic acids, enzymes, and other proteins of microorganisms. The biocidal activities of glutaraldehyde solutions are dependent on a variety of variables, such as pH, temperature, concentration at the time of use, the presence of inorganic ions, and the age of the solution (155). Aqueous solutions of glutaraldehyde are usually acidic and are not sporicidal in this form. Therefore, they need to be activated by adding an alkalinizing agent. These activated solutions, however, rapidly lose their activity because glutaraldehyde molecules polymerize at an alkaline pH. Therefore, the shelf life of such solutions is limited to 14 days unless the manufacturer recommends otherwise. To overcome this problem, some manufacturers have developed novel formulations with longer shelf lives (e.g., activated dialdehyde solutions containing 2.4 to 3.5% glutaraldehyde with a maximum reuse life of 28 days).

The activities of disinfectants increase as the temperature rises. Among eight disinfectants tested, glutaraldehyde was found to be the chemical most strongly affected by temperature (157). Some stable acid glutaraldehydes may be used at temperatures of 35 to 55°C at concentrations below 2%. Glutaraldehyde retains its activity in the presence of organic matter. A standard 2% aqueous solution of glutaraldehyde buffered to pH 7.5 to 8.5 is bactericidal, tuberculocidal, sporicidal, fungicidal, and virucidal. It rapidly kills both Gram-negative and Gram-positive vegetative bacteria. Longer exposure times are required to inactivate spores and mycobacteria. Spores of *Bacillus* and most *Clostridium* spp. are generally destroyed by 2% glutaraldehyde in 3 h, whereas spores of *Clostridium difficile* are eliminated more rapidly (158). In contrast, *Cryptosporidium parvum* oocysts remained viable and infectious after 10 h in a 2.5% glutaraldehyde solution (159). Several investigators have questioned glutaraldehyde's ability to inactivate mycobacteria. For example, Rubbo et al. (160) demonstrated that glutaraldehyde more slowly inactivated *M. tuberculosis* than did alcohols, formaldehyde, iodine, and phenol. Ascenzi (155) showed in the quantitative suspension test that 2% glutaraldehyde killed only 2 to 3 log units of *M. tuberculosis* in 20 min at 20°C. Similarly, Collins (161) reported that glutaraldehyde could not completely inactivate a standardized suspension of *M. tuberculosis* within 10 min. Nontuberculous mycobacteria such as *Myobacterium avium*, *Myobacterium intracellulare*, and *Mycobacterium gordonae* are more resistant to inactivation than *M. tuberculosis* (162). These and other data suggest that 20 min (at 20°C) is the minimum exposure time needed to reliably inactivate tuberculous and nontuberculous mycobacteria by 2% glutaraldehyde, provided that the contaminated item has been thoroughly cleaned before disinfection (20, 163). Glutaraldehyde-resistant mycobacteria have been isolated from endoscope washer-disinfectors (164, 165; see "Endoscopes" below). The virucidal activity of glutaraldehyde extends to the nonenveloped (hydrophilic) viruses, which are generally more resistant to disinfectants than are the enveloped (lipophilic) viruses. Numerous viruses were documented to be inactivated, including HIV, HAV, HBV, poliovirus type 1, coxsackievirus type B, yellow fever virus, and rotavirus (21, 155). The disadvantages of glutaraldehyde include the fact that it coagulates blood and can fix proteins and tissue to surfaces (20, 166). In addition, glutaraldehyde has a pungent and irritating odor, and its vapor at the level of 0.2 ppm irritates the eyes, throat, and nose. HCWs exposed to glutaraldehyde can develop allergic contact dermatitis, asthma, rhinitis, and epistaxis. Measures that may minimize employee exposure include covering immersion baths with tight-fitting lids, improved ventilation, ducted exhaust hoods or ductless fume hoods with vapor absorbents, personal protective equipment, and appropriate automated machines for endoscope disinfection (20, 167). Due to dilution, glutaraldehyde concentrations commonly decline during use in manual and automatic baths used for endoscopes (166). Test strips should be used to ensure that the glutaraldehyde concentration has not fallen below 1 to 1.5%. Equipment disinfected with glutaraldehyde and rinsed inadequately has caused serious clinical complications, including proctocolitis (colonoscopes) (168, 169) and keratopathy (ophthalmic instruments). Because the infectivity of prions can be stabilized when instruments are treated with formaldehyde before they are autoclaved (170), aldehydes are no longer recommended for disinfecting endoscopes in some European countries (e.g., France) (see "BSE and vCJD" below).

OPA

A 0.55% OPA solution has been approved as a high-level disinfectant by the FDA and by agencies in other countries. However, different countries or areas have set different exposure times for a 0.55% solution of OPA at 20°C to achieve high-level disinfection: 12 min in the United States, 10 min in Canada, and 5 min in Europe, Asia, and Latin America. Compared with glutaraldehyde, OPA has several advantages: (i) it does not require activation; (ii) it is compatible with many materials (i.e., similar to glutaraldehyde); (iii) it is more stable during storage and reuse as well as at a wide pH range of 3 to 9; (iv) it has low vapor properties; (v) its odor is barely perceptible; (vi) it is more rapidly mycobactericidal than glutaraldehyde *in vitro* and has good activity against glutaraldehyde-resistant strains at longer exposure times (171). However, 0.5% OPA is slowly sporicidal and does not inactivate all spores within 270 min of exposure (172). In addition, OPA stains proteins, skin, clothing, and instruments. OPA vapors may irritate the respiratory tract and eyes. At present, the effects of long-term exposure and safe exposure levels are not well defined. Therefore, OPA must be handled with appropriate safety precautions (i.e., gloves, fluid-resistant gowns, and eye protection), and it must be stored in containers with tight-fitting lids. If additional studies corroborate OPA's advantages, this compound may replace glutaraldehyde for many uses, especially endoscope disinfection. The new agent appears to be particularly useful in washer-disinfectors, where glutaraldehyde-resistant mycobacteria have emerged (165, 172).

Formaldehyde

Formaldehyde and its condensates are reviewed in depth elsewhere (173). Formaldehyde in aqueous solutions or as a gas has been used as a disinfectant and sterilant for many decades. Its use in the health care setting, however, has sharply decreased for several reasons. The irritating vapors and pungent odor produced by formaldehyde are apparent at very low levels (<1 ppm). Moreover, allergy to formaldehyde is fairly common. In addition, the Occupational Safety and Health Administration in the United States and the Health and Safety Executive of the United Kingdom indicated that formaldehyde vapors may be carcinogenic. Thus, the Occupational Safety and Health Administration limits the 8-h time-weighted average exposure in the workplace to a concentration of 0.75 ppm. Elevated levels of occupational exposures have been found among workers in dialysis units and gross anatomy laboratories (174). Consequently, formaldehyde and formaldehyde-releasing agents are used infrequently in health care institutions, despite this agent's broad-spectrum microbicidal activity. In fact, formaldehyde has been largely replaced by peracetic acid as an agent for disinfecting hemodialysis equipment and water dialysate tubing systems. Paraformaldehyde vaporized by heat is used to decontaminate biological safety cabinets.

Chlorine and Chlorine-Releasing Compounds

Due to its hazardous nature, chlorine gas is rarely used as a disinfectant. Among the large number of chlorine compounds commercially available, hypochlorites are the most widely used disinfectants. Hypochlorite has been used for more than a century and remains an important disinfectant. Rutala and Weber published an extensive review of uses for inorganic hypochlorite in health care facilities (175), and Karol reviewed the potential hazards and significant benefits of chlorine use (176). Aqueous solutions of sodium hypochlorite are usually called household bleach. Bleach commonly contains 5.25% sodium hypochlorite or 52,500 ppm available chlorine; a 1:10 dilution of bleach provides about 300 to 600 mg of free chlorine per liter. Alternative chlorine-releasing compounds frequently used in health care facilities include chloramine-T, sodium dichloroisocyanurate tablets, and chlorine dioxide. Demand-release chlorine dioxide is an extremely reactive compound and must be prepared at the point of use. It is used primarily to chlorinate potable water, swimming pools, and wastewater. In Europe, commercial chlorine dioxide preparations are available to disinfect instruments. In aqueous solution, all chlorine compounds release hypochlorous acid, the most likely active compound. The mechanism of microbicidal action of hypochlorous acid has not been fully elucidated, but it inhibits key enzymatic reactions within cells and denaturates proteins. Lowering the pH or raising the temperature or concentration increases its antimicrobial efficacy. Chlorine compounds have broad antimicrobial spectra including, at higher concentrations, bacterial spores and M. *tuberculosis*. Therefore, hypochlorite can be used as a high-level disinfectant for semicritical items. Concentrations of 100 ppm of available chlorine inactivate vegetative bacteria and viruses in 10 min. Suspension tests document that both enveloped and nonenveloped viruses, including HIV, HAV, HBV, herpes simplex virus types 1 and 2, poliovirus, coxsackievirus, and rotavirus are inactivated (175). In one study, a concentration of 100 ppm chlorine eliminated 99.9% of *Bacillus subtilis* endospores in 5 min (177). However, endospore-forming bacteria, mycobacteria, fungi, and protozoa usually are less susceptible to chlorine than other microorganisms, and high concentrations of chlorine (1,000 ppm) are required to completely destroy them. Despite this limitation, sodium hypochlorite solutions (500 ppm and 1,600 ppm) have been reported to decrease C. *difficile* environmental contamination and terminate outbreaks of infections caused by this organism (178). *Cryptosporidium* oocysts are particularly resistant to chlorine. These oocysts remain infective for several days in swimming pool water containing recommended chlorine concentrations, and because of their small size, they may not be removed efficiently by conventional pool filters. Outbreaks of *Cryptosporidium* infections have been associated with drinking water and swimming pools (179). Of note, chloramine-T and sodium dichloroisocyanurate seem to have less sporicidal action than does sodium hypochlorite. Hypochlorite is fast acting, nonstaining, nonflammable, and inexpensive. However, its use is limited because it is corrosive, inactivated by organic matter, and relatively instable. Sodium hypochlorite can injure tissue; however, this occurs rarely in health care facilities (175). Inhalation of chlorine gas may irritate the respiratory tract, resulting in cough, dyspnea, and pulmonary edema or chemical pneumonitis. The potential carcinogens trihalomethanes have been detected in chlorine-treated water, and high levels of trihalomethanes can be detected when hospitals hyperchlorinate their water systems (180).

Chlorine compounds have other important disadvantages. Blood or other organic matter substantially inactivates hypochlorites and other chlorine compounds. Consequently, items used for patient care and environmental surfaces must be cleaned before hypochlorite is used. In addition, biofilm (e.g., in the pipes of a water distribution system) also reduces the efficacy of chlorines significantly. Moreover, the free available chlorine levels in solutions can decay to 40 to 50% of the original concentration after the container has been opened for 1 month. Therefore, concentrations higher than those established in laboratory experiments should be used in practice. Loss of free chlorine can be minimized if the solutions are kept and used at room temperature, in dilution, in an alkaline pH range, and stored in closed opaque containers.

Depending on the concentrations employed, sodium hypochlorite is used in hospitals as a high-level disinfectant for selected semicritical devices (e.g., dental equipment and mannequins used for cardiopulmonary resuscitation training), as an intermediate-level disinfectant (e.g., hemodialysis equipment), and as a low-level disinfectant for environmental surfaces and hydrotherapy tanks. For example, the CDC recommends that HCWs use a 1:100 dilution (5,000 ppm) of hypochlorite to decontaminate spills of blood and certain other body fluids (181). Because chlorine can be inactivated by blood and other organic material, a full-strength solution or a 1:10 dilution will be safer unless the surface is cleaned before it is disinfected (182–184). Household bleach also can be used to disinfect tabletops, incubators, and spills in laboratories or to disinfect syringes used by drug addicts if sterile disposable syringes are not available (185). At low concentrations, chlorines (usually about 0.5 ppm free chlorine) are used to chlorinate the drinking water. Hyperchlorination of institutional water systems has controlled epidemics caused by *Legionella pneumophila* (180) but also corrodes the water distribution system (180). Stabilized solutions of chlorine dioxide appear to be less toxic and more efficacious than chlorine for controlling growth of legionellae (186). A growing number of municipal water treatment plants in the United States are using monochloramine as a residual disinfectant. Chloramination of drinking water has several advantages compared to the use of free chlorine, including decreasing the risk of Legionnaires' disease at the municipal level or in individual hospitals (187). However, outbreaks of *Cryptosporidium* infections have occurred in cities that use chloramines in their drinking water.

Hydrogen Peroxide

Hydrogen peroxide, a strong oxidizer, is used for high-level disinfection and sterilization. It produces destructive hydroxyl free radicals that attack membrane lipids, DNA, and other essential cell components. Although the catalase produced by anaerobic and some aerobic bacteria may protect cells from hydrogen peroxide, this defense is overwhelmed by the concentrations used for disinfection (188). Generally, a 3% hydrogen peroxide solution is rapidly bactericidal, but it kills organisms with high cellular catalase activity (e.g., S. *aureus* and S. *marcescens*) less rapidly. Surprisingly, 3% hydrogen peroxide was ineffective against vancomycin-resistant enterococci (19, 189). Spores are more resistant than vegetative bacteria to hydrogen peroxide. For example, a

3% solution of hydrogen peroxide destroyed 10^6 spores in six of seven exposure trials that were 150 min long; a 10% solution was always successful in 60 min (190). Higher concentrations of hydrogen peroxide (17.7 and 35.4%) killed *Bacillus subtilis* spores in 9.4 and 2.3 min, respectively (191). It has been found that 10% hydrogen peroxide was the most active of the seven chemical disinfectants tested against *B. subtilis* spores (192). However, other investigators found that the sporicidal activity of hydrogen peroxide was lower than those of peracetic acid and chlorine (193). Hydrogen peroxide's sporicidal activity can be enhanced by increasing the concentration or temperature or by using it in conjunction with ultrasonic energy, UV radiation, and some chemical agents such as peracetic acid (25, 152, 194). A 0.3% solution of hydrogen peroxide is able to inactivate HIV in 10 min (25), and a 3% concentration inactivates rhinovirus in 6 to 8 min at 37°C (194). However, a 6% solution was ineffective against poliovirus at 1 min (152). Hydrogen peroxide does not coagulate blood and does not fix tissues to surfaces. In fact, it may enhance removal of organic material from equipment. Hydrogen peroxide has a low toxicity for humans. It decomposes to oxygen and water, and therefore, it is environmentally safe. It is neither carcinogenic nor mutagenic. Concentrated solutions may irritate the eyes, skin, and mucous membranes. Hydrogen peroxide can be destroyed easily by heat or enzymes (catalase and peroxidases). Stabilized solutions can be used for high-level disinfection of semicritical items, considering the corrosive effects of hydrogen peroxide on copper, zinc, and brass (20). The FDA has approved commercial products containing either 7.5% hydrogen peroxide alone or combinations with peracetic acid as liquid sterilants and high-level disinfectants for processing reusable medical and dental devices (www.fda.gov) (195). Concentrations of 3 to 6% are used to disinfect ventilators, soft contact lenses (3% for 2 to 4 h) (196), and tonometer biprisms (20, 188, 197). Vaporized hydrogen peroxide is also used for plasma sterilization (see below). Despite its limited toxicity, hydrogen peroxide can damage human tissues. Patients exposed to endoscopes contaminated by residual hydrogen peroxide have developed pseudomembrane-like enterocolitis (pseudolipomatosis) (195). In addition, patients who were exposed to tonometer tips disinfected with hydrogen peroxide and rinsed improperly suffered corneal damage (197). Use of hydrogen peroxide to clean wounds and in dental regimens remains controversial (188).

Peracetic Acid

Peracetic acid (or peroxyacetic acid) is a more potent germicidal agent than hydrogen peroxide and was the most active agent in several *in vitro* studies (198, 199). Concentrations of ≤1% are sporicidal even at low temperatures. The mechanism of action of peracetic acid has not been fully elucidated, but its mechanism of action is likely to be similar to that of hydrogen peroxide and other oxidizing agents. Peracetic acid remains effective in the presence of organic matter. At low concentrations, it is considerably less stable than hydrogen peroxide; preparations with appropriate stability have been developed and are commercially available. Peracetic acid corrodes steel, galvanized iron, copper, brass, and bronze, and it attacks natural and synthetic rubbers. In addition, concentrated solutions can seriously damage eyes and skin. Furthermore, some investigators have raised concerns about the potential toxicity of the combination of peracetic and acetic acids (200). Feldman et al. reported that mortality rates in freestanding dialysis facilities that reprocessed dialyzers with peracetic and acetic acid were higher than in facilities that discarded dialysis filters or used formaldehyde for reprocessing (201). To date, investigators have not determined whether the higher death rate was caused by the disinfectants or associated with other practices at the facilities or with patient risk factors. Nevertheless, because peracetic acid has powerful germicidal activity and does not produce toxic residues, peracetic acid is very attractive for use in health care settings, most frequently in combination with hydrogen peroxide to disinfect hemodialyzers. The FDA lists several commercial products containing a combination of peracetic acid and hydrogen peroxide as high-level disinfectants and chemical sterilants. The use of peracetic acid for chemical sterilization of instruments and endoscopes (Steris System 1) is discussed below.

Alcohols

Alcohols are also excellent products for intermediate-level and low-level disinfection of small, clean surfaces, equipment, and the environment (e.g., rubber stoppers of medication vials, stethoscopes, and medication preparation areas). However, alcohols may damage rubber, certain plastic items, and the shellac mountings of lensed instruments after prolonged and repeated use (20). Moreover, alcohols are flammable (one should consider the flash point) and thus must not be used on large surfaces, particularly in closed, poorly ventilated areas. Alcohols cannot penetrate protein-rich materials. Therefore, a spray or a wipe with alcohol may not disinfect a surface contaminated with blood or other body fluids that has not been cleaned first. For mechanisms of action and antimicrobial activity see discussion under "Antiseptics."

Phenolics

Since Lister's pioneering use of phenol (carbolic acid) as an antiseptic, a large number of phenol derivatives (or phenolics) have been developed and marketed. Phenol derivatives originate when one of the hydrogen atoms on an aromatic ring is replaced by a functional group (e.g., alkyl, benzyl, phenyl, amyl, or chloro). The three phenolics most commonly used as constituents of disinfectants are *o*-phenylphenol, *o*-benzyl-*p*-chlorophenol, and *p*-*tert*-amylphenol. The addition of detergents to the basic formulation results in products that clean, dissolve proteins, and disinfect in one step. Phenolics at higher concentrations act as gross protoplasmic poisons, penetrating and disrupting the bacterial cell wall and precipitating the cell proteins (202). Lower concentrations of these compounds inactivate cellular enzyme systems and cause essential metabolites to leak from the cell. Phenol compounds at concentrations of 2 to 5% are generally considered bactericidal, tuberculocidal, fungicidal, and virucidal against lipophilic viruses (202). However, the manufacturers' efficacy claims have generally not been verified by independent laboratories or the EPA (20). A collaborative study by Rutala and Cole documented the fact that randomly selected EPA-registered phenolic detergents and quaternary ammonium compounds do not consistently meet the manufacturers' bactericidal label claims (203). Phenolics tested by the AOAC use-dilution method at the recommended use dilution failed to kill *P. aeruginosa* in 33 to 78% of laboratories. However, extreme variability of test results has been observed among laboratories testing identical products (203). Phenolics at in-use dilutions are not lethal to bacterial spores. Terleckyj and Axler found that a 2% phenolic killed a wide spectrum of clinically important fungi but did not kill *Aspergillus fumigatus* (204). Although 5% phenol inactivated both lipophilic and hydrophilic viruses, Klein and DeForest found that 12% *o*-phenylphenol was effective only against lipophilic viruses (21). Similarly, other investigators demonstrated little or no virucidal effect of a phenolic against coxsackievirus type B4, echovirus type 11, or poliovirus type 1 (205). Martin et al. showed that a 0.5% commercial phenolic formulation (2.8% *o*-phenylphenol and 2.7% *o*-benzyl-*p*-chlorophenol) inactivated HIV (25), but another commercial product containing phenolics at a final concentration of 1% did not completely inactivate cell-associated HIV suspended in blood (206). A phenol-based preparation (14.7% phenol diluted 1:256 in tap water) and a bleach dilution (800 ppm of available chlorine) reduced rotavirus numbers similarly and interrupted transfer of virus from disks to fingerpads (207). Phenolic compounds are relatively tolerant of anionic and organic matter. They are absorbed by rubber and plastics and leave a residual film, which may irritate skin and tissues. *p-tert*-Butylphenol and *p-tert*-amylphenol have been reported to depigment skin. Although differences between the various compounds exist, phenolics are degraded in wastewater at a lower rate than other germicides, which limits their use in Europe. Phenolic germicidal detergent solutions may be used for intermediate-level and low-level disinfection of surgical instruments and noncritical patient care items. These compounds are also appropriate for decontaminating the hospital environment, including laboratory surfaces. They should not be used to disinfect bassinets and incubators because they can cause hyperbilirubinemia in infants (20).

Quaternary Ammonium Compounds

A wide variety of quaternary ammonium compounds (quats) with antimicrobial activity have been introduced in the past decade. Some of the compounds used in health care settings are benzalkonium chloride, alkyldimethylbenzyl ammonium chloride, and didecyldimethyl ammonium chloride. Quats are cationic surface-active detergents, which appear to kill microorganisms by disrupting cell membranes, inactivating enzymes, and denaturing cell proteins (208). However, they have a limited antimicrobial spectrum. Products sold as hospital disinfectants are not sporicidal and are generally not tuberculocidal or virucidal against hydrophilic viruses. Scientific investigations using the AOAC use-dilution method have not reproduced the bactericidal and tuberculocidal claims made by the manufacturers (209). Consequently, HCWs should be suspicious of the claims on labels and of results from in-house evaluations that have not been verified by an independent laboratory. The overestimation of the germicidal activity may be related to incomplete inactivation of the compounds tested. In this case, the bacteriostatic (inhibitory) activity rather than the bactericidal activity is measured (208). The antimicrobial spectrum of quats may be improved by combining them with amines and biguanides or by using them at higher temperatures in washing machines.

Several outbreaks of infections have been associated with quat solutions contaminated in use by Gram-negative bacteria such as *Pseudomonas* spp. or *S. marcescens* or by *Mycobacterium abscessus* (210–212). The contaminated solutions were used as antiseptics on skin and tissue and to disinfect patient care supplies or equipment (i.e., cardiac catheters and cystoscopes). In fact, microbiology laboratories use the quat cetrimide in selective media to isolate *P. aeruginosa*.

In addition, it has been demonstrated that vaginal ultrasound probes can be contaminated with human and human papilloma virus DNA, despite disinfection with quats and use of a probe cover (213).

Quats have other disadvantages. Genes conferring resistance to quats have been detected in 6 to 42% of *S. aureus* isolates collected in Japan and Europe (40). Organic matter, anionic detergents (soaps), and materials such as cotton and gauze pads can reduce the microbicidal activities of quats. Despite these limitations, quats are nonstaining, odorless, noncorrosive, and relatively nontoxic. They are excellent cleaning agents, but sticky residue may build up on surfaces. On the basis of their limited antimicrobial spectra, they should be used in hospitals only for environmental sanitation of noncritical surfaces such as floors, furniture, and walls (20).

Other Germicides of Interest

Glucoprotamine, the conversion product of L-glutamic acid and cocopropylene-1,3-diamine, possesses a broad antimicrobial spectrum that includes vegetative bacteria, mycobacteria, fungi, and enveloped viruses (214, 215). A clinical study examining used specula from a gynecologic clinic demonstrated that the product killed >6 log units of vegetative bacteria, excluding spores (216). The manufacturer's data sheets indicate good compatibility of the compound with humans, the environment, and various materials. A commercial product, available in Europe, can be used to disinfect instruments and endoscopes.

Peroxygen compounds have proven efficacy against bacteria, bacterial spores, fungi, and a broad spectrum of viruses. A 1% concentration of a new commercial formulation containing peroxygen achieved a 10^5-fold killing of *B. subtilis* in 2 to 3 h in the absence of blood, but killing was poor in the presence of blood (217). Moreover, several investigators have found that peroxygen has poor mycobactericidal activity (164, 218). Besides other applications, these compounds may be suitable for disinfecting laboratory equipment and workbenches. Superoxidized water is prepared at the point of use by the electrolysis of NaCl solution, which generates hypochlorous acid and a mixture of radicals with strong oxidizing properties (219). Freshly generated solutions rapidly destroy bacteria, including spores and mycobacteria, fungi, and viruses in the absence of organic loading (220). A commercial adaptation of this process (i.e., Sterilox) has been marketed in Europe since 1999 and recently was approved by the FDA (see "Endoscopes" below) (219). Because Sterilox solutions are unstable, they should be used only once for high-level disinfection. Some investigators have claimed that superoxidized water is compatible with instruments and that it does not damage the environment, irritate the respiratory tract and skin, or corrode metal. However, others have reported that superoxidized water damages flexible endoscopes. Further studies are needed to explore the use of this new disinfectant in clinical settings.

Metals such as copper and silver ions inactivate a wide variety of microorganisms (221). Although further work is required to explore their use in health care, they currently are used to disinfect water and to prevent infections associated with medical devices (e.g., intravascular catheters impregnated with silver sulfadiazine). For example, copper-silver ionization systems are successfully used to minimize legionella colonization in water systems (222). Surfacine is a new silver-based surface germicide that may be applied to inanimate or animate surfaces. Surfacine immediately eliminates microorganisms from surfaces and also has long-term residual activity (223, 224).

Emergence of Resistance to Biocides

Microorganisms rarely become resistant to disinfectants. However, frequent use of sublethal concentrations of disinfectants can select for resistant strains (225–227). Mechanisms of resistance include acquisition of resistance plasmids, changes in the cell membrane (e.g., chlorhexidine in *Pseudomonas stutzeri*), capsule formation (*Klebsiella* spp.), and activation of the *nor*A efflux pump (*S. aureus*). A large proportion of household soaps now contain antibacterial agents (up to 45% in one study), which may increase the probability that resistant bacteria will emerge (228). Multiple outbreaks have been associated with soaps containing antibacterial agents such as chlorhexidine, hexetidine solution, or chlorxylenol (225–227). However, the concentrations of biocides used in the health care setting are much higher than the minimum biocidal concentrations *in vitro*. Therefore, resistance has not become a major problem in the clinical setting to date. However, a recent study demonstrated high-level resistance to glutaraldehyde (229).

Readers desiring more information about disinfectants and antiseptics (6, 230) and resistance to these agents should read several excellent articles (6, 230–232).

Inactivation of Emerging Pathogens and Antibiotic-Resistant Bacteria

New and emerging pathogens such as the causative agent of vCJD, noroviruses, SARS and Middle East respiratory syndrome coronaviruses, avian and swine influenza viruses, hypervirulent *C. difficile*, Panton-Valentine leukocidin-producing *S. aureus*, *Enterobactericeae* producing extended-spectrum beta-lactamases and/or carbapenemases, and drug-resistant nonfermenting Gram-negative bacilli threaten the public health. Only limited data exist regarding the susceptibility of emerging pathogens to commonly used disinfectants or sterilants. Surrogate microbes have been studied for some pathogens. Examples include feline calicivirus for noroviruses, vaccinia virus for variola virus, and *Bacillus atrophaeus* (formerly *B. subtilis*) for *Bacillus anthracis* (233). Other infectious agents that cannot be evaluated by standard testing procedures (e.g., hepatitis C virus [HCV]) have been tested by alternative methods, such as PCR. With the exception of prions, there is no evidence that emerging pathogens are less susceptible to approved standard disinfection and sterilization procedures than are comparable classical pathogens. Standard disinfection and sterilization procedures for patient care equipment as recommended in guidelines and in this chapter are adequate to disinfect or sterilize instruments or devices contaminated with blood and other body fluids (234). Hospital disinfectants registered by the EPA, other than one peroxygen compound, do not have specific claims for activity against noroviruses. Because noroviruses are nonenveloped, most quats do not have significant activity against them. Phenolic-based preparations have been found to be active *in vitro* against a surrogate virus of this group. However, concentrations two- to fourfold higher than those recommended for routine use by manufacturers may be required. In the event of a norovirus outbreak, the CDC recommends using a hypochlorite solution (minimum chlorine concentration of 1,000 ppm) to decontaminate hard, nonporous, environmental surfaces (http://www.cdc.gov/norovirus/index.html). SARS coronavirus and avian influenza virus are inactivated by sodium hypochlorite and a commercially available peroxygen compound (235); phenolic compounds and quats are less effective. A sporicidal germicide is required to efficiently eliminate *C. difficile* spores. In a recent study, glutaraldehyde (2%), peracetyl ions (1.6%, equivalent to 0.26% peracetic acid), and acidified nitrite demonstrated biocidal activity against *C. difficile* spores (236). Hypochlorite-based disinfectants have been used to disinfect environmental surfaces in areas with ongoing transmission of *C. difficile*. Daily disinfection with bleach wipes with 0.55% active chlorine reduced hospital-acquired *C. difficle* infections on units with high endemic incidence by 85% (237). Recent outbreaks with virulent strains may require more focus on environmental cleaning and disinfection (238, 239). There is some evidence that sporulation of *C. difficile* can be enhanced by contact with non-chlorine-based cleaning agents in subinhibitory concentrations (240). There are no data demonstrating that disinfectants used at recommended contact conditions and concentrations are less effective against antimicrobial-resistant bacteria than against antimicrobial-susceptible bacteria (234). Inactivation of prions, including those causing vCJD, is discussed below.

Mode of Application of Chemical Disinfectants

Cleaning and Disinfecting Surfaces and Floors

In general, the environment is not a primary reservoir for nosocomial pathogens. However, in some cases, environmental contamination may be important. Recent examples include respiratory syncytial virus (241) and the SARS coronavirus (242). The CDC's recent guidelines for environmental infection control in health care facilities recommend using an EPA-registered hospital detergent/disinfectant designed for general housekeeping purposes in patient care areas, especially in intensive care units, operating theaters, and emergency rooms, where blood, body fluids, or multidrug-resistant organisms may have contaminated surfaces (148). A one-step process is adequate in most areas, but a rinse step is necessary in nurseries and neonatal intensive care units, especially if a phenolic agent was used (243). Products with quats allow cleaning and disinfecting in one step, but residual quats on the surface may result in sticky, smeary surfaces. Other products may require a two-step approach (a cleaning step and a disinfection step), doubling the workload. "High-touch" surfaces (e.g., doorknobs, bed rails, and light switches) should be disinfected more frequently than "minimal-touch" surfaces. A simple detergent is adequate for cleaning surfaces for other patient care areas and in non-patient-care areas. Cleaning with a detergent is much more important than adding a disinfectant to the solution. In fact, several studies found that adding a disinfectant did not prolong the reduction in bacterial load on surfaces (146). Routine disinfection of environmental surfaces is necessary for all areas with patients in contact isolation (e.g., patients infected with MRSA). Twice-daily disinfection may be necessary to control an outbreak with vancomycin-intermediate *S. aureus* (239, 244).

In rare situations, routine disinfection of surfaces and floors is crucial: when cases of norovirus or clusters with *C. difficile* or MRSA are detected, an immediate switch from cleaning floors and surfaces to using a highly active disinfectant is warranted. Several studies demonstrate a correlation between contaminated surfaces and clinical cases (245–247). When patients with suspected norovirus infection vomit, immediate disinfection of the vomitus with highly concentrated bleach or an oxygen-release compound is crucial. Norovirus is highly contagious; in fact, 100 virions are sufficient to induce infection, but $>10^6$ virions are shed by infected patients.

The conventional disinfection methods may be limited by reliance on the operator to ensure appropriate selection, formulation, distribution, and contact time of the agent. "No-touch" automated room disinfection (NTD) systems have been introduced with the goal to reduce these problems. Available NTD systems include hydrogen peroxide (H_2O_2) vapor systems, aerosolized hydrogen peroxide, and ultraviolet radiation. The more frequently described H_2O_2 vapor systems and the aerosolized hydrogen peroxide require isolation of air vents and doors of the room to be decontaminated. Peroxide will be either aerosolized or vaporized. There is some evidence that the use of H_2O_2 vapor systems reduces transmission of pathogens that are associated with the environment in the endemic as well as epidemic setting (248–252). However, the cost-effectiveness of NTD systems will be affected by the degree to which they reduce transmission and requires further evaluation. There are few studies disclosing the cost, but further information can be found in a review by Otter et al. (253).

Disinfection of Medical Devices

Classification of Devices for Reprocessing

The principal goal of disinfection and sterilization of medical devices is to reduce the numbers of microorganisms on a device to a level that will prevent transmission of infectious organisms, with a considerable safety margin. The most conservative approach would be to reprocess all items and devices with overkill sterilization. Obviously, not all items must undergo the most vigorous process to eliminate any microorganisms. For example, items such as blood pressure cuffs that are used at nonsterile body sites do not need to be sterilized between patients. In contrast, only sterilization will eliminate any risk of infection for devices used in a normally sterile body site. In some cases, the best choice may be to use disposable items instead of reusable devices because reprocessing may be more expensive or does not provide the desired level of safety. The latter may apply to items in contact with neural tissue of a patient suffering from any form of CJD or with tonsils and other lymphatic tissues of persons with spongiform encephalopathy (bovine spongiform encephalopathy [BSE] or vCJD) (254–256). Therefore, devices must be classified to allow staff to define the appropriate method for disinfection and/or sterilization for each item. A classification system should balance the potential risks for transmission of infection (e.g., the infectious dose) and the resources available to achieve the necessary or desired level of antimicrobial killing. The most commonly used classification was proposed by Earle H. Spaulding in 1968 (257). He proposed three categories that are based on the devices' potential for transmitting infectious agents: critical, semicritical, and noncritical (Table 4). The CDC cites this classification in its "Guidelines for Environmental Infection Control in Health-Care Facilities" (http://www.cdc.gov/hicpac/pdf/guidelines/eic_in_hcf_03.pdf), as does the FDA for approval of sterilants and high-level disinfectants (see http://www.fda.gov/MedicalDevices/DeviceRegulationand Guidance/ReprocessingofSingle-UseDevices/ucm133514.htm). Most infection control professionals worldwide use this classification as well. However, this simple classification does not work perfectly for all devices. Even the definition of sterilization as the absence of any viable microorganisms must be revised to address the prions responsible for CJD and vCJD (258).

Critical items. Items that enter normally sterile parts of the human body, such as surgical instruments, implants, or invasive monitoring devices (Table 4), are classified as "critical items." Because items classified as critical carry the highest risk for the patient, sterilization is the preferred method for reprocessing these items. Autoclaving is the method of choice if the device is not heat labile. Alternative sterilization processes that use ethylene oxide or plasma require prolonged times, and the FDA has not approved them for use with instruments that have small dead-end lumens, which are difficult to sterilize. Liquid sterilization with a glutaraldehyde-based formulation or peracetic acid is acceptable if sterilization by one of the methods mentioned above is not feasible and the formulation and/or automated device has been cleared by the FDA.

Semicritical items. Semicritical objects come into contact with mucous membranes or nonintact skin and should be free of microorganisms except spores. Intact mucous membranes generally resist bacterial spores but are susceptible to other microorganisms such as vegetative bacteria (e.g., *M. tuberculosis*) or viruses (e.g., HIV and cytomegalovirus). Examples of semicritical equipment include anesthesia equipment, respiratory equipment, and endoscopes. These items should be processed with a high-level disinfectant such as glutaraldehyde, stabilized hydrogen peroxide, peracetic acid, or a chlorine compound. Chlorine compounds corrode items and, therefore, are rarely used to disinfect medical devices.

TABLE 4 Spaulding classification of devices

Clinical device	Definition	Example(s)	Infectious risk	Reprocessing procedure (FDA classification)
Critical device	Medical device that is intended to enter a normally sterile environment, sterile tissue, or the vasculature	Surgical instruments	High	Sterilization by steam, plasma, or ethylene oxide; liquid sterilization acceptable if no other methods feasible
Semicritical device	Medical device that is intended to come in contact with mucous membranes or minor skin breaches	Flexible endoscope	High, intermediate	Sterilization desirable; high-level disinfection acceptable
Noncritical device	Medical device that comes in contact with intact skin	Blood pressure cuff, electrocardiogram electrodes	Low	Intermediate or low level
Medical equipment	Device or component of a device that does not typically come in direct contact with the patient	Examination table	Low	Low-level disinfection, sanitizer

Noncritical items. Noncritical items (bedside tables, crutches, stethoscopes, furniture, and floors) come into contact with intact skin only. Intact skin is a very effective barrier against microorganisms, and therefore, these items and devices do not need to be sterilized. Such items pose a low risk for direct transmission of pathogens and can usually be cleaned at the bedside or at their point of use with a low-level disinfectant. For example, HCWs can disinfect their stethoscopes by wiping the surfaces with alcohol. However, noncritical devices can contribute to the transmission of pathogens by the indirect route. For instance, up to 60% of cultures of the environment near patients colonized or infected with vancomycin-resistant enterococci are positive for this organism (259). HCWs can contaminate their hands when they touch these surfaces. If they do not practice hand hygiene, they can spread these pathogens to devices or directly to other patients. Therefore, noncritical items must be decontaminated if they are likely to be contaminated with pathogenic organisms. The FDA also developed a classification based on safety considerations and the regulations manufacturers must meet before marketing a device. Medical products are listed as class I to III products (Table 5). Simple products (e.g., a tongue depressor) are classified as medical product class I, which must meet very simple requirements before being marketed legally. Class II products (e.g., autoclaves) require a premarket notification [510(k)] demonstrating that the device is at least as safe and effective as a legally marketed device. Class III devices are those that support or sustain human life and are of substantial importance in preventing impairment of human health (e.g., a pacemaker).

Due to the level of risk associated with class III devices, the FDA requires companies (section 515 of the Federal Food, Drug, and Cosmetic Act) to file a premarket approval application to obtain marketing clearance. The premarket approval application must contain sufficient valid scientific evidence documenting that the device is safe and effective for its intended use (258).

Endoscopes

Reprocessing endoscopes is probably the most challenging reprocessing task in health care. Multiple reports of outbreaks associated with insufficient reprocessing techniques or defects of the endoscope have been published (Table 6). However, ample data indicate that a sufficient level of safety can be achieved even with manual disinfection of endoscopes if the guidelines are strictly followed (260). Flexible endoscopes have intricate, sophisticated small parts that are difficult to clean but must be cleaned before they can be disinfected because organic material such as blood, feces, and respiratory secretions interfere with disinfection (261). Several studies have demonstrated the importance of cleaning in experimental studies with duck HBV, HIV, and *Helicobacter pylori* (262, 263). A large study in several centers in the United States found that 23.9% of the cultures of specimens from the internal channels of 71 gastrointestinal reprocessed endoscopes grew $\geq 10^6$ CFU of bacteria and that 78% of the facilities did not sterilize all biopsy forceps (264). Other studies have documented that up to 40% of the institutions do not follow published guidelines for endoscope disinfection (167, 265, 266), and reuse of disposable endoscopic accessories is common in the United States. These items frequently are not sterilized, and reprocessing protocols are not standardized. Therefore, reused disposable items might be a source of cross-transmission (261, 267). Currently, most high-level disinfectants approved by the FDA for reprocessing endoscopes contain >2% aldehyde with or without peracetic acid (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/ReprocessingofSingle-UseDevices/ucm133514.htm). However, aldehydes should only be used after completing the cleaning cycle because they may stain prions to the instruments. Endoscopes, which are semicritical items, must be immersed in ≥2% glutaraldehyde for ≥20 min to achieve the necessary level of disinfection. These parameters are sufficient to kill ≥3 log units of mycobacteria, the most-resistant vegetative bacteria. Glutaraldehyde-resistant mycobacteria have been identified (164). Several authors raised concerns that *C. difficile* may not be fully inactivated by standard reprocessing procedures. However, transmission of *C. difficile* by contaminated endoscopes has not been reported to date. Moreover, cryptosporidia withstand several hours of exposure to glutaraldehyde (159) but do not survive on dry surfaces (268). Therefore, drying before storing reprocessed items is part of the process and should not be cut to save time, e.g., in endoscopy units. The glutaraldehyde concentration in commercial cleaner-disinfectors can decrease by more than 50% after 2 weeks, which may promote the emergence of resistant bacteria (165). Higher concentrations of glutaraldehyde (3.2% instead of 2%) appear to be safe for endoscopes and achieve the required ≥3-log-unit killing with a higher margin of safety than achieved with the standard concentration (269). OPA and peracetic acid plus hydrogen peroxide can be

TABLE 5 Principles of medical device classification

Classification	FDA regulation	Premarket requirements by the FDA	Proposed classification by Global Harmonization Task Force[a]	Example(s)
Class I	Least regulated, requires fewest regulations	None	A	Band-Aid, tongue depressor
Class II	Must meet federal performance standards	Premarket notification [510(k)]	B	Hypodermic needles, suction equipment
			C	Orthopedic implants
Class III	Implanted and life-supporting or life-sustaining devices are required to have FDA approval for safety and effectiveness	Premarket approval	D	Pacemaker

[a]Details available at http://www.imdrf.org/docs/ghtf/final/sg1/technical-docs/ghtf-sg1-n77-2012-principles-medical-devices-classification-121102.pdf.

TABLE 6 Outbreaks and pseudo-outbreaks associated with contaminated endoscopes or instruments for minimally invasive procedures

Microorganisms	No. of cases	No. of deaths	Yr of publication	Problem identified	Type of outbreak	Reference(s)
K. pneumoniae	7	0	2010	Failure to dry the duodenoscope after resprocessing	Mixed	347
P. aeruginosa	20	0	2009	Failure of automatic endoscope reprocessor and noncompliance	Mixed	335
P. aeruginosa	7	0	2008	Failure of automatic endoscope reprocessor and noncompliance	Infections	336
P. aeruginosa	17	Not reported	2006	Inadequate processing and storage of a flexible bronchoscope	Mixed	337
K. pneumoniae, *Proteus vulgaris*, *Morganella morganii*	11	0	2005	Loose port of the bronchoscope's biopsy channel	Mixed	338
P. aeruginosa	16	0	2005	Defective biopsy forceps	Mixed	261
P. aeruginosa	3	0	2004	Probable defective endoscope	Infections	339
P. aeruginosa	39	3	2003	Loose biopsy port cap in the bronchoscope	Infections	5
P. aeruginosa	18	0	2001	Improper connection to liquid sterilization device	Mixed	340
M. *xenopi*	58	0	2001	Inappropriate disinfection of microsurgical instruments, tap water rinse after disinfection	Infections	151
P. aeruginosa	11	2	2000	Failure of washer-disinfector, purchased without expert advice, poor maintenance	Infections	3, 341
P. aeruginosa, mycobacteria	29	0	1999	Problems related to the use of Steris System 1 processor	Mixed	271
HCV	2	0	1997	Cleaning, immersion	Infections	342
M. *tuberculosis*	2	0	1997	Cleaning, immersion	Infections	343
M. *tuberculosis* (multidrug resistant)	5	1	1997	Cleaning, immersion	Infections	344
P. aeruginosa	23	0	1996	Failure of washer-disinfector	Pseudo-outbreak	345
Nontuberculous mycobacteria	4	0	1992	Failure of washer-disinfector	Pseudo-outbreak	346
Multiple microorganisms	377	7	1993	Cleaning, immersion, use of tap water, poorly designed washer-disinfector	Infections	4 (review)

used to disinfect endoscopes. Because the latter might corrode some endoscopes, reprocessing staff should ensure that the manufacturer of the endoscope approves this disinfectant for reprocessing. Automated washer-disinfectors specifically for endoscopes were developed, in part, to reduce the work needed to reprocess endoscopes and to decrease the risk of human errors during manual reprocessing. These machines rinse the endoscopes, clean them in several steps, and run a full-cycle disinfection process. The time endoscopes are exposed to disinfectants is set by the machine and cannot be shortened, as it can be by busy staff manually reprocessing endoscopes. However, endoscope washers can become contaminated with pathogenic bacteria. For example, one study found Gram-negative bacteria and/or mycobacteria in 27% of cultures of specimens obtained before the final alcohol rinse and in 10% of cultures of specimens obtained thereafter. In the same study, 37 and 27% of the manually disinfected endoscopes remained contaminated at the same time points (270). In 1992, Olympus recalled (recall no. Z-039/040-2 by the FDA) its 835 model endoscope washers because the design allowed the internal tanks and tubing to become colonized by waterborne organisms such as *Pseudomonas* spp. In 1999, CDC reported three outbreaks related to the Steris System 1 (271). This device is supposed to sterilize the endoscopes, but they must first be cleaned manually (272). See Table 6 for a summary of outbreaks related to endoscopes, including those related to contaminated washer-disinfectors. Newer washer-disinfectors should continuously monitor the pressure in all channels to detect debris blocking the channels, provide adapters for all types of endoscopes, use an appropriate disinfection process with an FDA-approved disinfectant, use filtered water or sterile water for rinsing, and have a built-in automatic disinfection process. These washer-disinfectors can help staff trace problems by monitoring and documenting the disinfecting process in a manner similar to that used by autoclaves. To avoid problems, knowledgeable staff should review currently marketed machines before purchasing a washer-disinfector to ensure that the one they choose is appropriate for their needs (273). To facilitate this process, the FDA recommends that the manufacturer provide a list of all brands and models of endoscopes that are compatible with the washer-disinfector and highlight limitations associated with processing of certain brands and models of endoscopes and accessories. Preferably, the manufacturer should identify endoscopes and accessories that cannot be reliably reprocessed in the device (negative list). In addition, HCWs should be trained to use the equipment and monitored subsequently to ensure that they follow the protocol exactly. Although this is not yet mandatory, it is prudent to regularly culture the rinse water of washer-disinfectors for pathogens such as *Pseudomonas* spp. and *Mycobacterium* spp. to identify problems before clinical cases occur. In Europe, validation of the whole procedure is necessary to ensure that it complies with the requirements of the European Standard EN ISO 15883 parts 1, 4, and 5 for automated endoscope reprocessing (274). However, outbreaks may occur despite negative routine culture results (159, 268).

If washer-disinfectors recycle water, residual glutaraldehyde may remain on the endoscopes. Manual reprocessing is more prone to leave residual glutaraldehyde on endoscopes than are automated washer-disinfectors (275). Thus, endoscopes that are manually disinfected should be thoroughly rinsed to remove any residual disinfectant, specifically glutaraldehyde. Patients exposed to residual glutaraldehyde can develop colitis (168, 169). Reprocessed endoscopes should be stored vertically (to facilitate drying) in a cabinet (to protect them from dust and secondary contamination). Drying cabinets with a heat fan, which keep the endoscope dry in a clean-air environment, are available. Reprocessed endoscopes that are stored for days or weeks before use probably should be reprocessed again, or alternatively, the channels should be rinsed with spore-filtered alcohol (70% g%) if this agent is compatible with the instrument. In France, reprocessing is mandatory if the reprocessed endoscope has not been used within certain time limits. However, the necessity of these precautions has not been established. Guidelines for infection prevention and control in flexible endoscopes have been updated (167) and should be consulted before choosing a method and/or disinfectant for reprocessing. A checklist adapted from the FDA recommendations may help staff reprocessing endoscopes avoid errors (http://www.fda.gov/MedicalDevices/Safety/AlertsandNotices/PublicHealthNotifications/ucm062282.htm) (Table 7).

TABLE 7 Checklist to avoid reprocessing errors of endoscopes[a]

1	All staff must comply with the manufacturer's instructions for cleaning endoscopes
2	Determine whether your endoscope is suitable for reprocessing in an automatic washer-disinfector, which is the preferred method
3	Compare the reprocessing instructions provided by the endoscope and washer-disinfector manufacturers and resolve any conflicting recommendations
4	Follow the instructions provided by the manufacturers of the endoscopes and the chemical germicides
5	Consider drying endoscopes with alcohol
6	Monitor adherence to the protocols for reprocessing endoscopes
7	Provide comprehensive, intensive training for all staff reprocessing endoscopes; keep records of persons attending training
8	Endoscopes sent for repairs should be labeled as "contaminated equipment for repair"
9	Implement a comprehensive quality control program

[a] Adapted from the FDA (http://www.fda.gov/MedicalDevices/Safety/AlertsandNotices/PublicHealthNotifications/ucm062282.htm).

An updated list of sterilants and high-level disinfectants approved by the FDA in a 510(k) with general claims for processing reusable medical and dental devices can be found on the FDA website (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/ReprocessingofSingle-UseDevices/ucm133514.htm). Of note, more than 20% of all damage to endoscopes is associated with disinfecting agents. Therefore, staff members who reprocess these items must ensure that the instruments and the disinfectant are compatible (267). More detailed information is available in the new CDC guideline (www.cdc.gov/hicpac/Disinfection_Sterilization/acknowledg.html).

Dental Equipment

Critical and semicritical dental instruments should be sterilized; if they will not be used immediately, they should be packaged before they are sterilized. All high-speed dental handheld pieces should be sterilized routinely between patients. Handheld pieces that cannot be heat sterilized should be retrofitted to attain heat tolerance; if this is not feasible, they should not be used. The adequacy of sterilization cycles should be verified by periodically (e.g., at least weekly) including a biological indicator with the load. This recommendation is rarely followed in Europe (276). In fact, 33% of British dental practices do not have a policy on general disinfection and sterilization procedures and only 3% own a vacuum autoclave (277), and 52.9% of the Irish dentists who responded to a survey did not autoclave their dental handpieces and only 44.7% disinfected impressions before sending them to the laboratory (278). Environmental contamination can be a problem in dental offices (279). For example, *Legionella* spp. can contaminate the air-water syringes and high-speed outlets. Water used on patients infected with bloodborne pathogens can contaminate surfaces and equipment. In fact, Piazza et al. found that more than 6% of samples from workbenches, air turbine handheld pieces, holders, suction units, forceps, and dental mirrors were positive by PCR for HCV (280). Therefore, infection control issues, particularly in regard to HCV and HBV, may be more important in dentistry than has been appreciated previously. The CDC and the American Dental Association (ADA) have published guidelines for infection control in dental settings (281, 282). The ADA recommends that metal and porcelain equipment be immersed in glutaraldehyde or exposed to this disinfectant, that removable dentures and acrylic or porcelain be disinfected with iodophors or chlorine compounds, and that wax rims or bite plates be disinfected with a spray containing iodophors. Additional information can be found on the website of the ADA (http://catalog.ada.org/ProductCatalog/601/OSHA-Infection-Control/The-ADA-Practical-Guide-to-Effective-Infection-Control/P692).

Guidelines for Choosing a Disinfectant

Rutala and Weber have published guidelines for the selection and use of disinfectants and recommendations on the preferred method for disinfection and sterilization of patient care items (20, 283). The CDC issued guidelines for environmental infection control in health care facilities, including recommendations for cleaning and disinfection (148) and updated its recommendation in 2008 (www.cdc.gov/hicpac/Disinfection_Sterilization/acknowledg.html) (142).

When choosing a disinfectant, individuals responsible for infection control should review its effectiveness against the expected spectrum of pathogens (Tables 3 and 8) to ensure that it is adequate for the intended purpose. In addition, staff must ensure that the disinfectant is compatible with the devices it is intended to disinfect and that devices that are immersed longer than recommended will not be damaged. The latter is important because staff might forget to remove instruments, for example, during weekends or night shifts. Prolonged exposure to a disinfectant may damage the instrument. Staff should also consider the toxicity, odor, compatibility with other compounds, and residual activity of disinfectants before choosing them (Table 8). Advice from health care professionals of different institutions is very helpful to learn about their experience and to uncover problems such as interactions with detergents, unexpected coloring, odors, and, last but not least, emotions elicited. A new disinfectant used for environmental surfaces may interact with those used in the past and temporarily release unpleasant odors. Written infection control standards for environmental surfaces help to avoid incompatibilities. It is prudent to contact colleagues already using a disinfectant before introducing it in a health care facility. Once staff have identified a product that meets a facility's needs, only strong evidence from good studies should lead to a change to a new product (e.g., the product has improved activity or works faster).

Disinfection by Heat versus Immersion in Germicides

Disinfection by heat has become much more common than in the past and has replaced disinfection with germicides

TABLE 8 Overview of common disinfectants[a]

			Active against:						Important characteristics									
Germicide	Use dilution	Level of disinfection	Bacteria	Lipophilic viruses	Fungi	Small or hydrophilic viruses	*M. tuberculosis*	Bacterial spores	Shelf life of >1 wk	Corrosive or deleterious effect	Residue	Inactivated by organic matter	Skin irritant	Eye irritant	Respiratory irritant	Toxic	Environmental concerns	Application in hospitals
Glutaraldehyde	2–3.2%	High/CS	+	+	+	+	+	+	+	−	+	−	+	+	+	+	−	Endoscopes
Hydrogen peroxide	3–25%	High/CS	+	+	+	+	+	±	+	±	−	±	+	+	−	+	−	Contact lenses
Chlorine	100–1,000 ppm free chlorine	High	+	+	+	+	+	±	+	+	+	+	+	+	+	+	±	Selected semicritical devices
Isopropyl alcohol	60–95%	Intermediate	+	+	+	±	+	−	+	±	−	±	±	+	−	+	−	Small-area surfaces
Glucoprotamine	1.5–4%	Intermediate	+	+	+	+	+	−	+	−	−	−	+	+	−	−	−	Diagnostic instruments
Phenolic compounds	0.4–5% aqueous	Intermediate	+	+	+	±	+	−	+	−	+	−	+	+	−	+	+	Surgical instruments
Iodophors	30–50 ppm free iodine	Intermediate	+	+	+	+	±	−	+	±	+	+	±	+	−	+	−	Medical equipment
Quaternary ammonium compounds	0.4–1.6% aqueous	Low	±	+	±	−	−	−	+	−	+	+	+	+	−	+	−	Disinfection in food preparation areas and floors

[a]Data from references 6, 20, 149, 226, and 329. Abbreviations: CS, chemical sterilant; +, yes; −, no; ±, variable results. Efficacy of the disinfectants is based on an exposure time of less than 30 min at room temperature. Spores require prolonged exposure times (up to 10 h) unless used with a machine at higher temperatures.

for many applications in European health care facilities (284). The advantages of these devices are obvious: (i) the processes are automated and are monitored and documented in a manner similar to that for sterilization; (ii) microorganisms have not developed resistance to these processes; (iii) the cost per load is probably less than the cost of germicides. In addition, studies by Gurevich et al. (285) indicate that pasteurization with a germicide is more effective than pasteurization without a germicide. However, washers include a cleaning process with an average reduction of 4 log units, coupled with heat disinfection (5-log-unit killing; washer-disinfectors such as the AMSCO Reliance 430 achieve an inactivation factor of >5 log units) (2, 51), resulting in a total reduction of 8 to 9 log units. This surpasses any international requirements for high-level disinfection. Thermal disinfection has several disadvantages. First, the cost to purchase and install the equipment is much higher than for systems using a germicide. Second, considerable power is needed to heat the water. Third, some non-spore-forming microorganisms such as enterococci resist temperatures of up to 71°C for 10 min. Thus, recommendations such as those in the United Kingdom (the Department of Health requires 65°C for 10 min, 71°C for 3 min, or 80°C for 1 min) may not be adequate for these organisms (286). Medical washer-disinfectors that are intended to clean, disinfect at a low or intermediate level, and dry surgical instruments, anesthesia equipment, hollowware, and other medical devices are exempt from the premarket notification procedures in subpart E of part 807 of the chapter subject to §880.9 (287). The ISO provided standards for these processes in ISO norm 15883, which defines the standards for washer-disinfectors by heat with and without the addition of disinfectants. This organization has not defined a temperature at which these devices must work but rather allows manufacturers to choose a temperature in a given range at which their devices should operate. In the United States, hot-water pasteurization is generally performed at 77°C for 30 min (288), but few scientific data support use of a particular temperature. ISO 15883 introduces the A_0 concept, which is based on the fact that a defined temperature will generate a predictable lethality effect against microorganisms. Corresponding exposure temperatures and time periods that achieve high-level disinfection can be calculated assuming the presence of particularly heat-resistant microorganisms in numbers in excess of those likely to be encountered on the medical devices to be processed. ISO 15883 introduces the term A_0 for moist heat disinfection (thermal disinfection). The A_0 value of a moist heat disinfection process denotes the lethality effects expressed in terms of the equivalent time in seconds at a temperature of 80°C delivered by the process to the medical device with reference to microorganisms possessing a z value of 10. Given a predefined A_0, equivalent killing of microorganisms is achieved if the following formula is followed: $A_0 = \Delta T \Sigma 10^{(T-80)/z} t$, where T is the temperature in degrees Celsius, and t is time in seconds. An A_0 value of 600, which can be achieved at 80°C over 10 min, 90°C over 1 min, or 70°C over 100 min, is the minimum requirement for noncritical medical devices (287, 288). An A_0 value of at least 3,000, which can be achieved by exposure to hot water, e.g., at 90°C (the medical device must tolerate this temperature for >5 min), must be employed for medical devices contaminated with heat-resistant viruses such as rotavirus and HBV. An A_0 value of at least 3,000 is also appropriate for high-level disinfection of all semicritical devices. The test procedure based on the A_0 concept has been highly reproducible and found to be suitable to test washer-disinfectors (289, 290).

STERILIZATION

Principles, Definitions, and Terms

As outlined in Table 2, sterilization is not a relative term but defines the complete absence of any viable microorganisms, including spores. However, this absence cannot be proven by current microbiological techniques (291). Therefore, sterilization can be defined as a closely monitored, validated process used to render a product free of all forms of viable microorganisms, including all bacterial endospores. To test the ability of sterilization systems to meet the latter definition of sterilization, manufacturers developed a worst-case scenario that allows the process (log killing) to be quantified and estimates the probability of process failure. Large safety margins were included in this test, which is based on the assumption that items are heavily contaminated with spores, soil, and proteins. It is important to note that, while these conditions are used for testing, in clinical practice, items that are heavily soiled should not be sterilized and such a scenario would represent a critical failure of the reprocessing cycle. Any device undergoing sterilization first must undergo an appropriate cleaning process. A manufacturer must demonstrate that a sterilizer is effective against a wide range of clinically important microorganisms before being approved by the FDA. In addition, proof of efficacy must be performed with organisms (usually bacterial spores) that have been shown to be the most resistant to the new technology. A validated and reliable biological indicator must be developed, and studies must establish that sterility will be consistently achieved when critical process parameters operate within a defined range. This assures the operator that as long as there is no operational error or equipment failure, sterility is achieved. Several guidelines are essential documents for staff needing to understand reprocessing and sterilization of medical devices. ISO 14937 provides general criteria for characterizing a sterilizing agent and for the development, validation, and routine control of a sterilization process for medical devices. ISO 11134 (moist heat) and ISO 11135 (ethylene oxide) documents describe the standards for use of these methods of sterilization in the industrial setting in the United States. The American National Standard Institute/Association for the Advancement of Medical Instrumentation (ANSI/AAMI) published adaptations of these standards for health care facilities: standard 46 (moist heat) and standard 41 (ethylene oxide) (Table 2). In Europe, EN 550, EN 554, and EN 285 define the standards for steam and ethylene oxide sterilization. ISO 14161 provides guidance that staff can use when selecting and using biological indicators and when interpreting the results of these tests. ISO 17664 specifies which information medical device manufacturers must provide so that the medical device can be processed safely and continue to function properly. Readers are referred to other publications for additional information about sterilization (6, 291, 292). Hot-air sterilization does not belong to the state-of-the-art technologies, but it is still used in many countries. However, the distribution of dry heat to the instruments requires long exposure times. Temperatures of >185°C resinify paraffin, destroying the lubricating function of instruments, and higher temperatures are corrosive, resulting in loss of hardness. Therefore, hot-air sterilization has largely been replaced by better, safer, and faster technologies.

Monitoring

Any sterilization process must be monitored by mechanical, physical, chemical, and facultatively biological methods. Before routine use, the performance of the machine should be validated with the most difficult load used at the institution to ensure safety of the process. In routine use, a printout of the physical parameters (e.g., temperature and pressure) during sterilization should be kept for documentation purposes. In addition, chemical indicators placed on the tested items change color if they are exposed to adequate temperatures and exposure times. They are inexpensive and easy to use and provide a visual indication that the item has been exposed to the sterilization process. Good clinical indicators are able to identify a sterilizer failure. However, some are too sensitive, giving false-positive results (293, 294), which may cause unnecessary recalls of adequately sterilized items. Less-sensitive chemical indicators do not detect small deviations in the process. In 1963, Bowie and Dick determined that if residual air remained in a sterilizer after the vacuum phase and there was only one package in the chamber, the air would concentrate in that package (295). They developed the Bowie-Dick test to determine whether steam penetration and air removal occurred successfully. This test does not provide information about the sterilization process.

Biological indicators are the best monitors of the sterilization process. If the spores in commercially available standard biological indicators do not grow during an appropriate incubation period, the results indicate that the process was able to kill $\geq 10^6$ CFU. For flash sterilization, the Attest Rapid Readout biological indicator detects the presence of a spore-associated enzyme, α-D-glucosidase, and permits staff to assess the efficacy of sterilization within 60 min (296). Staff should investigate positive biologicals because they can provide the only indication that something is wrong with the sterilization process (297).

An important question is whether a load can be distributed before the final results of the biological indicator are available (i.e., parametric release). The Joint Commission on Accreditation of Healthcare Organizations standard allows the use of appropriate chemical indicators without routine use of a biological indicator. A common approach is to use the sterilized items if the physical and chemical parameters of adequate sterilization were met without awaiting the culture results from the biological indicators. In Europe, routine use of biological indicators is not required if the sterilizer has undergone testing by a validation procedure used for industrial steam sterilization (EN 285, EN 550, EN 554, or EN 556). Most sterilizers in European hospitals probably do not meet these very strict requirements (298), and consequently, biological indicators are used regularly to ensure that they are working properly. These industrial standards for validation of steam sterilization will be implemented in health care organizations, but this change is controversial because of the associated expenses. The future is likely to involve parametric release with regular validation and/or commissions of the equipment. Legal aspects will probably determine the outcome of this discussion, and lawyers are likely to accept nothing but a zero risk. However, the goal of a zero risk for contamination in central sterilization services will probably contribute to excessive health care costs. Therefore, standards for sterilization should exclude a risk for contamination after the reprocessing cycle but should avoid steps that are performed only for legal reasons.

Packaging, Loading, and Storage

Items that are clean and dry should be inspected and then wrapped and packaged (or put in containers) before sterilization. Wrappers should allow steam or gas to penetrate into the package but should protect the items from recontamination after sterilization. For steam sterilization, muslin as the only wrapper has limitations and handling of items made of muslin leads to contamination (299). Items should be labeled with information such as expiration date, type of sterilization, and identification code for traceability.

Steam Sterilization

The most reliable method of sterilization is one that uses saturated steam under pressure. It is inexpensive, nontoxic, and very reliable.

Steam penetrates fabrics, and its inherent safety margin is much higher than that of any other sterilization technique. Therefore, it should be used whenever possible. Obviously, other techniques must be used for heat-sensitive items. Steam irreversibly coagulates and causes denaturation of microbial enzymes and proteins. Three parameters are critical to ensure that steam sterilization is effective: the amount of time the items are exposed to steam, the temperature of the process, and moisture. Unlike time and temperature, the moisture condition in the autoclave cannot be directly determined. The D-value determines the time required to kill 10^6 CFU of the spores most resistant to the sterilization process under study. Devices or instruments must reach the desired temperature, which is not necessarily identical to the temperature displayed on the autoclave's gauge. A drop of only 1.7°C (3°F) increases the time required to sterilize an item by 48%. Without moisture, a temperature of 160°C is required for dry-heat sterilization. Dry air does not provide steam for condensation, and the heat transfer to objects is slower than when moisture is present. Pressurized steam quickly transfers energy to the sterilizer load and causes more rapid denaturation and coagulation of microbial proteins. In addition, pressurizing the steam allows one to achieve dry 100% saturated steam. Thus, there is no mist that could cause the packaging and/or the items to become wet.

Residual air in the autoclave interferes with the sterilization process. The amount of air within the sterilizer can be estimated by comparing the chamber pressure with the saturated steam pressure calculated from the average chamber temperature. A measured pressure greater than the calculated saturated pressure indicates the presence of residual air in the chamber. Such monitoring devices are common in the United Kingdom.

Several types of autoclaves are available: gravity displacement steam sterilization, prevacuum steam sterilization, and steam flash-pressure pulsing steam sterilization autoclaves. The sterilization process is less consistent in gravity displacement steam sterilizers than in the other sterilizers (300). For example, gravity displacement autoclaves are more likely than the other systems to leave residual air in the chamber before the steam is introduced. Prevacuum sterilizers resolved part of this problem and cut the cycle time in half. However, the effectiveness of sterilization still can be compromised by small leaks (1 to 10 mm Hg/min) in the sterilizer (291). The most current technology is the steam flash-pressure pulsing steam sterilization technique. Because air leaks do not decrease the effectiveness of the process, it nearly eliminates the problem of air in the chamber and it reduces the thermal lag upon heating of the load to the desired exposure temperature (300).

The process of sterilization has several cycles: conditioning, exposure, and drying. Common cycles for steam sterilization in prevacuum or flash-pressure pulsing steam sterilizers are 121°C for 15 min (121°C for 30 min in a gravity displacement sterilizer) or 132°C for 4 min. EN 554 requires steam sterilizers to provide this temperature throughout the entire chamber within a narrow margin (0 to +3°C).

Flash sterilization is an emergency process used, for example, after a surgical instrument is dropped but needs to be immediately available during a procedure (301). Unwrapped devices are exposed to pressurized steam for 3 min, usually in the operating suite, sometimes without a biological indicator. The autoclaves employed are gravity displacement sterilizers that have the problems mentioned above. If HCWs are in a hurry, they may not clean the item properly, which will prevent proper sterilization. In addition, because the items are not wrapped, they can be contaminated easily when they are transported to the operating room. Even properly wrapped sterile items can become contaminated if they are transported several times (299). Moreover, some patients have been injured by items that were flash sterilized (302). Therefore, flash sterilization is controversial and several investigators have suggested that it should be used only in emergency situations when no other device is available. Flash sterilization should not replace standard sterilization protocols (303) and should not be used to save time instead of sterilizing items by the standard methods or because the health care facility does not want to purchase an extra instrument set (103). If flash sterilization of an implantable device is unavoidable, records must be kept (i.e., load identification, patient's name/hospital identifier, and biological indicator result) to facilitate epidemiological tracking.

Ethylene Oxide Gas

Temperature- and/or pressure-sensitive items have been sterilized traditionally with ethylene oxide in a standard gas. Ethylene oxide inactivates all microorganisms, including spores, probably by an alkylation process. *B. subtilis* bacterial spores are among the most resistant, and therefore, these are used as a biological monitor for this process. A new rapid-readout ethylene oxide biological indicator indicates an ethylene oxide sterilization process failure by producing fluorescence, which is detected in an autoreader within 4 h of incubation at 37°C, and a color change related to a change in pH of the growth media within 96 h of continued incubation (224).

The process of sterilizing items with ethylene oxide begins by adding nitrogen gas to remove air or by evacuating the chamber. Items are then exposed to ethylene oxide at 55°C (130°F). Six variable but interdependent parameters—gas concentration, vacuum, pressure, temperature, relative humidity, and time of exposure—must be controlled when ethylene oxide is used. The gas concentration cannot be measured online, limiting the extent of monitoring. Therefore, the concentration should be validated as outlined in ISO 11135.

Ethylene oxide sterilization has several disadvantages. It is useful only as a surface sterilizer because it does not reach blocked-off surfaces. In addition, ethylene oxide is flammable, explosive, and carcinogenic to laboratory animals, and it requires special safety precautions. Moreover, items sterilized by ethylene oxide must be aerated for approximately 12 h to remove any traces of the gas. Thus, the entire process takes >16 h, but modern sterilizers can run at shorter cycles. Furthermore, toxic residues can be trapped in the wrapper or the items. Polyvinyl chloride and polyurethane absorb ethylene oxide readily and require long periods to dissipate the oxide. The wrapper should be a barrier against recontamination after sterilization, but it also can prevent ethylene oxide from reaching the item. Therefore, only materials with documented ethylene oxide penetration and dissipation properties should be used as wrappers.

The future of ethylene oxide in sterilization is limited, mainly due to its toxicity. However, no currently available technology, including plasma sterilization (see below), can replace sterilization with ethylene oxide entirely. In addition, sterilization with ethylene oxide does not fail as frequently as sterilization with plasma when residual proteins and/or salts are present on the items (146).

Plasma Sterilization

The low-temperature plasma is produced in a closed chamber with deep vacuum, an electromagnetic field, and a chemical precursor (hydrogen peroxide or a mixture of hydrogen peroxide and peracetic acid). The resulting free radicals, the chemical precursors, and the UV radiation are thought to be the products that rapidly destroy vegetative microorganisms including spores.

The Sterrad 100 sterilizer was the first plasma sterilizer for use in health care facilities and has been on the market in Europe since 1990 and in the United States since 1993. In August 1997, the Sterrad 100 system was approved to sterilize certain surgical instruments with long lumens, such as those used in urologic, laparoscopic, and arthroscopic procedures, including instruments with single stainless steel lumens of ≥3 and <400 mm in length. The Sterrad 100S has since replaced the Sterrad 100. The Sterrad 100S adds one sterilization cycle and thereby fulfills the requirement to kill 10^6 spores halfway through the cycle. A smaller device, the Sterrad 50, has been independently tested for efficacy (304). Other sizes, e.g., the large Sterrad 200, approved by the FDA in 2003, can sterilize small lumens (single stainless steel lumens with an inside diameter of 1 mm or larger or Teflon/polyethylene lumens with an inside diameter of 6 mm or larger). The new Sterrad NX system, approved by the FDA in April 2005, is the fastest low-temperature hydrogen peroxide gas plasma sterilizer yet. This system employs a new vaporization system that removes most of the water from the hydrogen peroxide, improving diffusion of peroxide into lumens. Consequently, a broad range of instruments, including single-channel flexible endoscopes, can be processed within 38 min. In 2001, the FDA cleared biological indicators suitable for plasma sterilization.

Regardless of the model, the basic steps are the same. Medical instruments are placed in the sterilization chamber, a strong vacuum is created in the chamber, and a solution of 59% hydrogen peroxide and water is automatically injected from a cassette into the sterilization chamber. The solution vaporizes and diffuses throughout the chamber, surrounding the items to be sterilized. Radiofrequency energy is applied to create an electric field, which in turn generates the low-temperature plasma, inducing free radicals. The combination of the diffusion pretreatment and plasma phases sterilizes the item while eliminating harmful residuals. At the end of the cycle, the radiofrequency energy is turned off, the vacuum is released, and the chamber is filled with filtered air, returning it to normal atmospheric pressure.

Plasma sterilizers have several disadvantages. First, materials that absorb too much hydrogen peroxide (e.g., cellulosics and some nylons such as those from connectors, cables, and insulators), materials that catalytically decompose hydrogen peroxide (e.g., copper and nickel alloys from electrical wire, solder, and surgical instruments), and materials that react with hydrogen peroxide (such as organic dyes [colored anodized aluminum] and organic sulfides of solid lubricant in endoscopic devices) cannot be sterilized in a Sterrad. Second, the cassettes required to run the device and the special nonmuslin wrapper are relatively expensive.

Low-Temperature Sterilization by Ozone

The 125L Ozone Sterilizer (TSO3, Quebec, Canada) uses medical-grade oxygen, water, and electricity to generate ozone within the sterilizer to provide an efficient sterilant without producing toxic chemicals or using high temperatures (it runs at 25 to 35°C). Ozone forms when oxygen is submitted to an intense electrical field that separates oxygen molecules into atomic oxygen (O), which in turn combines with other oxygen molecules (O_2) to form triatomic oxygen (O_3), or ozone, providing a sterility assurance level of 10^{-6} in approximately 4 h. At the end of the cycle, the oxygen and water vapor safely vent directly into the room. The sterilization chamber has a capacity of 125 liters. Processed medical instruments require no aeration at the end of the sterilization cycle. Medical devices are packaged in a TSO3 sterilization pouch or in anodized aluminum sterilization containers. The TSO3 OZO-TEST self-contained biological and chemical indicators should be used to evaluate the machine's performance. An ozone sterilizer can be installed as a free-standing unit or recessed behind a wall. These devices are used primarily in Canada. These sterilizers are approved by the FDA, but few health care facilities in the United States use them.

Liquid Sterilization

The FDA approved the Steris System 1 in 1988, but it is not considered a sterilizer in Europe (305). The machine is designed to sterilize immersible devices, including flexible endoscopes, with 35% liquid peracetic acid (an FDA-approved sterilant that is sporicidal) (306, 307), supplemented with buffering, anticorrosion, wetting, and surface-active agents. Peracetic acid is automatically diluted with sterile filtered water, and the items are exposed for 12 min. The entire sterilization process takes approximately 30 min at ca. 50°C. Items can be used immediately after the process is completed and do not need to be aerated.

Clinical studies of the Steris System 1 have been performed with bronchoscopes, hysteroscopes, colonoscopes, and rigid endoscopes (272, 308). Independent efficacy tests demonstrated some failures (272). Exposure time and temperature are monitored electronically, and conductivity is used as a surrogate marker for peracetic acid concentration. However, the machine can complete its cycle normally and print a report stating that the concentration of peracetic acid was in the normal range when it was run intentionally without peracetic acid (309). Commercially available spores can be used for monitoring sterilization (310), but false-positive test strips can occur as a result of improper use of the clip used to attach the test strips (311). Other disadvantages of this system include the high cost of purchasing and using the equipment, which is considerably greater than the cost of purchasing and using systems for high-level disinfection with glutaraldehyde (312). In addition, the device does not clean the items. Thus, the cleaning step adds to the overall time of reprocessing the items. The Steris System 1, like all other nonsteam sterilizers, cannot meet the requirements for sterilization if residual debris and/or proteins are present on the items. The system has been considerably improved over the last decade, but the changes have not yet been approved by the FDA, which has issued a letter of concern (http://www.fda.gov/ICECI/EnforcementActions/WarningLetters/2008/ucm1048303.htm).

REUSE OF SINGLE-USE DEVICES

Current FDA policy states that the responsibility for the safety and performance of reprocessed single-use devices lies with the reprocessor, not the original manufacturer. The FDA considers the hospital to be the manufacturer of a single-use device if it has been resterilized. Therefore, the reprocessor must ensure that the reprocessed items are sterile and not contaminated with toxic substances such as endotoxins or residual ethylene oxide and that the product's integrity, composition, and function are essentially identical to those of a new product. Most hospitals cannot afford to generate appropriate data on the quality and performance of reprocessed single-use items. In addition, if a manufacturer changes the product, the reprocessor would need to redo the analyses before the device could legally be marketed after reprocessing (management of change).

The FDA published a final guidance on this topic (see website for details: http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/ReprocessingofSingle-UseDevices/default.htm).

Some institutions resterilize items that have not been used on patients but that, for instance, were dropped and/or whose package was damaged. Even this approach can be problematic. For example, the FDA published an alert documenting that the quality of an implant that was originally sterilized with ethylene oxide and resterilized with steam was impaired by the reprocessing method. In addition, the quality, product integrity, and performance of many plastic or rubber products after reprocessing are unknown. Moreover, the FDA does not allow health care facilities that send equipment and supplies to a reprocessing company to transfer full responsibility to that company (see the full text at http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/ReprocessingofSingle-UseDevices/default.htm). Furthermore, if a hospital reprocesses a single-use device, the hospital is responsible for ensuring that the device complies with all applicable FDA labeling requirements, even if the device is exempt from the premarket requirements. If the hospital does not ensure that the device complies with FDA labeling requirements, the device is misbranded, and the hospital may be considered in violation of section 301(k) of the Act. As of 14 August 2001 and of 14 February 2002, FDA enforced premarket filing requirements for reprocessed class II devices (i.e., moderate-risk devices such as a cardiac mapping catheter used to map electrical activity of the heart).

In many countries throughout the world, health care facilities reprocess single-use items (sometimes illegally) because resources are limited and this may be the only way to provide patients with access to state-of-the-art health care. We believe that new reprocessing technologies using washer-disinfectors coupled with highly effective low-temperature sterilizers can kill all microorganisms, even in narrow lumens such as cardiac catheters. In fact, a commercial reprocessor in Germany legally has reprocessed >4 million single-use items without any published serious side effects, saving between 30 and 50% of the cost for a new item. With the expertise of an infection control professional, the health care facilities may provide the desired level of microbiological and toxicological safety. However, they probably cannot ensure that the design and function of the device are still adequate. Thus, in the United States and countries with similar regulations on quality assurance programs, reuse of single-use devices may not be cost-effective. In addition, organizations that sell used single-use devices to patients and/or insurance companies as new devices will encounter legal and ethical issues. Continuous improvements of new devices also impede reprocessing of single-use devices on a large scale. However, financial restriction may change the current beliefs; the reader should consult the FDA website and experts in the field before considering reprocessing single-use devices.

BSE AND vCJD

CJD has been identified on all continents and is thought to occur worldwide. The incidence of CJD is estimated to be about 1 case per 10^6 persons per year. Most cases of CJD are sporadic; <10% of CJD cases may be related to a genetic autosomal dominant predisposition, and few nosocomial cases are related to use of contaminated tissue or contaminated human growth hormone. The emergence of vCJD has brought about a major medical and economic crisis in Europe (313–315). As of 2014, no curative therapy is available for CJD or vCJD; however, several approaches have been investigated with limited success (316). It is generally accepted that eating BSE agent-contaminated meat is the cause of vCJD (314, 317).

The agent causing vCJD is not a classic microorganism but an altered prion protein (258, 318–320). Its origin remains obscure, but the BSE agent from cattle is most probably responsible for vCJD in humans. vCJD has a different clinical presentation and occurs at a much younger age (321). The mean age at death from vCJD is 28 years; only 6 of 90 patients died at the age of 50 years or older (322). Among several hypotheses that may explain why this age group is most affected is that the incubation period is shorter in the young than in the elderly or they are more susceptible to infection. In the United Kingdom, the number of people exposed to potentially infective doses through food may be extremely high. All patients genotyped so far are homozygous for methionine on codon 129 of the prion protein gene. It is postulated that heterozygous individuals may have much longer incubation times before vCJD becomes evident. Therefore, asymptomatic carriers may pose a risk for transmission if they undergo routine surgery and instruments are not reprocessed by a prion-safe program. A recent study of appendix samples by the United Kingdom Health Protection Agency estimates 1 in 2,000 silent prion infections in the United Kingdom population (323).

Patients suffering from vCJD harbor large numbers of prions in their tonsils and spleen before they have signs, symptoms, and pathological findings of the disease. In contrast, patients with sporadic CJD suffer from spongiform encephalopathy long before the prion spreads into muscles and lymphoid tissue (324). Consequently, the United Kingdom has developed very strict precautions; for example, it was required that all tonsillectomies be performed with disposable instruments. In 2002, this practice was discontinued because serious complications arose when disposable instruments were used. However, none of the samples, including 276 samples initially reactive in one enzyme immunoassay, that were investigated by immunohistochemistry or immunoblotting was positive for the presence of CJD.

The fact that the vCJD prion agent is found in lymphoid tissue and tonsils indicates that prions are not restricted to neural tissue (316). Lymphatic organs typically show early accumulation of prions, and B cells and follicular dendritic cells are required for efficient neuroinvasion. The actual entry into the central nervous system probably occurs via peripheral nerves, and the prions accumulate in neural tissue once inflammation of the lymphoid tissue is in progress (325).

Experimental evidence from animal models indicates that blood can contain prion infectivity, which suggests a potential risk for BSE transmission via proteins isolated from human plasma (326). Three cases of probable transmission by blood transfusion raise more concern about the safety of the blood supply (327).

Previously, problems with reprocessing instruments used on patients with CJD were limited to invasive instruments that came into contact with neural tissue, predominantly instruments used in neurosurgery. However, as noted above, vCJD is highly lymphotropic, so that any instruments used on lymphoid tissues may be contaminated with prions (325). As outlined above, appropriate reprocessing of surgical items includes cleaning, disinfection, and sterilization. Aldehydes enhance the resistance of prions and abolish the inactivating effect of autoclaving (170). Therefore, aldehydes are no longer recommended for disinfecting surgical instruments in Europe before they have undergone a thorough cleaning process. In France, aldehydes are no longer used for endoscope reprocessing, despite evidence that peracetic acid may stain prions as well (328). Small resistant subpopulations of infective prions may survive autoclaving at 132 to 138°C. These resistant subpopulations are not inactivated by simply reautoclaving, and they have biological characteristics that differentiate them from the main population (329). The worst-case scenario is that the agent for vCJD might become self-replicating when it contaminates surgical instruments. Therefore, prions challenge reprocessing techniques like never before.

The minimum requirements for decontamination procedures and precautions for materials potentially contaminated with the agent that causes CJD or, still more, vCJD, remain unknown. However, it is clear that dry heat (160°C for 24 h), formaldehyde sterilization, and standard steam sterilization do not sterilize prion-contaminated items (330). The scientific uncertainties and lack of data do not allow agencies such as national health departments, the WHO, or the CDC to formulate guidelines that are completely evidence based, and this explains why various countries have taken different approaches to addressing issues of reprocessing instruments. In January 2001, the British government spent the equivalent of $300 million to improve reprocessing techniques in Central Sterilization Services and required the use of disposable instruments for tonsillectomies. The French Public Health Office published its recommendations on 14 March 2001. They require all surgical instruments with potential exposure to lymphatic tissue, the central nervous system, or the eyes to be soaked in sodium hypochlorite for 1 h or NaOH for 1 h and sterilized at 134°C for 18 min. If instruments do not tolerate this aggressive approach, they must be cleaned twice; treated with various chemicals such as peracetic acid, iodophors, 3% sodium dodecyl sulfate, or 6 M urea; and autoclaved at 121°C for 30 min. In Germany, it is recommended to decontaminate instruments with NaOH, NaOCl, or guanidine thiocyanate or to use medical washer-disinfectors with a superalkaline cleaner (pH > 10) followed by sterilization at 134°C for 5 min. Since 2002, Switzerland requires all surgical instruments to be sterilized at 134°C for 18 min. The background of the Swiss recommendation is that the usual rendering process for carcasses, which was discontinued, resulted in only a 1-log-unit reduction of the infectious particles (331). Therefore, a reduction in the number of infectious particles may suffice to stop transmission.

CDC recommends that instruments exposed to potentially prion-contaminated items be autoclaved for 1 to 1.5 h at 132 to 134°C, immersed in 1 N sodium hydroxide for 1 h at room temperature, or immersed in sodium hypochlorite 0.5% (at least 2% free chlorine) for 2 h at room temperature.

See the CDC website for further information (http://www.cdc.gov/ncidod/dvrd/vcjd/index.htm).

In the United States, one patient who was a former resident of the United Kingdom has been diagnosed with vCJD, and the first case of BSE in cattle was identified in 2003. However, more cases may occur because 37 tons of "meals of meat or offal" that were "unfit for human consumption" was sent from the United Kingdom to the United States in 1997, well after the government banned imports of such risky meat (332).

High-risk patients are patients with suspected CJD and their family members, patients treated with pituitary extracts, and patients who received cornea transplants. In addition, items should be considered contaminated with prions if a brain biopsy for the diagnosis of CJD is requested. Instruments used in such procedures should be discarded or placed under quarantine until the histopathological diagnosis is known. The incidence of vCJD in the United Kingdom is decreasing rapidly, indicating that current reprocessing techniques suffice. However, knowledge about this topic is increasing rapidly over time and our current understanding may be shown to be false in the future (254). In May 2005, British officials published an excellent assessment of the risk for contaminating surgical instruments with prions (https://www.gov.uk/government/publications/guidance-from-the-acdp-tse-risk-management-subgroup-formerly-tse-working-group). The key observation in this report is that, on average, 0.2 mg of protein remains on surgical instruments despite "standard cleaning and disinfection," which was sufficient to cause an experimental case of CJD. Therefore, more research and new methods of cleaning and disinfection are needed for surgical instruments.

In laboratories, all samples should be handled using gloves and the processing of suspected specimens should be performed while wearing protective covering, e.g., gloves and disposable gowns or aprons (333). Masks and eyewear may be appropriate if splashing or aerosols are anticipated. Contaminations or spills should be decontaminated immediately with NaOH. To minimize environmental contamination, disposable cover sheets could be used on work surfaces (12).

The reader is referred to the websites of the CDC, the FDA, and the WHO to obtain the most recent updates on this topic. In addition, the Society for Healthcare Epidemiology of America published detailed guidelines on this topic (12). More information about prions can be found in chapter 109 of this *Manual*.

In conclusion, monitoring the processes of antiseptics, cleaning, disinfection, and sterilization is an essential part of infection control programs. The wide variety of applied techniques, germicidal agents, and procedures not only requires a broad understanding of the material, but it also needs well-trained personal to avoid incidents that result in damage to patients or equipment.

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Biothreat Agents

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The ideal qualities for a successful biothreat agent are a high rate of illness in exposed persons/animals, a high case fatality rate, a short incubation period, and a paucity of immunity in the targeted population. In addition, success is also influenced by the availability of treatment, the ability of the agent to transmit from person to person, the ease with which the agent can be produced and disseminated, and a disease that is difficult, at least initially, to recognize clinically or to diagnose.

General clues that one may be dealing with an unrecognized biothreat event include a large outbreak of illness with a high death rate, a recognized case(s) of an uncommon disease, illness in a geographic region where the disease is not endemic, disease out of its usual seasonality, simultaneous outbreaks of the same disease in various parts of the country/world, and the presence of sick and dying animals. The Centers for Disease Control and Prevention (CDC) have classified potential biothreat agents into categories depending on the basis of their threat to national security, with those organisms belonging to category A being the most serious threats, due to their unique features in causing diseases capable of mass destruction (Table 1). These and other agents are discussed in this chapter.

The LRN

The Laboratory Response Network (LRN) was established through the work of the Department of Health and Human Services (HHS) and the CDC, in accordance with Presidential Decision Directive 39. This directive, signed by President Bill Clinton on 22 May 1998, made the fight against terrorism a top national security priority and defined policies regarding the federal response to threats or acts of terrorism involving nuclear, biological, and/or chemical material and/or weapons of mass destruction. Specifically, this directive outlined the United States' efforts to "deter, defeat and respond vigorously to all terrorist attacks on our territory and against our citizens, or facilities, whether they occur domestically, in international waters or airspace or on foreign territory. The United States regards all such terrorism as a potential threat to national security as well as a criminal act and will apply all appropriate means to combat it. In doing so, the U.S. shall pursue vigorously efforts to deter and preempt, apprehend and prosecute, or assist other governments to prosecute, individuals who perpetrate or plan to perpetrate such attacks" (http://www.fas.org/irp/offdocs/pdd39.htm).

Through a collaborative effort involving the Federal Bureau of Investigation and the Association of Public Health Laboratories (APHL), the LRN was established in August 1999. Its objective was to improve and ensure an effective laboratory response to bioterrorism. The LRN worked toward this goal by improving the public health infrastructure, increasing laboratory capacity and staffing, and training laboratory personnel in advanced diagnostic technologies. The LRN forms an integrated network of more than 150 local and state public health, federal, and military laboratories (representing all 50 states, Australia, Canada, the United Kingdom, Mexico, and South Korea) that can respond to bioterrorism, chemical terrorism, and other public health emergencies.

The LRN states its mission as follows: "The LRN and its partners will develop, maintain and strengthen an integrated national and international network of laboratories that can respond quickly to needs for rapid testing, timely notification and secure messaging of results associated with acts of biological or chemical terrorism and other high priority public health emergencies" (http://www.bt.cdc.gov/lrn/).

There are an estimated 25,000 private and commercial laboratories (including clinical, food testing, veterinary diagnostic, and environmental testing laboratories) in the United States, some of which serve as critical sentinel laboratories. While most of these laboratories do not perform confirmatory testing, they represent the first contact with patients and are in a position to alert public health officials of possible biothreat agents. These laboratories conduct tests to rule out other diseases and send possible biothreat samples/organisms to the appropriate LRN reference laboratories for confirmatory testing. The LRN structure is shown in Fig. 1.

LRN sentinel laboratories are certified to perform high-complexity testing under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) by the Centers for Medicare and Medicaid Services (CMS) for the applicable microbiology specialty. Laboratory in-house testing includes Gram stains and at least one of the following: lower respiratory tract, wound, or blood culture. LRN reference laboratories are responsible for investigation and/or referral of specimens to LRN national laboratories. LRN reference laboratories consist of over 100 state and local public health, military, federal, and international laboratories (veterinary, agriculture, food, and water testing laboratories) in and outside the United States. LRN national laboratories, including

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TABLE 1 HHS and USDA select agents and toxins (with biothreat categories)[a,b]

HHS select agents and toxins	Overlapping select agents and toxins	Other critical agents for public health preparedness
Abrin	*Bacillus anthracis** (A)	Western equine encephalitis virus (B)
Botulinum neurotoxins* (A)	*Bacillus anthracis* Pasteur strain	Epsilon toxin of *Clostridium perfringens* (B)
Botulinum neurotoxin-producing species of *Clostridium** (A)	*Brucella abortus* (B)	*Salmonella* species (B)
Conotoxins (short, paralytic alpha conotoxins containing the following amino acid sequence: $X_1CCX_2PACGX_3X_4X_5X_6CX_7$)	*Brucella melitensis* (B)	*Shigella dysenteriae* (B)
Coxiella burnetii (B)	*Brucella suis* (B)	*Escherichia coli* O157:H7 (B)
Crimean-Congo hemorrhagic fever virus	*Burkholderia mallei** (B)	*Vibrio cholerae* (B)
Diacetoxyscirpenol	*Burkholderia pseudomallei**	*Cryptosporidium parvum* (B)
Eastern equine encephalitis virus (B)	Hendra virus	Hantavirus (C)
Ebola virus* (A)	Nipah virus (C)	Yellow fever virus (C)
*Francisella tularensis** (A)	Rift Valley fever virus	Multidrug-resistant *Mycobacterium tuberculosis* (C)
Lassa fever virus (A)	Venezuelan equine encephalitis virus	
Lujo virus		
Marburg virus* (A)		
Monkeypox virus		
Reconstructed replication-competent forms of the 1918 pandemic influenza virus containing any portion of the coding regions of all eight gene segments (reconstructed 1918 influenza virus)		
Ricin (B)		
Rickettsia prowazekii		
Severe acute respiratory syndrome-associated coronavirus (SARS-CoV)		
Saxitoxin		
South American hemorrhagic fever viruses (Chapare, Guanarito, Junin, Machupo, and Sabia viruses)		
Staphylococcal enterotoxin A, B, C, D, and E subtypes (B)		
T-2 toxin		
Tetrodotoxin		
Tick-borne encephalitis complex (flavi-) viruses (Far Eastern subtype, Siberian subtype) (C)		
Kyasanur Forest disease virus		
Omsk hemorrhagic fever virus		
Variola major virus (smallpox virus)* (A)		
Variola minor virus (alastrim)*		
*Yersinia pestis** (A)		

[a] *, Tier 1 agent. Select agent regulations were revised in October 2012 to designate "tier 1" agents with a documented risk of causing a high-consequence event higher than those with other biological select agents and toxins. Criteria for tier 1 status were as follows: (i) ability to produce a mass casualty event or devastating effects to the economy, (ii) communicability, (iii) low infectious dose, and (iv) history of or current interest in weaponization based on threat reporting. See http://www.selectagents.gov/select%20agents%20and%20toxins%20list.html.

[b] Note that the CDC divides bioterrorism agents into three categories (A, B, and C) depending on how easily they can be spread and the severity of illness or rate of death they cause. Category A agents are considered the highest risk, as they can easily be disseminated or transmitted from person to person, result in high mortality rates, have the potential for major public health impacts, might cause public panic and social disruption, and require special action for public health preparedness. Category B agents are the second-highest-priority agents and include those that are moderately easy to disseminate, result in moderate morbidity rates and low mortality rates, and require specific enhancements of the CDC's diagnostic capacity and enhanced disease surveillance. Category C agents are the third-highest-priority agents and include emerging pathogens that could be engineered for mass dissemination in the future because of availability, ease of production and dissemination, and potential for high morbidity and mortality rates and major health impacts.

those operated by the CDC and the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), have specialized infrastructure, training, and biosecurity that are necessary for handling highly infectious biological agents. These laboratories are responsible for specialized strain characterizations, susceptibility testing, and bioforensics.

The American Society for Microbiology (ASM), along with the APHL and the CDC, has been instrumental in the development of sentinel-level laboratory testing protocols for the detection of biothreat agents. These protocols were developed to allow LRN sentinel laboratories to rapidly rule out or refer suspected biothreat agents to the appropriate LRN reference laboratory. Along with the CDC and APHL, ASM serves as a resource for training and education for microbiologists in hospital and commercial laboratories regarding their roles and responsibilities as LRN sentinel laboratories. These protocols and other useful information regarding bioterror agents

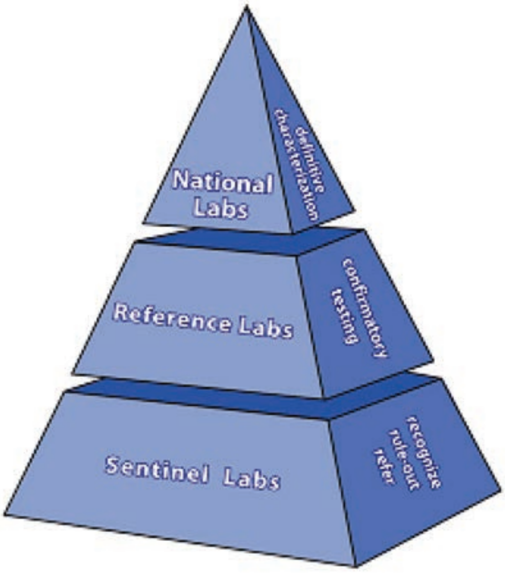

FIGURE 1 Structure of the Laboratory Response Network. doi:10.1128/9781555817381.ch14.f1

can be found at the following ASM website page: http://www.asm.org/index.php/guidelines/sentinel-guidelines.

The College of American Pathologists (CAP), together with the CDC, also serves an important role in promoting the diagnostic capability and capacity of clinical and public health laboratories by developing the Laboratory Preparedness Exercise (LPX) proficiency test. There are additional sources of proficiency testing for laboratory bioterrorism preparedness, such as the Wisconsin State Laboratory of Hygiene (http://www.slh.wisc.edu/proficiency/). These proficiency programs help to ensure the capability of participating sentinel laboratories to rule out and/or refer biological threat agents to the appropriate LRN reference laboratory.

A directory of LRN public health reference laboratories is available at http://www.cdc.gov/mmwr/international/relres.html.

Federal Select Agent Program

The Federal Select Agent Program was established due to a heightened concern about the ease with which disease-causing agents could be obtained. Section 511 of the Antiterrorism and Effective Death Penalty Act of 1996 (Public Law 104-132) directed the HHS to establish a list of biological agents and toxins that could threaten public health and safety, procedures for governing the transfer of those agents, and training requirements for entities working with these "select agents." HHS delegated the authority to implement this Act to the CDC, which then established the CDC Select Agent Program.

Congress significantly strengthened oversight of select agents after the anthrax attacks of 2001 by passing the USA Patriot Act and the Bioterrorism Act, which together restricted access to select agents and increased safeguards and security measures, as well as oversight of the possession and use of select agents. In addition to strengthening the regulatory authorities of the CDC, the Bioterrorism Act also granted comparable regulatory authorities to the U.S. Department of Agriculture (USDA) over select agents that pose a severe threat to animal and plant health or products. The USDA's Animal and Plant Health Inspection Service (APHIS) then established the Agricultural Select Agent Program. These two programs constitute the Federal Select Agent Program.

The Program greatly enhances the nation's oversight of the safety and security of select agents by developing, implementing, and enforcing the Select Agent Regulations, maintaining a national database, inspecting entities that possess, use, or transfer select agents, and ensuring that all individuals who work with these agents undergo a security risk assessment performed by the Federal Bureau of Investigation/Criminal Justice Information Service. The list of agents that the Federal Select Agent Program regulates is shown in Table 1. The list is reviewed by the CDC and APHIS at least every 2 years to determine if agents need to be added to or deleted from the list. Most recently (2012), *Coccidioides* species were deleted from the select agent list. Refer to the ASM website for flowcharts and additional information on bioterror agents and preparedness: http://www.asm.org/index.php/issues/sentinel-laboratory-guidelines.

CDC Bioterrorism Agents and Diseases: Categories and Definitions

The CDC divides bioterrorism agents into three categories (A, B, and C) depending on how easily they can be spread and the severity of illness or rate of death they cause (Table 1) (http://www.bt.cdc.gov/bioterrorism/overview.asp). Category A agents are considered the highest risk, as they can easily be disseminated or transmitted from person to person, result in high mortality rates, have the potential for major public health impacts, might cause public panic and social disruption, and require special action for public health preparedness. Agents included in this category are *Bacillus anthracis*, *Clostridium botulinum* toxin, *Yersinia pestis*, variola virus (the agent of smallpox), *Francisella tularensis*, and hemorrhagic fever viruses (HFVs), such as filoviruses (e.g., Ebola virus and Marburg virus) and arenaviruses (e.g., Lassa virus and Machupo virus). Characteristics of several of these agents are listed in Table 2.

Category B agents are the second-highest-priority agents and include those that are moderately easy to disseminate, result in moderate morbidity rates and low mortality rates, and require specific enhancements of the CDC's diagnostic capacity and enhanced disease surveillance. Category B agents include *Brucella* species (*Brucella abortus*, *Brucella melitensis*, and *Brucella suis*), epsilon toxin of *Clostridium perfringens*, food-safety threats (e.g., *Salmonella* species, *Escherichia coli* O157:H7, and *Shigella* species), *Burkholderia mallei*, *Burkholderia pseudomallei*, *Chlamydia psittaci*, *Coxiella burnetii*, ricin toxin from *Ricinus communis* (castor beans), staphylococcal enterotoxin B (SEB), *Rickettsia prowazekii*, viral encephalitis viruses (such as alphaviruses, e.g., Venezuelan equine encephalitis [VEE] virus, Eastern equine encephalitis [EEE] virus, and Western equine encephalitis [WEE] virus), and water-safety threats (e.g., *Vibrio cholerae* and *Cryptosporidium parvum*).

Category C agents are the third-highest-priority agents and include emerging pathogens that could be engineered for mass dissemination in the future because of availability, ease of production and dissemination, and potential for high morbidity and mortality rates and major health impacts. These emerging pathogens are covered in their individual organism chapters.

TABLE 2 Summary of biothreat agent characteristics

Characteristic	*B. anthracis*	*Y. pestis*	*Burkholderia pseudomallei* and *B. mallei*	*F. tularensis*	*Brucella* spp.	Variola virus (smallpox)
Gram stain morphology	Large Gram-positive rod, nonmotile. From blood agar (BA): no capsule, central to subterminal spores that do not enlarge the cell. From blood: capsule, no spores.	Plump Gram-negative rod. Gram stain: ± bipolar or "safety pin" appearance. Wright-Giemsa stain: bipolar or "safety pin" appearance.	*B. pseudomallei*: small Gram-negative rod. *B. mallei*: small Gram-negative coccobacillus. Gram stain: ± bipolar or "safety pin" appearance (*B. pseudomallei*). Wright-Giemsa stain: bipolar or "safety pin" appearance (*B. pseudomallei*).	Minute Gram-negative coccobacillus, poorly staining, smaller than *Haemophilus influenzae*, pleomorphic.	Tiny Gram-negative coccobacillus, faintly staining.	
Growth	Standard conditions, extremely rapid.	28°C optimum, without agitation; 35–37°C, more slowly.	35–37°C, ambient atmosphere, though CO_2 is acceptable.	Aerobic conditions. Growth is best on media containing cysteine, such as BCYE, but will often grow initially on chocolate agar or BA.	Grows in blood culture media, can require blind subculturing.	Grows in most cell lines, unusual or unrecognizable cytopathic effect (CPE)
Colonial morphology (BA)	Nonhemolytic, ground glass, irregular/wavy edges, tenacious, "beaten egg whites" when touched with loop.	Nonhemolytic, pinpoint colonies at 24–48 h, "fried egg" or "hammered copper" or shiny at 48–72 h.	*B. pseudomallei*: small, smooth creamy colonies in first 1 to 2 days, gradually changing after a few days to dry, wrinkled colonies similar to those of *Pseudomonas stutzeri*. *B. mallei*: smooth, gray, translucent colonies without pigment.	Does not passage well on BA.	Small colonies, punctate after 48 h, nonhemolytic.	
Tests	Catalase (+)	Catalase (+), oxidase (−), urease (−). On MacConkey agar (MAC): lactose (−), indole (−).	Catalase (+), colistin (10 μg) and polymyxin B (300 U) (resistant), motility (+, *B. pseudomallei*; −, *B. mallei*), indole (−), oxidase (+, *B. pseudomallei*; ±, *B. mallei*). On MAC: lactase (−, *B. pseudomallei*; − or no growth, *B. mallei*).	Catalase (weakly +), oxidase (−), β-lactamase (+), satellites (−). On MAC: no growth.	Oxidase (+), catalase (+), urease (+) (though some strains are negative), satellites (−). On MAC: poor to no growth.	CPE can be passed.

BIOTHREAT AGENTS AND INFECTIONS

Category A Agents

Anthrax (*Bacillus anthracis*)

Significance

The biothreat associated with the inhalation of spores from *B. anthracis* has been known for decades, and in the past the organism was developed as a potential military weapon by many countries, including the United States (1, 2). In 2001, envelopes containing confirmed anthrax spores were mailed to southern Florida, New York City, and Washington, D.C., resulting in 22 cases of anthrax (11 inhalational cases, with 5 deaths, and 11 cases of cutaneous anthrax) (2).

Transmission and Disease

B. anthracis is a large, aerobic or facultative anaerobic, spore-forming, Gram-positive bacillus that naturally inhabits the soil. Four forms of the disease can occur: cutaneous (>95% of all human anthrax cases), inhalational, gastrointestinal, and meningitis forms. The inhalational form (acquired through inhalation of organism spores) is primarily associated with bioterror attacks and causes pulmonary anthrax. In addition, anthrax meningitis can occur as a result of a bioterror event. Any case of inhalational or meningeal anthrax should prompt suspicion of possible bioterrorism. Human-to-human transmission of anthrax has not been reported. See ASM's sentinel-level clinical microbiology laboratory guidelines for anthrax (http://www.asm.org/index.php/guidelines/sentinel-guidelines). Additional information regarding *B. anthracis* can be found in chapter 26.

Botulism (*Clostridium botulinum*)

Significance

Clostridial toxins are among the most powerful neurotoxins known. Botulinum toxin acts at the neuromuscular junction and at the peripheral autonomic synapses, resulting in neuromuscular weakness and autonomic dysfunction (3). A bioterror attack using botulinum toxin could occur through ingestion or inhalation of the toxin, such as contamination of a food or air supply. Many countries, including Japan, the United States, the former Soviet Union, and Iraq, have, at some point, developed botulinum toxin for biological warfare (3).

Transmission and Disease

C. botulinum is an anaerobic, spore-forming, Gram-positive bacillus that is found naturally in the environment (soil and water). The organisms primarily associated with human disease produce toxin types A, B, and E. Botulism is naturally acquired from contaminated food products processed by methods that do not destroy spores. This allows bacteria contained in the food to become vegetative and toxins to be produced. The clinical presentation associated with a bioterror attack with botulinum would include gastrointestinal symptomology (nausea, vomiting, and diarrhea) followed by neurological signs of dry mouth and blurred and/or double vision. In cases of severe disease, symptoms include difficulty swallowing, voice impairment, and peripheral muscle weakness. If the muscles of the respiratory tract are involved, respiratory failure and death may result. See ASM's sentinel-level clinical microbiology laboratory guidelines for botulism (http://www.asm.org/index.php/guidelines/sentinel-guidelines). Additional information regarding *C. botulinum* can be found in chapter 53.

Plague (*Yersinia pestis*)

Significance

The first reported use of *Y. pestis* as a bioweapon was in the 1300s, as the Tartars, invading the city of Kaffa (now Feodosiya, Ukraine), catapulted bodies of plague victims over the city walls, resulting in an epidemic (4). Many countries have developed this agent as a bioweapon, including the former Soviet Union and Japan (5). An incident involving a white supremacist who fraudulently obtained cultures of *Y. pestis* from the American Type Culture Collection prompted the U.S. Congress to pass a 1996 regulation governing the acquisition, transfer, and use of agents that could be used for bioterrorism purposes (6).

Transmission and Disease

Y. pestis is a slow-growing (optimal growth is at 28°C; up to 5 days of incubation is recommended), plump, Gram-negative coccobacillus/bacillus and is part of the *Enterobacteriaceae* family. Plague is a worldwide zoonotic disease that is transmitted between animals via infected fleas. There are three forms of plague: bubonic, septicemic, and pneumonic. The pneumonic form would be the most likely disease manifestation seen in the event of a bioterror attack. See ASM's sentinel-level clinical microbiology laboratory guidelines for plague (http://www.asm.org/index.php/guidelines/sentinel-guidelines). Additional information regarding *Y. pestis* can be found in chapter 39.

Smallpox (Variola Major)

Significance

An excellent overview of smallpox and its historical use as a biothreat weapon is given by Rotz et al. (7). Briefly, one of the first possibilities of smallpox being used as a biothreat weapon dates back to the 1600s, when the Spanish supplied the natives of South America with smallpox-contaminated clothing in an effort to gain control of their lands. Then, in the 1760s, the North American British forces gave smallpox-contaminated blankets to the native Indians in an effort to reduce their populations and gain control of land. In more recent times, the Russians associated with Bioperparat worked with many potential biothreat agents, including smallpox, for use in intercontinental missiles (8). Today, concern still exists regarding illegitimate stores of the agent and their potential use in bioterrorism, especially because routine vaccination has not been in place for decades, leading to a susceptible populace.

Transmission and Disease

Variola virus belongs to the genus *Orthopoxvirus* in the family *Poxviridae*. Variola virus is a large, brick-shaped virus measuring approximately 302 to 350 nm by 244 to 270 nm and is comprised of a single linear double-stranded DNA genome. As there is no known disease associated with smallpox in the world, even one case would prompt the suspicion of a bioterror incident. Human transmission occurs primarily by inhalation of airborne particles containing the virus, but transmission may occur via fomites (contaminated bedding or clothing) as well. Disease consists of symptoms such as malaise, fever, chills, vomiting, headache, and backache, with the eventual formation of a papular rash on the face, hands, and arms, later spreading to the legs and, lastly, the trunk. The mortality due to smallpox in the unvaccinated population is approximately 30% (9). See ASM's sentinel-level clinical microbiology laboratory guidelines for unknown viruses (http://

www.asm.org/index.php/guidelines/sentinel-guidelines). Additional information regarding smallpox can be found in chapter 107.

Tularemia (*Francisella tularensis*)

Significance

The highly infectious nature of *F. tularensis*, due to low inhalation inocula and a very susceptible human population, as well as its substantial morbidity and mortality in untreated patients, makes this bacterium a potential agent of bioterrorism. The Japanese began experiments on human prisoners for use of this agent as a military weapon in the early 1930s, and in 1955 the United States used volunteers and military personnel to test the effects of inhaled organisms (10). In the 1990s, Russia developed antibiotic- and vaccine-resistant strains of *F. tularensis* (http://www.pbs.org/wgbh/nova/bioterror/agen_tularemia.html).

Transmission and Disease

F. tularensis is an extremely small, fastidious, pleomorphic, Gram-negative coccobacillus. Aerosolized particles containing the organism account for the acquisition of pneumonic tularemia, the primary disease that would be associated with a bioterror attack; however, patients inhaling aerosolized organisms may present with other forms of tularemia (ulceroglandular, glandular, oculoglandular, pharyngeal, and typhoidal). Respiratory failure or shock causes most fatalities from pneumonic disease (http://www.pbs.org/wgbh/nova/bioterror/agen_tularemia.html). Two subspecies of *F. tularensis*, *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B), are of clinical importance in causing tularemia. See ASM's sentinel-level clinical microbiology laboratory guidelines for *F. tularensis* (http://www.asm.org/index.php/guidelines/sentinel-guidelines). Additional information regarding *F. tularensis* can be found in chapter 46.

Diseases Caused by HFVs

Significance

Hemorrhagic fever viruses (HFVs) were developed as biological weapons by both the United States and the former Soviet Union. The United States stopped this program and destroyed all weapons in the late 1960s.

Transmission and Disease

The HFVs comprise four families: the *Arenaviridae*, *Bunyaviridae*, *Filoviridae*, and *Flaviviridae*. The clinical course following exposure varies with the virus causing the infection, but typically patients have symptoms of fever, myalgia, and malaise followed by exacerbation of symptoms and development of prostration and hemorrhage, with central nervous system (CNS) depression. Patients who develop shock with extensive hemorrhage and CNS damage generally have poor outcomes (11).

See ASM's sentinel-level clinical microbiology laboratory guidelines for unknown viruses (http://www.asm.org/index.php/guidelines/sentinel-guidelines). Additional information regarding HFVs can be found in section IV of this *Manual*.

Category B Agents

Brucellosis (*Brucella* Species)

Significance

B. suis was the first biological agent to be utilized in the biological warfare program in the United States during the mid-1950s. *Brucella* species are considered among the agents less likely to be utilized in a bioterrorism attack, in part because brucellosis results in a high morbidity but low mortality. However, it remains a threat because the disease process is long and can be incapacitating. *Brucella* species listed as category B agents are *B. abortus*, *B. melitensis*, and *B. suis*.

Transmission and Disease

Brucellosis is a zoonotic infection, with four species being recognized as causing infection in humans: *B. abortus* (cattle), *B. melitensis* (goats, sheep, and camels), *B. suis* (pigs), and *Brucella canis* (dogs). In addition, it is a common laboratory-acquired infection. *Brucella* organisms are small, aerobic, Gram-negative coccobacilli that grow slowly (2 to 3 days for initial isolation). *Brucella* can cause both acute and chronic infections. Brucellosis is a disease of nonspecific symptoms, consisting of fever, sweats, headache, anorexia, back pain, and weight loss, which can last for months and relapse after discontinuation of therapy (12). The chronic form of the disease can mimic miliary tuberculosis, with suppurative lesions in the liver, spleen, and bone. Brucellosis has a mortality of 5% in untreated individuals. See ASM's sentinel-level clinical microbiology laboratory guidelines for *Brucella* (http://www.asm.org/index.php/guidelines/sentinel-guidelines). Additional information regarding *Brucella* species can be found in chapter 47.

Glanders and Melioidosis (*Burkholderia mallei* and *B. pseudomallei*)

Significance

It is believed that *B. mallei* was used to infect large numbers of Russian horses and mules on the Eastern Front during World War I and that the Japanese infected horses, civilians, and prisoners of war during World War II. The United States studied this agent as a possible biothreat agent in 1943 and 1944 but did not weaponize it. The former Soviet Union is also believed to have been interested in *B. mallei* as a potential biothreat agent (13).

Transmission and Disease

Glanders and melioidosis are related diseases produced by bacteria of the *Burkholderia* species, which are nonsporulating, obligately aerobic, Gram-negative bacilli. They produce similar diseases which consist of several forms: localized infections, pulmonary infections (pneumonia, pulmonary abscesses, and pleural effusions), septicemia, and a chronic form (multiple abscesses in internal organs). Both *B. mallei* and *B. pseudomallei* are considered potential biothreat agents in the aerosolized/pulmonary form in that they are highly infectious by inhalation and because of their resistance to many routine antibiotics (http://emedicine.medscape.com/article/830235-overview). See ASM's sentinel-level clinical microbiology laboratory guidelines for *Burkholderia* species (http://www.asm.org/index.php/guidelines/sentinel-guidelines). Additional information regarding *Burkholderia* species can be found in chapter 43.

Q Fever (*Coxiella burnetii*)

Significance

C. burnetii has a very low infective dose and is resistant to the effects of drying and heat. Humans are very susceptible to infection with this organism, and a single organism is capable of causing disease in a susceptible person. In the

1960s, the U.S. military considered *Coxiella* an excellent "incapacitating" agent, as the disease is debilitating but rarely lethal, and envisioned using this agent to cripple enemy forces. Russia, and possibly also Iraq, has also developed and tested the Q fever agent as a biothreat agent, and the cult Aum Shinrikyo obtained *C. burnetii* but was unsuccessful in weaponizing it.

Transmission and Disease

C. burnetii is a pleomorphic, Gram-negative, intracellular coccobacillus that cannot be cultured on routine bacteriologic media. Q fever is a zoonotic disease seen primarily in parturient goats, sheep, and cattle. Aerosolized contaminated dust particles are the source of human infection, and the infectious dose is very low. There are three clinical presentations of this infection: atypical pneumonia, progressive pneumonia (of a rapid nature), and as an incidental finding in a patient with fever and pneumonia. The last description is the most common form of the disease (14). Q fever may present as a chronic infection. See ASM's sentinel-level clinical microbiology laboratory guidelines for Q fever (http://www.asm.org/index.php/guidelines/sentinel-guidelines). Additional information regarding Q fever can be found in chapter 66.

Staphylococcal Enterotoxins

Significance

Staphylococcal enterotoxin B (SEB) was part of the United States' bioweapons program until the early 1970s. SEB was thought to be useful as a weapon due to its ease of aerosolization, stability, and ability to produce multisystem organ failure and death if inhaled in large amounts. Even if inhaled in smaller amounts, this toxin produces a debilitating disease that takes up to 2 weeks for recovery.

Transmission and Disease

SEB is one of several enterotoxins produced by *Staphylococcus aureus*, which is a ubiquitous, nonmotile, Gram-positive coccus found on the skin and mucous membranes of humans and animals. SEB is also a potent superantigen capable of massive overstimulation of the immune system, resulting in an overwhelming inflammatory response and an endotoxin-like shock which can result in multisystem organ failure and death (15). See ASM's sentinel-level clinical microbiology laboratory guidelines for staphylococcal enterotoxin B (http://www.asm.org/index.php/guidelines/sentinel-guidelines). Additional information regarding SEB can be found in chapter 21.

Epsilon Toxin (*Clostridium perfringens*)

Significance

Because of its potency—nearly equal to that of botulinum neurotoxin (16)—epsilon toxin of *C. perfringens* is considered a potential bioterrorism agent.

Transmission and Disease

C. perfringens is a spore-forming, Gram-positive bacillus that naturally inhabits the soil. *C. perfringens* produces several toxins, including epsilon toxin, which is synthesized by toxinotypes B and D. *C. perfringens* toxinotypes B and D are animal pathogens. Epsilon toxin forms pores in the plasma membranes of eukaryotic cells, but its mode of action is not fully elucidated. In animals, high levels of epsilon toxin are produced in the intestine. The toxin then spreads via the circulatory system to various organs, where it causes edema and hemorrhage. It is fast acting, causing death within hours or minutes of exposure (17). Documented human cases of enterotoxemia due to epsilon toxin are very rare, with only two reported cases, both reported in 1955 (16). Because of its rarity, any case of enterotoxemia due to epsilon toxin should prompt suspicion of possible bioterrorism. As a biothreat agent, epsilon toxin would likely be delivered as an aerosol or through water- or foodborne routes. Additional information regarding *C. perfringens* and epsilon toxin can be found in chapter 53.

Psittacosis (*Chlamydia psittaci*)

Significance

C. psittaci survives for long periods in dry environments, is spread naturally through the aerosol route, and has the potential to cause severe disease in humans. For these reasons, it is listed as a potential bioterrorism agent.

Transmission and Disease

C. psittaci is an obligately intracellular bacterium that causes respiratory disease in birds. Human infections (psittacosis) are usually associated with exposure to bird secretions. Gardening and lawn mowing have been linked with psittacosis, and person-to-person transmission may occur (18). *C. psittaci* infection in humans usually presents as a mild to severe respiratory infection, with a wide range of symptoms. Signs and symptoms include fever, headache, muscular aches, nonproductive cough, and difficulty breathing. Extrapulmonary complications may also occur. As a biothreat agent, *C. psittaci* would likely be spread through the aerosol route. Additional information regarding *C. psittaci* can be found in chapter 63.

Epidemic Typhus (*Rickettsia prowazekii*)

Significance

Among the characteristics that make *R. prowazekii* a potential bioterrorism agent are its aerosol transmission, high infectivity, stable extracellular forms, and high virulence. *R. prowazekii* was developed as a biological weapon by several countries during the 1930s and 1940s (19).

Transmission and Disease

R. prowazekii is an obligately intracellular bacterium that relies on an arthropod host (human body louse) as its principal vector. The classic epidemiologic cycle of epidemic typhus requires the feeding of an infected louse on a susceptible human host. Symptoms include high fever, myalgia, and arthralgia. Because of the nonspecific symptoms, infections may go undiagnosed or be treated with ineffective antibiotics. Among untreated patients, the mortality rate can reach 20% (20). Human-to-human transmission of *R. prowazekii* has not been reported. As a biothreat agent, *R. prowazekii* would likely be spread through the aerosol route or through infected lice. Additional information regarding *R. prowazekii* can be found in chapter 64.

Viral Encephalitis (Alphaviruses)

Significance

Among the alphaviruses, Eastern equine encephalitis (EEE) virus, Venezuelan equine encephalitis (VEE) virus, and Western equine encephalitis (WEE) virus share several characteristics related to their joint classification as potential bioterrorism agents: route of transmission, morbidity in humans and equines, and cell culture infectivity.

Transmission and Disease

The genus *Alphavirus* is a member of the family *Togaviridae*. There are 29 species in the genus *Alphavirus*, including EEE, VEE, and WEE viruses (21). Alphaviruses infecting humans are arthropod borne and cause encephalitis in humans. Diseases can range from mild fever and myalgias to fatal encephalitis. Survivors of encephalitis often suffer from permanent neurologic sequelae. As biothreat agents, alphaviruses would likely be spread through the aerosol route or through infected mosquitoes.

Additional information regarding alphaviruses can be found in section IV of this *Manual*.

Food- and Water-Safety Threats (Bacteria, Viruses, and Protozoa)

Significance

Contamination of food and water supplies is a real as well as potential route for the dispersal of bioterrorism agents because of the ability to infect a large number of individuals. Food- and waterborne agents considered to be potential bioterrorism agents include *Salmonella*, *Shigella*, toxigenic strains of *Escherichia coli*, *Campylobacter*, *Vibrio cholerae*, and *Vibrio parahaemolyticus*, *Listeria monocytogenes*, *Yersinia enterocolitica*, noroviruses, hepatitis A virus, *Cryptosporidium parvum*, *Cyclospora cayetanensis*, *Entamoeba histolytica*, *Giardia duodenalis* (formerly *Giardia lamblia*), microsporidia, and *Toxoplasma gondii* (22). At least two of these agents have been used in documented cases of intentional contamination and bioterrorism. In one event, pastries were intentionally contaminated with a laboratory stock culture of *Shigella dysenteriae*, infecting 12 (27%) of 45 laboratory staff members (23). In another event, members of a religious commune intentionally contaminated salad bars in several restaurants with *Salmonella* (24).

Transmission and Disease

The food- and waterborne agents listed above share some features, such as the fecal-oral route of transmission (especially secondary cases), yet they also have unique characteristics. Characteristics that are compatible with bioterror use include a low infectious dose (*Shigella*, norovirus, hepatitis A virus, and *C. parvum*) and environmental stability (norovirus, hepatitis A virus, and *C. parvum*), including in aerosols (norovirus). While most of these agents cause an acute or protracted gastroenteritis, some are associated with additional syndromes, such as septicemia (*Salmonella enterica* serotype Typhi and *L. monocytogenes*), Guillain-Barré syndrome (*Campylobacter jejuni*), and hemolytic-uremic syndrome (*E. coli* serotype O157:H7). Some cause significant morbidity and mortality in immunocompromised persons, including those with AIDS (*L. monocytogenes*, microsporidia, *T. gondii*, and *C. parvum*). A challenge with the prompt identification of bioterrorism events involving food- and waterborne agents is the tendency of these agents to produce large outbreaks of disease naturally. Epidemiological evidence may be necessary to indicate suspicion of bioterrorism.

In summary, there is a very real possibility that the laboratory will be the first to recognize an act of bioterrorism. In a suspected or confirmed bioterrorism event, immediate and effective communication with all appropriate institutional and medical personnel and public health officials is imperative. The laboratory may be called upon to assist in the diagnosis and management of patients who have been exposed overtly or covertly to a bioterrorism agent. The laboratory needs to promptly assist clinicians by providing them with accurate information on the selection, collection, and transport of specimens. In addition, the laboratory must handle these specimens in a manner that will result in the greatest probability of success in establishing a diagnosis and minimizing the exposure of hospital personnel and patients to infectious agents.

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The Human Microbiome

JAMES VERSALOVIC, SARAH K. HIGHLANDER, AND JOSEPH F. PETROSINO

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The total number of bacteria in the human body is estimated to be at least 10 times greater than the number of human cells (1, 2), and recent studies, particularly the efforts of the NIH Human Microbiome Project (HMP) Consortium, have led to a greater understanding of the identity and distribution of microorganisms that constitute these microbial populations. In particular, implementation of next-generation sequencing (NGS) has helped to illuminate how these bacteria contribute to and are affected by human health and disease. Significant progress in cataloging and characterizing these microorganisms and genes has been made in recent years, thanks to NGS approaches, and microbiome studies have expanded beyond the gut to other body sites. NGS DNA sequencing platforms have made it possible to sequence the DNA of the collective genome (or metagenome) of entire microbial communities (Fig. 1) from different body sites in health and disease and in different life stages, effectively enabling the characterization of the "human microbiome" (see Table 1 for basic definitions).

INTRODUCTION TO THE HUMAN MICROBIOME PROJECT

In 2007, an NIH Roadmap for Medical Research project called the Human Microbiome Project (HMP) was initiated (http://nihroadmap.nih.gov/hmp/). The overarching goal of the HMP is to develop tools and resources for characterization of the human microbiota and to relate this microbiota to human health and disease. The HMP is leveraging the constantly advancing sequencing and bioinformatic technologies to address the following broad goals:

- Determining whether individuals share a core human microbiome
- Understanding whether changes in the human microbiome can be correlated with changes in human health and disease
- Developing the new technological and bioinformatic tools needed to support these goals
- Addressing the ethical, legal, and social implications raised by human microbiome research

A comprehensive clinical protocol outlined standardized sampling (3) and nucleic acid extraction procedures for the two clinical sampling centers (one at Baylor College of Medicine [BCM] and one at Washington University, St. Louis, MO). Further efforts were made to standardize how samples were processed, sequenced, and analyzed (4). The progress within the HMP culminated in the publication of two flagship manuscripts (5, 6) and more than 30 companion papers (e.g., see http://www.ploscollections.org/hmp), beginning in June 2012, that describe the initial observations associated with the HMP healthy cohort and numerous derivative studies that examine specific facets of these more deeply characterized communities (Fig. 1).

Among initial microbiome comparisons, analyses of samples across 18 body sites confirmed high interindividual variation (7) and determined that even rare organisms in these communities are important reservoirs of genetic diversity (8). Additionally, data from the large HMP cohort suggest that the composition of the gut microbiome is most often characterized by smooth abundance gradients of key organisms (6) and does not necessarily cluster subjects into discrete types. However, microbial communities found in other niches, such as the vagina, can exhibit such clustering (4). While the microbial communities varied among subjects, the metabolic pathways encoded by these organisms and necessary for human-commensal viability were consistently present, forming a functional "core" to the microbiome at all body sites (6, 9, 10). Although the pathways and processes of this core were consistent, the specific genes associated with these pathways varied. Microbial sugar utilization, for example, was enriched for metabolism of simple sugars in the oral cavity, complex carbohydrates in the gut, and glycogen/peptidoglycan degradation in the vaginal microbiome (10).

TECHNIQUES FOR THE STUDY OF THE HUMAN MICROBIOME

Early studies of the human microbiome relied on culture-dependent methods; however, it is now known that the majority of microorganisms from the human body cannot be cultured *in vitro*. Most current techniques for characterization of a metagenomic sample are PCR based and target the highly conserved bacterial small-subunit 16S rRNA gene. Portions of the gene can be amplified and fingerprinted by using electrophoretic techniques, such as terminal restriction fragment length polymorphism (TRFLP) analysis and denaturing gradient gel electrophoresis. Full-length 16S rRNA genes or segments of these genes can be amplified prior to microarray analyses or DNA sequencing studies on

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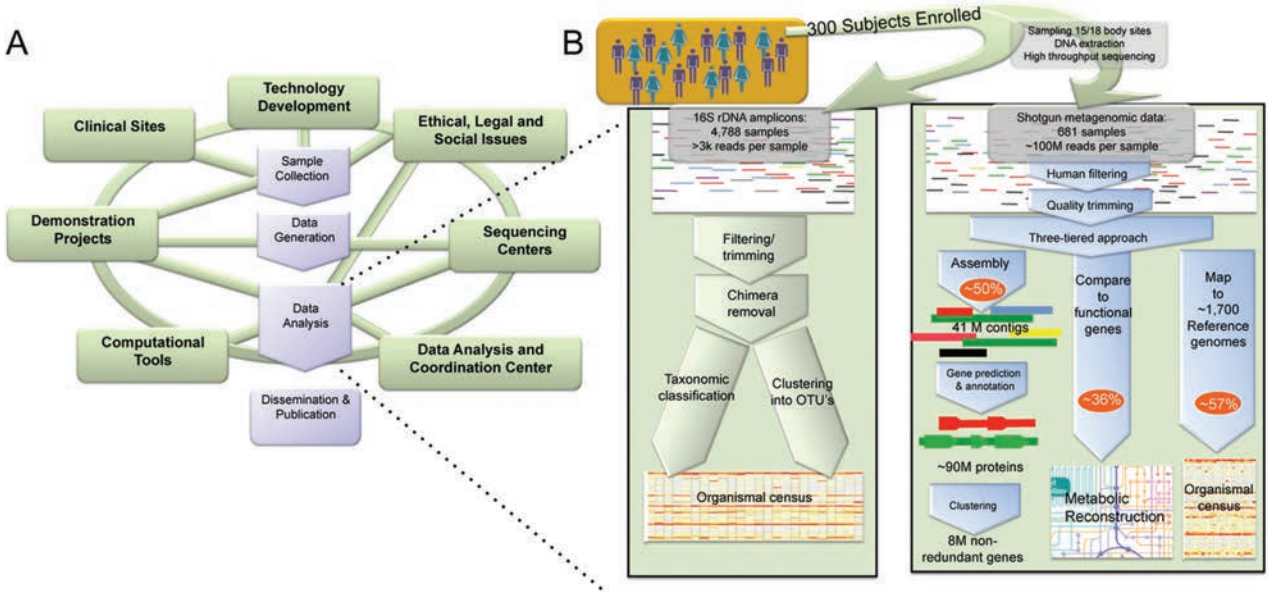
A
Clinical Sites
Technology Development
Ethical, Legal and Social Issues
Sample Collection
Demonstration Projects
Data Generation
Sequencing Centers
Data Analysis
Computational Tools
Data Analysis and Coordination Center
Dissemination & Publication
B
300 Subjects Enrolled
Sampling 15/18 body sites
DNA extraction
High throughput sequencing
16S rDNA amplicons:
4,788 samples
>3k reads per sample
Filtering/ trimming
Chimera removal
Taxonomic classification
Clustering into OTU's
Organismal census
Shotgun metagenomic data:
681 samples
~100M reads per sample
Human filtering
Quality trimming
Three-tiered approach
Assembly
~50%
41 M contigs
Gene prediction & annotation
~90M proteins
Clustering
8M non-redundant genes
Compare to functional genes
~36%
Metabolic Reconstruction
Map to ~1,700 Reference genomes
~57%
Organismal census

FIGURE 1 Model for microbiome data generation and analyses. (A) Various components (green) and processes (lavender) involved in microbiome projects. (B) Processes of sequencing data generation/editing and data analyses (bioinformatic strategies). Adapted from reference 128 with permission from the Public Library of Science. doi:10.1128/9781555817381.ch15.f1

NGS platforms (e.g., the 454 platform and, more recently, the Illumina MiSeq and Life Technologies Ion Torrent platforms). 16S rRNA gene sequences that are ≥97% identical are considered to be within the same species, while those that are ≥95% identical are within the same genus.

Culture-dependent and culture-independent surveys have shown that the human body hosts four predominant phyla (Table 2). The four phyla, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria*, are the most highly represented phyla across all body sites (Fig. 2). In a recent study, these four phyla comprised 92.3% of bacterial DNA sequences analyzed from multiple human sources, including hair, oral cavity, skin, and gastrointestinal (GI) tract (11). The predominant phyla vary by anatomical site, and presumably the host milieu has a crucial role in shaping the composition of microbial communities at each site. Recent efforts have been aimed at expanding the DNA sequence representation within each phylum so that more comprehensive phylogenetic assessments can be performed in the future (12).

HUMAN MICROBIOME STUDIES

The Oral Microbiome

Each human body site is characterized by distinctive patterns of microbial composition (Fig. 2). The oral cavity includes ecologic niches rich in microbial diversity, such as saliva, gingival crevices, the tongue surfaces, and the posterior pharynx, and is colonized with hundreds of bacterial species. An estimated 500 or more different species reside in the human oral cavity (8, 13). Comprehensive sampling of the oral cavity (nine distinct sites), including plaque and saliva, was included in the HMP (5, 6). The human oral microbiome matches or exceeds the intestinal microbiome, and those of all other body sites sampled in the HMP, in terms of microbial diversity within individuals (alpha diversity) (6). The HMP has estimated the presence of approximately 70 distinct bacterial genera in the oral cavities of most adults (6). The most abundant oral bacterial genera include *Actinomyces*, *Bacteroides*, *Prevotella*, *Streptococcus*, *Fusobacterium*, *Leptotrichia*, *Corynebacterium*, *Veillonella*,

TABLE 1 Basic terminology of the human microbiome

Name	Terminology
Microbiome	Assemblage of microbes (archaea, bacteria, fungi, parasites, and viruses/phages), constituting a microbial community and occupying a specific habitat.
Metagenome	Collection of genomic DNA (genetic capacity) of an entire microbiome and its host DNA.
Metatranscriptome	The genes expressed within a microbiome.
Metabolome/metabonome	Metabolites produced by individual microbes (metabolome) or the entire microbiome (metabonome).
Alpha and beta diversity	Microbial species diversity within single samples or human individuals (alpha diversity) and between multiple samples or individuals (beta diversity) at a single body site.
Operational taxonomic unit	Bacterial taxon defined by its 16S rRNA gene sequence or whole-genome sequence. The taxon may or may not have a formally designated species name.
Canonical pathogens	Pathogens that cause infections as single infectious agents in a healthy human host.

TABLE 2 Predominant phyla in healthy individuals by body site

Body site	Predominant phylum or phyla[a]	Reference(s)
Colon (stool)	*Bacteroidetes*, *Firmicutes*, *Actinobacteria*	6
Esophagus	*Firmicutes*, *Bacteroidetes*, *Actinobacteria*	44
Oral cavity	*Firmicutes*, *Actinobacteria*, *Bacteroidetes*	6, 126
Placenta	*Proteobacteria*, *Tenericutes*	85
Skin, dry	*Proteobacteria*, *Actinobacteria*, *Bacteroidetes*	110
Skin, moist	*Actinobacteria*, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*	110
Skin, sebaceous	*Actinobacteria*, *Firmicutes*, *Proteobacteria*	110
Small intestine	*Firmicutes*	2, 53
Stomach	*Proteobacteria*, *Firmicutes*, *Actinobacteria*	51, 52
Upper respiratory tract	*Firmicutes*, *Proteobacteria*, *Actinobacteria*	6, 95
Vagina	*Firmicutes*, *Actinobacteria*, *Fusobacteria*	6, 88, 127

[a] Phyla are listed in order of predominance at each body site, based on the cited reference(s).

Rothia, *Capnocytophaga*, *Selenomonas*, *Treponema*, and TM7. Additionally, *Methanobrevibacter* taxa from the *Archaea* domain are present in the human oral cavity. Different sites within the oral cavity have various degrees of biological diversity, with plaque and saliva specimens containing the greatest relative diversity (8).

New concepts have emerged because of these pioneering studies, including relative differences in biological diversity between body habitats. Relative to human skin, several sites in the human oral cavity demonstrated relatively greater richness in terms of microbial ecology within individuals but lower beta diversity or a greater similarity of microbial communities between individuals (5, 6). Health-associated microbial communities may protect against infection, but the oral cavity also harbors organisms that are implicated in both local and systemic diseases, including periodontal diseases (14), endocarditis (15), and aspiration pneumonia (16). The relative preponderance of health- or disease-associated mi-

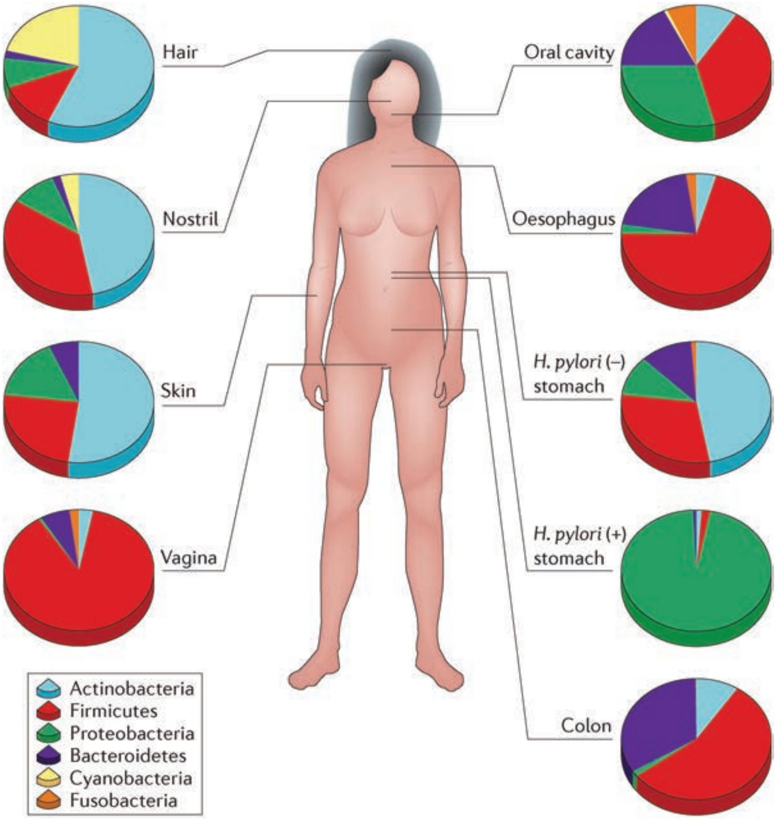

FIGURE 2 Differences in microbial composition by body habitat. The figure shows the relative abundances of six different bacterial phyla at eight different body sites. The relative abundances were obtained by next-generation DNA sequencing of 16S rRNA genes. Adapted from reference 129 with permission from the Nature Publishing Group. doi:10.1128/9781555817381.ch15.f2

crobes combined with human genetic susceptibilities may ultimately account for different disease phenotypes.

Periodontitis is an infectious and inflammatory disease condition that is associated with disturbances in microbial ecology among human adult tooth-borne microbiomes. Socransky et al. proposed that periodontitis is the result of complexes or consortia of pathogens (17). The so-called red complex, containing *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, is the pathogenic group most strongly associated with this disease process (17, 18). Periodontitis is an independent predictor and comorbidity factor contributing to different disease conditions, including preterm birth, cardiovascular disease, pulmonary disorders, diabetes, and obesity (19). Progressive periodontitis in pregnant women has been reported to increase the risk of severely adverse pregnancy outcomes (20, 21). These associations underscore the significance of the oral microbiome to the systemic health status and predisposition to specific diseases.

Microbial community profiling by 16S rRNA gene sequencing and other methods has been used to evaluate the oral microbiome. These methods include denaturing gradient gel electrophoresis (22–24), TRFLP analysis (25–28), and checkerboard DNA-DNA hybridization, where 45 DNA samples can be queried against 30 to 40 DNA probes (29). Socransky and coworkers applied checkerboard DNA-DNA hybridization to examine microbial communities in supragingival (30) and subgingival (17) plaque and microbial succession patterns (shifts) during dental biofilm redevelopment in health and periodontitis (31, 32). In both studies, distinct complexes were identified by principal component and correspondence analyses and were assigned to color groups. The communities in supragingival plaque samples from 187 subjects (4,475 samples in total) clustered into six groups, including the aforementioned pathogen-associated red complex. One bacterial pathogen, *Aggregatibacter actinomycetemcomitans*, has been linked to a highly aggressive periodontitis (localized aggressive periodontitis) in Africans (19). The uncultured bacterial taxon TM7 has been associated with periodontitis based on whole-genome shotgun (WGS) sequencing studies, but this disease association has yet to be evaluated fully in a population-based cohort.

As in the HMP, other studies have examined microbial colonization and succession at different sites in the oral cavity. Aas et al. (33) examined nine oral sites: tongue dorsum, tongue lateral sides, buccal epithelium, hard palate, soft palate, supragingival plaque, subgingival plaque, maxillary anterior vestibule, and tonsils. A total of 2,589 16S rRNA gene clones from five subjects were evaluated; this sequencing effort resulted in 141 predominant species and 13 new phylotypes. All sites contained the *Gemella*, *Granulicatella*, *Streptococcus*, and *Veillonella* genera. The tonsils were found to have the greatest microbial diversity and varied significantly among subjects. Organisms implicated in bacterial endocarditis, such as *Streptococcus mitis*, *Streptococcus oralis* (34), and *Granulicatella adiacens* (35), were found in each healthy subject, which supports the concept that such oral microbes may potentially translocate into the bloodstream secondary to dental procedures. A central concept that emerged from the HMP was that opportunistic pathogens were much more prevalent than canonical pathogens and were present across multiple body sites in a given individual (6). Individuals with breaches in mucosal integrity or immunocompromised status may be vulnerable to opportunistic pathogens present in the human microbiome.

A human oral microbe identification microarray (http://mim.forsyth.org) that contains 16S rRNA gene oligonucleotide probes representing approximately 300 oral bacterial species was described previously (36). While the presence of more periodontal pathogens was observed in the diseased subjects, the main conclusion from the study was that increased microbial diversity was associated with diseased subgingivae. The salivary microbiome has also been studied (37, 38). Samples from 120 healthy subjects from 12 geographic locations were analyzed by Sanger sequencing of the V4 to V6 regions of the 16S rRNA gene. A total of 101 genera were identified, and the numbers of bacterial genera per subject ranged from 6 to 30. The most prevalent genus was *Streptococcus* (23.7%), consistent with the predominance of *Streptococcus* in the esophagus and stomach of healthy individuals.

The Gastrointestinal Microbiome

The human GI tract encompasses numerous different anatomical sites, including the esophagus, stomach, small intestine, colon, rectum, and anus. Each of these sites is colonized by different numbers and populations of microbes.

Esophageal Microbiome

The esophagus is colonized by bacteria that are introduced from the oropharynx by swallowing or from the stomach by reflux. Early studies focused on surveys of cultured bacteria (39–43). Limited numbers of bacteria were examined, and in nondiseased subjects, aerobic and anaerobic Gram-positive organisms predominated. Only three metagenomic surveys of the esophageal microbiota have been reported. A study by Pei et al. (44) showed that the distal esophageal microbiomes of four adults had compositions similar to that of the oropharynx, with the exception that no spirochetes were found in the esophagus. As in the oral cavity, *Streptococcus* was the dominant genus in the healthy esophageal microbiome. Bacterial DNA sequences were categorized into the following six phyla or groups: *Firmicutes* (70%), *Bacteroidetes* (20%), *Actinobacteria* (4%), *Proteobacteria* (2%), *Fusobacteria* (2%), and TM7 (1%). This distribution highlights the preponderance of *Firmicutes* and *Bacteroidetes* in the GI tract. Thirty-six new species were discovered, and Chao 1 (45) analysis estimated that the esophageal community contains about 140 species-level operational taxonomic units (OTUs) (44). The same group examined differences in the esophageal microbial communities in patients with esophagitis or Barrett's esophagus (intestinal metaplasia) (46, 47). Two distinct microbiomes were seen in healthy and diseased subjects. Healthy individuals had esophageal microbiomes that were composed predominantly of streptococci, while the diseased patients' microbiomes had larger numbers of Gram-negative anaerobes and increased bacterial diversity (47). A similar shift in terms of bacterial composition was reported in an earlier study of esophageal carcinoma (41).

Gastric Microbiome

The low pH and rapid peristalsis in the stomach suppress persistent colonization by many bacteria. The stomach and small intestine each are thought to contain about 100 microorganisms/ml, but the organismal counts can increase to 10^5 per ml following a meal (48). The best-studied and most dominant member of the stomach microbiota is *Helicobacter pylori* (49). Culture-dependent methods have revealed other genera, such as *Lactobacillus*, *Streptococcus*, and *Staphylococcus*, as well as members of the *Enterobacteriaceae*, in the stomach (50), although a metagenomic analysis of gastric biopsy specimens revealed far greater diversity (128 phylotypes) than had been appreciated previously by culture-based approaches (51). The most prevalent genera in the gastric microbiome, besides *H. pylori*, include several genera found commonly in the oral cavity and esophagus. Bacterial genera found in the human stomach include *Prevotella*, *Streptococcus*, *Veillonella*, and *Rothia*. The genus *Streptococcus* is a predominant genus in the stomach when *H. pylori* is absent (52).

Small Intestinal Microbiome

Like the stomach, the proximal small intestine (duodenum and jejunum) is colonized by relatively limited numbers of bacterial species. Acidic pH and bile in the proximal to mid-small intestine inhibit bacterial colonization. Bacterial communities in the small intestine typically consist of lactobacilli, enterococci, Gram-positive aerobes, and facultative anaerobes, and the genus *Streptococcus* seems to be the dominant genus in the duodenum and jejunum (2, 50, 53). This portion of the GI tract has been assessed mainly by culture-dependent methods, with a relative paucity of molecular data. A recent quantitative-PCR-based study described differences in microbial composition between ileostomy specimens and intact small intestinal tissue (54). In the ileum, the microbial composition becomes complex and approaches that of the colon in terms of species richness and the nature of predominant bacterial genera.

Colonic Microbiome

The human colon contains a rich collection of microbial communities, with numerical estimates ranging from 10^{11} to 10^{12} bacteria/g, and likely exceeding 1,000 bacterial species (6, 8) per individual, the majority of which belong to the phyla *Bacteroidetes* and *Firmicutes* (6, 55, 56). Other phyla with sufficient representation in the intestine include *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. In patients with different GI diseases, bacterial population shifts occur such that *Proteobacteria* become relatively more abundant (57). Comprehensive evaluation of self-collected stool specimens from healthy adults documented high degrees of species richness and microbial diversity in the human intestine (5, 6) relative to other body habitats. The intestine is characterized by a long "tail" of rare, unknown bacterial taxa that accounts for the majority of species or OTUs in the gastrointestinal tract (8). In contrast, in the absence of nucleic acid amplification, the healthy intestinal microbiome contains 70 to 100 known bacterial taxa, on average, based on whole-genome shotgun sequencing (6, 58). In terms of known gut bacteria, the dominant genus by relative abundance in stool is *Bacteroides*. A relatively abundant signature species is another organism from the phylum *Bacteroidetes*, known as *Prevotella copri* (6), and these signature organisms vary between different body habitats. The spatial distribution of gut microbes also depends on the mucosal versus luminal locations of these communities (56). The mucosa-associated microbiomes differ in composition from those found in stool specimens.

Bacteria from the distal gut are critical to host nutrition and may play key roles in health and disease. Microbe-derived carbohydrate fermentation by-products, such as short-chain fatty acids (SCFAs) (e.g., butyrate, acetate, and propionate), provide 10% or more of the body's metabolic requirements (59). Butyrate, produced by clostridial clusters IV and XIVa, is the primary energy source of the colonic epithelium, and this SCFA has been reported to possess anticancer features (60–62). SCFAs may play a role in preventing infection by pathogens such as *Salmonella enterica* serovar Typhimurium (63) and enterohemorrhagic *Escherichia coli* O157:H7 (64). Colonic bacteria further contribute to nutrition by synthesizing amino acids (65) and vitamins (e.g., vitamins K and B_{12}, biotin, folic acid, and pantothenate) (66, 67). Three basic enterotypes or classes of gut microbiomes have been described, with relatively distinct features in terms of bacterial composition and function (68). Enterotypes 1, 2, and 3 are enriched for different genera, such as *Bacteroides*, *Prevotella*, and *Ruminococcus*, respectively. The same enterotypes are differentially enriched for specific pathways, such as biotin and thiamine biosynthesis (68), and these genetic profiles highlight differences in vitamin and metabolite production by the microbiome. This classification system may be a useful framework for considering basic differences in human populations and susceptibility to disease. For example, differences in biosynthetic capacity, including vitamin metabolism, may have nutritional implications for human health. It is worth noting that the concept of enterotypes in healthy humans has been challenged by alternative concepts, such as that of enterogradients (55). Gut microbial communities may undergo rapid shifts in composition due to dietary changes (69), but enterotypes remained relatively stable despite a 10-day dietary intervention (70). WGS sequence reads from fecal samples of two individuals were analyzed in depth (71). More than 50,000 open reading frames were predicted and examined for enrichment of COG (clusters of orthologous groups) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways. Analysis of these pathways revealed enzymes required for degradation of plant polysaccharides, production of SCFAs, vitamin biosynthesis, methanogenesis, and degradation of toxic plant phenolics. These results and those of other studies highlight the metabolic capabilities of the gut microbiota.

The gut microbiome appears to have a fundamental impact on human body metabolism, and several studies have highlighted differences in gut microbial populations in obesity and metabolic syndrome. Ley et al. compared the microbiomes in stool samples from obese human subjects placed on fat-restricted and carbohydrate-restricted diets (72). Regardless of the diet type, the percentage of *Bacteroidetes* organisms increased with weight loss, while the percentage of *Firmicutes* organisms decreased. A different approach is to examine the relative numbers of different genes in the entire intestinal microbiome or microbial gene richness as a biomarker of human health. Using this approach, individuals have been classified as either high-gene-count (HGC) or low-gene-count (LGC) individuals, and these gene counts seem to affect health status and disease susceptibility. LGC and HGC individuals yielded average quantities of 380,000 and 640,000 bacterial/phage genes, respectively (73). HGC individuals were proposed to have a greater variety of microbial metabolic functions, a relatively robust gut microbiome, and better overall health status, including a lower prevalence of obesity and metabolic disorders (73).

Metagenomic studies aimed at understanding the microbial contributions to chronic immune-mediated disorders, such as inflammatory bowel disease (IBD) and type 2 diabetes, have been reported. IBD is likely to be multifactorial in pathogenesis, with factors including host genetics, immune responses, environmental factors such as diet, and the gut microbiome (74). Crohn's disease and ulcerative colitis are associated with diminished fecal diversity. A PCR-based survey indicated that biopsy samples from patients with IBD were depleted of commensal bacteria belonging to the *Firmicutes* and *Bacteroidetes* phyla (75). In particular, *Clostridiales* members were diminished in relative abundance, including many bacterial taxa in clostridial cluster XIVa, which contains butyrate-producing organisms. Subsequent studies revealed deficiencies of *Faecalibacterium prausnitzii* in patients with IBD (75–78). Beyond studies of microbial composition, differences in the relative abundances of metabolic pathways were found in patients with IBD (79). Oxidative stress pathways were relatively enriched in the IBD microbiome, whereas several microbial metabolic pathways associated with carbohydrate metabolism and amino acid biosynthesis were enriched in healthy subjects. IBD genotype (e.g., NOD2 status) and phenotype have been associated with specific compositional changes in the intestinal microbiome (80). Differences in gut microbial composition have been described for type 2 diabetes, a chronic disorder with autoimmune and metabolic components. Metagenome-wide association studies (MGWAS) have identified genetic signatures in the metagenome which are associated with type 2 diabetes (81). These studies strongly suggest that the microbiota in the gut may contribute to activation of signaling pathways required for the development of human diseases such as diabetes.

The Vaginal Microbiome

The vaginal microbiota plays an important role in preventing genital and urinary tract infections. The composition of the vaginal microbiota varies with age, pH, and hormonal levels (82). The vaginal microbiome is dynamic and changes during the menstrual cycle (83) and during pregnancy (84). Interestingly, the placental microbiome that develops during pregnancy has also been described (85), and the microbial composition of the placental microbiome is distinct from that of the vaginal microbiome. The placental microbiome is most similar in metagenomic composition to the oral microbiome. In the vagina, lactobacilli are considered the most prevalent organisms in healthy premenopausal women (86, 87), and these are considered protective for the host based on their proposed role in suppression of pathogen colonization. Such effects may result from mucous adherence by lactobacilli, production of organic acids and reduction of vaginal pH, and production of antimicrobial compounds that prevent pathogen proliferation (88). In addition to the lactobacilli, the predominant cultured vaginal microbes in the clinical microbiology laboratory are *Mobiluncus* spp., *Gardnerella vaginalis*, *Bacteroides* spp., *Prevotella* spp., and *Mycoplasma hominis* (89).

Microbial community profiling studies have demonstrated that the vaginal microbiota is highly variable, and fluctuations in composition presumably depend on differences in sexual and hygienic practices, in addition to host genetics. In the HMP, three sites in the vagina were studied, and differences were documented among these sites (6). The vagina is relatively limited in terms of microbial diversity within and between individuals. *Lactobacillus* is the predominant member of the vaginal community in most individuals, but in some cases, vaginal lactobacilli may be undetectable. The microbiota of eight healthy women with three different "grades" of vaginosis was examined by sequencing the V1 to V3 regions of the 16S rRNA gene by the Sanger method (90). The vaginal bacterial composition associated with each of these grades was quite distinct: grade 1 individuals (healthy) were almost exclusively colonized by *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii*; grade 2 subjects had *Lactobacillus iners*, *Atopobium vaginae*, *Prevotella bivia*, and *Sneathia sanguinegens*; and grade 3 subjects were predominantly colonized with *A. vaginae* or *Peptostreptococcus anaerobius*. Several studies have associated an altered vaginal microbiota with an increased risk of viral coinfection (91–93).

Previous studies showed that the microbial composition of the vaginal microbiota varies among healthy Caucasian women (94). The number and distribution of phylotypes differed among the five subjects. Two females had exclusively or nearly exclusively *L. crispatus*, one had *L. iners* as the predominant species, and one was predominantly colonized by *A. vaginae*. The fifth subject had seven phylotypes, predominantly *L. iners*, but also significant amounts of *A. vaginae*, *Megasphaera*, and *Leptotrichia*. Like lactobacilli, *Leptotrichia* and *Atopobium* are lactic acid producers. Different microbial communities may manifest shared functions. A relatively large study included 396 asymptomatic North American women and documented the presence of five groups based on vaginal microbiome composition (87). Four of the five groups were dominated by different *Lactobacillus* species, whereas the fifth group contained larger proportions of strict anaerobes. The proportions of each microbiome group varied among four ethnic groups included in this study and highlighted the interrelationships between the host genotype/phenotype and vaginal microbiome composition (87). Such differences in microbial communities may contribute to differences in disease susceptibility.

The Microbiome of the Respiratory Tract

The healthy nares and nasopharynx contain streptococci, staphylococci, corynebacteria, *Moraxella* spp., *Neisseria* spp. (including *Neisseria meningitidis*), and *Haemophilus* (95). The HMP documented the relative predominance of the genera *Corynebacterium*, *Moraxella*, *Propionibacterium*, and *Staphylococcus* in the anterior nares (6). Potential pathogens often colonize the nares and nasopharynx. Microorganisms associated with inner ear infections of children (e.g., *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*) have been found in the nasopharynx (96). The carriage rate for *Staphylococcus aureus* has been estimated to be approximately 30%, with methicillin-resistant *S. aureus* representing 1.5% of the strains isolated (97). Studies of the human microbiome reported that *Staphylococcus aureus* was a prominent species in the anterior nares (6), and this species represents a possible pathogen in its human host. Changes in airway microbial composition have been associated with different disease states involving the respiratory tract. Microbial diversity was greater in patients with tuberculosis than in healthy controls (98), and a core cystic fibrosis-associated microbiome including seven bacterial genera has been proposed (99). The respiratory tract is relatively understudied in terms of its microbiome, but it is becoming clear that significant shifts in composition can be associated with different disease phenotypes.

The Skin Microbiome

The skin microbiome is the first line of defense against infection and plays a role in modulating the inflammatory response. Minutes after birth, colonization of the initially sterile skin habitat begins to occur, as newborns are first colonized with similar, low-diversity microbiomes at multiple body sites (100, 101). Members of the environmental microbiota then begin to colonize different regions of the skin as they acquire distinct moisture, temperature, and glandular characteristics, giving rise to habitats with increasingly diverse microbiotas (102). These skin-associated microbial communities continue to change in puberty, depending on age, gender, and environmental exposures (103–107). Metagenomic studies using 16S rRNA gene sequencing in adults showed that the vast majority of skin (as well as

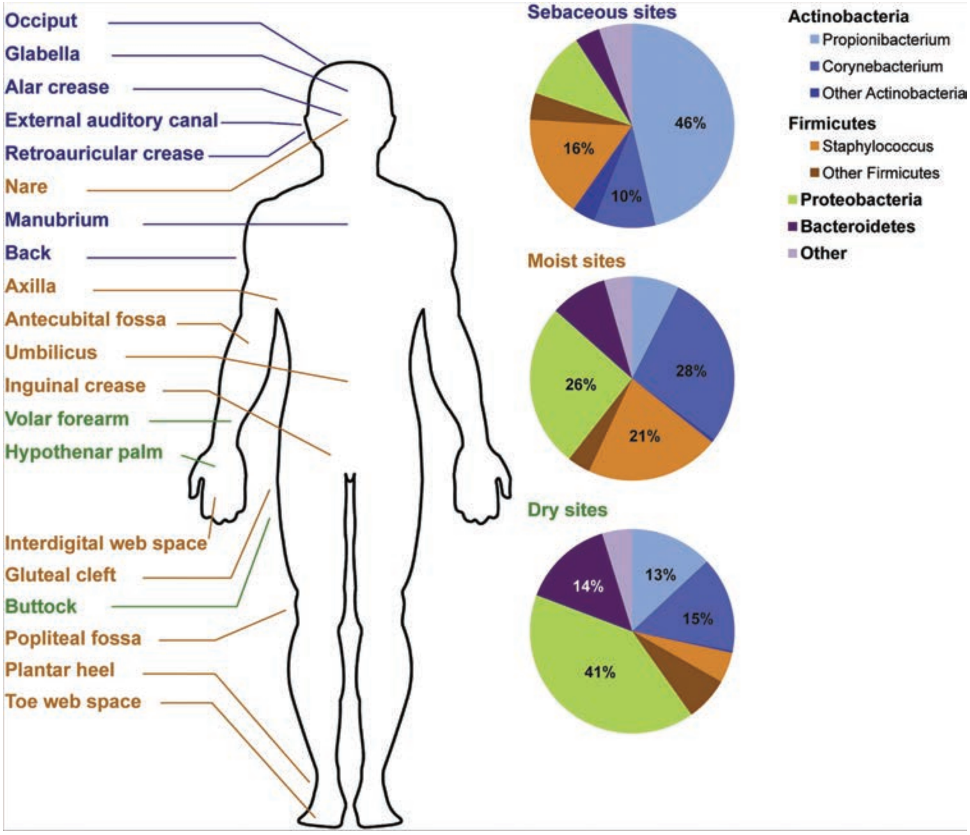

FIGURE 3 Bacterial compositions at different skin sites. The relative abundances of different bacterial phyla and genera are depicted in terms of the type of skin site (dry, moist, or sebaceous). The data were generated largely by 16S rRNA gene sequencing of organisms on swab samples obtained from different skin sites. Adapted from reference 130 with permission from Elsevier. doi:10.1128/9781555817381.ch15.f3

gut) commensals are grouped into four dominant phyla: *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*. These phyla contain thousands of distinct species (103, 108, 109). For instance, the palm microbiome comprised 4,742 distinct species in 51 healthy subjects, with an average of 158 species colonizing a single palm (103). Surveys of more than 20 different skin sites showed that similar habitats, such as the axillae and the popliteal fossae, share similar microbial compositions (Fig. 3) (110). For instance, in all individuals, *Propionibacterium* species dominate sebaceous areas, such as the back, forehead, and retroauricular crease (6), whereas *Staphylococcus* and *Corynebacterium* species dominate moist areas, such as the axillae (Fig. 3). Unexpectedly, *Proteobacteria*, typically thought to appear on skin only as gastrointestinal contaminants, were found in dry skin habitats, such as the forearm and leg. Diversity and variance fluctuate in a body-site-dependent manner. For example, the antecubital fossa is characterized by fewer distinct species than those at other skin sites. This relatively low alpha diversity is contrasted with a higher beta diversity in comparing the antecubital fossa communities between individuals (6, 111, 112). The skin microbiota generally protects individuals against colonization by pathogens, but skin microbes may be pathogenic if these organisms penetrate the skin in a susceptible host. For example, *Staphylococcus epidermidis* is a common skin colonizer. However, this species may cause infections in immunocompromised patients or those with indwelling devices. Conversely, *Pseudomonas fluorescens* is thought to be a protective skin organism, because it produces the polyketide antibiotic mupirocin (113), which is active against Gram-positive bacteria, including methicillin-resistant *S. aureus* (108–110, 114). The skin microbiome varies substantially between individuals, and colonizing microbes may produce antimicrobial compounds that shape microbial communities.

REFERENCE STRAIN GENOMES

A critical component of the HMP has been the generation of genome sequences of microbes and viruses that inhabit the human body to create a catalog of reference genomes (115). This catalog serves as a reference data set for metagenomic analyses. Bacterial genome sequences (draft or higher quality) from nearly 5,000 nonpathogenic strains that inhabit the human body have been deposited in the NCBI database, and nearly 2,000 were contributed by the HMP Consortium. The HMP goal is to contribute 3,000 reference genomes (bacterial, fungal, and viral) to the catalog. Community input was sought to nominate strains and isolates for sequencing, and an effort was made to distribute isolates across the five major body sites, although strains from the gastrointestinal tract and oral cavity are overrepresented. These genomes have been added to the comprehensive human microbiome catalog (http://www.hmpdacc.org/catalog/).

The Roche 454 long-tag, paired-end sequencing strategy, originally used by the HMP Consortium for reference genome sequencing, has been replaced by high-coverage (ca. 100× to 400×), short-read sequencing on the Illumina HiSeq platform. Reads are assembled using various assemblers, such as Velvet, ALLPATHs, or the Celera Assembler. Nearly all genome sequences that are currently being deposited are draft WGS sequences that consist of one to dozens of unordered scaffolds. To address the draft nature of these sequences, a set of nomenclature, criteria, and standards for different levels of genome finishing was established (115, 116). The five levels are as follows: (i) standard draft, (ii) high-quality draft, (iii) improved high-quality draft, (iv) annotation-directed improvement, and (v) finished sequence. The levels range from basic assembled contigs (standard draft) to a gold standard complete genome that is assembled into one contiguous piece (finished sequence), has less than 1 error per 100,000 bp, and has all repetitive sequences (including rRNA operons) correctly ordered and placed. Most HMP reference genomes (ca. 70%) are high-quality drafts, defined as having >90% of the genome contained in contigs longer than 500 bp, >90% of the genome covered >5-fold, an average contig length of >5 kb, a contig N_{50} of >5 kb, and a scaffold N_{50} of >20 kb.

The HMP Consortium evaluated methods for gene finding using algorithms such as Glimmer (117) and GeneMark (118). Gene calling was established based on evidence from BLAST searches against the NCBI's bacterial nonredundant nucleotide and protein databases and alignments to proteins in the Pfam database (http://pfam.sanger.ac.uk/). An automated annotation pipeline was established using tools such as tRNAscan-SE to identify tRNA genes (119) and RNAmmer (120) and Rfam (121) to predict noncoding RNA genes. Gene product annotation is based on evidence from programs such as InterProScan (122), PSORTb (123), and KEGG (http://www.genome.jp/kegg/). Uniform protein naming follows Enzyme Commission nomenclature and descriptors suggested by the JCVI Prokaryotic Annotation Pipeline (124).

The utility of the HMP Reference Genomes Catalog was demonstrated by the Human Microbiome Consortium in a read-mapping exercise. A total of 3.5 Tb of Illumina WGS data were generated from 681 microbiome samples from the HMP cohort (5). Following trimming and filtering of human DNA contamination, these reads were aligned to a carefully curated nonredundant reference genome set that contained 2,265 complete and draft genomes plus plasmid sequences that included the sequences of 800 HMP reference genomes (http://hmpdacc.org/HMREFG). In this case, 57.6% of the 35 billion reads were aligned to a reference genome in the HMREFG data set. Furthermore, 26% of these reads could be aligned to one of 223 select HMP reference genomes (5). This result is encouraging, but the coverage is far less than 100%, indicating the importance of continuing reference genome sequencing.

SUMMARY AND CONCLUSIONS

The human microbiome includes a diverse collection of human-associated microbes that represent a small fraction of the total microbial "universe" in other animals, life forms, and the environment. This human microbiome can be defined due to the rapid development of advanced DNA sequencing technologies coupled with bioinformatic strategies. Bioinformatic tools include augmented phylogenetic sequence databases, annotation and genome assembly tools, and refined functional approaches to aggregate sequence data. Metagenomic strategies are being applied to provide glimpses into the variation in microbial composition and function at different body locations and among different individuals.

Future approaches in medical microbiology will be shaped in part by developments in the fields of metagenomics and human microbiome research. The identification of single agents of infection will be supplemented by techniques exploring the relative compositions of microbiomes in the context of different infections and other disease states. Differences in microbial composition that are associated with noninfectious immune-mediated disorders may extend the "reach" of the medical microbiology laboratory into other areas of human medicine in the future. Finally, pathogen discovery efforts (see chapter 16) will be enhanced by new metagenomic strategies, and these studies may uncover single etiologic agents of infections as well as changes in microbial communities at specific body sites that may contribute to infectious diseases.

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Microbial Genomics and Pathogen Discovery*

JENNIFER K. SPINLER, PEERA HEMARAJATA, AND JAMES VERSALOVIC

16

In recent years, our understanding of microbial diversity has grown tremendously as many previously unidentified bacterial, archaeal, and viral species have been discovered and sequenced. In the era of the human microbiome and metagenomics (chapter 15), large-scale DNA sequencing projects and advances in bioinformatics have yielded abundant data regarding human-associated microbes. As human microbiology rapidly expands beyond its past framework of cultured pathogens in the medical microbiology laboratory, opportunities for detection and identification of novel human pathogens associated with infectious diseases abound. In this chapter, we focus on specific or defined sets of pathogens associated with human infections, in contrast to microbial components of disease and microbial ecology (topics covered in chapter 15). We begin with an overview of historical methodologies, followed by a brief description of the evolution of nucleic acid sequencing technologies. Finally, we describe how microarrays, nucleic acid sequencing technologies, and mass spectrometry are profoundly reshaping strategies aimed at pathogen discovery and identification. A timeline of these scientific advances is depicted in Fig. 1.

Our ability to visualize human microbes first arose in the 17th century when Anton van Leeuwenhoek created the microscope and observed human-associated bacteria in the human oral cavity, which provided the first physical evidence of the diversity and ubiquity of microbes in the world. Another giant leap occurred in the 19th century when Robert Koch first demonstrated that bacteria could be grown in pure culture, beginning with the analysis of blood from cows infected with the anthrax agent. He subsequently became most widely known for the postulates regarding microbial disease causation that bear his name. For decades following, microbiologic culture and microscopy were the only tools available to directly examine human microbes. In the last 2 decades of the 20th century, however, DNA amplification of consensus rRNA gene sequences and other genetic targets revealed a vast diversity of bacteria, viruses, and other microbes that had eluded cultivation in the laboratory. Today, DNA and RNA sequencing technologies have evolved to the point that it is feasible to comprehensively define the collection of microbes present in humans (i.e., the human "microbiome") or any other ecological niche in a fashion that is independent of microbiologic culture or microscopy.

A BRIEF HISTORY OF PATHOGEN DISCOVERY

Classical Methods

Classical methods of microbial discovery relied on the ability to culture microorganisms in the laboratory and to directly visualize microbial morphology by microscopy. Body fluid or tissue specimens associated with specific disease phenotypes and considered to be of infectious origin are still used today to inoculate microbiologic growth media and cultivate the microbe(s) present in the sample. In the case of suspected bacterial agents, selective or nonselective growth media can be utilized, and biochemical or serological tests can facilitate identification of cultured isolates. Primary or immortalized human and mammalian cell lines are inoculated for suspected viruses, and viruses have been identified by cytopathic effects (light microscopy), immunological methods (serological reactions based on different antisera), and viral particle morphology (electron microscopy). For pathogen discovery and biomedical research purposes, clinical specimens have been used for decades to infect animal models and prove Koch's postulates. These classical methods have been very useful and resulted in the discovery of many currently accepted human pathogens; examples include *Bacillus anthracis*, *Mycobacterium tuberculosis*, *Yellow fever virus*, and *Poliovirus*. However, there are two fundamental limitations with conventional approaches: (i) these methods depend on the ability of microbes to grow on media substrates in the laboratory; and (ii) even if the microbe can be cultivated, unknown candidate agents may not be unambiguously identified.

Molecular Strategies: Pathogen Discovery

In the 1980s, scientists began to apply DNA amplification technologies to detect microbes present in human specimens if existing knowledge about DNA sequences in microbial genomes could be targeted. The recognition that selecting PCR primers designed to highly conserved regions in a set of sequences (e.g., multiple bacteria or several viruses from a common taxonomic group) could enable the detection of previously unidentified microbes provided a novel approach for the identification of microbes. One of the broadest applications of this technique has been the design of primers to

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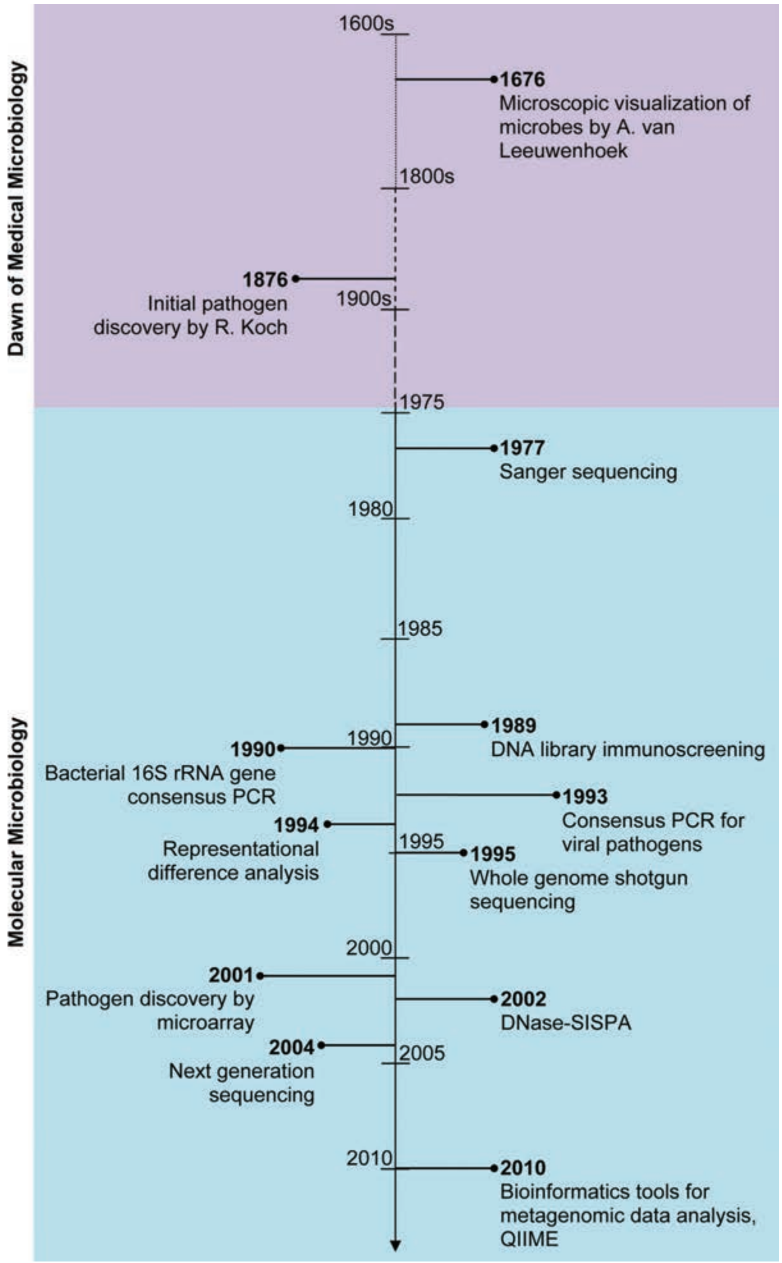
Dawn of Medical Microbiology
Molecular Microbiology
1600s
1676
Microscopic visualization of microbes by A. van Leeuwenhoek
1800s
1876
Initial pathogen discovery by R. Koch
1900s
1975
1977
Sanger sequencing
1980
1985
1989
DNA library immunoscreening
1990
1990
Bacterial 16S rRNA gene consensus PCR
1993
Consensus PCR for viral pathogens
1994
Representational difference analysis
1995
1995
Whole genome shotgun sequencing
2000
2001
Pathogen discovery by microarray
2002
DNase-SISPA
2004
Next generation sequencing
2005
2010
2010
Bioinformatics tools for metagenomic data analysis, QIIME

FIGURE 1 A timeline depicting major landmarks in medical microbiology and molecular microbiology that have enhanced pathogen discovery. doi:10.1128/9781555817381.ch16.f1

the conserved 16S rRNA gene, which enables the detection of most members of the bacterial domain depending on the selection of specific primer pairs. Alternatively, more specific primers can be selected that are conserved within a given taxon (family, genus, or species) to identify a targeted set of microbes. The ability to design highly conserved primers depends on the existence of sufficient amounts of DNA sequencing data, and recent advances in the Human Microbiome Project have contributed greatly to a refinement of primer selection and amplification strategies for bacterial genera and species. Different microbes have been discovered by using PCR in conjunction with the classical methods of microbial detection. With the advent of Sanger sequencing, entire 16S rRNA genes could be sequenced, including the *Escherichia coli* 16S rRNA gene in 1978 (1).

Relman and coworkers described the first examples of using consensus PCR primers to identify the causative agents of specific human diseases. Bacillary angiomatosis was commonly considered to be infectious in origin. A putative agent could be visualized in tissue sections following staining, but efforts to culture the organism had failed. Amplicon sequencing using 16S rRNA gene consensus primers demonstrated that a previously uncharacterized rickettsia-like bacterium was present in tissue samples of patients with bacillary angiomatosis (2). The bacterium was identified as a member of the genus *Bartonella*. A similar approach was subsequently applied to address the etiology of Whipple's disease. Whipple's disease was first described in 1907 as a rare systemic disorder that primarily caused malabsorption but could affect any part of the body. Consensus PCR using primers targeting bacterial 16S ribosomal genes resulted in the identification of an uncharacterized actinomycete, which was classified as *Tropheryma whipplei* (3). These two cases demonstrated the power of molecular pathogen discovery methods.

Conserved PCR primers and nucleic acid probes have been applied to virus discovery. However, since no universally conserved sequence akin to 16S rRNA sequences in bacteria is present in all viruses, different consensus nucleic acid sequences must be identified for different classes of viruses and consensus primers designed for each viral taxon of interest. A seminal example of using consensus PCR to identify a viral pathogen occurred during the emergence of hantavirus pulmonary syndrome in 1993 (4). In the course of investigating an unusual outbreak of a lethal pulmonary disease in otherwise healthy young adults in the southwestern United States, extensive testing by classical microbiological methods ruled out the most likely candidates known to cause severe respiratory disease. Serological tests revealed that patient sera were cross-reactive with known hantaviruses. PCR primers were designed to conserved regions of known hantavirus sequences, which were then used to amplify nucleic acids extracted from tissue samples isolated from dying patients. Sequencing of the amplicon generated by the primers resulted in the identification of a novel member of this family, which was ultimately named *Sin Nombre virus*.

Since these seminal applications of consensus PCR for the identification of bacterial and viral pathogens, many applications of nucleic acid amplification for pathogen discovery have been published. Consensus PCR, either alone or in conjunction with microbiologic culture, microscopy, and antigen detection methods, has been used to detect novel microbes, as illustrated by the recent discoveries of new groups of rhinoviruses (5–9), parechoviruses (10–15), *Chapare virus* (arenavirus) (16), *Bundibugyo ebolavirus* (17), the Middle East respiratory syndrome coronavirus (18), and an entirely new bacterium associated with cord colitis syndrome (19). However, broad-range PCR strategies often failed to identify a specific infectious agent. In these examples, the authors had robust hypotheses regarding the nature of the microbes of interest (i.e., bacterium versus virus) or which specific candidate viral taxon might be present. Often, leading candidates or phylogenetic groups of interest may not be apparent, limiting the application of specific molecular strategies. Outside of the bacterial kingdom, the relative paucity of consensus probes or primers for archaea, eukaryotic microbes, and viruses emphasizes the limits of targeted molecular strategies. For these reasons, more comprehensive genomics-based strategies have been applied to pathogen discovery and in-depth characterization.

Early Applications of Microbial Genomics: Pathogen Discovery

The discoveries of *Hepatitis C virus* (HCV) and *Human herpesvirus* 8, also named Kaposi's sarcoma-associated herpesvirus, represented two breakthroughs in the application of genomics-based molecular methods for pathogen discovery. In 1989, the identification of HCV in patients with non-A, non-B (NANB) hepatitis relied on a DNA library-based immunoscreening strategy (20). A randomly primed cDNA library was made from material from infected animals and screened using patient serum from NANB hepatitis patients with the goal of identifying cDNA clones that generated peptide sequences recognized by the patient sera. More than 1 million clones were screened, and a single clone reacted specifically with NANB hepatitis patient sera. From this initial cDNA clone fragment, the entire HCV genome was eventually sequenced. HCV is now recognized as being responsible for the vast majority of cases of NANB hepatitis. In 1994, human herpesvirus 8 was discovered in the lesions of AIDS-associated Kaposi's sarcoma (21). The identification of Kaposi's sarcoma-associated herpesvirus relied on representational difference analysis, a subtractive hybridization-based method, to enrich for and identify unique sequences present in Kaposi's sarcoma lesions but not in healthy tissue controls. While these two examples demonstrated the potential utility of early genomics-based strategies, subsequent success stories were rare, most likely due to the technical challenges associated with first-generation genomics-based strategies.

By the end of the 20th century, classical microbiologic culture-based methods for microbial discovery had been augmented by early molecular and genomics-based strategies, such as consensus PCR, library immunoscreening, and representational difference analysis. In parallel, reference genome sequencing of specific microbes was becoming feasible, setting the stage for the convergence of pathogen discovery and microbial genomic sequencing efforts. Further developments in genomics-based strategies were needed to advance pathogen discovery in the 21st century.

RECENT HISTORY OF MICROBIAL GENOMICS

Microbe sequencing in the 20th century relied exclusively on Sanger dideoxy sequencing, the dominant sequencing strategy since its invention in 1977. From its initial incarnation using slab-gel electrophoresis, incremental advances in sequencing capacity evolved as sequencing transitioned to capillary electrophoresis.

Formally, the era of microbial genomics began with the complete sequencing of the *Haemophilus influenzae* genome in 1995. However, it was recognized almost 2 decades earlier that an organism's genomic sequence could serve to classify and define the relatedness of both prokaryotic and eukaryotic organisms (22). When the *H. influenzae* genome was sequenced, this bacterium became the first free-living organism to have its genome sequenced in its entirety (23). This was a landmark achievement, notable also because of the use of a "shotgun" strategy to assemble the complete genome. "Shotgun" refers to the random fragmentation and cloning of DNA fragments followed by computational assembly of the overlapping regions to generate a complete genome sequence. Based on this proof of principle, genomes of larger microbes and eukaryotic organisms were subsequently sequenced. The following year, *Saccharomyces cerevisiae* was the first eukaryotic organism to be fully sequenced (24), and then in 1998, the first multicellular eukaryotic genome to be sequenced, that of *Caenorhabditis elegans*, was published (25). Since then, the complete genomes of many human and animal pathogens have been sequenced, including notable pathogens such as *M. tuberculosis* (26), *Yersinia pestis* (27), and *Plasmodium falciparum* (28). In 2004, the complete 1.2-

Mb genome of mimivirus, the largest known virus, was published (29).

Next-generation DNA sequencing modalities have revolutionized the depth and throughput of sequencing applications in medical microbiology. Three major platforms in current use are 454 (454 Life Sciences, a Roche Company, Branford, CT), HiSeq and MiSeq (Illumina, San Diego, CA), and Ion Torrent/PGM (Life Technologies, Carlsbad, CA). Key characteristics of these platforms include the fact that all of them have exponentially increased the raw sequence generation capacity and dramatically decreased the cost per base relative to Sanger sequencing. For a more detailed description of each of these platforms and capabilities, see reference 30. With these increases in sequencing capacity, the sequencing of microbial genomes has become routine. Next-generation DNA sequencing platforms have enabled scientists to comprehensively explore the microbial diversity of the human microbiome and the viral diversity, the “virome,” present in humans (see chapter 15). These efforts have vastly expanded the world of sequenced microbes, and sequences have been deposited in various databases (e.g., http://www.ncbi.nlm.nih.gov/genome/ and http://hmpdacc.org).

THE MERGING OF PATHOGEN DISCOVERY AND MICROBIAL GENOMICS

The fields of pathogen discovery and microbial genomics have converged in the past 2 decades so that more pathogens can be detected and identified (Fig. 1). Two basic molecular approaches for massively parallel analysis have recently emerged that are capable of defining the spectrum of microbes present in clinical specimens: microarrays and sequencing-based detection. Both strategies benefited greatly from the increased focus on human and microbial sequencing. Multiple databases of nucleic acid sequences provide the substrate from which consensus PCR primers for sequencing applications and probes for microarrays have been designed. DNA sequencing-based approaches have evolved rapidly in the past decade and have resulted in next-generation DNA sequencing pipelines for pathogen discovery and detection. As sequencing technologies are rapidly supplanting microarray-based hybridization strategies, microarrays continue to represent a useful option for bacterial and viral detection. Data analysis or bioinformatics tools and pipelines are also being refined to meet the challenge of genomics and “big data” in the clinical laboratory, whether the data are generated by microarray or sequencing platforms.

Microarray-Based Approaches

Microarrays were initially developed in the early 1990s to analyze gene expression patterns of a single organism in a highly parallel fashion. For example, early studies analyzed every gene present in the yeast *S. cerevisiae* (31). Regardless of the technical format, the fundamental principle driving all microarrays is that of nucleic acid hybridization. If a given sample contains sequences that are complementary to those represented on the array, hybridization should occur. Thus, the target sequences to be detected by a given microarray are limited only by the availability of DNA sequences from which probes can be designed. As DNA microarray technology evolved in the late 1990s, it became clear that microarrays might make excellent microbial diagnostic tools. The inherently highly parallel nature of microarrays, coupled with the expanding amount of microbial sequencing data, made it possible to design microarrays with the capacity to simultaneously detect a wide range of microbes.

The first significant effort to develop a microarray for broad-range pathogen identification focused on detecting all known respiratory viruses (32). This microarray contained ~1,600 oligonucleotides representing ~140 viruses from all virus families with members known to cause respiratory disease. A more comprehensive array, the ViroChip, was designed using 70-mer oligonucleotides derived from every fully sequenced viral genome in GenBank at the time (33). The oligonucleotide probes selected were highly conserved 70-mers from each viral taxon, enabling the detection of known and unknown family members through cross-hybridization. Following the random amplification of extracted nucleic acids, samples are labeled with fluorescent dyes (typically Cy3 or Cy5). The resulting hybridization patterns on the microarray can be interpreted to infer the presence or absence of known or novel viruses. A variety of approaches for microarray data analysis, including visual inspection, hierarchical clustering (34), and several customized bioinformatics programs, such as GreeneLAMP (35), E-Predict (36), DetectiV (37), PhyloDetect (38), and VIPR (39) and VIPR HMM (40), have been developed.

The ViroChip was successfully utilized to identify a novel coronavirus as the causative agent for the severe acute respiratory syndrome outbreak in 2003 (33, 41, 42). Other broad-range DNA microarrays, in addition to the ViroChip, have been described. GreeneChip, a pan-microbial microarray that contains probes for viruses, bacteria, and parasites (35), has been successfully used in a variety of diagnostic scenarios to detect known or unexpected viruses (43–47). Lawrence Livermore Microbial Detection Array is a high-density microarray that contains target probes against every bacterium and virus whose full genome sequence was available at the time of development (48, 49). This array, in combination with multiple displacement amplification-based whole-genome amplification via isothermal amplification by the ϕ29 polymerase, was successfully used to detect the presence of many DNA and RNA viruses in various types of clinical specimens (50). PathChip is another pathogen microarray option that utilizes random-tagged PCR prior to hybridization, and has been used to identify viral pathogens in nasal wash specimens collected from pediatric patients with respiratory tract infections (51). DNA microarrays can also be used for subtyping of viruses such as influenza A (52).

On the bacterial front, microarrays with probes targeting detection of 16S rRNA gene sequences have been developed to define the bacterial diversity present in a given sample. The PhyloChip, a high-density 16S rRNA gene microarray containing ~300,000 probes, was used to examine microbial diversity in three environmental samples, including urban aerosol, subsurface soil, and subsurface water (53). This study demonstrated that this DNA microarray could reveal a broad range of microbial diversity. A second microarray containing 10,500 probes, including 9,000 taxonomically specific probes targeting the 16S rRNA gene, was used to perform a systematic and quantitative study of bacterial colonization in the infant gastrointestinal tract (54). These microarray results compared favorably with data generated by sequencing-based techniques, demonstrating the utility of microarrays. Microarray-based assays have been successfully utilized to identify bacterial pathogens in several types of infections, such as bacteremia (55–57), genitourinary infections (58, 59), and infectious diarrhea (60).

Fungal identification strategies by microarrays have utilized targets such as the 28S rRNA gene and internal transcribed spacer regions within rRNA gene clusters. Microarray technology has been used to identify common fungal pathogens in humans, including microbes in the genera *Candida*, *Aspergillus*, and *Fusarium* (61, 62).

DNA microarrays aimed at microbial diagnostics have benefited immensely from the genomics era, which has spawned a wealth of microbial sequences available in public DNA databases. As demonstrated by successful examples using the ViroChip, microarrays can be a robust platform for virus and pathogen discovery. However, a clear limitation of all hybridization-based strategies for pathogen discovery, including DNA microarrays, is that sequences must be known and well characterized. DNA sequencing strategies, by contrast, offer a relatively open-ended strategy for pathogen discovery that does not rely on the development of specific probes in advance. The data generated by DNA sequencing platforms can be used as virtual "probes" with DNA databases for identification of unexpected pathogens.

Sequencing-Based Approaches

Pathogen discovery and microbial sequencing techniques merged with the discovery of *Human metapneumovirus* (HMPV) in 2001, which combined classic viral culture with a molecular strategy, termed random arbitrarily primed PCR (63). Efforts to culture respiratory secretions from children suffering respiratory tract infections led to the identification of a putative unidentified virus that could be passaged in several mammalian cell lines. Arbitrary primers were used to generate PCR amplicons, and those amplicons that were uniquely present in infected cells were selectively sequenced by Sanger sequencing methods. Multiple fragments having limited sequence identity to avian pneumoviruses were detected, indicating that a novel virus, now known as HMPV, was present in the infected cells. Seroprevalence studies indicated that by the age of 5 to 10 years, most individuals were antibody positive, suggesting that this virus is a common infection acquired in childhood. HMPV can cause severe respiratory infections similar in presentation and case severity to respiratory syncytial virus, including pneumonia and bronchiolitis, and is responsible for 5 to 10% of hospitalizations of patients with respiratory tract infections (64).

In the same year, a candidate independent sequencing strategy for identification of novel viruses, termed DNase-SISPA, was described (65), and it was the methodological basis for discovering novel viruses for nearly a decade. The experimental strategy relied on sequence-independent single primer amplification (SISPA), wherein an adaptor containing a primer-binding sequence is ligated to both ends of a cDNA fragment and a single primer is then used for PCR. To enrich specifically for viral nucleic acids present in virions, the clinical specimen is first subjected to ultracentrifugation to collect the virions, which are subsequently treated with DNase to degrade any cellular nucleic acids that are not protected within the viral capsids. Following this enrichment, the sample is extracted for DNA or RNA and amplified using SISPA. The enrichment steps in this protocol are necessary to increase the chances of sequencing a virus-derived sequence, given the labor and costs of performing extensive Sanger sequencing on the unenriched sample. In this proof-of-concept study, two novel bovine parvoviruses were identified (65). DNase-SISPA and variations of this method were successfully applied to discover many potentially pathogenic viruses with low sequence homology to known viruses during the next several years (Table 1). The identification of these novel viruses highlights the utility of DNA sequencing for viral detection.

Advances in virus discovery continued with the combination of DNase-SISPA-based methods and high-throughput Sanger sequencing. *Human bocavirus* (HBoV) was discovered in 2005 (66) from respiratory secretions pooled from multiple patients with an unexplained respiratory illness. Amplicons of 600 to 1,500 bp were cloned and sequenced using high-throughput Sanger sequencing with one 384-well plate. Phylogenetic analysis demonstrated that this novel genome is a previously uncharacterized species of the genus *Bocavirus*, HBoV. Subsequent studies of this virus have demonstrated that HBoV is frequently detected in children with respiratory tract infections, children with asthma exacerbations, and children with acute gastroenteritis. Seroepidemiology studies have confirmed infection in a Japanese cohort, with 71.1% overall prevalence with exposure by age 6 (67), while a second study in Sweden reported a lower rate, 33%, in a cohort of children with acute wheezing (68). The application of the same method with multiple specimen types resulted in the discovery of several novel viruses that have yet to be confirmed as pathogens (see Table 1). These discoveries demonstrate that sequence-independent amplification followed by limited Sanger capillary sequencing (typically ≤384 clones) is a robust method for identification of novel viruses present in clinical specimens.

The advent of next-generation sequencing (NGS) technology increased the sequencing depth of specimens, allowing for detection of microbes present at lower titers as well as facilitating the generation of complete genomes of novel microbes. The 2008 discovery of *Merkel cell polyomavirus* (MCPyV) was the first study describing the identification of a novel virus using NGS on the Roche 454 FLX platform (69). In this instance, cDNA libraries made from Merkel cell carcinoma tumors were sequenced, and from the ~382,000 high-quality sequence reads generated, one fragment had detectable sequence similarity to a known polyomavirus. Further analysis demonstrated that a highly divergent polyomavirus genome, that of MCPyV, was present in the majority of the Merkel cell carcinoma tumors examined. Subsequent studies have corroborated this finding, and mapping of integration sites demonstrated that the virus was clonally integrated in the respective tumors. Given the very low abundance of MCPyV mRNA sequences in these samples, the detection of viral transcripts would not have been possible without the use of the next-generation platform, which enabled deep sequencing of the specimens in a cost-effective manner. For other novel tumor-associated viruses that have been identified using NGS technology, see Table 2.

NGS by 454 FLX has played a pivotal role in defining the etiology of several unexplained infectious illnesses. The first was a mysterious case cluster of five patients with undiagnosed hemorrhagic fever (70). RNAs from two postmortem liver biopsy samples and one serum sample were randomly amplified and sequenced, and the analysis of ~300,000 sequences yielded nine fragments with limited sequence similarity to viruses in the genus *Arenavirus*. Phylogenetic analysis of the novel Lujo virus demonstrated that it branched from the Old World arenavirus complex and had the greatest similarity to Mobala virus, Lassa fever virus, and *Tamiami virus*, with 67 to 74% amino acid identity in the nucleoprotein. Further examination of the receptor-binding portion of G1 demonstrated that Lujo virus is equally distant from the Old World and New World arenaviruses. Other elusive etiologic viral agents of unexplained gastroenteritis have recently been identified using 454 FLX, and they are summarized in Table 2.

TABLE 1 Novel viruses discovered by Sanger sequencing methods

Virus	Specimen type	Discovery method	Disease	Reference(s)
Human metapneumovirus	Respiratory secretions	Viral culture, random arbitrarily primed PCR, and Sanger sequencing	Severe respiratory infections	63
Bovine parvoviruses	Bovine serum	DNase-SISPA	Contaminants of commercial bovine serum	65
Human coronavirus NL63	Respiratory samples	Virus discovery cDNA-amplified fragment length polymorphism	Croup	111
Parvovirus 4	Plasma	DNase-SISPA	Viral sepsis of immunocompromised patients	112
Anellovirus SA1	Plasma	DNase-SISPA	Unknown	112
Anellovirus SA2	Plasma	DNase-SISPA	Unknown	112
Anellovirus	Healthy donor blood	DNase-SISPA	Unknown	113
Anellovirus	Healthy donor blood	DNase-SISPA	Unknown	113
Human bocavirus	Respiratory secretions	DNase-SISPA + high-throughput Sanger sequencing	Respiratory tract infections, asthma exacerbation, and acute gastroenteritis	66
KI polyomavirus	Respiratory secretions	DNase-SISPA + high-throughput Sanger sequencing	Unknown	114
WU polyomavirus	Respiratory secretions	DNase-SISPA + high-throughput Sanger sequencing	Unknown	115
Saffold virus	Stool	DNase-SISPA + high-throughput Sanger sequencing	Unknown	13, 116
Astrovirus MLB1	Stool	DNase-SISPA + high-throughput Sanger sequencing	Unknown	117
Human cosavirus A1	Stool	DNase-SISPA + high-throughput Sanger sequencing	Nonpolio acute flaccid paralysis	118–120
Human bocavirus 2	Stool	DNase-SISPA + high-throughput Sanger sequencing	Nonpolio acute flaccid paralysis and acute gastroenteritis	121, 122
Human bocavirus 3	Stool	DNase-SISPA + high-throughput Sanger sequencing	Acute gastroenteritis	121
Bat polyomaviruses	Bat blood, major organs, and rectal and oral swabs	Seminested PCR + Sanger sequencing	Unknown	96

Sequencing-based discovery of novel bacterial pathogens can be performed by sequencing specific gene targets or using a combination of whole-genome shotgun sequencing (WGS) and a range of data analysis tools. These tools may be applied to the assembly and analysis of bacterial genomes from either pure culture or patient specimens. A workflow for implementing bacterial WGS-based molecular diagnostics is depicted in Fig. 2. In 2013, a novel species of *Bartonella* was identified from a patient with chronic bartonellosis by combining culture methods, PCR amplification, and Sanger sequencing of three gene fragments important for speciation. Blazes et al. targeted the *rrs*, *gltA*, and *rpoB* genes, and based on sequence analysis using BLAST, ClustalW, and MEGA5 software, they showed that the sequence similarities to other known *Bartonella* spp. were below the standard similarity ranges for *Bartonella*, and identified this novel isolate as "*Candidatus* Bartonella ancashi" (71). In the same year, Bhatt et al. (19) applied WGS to DNA isolated from formalin-fixed, paraffin-embedded biopsy specimens from patients with cord colitis in an effort to identify a novel etiologic agent for the disease. Previous theories for the etiology of cord colitis assumed it was a manifestation of graft-versus-host disease as opposed to a distinct clinical syndrome. Their efforts resulted in the identification of a novel bacterial draft genome, *Bradyrhizobium enterica*, which was absent from control specimens and associated with all cord colitis specimens they analyzed (Fig. 3).

NGS has also been applied to areas of molecular epidemiology and pathogen emergence. In 2011, Wright et al. (72) used NGS to quickly complete the whole-genome characterization of an anthrax-like agent within days of its recovery from antemortem cultures. The etiologic agent was determined to be *Bacillus cereus*, not *B. anthracis*. Rapid genome sequencing and analysis of the causative agent of this fatal,

TABLE 2 Novel viruses discovered by NGS methods

Virus	Specimen type	Discovery method	Disease	Reference(s)
Merkel cell polyomavirus	Merkel cell carcinoma tumors	Roche 454 FLX	Merkel cell carcinoma	69
Epstein-Barr virus type 1	Nasopharyngeal carcinoma tumor biopsy	Illumina Genome Analyzer IIx	Nasopharyngeal carcinoma	123
CNS[a] lymphoma viruses (Epstein-Barr virus, cytomegalovirus, JC virus, and HIV)	RNA from primary CNS lymphomas	Next-generation transcriptome sequencing	CNS lymphomas	124
Lujo virus	RNA from postmortem liver biopsy	Next-generation transcriptome sequencing	Hemorrhagic fever	70
Human klassevirus 1	Pediatric stool	Roche 454 FLX	Diarrhea	125, 126
Astrovirus VA1	Stool	GS-FLX Titanium	Acute gastroenteritis	127
Middle East respiratory syndrome coronavirus	Tracheal aspirates and nasopharyngeal or throat swabs	Illumina	Middle East respiratory syndrome	18
Mamastrovirus, *Bocavirus*, *Circovirus*, *Iflavirus*, and *Orthohepadnavirus*	Bat thoracic and abdominal organ specimens	DNase-SISPA + Solexa sequencing	Unknown	95
Miniopterus schreibersii papillomavirus	Bat rectal and oral swabs	454 sequencing	Unknown	97

[a] CNS, central nervous system.

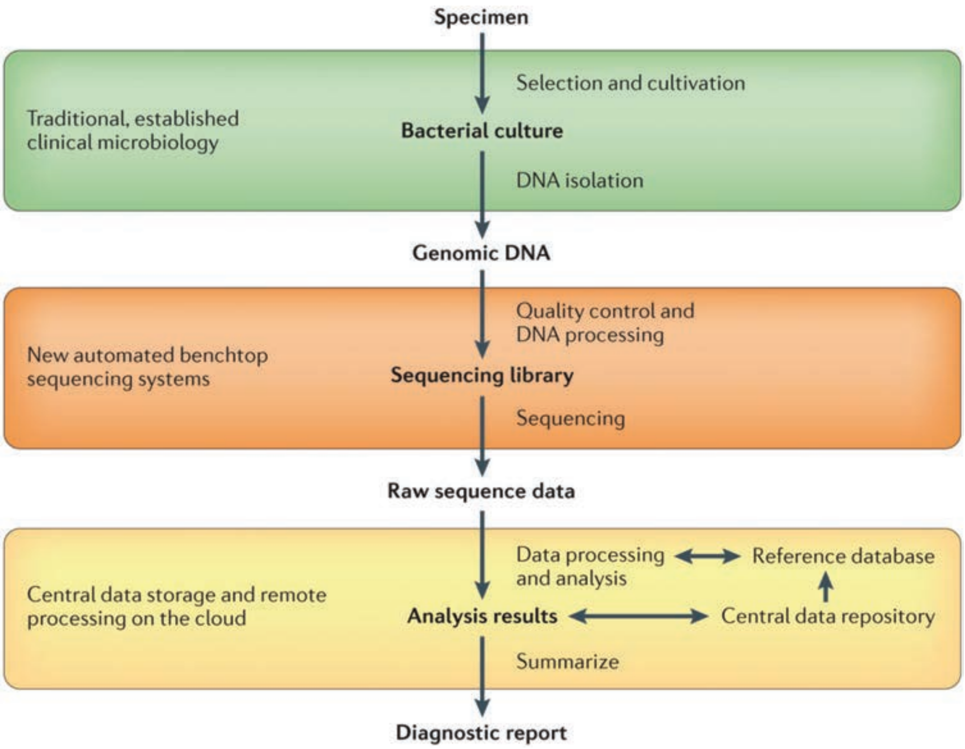
Specimen
Traditional, established clinical microbiology
Selection and cultivation
Bacterial culture
DNA isolation
Genomic DNA
New automated benchtop sequencing systems
Quality control and DNA processing
Sequencing library
Sequencing
Raw sequence data
Central data storage and remote processing on the cloud
Data processing and analysis
Reference database
Analysis results
Central data repository
Summarize
Diagnostic report

FIGURE 2 A proposed workflow for bacterial, WGS-based molecular diagnostics. This workflow uses benchtop sequencing for data generation and implements cloud storage for central data storage and remote data processing. Double-headed arrows indicate constant updating of the central data repository and reference database. Reprinted from reference 128 with permission of Nature Publishing Group. doi:10.1128/9781555817381.ch16.f2

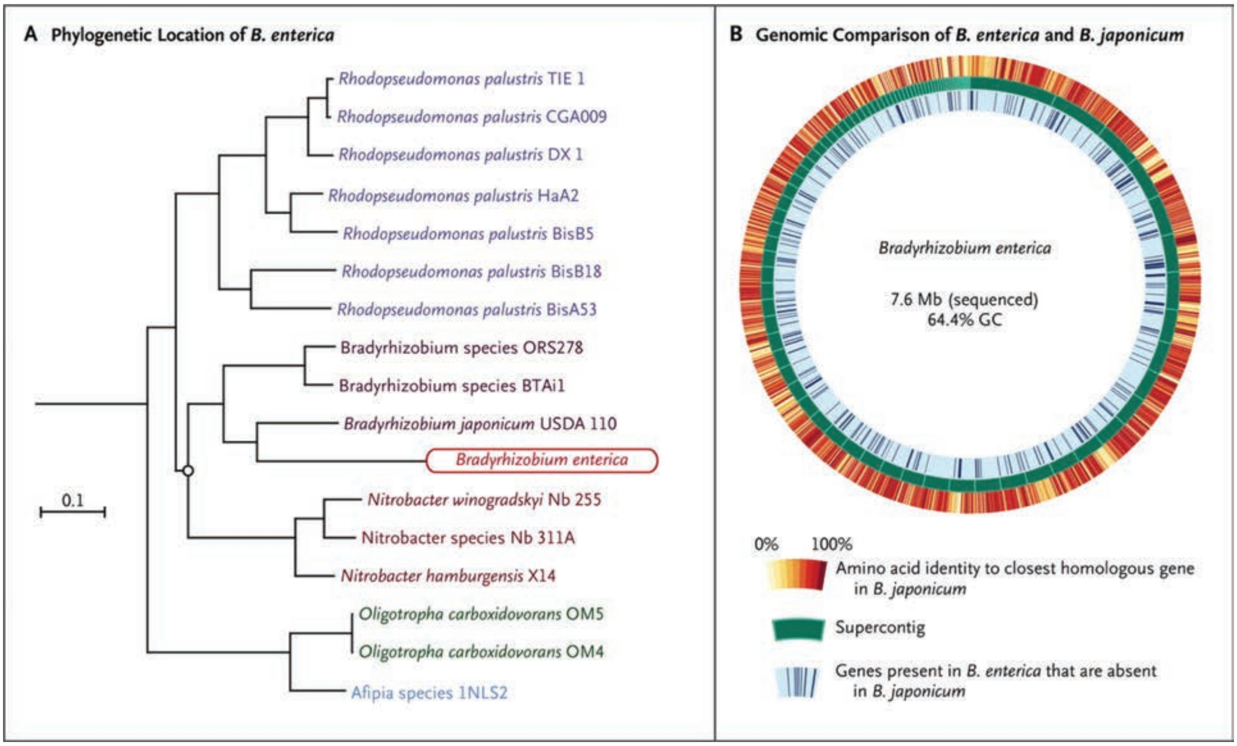
A Phylogenetic Location of B. enterica
Rhodopseudomonas palustris TIE 1
Rhodopseudomonas palustris CGA009
Rhodopseudomonas palustris DX 1
Rhodopseudomonas palustris HaA2
Rhodopseudomonas palustris BisB5
Rhodopseudomonas palustris BisB18
Rhodopseudomonas palustris BisA53
Bradyrhizobium species ORS278
Bradyrhizobium species BTAi1
Bradyrhizobium japonicum USDA 110
Bradyrhizobium enterica
0.1
Nitrobacter winogradskyi Nb 255
Nitrobacter species Nb 311A
Nitrobacter hamburgensis X14
Oligotropha carboxidovorans OM5
Oligotropha carboxidovorans OM4
Afipia species 1NLS2
B Genomic Comparison of B. enterica and B. japonicum
Bradyrhizobium enterica
7.6 Mb (sequenced)
64.4% GC
0% 100%
Amino acid identity to closest homologous gene in B. japonicum
Supercontig
Genes present in B. enterica that are absent in B. japonicum

FIGURE 3 Phylogenetic analysis and genome assembly of *Bradyrhizobium enterica*, a novel organism associated with cord colitis. Assembly of the *B. enterica* genome included unmappable reads generated from WGS of samples from patients with cord colitis. (A) Multisequence alignment of 400 core protein-coding genes was used to generate a rooted phylogenetic tree showing the predicted evolutionary relationships between *B. enterica* and related species. (B) Genomic comparison of *B. enterica* to *Bradyrhizobium japonicum*. Dark blue lines on the inner circle depict genes present in the genome of *B. enterica* that are absent in the genome of *B. japonicum*. The outer circle depicts amino acid sequence identity of each *B. enterica* predicted protein to its closest homologue in *B. japonicum*. The middle circle is a representation of the contig assembly, with the borders of each contig (dark green) outlined in light green. Reprinted from reference 19 with permission of Massachusetts Medical Society. doi:10.1128/9781555817381.ch16.f3

anthrax-like pneumonia effectively determined the etiology of the infection. WGS was first applied as a genome-based molecular epidemiological tool in 2013. Roetzer et al. (73) assessed WGS-based genotyping as a means to track the spread of *M. tuberculosis* in a metropolitan area and follow its decade-long clonal expansion to determine the short-term evolution of the *M. tuberculosis* genome. The results showed that genotyping of an *M. tuberculosis* outbreak using WGS methods was more robust than conventional methods like IS*6110* restriction fragment length polymorphism, spoligotyping, and mycobacterial interspersed repetitive-unit–variable-number tandem-repeat typing, which examine <1% of the genome. Through these efforts, Roetzer and colleagues were able to identify a large outbreak and trace the transmission and evolution of an *M. tuberculosis* clone of the Haarlem lineage. Additionally, WGS has been used to investigate the emergence of hypervirulent and invasive group A *Streptococcus* (GAS) strains in a single human host. Flores et al. (74) analyzed genome sequences generated on the MiSeq platform (Illumina) of paired noninvasive and invasive GAS isolates from a patient with a skin/soft tissue infection. Whole-genome comparisons of these paired isolates showed that emergence of an invasive GAS phenotype originated from mutations that countered the established paradigm and enhanced the importance of understanding the molecular evolution of pathogenicity. Having the ability to apply NGS to molecular epidemiology and pathogen evolution could significantly enhance our ability to effectively manage and control public health threats.

Studying historical pathogens can provide scientists with important information to counteract potentially disastrous outbreaks. To sequence the 1918 influenza virus, cDNA libraries were made from highly degraded RNA isolated from nearly century-old, formalin-fixed and paraffin-embedded lung tissue. The whole genome of the 1918 pandemic influenza A virus was sequenced at 3,000-fold coverage in a single run using the Illumina Genome Analyzer IIx platform (75). This strategy represented a significant advance over the first sequencing attempt, which took ~9 years using Sanger methods (76). Both 454 and SOLiD NGS methods have been used to identify a previously unknown family of mobile genetic elements, the transpovirons (77). The transpovirons are linear mobile genetic elements that infect large viruses, contain genetic elements similar to virophages, and contribute to the evolution of large DNA viruses and interviral gene transfer. Additionally, high-throughput sequencing methods have been used to identify bacterial defense mechanisms, or toxin-antitoxin gene pairs, against bacteriophages. WGS of 1.5 million genes on a Sanger platform aided in the identification of hundreds of toxin-antitoxin pairs important for neutralizing the effects of bacteriophage toxins (78). Phage display is extremely useful in identifying protein-protein interactions between unknown factors, but has historically been very labor-intensive. The application of NGS to phage display has enabled the analysis of phage libraries to increase from hundreds of clones to thousands or more. The HiSeq/MiSeq (79–82) and 454 (83–85) platforms have been successfully applied to phage display. Additionally, NGS methods have been combined with other methods like proteomics and metabolic modeling to study clinically relevant pathogens like *Listeria monocytogenes* (86), *Helicobacter pylori* (87), and *Francisella tularensis* (88). The application of NGS techniques to these areas of interest can provide timely and valuable information with regard to emerging and reemerging pathogens.

NGS platforms have created the burden of analyzing extremely large data sets ("big data"), which has resulted in the demand for innovation in bioinformatics and data analysis tools. A major impetus for bioinformatics tool development resulted from the generation of massive quantities of sequencing data by the Human Microbiome Project (89). Bioinformatics tools were developed using an open-source software pipeline, QIIME (Quantitative Insights Into Microbial Ecology) (90). The QIIME workflow can process millions of sequences, conduct sequence alignments and phylogenetic comparisons, and provide graphical displays that can be used on platforms ranging from laptops to high-performance computing clusters. In an effort to control or predict outbreaks of emerging or reemerging pathogens, scientists have developed data analysis tools that act as surveillance mechanisms for predicting potential species crossovers and dominant influenza strains. Two automated data analysis platforms have been developed for identifying novel pathogens from long-read NGS platform data, VirusHunter (91) and PathogenFinder (92). VirusHunter enables the automated mining of Roche 454 and other long-read NGS platform data to identify novel viruses, while PathogenFinder works to predict bacterial pathogenicity from NGS data. Batch-Learning Self-Organizing Map (BLSOM) (93) is an analysis tool that has been applied to track sequence changes in influenza virus genomes after viruses invaded human hosts from other mammalian or avian hosts. This analysis tool is capable of surveying millions of genomic sequences to identify potentially novel influenza A and B strains. In addition to these newly developed pipelines, there are preexisting well-known alignment algorithms (BLAST, BLAT, BWA, BWA-SW, BWA-MEM, BFAST, Bowtie2, Novoalign, GSNAP, SHRiMP2, and STAR) that can be used to characterize DNA or RNA sequences from NGS data, and these tools have recently been evaluated for their accuracy at identifying divergent viral sequences (94). Researchers have surveyed viral metagenomic sequence data from bat specimens, an important reservoir for emerging pathogenic viruses, using BLAST analysis tools and have discovered several novel viruses (Table 2) that could potentially pose a zoonotic health threat (95–97). When considering the ability to sequence century-old genomes and to perform comprehensive molecular surveillance with DNA sequencing data, NGS provides opportunities for advancing diagnostic microbiology in the era of genomics and metagenomics.

ASSESSING THE ROLE OF PATHOGENICITY

A broad range of microbial diversity is being uncovered, and many more microbes remain to be discovered. The pace at which new microbes (and viruses in particular) in clinical specimens are being discovered is growing exponentially. The challenge that faces the scientific community is defining the relevance of the growing list of new microbes to human infections. In 1890, Robert Koch published a set of postulates in an attempt to standardize the evidence needed to demonstrate a causal role for a microbe in a disease (98). Koch's postulates are well known to this day and, despite being more than 100 years old, still serve as guidelines for proof of causality. They are as follows.

1. The parasite occurs in every case of the disease in question and under circumstances which can account for the pathological chances and clinical course of the disease.
2. The parasite occurs in no other disease as a fortuitous and nonpathogenic parasite.
3. After being fully isolated from the body and repeatedly grown in pure culture, the parasite can induce disease anew.

A major challenge in the fulfillment of Koch's postulates, especially in a molecular era, is that many microbes cannot be grown in pure culture. Another limitation is that microbes that have either a carrier state or can cause subclinical infections, such as *Neisseria meningitidis* and *M. tuberculosis*, violate Koch's postulates. Other scenarios that limit the applicability of Koch's postulates include cases in which coinfection with more than one microbe causes disease or situations in which the host genetic background contributes to the disease state.

Koch's postulates have been reconsidered in various publications. Bradford Hill (99) and Alfred Evans (100) proposed broader criteria for causation, including epidemiological and immunological data. Most recently, a guide for disease causality that accounts for molecular methods of microbial detection has been proposed by Fredericks and Relman (101). These revisions of Koch's postulates have remained focused on the traditional concept that disease arises from the presence of a foreign microbe (and the biological consequences of its presence). However, in the genomics era, the concept of a "pathogen" and how it causes disease must be reimagined in the 21st century. With the advent of the Human Microbiome Project (89), we have learned about the intricate relationships between human hosts and the complex microbial communities that reside within them. The human intestinal microbiome contains sets of genes encoding core microbial functions and stable microbial communities contributing to the function and integrity of the gastrointestinal tract. Perturbations of the composition of microbial communities, known as dysbiosis, may result in increased susceptibility to infectious diseases (102). With this observation, it is possible that some "diseased" states may result from disruptions in the microbiome. The pathogenesis of a particular disease may involve more than just the presence of a pathogenic microbial species, and may include intrakingdom interactions between microbes and interkingdom interactions between microbes and host. Scientists have begun to elucidate the complex role of microbial communities in several disease states such as obesity (103), inflammatory bowel disease (104), autoimmunity (105), and atopy (106) by comparative analysis of the microbiomes of healthy and diseased populations.

With the advancement of NGS technologies and the large amounts of data generated by each sequencing run, careful consideration must be taken before reporting a microorganism as the cause of infection in a particular case. When analyzing data from WGS or metagenomic studies, the presence of genetic elements associated with virulence does not identify a microorganism as an etiologic agent in an infection. To determine the pathogenicity of an organism or virulence factors identified by metagenomic analyses, several important factors such as site of specimen collection, host status, and clinical presentation must be carefully examined (107, 108).

CONCLUSIONS

Since the era of the microbe hunters in the late 19th and early 20th centuries, mankind has been interested in the discovery and eradication of human pathogens. Traditional microbiologic techniques that relied substantially on laboratory cultivation have been supplanted in part by molecular methods including nucleic acid amplification and sequencing. Advances in microarrays and nucleic acid sequencing culminated in many discoveries of previously uncultured pathogens. Microbial genomic sequencing has yielded a refined understanding of molecular epidemiology of various pathogens, and has assisted with the refined stratification of viral and bacterial pathogens. NGS strategies have been applied to diagnosis of infections in real time in the clinical laboratory (72, 109), and such applications are expected to grow, especially in reference and public health laboratory settings (110). The ability to rapidly scan complex collections of microbial and viral genetic information for compositional and functional information will continue to profoundly impact our understanding of pathogenesis and the development of new diagnostic tests in this era of metagenomic medicine.

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Taxonomy and Classification of Bacteria

PETER A. R. VANDAMME

17

> A hitherto undetected similarity exists between Lewis Carroll's Alice and taxonomists, and bacterial taxonomists in particular. Alice lived (or dreamed) in a world of fantasy in which the eating or drinking of various delicacies gave her the ability to magnify or minimize characters, and she not only upset preconceived ideas of relationships in size, but also disturbed the significance or importance attached to hereditary factors (such as monarchy) and the normal (as distinct from the decapitate) state of the whole organism. Taxonomists also seem to do these things, though they do not need to bite mushrooms or drink magical concoctions in the laboratory. Like the people of Wonderland they tend to be argumentative, and one taxonomist seldom sees a problem through the same spectacles (whether rose tinted or not) as his colleagues. Again like Alice, the taxonomist makes an individual approach to the world around him, and while each is extremely conservative in his views about bacteria, he is by inclination a radical if not an anarchist, and an opportunist if not a dreamer.

With these words, S. T. Cowan introduced a sparkling essay on bacterial taxonomy in 1970 (1). His contributions to the practice of bacterial taxonomy, written in the 1960s and 1970s (1–3), should be read by everyone interested in this field, also now that genomics has moved the species concept from obscurity into the spotlight of microbiology's highest-impact journals (4–7). "Taxonomy" is generally considered a synonym of "systematic" and is traditionally divided into classification (the orderly arrangement of organisms into taxonomic groups on the basis of similarity), nomenclature (the labeling of the units), and identification (the process of determining whether an unknown belongs to one of the units defined). It is generally accepted that bacterial classification should reflect as much as possible the natural relationships among bacteria. For a long time, these were considered the phylogenetic relationships as encoded in highly conserved macromolecules such as 16S or 23S rRNA genes (8), but nowadays, whole-genome comparisons offer new and exciting opportunities for the study of these natural relationships. Boundaries are nevertheless made by humans, and every classification of bacteria is artificial. Classification serves very practical purposes, i.e., recognition of organisms that were encountered previously and categorization of new ones into a logical and tractable system. In this era of whole-genome sequence analysis, it is more than ever obvious that the genomes of microbes undergo change, sometimes considerably. Although the extent of lateral gene transfer (LGT) is controversial, it does not alter our need to identify organisms, particularly in the context of epidemiological studies and surveillance, as identification yields a tremendous amount of accompanying information. Science indeed has a way of making itself useful, and the useful application of classification is identification (2).

CLASSIFICATION OF BACTERIA

Early classification systems used mainly morphological and biochemical criteria to delineate the species of bacteria. This type of classification was monothetic, as it was based on a unique set of characteristics necessary and sufficient to delineate groups. These early classification systems were replaced by theories of so-called natural systems, which were the phenetic and phylogenetic classifications. In the former, relationships between bacteria were based on the overall similarity of both phenotypic and genotypic characteristics. Phenetic classifications demonstrate the relationships among organisms, as they exist without reference to ancestry or evolution. In phylogenetic classifications, relationships are described by ancestry, not according to their present properties.

Special-purpose and general-purpose classification systems are the main categories of classification systems. Special-purpose classification systems are objectively determined and do not fit a preconceived idea. For instance, the separation between the very closely related species *Escherichia coli* and *Shigella dysenteriae* or between *Bordetella pertussis* and *Bordetella bronchiseptica* does not conform to the general ideas of present-day species delineation but fits primarily a practical and historical purpose. Yet, nowadays, most taxonomists favor a general-purpose classification system that is stable, objective, and predictive and that can be applied to all bacteria. The classifications obtained with a general-purpose classification system do not fit a single purpose but attempt to reflect the natural diversity among bacteria. The best way to generate such general-purpose classifications is by combining the strengths of both phenetic and phylogenetic studies, a practice nowadays often referred to as polyphasic taxonomy (9).

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Criteria for Species Delineation

The criteria used to delineate species have developed in parallel with technology. The early classifications were based on morphology and biochemical data. When evaluated by means of our present views, many of these early phenotype-based classifications generated extremely heterogeneous assemblages of bacteria. Individual species were characterized by a common set of phenotypic characters and differed from other species in one or a few characters that were considered important. The introduction of computer technology allowed comparison of large sets of characteristics for large numbers of strains, forming the basis for phenetic taxonomy. Such numerical analyses of phenotypic characters yielded superior classifications in terms of objectivity and stability. Yet the principles and practices of numerical taxonomy, whereby taxa are classified from comparisons of large strain sets using large numbers of equally weighted features, have largely been abandoned (10). Gradually, chemotaxonomic and genotypic methods were introduced into classification systems. Numerous different chemical compounds were extracted from bacterial cells, and their suitability for use in the classification of bacteria and the definition of species has been analyzed (11).

In 1987, the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics (12) stated that taxonomy should be determined phylogenetically and that the complete genome sequence should therefore be the standard for species delineation. Whole-genome DNA-DNA hybridization analysis was the best approach to the sequence standard for several decades and represented the best applicable procedure. A bacterial species was defined as a group of strains, including the type strain, that share 70% or greater DNA-DNA relatedness, with a ΔT_m of 5°C or less (T_m is the melting temperature of the hybrid, as determined by stepwise denaturation; ΔT_m is the difference in T_m [in degrees Celsius] between the homologous hybrid and the heterologous hybrid formed under standard conditions) (12). This species definition was based on a large amount of empirical data, including both DNA-DNA hybridization data and other characteristics. The designated type strain of a species serves as the name bearer of the species and as the reference specimen. It was also recommended that phenotypic and chemotaxonomic features should agree with this definition. Preferentially, several simple and straightforward tests should endorse the species delineation based on DNA-DNA hybridization values. Groups of strains that were delineated by means of DNA-DNA hybridization studies as distinct species but that could not be distinguished by phenotypic characteristics should not be named.

The Polyphasic Species Concept

A wide variety of cellular components have been used to study relationships among bacteria and to design classifications (13). The information present at the DNA level has been analyzed by estimations of the DNA base composition and the genome size, whole-genome DNA-DNA hybridization, restriction enzyme analysis, and, increasingly more often, direct sequence analysis of various genes. rRNA fractions have been studied intensively, particularly because they serve as phylogenetic markers. Various chemical compounds, including fatty acids, mycolic acids, polar lipids, polysaccharides, sugars, polyamines, and respiratory quinones, as well as, again, a tremendous number of expressed features (data derived from, e.g., morphologic, serologic, and enzymologic studies), were all used to characterize bacteria (11, 14). Several of these approaches have been applied to taxonomic analyses of virtually all bacteria. Others were performed with a limited number of organisms because they are laborious, time-consuming, or technically demanding or because they were relevant only for a particular group.

The term "polyphasic taxonomy" was coined by Colwell (15) in 1970 and described the integration of all available genotypic, phenotypic, and phylogenetic information into a consensus type of general-purpose classification. It departs from the assumption that the overall biological diversity cannot be encoded in a single molecule and that the variability of characters is group dependent; it integrates several generally accepted ideas for the classification and reclassification of bacteria. Polyphasic taxonomy is phylogeny based and uses sequence analysis and signature features of rRNA for the deduction of a phylogenetic framework for the classification of bacteria (5, 9). The next step in the process of classification is the delineation of individual species—and other taxa—within these phylogenetic branches. Despite its drawbacks, DNA-DNA hybridization still forms the cornerstone of species delineation. However, the threshold value for species delineation should be allowed variation. This polyphasic approach is pragmatic; for instance, *B. pertussis*, *Bordetella parapertussis*, and *B. bronchiseptica*, which share DNA-DNA hybridization levels of >80%, are considered three distinct species because they differ in many phenotypic and chemotaxonomic aspects (16). It is essential that the boundaries of species demarcation be flexible in order to achieve a classification scheme that facilitates identification.

The application of numerous other types of analyses of genotypic, chemotaxonomic, and phenotypic characteristics of bacteria to the delineation of bacteria at various hierarchical levels represents the third component of polyphasic taxonomy (9). The goal is to collect as much information as possible and to evaluate all results in relation to each other in order to draw useful conclusions. An additional advantage is that once the taxonomic resolution of these approaches has been established for a particular group of bacteria through the analysis of taxonomically well-characterized strains, they may be used as alternative tools to identify new isolates at different taxonomic levels. It should be noted that the resolution of these alternative methods is often group dependent. For instance, cellular fatty acid analysis is useful for the accurate identification of strains of many bacterial species to the species level. In certain bacterial groups, however, the cellular fatty acid profile may be indicative of the genus or a group of phylogenetically related genera but not of a particular species within one of these genera.

The contours of a polyphasic bacterial species are obviously less clear than the ones defined by Wayne et al. (12), and this lack of a rigid definition has been contested, as it allows too many interpretations (10, 17, 18). Polyphasic classification is empirical and contains elements from both phenetic and phylogenetic classifications. There are no strict rules or guidelines, and the approach integrates any significant information on the organisms, resulting in a consensus type of classification. In this respect, its main weakness is indeed that it relies on common sense to draw its conclusions. The bacterial species appears as a group of isolates in which a steady generation of genetic diversity resulted in clones characterized by a certain degree of phenotypic consistency, by a significant degree of DNA-DNA hybridization, and by a high level of 16S rRNA sequence similarity.

The species is the most important and, at the same time, the central element of bacterial taxonomy. There are at present no rules for the delineation of higher hierarchical ranks such as genus, family, and order. Although there is an expectation that at the generic level taxa should be supported by phenotypic descriptions (5, 10, 13), in practice, higher ranks are mostly delineated on the basis of 16S rRNA sequence comparison and stability analyses of the clusters that are obtained (19, 20). The latter has weakened the emphasis on phenotypic descriptions of taxa. Nevertheless, analyses of conserved signature indels and conserved signature proteins demonstrate that groups of prokaryotes ranging from phylum to genus levels can be recognized and support a tree-like vertical inheritance of the genes containing these molecular signatures that is consistent with phylogenetic trees (21).

Multilocus Sequence Analysis: A Short-Lived Alternative to DNA-DNA Hybridization Experiments?

In 2002, a new ad hoc committee for the reevaluation of the species definition in bacteriology made various recommendations in light of developments in methodologies available to systematists (22). One of the particularly interesting developments was multilocus sequence analysis (MLSA). In contrast with multilocus sequence typing (MLST), a specific tool designed for molecular epidemiology and for defining strains within named species, whereby similarities and differences are usually measured as differences in allelic profiles, MLSA employs phylogenetic procedures based on the nucleotide sequences of the alleles instead to reveal similarities between strains representing different species and genera (6, 23). Many examples of such studies have been published, and in general the clusters delineated correlated well with species demarcated by DNA-DNA hybridization experiments (6). It is, for instance, noteworthy that the DNA-DNA hybridization results that demonstrated that *Yersinia pestis* and *Yersinia pseudotuberculosis* represented a single species were mirrored in the MLSA tree, where *Y. pestis* clusters among *Y. pseudotuberculosis* strains; the same observation was made for *Burkholderia mallei* and *Burkholderia pseudomallei* (24, 25). There is no universal MLSA cutoff or descriptor of clusters that characterizes species, nor are ecological features consistently available to distinguish natural clusters that could be used to define species. Therefore, MLSA thresholds for species delineation must be validated through the analysis of taxonomically well-characterized reference strains (13) before this method can replace DNA-DNA hybridization experiments in the frame of polyphasic taxonomic studies (see, e.g., reference 26). Especially for depicting relationships within and between closely related species, this approach has a resolution superior to the traditional 16S rRNA gene sequence analysis. The deduced phylogenetic trees not only provide a phylogenetic backbone but also reveal intraspecies relationships at a level where comparative 16S rRNA sequence analysis is no longer discriminatory.

The number and lengths of gene fragments to be used in MLSA studies have not been systematically studied, although typically six to eight genes are analyzed (27). Housekeeping genes are preferentially used because they evolve relatively slowly and most of the variation that accumulates in these genes is considered selectively neutral. Sequence diversity, however, often precludes the development of primer sets that can be used for studying multiple genera or even species. Although very appealing for its resolution, portability, and throughput capacity, MLSA thus suffers from the difficulty of developing widely applicable schemes. As the costs of whole-genome sequencing continue to decline, it is conceivable that extracting MLSA sequences (or MLST alleles) from draft genome sequences will become more straightforward and affordable than performing traditional MLST or MLSA analyses (28).

Toward a Genomic Threshold for Species Definition

For several decades, bacterial taxonomists have considered whole-genome information the standard for determining taxonomy. The number of whole-genome sequences is increasing rapidly and allows assessment of genome-level variation within and between species. It has become clear that in addition to nucleotide substitutions, other genetic forces such as gene loss, gene duplication, chromosomal rearrangements, but also LGT shape the genome and that considerable fractions of the genome of any particular strain may be unique to that strain (29). One of the more significant recent discoveries in bacterial genomics is that bacterial species appear to comprise a set of core and accessory genes, with only the former present in all isolates of that species and with the sum of the two components forming the species pan-genome (30). The origin, composition, and size of bacterial pan-genomes and whether they are finite or infinite have been the source of debate (31).

In particular, the extent to which LGT occurs and its consequences for bacterial evolution, a biological species concept of bacteria, and eventually a bacterial species definition have been debated vigorously. Although recognized decades ago, LGT was long viewed as a minor phenomenon that did not jeopardize the general idea of vertical evolution as depicted in phylogenetic trees based on rRNA or other conserved genes. An overwhelming disruptive influence of LGT also did not correspond with daily diagnostic practices during which numerous bacterial isolates can be recognized as belonging to long-established species. However, MLSA and especially phylogenomics studies have shown that numerous genes have different evolutionary histories, and the ribosomal tree of life has been referred to as "the tree of one percent" (of all genes in microbial genomes) (32, 33). Microbial evolution seemed better modeled in the form of a dynamic network of evolution in which the nodes were bacterial and archaeal genomes and the edges were the fluxes of genetic information between the genomes (33). Yet there is now compelling evidence that a considerable set of genes share a highly significant phylogenetic signal that can be used to reconstruct bacterial phylogeny (34). The traditional tree of life can be replaced with a common and coherent phylogenetic trend of many genes, called the phylogenetic forest or the statistical tree of life (33, 34). Several studies of complete genomes have suggested universal sets of protein-coding genes that may be useful for a phylogenomic species delineation in microbial taxonomy, and genes that encode components of the translation system in particular show substantial congruency between each other and with the standard rRNA tree (35–39). It also became apparent that not only does the frequency of LGT vary dramatically among bacteria (an observation that was apparent more than a decade ago through numerous DNA fingerprinting studies) but that LGT should be considered a cohesive force in evolution rather than a disruptive force leading to a genetic melting pot (18, 40, 41), as the requirement for physical proximity, the homology dependence for successful recombination, and the fitness reduction in the recipient organism all favor recombination between closely related bacteria (40). Finally, the differential existence of an accessory genome is clearly compatible with LGT-driven environmental adaptation. In a study of the evolutionary origin of the genomic repertoires in *Gammaproteobacteria*, Lerat et al. (42) concluded that gene acquisition was very common based on the large number of genome- or clade-restricted gene families. However, beyond their initial acquisitions, few gene histories conflicted with the organismal tree. The picture emerged that bacterial lineages are constantly subjected to the input of new genes from a large available pool, which may be of bacteriophage origin, and that resident genes are continually lost. The diversity of gene families unique to single genomes indicates that the pool of available genes is very large, allowing the rate of gene acquisition to be both high for a genome and very low for a particular gene. A taxonomically particularly interesting study was published by Lefebure et al. (43), who analyzed 96 genome sequences derived from two closely related sympatric sister species of pathogenic bacteria, *Campylobacter coli* and *Campylobacter jejuni*. The results showed that both species had finite pan-genomes and that there are unique and cohesive features to each of their genomes defining their genomic identity. The two species have a similar pan-genome size; however, *C. coli* has acquired a larger core genome and each species has evolved a number of species-specific core genes, possibly reflecting different adaptive strategies, in spite of their occurrence in the same niche (the gastrointestinal tract of several hosts). Genome-wide assessment of the level of LGT within and between the two sister species, as well as within the core and noncore genes, demonstrated a resistance to interspecies recombination in the core genome of the two species and therefore provided persuasive support for the core genome hypothesis for bacterial species.

How can all of this information be used in a new species definition? Studies by Konstantinidis and Tiedje (29) and Goris et al. (44) revealed the average nucleotide identity (ANI) of the shared genes between two strains to be a robust means to compare genetic relatedness. ANI values of ~95% corresponded to the traditional 70% DNA-DNA hybridization standard of the current species definition, and several recently published descriptions of novel bacterial species have incorporated the ANI analysis of draft whole-genome sequences (a minimum of 20% of the randomly sequenced complete genome must be available) to replace DNA-DNA hybridization experiments (5, 45). At the 95% ANI cutoff, current species include only moderately homogeneous strains, apparently as a result of the strains having evolved in different ecological settings. A large fraction of the differences in gene content within species was associated with bacteriophage and transposase elements, revealing an important role of these elements during bacterial speciation. These findings were consistent with a definition for species that would include a more homogeneous set of strains than that provided by the current definition and one that considers the ecology of the strains in addition to their evolutionary distance (46). Goris et al. (44) also demonstrated that the 70% DNA-DNA reassociation threshold corresponded with 69%-conserved DNA, or, when the analysis was restricted to the protein-coding portion of the genome, 85%-conserved genes.

ANI values detect the DNA conservation of the core genome, whereas conserved DNA calculates the proportion of DNA shared by two genomes. Both estimates of intraspecies similarity therefore do not necessarily correlate, and for that reason, Deloger et al. (62) introduced the maximal unique match (MUM) index values, which take into account both criteria of diversity. MUM index values represent a calculation for genomic distances that is based on the number of maximal unique and exact matches of a given minimal length shared by the two genomes being compared. MUM index values correlate better with the ANI than with the DNA content and group strains in a way that is congruent with MLSA trees.

Major Groups of Bacteria

The tree of life, based on comparative small-subunit rRNA studies, comprises three lines of descent that are nowadays referred to as the domains *Bacteria*, *Archaea*, and *Eucarya* (8). There are currently 34 formally named phyla in the *Bacteria*, which are further subdivided into numerous taxa (47; http://www.ncbi.nlm.nih.gov/Taxonomy/tax.html/). However, a large number of uncultured bacteria represent additional phylogenetic lineages awaiting formal classification, and alternative phylogenetic classification systems (and thus alternative nomenclatures) have been proposed in which, for instance, more-comprehensive data sets, including multiple molecular sequences and cell envelope and ultrastructural characteristics, rather than reliance solely on rRNA sequence similarity, define the units of classification (48).

The present division into phyla is largely based on the classification proposed in the second edition of *Bergey's Manual of Systematic Bacteriology* (20). However, *Bergey's Manual of Systematic Bacteriology* is not an official document endorsed by the International Committee on Systematics of Prokaryotes, and the nomenclature and classification schemes presented are no more than recommendations by the editors and authors of that manual. There is no official or valid publication of classification schemes in bacterial taxonomy, and the International Committee governs only the rules of nomenclature of prokaryotes and matters relating to the Bacteriological Code (47). Recently, Gribaldo and Brochier-Armanet (49) called for a general discussion on the higher taxa of prokaryotes, to lay down common rules for the establishment of names for such taxa and to broaden the scope of the *International Code of Nomenclature of Prokaryotes* to include the higher taxa. In addition to 16S rRNA-based distinctiveness, the use of biological signatures, such as the occurrence of unique features in terms of key cellular processes identified by distinctive gene distributions and the availability of at least one cultivated representative and a complete genome sequence, was recommended to define phyla (49).

In the traditional (rRNA gene sequence-based) view of higher-order taxonomy and phylogeny, three phyla, the *Proteobacteria*, the *Firmicutes* (Gram-positive organisms with low G+C contents, including *Bacillus*, *Clostridium*, *Staphylococcus*, *Mycoplasma*, and the classical lactic acid bacteria, such as *Enterococcus*, *Streptococcus*, and *Lactobacillus*), and the *Actinobacteria* (Gram-positive organisms with high G+C contents, including *Bifidobacterium*, *Mycobacterium*, and *Corynebacterium*), comprise the large majority of clinically relevant species. The *Bacteroidetes* (*Bacteroides*, flavobacteria, and sphingobacteria), the *Spirochaetes* (spirochetes and leptospiras), and the *Chlamydiae* (chlamydias) represent some of the other phyla. A detailed overview is given by Garrity and Holt (20) in their introductory chapter to the second edition of *Bergey's Manual of Systematic Bacteriology*. That edition is structured in an order based on the topology of the 16S rRNA phylogenetic tree.

The largest phylum by far is the *Proteobacteria*, which contains five main clusters (classes) of genera that are referred to with the Greek letters alpha, beta, gamma, delta, and epsilon. More recently, two additional classes within the *Proteobacteria* have been reported, the "*Zetaproteobacteria*" (a name that has not been validly published and therefore is formally written between quotation marks) and the *Acidothiobacillia* (50, 51). The *Proteobacteria* comprise the majority of the known Gram-negative bacteria of medical, industrial, and agricultural significance. This phylum includes *Brucella*, *Ehrlichia*, and *Rickettsia* (*Alphaproteobacteria*); *Burkholderia*, *Bordetella*, and *Neisseria* (*Betaproteobacteria*); *Aeromonas*, *Legionella*, *Vibrio*, and the family *Enterobacteriaceae* (*Gammaproteobacteria*); and *Campylobacter* and *Helicobacter* (*Epsilonproteobacteria*). The *Deltaproteobacteria*, "*Zetaproteobacteria*," and *Acidothiobacillia* comprise a variety of mainly environmental bacteria that have little clinical relevance.

Uncultured Bacteria

The classification and nomenclature of uncultured bacteria that are only minimally characterized by morphological characteristics or by differences in molecular sequence are outstanding challenges in bacterial classification (52). A category that formally classifies incompletely described prokaryotes has been recognized (53). "*Candidatus*" is considered a taxonomic status for uncultured candidate species for which relatedness has been determined (for instance, for which phylogenetic relatedness has been determined by amplification and sequence analysis of prokaryotic RNA genes by use of universal prokaryotic primers) and whose authenticity has been verified by *in situ* probing or a similar technique for cell identification. In addition, it is also mandatory that information concerning phenotypic, metabolic, or physiological features be made available. The latter data may serve as a starting point for further investigation and eventual description and naming. With the advent of genomics, these original concerns may appear trivial now that we have the technical means to study large genomic fragments of uncultured microbes by shotgun cloning and sequencing of bulk DNA extracted from mixed communities (54, 55) or by DNA amplification from single cells (56), enabling sequencing from uncultured microorganisms from the environment (57). In particular, the metagenomics field has opened a fascinating new window for studying the uncultured microbial diversity in a range of ecosystems, including those of the human body (see chapter 15). Exploring the diversity of such ecosystems by comparative rRNA sequence analysis is based on identification of phylotypes, which are defined as groups of 16S rRNA gene sequences with a certain level of similarity. The cutoff values of rRNA sequence similarity that are used for phylotype definition are not consistent. In studies of the diversity of microbes in the human gastrointestinal tract, these values vary between 97 and 99% (58). The higher the cutoff value, the higher is the number of distinct phylotypes and thus estimated species richness. Regardless of the cutoff value used, the resulting diversity estimates should be considered rough indicators of the microbial diversity present, as bacteria with rRNA sequences that are 99% similar may still encompass multiple species and considerable ecological and genomic heterogeneity (6).

IDENTIFICATION

Identification is part of taxonomy. It is the process whereby an organism is recognized as belonging to a known taxon (species, genus, or higher taxonomic rank) and designated accordingly. It relies on a comparison of the characters of an unknown with those of established units in order to name it appropriately. This implies that identification depends on adequate characterization.

As part of identification strategies, dichotomous keys based on morphological and biochemical characteristics have only partly been replaced by other methods. Taxonomic studies provide an impressive array of alternative techniques derived from analytical biochemistry and molecular biology for examination of numerous cellular compounds (11, 13). Various identification approaches are discussed in chapters 4 through 6 of this *Manual*. Each of these techniques is useful for characterization and hence identification of bacteria. Databases of many types of biological data, including rRNA and other gene sequences, whole-cell fatty acid components, and matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectra, or miniaturized series of phenotypic characteristics may allow identification of many isolates. Yet the success of these databases also depends on the completeness of the databases, the exactness of the methods, and how carefully the individual entries have been delineated.

CLASSIFICATION AND IDENTIFICATION METHODS

In principle, all genotypic, phenotypic, and phylogenetic information can be used to classify bacteria. Genotypic information is derived from the nucleic acids present in the cell, whereas phenotypic information is derived from proteins and their functions, different chemotaxonomic markers, and a wide range of other expressed features. In the present polyphasic-species definition, a minimal taxonomic study consists of sequence analysis to determine the phylogenetic position of the unknown species (the 16S rRNA gene is most commonly used), DNA-DNA hybridizations to determine its precise level of relatedness toward its nearest phylogenetic neighbors, and biochemical characterization to distinguish the new taxon from the established ones. Typical for the process of polyphasic taxonomy is that information from other approaches is used to classify bacteria at different taxonomic levels. When working one's way through lists of methods, it is of primary interest to understand at which level these methods carry information and to realize their technical complexity, i.e., the amount of time and work required to analyze a certain number of isolates. The validation of a new classification or identification tool involves a determination of its taxonomic resolution by means of well-characterized reference strains. As a proof of concept, such validation studies mostly start with the analysis of type strains only, but because of the genotypic variability of bacterial species (both in gene content and in gene sequences), the true value of new classification or identification tools can appropriately be assessed only through the analysis of multiple well-characterized reference strains and subsequent validation using new isolates. The list of methods given below is not meant to be complete or to describe all of their aspects. It comprises the major categories of taxonomic techniques required to classify and identify bacteria and focuses on novel developments.

DNA-DNA Hybridization Studies

Although contested for its technical difficulties and for the inability to build cumulative databases, at present, DNA-DNA hybridization is still acknowledged as the reference method to establish relationships within and between species. Different DNA-DNA hybridization procedures have been described, both traditional and miniaturized versions (9, 59–61). In DNA-DNA hybridization studies, it is commonly unclear if hybridizations were performed under optimal, stringent, or suboptimal conditions. The stringency of the reaction is determined by the salt and formamide concentrations, by the temperature, and by the molar percentages of G+C of the DNAs used. DNA-DNA hybridizations performed under standard conditions are not necessarily optimal or stringent for all bacterial DNAs. As a standard, optimal conditions for hybridizations should be preferred because the optimal temperature curve for hybridization is rather broad (~5°C) (9).

Whole-Genome Sequence-Based Methods

The percentage of conserved DNA (pcDNA) method introduced by Goris et al. (44) and the MUM index of Deloger et al. (62) can be regarded as *in silico* imitations of DNA-DNA hybridization. Both similarity measures are based on finding subsequences that are conserved in the genomes under comparison. However, pcDNA traces inexact matches using a BLAST approach (63), whereas the MUM index uses the MUMmer software (62) to find all maximal unique exact matches (MUMs) shared by the genomes. MUMmer applies suffix trees as an index structure to speed up the search process. As a result, computing the MUM index is ~100 times faster than the pcDNA method (64). Both methods require complete or nearly complete genome sequences, as they are highly sensitive to large sequence stretches missing in draft genomes.

The ANI method (5, 44, 65) and core gene identity (CGI) method (66) are both natural extensions to MLSA in case complete or draft genome sequences are available. Whereas MLSA computes similarity values as the average similarity among a limited set of orthologous protein-coding genes shared among all organisms studied, ANI and CGI maximize the information content by taking all orthologous protein-coding genes of the genomes into consideration. The main difference between the approaches is that ANI computes a pairwise genome similarity based on all orthologous genes shared between two genomes, while CGI restricts the computation of pairwise genome similarity to orthologous genes shared by all genomes under study.

The present state of the art demonstrates that both pcDNA and MUM index parameters are equivalent (because they have the same taxonomic resolution) but superior (because they are sequence based and thus cumulative) approaches to the traditional DNA-DNA hybridizations for the species-level classification of bacteria. Similarly, the ANI and CGI parameters are equivalent but superior tools for studying their phylogeny. The latter techniques are superior compared with traditional 16S rRNA-based approaches because they are based on a much larger part of the genome and because they have a better resolution for discriminating closely related bacteria. Each of these methods is starting to be used in taxonomic studies of different bacteria (45, 64, 66, 67), and it can be anticipated that the practice of DNA-DNA hybridization experiments as the cornerstone for species delineation in taxonomic studies of bacteria will be abandoned fairly soon.

rRNA Studies

rRNA is the best single target for studying phylogenetic relationships because it is present in all bacteria, it is functionally constant, and it is composed of highly conserved as well as more variable domains (8). The components of the ribosome (rRNA and ribosomal proteins) have been the subjects of phylogenetic studies for several decades, and direct sequencing of partial or nearly entire 16S or 23S rRNA molecules has become common practice. The larger the conserved elements examined, the more information they bear and the more reliable the conclusions become. International databases comprising published and unpublished partial or complete sequences have been constructed (68) but have also accumulated poor-quality sequences and sequences that are not accurately or even not correctly labeled. For these and other reasons, several initiatives for providing the scientific community with curated 16S rRNA databases have been undertaken (e.g., see references 69–71). The "All-Species Living Tree" project (69; http://www.arb-silva.de/projects/living-tree/) aims to reconstruct a single 16S rRNA tree harboring all sequenced type strains of the hitherto classified species of *Archaea* and *Bacteria*. Sequences are selected manually due to a high error rate in the names and information fields provided for the publicly deposited entries. A most useful tool to track the identity of strains for which sequences are deposited is the StrainInfo bioportal (72; http://www.straininfo.net/), which brings together the biological material kept at multiple biological resource centers into a single portal interface, with direct pointers to the relevant information at the culture collections' websites. This information is automatically linked to related sequences in the public domain and refers to all known scientific publications that deal with the organism. To support the taxonomic depth of the information provided by the StrainInfo bioportal, all taxonomic names appearing in the bioportal are fully integrated with and linked out to key taxonomic information sources.

rRNA sequence analysis not only is used to determine relationships among genera, families, and other higher ranks but often replaces DNA-DNA hybridization studies for the delineation of species in taxonomic practice. Such application of rRNA similarity data may not be appropriate, as some genera, for instance, *Bacillus*, *Burkholderia*, *Corynebacterium*, *Mycobacterium*, and many others, comprise clusters of closely related species that exhibit nearly identical 16S rRNA gene sequences. Stackebrandt and Goebel (73) reported that organisms sharing >98.5% rRNA similarity may or may not belong to a single species and that the resolution of 16S rRNA sequence analysis for determination of the degree of relatedness between closely related organisms is generally low. There is no single threshold value of 16S rRNA similarity for species recognition (73). However, organisms with <97% 16S rRNA sequence similarity do not give a DNA-DNA reassociation level of >60%, no matter which DNA-DNA hybridization method is used. Subsequent studies revealed that for the majority of organisms, the 97% cutoff value could be raised to 98.7 to 99% (74). Nevertheless, other studies extended the observations on intraspecies 16S rRNA divergence considerably, as differences in 16S rRNA gene sequence of up to 4.5% were reported among strains of several species belonging to the *Epsilonproteobacteria* (75, 76).

In spite of its limitations, rRNA sequence analysis is commonly used for the identification of bacteria (77, 78), and commercial identification systems based on analysis of rRNA gene sequences are available (e.g., MicroSeq 500 16S rDNA Bacterial Sequencing Kit; Perkin-Elmer Applied Biosystems, Foster City, CA). A fraction of the 5′-terminal region of the 16S rRNA gene (positions 60 to 110 of the *E. coli* numbering system) is one of the most informative or discriminating regions for closely related organisms (19).

Comparison of 16S rRNA gene sequences in many bacterial genera will lead to correct identification to the species level, but it is equally true that many taxonomic studies have revealed that comparative rRNA sequence analysis is often not sensitive enough to identify strains to the species level. There is a lack of knowledge not only of the strain-to-strain variation within a species but also of the interoperon variation within a single strain. Therefore, concluding that an unidentified isolate belongs to a particular species because it shares a high percentage of its 16S rRNA gene sequence with particular species or concluding that it represents a novel species because it occupies a unique position in the phylogenetic tree supported by a high bootstrap value or because it shares only 97% of its 16S rRNA sequence with its closest neighbor is premature in the absence of appropriate complementary data. This is even more true for partial sequence data, as partial rRNA gene sequences carry only limited information about the molecule and different parts of the gene may carry information about different taxonomic levels.

The interesting taxonomic properties of rRNA or rRNA gene molecules have been exploited in many ways. Although highly conserved, the rRNA genes also consist of variable domains that are particularly useful for diagnostic purposes and for mixed-community analyses (79–82). New technologies, exploiting the universal characteristics of the rRNA genes and their potential for species identification, emerge regularly. A growing number of studies report on the use of pyrosequencing (Biotage, Uppsala, Sweden), which provides rapid, short-read sequencing of 30 bases to classify, identify, and subtype bacteria, yeasts, and fungi (see, e.g., references 83 and 84). Turenne et al. (85) used single-stranded conformation polymorphism analysis of PCR amplicons to distinguish between organisms, and Yang et al. (86) used high-resolution melt analysis to characterize PCR products generated from three hypervariable regions of the 16S rRNA gene of clinically relevant bacterial pathogens and concluded that it allowed highly specific species identification. Still other approaches combine the diagnostic potential of 16S rRNA genes with the speed and discriminatory power of mass spectrometric analyses (see below).

Sequence Analysis of Protein-Encoding Genes

As an alternative to 16S rRNA gene sequence analysis, numerous other macromolecules have been examined for their potential as microbiological clocks and their applications as identification tools. Among others, various ribosomal proteins (37, 87, 88), chaperonin (88), RNA polymerases (89), RecA (90), and manganese-dependent superoxide dismutase (91) were shown to be valuable molecular chronometers. These alternative macromolecules should be widely or universally distributed among bacteria, they should not be transmitted horizontally, and their molecular evolution rate should be comparable to or somewhat higher than that of 16S rRNA, which would render them more suitable for differentiation of closely related organisms.

Potential pitfalls of overreliance on a single phylogenetic marker are illustrated in the taxonomic studies of species of the *Streptococcus bovis* group. *Streptococcus infantarius* subsp. *coli* was reclassified as the novel species *Streptococcus lutetiensis*, and another group of streptococci was proposed as the novel species *Streptococcus pasteurianus*, primarily on the basis of manganese-dependent superoxide dismutase gene sequences (92), while subsequent studies demonstrated that neither *S. lutetiensis* nor *S. pasteurianus* represented novel species (93). As mentioned above, studies of complete genome sequences have suggested universal sets of protein-encoding genes (35–39), and the analysis of multiple protein-encoding genes (not necessarily the most conserved ones) buffers the distorting effects of horizontal gene transfer or recombination events (65).

Other Genotypic Methods for Bacterial Classification

A range of different genotypic techniques has been used to characterize bacteria at various taxonomic levels. The molar percentage of guanine plus cytosine (the DNA base ratio or percent G+C value) is one of the classical genotypic characteristics and is part of the standard description of bacterial taxa. Generally, the range in G+C content observed among the strains of a species should not be >3%, and among the species of a genus it should not be >10%. In general, in the bacterial world the G+C content varies between 24 and 76%.

A tremendous number of molecular diagnostic methods, most of which are PCR based, have been developed. Most of these generate arrays of DNA fragments that are separated and detected in various ways, and appropriate software has been developed for pattern recognition and analysis and for database construction. One of these DNA fingerprinting methods, amplified fragment length polymorphism analysis (94), is useful for the classification of strains at the species and genus levels. The basic principle of amplified fragment length polymorphism analysis is restriction fragment length polymorphism analysis, modified by using PCR-mediated amplification to select particular DNA fragments from the pool of restriction fragments. This selective amplification process results in an array of about 30 to 40 DNA fragments, some of which are species (or even genus) specific, while others are strain specific (95). PCR-based typing methods that use random or repetitive elements as primers have been applied to strain characterization of a wide variety of bacteria (96–98). Although primarily applied for infraspecies strain comparisons, these techniques are useful in classification as well.

Phenotypic Methods

Phenotypic methods comprise all those that are not directed toward DNA or RNA and therefore also include the chemical or chemotaxonomic techniques. The classical phenotypic tests traditionally constituted the basis for the formal description of bacterial species, subspecies, genera, and families. While genotypic data are used to allocate taxa to a phylogenetic tree and to draw the major borderlines in classification systems, phenotypic consistency is required to generate useful classification systems and may therefore influence the depth of a hierarchical line (12, 22). The paucity or variability of phenotypic characteristics for certain bacterial groups regularly causes problems in describing or differentiating taxa. For such bacteria, alternative chemotaxonomic or genotypic methods are required to reliably characterize strains.

The classical phenotypic characteristics of bacteria comprise morphological, physiological, and biochemical features. Individually, many of these characteristics are poor parameters for genetic relatedness, yet as a whole, they provide descriptive information for the recognition of taxa. The morphology of a bacterium comprises both cellular (shape; the presence of an endospore, flagella, and inclusion bodies; and Gram staining characteristics) and colonial (color, dimensions, and form) characteristics. The physiological and biochemical features comprise data on growth at different temperatures; growth in the presence of different pH values, salt concentrations, or atmospheric conditions; growth in the presence of various substances, such as antimicrobial agents; and the presence or activities of various enzymes and utilization of compounds, etc. Very often, highly standardized procedures are required to obtain reproducible results within and between laboratories.

Phenotypic data were the first to be analyzed by means of computer-assisted numerical comparison. In the 1950s, numerical taxonomy arose in parallel with the development of computers (99) and allowed comparison of large numbers of phenotypic traits for large numbers of strains. Data matrices showing the degree of similarity between each pair of strains and cluster analyses resulting in dendrograms revealed a general picture of the phenotypic consistency of a particular group of strains. Because such large numbers of characteristics reflect a considerable amount of genotypic information, it soon became evident that numerical analysis of large numbers of phenotypic characteristics was indeed taxonomically relevant.

In taxonomic practice, phenotypic characterization became compromised and sometimes more of a burden than a useful taxonomic activity. Frequently, phenotypic data are compared with literature data obtained using other conditions or methods. The need for continued phenotypic characterization at every taxonomic level not only to delineate taxa and appreciate their phenotypic coherence but also to evaluate their physiological and ecological functions cannot be denied. A minimal phenotypic description is not only the identity card of a taxon but also a key to its biology. Although accepted as necessary, differential phenotypic characters are often hard to find with a reasonable amount of effort and time.

Chemical Methods

The term “chemotaxonomy” refers to the application of analytical methods to the collection of information on various chemical constituents of the cell to classify bacteria. As with the other phenotypic and the genotypic techniques, some of the chemotaxonomic methods have been widely applied to vast numbers of bacteria, whereas others were so specific that their application was restricted to particular taxa. The markers studied include whole-cell protein profiles, isoprenoid quinones, cytochromes, peptidoglycans, polyamines, polar lipids, pigments, particular enzymes, sterols, and hopanoids (11). Very often, analytical difficulties have been the main restrictions to their wide-scale application.

Cell Wall Composition

The distinction between Gram-negative and Gram-positive types of bacteria is still one of the characteristics that are first analyzed in order to guide subsequent characterization and identification steps. The determination of the cell wall composition has traditionally been important for Gram-positive bacteria. The peptidoglycan type of cell wall of Gram-negative bacteria is rather uniform and provides little information. The cell walls of Gram-positive bacteria, in contrast, contain various peptidoglycan types that may be genus or species specific (100). The most valuable information is derived from the type and composition of the peptide cross-link between adjacent chains in the polymer network. A variable that received little attention is the degree of N and O acetylation of the amino sugars of the glycan chain. The analytical procedure is time-consuming, although a rapid screening method has been proposed. Membrane-bound teichoic acid is present in all Gram-positive species, but cell wall-bound teichoic acid is present in only some Gram-positive species. Teichoic acids can easily be extracted and purified and can be analyzed by gas-liquid chromatography (101).

Cellular Fatty Acid Analysis

More than 300 fatty acids and related compounds are present in bacterial cells. Polar lipids are the constituents of the lipid bilayer of bacterial membranes and have been frequently studied for classification and identification purposes. Other types of lipids, such as sphingophospholipids, occur in only a restricted number of taxa and were shown to have taxonomic value within these groups (11). Variations in chain lengths, double-bond positions, and substituent groups are very useful for the characterization of bacterial taxa (102). Mostly, the total cellular fatty acid fraction is extracted, but particular fractions, such as the polar lipids, have also been analyzed. The cellular fatty acid methyl ester composition is a stable parameter provided that highly standardized culture conditions are used. The methylated fatty acids are typically separated by gas-liquid chromatography, and both the occurrence and the relative amounts of methylated fatty acids characterize bacterial fatty acid profiles.

MALDI-TOF and Other Mass Spectrometric Methods

The first reports involving the use of MALDI-TOF mass spectrometry (MS) were published in the late 1980s, and its application has increased exponentially. By now, this methodology and its many applications have revolutionized the routine practices in clinical microbiology. In MALDI-TOF MS, the sample is mixed with a matrix that is chosen such that it specifically absorbs a laser beam. The resulting high-energy impact is followed by the formation of ions that are extracted through an electric field and that are subsequently focused and detected as an m/z (mass/charge) spectrum. Typically, high-abundance peptides, like those derived from ribosomal protein fractions, that are of low mass and ionize readily are observed in the spectra (103). The simplicity and speed of analysis represent part of its strength, and the whole process can be highly automatized. These features make the approach particularly attractive to research laboratories that routinely deal with the analysis and identification of large numbers of bacterial isolates. In microbiology, MALDI-TOF MS is primarily being used for species-level identification of various bacteria and fungi, but its potential for infraspecific typing of bacteria is being explored (103, 104). The technique is nowadays used for an increasing range of applications, including the analysis of mixed cultures, the differentiation between antimicrobial-resistant and -susceptible strains, direct identification of bacteria and yeasts in clinical specimens, and the rapid grouping of bacterial species in large collections of isolates (105).

The potential of MALDI-TOF MS for bacterial identification has also been used in a number of alternative ways. These include the fast and accurate differentiation of PCR products according to their lengths and rapid analysis of PCR products and restriction fragment length polymorphism patterns of microbial samples for size determination of double-stranded amplicons and restriction fragments (106, 107). Because of limitations of these approaches by length heterogeneities of specific marker genes that diminish their discriminatory power, von Wintzingerode et al. (108)

combined base-specific cleavage of amplified 16S rRNA genes with MALDI-TOF MS. In this process, 16S rRNA gene signature sequences are amplified in the presence of dUTP instead of dTTP, followed by strand separation and uracil-DNA-glycosylase-mediated cleavage at each T-specific site. Fragment pattern detection was performed by MALDI-TOF MS and proved useful for the identification of several bacteria, including *Bordetella* and *Mycobacterium* strains (108, 109).

Other studies report on the use of surface-enhanced laser desorption ionization-TOF MS for the identification of bacteria (110–112). Surface-enhanced laser desorption ionization is distinguished from MALDI in its use of an active sample probe—the ProteinChip array—that has an adsorptive surface that allows bacterial lysates to be subjected, without prior treatments, to on-chip sample preparation steps, such as selective washing and desalting. This procedure minimizes sample losses, while speeding up and simplifying sample preparation, compared to the standard methods normally employed prior to the use of MALDI. Furthermore, the active capture of the proteins by the protein chip array ensures nondiscriminatory binding of target proteins, which in turn improves the reproducibility and allows both peak mass-to-charge ratios and intensity to be used in sample characterization.

Finally, the Ibis T5000 biosensor technology (Bruker Daltonics Inc., Billerica, MA) uses broad-range PCR primers that target conserved regions of bacterial genomes, such as ribosomal sequences and conserved elements from essential protein-coding genes (i.e., housekeeping genes), and is designed to rapidly detect and identify a variety of pathogens without prior knowledge of the pathogen's nucleic acid sequence (113). The use of such broad-range priming targets across the widest possible grouping of organisms enables amplification of most species within a group. The T5000 biosensor uses electrospray ionization MS to analyze the products of broad-range PCR, which allows for the precise determination of the molecular mass of the PCR products. These high-precision mass measurements are then used to unambiguously derive base compositions of the PCR products, which are compared to a database for identifying the organism. This technology allows for a multilocus identification of bacteria in the samples with significantly less time and effort than sequencing and performs well with samples from a variety of clinical and environmental matrices, including blood, serum, various tissues, and even mosquito homogenates (114).

FTIR Spectroscopy

Fourier transform infrared (FTIR) spectroscopy is used for the identification of substances in chemical analyses. In general, the wave number, the reciprocal of the wavelength, is used as the physical unit. FTIR spectroscopy involves the observation of vibrations of molecules that are excited by an infrared beam. Molecules are able to absorb the energy of distinct light quanta and start a rocking or rotation movement. An infrared spectrum represents a fingerprint that is characteristic for any chemical substance. The composition of biological material and, thus, of its FTIR spectrum is exceedingly complex, representing a characteristic fingerprint. Naumann and coworkers suggested identifying microorganisms by FTIR spectroscopy (115). In principle, a reference spectrum library is assembled based on well-characterized strains and species. The FTIR spectrum of any unidentified isolate is then measured under the same conditions as those used for the reference spectra and is compared to spectra in the reference spectrum library. FTIR spectroscopy is now used for the identification and typing of a growing number of bacteria and yeasts (116–119).

Conclusions

The scientifically and economically ideal identification technique remains beyond reach. Cowan's (2) intuitive approach (which is used when the identity of the unknown is anticipated) and the stepwise method (which involves the use of dichotomous keys) suffice for numerous isolates and require only simple, rapid, and inexpensive biochemical tests. Cowan's views are easily adapted to modern methodology. If this first-line approach fails, alternative procedures are required and available. For several reasons, including the comprehensiveness of the public databases, complete 16S rRNA gene sequence analysis is the most straightforward and obvious choice for establishing a rough identity of an isolate, although it often fails to differentiate closely related species. Much of its superiority is based on its robust capacity to reveal the phylogenetic neighborhood of the organism studied, which is information not provided by any of the other current identification protocols. This information will direct the additional analyses required for final identification to the species level. Accurate species-level identification is thus very often a two-step process in which an unknown is first assigned to a particular group, after which it can be accurately identified at the species level. The former can be achieved through sequence analysis of 16S rRNA genes using near-universal primers, the latter through an appropriate selection of housekeeping genes and specific primers once the tentative identity of an unknown is established.

NOMENCLATURE

> Nomenclature is the supreme generator of heat, bad temper and ill-will among taxonomists and every kind of microbiologist; the reason is that in matters of nomenclature we are all conservative. We hate change (3).

Valid Publication of Bacterial Names

The *International Code of Nomenclature of Bacteria* (120) includes rules on how to name bacteria at different taxonomic ranks. The aim of nomenclature is to ensure that an organism is tagged with a unique name that carries valuable information. Prior to 1980, a proposal of a new bacterial taxon could be validly published in any microbiological book or journal, and the authors of the relevant sections of the successive editions of *Bergey's Manual of Determinative Bacteriology* had to attempt to give a complete list of the members of any particular genus or group of genera. The unavailability of type strains and the fact that microbiologists from different disciplines were not always familiar with one another's work caused great difficulty. All too often a worker would discover several years later that "his" or "her" organism had in fact been described earlier under a different name.

To overcome such problems and others, 1 January 1980 was chosen as a new starting date for bacterial nomenclature. At that time, the Approved Lists of Bacterial Names were published on behalf of the Judicial Commission of the International Committee on Systematic Bacteriology (121). Only those names included on these lists had standing in bacterial nomenclature, and names of taxa were to be included only if they were adequately described and if a type strain was available. From then onwards, all new names were validly published only in the *International Journal of Systematic Bacteriology* (now the *International Journal of Systematic and Evolutionary Microbiology*). Names can effectively be published in other journals and then validated subsequently by announcement in the Validation Lists in the *International Journal of Systematic and Evolutionary Microbiology*. Complete overviews of validly published names can easily be obtained through Internet sites such as http://www.bacterio.net/. One of the conditions for valid publication of names is that type strains of novel species must be deposited in two public culture collections in different countries. In case different names for the same organism are validly published, nomenclatural priority goes to the name that was validated first. As a result of this practice, all validly named species in any particular group can easily be traced and reference strains are available.

Why Do Names Change?

There have been more important causes for the modification of bacterial names than the occasional detection of synonymy. As described above, our present view on bacterial classification is phylogeny based. With the advent of rRNA-DNA hybridization in the 1970s and subsequently of rRNA gene sequencing, taxonomists had a new framework in which they could revise classification schemes. The classical—and extreme—example is the revision of the taxonomy and nomenclature of the genus *Pseudomonas*, which has been proceeding painstakingly slowly over several decades. The most important reason for this slow progress is that, through the work of De Vos and De Ley (122), it became clear not only that the genus *Pseudomonas* consisted of five major species clusters but also that these clusters formed a polyphyletic part of a major group of bacteria now known as the *Proteobacteria*. Revision of the taxonomy of the pseudomonads had to consider the relationships of the various subbranches toward their numerous respective neighbors (123).

The modification of our view on classification has been by far the most important reason for name changes. However, various forms of poor taxonomic practice, like the erroneous reliance on 16S rRNA similarity data rather than the level of DNA-DNA hybridization (76) or the analysis of some, rather than all, relevant species (124), also invoke a lot of changes, and hence irritation. An important reason for name changes is the lack of criteria for genus delineation. For instance, immediately after the original description of the genus *Burkholderia* (124), the same group of authors reclassified two of its species (*B. solanacearum* and *B. pickettii*) into the new genus *Ralstonia* (125). At present, the genus *Burkholderia* contains >70 validly named species, many of which have been isolated from soil and water samples (see chapter 43). A large phylogenetic cluster of soilborne and plant-associated *Burkholderia* species is considered beneficial to plants, but their classification in the genus *Burkholderia* hampers their exploitation as biological control or plant growth promotion agents. Although several of the opportunistic pathogens of the *Burkholderia* genus also have plant-beneficial properties, the phylogenetic distinctiveness of the misleadingly called "plant-beneficial group" has been used to argue for splitting the *Burkholderia* genus (126, 127).

Also, the intrinsically inadequate descriptions of species that comprise only a single isolate, the so-called one-strain taxa (species [or genera] that are proposed on the basis of data for only one strain), have probably caused more problems than they have solved, and this is definitely the case in the context of diagnostic microbiology. It is not possible to estimate the variability of the phenotype in the case of a species with one strain or in the case of a genus with one species and one strain, for which many recent examples exist. In diagnostic microbiology, it is well known that a species is characterized by a certain degree of variability. This variability can be measured by both phenotypic and genotypic criteria and may be revealed by simple biochemical testing or sophisticated genomic-fingerprinting techniques. In the absence of sufficient strains for quantitation of the range of divergence within a species, it will be difficult or impossible to identify new isolates of this species without DNA-DNA hybridization experiments. A classification based on results obtained with a single strain cannot be stable. Indeed, already the detection of a second strain will inevitably necessitate revision of the original species description.

As a concluding remark, it should be mentioned that there is no "undo" function in bacterial nomenclature. A name that was validly published remains valid regardless of the number of modifications it undergoes thereafter. For instance, the changes of the name *Pseudomonas maltophilia* to *Xanthomonas maltophilia* and finally to *Stenotrophomonas maltophilia* (128) may be reasonable to some taxonomists, but the changes, particularly the most recent, have been refuted by many clinical microbiologists. As these three names were all proposed according to the rules of bacterial nomenclature, they were all validated, and the use of each of them is correct and valid.

CONCLUSIONS

There seems to be a clear and bright future for bacterial taxonomy in the genomics era. A much broader range of taxonomic studies of bacteria has gradually replaced the former reliance on morphological, physiological, and biochemical characterization. This polyphasic taxonomy takes into account all available phenotypic and genotypic information and integrates it in a consensus type of classification, framed in a general phylogeny derived from 16S rRNA gene sequence analysis. The bacterial species appears as a group of isolates that originated from a common ancestor population in which a steady generation of genetic diversity resulted in clones that had different degrees of recombination and that were characterized by a certain degree of phenotypic consistency, a significant degree of DNA-DNA hybridization, and a high degree of 16S rRNA gene sequence similarity (9). Whole-genome sequences will make the practice of polyphasic taxonomy even more complete, and several genomic parameters like pcDNA, MUM index, and ANI values are ready to replace DNA-DNA hybridization as the cornerstone of species description.

While the old DNA-DNA hybridization species threshold is being replaced by MLSA or ANI value-based thresholds (5, 13, 23, 44), a fair reappraisal of bacterial taxonomy and the species definition will require more than a mere methodological translation of threshold levels. Several considerations should be made. First, whole-genome sequence studies confirm that in the space of microbial diversity many cores can be distinguished (see, e.g., reference 43), and these cores very often correspond with established species that are phenotypically but not necessarily ecologically coherent. It would be wrong to let the discussion be steered by an attempt to hold on to species boundaries defined by DNA-DNA hybridization experiments. A new discussion on the bacterial species concept, directed by insights in genome evolution and metagenomics but also by practical concerns (classification should remain practical and facilitate identification), is badly needed (18, 33, 129) and should yield whole-genome sequence-based thresholds, irrespective of their degree of correlation with DNA-DNA hybridization thresholds.

A second key point in the discussion of a modern bacterial species definition was raised by Sutcliff et al. (10), who called for a significant reappraisal of the procedures used to describe novel prokaryotic taxa, including the introduction of new publication formats. They rightly pointed out that although progress in the description of new microbial taxa is being made at accelerating rates, there is an enormous backlog of work and conservative estimates suggest that the presently described bacterial species (<10,000) may represent significantly less than 0.1% of the existing microbial diversity (130). Thus, even if progress in describing new taxa can be accelerated considerably over current rates, the challenge of adequately describing prokaryotic diversity could take several centuries (10, 130)! Consequently, a key question for the systematics community is to consider how we should continue to describe new prokaryotic taxa if we want to uncover more than the tip of the iceberg in the next few decades. Conflicting with this need to dramatically change our throughput capacity to describe new prokaryotic taxa, comprehensive guidelines for the description of novel species have been published by leading scientists of the systematics community (13). In light of the vast unreported microbial diversity, this is counterproductive, and one must reassess how much characterization is enough (10). Other practical considerations in this respect are that the number of bacterial taxonomists in Western countries is in sharp decline and that the extent of new species descriptions is primarily constrained by journal capacities rather than taxonomists' activities (130). A revitalization of the practice of prokaryotic systematics is indeed badly needed (10, 130)!

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Specimen Collection, Transport, and Processing: Bacteriology*

ELLEN JO BARON

18

One of the key principles of good specimen collection is to avoid introduction of colonizing bacteria surrounding the site of infection or on the skin or mucous membranes near the infectious site. This is done by disinfecting the surface skin and aspirating material, by using sheathed collection devices, or by collecting samples during surgical procedures. For sites naturally colonized by more than one species, selective methods are used to detect specific pathogens and prevent contamination with nonpathogenic commensals. More detailed information about sample collection and handling can be found in the Infectious Diseases Society of America (IDSA) and American Society for Microbiology (ASM) guideline document (1).

Specimens for bacteriologic culture should be collected as soon as possible after the onset of disease and before the initiation of antimicrobial therapy or as soon after the start of therapy as possible. A second specimen may be necessary because of poor specimen quality or inadequate transport conditions that affected the first specimen, but is otherwise rarely required for diagnosis of an acute infectious disease. Exceptions include the collection of multiple blood specimens for culture and those obtained for additional studies other than those originally requested.

Microbiologists must help caregivers to choose, collect, and transport the specimen in a manner that optimizes the laboratory's diagnostic testing activities. A common mechanism today is to provide a written or online laboratory specimen collection guide (2); for an example, see http://www.stanfordlab.com/pages/microbiology.htm.

GENERAL PRINCIPLES OF SPECIMEN COLLECTION AND TRANSPORT

The Specimen Must Represent the Infected Site

The choice of specimen to be collected for laboratory diagnosis is based on the site of the infection and the nature of the suspected bacteria. Table 1 lists anatomic sites with appropriate and inappropriate clinical specimens (3). General specimen selection and collection guidelines include proper labeling of the sample to include two patient identifiers, the source of the sample, and information on who collected the sample and at what time collection occurred.

Suspected Agent of Bioterrorism or Intentional Release of Biological Agent

Specimens from suspected acts of terrorism are important legally, and a chain of custody must be employed. See the ASM Laboratory Response Network Sentinel Level Clinical Laboratories website (http://www.asm.org/index.php/guidelines/sentinel-guidelines) and chapter 14 for more in-depth information.

Use of the Best Collection Method (Table 2)

Swabs

Swabs are appropriate when a large volume of sample is not necessary, such as from surface wounds (where the organism load is high) or oropharyngeal samples (where the presence of even one colony of *Streptococcus pyogenes* is clinically relevant) and where there will be a limited number of media to inoculate. Swabs should be collected carefully to avoid touching noninvolved surfaces or mucosae, which harbor contaminating bacteria, and they should be rolled or rubbed vigorously over the infected surface to maximize adsorption of the infecting agent.

Flocked swabs have been proven to be superior to fiber swabs for collection of nasopharyngeal samples for detection of respiratory viruses (4–6). In one evaluation using seeded samples, flocked swabs in a transport medium even preserved several anaerobic bacteria when refrigerated for up to 24 h (7). They have the advantage of being usable in automated inoculation instruments with potential improvement in culture yield (8).

Swabs may be transported dry (with desiccant to enhance survival) for recovery of *Corynebacterium diphtheriae* and *S. pyogenes* only (9, 10).

Swabs should not be used when multiple media must be inoculated, as the sample must be diluted to reach sufficient volume. Swabs are also not recommended for anaerobic cultures (Table 3), or for fungal cultures, because hyphal elements are not picked up. Swabs may be used to collect material from surface lesions for detection of mycobacteria, such as *Mycobacterium marinum*, but if there is fluid or tissue, that material should be obtained for culture, not a swab of

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*This chapter contains some information presented by Richard B. Thomson, Jr., in chapter 16 of the 10th edition of this *Manual*.

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TABLE 1 Selection of common clinical specimens for bacterial culture[a]

Anatomic site	Clinical specimen	
	Appropriate	Inappropriate
Lower respiratory tract	Freshly expectorated mucus and inflammatory cells (pus), sputum Bronchoalveolar lavage fluids Endotracheal aspirates	Saliva, oropharyngeal secretions, sinus drainage from nasopharynx; bronchial washes
Sinus	Secretions collected by direct sinus aspiration, or washes, curettage, and biopsy material collected during endoscopy	Nasal or nasopharyngeal swab, nasopharyngeal secretions, sputum, and saliva
Urinary tract	Midstream urine, urine collected by "straight" catheterization, urine collected by suprapubic aspiration, urine collected during cystoscopy or other surgical procedure	Urine from Foley catheter collection bag, "bagged" urine from infants
Superficial wound	Aspirations of pus or local irrigation fluid (nonbacteriostatic saline), swab of purulence originating from beneath the dermis	Swab of surface material or specimen contaminated with surface material, irrigation with saline-containing preservative
Deep wound	Purulence, necrosis, or tissue from deep subcutaneous site	Specimen contaminated with surface material
Gastrointestinal tract	Freshly passed stool, washes, or feces collected during endoscopy; rectal swab (in selected cases)	Rectal swab, specimen for bacterial culture if diarrhea developed after patient in hospital for >3 days
Venous blood	Two to four blood specimens collected from separate venipunctures before initiation of antibiotics, each containing ~20 ml of blood for patients >90 lb (see Table 9 for pediatric volumes); antisepsis with iodine-containing compound or chlorhexidine	Clotted blood; one or more than four blood specimens collected within a 24-h period; volume of blood <20 ml per culture (i.e., per venipuncture); antisepsis with alcohol only (adults)

[a] Reprinted from reference 3 with permission.

the fluid. In all cases, the actual specimen (tissue, aspirate, or biopsy) is preferred over a swab of the specimen (1).

Swabs from the surface of decubitus ulcers are not recommended for culture, as they are likely to yield colonizing bacteria that have superinfected the surface, making interpretation of results difficult (11, 12). Swabs collected through the nose for diagnosis of sinusitis are other specimens of dubious value. If the swabs have been endoscopically obtained, they may be free of nasal mucosal contamination and reveal infecting bacteria, but they will not recover fungi, which are the etiological agents of chronic sinusitis in ~25% of cases (13).

Aspirates

For abscess contents, body fluids, and other fluid collections below the skin, aspirates obtained through disinfected intact skin are preferred over swabs. The skin should be thoroughly cleaned with 70% alcohol, followed by disinfection with tincture of iodine or chlorhexidine, which must be completely dry before inserting the needle. An angel wing type of device can be used to safely inject the fluid in the syringe into a transport container. If anaerobes are being considered, the transport container must be free of air. Anaerobic transport vials with a nitrogen atmosphere above a gel containing oxygen-scavenging components are commercially available. Special small anaerobic transport vials containing specific anaerobic transport media for endodontal samples are also available. Only laboratories with expertise in diagnosis of dental infection should attempt to culture this type of specimen.

Normally sterile fluids (such as pleural, synovial, and pericardial fluids) are aspirated, but in addition to placing some of the fluid into a sterile tube or a citrated or EDTA tube (to prevent clot formation) for immediate Gram stain evaluation, a quantity (preferably 10 ml) should be injected into either an aerobic blood culture bottle or a Wampole Isolator (Alere Inc., Waltham, MA) tube directly at the patient's bedside (14, 15).

Complications occur in the clinical setting of intestinal tract perforation when microorganisms other than the expected *Enterobacteriaceae*, anaerobes, and enterococci are involved. Consensus standards include recommendations for when and how cultures from intra-abdominal sites should be handled (16, 17). Standard empirical therapy may not adequately treat *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida* spp., or multidrug-resistant bacteria. Laboratory policies on the extent of identification of isolates based on an arbitrary number (such as 3 or fewer) should be modified with appropriate clinician justification and discussion.

Tissues

Biopsied tissue should be obtained by the surgeon using aseptic technique. Surface skin should be disinfected with chlorhexidine before the skin is cut. Once the first incision is made, which may inadvertently cut through a subsurface microcolony of bacteria (*Propionibacterium acnes* and *Staphylococcus epidermidis*), surgeons should switch to a fresh, sterile scalpel. Tissue should be placed into an anaerobic transport vial. If anaerobes are not considered, tissue may be placed into a sterile tube or cup with a tight lid. Tissue from draining sinus lesions should be obtained by curetting deep within the interior of the tract after thorough surface skin disinfection. Swabs from the drainage are likely to harbor surface bacteria that may not be associated with the underlying infection. Bone biopsy samples to diagnose osteomyelitis should be obtained directly from the bone itself after debridement of the overlying, usually superinfected tissue (12,

TABLE 2 Collection, transport, and storage guidelines for microbiological laboratory diagnostic studies[a]

Specimen type	Collection guideline(s)	Transport[b] device and/or minimum vol	Transport time	Storage time and temp	Replica limits	Comment(s)
Abscess						
General	Remove surface exudate by wiping with sterile saline or 70% alcohol.					Tissue or aspirate is always superior to a swab specimen. If swabs must be used (aerobic culture only), collect two, one for culture and one for Gram staining. Preserve material on swab by placing in Stuart's or Amies medium.
Open	Aspirate if possible or pass a swab deep into the lesion to firmly sample the lesion's "fresh border."	Swab transport system	≤2 h, RT	≤24 h, RT	1/day/source	A sample of the base of the lesion and one of the abscess wall are most productive.
Closed	Aspirate abscess material with needle and syringe. Aseptically transfer *all* material into anaerobic transport device.	Anaerobic transport system, ≥1 ml	≤2 h, RT	≤24 h, RT	1/day/source	Contamination with surface material will introduce colonizing bacteria not involved in the infectious process. Do not use syringe for transport.
Bite wound	See Abscess.					Do not culture animal bite wounds ≤12 h old (agents are usually not recovered) unless signs of infection are present.
Blood	Disinfect culture bottle; apply 70% isopropyl alcohol or chlorhexidine to rubber stoppers and wait 1 min. Palpate vein before disinfection of venipuncture site. Disinfection of venipuncture site: 1. Cleanse site with 70% alcohol. 2. Swab concentrically, starting at the center, with tincture of iodine or chlorhexidine. 3. Allow the disinfectant to dry. 4. *Do not palpate vein at this point without sterile glove*. 5. Collect blood. 6. After venipuncture, remove iodine from the skin with alcohol.	Blood culture bottles for bacteria; adult, 20 ml/set[c] (higher vol most productive); infant and child, 1–20 ml/set depending on weight of patient (see Table 9)	≤2 h, RT	≤2 h, RT or per instructions	Four sets in 24 h	Acute febrile episode: two sets[c] from separate sites, all within 10 min (before antimicrobials) Nonacute disease: antimicrobials will not be started or changed immediately: two to four sets from separate sites, all within 24 h at intervals no closer than 3 h (before antimicrobials) Endocarditis, acute: three sets from three separate sites, within 1–2 h, before antimicrobials if possible Fever of unknown origin: two to four sets from separate sites. If negative at 24–48 h, obtain two or three more sets. Pediatric: collect immediately; rarely necessary to document continuous bacteremia with hours between cultures
Bone marrow aspirate	Prepare puncture site as for surgical incision.	Inoculate blood culture bottle or a 1.5-ml lysis-centrifugation tube.	≤24 h, RT if in culture bottle or tube	≤24 h, RT	1/day	Small volumes of bone marrow may be inoculated directly onto culture media. Routine bacterial culture of bone marrow is rarely useful.

Burn	Cleanse and debride the burn.	Tissue is placed into a sterile screw-cap container. Aspirate or swab exudates should be transported in sterile container or swab transport system.	≤24 h, RT	≤24 h, RT	1/day/source	A 3- to 4-mm punch biopsy specimen is optimum when quantitative cultures are ordered. Process for aerobic culture only. Quantitative culture may or may not be valuable. Cultures of surface samples of burns may be misleading.
Catheter						
Intravenous	1. Cleanse the skin around the catheter site with alcohol. 2. Aseptically remove catheter and clip 5 cm of distal tip directly into a sterile tube. Some elect to culture the 5-cm intracutaneous portion to evaluate for soft tissue infection. 3. Transport immediately to microbiology laboratory to prevent drying.	Sterile screw-cap tube or cup	≤15 min, RT	≤2 h, 4°C	None	Controversial whether culture of catheter tips is clinically relevant Acceptable intravenous catheters for semiquantitative culture (Maki roll method): central, CVP, Hickman, Broviac, peripheral, arterial, umbilical, hyperalimentation, Swan-Ganz
Foley	Do *not* culture, since growth represents distal urethral flora.					Not acceptable for culture
Cellulitis, aspirate from area of	1. Cleanse site by wiping with sterile saline or 70% alcohol. 2. Aspirate the area of maximum inflammation (commonly the center rather than the leading edge) with a needle and syringe. Irrigation with a small amount of sterile saline may be necessary. 3. Aspirate saline into syringe and expel into sterile screw-cap tube.	Sterile tube (syringe transport not recommended)	≤15 min, RT	≤24 h, RT	None	Yield of potential pathogens in minority of specimens cultured
CSF	1. Disinfect site with iodine or chlorhexidine preparation. 2. Insert a needle with stylet at L3-L4, L4-L5, or L5-S1 interspace. 3. Upon reaching the subarachnoid space, remove the stylet and collect 1–2 ml of fluid into each of three leakproof tubes.	Sterile screw-cap tubes Minimum amt required: bacteria, ≥1 ml; acid-fast, ≥5 ml	Bacteria: never refrigerate; ≤15 min, RT	≤24 h, RT	None	Obtain blood for culture also. If only one tube of CSF is collected, it should be submitted to microbiology first; otherwise, submit tube 2 to microbiology. Aspirate of brain abscess or a biopsy may be necessary to detect anaerobic bacteria or parasites.

(*Continued on next page*)

TABLE 2 Collection, transport, and storage guidelines for microbiological laboratory diagnostic studies[a] *(Continued)*

Specimen type	Collection guideline(s)	Transport[b] device and/or minimum vol	Transport time	Storage time and temp	Replica limits	Comment(s)
Decubitus ulcer	A swab is not the specimen of choice. 1. Cleanse surface with sterile saline. 2. If a sample biopsy is not available, aspirate inflammatory material from the base of the ulcer.	Sterile tube/container (aerobic) or anaerobic system (for tissue)	≤2 h, RT	≤24 h, RT	1/day/source	Since a swab specimen of a decubitus ulcer provides no clinical information, it should not be submitted. A tissue biopsy sample or needle aspirate is the specimen of choice.
Dental culture: gingival, periodontal, periapical, Vincent's stomatitis	1. Carefully cleanse gingival margin and supragingival tooth surface to remove saliva, debris, and plaque. 2. Using a periodontal scaler, carefully remove subgingival lesion material and transfer it to an anaerobic transport system. 3. Prepare smear for staining with specimen collected in the same fashion.	Anaerobic transport system	≤2 h, RT	≤24 h, RT	1/day	Periodontal lesions should be processed only by laboratories equipped to provide specialized techniques for the detection and enumeration of recognized pathogens.
Ear						
Inner	Tympanocentesis reserved for complicated, recurrent, or chronic persistent otitis media. 1. For intact ear drum, clean ear canal with soap solution and collect fluid via syringe aspiration technique (tympanocentesis). 2. For ruptured ear drum, collect fluid on flexible shaft swab via an auditory speculum (aerobic culture only).	Sterile tube, swab transport medium, or anaerobic system	≤2 h, RT	≤24 h, RT	1/day/source	Results of throat or nasopharyngeal swab cultures are not predictive of agents responsible for otitis media and should not be submitted for that purpose.
Outer	1. Use a moistened swab to remove any debris or crust from the ear canal. 2. Obtain a sample by firmly rotating swab in the outer canal.	Swab transport	≤2 h, RT	≤24 h, 4°C	1/day/source	For otitis externa, *vigorous* swabbing is required since surface swabbing may miss streptococcal cellulitis.
Eye						
Conjunctiva	1. Sample both eyes with separate swabs (premoistened with sterile saline) by rolling over each conjunctiva. 2. Medium may be inoculated at time of collection. 3. Smear may be prepared at time of collection. Roll swab over 1- to 2-cm area of slide.	Direct culture inoculation: BAP and CHOC Laboratory inoculation: swab transport	Plates: ≤15 min, RT Swabs: ≤2 h, RT	≤24 h, RT	None	If possible, sample both conjunctivae, even if only one is infected, to determine indigenous microbiota. The uninfected eye can serve as a control with which to compare the agents isolated from the infected eye. If cost prohibits this approach, rely on the Gram stain to assist in interpretation of culture.

Corneal scrapings	1. Specimen is collected by an ophthalmologist. 2. Using a sterile spatula, scrape ulcers or lesions, and inoculate scraping directly onto medium. 3. Prepare two smears by rubbing material from spatula onto 1- to 2-cm area of slide.	Direct culture inoculations: BHI with 10% sheep blood, CHOC, and inhibitory mold agar	≤15 min, RT	≤24 h, RT	None	If conjunctival specimen is collected, do so before anesthetic application, which may inhibit some bacteria. Corneal scrapings are obtained after anesthesia. Include fungal media. Scrapings for virus isolation and ameba detection should be submitted in a sterile container.
Vitreous fluid aspirates	Prepare eye for needle aspiration of fluid.	Sterile screw-cap tube or direct inoculation of small amount of fluid onto media	≤15 min, RT	≤24 h, RT	1/day	Include fungal media. Anesthetics may be inhibitory to some etiologic agents.
Feces						
Routine culture	Pass specimen directly into a clean, dry container. Transport to microbiology laboratory within 1 h of collection or transfer to Cary-Blair holding medium.	Clean, leakproof, wide-mouthed container, or Cary-Blair holding medium (>2 g)	Unpreserved: ≤1 h, RT Holding medium: ≤24 h, RT	≤24 h, 4°C ≤48 h, RT or 4°C	1/day	Do not perform routine stool cultures for patients whose length of hospital stay is >3 days and the admitting diagnosis was not gastroenteritis without consultation with physician. Tests for *C. difficile* should be considered for these patients. Swabs for routine pathogens are not recommended except for infants (see Rectal swab).
C. difficile	Pass liquid or soft stool directly into a clean, dry container. Soft stool is defined as stool assuming the shape of its container. Swab specimens are not recommended for toxin testing.	Sterile, leakproof, wide-mouthed container, >5 ml	≤1 h, RT 1–24 h, 4°C >24 h, −20°C or colder	2 days, 4°C for culture or NAAT 3 days, 4°C or longer at −70°C for toxin test or NAAT	One or two specimens may be necessary to detect low toxin levels.	Patients should be passing ≥3 liquid or soft stools per 24 h. Testing of formed or hard stool is not recommended unless patient has ileus. Freezing at −20°C or above results in rapid loss of cytotoxin activity.
E. coli (O157:H7) and other Shiga toxin-producing serotypes	Pass liquid or bloody stool into a clean, dry container.	Sterile, leakproof, wide-mouthed container, or Cary-Blair holding medium (>2 g)	Unpreserved: ≤1 h, RT Swab transport system: ≤24 h, RT or 4°C	≤24 h, 4°C ≤24 h, RT	1/day	Bloody or liquid stools collected within 6 days of onset from patients with abdominal cramps have the highest yield. Shiga toxin assay for all EHEC serotypes is better than sorbitol-MacConkey or chromogenic agar culture for 0157:H7 only.
Leukocyte detection	Send in clean, dry container.	For direct methylene blue smear, no transport medium	For direct smear, <2 h For lactoferrin tests, <48 h	For lactoferrin, 2–8°C		Controversial: some authors believe that this procedure provides results of little clinical value.

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TABLE 2 Collection, transport, and storage guidelines for microbiological laboratory diagnostic studies[a] *(Continued)*

Specimen type	Collection guideline(s)	Transport[b] device and/or minimum vol	Transport time	Storage time and temp	Replica limits	Comment(s)
Rectal swab	1. Carefully insert a swab approx. 1–1.5 in. beyond the anal sphincter. 2. Gently rotate the swab to sample the anal crypts. 3. Feces should be visible on the swab for detection of diarrheal pathogens.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	Reserved for detecting *N. gonorrhoeae*, *Shigella*, *Campylobacter*, herpes simplex virus, and anal carriage of group B *Streptococcus* and other beta-hemolytic streptococci, or for patients (usually children) unable to pass a specimen.
Fistulas	See Abscess.					
Fluids: abdominal, amniotic, ascites, bile, joint, pericardial, peritoneal, pleural, synovial	1. Disinfect overlying skin with iodine preparation. 2. Obtain specimen via percutaneous needle aspiration or surgery. 3. Always submit as much fluid as possible; *never* submit a swab dipped in fluid.	Anaerobic transport system, sterile screw-cap tube, or blood culture bottle for bacteria. Transport immediately to laboratory. Bacteria, >1 ml	≤15 min, RT	≤24 h, RT Pericardial fluid and fluids for fungal cultures, ≤24 h, 4°C	None	Amniotic and culdocentesis fluids should be transported in an anaerobic system and need not be centrifuged prior to Gram staining. Other fluids are best examined by Gram staining of a cytocentrifuged preparation. One aerobic blood culture bottle inoculated at bedside is highly recommended.
Gangrenous tissue	See Abscess.					Discourage sampling of surface or superficial tissue. Tissue biopsy or aspirates should be collected.
Gastric						
Wash or lavage for mycobacteria	Collect in early morning before patients eat and while they are still in bed. 1. Introduce a nasogastric tube into the stomach. 2. Perform lavage with 25–50 ml of chilled sterile, distilled water. 3. Recover sample and place in a leakproof, sterile container.	Sterile, leakproof container	≤15 min, RT, or neutralize within 1 h of collection	≤24 h, 4°C	1/day	The specimen must be processed promptly, since mycobacteria die rapidly in gastric washings. Neutralize when holding for >1 h with sodium bicarbonate.
Biopsy for *H. pylori*	Collected by gastroenterologist during endoscopy	Sterile tube with transport medium	<1 h, RT	≤24 h, 4°C	None	Culture may be needed for antimicrobial testing.
Genital: female						
Amniotic fluid	Aspirate via amniocentesis, or collect during cesarean delivery.	Anaerobic transport system, ≥1 ml	≤2 h, RT	≤24 h, RT	None	Swabbing or aspiration of vaginal secretions is *not* acceptable because of the potential for contamination with commensal vaginal biota.
Bartholin gland secretions	1. Disinfect skin with iodine preparation. 2. Aspirate fluid from ducts.	Anaerobic transport system, ≥1 ml	≤2 h, RT	≤24 h, RT	1/day	

Cervical secretions	1. Visualize the cervix using a speculum without lubricant. 2. Remove mucus and secretions from the cervical os with swab and discard the swab. 3. Firmly yet gently sample the endocervical canal with a new sterile swab.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	See text for collection and transport need for *C. trachomatis* and *N. gonorrhoeae*.
Cul-de-sac fluid	Submit aspirate or fluid.	Anaerobic transport system, >1 ml	≤2 h, RT	≤24 h, RT	1/day	
Endometrial tissue and secretions	1. Collect transcervical aspirate via a telescoping catheter. 2. Transfer entire amount to anaerobic transport system.	Anaerobic transport system, ≥1 ml	≤2 h, RT	≤24 h, RT	1/day	
Products of conception	1. Submit a portion of tissue in a sterile container. 2. If obtained by cesarean delivery, immediately transfer to an anaerobic transport system.	Sterile tube or anaerobic transport system	≤2 h, RT	≤24 h, RT	1/day	Do not process lochia, culture of which may give misleading results.
Urethral secretions	Collect at least 1 h after patient has urinated. 1. Remove old exudate from the urethral orifice. 2. Collect discharge material on a swab by massaging the urethra against the pubic symphysis through the vagina.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	If no discharge can be obtained, wash the periurethral area with povidone-iodine soap and rinse with water. Insert a small swab 2–4 cm into the urethra, rotate swab, and leave swab in place for at least 2 s to facilitate absorption.
Vaginal secretions	1. Wipe away old secretions/discharge. 2. Obtain secretions from the mucosal membrane of the vaginal wall with a sterile swab or pipette. 3. If a smear is also needed, use a second swab.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	For IUD, place entire device into a sterile container and submit at RT. Gram stain, not culture, is recommended for the diagnosis of BV. For detection of group B streptococcal colonization, also add rectal swab.
Genital: female or male lesion	1. Clean with sterile saline and remove lesion's surface with a sterile scalpel blade. 2. Allow transudate to accumulate. 3. While pressing the base of the lesion, *firmly* rub base with a sterile swab to collect fluid.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	For dark-field examination to detect *T. pallidum*, touch a glass slide to the transudate, add coverslip, and transport immediately to the laboratory in a humidified chamber (petri dish with moist gauze). *T. pallidum* cannot be cultured on artificial media.

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TABLE 2 Collection, transport, and storage guidelines for microbiological laboratory diagnostic studies[a] *(Continued)*

Specimen type	Collection guideline(s)	Transport[b] device and/or minimum vol	Transport time	Storage time and temp	Replica limits	Comment(s)
Genital: male						
Prostate	1. Cleanse the urethral meatus with soap and water. 2. Massage the prostate through the rectum. 3. Collect fluid expressed from the urethra on a sterile swab.	Swab transport or sterile tube for >1 ml of specimen	≤2 h, RT	≤24 h, RT	1/day	Pathogens in prostatic secretions may be identified by quantitative culture of urine before and after massage. Ejaculate may also be cultured.
Urethra	Insert a small swab 2–4 cm into the urethral lumen, rotate swab, and leave it in place for at least 2 s to facilitate absorption.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	
Pilonidal cyst	See Abscess.					
Respiratory, lower						
Bronchoalveolar lavage, brush, or wash; endotracheal aspirate	1. Collect washing or aspirate in a sputum trap. 2. Place brush in sterile container with 1 ml of saline.	Sterile container, >1 ml	≤2 h, RT	≤24 h, 4°C	1/day	A total of 40–80 ml of fluid is needed for quantitative analysis. For quantitative analysis of brushings, place brush into 1.0 ml of saline.
Sputum, expectorated	1. Collect specimen under the direct supervision of a nurse or physician. 2. Have patient rinse or gargle with water to remove excess oral biota. 3. Instruct patient to cough deeply to produce a lower respiratory specimen (not postnasal fluid). 4. Collect in a sterile container.	Sterile container, >1 ml Minimum amt: bacteria, >1 ml	≤2 h, RT	≤24 h, 4°C	1/day	For pediatric patients unable to produce a sputum specimen, a respiratory therapist should collect a specimen via suction. The best specimen from all patients should have ≤10 squamous cells/100× field (10× objective and 10× ocular).
Sputum, induced	1. Have patient rinse mouth with water after brushing gums and tongue. 2. With the aid of a nebulizer, have patients inhale ~25 ml of 3–10% sterile saline. 3. Collect in a sterile container.	Sterile container, >1 ml	≤2 h, RT	≤24 h, RT	1/day	Same as above for sputum, expectorated

Respiratory, upper						
Oral	1. Remove oral secretions and debris from the surface of the lesion with a swab. Discard this swab. 2. Using a second swab, vigorously sample the lesion, avoiding any areas of normal tissue.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	Discourage sampling of superficial tissue for bacterial evaluation. Tissue biopsy specimens or needle aspirates are the specimens of choice.
Nasal	1. Insert a swab, premoistened with sterile saline, approx. 1–2 cm into the nares. 2. Rotate the swab against the nasal mucosa.	Swab transport	≤2 h, RT	≤24 h, RT	1/day	Anterior nose cultures are reserved for detecting staphylococcal carriers or for nasal lesions.
Nasopharynx	1. Gently insert a small swab (e.g., calcium alginate or nasopharyngeal flocked swab into the posterior nasopharynx via the nose. 2. Rotate swab slowly for 5 s to absorb secretions.	Direct media inoculation at bedside or examination table, swab transport	Plates: ≤15 min, RT Swabs: ≤2 h, RT	≤24 h, RT	1/day	
Throat or pharynx	1. Depress tongue with a tongue depressor. 2. Sample the posterior pharynx, tonsils, and inflamed areas with a sterile swab.	Swab transport (dry swab with or without silica gel is good for *S. pyogenes* and *C. diphtheriae*)	≤2 h, RT	≤24 h, RT	1/day	Throat swab cultures are contraindicated in patients with epiglottitis. Swabs for *N. gonorrhoeae* should be placed in charcoal-containing or other specific transport medium and plated ≤12 h after collection (36). Bio-Bags and the GonoPak are better for transport at RT.
Tissue	Collected during surgery or cutaneous biopsy procedure	Anaerobic transport system or sterile, screw-cap container. Add several drops of sterile saline to keep small pieces of tissue moist.	≤15 min, RT	≤24 h, RT	None	Always submit as much tissue as possible. If there is excess tissue, save a portion of surgical tissue at −70°C in case further studies are needed. Never submit a swab that has been rubbed over the surface of a tissue. For quantitative study, a sample of 1 cm^3 is appropriate.
Urine						
Male and female, first void (for *Chlamydia* and *N. gonorrhoeae* NAAT)		Sterile tube or transport medium specified by NAAT manufacturer	Unpreserved: ≤2 h, RT; ≤24 h, 4°C	As specified by NAAT manufacturer	1/infectious episode	Do not collect >30 ml for NAAT.

(Continued on next page)

TABLE 2 Collection, transport, and storage guidelines for microbiological laboratory diagnostic studies[a] *(Continued)*

Specimen type	Collection guideline(s)	Transport[b] device and/or minimum vol	Transport time	Storage time and temp	Replica limits	Comment(s)
Female, midstream (for bacterial culture)	1. While holding the labia apart, begin voiding. 2. After several milliliters has passed, collect a midstream portion without stopping the flow of urine. 3. The midstream portion is used for bacterial culture.	Sterile, wide-mouthed container, ≥1 ml, or urine transport tube with boric acid preservative	Unpreserved: ≤2 h, RT Preserved: ≤24 h, RT	≤24 h, 4°C	1/day	Urine is toxic to cell lines and is therefore not the specimen of choice for chlamydial culture. Cleansing before voiding does not improve urine specimen quality; i.e., midstream urine samples are equivalent to clean-catch midstream urine samples (272).
Straight catheter	1. Thoroughly cleanse the urethral opening with soap and water. 2. Rinse area with wet gauze pads. 3. Aseptically, insert catheter into the bladder. 4. After allowing ~15 ml to pass, collect urine to be submitted in a sterile container.	Sterile, leak-proof container or urine transport tube with boric acid preservative	Unpreserved: ≤2 h, RT Preserved: ≤24 h, RT	≤24 h, 4°C	1/day	Catheterization may introduce urethral biota into the bladder and increase the risk of iatrogenic infection.
Indwelling catheter	1. Disinfect the catheter collection port with 70% alcohol. Clamp catheter below port and allow urine to collect in tubing for 10–20 min. 2. Use needle and syringe to aseptically collect 5–10 ml of urine. 3. Transfer to a sterile tube or container.	Sterile, leak-proof container or urine transport tube with boric acid preservative	Unpreserved: ≤2 h, RT Preserved: ≤24 h, RT	≤24 h, 4°C	1/day	Patients with indwelling catheters always have bacteria in their bladders. Do not collect urine from these patients unless they are symptomatic.
Wound	See Abscess.					

[a] Abbreviations: BAP, blood agar plate; BHI, brain heart infusion; CHOC, chocolate agar; CVP, central venous pressure; EHEC, enterohemorrhagic *E. coli*; RT, room temperature.

[b] All specimen containers are to be transported in leakproof plastic bags having a separate compartment for the requisition.

[c] One set usually refers to one culture with both aerobic and anaerobic broths.

TABLE 3 Suitability of various specimens for anaerobic culture

Acceptable material (method of collection)	Unacceptable material
Aspirate (by needle and syringe)	Bronchoalveolar lavage washing
Bartholin's gland inflammation/secretions	Cervical secretions
Blood (venipuncture)	Endotracheal secretions (aspirate)
Bone marrow (aspirate)	Lochia secretions
Bronchoscopic secretions (protected specimen brush)	Nasopharyngeal swab
	Perineal swab
Culdocentesis fluid (aspirate)	Prostatic or seminal fluid
Fallopian tube fluid or tissue (aspirate/biopsy sample)	Sinus washings or swabs
IUD for *Actinomyces* spp.	Sputum (expectorated or induced)
Nasal sinus (aspirate)	Stool or rectal swab samples
Placenta tissue (via cesarean delivery)	Tracheostomy secretions
Stool for *C. difficile*	Urethral secretions
Surgical site (aspirate, tissue)	Urine (voided or from catheter)
Transtracheal aspirate	Vaginal or vulvar secretions (swab)
Urine (suprapubic aspirate)	

18). Infected bone is soft enough to easily be removed by curetting or sometimes by aspiration with a large-bore needle.

Sputum

Expectorated sputum is the best noninvasive sample for diagnosis of pneumonia, a disease of the distal lung alveolar spaces. Endotracheal aspirates are more likely to contain upper respiratory microbes and saliva from the upper tract and may not represent the bacteria causing the pneumonia (19). For these reasons, respiratory tract secretions from patients other than those with cystic fibrosis should be evaluated microscopically for the presence of squamous epithelial cells (SECs) and bacteria (20, 21). Sufficiently hydrated patients with pneumonia should be able to produce a good sputum specimen if properly coached by the collecting staff. The patient should be sitting up, should rinse out his mouth with water, and should be encouraged to bring up a deep cough from the lungs. Sometimes mild percussion on the chest can help dislodge deep phlegm. If a patient cannot produce an acceptable sputum sample the first time, it is very unlikely that a repeat collection will yield a good specimen, and it is probably better to call respiratory therapy to try to obtain a specimen. For some respiratory diseases, such as pneumococcal pneumonia and Legionnaires' disease, the inability to obtain reliable sputum has spurred the development of urinary antigen tests with relatively good sensitivity (depending on the assay) and variable specificity (22–25).

Urine

Urine can be collected by midstream collection, catheterization (straight/in-out or indwelling), cystoscopic collection, or suprapubic aspiration. Foley catheter tips should not be submitted or accepted for culture, since they are always contaminated with members of the urethral microbiota and quantitation is not possible. A first-voided morning urine is optimal, since in most cases bacteria have been multiplying in the bladder for a number of hours. Cleansing of the periurethral areas has not been shown to improve the quality of urine culture, but midstream collection is still recommended (26).

Feces (Stool)

Stool is always superior to a rectal swab for bacterial testing, but if a swab is the only sample available, the swab should be inserted deep enough into the rectum to encounter some stool (appear brown). Swabs should always be submitted in transport media to preserve viability of pathogens. Some pathogens, especially *Shigella*, are labile in stool, so stool not submitted in appropriate transport medium (e.g., Cary-Blair medium) should be inoculated to media without delay. Testing more than one stool for bacterial pathogens is usually not productive. Fresh stool should be examined visually, and the areas showing blood, pus, or mucus should be sampled preferentially. Diarrheal stool is loose and takes the shape of the container. Although formed stool may be tested for some pathogens, it is less productive, and for *Clostridium difficile* in particular, testing formed stool (other than in cases of physician-documented ileus) can lead to recovery of organisms that are colonizers and not causing disease.

Prosthetic Devices

The device itself may be sent to the laboratory for microbiological studies. Sonication in a volume of liquid has been shown to facilitate detection of organisms forming a biofilm on prostheses (27, 28). Other studies have shown that sonication at 22°C for 7 min yields the best results (29). When the prosthesis is not removed and the infection is suspected in the site of a prosthetic joint, isolation of the same species from at least three of five separate tissue biopsies from the infected site can help differentiate contamination from true infection (30, 31). Rapid molecular methods have been used with some success to determine appropriate therapy before culture results are available (32).

Volume of Sample

In general, the more material available for testing, the better. This is true especially when more than one type of culture is requested. However, receiving more than the volume necessary to accommodate all requests could be problematic, particularly if less-trained staff choose the section to use for microbiological studies. Caregivers should be encouraged not to send large bags of fluids, except for peritoneal dialysate (from which the laboratory staff should remove 50 ml to concentrate by centrifugation), or very large pieces of tissue, as that requires more manipulation in the laboratory than necessary and could introduce contaminants or result in a less productive part of the sample being tested. Since a drop of material (~0.05 ml) is the minimum amount necessary to inoculate an agar plate or an enrichment broth, at least

1 drop per medium used should be received in the laboratory. As a general rule, at least 0.5 ml or 0.5 g of material should be received for routine bacterial culture, and more is necessary for additional studies. If the volume received is not sufficient to perform all requests or to inoculate all the usual media, the physician should be contacted to prioritize the requests. This not only helps the microbiologist to perform the appropriate test but also alerts the physician that the sample was problematic and could serve as a motivator for more adequate specimens in the future. Problems with volume often occur when fine-needle biopsy samples from interventional radiology are sent for microbiological studies, as further discussed below.

Specimen Maintenance during Transport

The type of test to be performed determines the nature of the transport system. The performance of various swab types and transport media should be verified prior to implementation. Manufacturers are required to do this, and laboratories often perform limited verifications. Selected criteria are important for the laboratory and should be monitored. For example, some nonviable bacteria in transport media (allowed per U.S. Food and Drug Administration [FDA] specifications) may be seen in initial Gram stains, so microbiologists may wish to sample several lots of the product to ascertain a low burden of dead but stainable organisms. The Clinical and Laboratory Standards Institute has developed standards for evaluating transport devices (33). Table 2 summarizes transport recommendations for routine bacteriology.

1. Transport specimens to the laboratory as soon as possible after collection. If transport will require more than 2 h (some organisms require a shorter time period), either a specific transport medium or refrigeration is required (Table 2) (1). For samples to be tested by molecular methods only, refrigeration or even freezing (the lower the temperature, the better; –80°C is best) is acceptable. Be sure to use the manufacturer's recommended or companion transport materials for commercial molecular assays. Any deviation from the product insert in sample collection and handling for any *in vitro* diagnostic test requires the laboratory to perform a relatively extensive verification of the performance of the assay.
2. Inoculate bacterial culture media within 24 h even if appropriate holding medium or refrigeration is maintained.
3. Small volumes of fluid (<1 ml) or tissue (<1 cm^3) should be submitted within 15 to 30 min to avoid evaporation, drying, and exposure to ambient conditions. A few drops of nonbacteriostatic saline may be added for hydration. Larger volumes and those specimens in holding medium may be stored for as long as 24 h.
4. Bacteria that are especially sensitive to ambient conditions include *Bordetella pertussis*, *Shigella* spp., *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, and anaerobes. Reliable detection of these species requires immediate processing. Delays of up to 6 h result in some loss of CFU when transport media are used (34). Longer delays, even with the use of transport media, result in significant loss of organisms. For delays beyond 6 h, refrigeration improves recovery; however, specimens containing anaerobes should be stored at ambient temperatures (35, 36).
5. Moving clinical specimens and infectious substances from one laboratory to another, regardless of the distance and vehicle used, requires adherence to specimen packaging and labeling instructions mandated by the federal government. Materials for transport must be labeled properly, packaged so as to be able to absorb and contain any liquid released during a break or spill, and protected during transport. See the Centers for Disease Control and Prevention (CDC) website (http://www.cdc.gov/od/eaipp/shipping/) and the International Air Transport Association (http://www.iata.org/publications/dgr/Pages/other-dgr-products.aspx) for more information.
6. Some fastidious microbes or special testing protocols have more unusual transport requirements (Table 4). The laboratory test guide or manual should include clear instructions for special transport methods (37). If specimens are to be shipped to a distant reference laboratory, the reference laboratory's conditions and requests must be met, and in many instances the laboratory provides transport devices to users. Continuous dialogue with physicians and other caregivers ensures that the appropriate information on collection and transport is accessed before specimen collection begins.

LABORATORY SAFETY ISSUES REGARDING BACTERIAL PATHOGENS

Workers should always maintain a level of suspicion that any specimen could harbor an infectious agent. Physicians may fail to alert the laboratory when they suspect the possibility of any dangerous pathogen, including *Coccidioides immitis/posadasii*, *Brucella*, etc. Some bacteria, such as *Mycobacterium tuberculosis*, and potential bioweapons on the list of select agents, such as *Francisella tularensis*, pose a threat to health care workers (38). Some precautions can be taken even before laboratory activities occur. These include obtaining proper vaccines, such as those for *N. meningitidis* and more unusual agents if the laboratory is expected to encounter them. It is prudent to obtain baseline sera from each worker and store it in an ultralow-temperature freezer to have a basis for comparison in the event of a future exposure. Workers with conditions that place them at higher risk for some infections (immunocompromised persons and those in certain ethnic groups) may wish to avoid those tasks or laboratory assignments that could place them at risk. Any laboratorians who handle specimens or who might have to enter an environment where aerosols are possible must be fit tested for an N95 mask or similar protective face shield respirator. These masks should be worn in any situation where aerosols are possible. The Occupational Safety and Health Administration mandates the Respiratory Protection Standard specifically for potential tuberculosis or severe acute respiratory syndrome coronavirus exposures, but other regulatory agencies and most employers have extended these rules to other respiratory hazards (https://www.osha.gov/SLTC/respiratoryprotection/standards.html). The CDC's most recent biosafety manual, found at http://www.cdc.gov/biosafety/publications/bmbl5/bmbl.pdf, has guidelines for research laboratories. A CDC-produced *Morbidity and Mortality Weekly Report* relates more specifically to clinical laboratories (http://www.cdc.gov/mmwr/preview/mmwrhtml/su6101a1.htm). Chapter 12 of this *Manual* contains a more in-depth discussion of biosafety in the laboratory.

Engineering Controls

Laboratory environments should be designed to protect workers from biohazards inherent in the nature of the work. Ready access to properly maintained and certified biosafety level 2 (BSL2) laminar-flow biological cabinets and good training in the proper use of such equipment

TABLE 4 Specimen management for infrequently encountered organisms[a]

Organism	Specimen(s) of choice	Transport issues	Comment(s)
B. henselae (cat scratch fever)	Blood, tissue, lymph node aspirate	1 wk at 4°C; indefinitely at −70°C	May see organisms in or on erythrocytes with Giemsa stain. Use Warthin-Starry silver stain for tissue. SPS is toxic.
Borrelia burgdorferi (Lyme disease)	Skin biopsy at lesion periphery, blood, CSF	Keep tissue moist and sterile; hand carry to laboratory if possible	Consider PCR in addition to culture. Culture yield is low. Warthin-Starry silver stain for tissue. AO, Giemsa for blood and CSF.
Borrelia spp. (relapsing fever)	Blood smear (blood)	Hand carry to laboratory if possible	Use direct wet mount in saline for dark-field microscopy. Stain with Wright's or Giemsa stain. Blood culture is unreliable.
Coxiella (Q fever),[b] *Rickettsia* (spotted fevers, typhus)	Serum, tissue (blood)	Blood and tissue are frozen at −70°C until shipped	Refer isolation to reference laboratory. Serologic diagnosis is preferred.
Ehrlichia and *Anaplasma* spp.	Blood smear, skin biopsy, blood (with heparin or EDTA anticoagulant), CSF, serum	Material for culture sent on ice; keep tissue moist and sterile; hold at 4–20°C until tested or at −70°C for shipment; transport on ice or frozen for PCR	Serologic diagnosis preferred. Fix smear in methanol. Tissue stained with FA or Gimenez stain. Refer isolation to reference laboratory. CSF for direct examination and PCR.
Francisella spp. (tularemia)[b]	Lymph node aspirate, scrapings, lesion biopsy sample, blood, sputum	Rapid transport to laboratory or freeze; ship on dry ice	Send to reference laboratory. Serology helpful. Gram stain of tissue is not productive. IFA available. Culture effective 10% of the time.
Leptospira spp.	Serum, blood (citrate-containing anticoagulants should not be used), CSF (1st wk), urine (after 1st wk)	Blood, <1 h; urine, <1 h or dilute 1:10 in 1% bovine serum albumin and store at 4–20°C	Serology most helpful. Acidic urine is detrimental. Dark-field microscopy and direct FA available. Warthin-Starry silver stain for tissue.
Streptobacillus spp. (rat-bite fever; Haverhill fever)	Blood, aspirates of joint fluid	High-volume bottle preferred	Do not refrigerate. Requires blood, serum, or ascitic fluid for growth. SPS is inhibitory. AO staining is helpful.

[a] Abbreviations: AO, acridine orange; FA, fluorescent antibody; IFA, indirect fluorescent antibody.

[b] Laboratory safety hazard. Class II biologic safety cabinet required. Also see chapter 12 and chapters 66 (*Coxiella*) and 46 (*Francisella*) of this *Manual*.

are important and should be encouraged, even in resource-poor laboratory settings. The laboratory environment should have good lighting over work surfaces and sufficient air exchanges to decrease exposure for workers (12 exchanges per hour has been recommended). When BSL2 cabinets are not available, all manipulations that may result in aerosol formation, and those involving samples containing blood or body fluids (such as blood culture bottles), should be performed behind a Plexiglas or other clear shield. As many devices as possible to prevent contamination or acquisition of pathogens should be employed, including showers, eyewashes, and hands-free faucets at all hand-washing sinks. Specimen containers should be carried within the laboratory in trays with solid sides and bottoms to prevent spillage if dropped accidentally. Staff should try to avoid carrying bottles or groups of tubes in their hands. Counters should be solid with backstops, and in BSL2 or -3 rooms, the ceiling and walls should be smooth and waterproof and the floor should be smooth with coved edges. See chapter 12 for more information.

Personal Protective Equipment

Workers should be allowed to wear gloves on the bench if they wish, and gloves should be changed often. Gloves should always be worn when handling original patient samples. Whether gloved or not, workers must wash their hands with alcohol gel or soap and water before leaving the laboratory, after removing gloves, and often at other times during normal work tasks. If splattering or aerosol is possible, face shields or goggles should be freely provided by the employer.

Education and a Culture of Safety

Supervisors, managers, and directors should create an atmosphere where workers maintain an environment of safe working habits and are constantly reminded of their responsibilities in this area. Everyone should know what to do in the event of a spill or accidental release in the laboratory, and drills should be conducted periodically to reinforce proper responses (38).

HANDLING OF SPECIMENS IN THE LABORATORY

Documentation of Arrival, Condition, and Appropriate Sample for Test Ordered

Upon arrival of specimens in the laboratory, the time and date of receipt should be recorded. Subsequently, the time of plating, which may differ substantially from the time of receipt, should be recorded. At the time of receipt, all specimens and requisitions should be carefully inspected. Specimens must be labeled and accompanied by a requisition reflecting the physician's order. The requisition must include the following information: patient name, age, sex, identifying number (such as social security number or unique registration/billing number), and location (hospital room, physician's office address, etc.); ordering physician's name; specimen source (detailed); date and time of collection; and test ordered. If an electronic system is being used, such as a barcode data entry system, all of the relevant information mentioned in this section must be captured. One good quality assurance activity is to document that correct and complete information is captured during the accessioning process. If the information is incomplete, laboratory personnel must call the collecting location and request the missing information. If a specimen is mislabeled or no patient name is provided, another specimen should be collected. Relabeling of a specimen is allowed only if another specimen cannot be collected, such as for tissue collected during a surgical procedure. Laboratory procedures must clearly state the exceptions that are allowed, the steps needed to verify and document exceptions, and the individuals responsible for relabeling. When relabeling has occurred, the course of events must be outlined in the laboratory report so the physician interpreting the results is aware of potential errors. Speedy initial inspection of incoming samples can allow recollection or notification of problems in an actionable time frame.

Laws governing specimen labeling can be reviewed at the Clinical Laboratory Improvement Amendments website (http://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/Downloads/apcsubk1.pdf). Specimens from outpatient facilities require additional information for Medicare and Medicaid billing. Patient diagnosis, in the form of an International Classification of Diseases, 9th revision or newer 10th revision code, is needed to confirm the justification for a particular test. If a test is not deemed necessary for a specific diagnosis, the patient must sign an advanced beneficiary notice documenting that the test is not considered necessary and that if it is performed, the patient will be required to pay the test charge. Medicare and Medicaid compliance rules also can be reviewed at the Centers for Medicare and Medicaid Services website (http://www.cms.hhs.gov).

Specimen Rejection

In spite of acceptable labeling, some specimen collection sites, transport containers, or transport conditions render the specimen unacceptable for processing (39). Table 2 lists acceptable criteria for specimen management based on collection or transport conditions and times. When specimens fall outside these limits (too long in transit, incorrect temperature, not in proper transport medium, wrong site for requested test, etc.), new specimens should be collected whenever possible. To protect the safety of laboratory workers, specimens that leaked or those that are grossly contaminated on the outside of the container should also be rejected. In addition, specimens may be rejected because of poor quality of specimen material collected or because it is the wrong specimen type for the test requested, rather than the conditions of transport (Tables 3 and 5). Specimen quality is evaluated by examining the quantity and cellular composition. Although the quantity of many specimens is limited by the collection method or physical size of the infected area, some specimens, such as urine, stool, and sputum, are available in abundance. If another specimen can be collected easily with a larger volume, it is appropriate and necessary to request new or additional material. If the specimen volume is less than needed for essential tests, small volumes of liquid specimens can be extended by adding 0.5 to 1 ml of sterile saline or a nutrient broth. It is important to add just enough liquid to provide specimen for all tests requested. A comment such as "Specimen has been diluted to allow performance of requested tests" should be added to the results documenting this action. In some cases, too much sample should also be considered for rejection. For example, if >30 ml of urine is received for *Chlamydia trachomatis* and *N. gonorrhoeae* molecular testing, the dilution of rare infectious particles may yield false-negative results. When blood culture bottles are overfilled, the excess blood volume may overwhelm the ability of sodium polyanethol sulfonate (SPS) to adequately anticoagulate the blood or remove inhibitors.

TABLE 5 Screening specimens requested for routine bacterial culture to ensure quality[a]

Specimen	Screening method	Results of screen	
		Acceptable for culture	No further testing; request another sample
Sputum	Microscopic examination of Gram-stained smear	<10 SECs/average 10× field	>10 SECs/average 10× field
Endotracheal aspirate	Microscopic examination of Gram-stained smear	<10 SECs/average 10× field and bacteria seen in at least 1 of 20 oil immersion fields	>10 SECs/average 10× field and no bacteria seen in 20 oil immersion fields
Bronchoalveolar lavage fluid	Microscopic examination of Gram-stained smear	<1% of cells present are SECs	>1% of cells present are SECs
Superficial wound	Microscopic examination of Gram-stained smear	<2+ SECs, PMNs present	>2+ SECs and no PMNs
Stool for bacterial pathogens	Days in hospital	≤3 days	>3 days (exception: physician provides good rationale)
Urine	Urinalysis, Gram stain of urine sediment	Positive dipstick leukocyte esterase test result or seeing >10 PMNs/mm^3 is an indicator of possible infection, but no method has yet proved truly reliable. One bacterium per oil power field corresponds to 10,000 CFU/ml in the urine.	Growth of three or more potential pathogens usually indicates biofilm on indwelling catheter or fecal contamination. Mixed fecal morphologies on Gram stain may indicate fistula into bladder from gastrointestinal tract.

[a] Modified from reference 3 with permission.

Specimens determined to have gross bacterial contamination from members of the normal microbiota, indicated by an abundance of SECs visualized on the initial Gram stain, should be rejected. Specimens are rejected by contacting the patient's caregiver, explaining the reason for rejection, and requesting a replacement specimen of acceptable quality. Timely notification and collection of replacement are necessary, especially in instances where antimicrobial therapy has been initiated. Regardless of the reason for rejection, it may be more politically palatable to state that the specimen cannot be accepted due to inability to correctly interpret the results, rather than to use the word "rejected" (N. Cornish, personal communication). Specimen rejection criteria should be reviewed by appropriate laboratory and medical staff representatives before becoming policy. Examples of acceptable and unacceptable specimens based on screening criteria are listed in Table 5.

Handling Samples That Are To Be Processed at a Remote Site

Transport and processing time limits suitable for laboratories near the specimen collection site are not appropriate for remote laboratories. Specimens must be handled in the same manner as if they are to be transported via public or private mail service. See the CDC Office of Health and Safety site and the Pipeline and Hazardous Materials Safety Administration site (http://www.phmsa.dot.gov/hazmat/standards-rulemaking/hmic) for the latest guidelines. The IDSA, recognizing the problem, has suggested guidelines for providing the best patient care under less-than-ideal circumstances (40). Managing movement of specimens using temperature-controlled boxes, reliable courier service, and good tracking systems is a challenge facing many providers today.

Prioritization

For busy laboratories that receive many samples over a short period, it is helpful to use a priority list to assist accessioning staff to determine in which order samples should be processed. Specimens ordered for STAT tests, such as Gram stains on specimens from patients still in the operating rooms, should receive the highest priority. A sample priority list is shown in Table 6.

TABLE 6 Suggested priority list for accessioning samples received for bacteriology studies[a]

1. STAT requests (in order: operating room, pediatric intensive care units, emergency department)
2. CSF for routine culture and Gram stain
3. Rapid antigen detection tests (group A *Streptococcus*, *Campylobacter*, others)
4. Endotracheal aspirates in Lukens trap (patients on transplant and neonatal and pediatric intensive care units only)
5. Acid-fast specimens (if arrive near the processing cutoff time)
6. Bronchoalveolar lavage samples
7. Tissues from operating room
8. Body fluids (not CSF)
9. Blood cultures
10. Tissues and aspirates (not from operating room)
11. Fresh stools not in transport media (place into transport medium)
12. Sputa, other tracheal aspirates
13. Swabs in transport tubes
14. Urine samples
15. Stools in transport medium, including Cary-Blair, viral transport medium, and sodium acetate-acetic acid formalin

[a] Virology, mycology, and molecular test specimens may also need to be included in the list depending on the laboratory's policies and sample-handling personnel distribution.

Initial Sample Handling

1. Swabs that will be inoculated onto only one or two plates can be rolled directly onto the agar surface, inoculating the least inhibitory medium first. If a Gram stain is requested or if numerous media must be inoculated, the swab can be vortexed in 0.8 to 1.0 ml of sterile saline or Trypticase soy broth and drops of the suspension can be used to prepare the slide and inoculate media. Swabs should also be used to dip into stool, but usually not for sputum unless the sample is uniformly viscous without visible saliva. Sputum is best picked up from the container with wooden sticks, which do not adsorb the fluid sections but stick to the more purulent portions, where the pathogens are likely to be.

2. Clear fluids other than urine should be concentrated on a slide using a cytocentrifuge. If a cytocentrifuge is not available, samples of >1 ml should be centrifuged (1,500 to 2,500 × *g*) and the supernatant removed with a pipette down to 1.0 ml. This sediment is mixed thoroughly (vortex or pipette) and used for testing. Only if the material is thick, bloody, or frank pus can it be placed onto the slide without concentration, and it should be smeared thinly, similar to making a thin blood film.

3. Tissue and bone should be minced (using two sterile scalpels), and if fungus cultures are requested, a small piece of intact tissue should be inoculated into the surface of each agar medium (see chapter 114) in addition to the standard inoculum. Tissue and bone for bacteriology cultures should also be ground up in a mortar and pestle, or tissue may be dispersed in an automated stomacher (Seward Limited, Worthing, West Sussex, United Kingdom) or grinder (41). A drop of this suspension should be streaked to obtain isolated colonies.

4. A Gram stain should be examined for all fluids and tissues and some swabs, such as those received for diagnosis of bacterial vaginosis (BV). Gram stains can deliver rapid, actionable results to clinicians; can determine if a specimen is acceptable for culture based on cellularity; and should be used to aid the microbiologist in interpretation of the culture (42). Table 7 suggests specimen types appropriate for Gram stain. Infection gives rise to purulence (abundant polymorphonuclear cells), blood, necrosis, and mucus (mucous membrane specimens). In general, gross examination of the specimen should identify yellow to tan purulence, red to rust-colored blood, clear and tenacious mucus, and brown to black discoloration of necrotic tissue. Portions for smear and culture should be taken from these areas. It may be beneficial to ask the assistance of a surgical pathologist when choosing the best portion of excised tissue for examination (43). Two smears of thick material such as sputum or tissue can be prepared by placing sufficient material between two glass slides and pressing the slides together while sliding them back and forth lengthwise to spread the material evenly one cell layer thick and to break up mucous strands. Ideally, microscopic examination of smears, using a 10× microscope objective, should demonstrate many polymorphonuclear cells and few or no SECs that would suggest cutaneous or mucocutaneous contamination with members of the normal bacterial microbiota. Specimens in which tissue necrosis is present also may show elastin fibers (Fig. 1) in stained smears.

TABLE 7 Recommendations for Gram stain and plating media for bacteriology specimens or organisms

Specimen or organism	Gram stain	Aerobic media[a]	Anaerobic media[a,b]	Comments
Body cavity fluids	x (separate fluid specimen needed)			Blood culture bottles should be used to incubate large volumes of specimens for all body cavity fluids, following manufacturers' recommendations regarding supplements, etc.
CSF (routine)	x	B, C		
CSF (shunt)	x	B, C, Th (selectively)		
Pericardial	x	B, C	BBA	
Pleural	x	B, C	BBA	
Peritoneal	x	B, C, Mac, CNA	BBA, LKV, BBE	
CAPD	x	B, C, Th	BBA	
Synovial	x	B, C		
Bone marrow	x	B, C	BBA	
Catheter tip		B		
Ear external fluid/swab	x	B, C, Mac		
Ear internal fluid	x	B, C	BBA	
Eye	x	B, C		
Gastrointestinal tract				
Feces		B, Mac, HE, Ca, EB (optional); sorbitol-Mac/chromogenic agar/ Shiga toxin testing		C. *jejuni/coli* in 5% O_2–10% CO_2–85% N_2 at 42°C for all gastrointestinal tract specimens
Rectal swab		B, Mac, HE, Ca, EB		
Genitourinary tract				
Vaginal/cervix	x	B, TM		Gram stain is method of choice for diagnosis of BV.
Urethra/penis	x	TM		
Other	x	B, C, Mac, TM, CNA	BBA, LKV, BBE	
Group B streptococcal screen (vaginal/anal screen)		Selective broth, subculture to B		
Lower respiratory tract				
Sputum	x	B, C, Mac (BCSA for cystic fibrosis)		
Tracheal aspirate	x	B, C, Mac		
Bronchoalveolar lavage fluid	x	B, C, Mac, CNA	BBA, LKV	Protected bronchoscopic brushing (in anaerobic transport) required for anaerobic culture
Bronchoalveolar brushing, washing	x	B, C, Mac		
Tissue	x	B, C, Mac, CNA, Th	BBA, LKV, BBE	
Upper respiratory tract				
Nasopharynx		B, C		
Nose		B, chromogenic agar		
Throat		B or SSA		
Urine		B, Mac or chromogenic agar		
Wound or abscess				
Swab	x	B, C, Mac, CNA	BBA, LKV, BBE	
Aspirate	x	B, C, Mac, CNA	BBA, LKV, BBE	
B. *pertussis* and B. *parapertussis*		Regan-Lowe		
Brucella spp.		B, C		
C. *diphtheriae*		Cystine-tellurite or Loeffler's serum		
C. *difficile*		CCFA		NAAT more sensitive and specific
E. *coli* O157:H7 (EHEC, STEC)		Sorbitol-Mac/ chromogenic agar		Shiga toxin EIA or NAAT more sensitive
F. tularensis		C or BCYE		
H. ducreyi		C + vancomycin (3 µg/ml)		Gram stain resembling "school of fish"

(Continued on next page)

TABLE 7 Recommendations for Gram stain and plating media for bacteriology specimens or organisms (*Continued)*

Specimen or organism	Gram stain	Aerobic media[a]	Anaerobic media[a,b]	Comments
H. pylori	x	BHI, others (see chapter 57)		Campylobacter gaseous atmosphere at 35–37°C
Legionella spp.		BCYE		
Leptospira		Fletcher's medium or EMJH		30°C for up to 13 wk
N. gonorrhoeae		TM		
Vibrio spp.		TCBS		
Y. enterocolitica		CIN		

[a] Abbreviations: B, blood agar; BBA, brucella blood agar; BBE, *Bacteroides* bile esculin agar; BCSA, *Burkholderia cepacia* selective agar; BCYE, buffered charcoal-yeast extract agar; BHI, brain heart infusion; C, chocolate blood agar; Ca, campylobacter agar; CAPD, fluid from chronic ambulatory peritoneal dialysis; CCFA, cycloserine-cefoxitin-fructose agar; CIN, cefsulodin-Irgasan-novobiocin agar; CNA, colistin-nalidixic acid agar; EB, enrichment broth; EHEC, enterohemorrhagic *E. coli*; EMJH, Ellinghausen-McCullough-Johnson-Harris medium; HE, Hektoen enteric agar; LKV, laked blood agar with kanamycin and vancomycin; Mac, MacConkey agar; SSA, group A *Streptococcus* selective agar; TCBS, thiosulfate-citrate-bile salts-sucrose agar; Th, thioglycolate broth; TM, Thayer-Martin agar.

[b] Set up anaerobic culture upon request, if specimen is collected and transported appropriately. Call physician if appropriate specimen does not have request for anaerobic culture.

Lower respiratory tract specimens are likely to show alveolar macrophages and Curschmann's spirals (Fig. 2), indicating that secretions have originated from the distal airways. Curschmann's spirals are casts of bronchioles found in patients with chronic lung disease caused most commonly by asthma and cigarette smoking. Although elastin fibers are present in noninfected surgical wounds and specimens from areas of tissue damage, they are also found in infected tissue where necrosis has occurred.

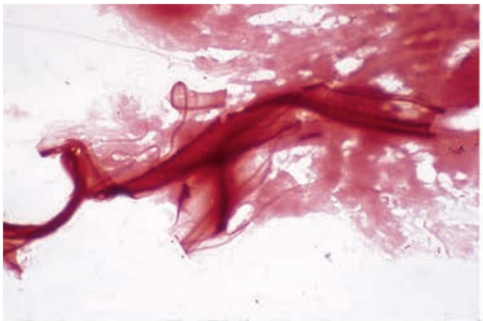
5. Based on the site of the sample and the physician's requests, each specimen should be inoculated to an appropriate medium chosen to recover suspected pathogens (Table 7). In this era of diminishing resources, two relatively recent innovations improve this process: chromogenic agars and automated plate inoculators. Chromogenic agars produce colonies that can be identified to genus or sometimes even species level directly from the appearance of the colonies without any further testing. Not only are they available for urinary tract pathogens (44) and some stool pathogens (45, 46), but also they are used widely for screening samples for resistant bacteria, such as methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), and multidrug-resistant Gram-negative rods (47, 48). These special media are discussed further in chapters covering organisms for which chromogenic media are available. In most cases, they are cost-effective by reducing both time and reagents used to identify the organisms in the conventional manner (44).

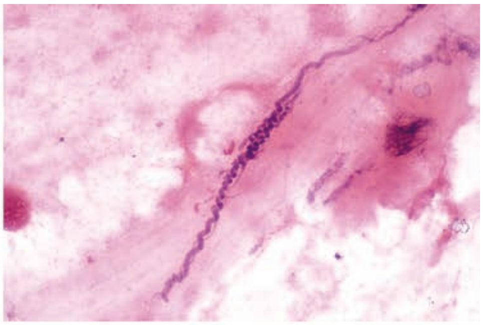
At least four automated media inoculators are available, and all have been reported to perform at least as well as manual plating and to reduce workload for short-staffed laboratories (49). Some require that the sample be inoculated onto the plate, e.g., Isoplater (Vista Technology, Edmonton, Alberta, Canada); others handle the container containing any liquid specimen, e.g., MicroStreak (LabTech Systems, Adelaide, South Australia, Australia) and InocuLAB (Dynacon, Mississauga, Ontario, Canada). Streaking patterns vary from spiral to quadrant. Some recent instruments even manage to handle Eswabs (swabs submitted in liquid transport media), e.g., WASP, or Walk-Away Specimen Processor (Copan Inc., Murrieta, CA), and other liquid sample devices, e.g., InoqulA (BD Kiestra, Franklin Lakes, NJ) and PREVI Isola (bioMérieux, Durham, NC) (50). The calculated estimated return on investment for one of the instruments was approximately 4 years (http://www.vistatechnology.com). These instruments are seeing increasing adoption as laboratory staffing becomes leaner.

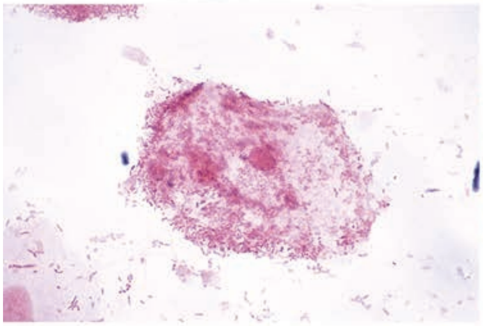
The use of enrichment broth is controversial, but it should be conservative. Table 7 presents our recommendations. For many samples, such as swabs, broth cultures are more likely to yield contaminating members of the skin microbiota than pathogens (51). When fastidious species or organisms that may be present in small numbers are clinically important, enrichment broths make sense. When simultaneously inoculated plates yield growth, it is rarely useful or efficient to continue to work with the broth. When initial plates do not show growth in the face of a sample with polymorphonuclear leukocytes (PMNs) or other suggestions of an infectious process, the broth should be held for at least 5 days, and sometimes up to 14 days (looking for *Actinomyces* in eye samples or *P. acnes* in prosthetic joint infection fluids, for example). Broths inoculated with cerebrospinal fluid (CSF) samples obtained from patients with indwelling shunts, joint fluids (should be injected directly into blood culture bottles), pericardial effusions, and other important fluids should be held for 14 days. Growth of skin organisms, such as coagulase-negative *Staphylococcus* and *Propionibacterium* species, must be evaluated in concert with clinical criteria to avoid overinterpretation (52). In fact, for clean orthopedic surgeries, culture is not indicated (53).

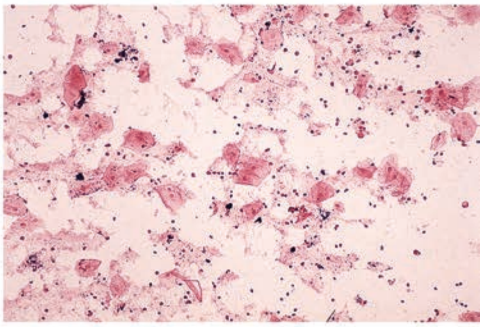
Once inoculated to the appropriate media based on the laboratory's protocols (Table 7), plates and broths are incubated in the appropriate atmosphere before they are examined for growth. Chocolate plates are always incubated in 5% CO_2, blood may be incubated in either air or CO_2 (depending on the requirements of the organisms to be recovered), and selective agar plates are best incubated in air, although MacConkey agar colonies appear virtually the same whether incubated in CO_2 or air. Special incubation atmospheres are mentioned in the chapters covering those species and in this chapter. Lighting a candle in an enclosed jar or metal box and allowing it to burn out naturally creates an excellent CO_2 atmosphere for good recovery of microaerophilic, capnophilic (requiring higher CO_2 concentration than air) bacteria. Unless otherwise noted, ideal temperatures for bacterial growth are 35 to 37°C.

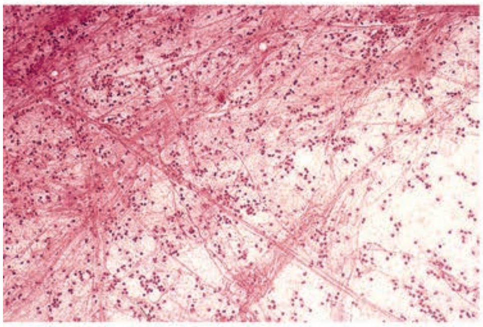
Specimens for anaerobic culture should be processed as soon as possible after arrival in the laboratory. Usual media include an anaerobic blood agar plate (CDC blood agar or brucella blood agar), a medium that inhibits Gram-positive and facultative Gram-negative bacilli such as KV blood agar (kanamycin-vancomycin), a differential or selective medium such as Bacteroides bile esculin, a Gram-positive

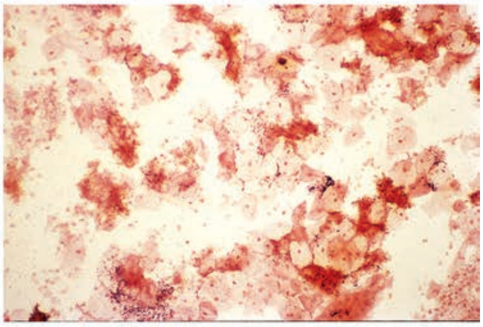


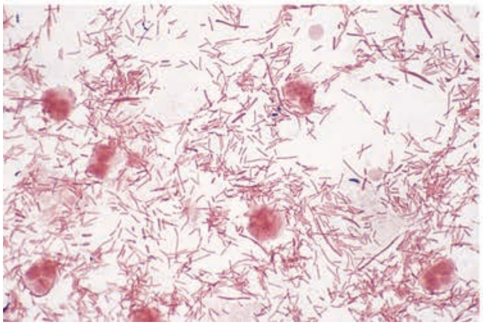












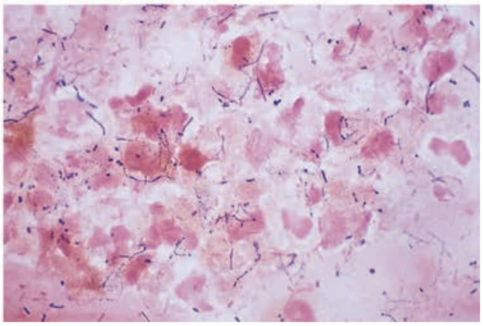


FIGURE 1 (row 1, left) Gram stain (×100) of surgical wound showing elastin fibers. doi:10.1128/9781555817381.ch18.f1.

FIGURE 2 (row 1, right) Gram stain (×1,000) of sputum showing Curschmann's spirals. doi:10.1128/9781555817381.ch18.f2.

FIGURE 3 (row 2, left) Gram stain (×1,000) of vaginal secretions showing clue cells. doi:10.1128/9781555817381.ch18.f3.

FIGURE 4 (row 2, right) Gram stain (×100) of unacceptable sputum specimen (grossly contaminated with members of the oropharyngeal microbiota) showing >10 SECs per low-power field. doi:10.1128/9781555817381.ch18.f4.

FIGURE 5 (row 3, left) Gram stain (×100) of acceptable sputum specimen showing <10 SECs per low-power field. doi:10.1128/9781555817381.ch18.f5.

FIGURE 6 (row 3, right) Gram stain (×100) of urine showing 4+ SECs, indicating gross contamination with vaginal or periurethral secretions and bacteria. doi:10.1128/9781555817381.ch18.f6.

FIGURE 7 (row 4, left) Gram stain (×1,000) of urine showing PMNs and 4+ Gram-negative bacilli. doi:10.1128/9781555817381.ch18.f7.

FIGURE 8 (row 4, right) Gram stain (×1,000) of a wound showing PMNs, mixed bacterial morphotypes suggesting aerobic and anaerobic bacteria, and both intra- and extracellular bacteria. This appearance suggests a mixed aerobic and anaerobic abscess or closed-space infection. doi:10.1128/9781555817381.ch18.f8.

selective medium (colistin-nalidixic acid blood agar or phenylethyl alcohol blood agar), and an enrichment broth such as chopped meat carbohydrate or thioglycolate (Table 7). Media should be incubated in an anaerobic environment immediately after inoculation. Incubation in anaerobic containers, such as GasPak jars (Becton Dickinson Microbiology Systems, Cockeysville, MD), AnaeroPack (Mitsubishi Gas Chemical America, Inc., New York, NY), or Bio-Bag Anaerobic Culture Set (Becton Dickinson Microbiology Systems) or in an anaerobic chamber is acceptable (54). A commercial product (Anoxomat; Mart Microbiology, Drachten, The Netherlands) can rapidly evacuate air and replace it with an anaerobic atmosphere in special jars for efficient anaerobic incubation. Anaerobes grow more slowly than aerobic or facultative bacteria. Jars or boxes should not be opened in air until after 48 h so that organisms in growth phase are not killed by exposure to oxygen (55). Anaerobic cultures should be held for at least 5 days before being reported as negative. Longer incubation (at least 7 days) of the broth is necessary for isolation of *Actinomyces*, *Propionibacterium* species from prosthetic joint infections, and some other fastidious anaerobes (56). See chapter 50 for further discussion.

6. For many reasons, it is best to retain specimens, even empty containers if the entire specimen was used. Sometimes additional tests are requested later after other results, such as histopathology, become available. Sometimes there is an error during laboratory processing and the original aliquot must be resampled. Other reasons include checking the label on the specimen, preparing a second smear if there is a question on interpretation of the first one, or examining the gross appearance of the sample. If possible, specimens should be stored in a refrigerator or ultracold freezer. A minimum of 5 days is recommended.

Culture Examination and Interpretation

Once colonies have grown on agar culture plates, microbiologists must differentiate potential pathogens requiring identification and antimicrobial testing from contaminants that represent members of the normal microbiota. Aids to interpretation are the relative quantities of each isolate, correlating culture results with Gram-stained smear results, and recognizing usual contaminants and pathogens from respective specimen sites. In general, when examining cultures of specimens from sites adjacent to normally colonized mucosae (e.g., sputum, urine, or superficial wounds), potential pathogens should outnumber indigenous members of the microbiota and should be seen in the direct Gram stain (3). When examining cultures of specimens from presumably sterile sites (e.g., CSF, joint fluids, other body fluids, and deep tissue), potential pathogens occur in any quantity and may or may not be seen in the direct Gram-stained smear. Specific criteria for identifying potentially significant isolates and contaminating members of the normal microbiota are addressed in the following sections of this chapter. It is a useful policy to save culture plates with significant growth for 1 week, allowing caregivers the opportunity to call to request further identification or additional antimicrobial testing when clinically indicated.

Specimens Received for Molecular Detection of Bacteria

Although blood has not yet been used widely for direct detection of bacteria using nucleic acid amplification tests (NAATs) or hybridization, such tests are being evaluated. Whole blood can be collected in EDTA tubes, in specially formulated acid-citrate vacuum tubes, or in other specialized tubes developed for NAATs. In cases in which plasma is the specimen for testing, removal of the liquid component from the red blood cells or using a plasma preparation tube as quickly as possible will provide the most accurate test results.

Tissue should be collected aseptically and submitted in a sterile container. Tissue is usually treated in some way to dissolve the matrix and release the nucleic acid contents of the microbe being sought; often this is done on an automated extraction instrument, or after initial grinding of the tissue, if necessary. If >24 h will pass before the test can be performed, tissue should be frozen at −70°C and transported to the laboratory on dry ice to prevent thawing. For culture, nasopharyngeal swabs and aspirates for *B. pertussis* or oropharyngeal swabs for *Mycoplasma pneumoniae* should be transported on ice, but room temperature transport is sufficient for molecular detection. Special swabs with proprietary transport media or first-void urine (<30 ml) are often sent for molecular detection of *N. gonorrhoeae* and *C. trachomatis*. The transport system should be specific for the type of test. Recommended procedures and transport systems vary widely depending on the laboratory and the test system employed. A recent guideline addresses these issues (1).

Reporting Results

"No growth"- and "normal microbiota"-type reports can be safely entered into a laboratory information system for routine reporting to be seen by the caregiver whenever the caregiver chooses to look. But reports that are important enough to result in a possible change of management or complex enough to require person-to-person discussion should be communicated directly to a caregiver via telephone or direct electronic messaging. In the case of acid-fast and fungal cultures, reports are often available days or weeks after the sample was received. Since the physician may no longer be actively following the case, it is especially important to telephone those results. Critical values, those that are immediately life-threatening, require the recipient of the call to write the result down and read it back to the caller. Each institution must develop its own critical-call procedure. Examples of critical values in bacteriology might include a positive CSF Gram stain, results suggestive of gas gangrene, a bioterrorism-related isolate, or a positive blood culture. Many microbiology results, including detection of a sexually transmitted infectious agent, detection of *M. tuberculosis*, or detection of a multidrug-resistant bacterium, should also be called to infection control personnel and/or local public health officials.

SPECIAL CONSIDERATIONS BASED ON SPECIFIC SPECIMEN TYPES (ALSO SEE TABLE 8)

Tissue Biopsy Samples

Tissue from a sterile site is usually obtained during a surgical procedure. Because this is among the most difficult and important specimens to get, it should be handled with utmost care and the surgeon should obtain sufficient volume for all possible tests, including histopathology and various microbiological studies. A 1-cm^3 piece should suffice for any microbiology requests. This is difficult with fine-needle aspirates and biopsy samples, which usually are minuscule. Tissue should be transported in a sterile container that maintains moisture. To avoid drying, small pieces of tissue can be moistened with a few drops of sterile, nonbacteriostatic saline. Alternatively, very small pieces of tissue can

TABLE 8 Usual etiologies of selected infectious disease syndromes

Disease	Etiologies
Central nervous system infection	
Acute meningitis, neutrophilic pleocytosis	*Streptococcus pneumoniae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus agalactiae*, *Haemophilus influenzae*, *Staphylococcus aureus*, Gram-negative rods[a], *Bacillus anthracis*
Acute meningitis, CSF shunt related	Coagulase-negative staphylococci, *Staphylococcus aureus*, *Propionibacterium* spp., Gram-negative enteric rods (e.g., *Escherichia coli* and *Klebsiella* spp.), Gram-negative nonfermenting rods (e.g., *Pseudomonas aeruginosa* and *Acinetobacter* spp.)
Chronic meningitis, predominantly lymphocytic pleocytosis	*Nocardia* spp., *Brucella* spp., *Leptospira interrogans*, *Mycobacterium tuberculosis*, *Treponema pallidum*, *Borrelia burgdorferi*
Gastrointestinal tract infection	
Infectious diarrhea[b]	*Salmonella* serotypes; *Shigella* spp.; *Campylobacter jejuni/coli*; *Campylobacter* spp. (other); STEC serotypes; *Clostridium difficile*; *Vibrio* spp.; *Aeromonas* spp.; *Pleisomonas shigelloides*; *Yersinia enterocolitica*; *E. coli* toxigenic, invasive, and effacing strains; *L. monocytogenes*; *Clostridium perfringens*; *Bacillus cereus*
Ingestion of preformed toxin[c]	*S. aureus*, *B. cereus*, *Clostridium botulinum*
Gastritis, gastric and duodenal ulcers	*Helicobacter pylori*
Genital tract infection	
Ulcers	*T. pallidum*, *Haemophilus ducreyi*, *Chlamydia trachomatis* (lymphogranuloma venereum), *Klebsiella granulomatis*
Urethritis	*Neisseria gonorrhoeae*, *C. trachomatis*
Vulvovaginitis	*N. gonorrhoeae* and *C. trachomatis* in prepubescent girls
BV	Overgrowth of vaginal biota with anaerobic bacteria
Cervicitis	*N. gonorrhoeae*, *C. trachomatis*
Endometritis	*Enterobacteriaceae* streptococci (groups A and B), enterococci, mixed anaerobic genera
Salpingitis, oophoritis	*N. gonorrhoeae*, *C. trachomatis*, mixed aerobic and anaerobic biotas
Pelvic abscess	Mixed aerobic and anaerobic biotas
Epididymitis	*N. gonorrhoeae*, *C. trachomatis*, *Enterobacteriaceae*, *P. aeruginosa*, various Gram-positive cocci
Ocular infections	
Conjunctivitis	*S. pneumoniae*, *S. aureus*, *H. influenzae*, *N. meningitidis*, *N. gonorrhoeae*, *C. trachomatis* (inclusion conjunctivitis), *C. trachomatis* (trachoma), others[d]
Keratitis	*S. aureus*, *S. pneumoniae*, *P. aeruginosa*, enterococci, *Streptococcus pyogenes* (group A), *Enterobacteriaceae*, *Pasteurella multocida*, coagulase-negative staphylococci (postsurgery), *Propionibacterium acnes* (especially postsurgery), rapidly growing mycobacteria, others[e]
Endophthalmitis	*S. aureus*, *P. aeruginosa*, *P. acnes*, *S. pneumoniae*, *N. meningitidis*, *Nocardia* spp.
Periorbital cellulitis	*S. aureus*, *S. pyogenes* (group A), *S. pneumoniae*, *H. influenzae*, *Clostridium* spp.
Otitis	
Otitis externa	*S. aureus*, *S. pyogenes* (group A), *P. aeruginosa*, *Vibrio alginolyticus*
Otitis media	*S. pneumoniae*, *H. influenzae*, *Moraxella catarrhalis*, *S. aureus*, rare pathogens: Gram-negative rods, anaerobes, aerobic actinomycetes
Respiratory tract infection	
Tracheitis, intubated patient	*Enterobacteriaceae*, *S. aureus*, *P. aeruginosa*, other nonfermenting Gram-negative rods
Bronchitis, community acquired	*S. pneumoniae*, *H. influenzae* (rarely, other *Haemophilus* species), *M. catarrhalis*, *S. aureus*, *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, *Bordetella pertussis*, *S. pyogenes* (group A); less commonly, same as hospital acquired
Bronchitis, hospital acquired	*Enterobacteriaceae*, *S. aureus*, *P. aeruginosa*, other nonfermenting Gram-negative rods; less commonly, same as community acquired
Pneumonia, community acquired	*S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *C. pneumoniae*, *M. pneumoniae*, *Legionella pneumophila*, *Nocardia* spp., *P. multocida*, aspiration (anaerobes); less commonly, same as hospital acquired
Pneumonia, hospital acquired	*Enterobacteriaceae*, *S. aureus*, *P. aeruginosa*, *L. pneumophila*, other nonfermenting Gram-negative rods, aspiration (anaerobes); less commonly, same as community acquired
Lung abscess	*S. aureus*, *Klebsiella pneumoniae*, *P. aeruginosa*, *S. pyogenes* (group A), anaerobes (aspiration pneumonia), *Nocardia* spp.
Empyema	*S. pneumoniae*; anaerobes; viridans group streptococci, especially *Streptococcus anginosus* group; *S. aureus*; *S. pyogenes* (group A); Gram-negative rods

(Continued on next page)

TABLE 8 Usual etiologies of selected infectious disease syndromes *(Continued)*

Disease	Etiologies
Osteomyelitis	*S. aureus*, *M. tuberculosis*, *P. aeruginosa*, other organisms depending on site
Urinary tract infection	
Prostatitis	*Enterobacteriaceae*, *P. aeruginosa*, enterococci
Urethral syndrome	Same as cystitis, but in lower numbers; *C. trachomatis*/*N. gonorrhoeae*; unknown, negative culture (about 15% of this disease group)
Cystitis	*Enterobacteriaceae*, especially *E. coli*; enterococci; *Staphylococcus saprophyticus* (women of childbearing age); nonfermenting Gram-negative rods; *Corynebacterium urealyticum* (patients with underlying urinary tract pathology)
Pyelonephritis	*Enterobacteriaceae*; enterococci; agents of bacteremia (descending infection), e.g., *S. aureus*

[a] Gram-negative rods, including *Enterobacteriaceae*, *P. aeruginosa*, and other nonfermenting Gram-negative rods.
[b] Disease caused by ingestion of bacteria followed by tissue invasion, toxin production, or other pathogenic mechanism.
[c] Disease caused by ingestion of preformed toxin.
[d] *Corynebacterium diphtheriae*, *M. tuberculosis*, *Francisella tularensis*, *T. pallidum*, *Bartonella henselae* (cat scratch), *P. multocida*, *Bacillus thuringiensis*, and *Moraxella lacunata*.
[e] *T. pallidum*, *N. gonorrhoeae*, *Moraxella* spp., *C. diphtheriae*, *Bacillus* spp., anaerobes, and nontuberculous mycobacteria.

be placed on a square of moistened sterile gauze. This serves to maintain moisture and allow easy identification by those receiving and processing the specimen. Large pieces of tissue, ~1 cm^3 or greater, maintain a reduced atmosphere in spite of brief aerobic transport. Oxygen-free transport may not be necessary in this circumstance, and the absence of anaerobic transport should not disqualify large pieces of tissue from anaerobic culture.

The laboratory may require the caregiver to prioritize the tests, as the volume will not be enough if several tests are ordered. The material can be emulsified and diluted in saline or broth, but that will compromise the sensitivity of any studies. Tissue is presumed to be uncontaminated by external or luminal microbes, but this is not always the case. Coagulase-negative staphylococci and *P. acnes* are particularly problematic in orthopedics, because they can cause slow, chronic infections in prosthetic joints and bones, sometimes manifesting years after placement of the prosthesis (52). A method to help the laboratory differentiate between contamination and true infection, especially useful for orthopedic joint prosthesis revision surgeries, is to collect as many as five separate pieces of tissue from different areas within the same surgical site. The laboratory should perform minimal processing of all five samples sufficient to detect staphylococci and propionibacteria in addition to complete processing of one sample as ordered by the physician. If three or more samples from the same general surgical field yield the same organism in culture, the organism is likely to be an etiological agent in that joint and not a contaminant (30, 31, 57). If anaerobic cultures are requested, the tissue must be transported in an anaerobic transport tube or vial, some versions of which maintain anaerobes extremely well even during overnight shipping (58). The same tissue can also be used for all other microbiological assays, as anaerobic transport vials are universal transport devices.

Cultures growing small numbers of bacteria not commonly associated with infection, such as coagulase-negative staphylococci, corynebacteria, propionibacteria, and saprophytic *Neisseria* spp., may represent contamination rather than true pathogens. One or two bacterial colonies growing on a single plate, of multiple plates inoculated, and not growing in broth culture, if used, generally represent contamination. Growth of one or two bacterial colonies on agar media not in the area of specimen inoculation or on streak lines in the second through fourth plate quadrants also is likely to represent contamination. In addition, bacteria considered contaminants are not detected in original Gram-stained smears. On the other hand, detection of the bacteria listed above as unlikely pathogens should always be reported when seen in the original Gram-stained smear, present in quantities above a few colonies, and when detected on or in multiple media.

Bone Marrow Transport and Culture

Bone marrow aspirates can be submitted for culture in lysis-centrifugation tubes (Wampole Isolator). The "pediatric" tube holds a maximum of 1.5 ml of specimen. One or more of the 1.5-ml tubes can be used. Aspirates may also be submitted in a sterile container containing anticoagulant. Sterile tubes with anticoagulant are less desirable since they use heparin, sodium citrate, or EDTA as an anticoagulant, all of which are more inhibitory than SPS (59, 60). Although SPS inhibits meningococci, gonococci, *Gardnerella vaginalis*, anaerobic cocci, and *Streptobacillus moniliformis*, these bacteria are unlikely to be detected in bone marrow aspirate specimens (61).

Although bone marrow aspirate cultures may be helpful in identifying disseminated fungal and mycobacterial diseases, they are unlikely to assist in the identification of usual bacterial diseases (62, 63). It is policy in some laboratories to consult with the ordering physician and suggest that routine bacterial culture is not necessary. In most cases, blood or other organ system culture is preferred for the identification of disseminated bacterial infections. Even if bone marrow culture is performed, direct Gram stain of bone marrow aspirates is not helpful and should not be a routine component of bacterial culture.

Lymph Nodes

Aspirates and biopsy samples from lymph nodes are usually sent to the laboratory for detection of nonbacterial infectious agents. However, studies of lymph nodes from patients with mycobacterial disease, cat scratch disease, plague (*Yersinia pestis*), brucellosis, or tularemia (*F. tularensis*) can be fruitful. Initial handling of the tissue itself and of the culture plates, whether showing growth or not, should be done with extreme care and employing all biosafety practices. Plates should be taped on both sides to allow circulation of atmosphere but not allow accidental opening. Manipulations of any colonies should be done with full biosafety standards employed until a highly virulent pathogen has been ruled out (also see chapter 14).

Necrotizing Fasciitis

Necrotizing fasciitis and gas gangrene (myonecrosis) are medical emergencies requiring immediate diagnosis and therapy that may include antimicrobials, surgical debridement, and the use of immunoglobulin and immunomediators to combat the fatal complications of severe septic shock (64, 65). Necrotizing fasciitis and gas gangrene are caused most commonly by toxin-producing *S. pyogenes*, other beta-hemolytic streptococci, *S. aureus*, *Clostridium* spp., and mixed aerobic and anaerobic bacteria (64, 66). Gram-stained smears generally show proteinaceous fluid, necrotic cell debris, rare or few PMNs (because of cell lysis), and the bacterial etiology. Culture should confirm the etiology and provide antimicrobial testing results where appropriate.

Cellulitis

Several studies have shown that aspirates from the leading edge of areas of cellulitis are not particularly fruitful. Injection of nonbacteriostatic saline and subsequent aspiration may be necessary to obtain enough material to culture (67, 68). Under the best of conditions, these specimens are unlikely to be positive (69). Blood cultures from patients with cellulitis also are unlikely to be positive (70). Tissue biopsy may be required if microbiological testing is deemed crucial for a rapidly progressing lesion.

Uncultivable Bacteria in Tissues

Some bacterial infections in tissue cannot be cultivated *in vitro* using standard microbiological media. These include tissue stages of syphilis caused by *Treponema pallidum*, donovanosis caused by *Klebsiella* (formerly *Calymmatobacterium*) *granulomatis*, rat-bite fever caused by *Spirillum minus*, and others. The organisms may be visualized by histopathologic stains. Newer molecular technologies are proving to be most reliable, especially for organisms causing genital ulcer disease and those found in biofilms (71, 72). The use of universal primer PCR to identify bacteria seen in tissue sections by histology, including *Tropheryma whipplei*, *Coxiella burnetii*, and *Bartonella henselae*, is gaining widespread favor (73, 74). Additional discussion can be found in chapter 48.

Placenta for Agents of BV

Placentas are sent to microbiology to determine the etiology of a preterm birth or if the obstetrician suspects an infection. A piece of tissue from the chorionic layer below the amnion should be obtained in an anaerobic transport vial. If the intact placenta is received in the laboratory, the top layer (amnion) of the placenta is cut using sterile scissors, and sterile forceps are used to pull it away from the underlying chorionic layer. Sterile scalpel or another sterile scissors is used to cut a small piece of the freshly exposed chorionic tissue for aerobic and anaerobic culture, and occasionally a piece can be placed into 2SP or SP4 transport medium for *Mycoplasma* or *Ureaplasma* culture. Molecular methods have not been productive (75). Chapter 62 has more information on detection of mycoplasmas.

Quantitative Culture

Tissue from traumatic wound or burn injury may be submitted for quantitative culture, with results of $\geq 10^5$ CFU/g being used to predict the likelihood of the development of wound-related sepsis (76). Limitations include the lack of reproducible results and the low predictive value compared to histologic examination of tissue. Swab cultures have been shown to correlate poorly with the biopsy culture results. Full-thickness punch biopsy samples should be collected for this process. To perform a quantitative culture, a portion of the specimen is weighed and homogenized in saline. The saline suspension is used to prepare serial dilutions for culture. Detailed procedures for quantitative tissue culture are given elsewhere (77).

Blood Cultures

Detection of bacteria and fungi circulating in the bloodstream is a major function of the microbiology laboratory (78–80). Key factors in obtaining optimal samples are thorough disinfection of the skin surface or the catheter port through which the blood will be obtained, an adequate volume of blood removed, and the appropriate timing of when to draw the blood, ideally before antibiotics have been administered. Blood obtained through an indwelling intravenous line is twice as likely to contain a surface organism contaminant, leading to a false-positive culture result, as is blood obtained through a peripheral skin site (81). It is important to recognize potential contaminants that grow, since treating contaminants as significant isolates is associated with unnecessary expense and dangers of antimicrobial misuse (82, 83). Common contaminants include coagulase-negative staphylococci, corynebacteria, *Bacillus* spp., and propionibacteria. In general, single cultures positive for these bacteria represent contamination. Multiple, separate cultures growing the same strain of these organisms are more likely to indicate a clinically significant bacteremia (78). Careful site preparation using 70% ethanol and chlorhexidine is critical to avoiding drawing up those contaminants, primarily coagulase-negative staphylococci (84). For patients for whom chlorhexidine is contraindicated, either tincture of iodine or povidone-iodine can be used after alcohol disinfection. All skin disinfectants are activated as they go from wet to dry on the skin surface, so a waiting period of 30 s (chlorhexidine) to 1 min (povidone-iodine) is necessary after application before the needle is inserted. Contamination rates of less than 2 to 3% are desired (85). Higher rates should be investigated and corrected by educational efforts (83, 85).

Another factor that aids the laboratory in interpreting blood culture results is to obtain two separate blood culture sets from two separate sites (78). Especially if one set has been obtained via a central or peripheral intravenous line, the second set should be drawn through well-prepared intact skin. Skin organisms involved in true sepsis should be recovered from both sites. Some hospital laboratories have a standard policy that blood culture orders always involve obtaining blood from two separate sites and inoculating at least two separate bottles. Recovery of all organisms is enhanced if two types of media are used. Most often, laboratories choose one aerobic and one anaerobic broth and atmosphere formulation (86). Even though the numbers of septicemias caused by strictly anaerobic bacteria are relatively low, the anaerobic medium can improve recovery of other facultative anaerobic bacteria as well and provides another opportunity to recover a greater range of bacteria. In addition, failure to detect an anaerobe can have serious consequences, as empirical therapy may not be adequate (87).

Traditional methods for culture of intravascular catheter tips are controversial for determining the source of a bacteremia because by the time the culture result is obtained, the catheter (if it was the source) has already been removed (88). These cultures should be performed by special request only, and only when concurrent blood cultures are obtained. The most common technique used to culture the exterior of the intravascular portion of a catheter is the semiquantitative method in which the 5-cm distal portion of the catheter is rolled across a blood agar plate four times (88). The catheter tip is discarded. Growth of >15 colonies is considered to be significant, i.e., implicating the catheter tip as the likely source of a bacteremia caused by the same organism simultaneously isolated from blood cultures. Interpretation of clinical importance is not possible without an accompanying positive blood culture. Soft tissue infections around a catheter insertion site should be cultured as a wound specimen using freshly expressed purulence that can be aspirated.

Some laboratories have adopted a method for determining if the indwelling intravenous (usually central) line is the source of a bacteremia without removing the line. Because many intravenous lines are removed unnecessarily, this method can preserve those lines not considered colonized, as determined by a higher number of organisms recovered in the blood obtained through the line than that obtained peripherally. Determining CFU is too labor-intensive, so a surrogate marker, the time to positivity in an automated blood culture system, has been used to arrive at similar conclusions, with the assumption that a faster time to a positive signal in the instrument, more than 2 h before the peripheral blood culture flags positive, relates to a higher original number of organisms in the culture and thus a colonized line requiring removal (89). Both catheter-tip surface cultures and time-to-positivity methods appear to predict catheter-related bloodstream infection better when used together than does either one used alone (90). Neither seems to work well to predict catheter-related candidemia (91).

The Isolator system is additionally utilized to recover fastidious organisms or those that do not multiply well in standard blood culture broth formulations or atmospheres. If specialized broth/atmospheres available for recovery of fungi and mycobacteria are not used routinely, laboratories should stock a supply of Isolator tubes and the centrifuge and capping instruments required to use the Isolator tubes. Because the white blood cells are lysed, intracellular organisms are released and better able to grow in cultures. The sediment containing the organisms can be inoculated onto any number of specialized media to enhance recovery. Infectious agents best recovered by the Isolator system include filamentous or dimorphic fungi (*Fusarium* and *Histoplasma capsulatum* are examples), some yeasts (*Cryptococcus* and yeasts from patients on antifungal therapy), *Bartonella* species, some *N. meningitidis* strains, *N. gonorrhoeae*, and other fastidious organisms that fail to grow in routine blood culture media or that fail to produce sufficient metabolites to trigger the detection system of an automated blood culture instrument (92). Direct plating of blood onto selective agar or performing a blind subculture after 4 days of incubation has been shown to recover *Legionella* from blood cultures better than the Isolator system (93). The Isolator system does not perform as well as other systems when recovering *S. pneumoniae*, other streptococci, *P. aeruginosa*, and anaerobes (94). Advantages of the Isolator system are the ability to inoculate the pellet to specific agar media when attempting to detect unusual etiologies of bacteremia, such as those caused by *F. tularensis* and *Bartonella* spp., and to provide colony counts, reported as CFU per milliliter of blood. Disadvantages of the system are the labor involved with initial processing and the potential for increased contamination that accompanies manipulation during processing (95).

Total volume of blood cultured is the major factor for identifying true-positive patients. For an adult, the most sensitive recovery requires at least 40 ml of blood (86). This is a small percentage of total blood volume. Yields from 20 ml and 30 ml of blood were 29.8 and 47.2% greater, respectively, than those from 10 ml of blood; yields from 40 ml were 7.2% greater than from 30 ml (86). In fact, more blood can and should be obtained from all patients than is currently practiced. Table 9 shows recommended blood culture volumes based on the weight of the patient and the corresponding percentage of the patient's total blood volume (96). Blood should be collected as quickly as possible after the culture is ordered and before antibiotics are given, if possible, and all the blood can be obtained at the same time (from different sites) (97, 98).

For automated blood culture systems, the standard 5-day protocol is sufficient to recover all the usual pathogens and some unusual ones, such as *Legionella* spp., and holding bottles for additional incubation time only increases the numbers of contaminants recovered (93, 99, 100). For patients with suspected bloodstream infections that fail to yield a pathogen with standard blood culture protocols, either the Isolator tube should be employed for culture or serological or molecular methods (which may not be available) must be attempted (78). However, blind subculture may be needed for *Legionella* recovery or if the patient is receiving antimicrobials at the time of blood collection, particularly for recovery of yeast species (99).

In most cases, timing of the blood culture with respect to the maximum temperature recorded for the patient during a febrile episode is less important than total volume cultured. This has been reinforced by results of a multicenter study (98). Although there was a slight benefit for patients <30 years old for blood cultures to be obtained >1 h after the maximum temperature, this was not seen with other age groups, and in general, only 10% of positive blood cultures were actually drawn at the time of the patient's peak temperature.

Semiautomated blood culture systems are present in nearly every clinical laboratory. Refer to chapter 4 for a complete discussion of manual and automated blood culture

TABLE 9 Recommended blood volumes to obtain for blood cultures[a]

Patient weight (lb)	Recommended blood vol per culture (ml)	Total blood vol for two cultures (ml)	Volume of blood equal to 1% of patient's total blood volume (ml)[b]
<18	1	2	2
18–30	3	6	6–10
30–60	5	10	10–20
60–90	10	20	20–30
90–120	15	30	30–40
>120	20	40	>40

[a] Data from reference 96.

[b] Blood volume calculated by assuming 85 ml/kg in newborns and 73 ml/kg in other patients. Two 20-ml blood specimens collected from an 80-kg adult (40 ml total) represent ~0.7% of the patient's total blood volume.

systems. The anticoagulant used in all blood culture systems is SPS and is known to be inhibitory to meningococci, gonococci, *Peptostreptococcus anaerobius*, *S. moniliformis*, and *G. vaginalis* (61), for which other methods of detection should be employed.

Positive blood culture bottles are evaluated initially by examining a Gram-stained smear of the broth (101). The report should include a description of the bacterial morphology and the Gram reaction. If a presumptive identification of the microorganism can be made, it may be added to the report. For example, a blood culture Gram stain report might state "Gram-positive cocci in clusters suggesting staphylococci." Specimens in positive blood culture bottles should be subcultured to media based on the organism seen in the Gram-stained smear. In addition, since 5 to 10% of all bacteremias are polymicrobial (contain more than one bacterial type), additional media are recommended. Anaerobic media and culture conditions should be used if the morphology of the organism seen in the Gram-stained smear is suggestive of an anaerobic bacterium or if the organism is recovered from an anaerobic culture bottle only. If a positively flagged bottle shows no organisms on Gram stain, a second smear should be prepared and stained using acridine orange, a DNA stain (102). If organisms are present, even if they have damaged or no cell walls, they will stain bright orange and can be recognized more easily.

Rapid sample preparation and improvements in matrix-assisted laser desorption ionization–time-of-flight mass spectrometry databases have made identification of organisms from positive blood cultures by mass spectrometry more feasible for clinical laboratories (103). Other molecular methods, performed directly from positive blood cultures and available from several manufacturers, are helping to shorten the time to result reporting. A PCR method for detection of a number of pathogens from blood obtained directly from the patient is available in Europe (Roche Molecular Diagnostics, Basel, Switzerland). See chapter 4 for more details on matrix-assisted laser desorption ionization–time-of-flight mass spectrometry and related technologies.

Gram staining a smear of peripheral blood or buffy coat layer may detect bacterial cells in the blood of patients with meningococcemia, *S. pneumoniae* infection, or overwhelming sepsis caused by other bacteria when the bacterial concentration in blood is very high (approaching 10,000 bacterial cells per ml of blood). In spite of published reports documenting the occasional use of direct staining of blood, the likelihood of results affecting patient management does not warrant use as a routine laboratory procedure.

Diagnosis of leptospirosis may prompt a special blood culture request (see below). A recommended method is to use 0.1 ml of heparinized whole blood or 0.2 ml from high-speed-centrifuged plasma sediment inoculated into Ellinghausen-McCullough-Johnson-Harris medium and incubated from a minimum of 1 month up to 3 months (104).

Cerebrospinal Fluid

CSF is collected for the diagnosis of meningitis (105). Bacterial meningitis can be divided into acute and chronic clinical presentations (106, 107). Acute meningitis with onset of symptoms within the previous 24 h is usually caused by pyogenic bacteria. Specific etiologies are related to age of the patient and whether the disease is community or nosocomially acquired. Chronic meningitis, with symptoms lasting at least 4 weeks, can have a wide variety of causes (Table 8). As with all clinical specimens, even those collected from a presumably sterile site, growth of contaminants occasionally does occur. The first tube collected by lumbar puncture, most likely to have a few skin organisms introduced during the procedure, should be used for chemistry tests; the second or third tube, less likely to contain the rare contaminant, should be used for microbiology studies.

CSF is usually obtained by lumbar puncture. Although bacterial stain and culture can be performed with as little as 0.5 ml of fluid, larger volumes are preferred since culture methods are more sensitive when low numbers of bacteria are concentrated by centrifugation before culture. A minimum of 5.0 ml and high-speed (3,000 × *g*) centrifugation are recommended for recovery of *M. tuberculosis*, although the yield may still be low (108). Filtration through a 0.45-μm-pore-size filter results in better concentration of *M. tuberculosis* in CSF. Specimens should be transported to the laboratory immediately in a sterile container maintained at room temperature. All smears should be prepared by cytocentrifugation (109), and cultures should be inoculated with 0.5 ml of CSF or sediment resuspended in 0.5 ml of CSF following centrifugation (1,500 × *g* for 15 min) when >1 ml of fluid is received. Media are inoculated in accordance with recommendations in Table 7. PCR and a commercially available probe amplification technology have been reported to be sensitive and specific for the detection of *M. tuberculosis* in CSF (110–112). Anaerobes are rarely found in CSF, even in cases of mixed anaerobic abscess of brain tissue. However, anaerobic incubation of CSF is necessary to recover microaerophilic streptococci that cause infection after trigeminal nerve injection. Broth enrichment should not be used routinely (51). *P. acnes* may be the cause of infection in patients with indwelling CSF shunts, hence the recommendation to inoculate an anaerobic broth such as thioglycolate or chopped meat carbohydrate with an aliquot of shunt fluid (113).

Smears should be Gram stained and results reported to the physician immediately. Results should include a description and semiquantitative enumeration of polymorphonuclear inflammatory cells and bacterial morphology. If the results are suggestive of a bacterial group, this too can be communicated. Because of low sensitivity and lack of effect on the care and management of patients (in the developed world), and because the cost of antigen testing is much higher than the cost of the Gram stain, direct antigen tests should be considered only when direct communication with the clinical service documents a specific need, such as prior antimicrobial therapy (114). However, in locations where empiric antibiotics are not standard of care and laboratory skill levels are low, bacterial antigen tests may be useful adjunctive tests (115). Note that cryptococcal antigen tests are still valuable. Direct nucleic acid amplification of panmicrobial RNA and DNA from CSF will likely completely replace the residual antigen tests in laboratories that can justify the expense; one company has already received funding for development of such a panel. Leptospires can be detected in CSF during the first 10 days of acute illness. CSF should be collected before initiation of antimicrobial therapy and while the patient is febrile. Direct detection of leptospires by dark-field examination of CSF is not recommended. CSF should be inoculated into Ellinghausen-McCullough-Johnson-Harris medium. An inoculum of 0.5 ml is recommended. Cultures should be incubated at room temperature for up to 13 weeks (37) (see chapter 58).

Eyes

Several types of specimens may be collected for the microbiological analysis of eye infections, including conjunctival scrapings obtained with a swab or sterile spatula for the diagnosis of conjunctivitis, corneal scrapings collected with a sterile spatula for the diagnosis of keratitis, vitreous fluid collected by aspiration for the diagnosis of endophthalmitis, and fluid material collected by aspiration or tissue biopsy for the diagnosis of periorbital cellulitis (116). Pathogenic bacteria potentially present in these anatomic sites are listed in Table 8 (117). Because the volume of specimen collected from corneal scrapings and vitreous fluid aspiration is very small, direct inoculation of agar culture plates and preparation of smears in the clinic or at the bedside is recommended (116). A close working association is needed between the laboratory and ophthalmologist to ensure a supply of appropriate culture media, correct technique for inoculation of media, and rapid transport of plates and smears to the laboratory. Media should be inoculated by rubbing the specimen onto a small area of the agar plates (swab) or wiping the material from the scraping off the spatula gently onto the agar surface, sometimes in the shapes of little C's. Plates are placed directly into the incubator without cross-streaking by laboratory personnel. This allows the plate reader to detect more easily airborne contaminants that settle on the plate during inoculation procedures that occur outside controlled laboratory conditions.

Media needed for the detection of usual pathogens should include chocolate agar for fastidious bacteria and most fungi (Table 7) (118). Media for other microorganisms (some fungi, viruses, mycobacteria, etc.) should be inoculated if deemed appropriate by the ophthalmologist and microbiologist and specifically ordered. Incubation at 35 to 37°C in 3 to 5% CO_2 is necessary.

C. trachomatis in Eyes

Detection of chlamydial nucleic acid (nucleic acid amplification [NAA]) in conjunctival scrapings is the test of choice in the diagnosis of inclusion conjunctivitis or trachoma, despite the lack of FDA-cleared tests in the United States (119, 120). Examination of conjunctival smears using direct fluorescent-antibody (DFA) staining with fluorescein-conjugated monoclonal antibodies is possible for those laboratories with the skilled staff, appropriate reagents, and UV microscope available. A less sensitive but readily available method is examination of Giemsa-stained conjunctival smears for intracytoplasmic, perinuclear inclusions within epithelial cells (121). Cell culture for the isolation of *C. trachomatis* is more time-consuming and technically demanding than DFA or NAA and less sensitive than molecular methods (122). Conjunctival scrapings and secretions for culture should be transported in 2SP medium (sucrose phosphate or sucrose phosphate glutamate) with bovine serum and antimicrobials (usually gentamicin, vancomycin, and nystatin or amphotericin B). Swabs with wooden shafts should be avoided, since constituents of the wood are toxic to chlamydiae. Specimens for culture should be refrigerated during short delays or stored at −70°C for delays longer than 48 h.

Ears

Nasopharyngeal cultures have poor predictability for detection of middle ear pathogens and should not be used for that purpose (123). Two types of ear specimens are received most commonly by the laboratory: swab specimens for the diagnosis of otitis externa and middle ear fluid specimens for the diagnosis of otitis media (Table 8). Potential pathogens at these two sites differ (124). Since anaerobic bacteria may be involved in middle ear infections (albeit rarely), anaerobic culture, if ordered, should be performed on properly collected aspirates that are transported in anaerobic transport vials. Middle ear fluid is less likely to be contaminated than external ear specimens. All isolates from middle ear aspirates should be reported and, if requested, antimicrobial testing performed on strains with unpredictable susceptibility to antimicrobials. Direct examination of Gram-stained smears of middle ear fluid is helpful and is recommended with all culture requests. Due to poor yield of bacterial cultures, physicians usually treat empirically.

External ear specimens may be contaminated with normal members of the microbiota of the skin or ear canal. Isolates of coagulase-negative staphylococci, diphtheroids, and viridans group streptococci may be listed as presumptive identifications without including results of antimicrobial testing. Even *S. aureus* is often recovered from patients without infection, so the presence of inflammation should be used to help guide the extent of workup (125). It is a useful policy to save the culture plates for 1 week, allowing physicians the opportunity to call to request further identification or antimicrobial testing when clinically indicated.

Genital Discharge, Exudates, and Lesions

Bacterial infections of genital tract sites produce various clinical syndromes, including vulvovaginitis, BV, genital ulcers, urethritis, cervicitis, endometritis, salpingitis, and ovarian abscess in females and urethritis, epididymitis, prostatitis, and genital ulcers in males. These diseases and their etiologies are listed in Table 8.

Many specimens are contaminated with members of the normal microbiota of the skin or mucous membranes. Pathogens such as *Haemophilus ducreyi*, *N. gonorrhoeae*, *Trichomonas vaginalis*, *T. pallidum*, and *C. trachomatis* are always significant. Other organisms such as *S. aureus*, beta-hemolytic streptococci, *Enterobacteriaceae*, and anaerobes are pathogenic only in certain clinical situations. The specimen source, relative quantity of potential pathogen compared to members of the normal microbiota, and Gram stain interpretation help the technologist determine which isolates require identification and antimicrobial testing. At a minimum, isolates from presumably sterile specimens and pure or predominant potential pathogens from specimens likely to be contaminated with members of the normal microbiota and containing PMNs should be identified and reported. Mixtures of anaerobes do not require individual identification and listing in most cases. Laboratories should avoid isolating, identifying, and performing antimicrobial testing on every bacterial isolate from all specimens (126). In addition to the excessive cost of this approach, unnecessary reporting of bacterial species contributes to excessive treatment of patients. Exact protocols for workup and reporting may require discussion and mutual agreement with knowledgeable clinicians in each practice environment.

Detection of *N. gonorrhoeae* and *C. trachomatis*

NAA methods are the state of the art for detection of *N. gonorrhoeae* and *C. trachomatis* in endocervical, vaginal, urethral, and urine specimens. Users must pay close attention to the types of specimens approved for use with each kit. Specimens should be collected using the procedures and devices recommended by the manufacturer. In addition, false-positive and false-negative reactions have been reported with some kits, particularly when nonvalidated sample types are tested, necessitating confirmation of results from some commercial assays (127–130).

Although eye, rectal, oropharyngeal, and abscess specimens are not FDA cleared for testing in the commercial amplification systems, molecular amplification is still the best method (131, 132). In fact, self-collected swabs by both men and women perform at least as well as those collected by health care personnel for molecular tests for *C. trachomatis* and *N. gonorrhoeae* (133). At this time, culture is still the only acceptable diagnostic procedure in some jurisdictions for medical-legal cases, but that should be changing soon (1).

Culture for *N. gonorrhoeae* is optimal when the specimen is directly inoculated to a selective medium, such as modified Thayer-Martin medium, and incubated immediately (35). Swab specimens (cotton swabs should be avoided because they may be toxic) should be placed in a transport system containing Stuart's or Amies medium and delivered to the laboratory as quickly as possible. A new document from the Association of Public Health Laboratories contains much useful information (http://www.aphl.org/AboutAPHL/publications/Documents/ID_2014Mar_Transportation-of-Specimens-for-Neisseria-gonorrhoeae-Culture.pdf). Some newer products have shown good ability to retain the viability of *N. gonorrhoeae* (34). Specimens for *N. gonorrhoeae* culture should be held at refrigeration temperature during transport (35, 36). As transport time increases, recovery by culture decreases. Specimens requiring more than 24 h for transport are unacceptable. Recent publications have shown that for specimens from ocular, pharyngeal, or anal sites, molecular methods are far superior to culture (132, 134, 135). In fact, one system outperformed other methods. Molecular tests are replacing culture even for genital samples, although the need for isolates to perform antimicrobial susceptibility testing and for patients refractory to empiric treatment is still important (130, 136). Follow guidelines from the test manufacturer for specimen collection and transport.

Although not a bacterium, *T. vaginalis* should now be routinely sought in genital infections due to the availability of commercial NAATs. Trichomoniasis is now known to be more common than either chlamydia or gonorrhea, primarily among women (137). See chapter 140 for more information.

BV and Vaginitis

BV occurs when conditions result in overgrowth of usual vaginal microbiota with various anaerobic genera, including *Mobiluncus* and *Bacteroides* (138). Although not characterized by a polymorphonuclear response, BV results in an increase in vaginal secretions that are relatively alkaline (pH > 4.5) compared to normal, the usual predominant microbiota of lactobacilli being replaced by anaerobes, and the presence of aromatic amines, which are detected by adding 10% potassium hydroxide and noting a pungent, fishy odor. In addition, excessive growth of the facultative bacterium *G. vaginalis* generally coincides with BV. Although *G. vaginalis* commonly is a member of the normal vaginal microbiota, the presence of increased concentrations that adhere to vaginal SECs, called clue cells, is characteristic of BV. Clue cells are SECs peppered with *G. vaginalis* bacteria, frequently showing heavier adherence toward the periphery of the cell and appearing like a doughnut (Fig. 3). New studies are showing that the bulk of bacteria actually associated with BV are identified only by molecular analysis, which at this time is beyond the scope of routine diagnostic laboratories (139). BV is still diagnosed best without culture (140, 141). Gram-stained smears should be examined and interpreted according to Table 10. In summary, until molecular amplification tests are available, BV should be diagnosed by performing a bedside pH and KOH "whiff test" and a laboratory Gram stain (142).

A combination probe assay (Affirm VPIII identification test; Becton Dickinson, Sparks, MD) is commercially available for the simultaneous detection of *Candida* spp., *G. vaginalis* (as a surrogate indicator for BV), and *T. vaginalis* in vaginal secretions, which in some studies outperforms more classical methods (143).

Screening for Group B *Streptococcus*

Pregnant women colonized vaginally or rectally with group B streptococci prior to delivery are at increased risk of infecting their newborns during delivery. Such women need intrapartum prophylaxis; currently 25% of all women receive antibiotics during labor in the United States, although the incidence of neonatal disease is only 0.3% (144). Current recommendations are for collecting vaginal and rectal swabs at 35 to 37 weeks of gestation for culture in a single enrichment broth such as LIM broth (145). This broth is incubated at 35°C for 18 to 24 h and subcultured to a blood agar plate the following day. Recent studies have shown that this approach misses ~10% of women who become colonized after their screening test, and a substantial number of women convert to negative and would not require prophylaxis (146–148). Real-time PCR performed on the enrichment broth culture has been used to improve sensitivity, but this approach still requires at least an 18-h turnaround time, not enough to allow timely use of intrapartum chemoprophylaxis for prevention of disease (149, 150). Newer PCR tests allow high sensitivity and specificity and rapid turnaround time, and some products can be performed by non-laboratory personnel at the time of delivery (151, 152). New recommendations are likely to include use of such tests for women who have not been screened previously or those with negative screening culture results (144, 145).

Dark-Field Examination for *T. pallidum*

Dark-field examination of tissues, tissue exudates, and material collected from chancres can be used to confirm the diagnosis of syphilis. For dark-field microscopy, the specimen should be examined within 20 min of collection to ensure motility of treponemes and should not be exposed to temperature extremes during transport to a dark-field microscope. The test requires a microscope equipped with a dark-field condenser and experienced personnel who are

TABLE 10 Diagnosis of BV using a Gram-stained smear of vaginal secretions, by the Vaginal Infection and Prematurity Study Group criteria[a]

Morphotype	No. of morphotypes seen per oil power field					Score
	None	≤1	1–5	5–30	>30	
Lactobacillus morphology	4	3	2	1	0	______
Gardnerella/Bacteroides spp.	0	1	2	3	4	______
Curved Gram-variable rods (*Mobiluncus* spp.)	0	1	2	3	4	______
Total score						______

[a] Adapted from reference 42. A total score of 0 to 3 is considered normal, 4 to 6 is intermediate, and 7 to 10 indicates BV.

able to recognize *T. pallidum* spirochetes based on the tightness and regularity of the spirals and on its characteristic slow, graceful, corkscrew movement (153). Only a few laboratories perform this test anymore. A DFA stain can be used and is performed on air-dried smears. The stability of the smear during transport and the easily identified, fluorescing treponemes make the DFA an attractive alternative to dark-field microscopy (154). Unfortunately, reagents for the DFA test are not commercially or widely available; it may be performed at some public health laboratories.

H. ducreyi

If infection with *H. ducreyi* is suspected, material from the base of the ulcer is collected and held at room temperature until it can be processed (155). One swab is used to prepare a smear for Gram staining. The presence of many small, pleomorphic Gram-negative bacilli and coccobacilli arranged in chains and groups ("school of fish") suggests *H. ducreyi* but is rarely seen (see chapter 36). Recovery of the organisms by culturing on an enriched medium such as GC agar containing 3 μg of vancomycin/ml, 1% hemoglobin, 5% fetal bovine serum, and 1% IsoVitaleX or Mueller-Hinton agar with 5% horse blood, 1% IsoVitaleX, and 3 μg of vancomycin/ml is necessary to confirm the diagnosis; culturing at 33°C yields better recovery than does culturing at 35°C (see chapter 36). Genital ulcer disease is much better diagnosed with molecular methods (71).

Actinomyces spp.

Certain organisms classically referred to as *Actinomyces* spp., such as *A. israelii*, may cause pelvic inflammatory disease in women who use intrauterine contraceptive devices (IUDs) (156) (also see chapter 52). An IUD submitted for culture should be placed in a sterile liquid medium (preferably reduced, such as thioglycolate) and vortexed, and the liquid medium should be used to inoculate aerobic and anaerobic culture media. Inflammatory debris and tissue attached to the IUD should be removed and cultured aerobically and anaerobically. Mixed infections with *Aggregatibacter actinomycetemcomitans* and *Actinomyces*, sometimes mistaken for malignancy, involve bone or other tissue sites (157). *Actinomyces* spp. produce small knots of intertwined bacterial filaments called grains or granules, which may be 1 mm or more in diameter. These grains should be crushed on a slide for staining (Gram stain is acceptable), and transferred to medium for culture. See chapter 52 for more information. The presence of branching Gram-positive filaments suggests *Actinomyces* in this clinical setting; culture confirms the diagnosis. Molecular methods have been found to be more sensitive in at least one study (158).

Lower Respiratory Tract

Specimens from the lower respiratory tract are submitted to determine the etiology of airway disease (tracheitis and bronchitis), pneumonia, lung abscess, and empyema (159–161). Table 8 gives a list of lower respiratory tract diseases and their common etiologies. Usual specimens submitted consist of expectorated sputum; induced sputum; endotracheal tube aspirations (intubated patients); bronchial brushings, washes, or alveolar lavages collected during bronchoscopy; and pleural fluid (162). Specimens should be delivered to the laboratory promptly and processed without delay (within 1 h of collection). If delays are unavoidable, the specimen should be refrigerated. *S. pneumoniae*, the most common etiology of bacterial pneumonia, is very susceptible to conditions outside the body and may be missed by culture when the sample is not plated immediately. A urinary antigen test for *S. pneumoniae* may be more sensitive in such cases (23, 163, 164). Given the difficulty of performing cultures for *Legionella pneumophila*, urinary antigen testing is recommended as an alternative method for diagnosis of legionellosis (165).

Lower respiratory tract secretions containing pathogens usually contain acute inflammatory cells (PMNs). Frequently, pathogenic bacteria are present within the PMNs. With the exception of aspiration pneumonia, characterized by mixed morphologies of bacteria including anaerobic species, pneumonia is caused by one or two organisms, and they should be predominant on Gram stain and culture. Endotracheal aspirates and sputum with numerous SECs are contaminated with upper respiratory tract organisms, and routine cultures cannot be used to determine the agent of the pneumonia. Quantitative cultures may enhance utility of endotracheal aspirates (166). Most bacterial lower respiratory tract disease is caused by inapparent aspiration of oropharyngeal contents. It follows that oropharyngeal microbiotas include the bacteria that cause lower respiratory tract disease. Detection of a potential pathogen in a grossly contaminated specimen may represent contamination with an oropharyngeal microbiota. The lack of usefulness of data from contaminated specimens has resulted in policies for screening and rejecting grossly contaminated respiratory tract specimens. There are many ways to assess the quality of respiratory tract specimens. A simple screening method involves assessment of SECs only (19, 167). SECs are found in the oropharynx but not in the lower respiratory tract. Increased numbers (defined as >10 per 10× objective microscopic field) indicate gross contamination with oropharyngeal contents, which includes usual members of the oral bacterial microbiota (Fig. 4). Acceptable sputum samples usually show numerous PMNs and rare SECs and often have mucous strands (Fig. 5). Table 5 lists respiratory tract specimens and usual screening policies. Respiratory tract specimens for the detection of *M. pneumoniae* and *Legionella* spp. should not be screened, as stated above, for adequacy. All specimens representing the lower respiratory tract are considered acceptable for the detection of these microorganisms (168). Different methods have been used to determine adequacy of samples for detection of *M. tuberculosis* (see chapter 30).

Bacteria should be reported when detected in Gram-stained smears if they are potential pathogens. Bacteria not in sufficient quantity or not representative of morphotypes resembling potential pathogens should be lumped together and reported as members of the normal respiratory microbiota. It is important to differentiate contaminating members of the respiratory microbiota from members of the respiratory microbiota causing aspiration pneumonia. Aspiration of relatively large amounts of oropharyngeal contents following loss of consciousness, paralysis of muscles involved with swallowing and breathing, or medical procedures such as intubation can result in infection of the airways with mixed members of the respiratory microbiota, leading to lung abscess and empyema (169). Gram stain of sputum from patients with aspiration pneumonia can be highly suggestive of the diagnosis. Stained smears show many PMNs and many mixed respiratory microbiota morphotypes, especially those suggesting streptococci and anaerobes. Much of the microbiota is intracellular. Aspiration pneumonia can be detected in hospitalized patients and those admitted directly from the community (170, 171).

Cultures of respiratory tract material should include a selective Gram-negative medium, such as MacConkey agar, sheep blood agar, and chocolate agar for the detection of *Haemophilus* spp. (Table 7). Culture plates should be incubated at 35°C in 3 to 5% CO_2 for 48 h before being reported as negative. Cultures are interpreted by examining the relative numbers and types of bacteria that grow and correlating these results to the Gram stain. Table 11 summarizes interpretative criteria used with respiratory tract specimens.

Special Considerations for Lower Respiratory Tract Specimens

Specimens Collected during Bronchoscopy

Bronchoalveolar lavage fluid and bronchial brush specimens from patients with suspected pneumonia should be cultured quantitatively to evaluate the significance of potential pathogens recovered (172, 173). Bronchial brush specimens, which contain approximately 0.01 to 0.001 ml of secretions, should be placed in 1 ml of sterile nonbacteriostatic saline or lactated Ringer's solution, which is less inhibitory to *Legionella* species. If anaerobes are to be cultured, the broth must be chopped meat or freshly boiled thioglycolate. The specimen should be delivered to the laboratory immediately. In the laboratory, the specimen is agitated on a vortex mixer, a smear is prepared by cytocentrifugation for staining with the Gram stain, and 0.01 ml of specimen is inoculated to appropriate medium by using a pipette or calibrated loop. Any growth of >10 colonies per plate of potential pathogens (corresponding to 10^6 CFU/ml of original specimen) appears to correlate with disease. Bronchoalveolar lavage results in the collection of 50 ml or more of saline from a larger lung volume. In the laboratory, a smear is prepared by cytocentrifugation and Gram stained (174). The Gram stain report should include a comment about the presence of SECs and intracellular bacteria. Grossly contaminated fluid (>1% of all cells are SECs) may have falsely elevated counts of potential pathogens. Intracellular bacteria are more likely to be potential pathogens. A 0.01- or 0.001-ml aliquot of bronchoalveoloar lavage fluid should be inoculated to agar media (Table 7). The recovery of <10,000 bacteria/ml suggests contamination. The recovery of >100,000 bacteria/ml suggests that the isolate is a potential pathogen. Detection of 10,000 to 100,000 bacteria per ml represents a "gray" zone (175). Counts of pathogens may be reduced by prior antimicrobial therapy or variations in "return" of lavage fluid during the bronchoscopy procedure (Table 11).

Legionella spp.

Legionella spp., especially *L. pneumophila*, are important causes of community- and hospital-acquired pneumonia (176) (also see chapter 49). Legionellosis can be diagnosed by culture, DFA staining of smears of respiratory secretions, detection of antigens in urine, or serological testing. Although culture has been preferred historically because, unlike other methods, it is not limited to detection of certain species or serotypes, many laboratories now offer urinary antigen testing instead. The urinary antigen tests for *L. pneumophila* serotype 1 are relatively sensitive (24, 177, 178). The immunochromatographic product used most commonly (BinaxNOW Legionella; Inverness Medical, Waltham, MA) was 95% sensitive with frozen urine samples (product insert) and 100% sensitive in a recent outbreak investigation in Germany (179). Antigen tests for other serotypes are not widely available. Before culture, respiratory samples should be diluted 10-fold in a bacteriologic broth, such as tryptic soy, or sterile water to dilute inhibitory substances that may be present in the specimen. Because legionellae grow slowly, optimal isolation from highly contaminated specimens, such as sputum, is achieved by decontaminating the specimens with acid before plating and special media are employed (180, 181). The specimen is diluted 1:10 in KCl-HCl buffer (pH 2.2) and incubated for 4 min at room temperature. It is important not to incubate the specimen for longer than 4 min because legionellae may themselves be killed by acid exposure. Specimens are inoculated onto buffered charcoal-yeast extract (BCYE) agar with and without antimicrobial agents (e.g., vancomycin, polymyxin B, and anisomycin) and often other agars (181). The cultures are incubated in humidified air at 35°C for a minimum of 5 days. Using a dissecting microscope, small colonies with a ground-glass appearance, typical of *Legionella* spp., can be detected after 3 days of incubation. NAA for *Legionella* is sensitive and specific, although no commercial product is available yet (182).

M. pneumoniae

M. pneumoniae is a common cause of pneumonia, referred to as primary atypical pneumonia. Because *M. pneumoniae* is fastidious and grows very slowly, a definitive diagnosis is often based on the results of serological or molecular detection tests. When culture is required, the specimen of choice is a throat swab; however, sputum or other respiratory specimens are also acceptable. The specimen should be placed immediately into a transport medium containing protein, such as albumin, and penicillin to reduce the growth of contaminating bacteria. Specimens may be stored in the transport medium for up to 48 h at 4°C or frozen for longer periods at −70°C. PCR methods have been used success-

TABLE 11 Interpretation of bacterial lower respiratory tract culture results[a]

Specimen	Likely to be significant	Not likely to be significant	Additional data suggesting that isolate is significant
Sputum—expectorated or induced	Predominant potential pathogen in Gram stain and culture. Neutrophils abundant.	Potential pathogen not present in Gram stain and only 1–2+ growth in culture. Neutrophils not abundant in Gram stain.	Potential pathogen within neutrophils (intracellular bacteria).
Endotracheal tube aspirate (165)	Predominant potential pathogen in Gram stain and culture. Neutrophils abundant.	Potential pathogen only 1–2+ growth in culture. Neutrophils not abundant in Gram stain.	Potential pathogen in quantities >10^6 CFU/ml. Potential pathogen within neutrophils (intracellular bacteria).
Bronchoalveolar lavage fluid	Predominant potential pathogen seen in every 100× field of Gram stain. Quantitative culture detects >10^5 CFU of potential pathogen/ml.	Potential pathogen not seen in Gram stain. Quantitative culture detects <10^4 CFU of potential pathogen/ml.	Potential pathogen within neutrophils (intracellular bacteria).

[a] Reprinted from reference 162 with permission.

fully to detect M. *pneumoniae* directly in respiratory tract specimens, including as a component of a new respiratory panel assay (183), the FilmArray Respiratory Panel (BioFire Diagnostics, Inc., Salt Lake City, UT) (http://www.biofiredx.com/pdfs/mediakit/PRESS%20RELEASE%20-%20BioFireRP1.7_FDAClearance-0043.pdf). Molecular detection by PCR or a related technique is the most sensitive method for the detection of M. *pneumoniae* (1).

Specimens from Patients with Cystic Fibrosis (Also See Chapters 42 and 43)

Respiratory samples (sputum, aspirates, or "gag sputa" [deep throat swabs found to reliably recover important pathogens in these patients]) from cystic fibrosis patients require additional media and a tremendous extra effort to detect the important pathogens found in these patients (184). *Burkholderia cepacia* complex, particularly some genomospecies, is especially important (185, 186). This organism grows well on routine media; however, selective media such as *B. cepacia* selective agar, *Pseudomonas cepacia* agar, and oxidative-fermentative polymyxin B-bacitracin-lactose agar are useful for optimal recovery from respiratory secretions (187). Comparative studies show *B. cepacia* selective agar to be superior (also see chapter 43) (187). *H. influenzae*, *S. aureus* (often difficult-to-recognize small-colony variants), and mucoid *P. aeruginosa* are also important to identify. Many laboratories now use selective agar or chromogenic agar for *S. aureus* detection in cystic fibrosis patient samples (188). Molecular methods are proving useful for detecting important organisms in cystic fibrosis patients (185).

Chlamydia and *Chlamydophila* spp.

Chlamydiae are important causes of respiratory illnesses in children and adults (189, 190). *C. trachomatis* can cause serious respiratory disease in newborn infants. *Chlamydia* (also called *Chlamydophila*) *pneumoniae* causes illness in all age groups, but most disease occurs in adolescents and young adults (also see chapter 63). *Chlamydophila psittaci* is primarily an animal pathogen but occasionally causes disease in humans exposed to sick animals. Lower respiratory tract secretions, in addition to nasopharyngeal washes, for the detection of chlamydiae are collected and transported to the laboratory immediately in a medium containing antimicrobial agents (e.g., gentamicin and nystatin). If delays in transport or processing occur, the specimen should be stored at 4°C for up to 48 h. Longer storage should be at −70°C or colder. Chlamydiae can be detected by rapid cell culture techniques (shell vial) using McCoy cells for *C. trachomatis* and *C. psittaci* and HEp-2 cells for *C. pneumoniae*. Most laboratories do not perform these types of cultures. As with *M. pneumoniae*, PCR is the most sensitive method for the detection of respiratory chlamydiae (191), and NAA detection of *C. pneumoniae* is commercially available as part of a multiplex PCR assay (183).

Nocardia spp.

Respiratory specimens for the detection of *Nocardia* spp. should be transported to the laboratory as soon as they are collected. For short delays, storage at 4°C is acceptable. Direct examination of a Gram-stained smear containing a *Nocardia* species shows thin, beaded Gram-positive branching filaments. The filaments are also partially acid fast when stained by the modified Kinyoun method (chapters 2, 19, and 29). Although *Nocardia* spp. grow readily on many common media such as sheep blood and chocolate agar plates, Sabouraud agar for fungi, and Lowenstein-Jensen medium for mycobacteria, better recovery may be seen on BCYE agar for *Legionella* and modified Thayer-Martin medium. Mycobacterial decontamination procedures reduce the recovery of *Nocardia*. Selective BCYE agar is optimal for culture from contaminated specimens (192). Although *Nocardia* spp. are detected commonly following 1 week of incubation, cultures are incubated for a total of 2 to 3 weeks at 35°C.

Upper Respiratory Tract

Upper respiratory tract specimens include the external nares, nasopharynx, throat, oral ulcerations, and inflammatory material from the nasal sinuses. Although few serious diseases involve these areas, many pathogens colonize or persist in these sites while causing symptomatic infection in deeper, less accessible sites (193).

Throat specimens are collected to diagnose pharyngitis. The most common etiological agent is *S. pyogenes*; however, other beta-hemolytic streptococci, *Arcanobacterium haemolyticum*, *N. gonorrhoeae*, *C. diphtheriae*, mycoplasmas, chlamydophilas, and *Fusobacterium necrophorum* may also occasionally cause pharyngitis. Special requests are required for most of these agents, although routine throat cultures reveal beta-hemolytic streptococci and *A. haemolyticum*. New data on *F. necrophorum* suggest that it should be sought (by anaerobic culture) for patients with chronic sore throat and that the standard swab is sufficient for recovery (194–196). Swab specimens should be placed in a standard transport carrier containing Amies or modified Stuart's medium. A selective agar for *F. necrophorum* can enhance detection (197). If only group A streptococci are to be detected, the swab can be sent dry with desiccant. Refrigeration is preferred if transport requires more than a few hours. Many rapid direct tests for group A streptococci are commercially available, including enzyme immunoassay (EIA), immunochromatographic assays, and nucleic acid-based probe assays (198). Rapid tests still lack sufficient sensitivity to be used without culture backup for negative results in the pediatric population (199). When a rapid test is requested, two throat swabs should be collected. If only one swab is received, the culture plate should be inoculated first. Material remaining on the swab is used for the direct test. If the rapid test is positive, the second swab can be discarded, but if the rapid test is negative, the second swab must be used for culture to confirm the negative direct test. The nucleic acid-based probe test is considered sensitive and specific enough by many to eliminate the need for confirmatory culture (200). A position paper by representatives of the American Academy of Family Physicians, the American College of Physicians-American Society of Internal Medicine, and the CDC and IDSA guidelines state that rapid tests do not require confirmatory culture when used with adult patients (199, 201). NAATs will likely become the test of choice in the future; commercial products available today have excellent sensitivity and specificity (202).

To culture group A streptococci, either horse or sheep blood agar or selective blood agar may be used. Selective agar makes the organism easier to visualize by inhibiting accompanying members of the microbiota but may delay the appearance of colonies of *S. pyogenes*. Cultures should be incubated for 48 h at 35°C in an environment of reduced oxygen achieved by incubating anaerobically, in 5% CO_2, or in air with multiple "stabs" through the agar surface. Stabbing the agar surface with the inoculating loop pushes inoculum containing streptococci below the surface, where the oxygen concentration is reduced compared to the ambient (203). These culture conditions allow the recovery of groups C and G streptococci, organisms that may cause pharyngitis but do not cause the serious sequelae associated with group A streptococci (204). Reports from such cultures may state "beta-hemolytic streptococci, not group A, isolated." Although relative numbers of colonies are usually reported (1+, 2+, etc.), this number may only reflect the quality of the sample and should not contribute to therapy decisions.

Throat specimens also are used to identify patients infected with *N. gonorrhoeae*. For best results, the specimen should be inoculated immediately to a selective medium, such as modified Thayer-Martin agar. Cultures are incubated at 35°C in the presence of 5% CO_2 for 72 h. Although no tests are yet cleared by the FDA and thus laboratories need to verify their use, NAATs are also the test of choice for detection of *N. gonorrhoeae* in the throat, due to their enhanced sensitivity (134). One recent assay uses two DNA targets to achieve better specificity (129). *A. haemolyticum* is usually recognized on the second day, when its characteristic hemolysis manifests (see chapter 28).

The external nares can be cultured to identify carriers of *S. aureus* by using a single swab inserted only as far as the tip to collect secretions from both the left and right nares. The nose should be gently pressed inward during collection to maximize contact of the swab. The usual carrier systems used for swab transport containing Amies or Stuart's medium are acceptable. In the laboratory, the specimen can be inoculated to a sheep blood agar plate; however, use of selective media such as colistin-nalidixic acid agar or a selective and differential chromogenic medium (see chapter 19) is helpful in differentiating *S. aureus* or MRSA from other members of the microbiota and useful when interpreting large numbers of specimens (205, 206). Mannitol salt agar is not as sensitive as the chromogenic media (44). Cultures should be incubated at 35°C for 1 to 2 days per product insert. Commercially available, FDA-cleared molecular tests for the detection of *S. aureus* and MRSA in nasal swabs are more sensitive than culture, and results are available much faster (47, 207–209).

Specimens for the recovery of *B. pertussis* and *Bordetella parapertussis* should be collected with a small-tipped Dacron swab. Cotton may be toxic to the organism. Swabs should be transported to the laboratory in special media. For delays of up to 24 h, Amies medium with charcoal can be used. If the transport time will exceed 24 h, Regan-Lowe transport medium should be used (210). Culture, DFA staining, and PCR have been used for detection, but PCR is the most sensitive for detecting *B. pertussis* and *B. parapertussis* (Dacron swabs are preferred for PCR tests) (211–213). Culture, primarily for epidemiological studies, is performed using Regan-Lowe charcoal agar containing 10% horse blood and cephalexin. Because a few strains of *B. pertussis* do not grow in the presence of cephalexin, the use of Regan-Lowe medium with and without cephalexin is recommended for optimal recovery (214). Cultures are incubated at 35°C for 5 to 7 days in a humid atmosphere. The DFA test can yield rapid results for *Bordetella* but should probably be discontinued in favor of PCR methods (215).

Nasopharyngeal swab specimens are used to identify carriers of *N. meningitidis*. Transport in a swab container with Amies or Stuart's medium is acceptable. Specimens should be inoculated as quickly as possible to sheep blood or chocolate agar; however, selective agars for pathogenic *Neisseria* spp., such as modified Thayer-Martin, are necessary if interference by normal members of the microbiota is expected. Culture plates are incubated for 72 h in a humidified atmosphere at 35°C in the presence of 5% CO_2.

Vincent's angina is an oral infection characterized by pharyngitis, membranous exudate, fetid breath, and oral ulcerations. Sometimes referred to as fusospirochetal disease or necrotizing ulcerative gingivitis, it is caused by *Fusobacterium* spp., *Borrelia* spp., and other anaerobes. Diagnosis is made by direct examination of a smear of a swab specimen collected from the ulcerated lesions and stained with the Gram stain (193). The presence of many spirochetes, fusiform bacilli, and PMNs is presumptive evidence of this disease. Culture or NAAT for *F. necrophorum* may also be attempted (195, 216). It should be noted that canker sores do not have a known microbial etiology and should not be cultured.

Inflammatory material from the nasal sinuses should be cultured to detect the etiologies of sinusitis. Nearly all cases of bacterial sinusitis follow a primary, upper respiratory tract viral infection. Bacteria are trapped in the sinus as a result of damage to the epithelial lining cells of the sinus, and inflammation and swelling narrow or close the nasal ostium, preventing normal drainage (217, 218). Specimens collected during endoscopic procedures by physicians specializing in otorhinolaryngology are optimal since they are sampled directly from the infected sinus, avoiding contamination by normal members of the microbiota in the nasal passages. Aspirates, washes, scrapings/debridements, and biopsy material should be kept moist and sent in a sterile container to the laboratory. Examination of Gram-stained smears can provide a rapid, presumptive identification of likely pathogens. Aerobic culture is needed in all cases; anaerobic transport and culture may be needed in cases of chronic sinusitis. Ventilator-associated sinusitis occurs in <10% of patients with nasotracheal intubation. Members of the nosocomial microbiota are implicated. Endoscopic inspection is needed to obtain acceptable specimens for culture (219). Although swabs obtained with endoscopic guidance correlate well with aspirates for recovery of bacteria, they do a poor job of recovering fungi, which have been implicated as etiological agents of chronic sinusitis (13). Recent evidence is casting doubt on fungi as a cause of this syndrome (220).

Special Considerations for Upper Respiratory Tract Specimens

A. haemolyticum

A. haemolyticum can cause pharyngitis and peritonsillar abscess (221). The organism can be recovered on media used to detect *S. pyogenes*. Colonies of *A. haemolyticum* are beta-hemolytic and easily confused with those of beta-hemolytic streptococci. Rapid differentiation can be accomplished with the Gram stain. *A. haemolyticum* is a diphtheroid-shaped Gram-positive rod (see chapter 28). Incubation of plates at 35°C for up to 72 h may be required for optimal detection.

C. diphtheriae

Culture of both throat and nasopharyngeal specimens is used in the diagnosis of diphtheria. When specimens are processed for culture without delay, no special transport medium or conditions are required. For transport to a reference laboratory, specimens should be sent dry in a container with desiccant (222). Alternatively, specimens collected on swabs may be placed in Stuart's or Amies medium for transport to the laboratory. Smears of specimens for *C. diphtheriae* can be stained with the Gram stain and examined for pleomorphic (diphtheroid morphology) Gram-positive rods. In addition, smears can be stained with Loeffler's methylene blue stain and examined for pleomorphic, beaded rods with swollen (club-shaped) ends and reddish purple metachromatic granules. Bacteria with these characteristics are suggestive of but not specific for *C. diphtheriae*. Specimens should be inoculated to Loeffler's serum and potassium tellurite media for the recovery of *C. diphtheriae*. Cultures are incubated for 2 days at 35°C in 5% CO_2 before being reported as negative. See chapter 28 for more information.

Epiglottitis

A throat swab specimen may be helpful in determining the etiology of epiglottitis, a rapidly progressing cellulitis of the epiglottis and adjacent structures with the potential for swollen tissues to cause airway obstruction. Epiglottitis in the United States was almost always caused by *H. influenzae* serotype b before widespread use of the *H. influenzae* type b vaccine, and now the disease is seen more often in adults. That species is still a cause of epiglottitis, even in vaccinated individuals, and certainly in areas of the world where the vaccine is not available. Other bacteria such as *S. pneumoniae*, *S. aureus*, *S. pyogenes*, and other beta-hemolytic streptococci are often involved (223, 224). The specimen should be collected by a physician only in a setting where emergency intubation can be performed immediately to secure a patent airway. Specimens should be inoculated onto enriched medium, such as chocolate agar, and incubated at 35°C in an atmosphere of 5% CO_2 for 72 h. Nearly 100% of patients with epiglottitis have a blood culture positive for the same bacterium, so blood cultures should be ordered as part of differential diagnostic testing.

Gastrointestinal Tract

Feces and in some cases rectal swab specimens are submitted to the microbiology laboratory to determine the etiological agent of infectious diarrhea or food poisoning. In fact, most diarrhea is caused not by bacteria but by viruses, parasites, immune-related disease, or other factors. Feces submitted for enteric pathogens should be collected in a clean container with a tight lid and should not be contaminated with urine, barium, or toilet paper. Because intestinal pathogens can be killed by the metabolism of other fecal bacteria rapidly acidifying the specimen, specimens should be transferred to Cary-Blair transport medium soon after collection. *Shigella* is the most labile, often becoming nondetectable within 30 min of collection. For *Vibrio cholerae*, other transport media such as alkaline peptone water are preferred (see chapter 41). Rectal swabs should be placed in a transport system containing an all-purpose medium such as Stuart's. Recently, NAATs for a panel of stool pathogens (not limited to bacteria) have become commercially available, and they will likely be used broadly in the near future (225). Samples for molecular testing should be collected as described by the manufacturer.

It should be standard practice in all laboratories to evaluate the appropriateness of stool culture or NAAT requests. It is well established that hospitalized patients who did not enter the hospital with diarrhea are unlikely to develop bacterial enterocolitis caused by bacterial agents other than *C. difficile* (226, 227). For this reason, stool from patients who have been hospitalized for more than 3 days should not be processed for enteric pathogens without consultation with and justification by the patient's physician or caregiver. A simple policy of rejecting stool for routine bacterial culture from patients hospitalized for more than 3 days and offering *C. difficile* testing for health care-acquired diarrhea is recommended. On the other hand, *C. difficile* infection is rapidly expanding as a community-acquired disease, sometimes following hospital discharge or the use of outpatient antimicrobial therapy, and sometimes in patients with no previous risk factors. Therefore, requests for *C. difficile* infection testing should not be rejected when ordered for outpatients (228, 229). After an initial negative NAAT for *C. difficile*, however, repeat testing is not routinely warranted, and use of NAATs for test of cure is inappropriate (230).

Fecal leukocyte examinations have been recommended for the differentiation of inflammatory diarrheas (fecal leukocyte positive) from secretory diarrheas (fecal leukocyte negative). Infectious, inflammatory diarrheas are caused by invasive bacteria, while secretory diarrheas result from toxin-producing bacteria, viruses, and protozoan pathogens (231). Unfortunately, fecal leukocytes degrade in feces, making accurate recognition and quantification difficult. A lactoferrin test can serve as a surrogate marker for fecal leukocytes (EZ Leuko Vue; TechLab, Blacksburg, VA) since lactoferrin is not degraded during normal transport and processing times. Lactoferrin-positive stool specimens are considered positive for fecal leukocytes. However, invasive pathogens may result in fecal leukocytes being intermittently present or unevenly distributed in stool specimens, and fecal leukocytes may not be present in severe *C. difficile* infection. Numerous studies have shown either fecal white blood cells or lactoferrin to be not useful for differentiating inflammatory from noninflammatory diarrhea (232, 233). For these reasons, use of any tests for fecal leukocytes should not be relied on to rule in or rule out acute, infectious diarrhea (231). The lactoferrin assay can be used in the evaluation of patients with inflammatory bowel disease (234).

Usual gastrointestinal pathogens are listed in Table 8 (227). Inclusion of less frequently encountered pathogens should be considered when epidemiological factors suggest an increased likelihood. This may require periodic surveys of one's community to establish which pathogens are most common, especially when considering the addition of selective media or toxin assay for the routine detection of campylobacters other than *Campylobacter jejuni* or *Campylobacter coli*, *Vibrio* spp., and *Yersinia enterocolitica*. Use of molecular multiplex panels could alleviate the need for selection.

Selective and differential media are used to detect *Salmonella* and *Shigella* spp. (Table 7). These should include one that is differential but not selective for these pathogens, such as MacConkey agar, and one that is a mildly selective medium, such as Hektoen enteric or xylose-lysine desoxycholate agar. In some settings, a highly selective medium such as salmonella-shigella agar is also included. In addition, enrichment broth, such as Gram-negative broth or Selenite F broth, may increase detection of *Salmonella* and is recommended for testing samples from sensitive populations such as food handlers. *Shigella* is generally not enriched. Subculture of Gram-negative and Selenite F broths to a mildly selective and differential medium after 6 to 8 h and 12 to 18 h of incubation, respectively, is necessary to prevent overgrowth of normal members of the microbiota and decreased usefulness of the broth (227). All agar plates should be incubated in air at 35 to 37°C for 2 days before being reported as negative. The decision whether to use a highly selective agar medium and an enrichment broth varies from one laboratory to another. Optimally, additional media are used for a trial period to determine their value, which is measured by the detection of strains not present on the two standard media. In settings where such a trial is not possible, the use of MacConkey agar, Hektoen enteric, or xylose-lysine desoxycholate agar and an enrichment broth is recommended.

C. jejuni and *C. coli* are detected in culture with a medium such as campylobacter agar with 10% sheep blood and selective antimicrobial agents (Table 7). Media are incubated at 42°C in a microaerophilic atmosphere of nitrogen (85%), carbon dioxide (10%), and oxygen (5%) for up to 3 days. Special enrichment broths do not increase the number of campylobacter-positive cultures significantly (235). Detection of other *Campylobacter* species may require media without antibiotics and 37°C incubation (236). EIA methods for detection of surface antigens of some *Campylobacter* species have been evaluated and shown to be relatively sensitive and specific (237). New molecular methods will probably replace culture for enteric pathogens, including *Campylobacter*, in the near future (238, 239). See chapter 56 for more information on methods of detection of fecal *Campylobacter* spp.

Special Considerations for Gastrointestinal Tract Specimens

Other Enteric Pathogens

A physician order for *Vibrio* spp., *Y. enterocolitica*, *Aeromonas* spp., and *Pleisomonas shigelloides* may be needed in some geographic locations or epidemiological situations, since incidence is so low in most parts of the United States that the routine use of selective media is not justified. Media used for these enteric pathogens include thiosulfate-citrate-bile salts-sucrose agar for vibrios, cefsulodin-Irgasan-novobiocin agar for *Y. enterocolitica*, and blood agar or selective blood agar to demonstrate hemolysis and provide a medium for oxidase testing for *Aeromonas* spp. and *P. shigelloides* (both oxidase positive) (227). All of these enteric pathogens grow on usual media, but detection is enhanced and simplified using specific selective media.

STEC

The prevalence of Shiga toxin-producing *E. coli* (STEC) varies in different parts of the United States and the rest of the world. In addition to *E. coli* O157:H7, many other serotypes are implicated. In fact, in the United States ~50% of STEC organisms, those capable of causing hemorrhagic colitis and hemolytic-uremic syndrome, are not serotype O157:H7 (240, 241). Shiga toxin EIAs and PCR assays detect all serotypes. Some Shiga toxin-producing strains may not harbor all the mechanisms needed to be fully pathogenic in humans. Routine culture of all stools on a selective medium and use of an EIA on an enriched broth (either Gram-negative broth or MacConkey broth) culture for detection of STEC have been recommended by the CDC (233). Recommendations from both the CDC and American Public Health Laboratory for universal STEC testing contain strategies for referral of non-O157 strains for further testing by public health laboratories (233, 242). A downloadable guide is available from the American Public Health Laboratory site (http://www.aphl.org/AboutAPHL/publications/Documents/FS_2012April_Guidance-for-PHLs-Isolation-and-Characterization-of-Shiga-Toxin-Producing-Escherichia-coli-STEC-from-Clinical.pdf). Either sorbitol-MacConkey agar, cefixime-tellurite-sorbitol-MacConkey agar, or a chromogenic agar can be used to detect O157 serotype strains. Note that non-O157 strains such as the recent European outbreak O104 strain may not be detected using conventional methods (243). New NAATs are available for specific toxin gene detection or detection of toxin-producing *E. coli* strains as part of a stool pathogen panel (239, 244).

C. *difficile*

C. difficile infection is diagnosed by detecting the organism or its toxins in stool, in conjunction with clinical criteria (228). The gold standard test for disease is the toxigenic culture, which requires direct plating on enrichment agar containing taurocholate, or alcohol or heat treatment to enrich for spores, followed by culture in broth and/or solid media (245, 246) (also see chapter 53). Colonies must then be tested by a cell culture cytotoxin assay for toxin production (247). Because toxins are labile, molecular testing for the gene encoding toxin B, the major virulence factor, is becoming the standard test (248–250). Cell culture cytotoxicity assay for the detection of toxin B, EIA for the detection of toxins A and B, and latex agglutination or EIA for the detection of glutamate dehydrogenase (an antigen associated with *C. difficile* and occasionally other bacterial species) are all methods that have been used for diagnosis (249). EIAs are now known to have low sensitivity, depending on the prevalent strain types, and specificity (245, 251).

C. difficile-associated gastrointestinal disease is now appearing in patients without any risk factors, even in patients who had no antibiotic exposure (229, 252). Testing should not be performed on formed stool, with the major exception of patients with ileus or toxic megacolon, or as a follow-up to therapy to confirm cure. Repeat testing is only appropriate if symptoms persist or recur more than 7 days after an initial intervention (230). Since 2002, outbreaks of severe *C. difficile* disease in North America and Europe have been reported due to an epidemic strain, BI/NAP-1/027 (250, 253). This strain is also now widely acknowledged to cause more quickly progressing and serious disease, so aggressive diagnosis and treatment are important. Other strains are also associated with increased severity of disease, including those producing binary toxin (254, 255).

S. aureus, MRSA, and *Bacillus cereus*

Stool specimens or gastric contents collected from persons with short-incubation food poisoning (2 to 6 h) can be evaluated for *S. aureus* and *B. cereus* (also see chapters 21 and 26). In general, investigation is beneficial for general public health, rather than a sick individual who recovers quickly, and is best performed by public health laboratories rather than hospital clinical microbiology laboratories. Specimens should be examined by Gram stain, and because both of these organisms may be present normally in food, quantitative cultures must be performed. A series of dilutions (10^{-1} to 10^{-5}) of the specimen are prepared in buffered gelatin diluent, and 0.1-ml samples of the undiluted specimen and each of the dilutions are plated onto colistin-nalidixic acid or phenylethyl alcohol blood agar. The presence of 10^5 CFU or more of *S. aureus* or *B. cereus* per g of specimen is of potential significance (256).

MRSA may be a cause of health care-associated antibiotic-associated diarrhea (257, 258). The overall incidence is unknown. Gram stain of smears of nonformed stool showing sheets of staphylococcal clusters in combination with appropriate clinical findings suggests the diagnosis. Diagnosis consists of the detection of heavy growth of MRSA in combination with the detection of staphylococcal enterotoxin in stool, available from some national reference laboratories and numerous public health laboratories (because *Staphylococcus* enterotoxin B is a category B bioterrorism agent). Greater recognition of this disease should confirm its significance and result in rapid diagnostic methods and appropriate treatment.

Clostridium botulinum

The clinical diagnosis of foodborne and infant botulism may be confirmed by detecting botulinum toxin, *C. botulinum*, or both in feces, although primary laboratories are now requested to submit samples to their appropriate public health laboratory for testing (also see chapter 53). In the United States when a case of botulism is suspected, investigators at the CDC should also be notified immediately. Optimally, 25 to 50 g of stool, 15 to 20 ml of serum, and a sample of suspect food should be collected (256). Botulinum toxin could be used as a biological weapon (see chapters 14 and 53). Unexpected numbers of cases or unusual presentations should be investigated. The ASM website has up-to-date guidelines on actions that should be taken in the event of a suspected case (http://www.asm.org/images/PSAB/Botulism_July2013.pdf).

Helicobacter pylori

H. pylori is an important cause of gastritis and peptic ulcer disease, and infection can lead to certain malignancies (259, 260). The organism can be observed in tissue sections by using hematoxylin and eosin, Giemsa, or Warthin-Starry silver staining. In addition, organisms can be visualized in touch preparations of dissected tissue stained with the Gram stain. As discussed above, the presence of *H. pylori* in stomach or small bowel lesions can be confirmed by culture, antigen detection, urease detection, or the detection of exhaled bacterial metabolite (*H. pylori* breath test) (261, 262). Tissue biopsy specimens collected during endoscopy are used for culture and urease detection. Specimens for culture should be placed in transport medium (a medium such as brucella broth containing 20% glycerol is best for transport and storage) and transported to the laboratory immediately, or refrigerated during delays (263). Lightly minced tissue is inoculated to freshly prepared blood agar and incubated in a humid, microaerophilic atmosphere (5 to 10% carbon dioxide, 80 to 90% nitrogen, and 5 to 10% oxygen) at 37°C for 7 days (Table 7). The addition of 5% hydrogen should improve the yield of *H. pylori*. Tissue for urease detection is placed as soon as possible into the detection system and processed as specified by the manufacturer. Stool for antigen detection should be collected and handled according to instructions from the manufacturer. Antigen tests are still useful; PCR methods have not yet become common practice (264). In some clinical situations, serological testing for *H. pylori* antibody may be necessary (see chapter 57). Serum should be collected and stored at refrigeration temperature for short periods (up to 1 week) or frozen at −70°C for longer periods, as for other antibody tests.

Screening for VRE or Beta-Hemolytic Streptococci

Identifying carriers of VRE for infection control purposes and group A streptococci during investigations of outbreaks of necrotizing fasciitis or streptococcal toxic shock requires collection of a rectal swab specimen. Carriers of VRE can be identified by culturing rectal swab or perirectal swabbed material, but rectal swabs are superior if molecular methods are used (265). Specimens are inoculated to selective enrichment broth, such as Enterococcosel broth with 6 μg of vancomycin/ml, or agar media such as colistin-nalidixic acid blood agar containing 6 μg of vancomycin/ml (see chapter 19). Culture methods detect both *vanA*- and *vanB*-containing strains, but further testing is required to differentiate them. The fact that *vanB* is also present in nonenterococci in the bowel complicates the use of new molecular tests for surveillance of VRE, as positive PCR results will need to have the presence of a *vanB*-producing *Enterococcus* confirmed (266). Chromogenic agars for VRE are available and can enhance the speed and sensitivity of detection of these organisms (267). Carriers of group A streptococci can be identified by culturing rectal swab specimens on sheep blood agar or selective streptococcal agars used to identify patients with streptococcal pharyngitis.

Small Bowel Bacterial Overgrowth Syndrome (268)

Occasional requests are received for quantitative culture to help with diagnosis of small bowel bacterial overgrowth syndrome, in which the numbers and types of organisms in the duodenum or jejunum increase to resemble the numbers in the colon ($>10^5$/ml) (269). At this time, it is unclear which method best aids in diagnosis of small bowel bacterial overgrowth syndrome (270). Laboratories receive duodenal or jejunal aspirate material in a sterile container. Because the test requested is to determine whether the number of CFU in the sample is above or below the threshold of 10^5 CFU/ml, a simple method such as thorough vortexing of the sample and inoculation of a blood agar plate using a 0.001-ml calibrated loop can be used. Counting colonies in the same manner as for urine cultures is sufficient.

Urinary Tract

Diseases of the urinary tract include prostatitis, urethral syndrome, cystitis, and pyelonephritis (271). Etiologies are summarized in Table 8. Urine, prostatic secretion, or urethral cell/secretion specimens are needed to diagnose these diseases. Urine can be collected by midstream (clean-catch) collection, catheterization (straight/in-out or indwelling), cystoscopic collection, or suprapubic aspiration. Foley catheter tips should not be submitted or accepted for culture since they are always contaminated with members of the urethral microbiota and quantitation is not possible. A first-voided morning urine is optimal, since in most cases bacteria have been multiplying in the bladder for a number of hours. Clean-catch urine, implying cleansing of periurethral areas, has not been shown to improve the quality of urine culture and is not recommended, although current guidelines still recommend clean-catch collection (271–273).

Urine specimens should be transported to the laboratory immediately and processed within 2 h of collection. If a delay occurs, specimens may be refrigerated for up to 24 h. Transport tubes containing boric acid are available to stabilize the bacterial population at room temperature for 24 h if refrigeration is not available (273). Boric acid-preserved urines are acceptable for dipstick leukocyte esterase testing (274).

Urine culture is the most common test performed by most microbiology laboratories, and most urine cultures are negative; i.e., no specific potential pathogen is detected. Screening methods are available that attempt to rapidly separate those specimens containing significant counts of bacteria from negative specimens. In general, screening methods compare well with specimens containing bacteria at $\geq 10^5$ CFU/ml but perform poorly when colony counts are lower. Screening urine specimens by staining with the Gram stain is rapid and economical with regard to reagents but is labor-intensive and requires a trained technologist. The presence of 1 or 2 bacteria of similar morphotype, or more, in each oil immersion field (100× objective lens) correlates with a count of 100,000 or greater by culture (275). Commercially available dipstick tests that detect leukocyte esterase (an enzyme produced by neutrophils) and nitrite (the result of bacterial nitrate reductase acting on nitrate in the urine) are rapid, inexpensive, and simple to perform, but their sensitivity is low in some patient populations. False-negative dipstick screening occurs because frequent voiding dilutes the concentrations of leukocyte esterase and nitrite in urine, enterococci and other less common urinary tract pathogens do not produce nitrate reductase, and many patients with asymptomatic bacteriuria do not have significant numbers of leukocytes in urine (276, 277). In spite of this, outpatient screening algorithms have been proposed that incorporate enzyme screening in a "reflexive" urine test: i.e., urinalysis is performed; if positive for leukocyte esterase or nitrate reductase, a culture is set up, and if negative, a culture is not done. Such screening works best for symptomatic patients, diabetics, and women older than 60 years (277). Active research continues to find an accurate, cost-effective, sensitive, and specific automated urine screening system that eliminates the need to culture urine samples that are not indicative of urinary tract infection (UTI). After decades, this goal has not been reached.

The standard for quantitative bacterial culture of urine is the inoculation of 0.01 or 0.001 ml of specimen using a calibrated plastic or wire loop to appropriate medium, usually sheep blood and MacConkey agars or cystine–lactose–electrolyte-deficient agar. The loop is dipped vertically into the well-mixed urine, just far enough to cover the loop, and the loopful of urine is spread over the surface of the agar plate by streaking from top to bottom in a vertical line and again from top to bottom perpendicular to this line in a back-and-forth fashion. Prior to plate inoculation, it is necessary to ensure that a film of urine fills the loop with no bubbles to alter the calibrated volume. The inoculum of urine is spread over the entire agar surface to simplify counting of colonies after growth. Urine cultures are incubated at 35°C for 24 to 48 h. Although most urinary tract pathogens grow readily on usual agar media, slowly growing pathogens and those inhibited by the presence of antimicrobials in the patient specimen may not appear after overnight incubation (16 h). One approach uses the results of the leukocyte esterase and nitrite tests to determine which cultures get incubated for a full 48 h. Urine cultures that are negative after overnight incubation but had one or both positive enzyme tests are incubated for an additional day. Those that had negative enzyme tests are reported as "no growth" in a final report (271).

Contamination of urine, defined as growth of colonizing skin, vaginal, or fecal microbes in the absence of UTI, is detected in approximately 5 to 40% of cultures. Contamination is not reduced by the use of central processing areas, refrigeration, urine screening systems, specimen preservatives, or insulated specimen transport (278). Using a minicatheter to collect urine directly from the bladder can circumvent some of these contaminating bacteria, and collecting a suprapubic urine sample with a transcutaneous needle aspiration guarantees an even more representative urine sample. Urine collected by attaching a plastic bag to a baby's perineal region (bagged urine) is never suitable for culture. Either catheter-obtained urine or a suprapubic aspirate of urine must be submitted to avoid a contaminated specimen from babies. Suprapubic aspirates should be handled in the same manner as sterile body fluids and may be cultured anaerobically.

Agar paddles are available for urine culture in settings where inoculation and incubation of conventional agar plates are not convenient or possible (279). A standard film of urine is distributed over the agar-covered paddle, usually by dipping the paddle into a jar of urine. The paddle is then reinserted into its plastic container for incubation. Following incubation, the density of growth is estimated by comparison to photographs or drawings. A preliminary identification of Gram positive or Gram negative can be determined by colony color and morphology, and when appropriate, the entire paddle can be forwarded to a reference laboratory for complete identification and antimicrobial testing of the isolate. Agar paddle culture of urine with approximate colony counts compares favorably with standard culture (279).

The urethra is colonized normally with many different bacteria from the vagina, skin, or feces. Because of this, voided urine often becomes contaminated during passage. Commensal bacteria are differentiated from potential pathogens by quantitative culture. Bacterial counts indicating "significant" bacteriuria (isolate is a likely pathogen) vary with the host and type of infection. Table 12 summarizes significant counts for common clinical situations (271). The urinary tract above the urethra had been thought to be sterile in healthy humans, but new genomic sequencing methods are revealing the predominance of nonculturable organisms in bladder urine in some patients (280). The implications of these findings are just being explored. For example, several studies have shown that *Actinobaculum schaalii* is an important cause of UTIs, especially in pediatric patients and patients >60 years old; efforts to recover this catalase-negative, pleomorphic Gram-positive coccobacillus (requires anaerobic or CO_2 incubation) should be undertaken in such patients with pyuria (287).

Severe UTI generally involves the kidneys (pyelonephritis) and results in bacteremia. Rapid diagnosis and administration of appropriate antimicrobial therapy are necessary. In this clinical setting, blood cultures are needed and a STAT Gram stain of the urine can be useful. The Gram stain provides an immediate indication of the quality of the urine and a preliminary identification of the likely pathogen. Specimens containing high numbers of SECs are likely to be grossly contaminated with members of the periurethral or vaginal microbiota and should be re-collected immediately, before antimicrobials inhibit growth of the true pathogen (Fig. 6) (3). Gram stain identification of a potential pathogen (usually Gram-negative rods such as in Fig. 7) confirms that empiric therapy is correct or may suggest a change based on an unexpected pathogen such as *S. aureus*.

Infectious prostatitis can be a difficult diagnosis. Prostatic secretions may be submitted for culture, but interpreting results when cultures yield low numbers of potential pathogens or mixed cultures is not clear-cut. Culture results should be correlated with Gram stain results. Organisms not recovered from routine cultures, such as mycoplasmas and chlamydiae, may be etiological agents in prostatitis. New molecular methods will likely provide assistance in the future (281).

Special Considerations for Urine Specimens

Leptospires

Leptospira interrogans can be recovered from blood and CSF during the acute stages of disease and from urine after the first week of illness and for several months thereafter. Urine should be processed as soon as possible after collection, because the acidity of urine harms the organisms. If a delay in processing is expected, urine should be neutralized with sodium bicarbonate, centrifuged (1,500 × *g* for 30 min), and resuspended in buffered saline before being used to inoculate media (see chapter 58). Alternatively, the urine may be diluted 1:10 in 1% bovine serum albumin and stored

TABLE 12 Interpretation of urine culture results[a]

Urine specimen and patient	Likely to be significant	Not likely to be significant	Additional data suggesting that isolate is significant
Midstream, female with cystitis	>10^2 CFU of potential pathogen/ml, urine LE[b] is positive	Quantity of potential pathogen ≤ quantity of contaminating members of the biota	
Midstream, female with pyelonephritis	>10^5 CFU of potential pathogen/ml, urine LE is positive	Quantity of potential pathogen ≤ quantity of contaminating members of the biota	Gram stain demonstrates potential pathogen in neutrophils and/or casts.
Midstream, asymptomatic bacteriuria	>10^5 CFU of potential pathogen/ml, LE is usually negative	<10^5 CFU/ml of potential pathogen; quantity of potential pathogen ≤ quantity of contaminating members of the biota	Confirm by repeating urine when clinically indicated.
Midstream, male with UTI	>10^3 CFU of potential pathogen/ml, urine LE is positive	<10^3 CFU/ml of potential pathogen; quantity of potential pathogen ≤ quantity of contaminating members of the biota	Gram stain demonstrates potential pathogen in neutrophils and/or casts.
Straight catheter, all patients	>10^2 CFU of potential pathogen/ml, urine LE positive for symptomatic patients	<10^2 CFU/ml of potential pathogen, urine LE is negative	Gram stain demonstrates potential pathogen in neutrophils and/or casts.
Indwelling catheter, all patients	>10^3 CFU of potential pathogens/ml (multiple pathogens may be present)	Bacteriuria detected in asymptomatic patients, urine LE is positive or negative	No reason to culture unless patient is symptomatic.

[a] Reprinted from reference 162 with permission.
[b] Abbreviation: LE, leukocyte esterase.

at 5 to 20°C. Undiluted urine and urine diluted 1:10, 1:100, and 1:1,000 in sterile buffered saline should be inoculated to Ellinghausen-McCullough-Johnson-Harris or equivalent medium, with and without neomycin (37). Cultures should be incubated at 30°C for at least 13 weeks (Table 7). Many clinicians also collect serum for serological tests for leptospirosis.

Bacterial Antigen Testing

Bacterial antigen testing kits, for the purpose of diagnosing bacterial meningitis, should not be used with urine specimens due to lack of strong correlation with meningitis. In particular, the FDA issued a product alert specifically cautioning against the use of the group B *Streptococcus* antigen kits with urine specimens because of the risk of both false-positive and false-negative results (282). As mentioned above in the sections on respiratory disease, urinary antigen testing is a recommended method for diagnosis of pneumococcal pneumonia and *Legionella* pneumonia.

Wounds

Superficial wound exudates and pus may be submitted on swabs, but this is inferior to biopsy samples or aspirates. Newer swab designs do allow better sample collection (7, 283). It is helpful to perform a Gram stain on all possible specimens to help determine how to interpret the culture, but with only one swab, this is difficult. The swab can be vortexed in <1.0 ml of sterile saline or broth and squeezed to express any moisture, and the suspension used to inoculate the plates and make a smear for Gram stain, but this is a labor-intensive activity. Therefore, two swabs or a flocked swab and transport medium system should be requested for samples collected on swabs (283). Examination of a Gram-stained smear reveals bacterial morphotypes, acute inflammatory cells (PMNs), intracellular bacteria, cell necrosis, and elastin fibers resulting from tissue necrosis (Fig. 8). The quality of a wound specimen can be evaluated by noting the relative number of polymorphonuclear cells and SECs (Table 5). Excess numbers of SECs suggest gross contamination with members of the cutaneous microbiota. It is acceptable to limit workup of bacterial isolates when the specimen shows gross contamination. An example of a limited workup would be to list by Gram stain morphology the isolates encountered, with a comment explaining that the physician must call if a replacement specimen cannot be collected and further identification and antimicrobial testing is clinically warranted. If swabs of ulcers or decubiti are received for culture, despite the laboratory's attempts to discourage such samples, a limited workup of the organisms most likely to cause an underlying osteomyelitis should be performed (11, 12). The bacteria of importance include beta-hemolytic streptococci, *S. aureus*, *P. aeruginosa*, *Proteus* spp., *E. coli*, and *Klebsiella* spp.

Autopsy Samples

Microbiology testing as a component of the autopsy examination has been controversial (284). Postmortem and agonal invasion of sterile tissues confuses the significance of positive culture results, prompting some to argue against microbiology testing. Others found that the postmortem examination continues to uncover a significant number of infectious diagnoses, whether in the community or university hospital setting, that were missed by modern high-technology medicine (285). The value of autopsy microbiology is further enhanced by its use to identify emerging diseases, etiologies of biological warfare, community outbreaks, nosocomial infections, and antimicrobial resistance and uncover the cause of death in organ transplant patients and others with immunocompromising conditions. Safety precautions designed to protect the pathologist and dissection assistants during autopsy procedures have been thoroughly reviewed (286).

To minimize contamination of postmortem specimens, the body should be moved to a refrigerated locker (4 to 6°C) as soon as possible after death. Limited movement of the body has been shown to decrease the incidence of false-positive postmortem cultures. Although it has been shown that cultures collected within 48 h of death from a refrigerated cadaver did not show an increase in false-positive results, tissue and fluid specimens, as a rule, should be taken from refrigerated bodies within 15 h of death (284). This serves to diminish the likelihood of postmortem overgrowth of contaminants and improve detection of true pathogens.

Specimens should be obtained by sterilizing the surface of the organ with a hot spatula or iron surface until the surface is thoroughly dry. Body fluids, including blood, should be collected first. For blood collection, the wall of the heart and large vessel should be seared and a sterile needle (18 to 20 gauge) inserted. A 20-ml volume, or as close to 20 ml as possible, should be collected and injected directly into aerobic and anaerobic blood culture bottles. Blood culture results obtained before opening the chest cavity by percutaneous subxyphoid aspiration have been shown to have greater interpretive value (less contamination but detection of relevant organisms). Most conclude that postmortem blood cultures rarely provide information that is not already known. Solid viscera should be sampled by immediately cutting blocks of tissue from the center of the seared area. Samples should be submitted to microbiology with a requisition providing a full explanation of the studies needed. Postmortem cultures can be very useful for detecting pathogens that are not considered members of the normal human microbiota, such as *M. tuberculosis*, *Brucella* spp., *B. pertussis*, some systemic fungi (*H. capsulatum*, *C. immitis/posadasii*, etc.), parasitic helminths, and agents of biological warfare. Tissue samples should be transported to the microbiology laboratory immediately in sterile tubes. The use of transport media and laboratory processing methods should follow recommendations for premortem specimens. An efficient way to avoid unnecessary workup of contaminating microorganisms is to issue a preliminary report to the pathologist who performed the autopsy listing organisms detected by colony or Gram stain morphology, such as "lactose-fermenting Gram-negative rod" or "Gram-positive cocci in clusters." This is accompanied with a notation that further identification and antimicrobial testing will not be performed unless there is consultation with the laboratory director or technologist conducting the culture investigation. Plates can be held for 1 week and discarded if no additional information is requested.

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Reagents, Stains, and Media: Bacteriology*

RONALD ATLAS AND JAMES SNYDER

19

REAGENTS

A number of classical and rapid tests are used for the identification of medically important bacteria. Below are brief descriptions of commonly performed tests and reagents used in clinical microbiology. See references 1–4 for more detailed descriptions of these tests and the reagents they use.

Biochemical Tests

Acetamide hydrolysis test (Nessler reagent)

Nessler reagent is used in the determination of acetamide hydrolysis. This test is useful in differentiating some Gram-negative bacteria. Acetamide agar or broth is inoculated. After incubation at 35 to 37°C until colonies or turbidity develops, 1 drop of Nessler reagent is added to 1 ml of broth or directly to the plate. A positive reaction is indicated by the formation of a red-brown sediment. Nessler reagent is prepared by dissolving 1 g of mercuric chloride in 6 ml of distilled water and then adding 2 or 3 drops of concentrated hydrochloric acid to dissolve the sediment. Separately, 2.5 g of potassium iodide is dissolved in 6 ml of distilled water and then added to the mercuric chloride solution. Then 6 g of potassium hydroxide is dissolved in 6 ml of distilled water and added to the mercuric chloride-iodide solution along with an additional 13 ml of distilled water. The solution is filtered using a sintered glass funnel (not a Nalgene filter). The Nessler reagent is stored in the dark and should be useful for several weeks. The solution should be checked for decomposition prior to use (any color change other than yellow indicates decomposition, and a fresh solution should be prepared). Nessler reagent is toxic if swallowed, inhaled, or absorbed through the skin. It presents a neurological hazard, may act as a carcinogen, and may be a reproductive hazard. It is corrosive and causes burns.

L-Alanine-7-amido-4-methycoumarin (Gram-Sure)

L-Alanine-7-amido-4-methylcoumarin is a fluorogenic compound that is used as an adjunct to the Gram stain to distinguish between Gram-negative and Gram-positive aerobic rods or coccobacilli. It is available from Remel (Thermo Fisher Scientific, Lenexa, KS) as reagent-impregnated disks—Gram-Sure. This rapid disk test is used most commonly with Gram-positive organisms that may appear Gram variable or Gram negative, such as *Bacillus* or *Lactobacillus*. The mechanism of the test is dependent on the presence of aminopeptidase in the cell walls of Gram-negative organisms, which hydrolyzes the reagent L-alanine-7-amido-4-methycoumarin in the disk from a nonfluorescent substrate to a blue fluorescent compound. A pure colony of overnight growth is inoculated into demineralized water and then inoculated onto the disk. The disk is incubated at room temperature for 5 to 10 min and then observed under long-wave UV light for blue fluorescence. Blue fluorescence is indicative of Gram-negative bacteria, and the absence of blue fluorescence is indicative of Gram-positive bacteria. Obligate anaerobes may fail to give expected results (5).

Alkaline phosphatase

Alkaline phosphatase is detected by the hydrolysis of a colorless phosphate-containing compound to a colored product, e.g., *p*-nitrophenol phosphate to *p*-nitrophenol, which is yellow; phenolphthalein phosphate to phenolthalein, which is red under alkaline conditions; or indolyl phosphate to indigo, which is blue. This test is useful in the diffentiation of *Staphylococcus* spp., and it is incorporated in several commercial identification systems.

Arginine arylamidase (L-arginine-4-methoxy-β-naphthalamide)

Arginine arylamidase is included as a diagnostic test in some commercial systems, e.g., the API test system of bioMérieux (Durham, NC). The substrate for this test is L-arginine-4-methoxy-β-naphthalamide. A negative test is colorless, and a positive test produces an orange color. This test is useful in differentiating staphylococcal species and various other bacteria.

Arginine dihydrolase

Arginine dihydrolase is detected by dense inoculation (McFarland standard of 4 to 5) of Moeller broth to which 1% arginine is added. After inoculation, the broth is overlaid with paraffin oil. Alkalinization, caused by the production of ammonia, is detected by the pH indicators. A positive

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*This chapter contains some information presented by Kimberle C. Chapin and Tsai-Ling Lauderdale in chapter 21 of the ninth edition of this *Manual*. In particular, Table 1 and the section on medium additives have been taken directly from that chapter. Descriptions of a number of stains and reagents were also based on material in that chapter.

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result is indicated by a purple color, which can be read after 4 h to up to 2 days.

Benzidine test (benzidine hydrochloride)

The benzidine test is useful for differentiating coagulase-negative *Staphylococcus* spp. This test is included in several commercial systems. It is based on the presence of iron-porphyrin compounds. Addition of a solution of 1 g of benzidine hydrochloride dissolved in 20 ml of glacial acetic acid, 30 ml of water, and 50 ml of 95% ethanol followed by addition of a 5% solution of hydrogen peroxide (H_2O_2) results in the formation of a blue-green to deep blue color for positive organisms.

Bile solubility test (deoxycholate)

The bile solubility test is used in the presumptive identification of *Streptococcus pneumoniae*. The key reagent in this test is sodium deoxycholate, which is a surface-active bile salt. The test may be run in a tube or on agar plates. The test is performed on alpha-hemolytic streptococcal colonies. A few drops of a 10% solution of sodium deoxycholate can be applied directly to the surface of a colony. The plate is then incubated for 30 min at 35°C. Pneumococcal colonies are lysed, whereas viridans group streptococci are not lysed. Alternatively, a heavy suspension of cells can be added to physiological saline solution (pH 7.0) and divided into two tubes. The 10% sodium deoxycholate solution is added to one tube, and sterile physiological saline is added to the other. The tubes are incubated at 35°C and are visually compared. If the organism is bile soluble, the tube containing the deoxycholate will exhibit reduced turbidity within 15 min and show an increase in viscosity along with clearing of the solution.

CAMP test (beta-lysin)

The CAMP test is used to identify group B beta-hemolytic streptococci based on their formation of a substance (CAMP factor) that enlarges the area of hemolysis formed by streptococcal beta-hemolysin. Hardy Diagnostics' (Santa Maria, CA) CAMP Spot Test Reagent is used as a rapid CAMP test method. The reagent, containing staphylococcal beta-lysin (also called beta-toxin, beta-hemolysin, or beta-staphylolysin), acts directly with the CAMP factor that is diffused into the medium around the suspect colony. The beta-lysin has a synergistic effect in the presence of CAMP factor, producing enhanced hemolysis of sheep erythrocytes. Enhanced hemolysis is visible within 30 min to 1 h of placing a drop of CAMP Spot Test Reagent next to an isolated beta-hemolytic *Streptococcus* colony.

Catalase test (H_2O_2)

H_2O_2 is used to detect bacterial production of catalase. A concentration of 15% is used for the differentiation of anaerobes, which do not produce catalase. A 30% peroxide concentration is used to test *Neisseria* spp. Cells from a colony are transferred to a clean glass slide, and a drop of hydrogen peroxide is added. Production of bubbles indicates a positive reaction. Blood must be avoided, as erythrocytes produce catalase and can cause a false-positive reaction. It is also possible to add a drop of hydrogen peroxide directly to a colony or slant as long as the medium does not contain blood. Immediate bubbling indicates a positive reaction.

Coagulase test (rabbit plasma)

Dehydrated rabbit plasma with EDTA is used to detect free or bound (clumping factor) coagulase produced by *Staphylococcus* spp. Human plasma is preferred for the detection of bound coagulase produced by *Staphylococcus lugdunensis* and *Staphylococcus schleiferi* but is not routinely used because it may contain antibodies against staphylococci. A heavy suspension of cells is added to a clean glass slide and mixed with a drop of distilled water. If agglutination does not occur spontaneously, the procedure can be performed by adding a drop of rabbit plasma to the suspension and mixing with a circular motion. The formation of visible white clumps indicates the presence of bound coagulase. Positive and negative controls should be run. The test can also be run in a test tube that detects both free and bound coagulase. For this test, 0.5 ml of rabbit plasma is added to a sterile tube. The tube is inoculated with a loopful of the test organism and incubated at 35°C for 4 h. Observations for clotting should be made within the first 4 h since some staphylococci produce fibrolysin, which can dissolve the clot. If no clotting is observed, however, the tube should be incubated overnight at room temperature and again observed for delayed clotting.

Decarboxylase tests

Bromcresol purple is used to detect the pH change due to decarboxylation of any of the amino acids lysine, ornithine, and arginine. Decarboxylase tests are useful for differentiating the *Enterobacteriaceae*. For the decarboxylase test, a broth at neutral or slightly acidic pH containing an individual amino acid being tested is inoculated for at least 24 h in most cases. The broth may contain the bromcresol purple or the test may be run after growth by adding a solution of bromcresol purple to a drop of the medium to determine if the pH is alkaline. A more rapid test has been described (6) that omits glucose from the medium and uses a starting pH of 5.5. In a positive result, the drop in pH is indicated by a change in color of bromcresol purple from yellow to purple.

Esculin hydrolysis (ferric ammonium citrate)

The hydrolysis of esculin to esculetin is detected using a 1% solution of ferric ammonium citrate. After incubation in esculin-containing medium for 1 to 2 days, a few drops of ferric ammonium citrate is added. The immediate formation of a brown-black color indicates a positive reaction. Esculin hydrolysis can also be determined using esculin agar, which is indicated by blackening after overnight incubation.

β-Galactosidase (*o*-nitrophenyl-β-D-galactopyranoside [ONPG])

ONPG at a concentration of 4 mg/ml is used to detect β-galactosidase activity. This enzyme facilitates growth on a carbon source like lactose by cleaving it into a molecule of glucose and a molecule of galactose, which the cells can catabolize and on which the cells can grow. The substrate ONPG is used in place of lactose. When the β-galactosidase cleaves ONPG, *o*-nitrophenol is released. This compound has a yellow color. This test is especially useful for identification of members of the family *Enterobacteriaceae*. ONPG-impregnated tablets can be used for this test.

β-Glucuronidase (*p*-nitrophenyl-β-D-glucopyranoside, 4-methylumbelliferyl-β-D-glucuronide [MUG])

Detection of β-glucuronidase activity can be accomplished using either a colorimetric substrate (*p*-nitrophenyl-β-D-glucopyranoside) or a fluorometric substrate (MUG). This test is useful for the rapid identification of *Escherichia coli*, members of the *Streptococcus anginosus* group, and other bacteria. For the colorimetric test, a solution of 0.1% (wt/vol) *p*-nitrophenyl-β-D-glucopyranoside (colorimetric substrate) in 0.067 M Sorensen phosphate buffer (pH 8.0) is prepared. Tubes containing 0.5 ml of the substrate solution are inoculated with a loopful of bacteria from an overnight culture. The tubes are incubated at 35°C and examined after 4 h for the appearance of a yellow color (liberated *p*-nitrophenol). In the fluorometric test, the substrate MUG yields the product 4-methylumbelliferyl, which fluoresces blue under long-wave UV light. The MUG test is normally used for the presumptive identification of *E. coli* and more recently for streptococcal strains. To prepare MUG for the fluorescent test, dissolve 50 mg of MUG in 10 ml of 0.05 M Sorensen phosphate buffer, pH 7.5. Dilute 1:16 of the stock MUG and add 1.25 ml to a vial containing 50 sterile paper disks. Allow the disks to be thoroughly saturated until no liquid remains in the vial. Spread the saturated disks out and allow to dry completely. The disks can be stored in a dark bottle at −20°C for 1 year or at 4°C for 1 month. Wet the disk with 1 drop of sterile water. Apply the organism to the disk using a wooden stick or loop and then incubate the disk for up to 2 h at 35°C. Shine a long-wave UV light on the disk. A positive reaction is indicated by blue fluorescence.

Hippurate hydrolysis (ninhydrin reagent) (ferric chloride)

Hippurate hydrolysis to benzoic acid and glycine is useful in the identification of group B streptococci (GBS), some *Listeria* spp., *Gardnerella vaginalis*, *Campylobacter jejuni*, and *Legionella pneumophila*. Ninhydrin reagent can be used to detect the production of glycine. Ninhydrin reagent (3.5%) is prepared by adding 3.5 g of ninhydrin to 50.0 ml of acetone and 50.0 ml of 1-butanol. The ninhydrin reagent is stored in the dark at room temperature. A 1% (wt/vol) solution of sodium hippurate is prepared in 0.067 M Sorensen phosphate buffer (pH 6.4). Tubes containing 0.5 ml of this solution are inoculated and incubated at 35°C for 2 h, after which 0.2 ml of the ninhydrin reagent is added. Development of a deep blue-purple color within 5 min indicates a positive reaction. For *L. pneumophila*, 0.5 ml of 1% sodium hippurate solution is inoculated with a loopful of organism and incubated at 35°C in ambient air for 18 to 20 h, after which 0.2 ml of ninhydrin reagent is added. The cells and ninhydrin are mixed and incubated for an additional 10 min at 35°C. The mixture is observed for 20 min for blue-purple color development, which is indicative of a positive reaction. Ferric chloride can also be used to detect hippurate hydrolysis. Ferric chloride reagent (12 g of $FeCl_3 \cdot 6H_2O$ in 100.0 ml of 2% HCl) is added to inoculated broth (e.g., heart infusion broth or Todd-Hewitt broth) supplemented with hippurate. An insoluble brown ferric benzoate precipitate indicates a positive hydrolysis reaction.

Indole test (Ehrlich reagent, Kovács reagent, *p*-dimethylaminocinnamaldehyde [DMACA])

The indole test is used for the determination of production of indole from deamination of tryptophan by tryptophanase. This reaction can be detected using Ehrlich reagent, Kovács reagent, or DMACA. To prepare Ehrlich reagent, add 1 g of *p*-dimethylaminobenzaldehyde to 95 ml of 95% ethyl alcohol. Then slowly add 10 ml of concentrated hydrochloric acid. Using Ehrlich reagent, first extract the indole by adding 1 ml of xylene to a 48-h-old tryptone broth or other tryptophan-containing broth medium. Shake the tube vigorously for 20 s and let stand for 1 to 2 min to allow the xylene extract to come to the top of the broth. Gently add 0.5 ml of the Ehrlich reagent down the side of the tube. Do not shake the tube. A red ring at the interface of the medium and the reagent phase within 5 min represents a positive test. Ehrlich reagent is preferred for organisms that produce small amounts of indole, such as nonfermenters and anaerobes. To prepare Kovács indole reagent, add 10 g of *p*-dimethylaminobenzaldehyde to 150 ml of either amyl or isoamyl alcohol. Then add 50 ml of concentrated hydrochloric acid. Add 5 drops of Kovács reagent to either 48-h-old 2% tryptone broth or an 18- to 24-h-old tryptophan broth culture. Do not shake the tube after the addition of reagent. A red color at the surface of the medium indicates a positive test. For the spot indole test, add 2 ml of concentrated HCl to 18 ml of distilled water. Allow the mixture to cool. Then add 200 mg of DMACA. Moisten a piece of Whatman no. 3 paper with a couple drops of the reagent. Remove a well-isolated colony from an 18- to 24-h-old culture onto a blood agar plate with a sterile inoculating loop or a wooden stick and smear it onto the moistened filter paper. Observe for a blue to blue-green color within 2 min, which indicates a positive reaction. No color change or a pinkish tinge is considered negative. This test should be used only on colonies from media containing sufficient tryptophan and no glucose. Colonies from media containing dyes (e.g., MacConkey or eosin-methylene blue [EMB] agar) may cause misleading results and should not be used. Colonies from mixed cultures should not be used, as indole-positive colonies can cause indole-negative colonies to appear weakly positive.

LAP test (leucine naphthylamide)

The LAP test detects the presence of leucine aminopeptidase (LAP). The substrate leucine naphthylamide is hydrolyzed by LAP to leucine and free naphthylamine. The LAP test is helpful in the presumptive characterization of catalase-negative, Gram-positive cocci (streptococci, enterococci, and streptococcus-like organisms). *S. pneumoniae*, *Streptococcus pyogenes*, *Pediococcus*, *Lactococcus*, and *Enterococcus* spp. are all LAP positive, while other beta-hemolytic streptococci are LAP negative. Disks are impregnated with leucine-β-naphthylamide or leucine-α-naphthylamide, which is hydrolyzed by the enzyme LAP, produced by LAP-positive organisms. This enzymatic activity results in the release of β-naphthylamine, which couples with DMACA reagent (Dalynn RP95), when it is added, to form a highly visible red color. To perform this test, aseptically place an LAP disk in a sterile petri dish and allow the disk to warm to room temperature. Moisten with a small amount of water. Inoculate with several colonies from an overnight culture plate. Incubate for 5 min at room temperature. Add 1 drop of DMACA and read within 1 min. Development of a red or pink color indicates a positive test.

Lysozyme test (lysozyme)

The lysozyme test measures the ability of organisms, such as *Nocardia*, to grow in the presence of lysozyme. A solution of 50 mg of lysozyme in 50 ml of 0.01 N HCl is used for this test. The solution is filter sterilized and can be stored refrigerated for up to a week. For the lysozyme test, add 5 ml of lysozyme solution to 95 ml of basal glycerol broth (peptone, 1 g; beef extract, 0.6 g; glycerol, 14.0 ml; distilled water, 200 ml). Dispense in 5-ml aliquots and keep refrigerated. Growth of the test organism in the lysozyme-supplemented glycerol broth is compared with the growth in the unsupplemented glycerol broth.

Nitrate reduction test (*N,N*-dimethyl-naphthylamine and sulfanilic acid)

The nitrate reduction test is used to determine the ability to reduce nitrate to nitrite or free nitrogen gas. This test involves the use of two reagents. Reagent B is prepared by adding 0.6 ml of *N*,*N*-dimethyl-naphthylamine to 100 ml of 5 N (30%) acetic acid. Reagent A is prepared by adding 0.8 g of sulfanilic acid to 100 ml of 5 N acetic acid. These reagents are stored in the dark under refrigeration. To perform the test, add 0.05 ml of reagent A to 10 drops of an overnight growth from the nitrate broth culture and incubate for 5 to 10 min. Then add 0.05 ml of reagent B and incubate for an additional 5 to 10 min. Incubation should be in the dark. (Note: reagents A and B may be mixed and added together as indicated in the ninth edition of this *Manual*, but this lowers the sensitivity of the test.) An organism may be reported as nitrate positive if a red or purple-magenta color develops in the medium within a few minutes after nitrate reagents A and B are added to the medium, indicating that the organism has reduced nitrate to nitrite. The absence of a red-purple color after the addition of both reagents does not automatically mean that the organism is unable to reduce nitrate. Strains may have reduced the nitrate to nitrite and then reduced the nitrite completely to nitrogenous gases that are not detected when nitrate reagents A and B are added to the medium. If the medium does not change color after the addition of sulfanilic acid and α-naphthylamine, a small amount ("knife point") of zinc dust is added to the incubated medium. The zinc dust will catalyze the reduction of nitrate to nitrite chemically. Thus, if the nitrate has not been reduced by the organisms, i.e., they are nitrate negative, it will be reduced by the zinc dust and a red color will develop in the incubated medium within 15 min. If no color develops in the incubated medium after the addition of zinc dust, the organisms not only have reduced nitrate to nitrite but have reduced nitrite to nitrogenous gases; these organisms are also nitrate positive.

Oxidase test (TMPD/DMPD)

The oxidase test is a test used in microbiology to determine if a bacterium produces certain cytochrome *c* oxidases (7). It uses disks impregnated with a reagent such as *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) or *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD), which is also a redox indicator. TMPD is more sensitive than DMPD and therefore generally the preferred reagent. The reagent is a dark blue to maroon color when oxidized and colorless when reduced. A modified oxidase test is used for the differentiation of *Micrococcus* and related organisms from most other aerobic Gram-positive cocci. Six percent TMPD (the same chemical used in Kovács oxidase reagent) dissolved in dimethyl sulfoxide is used as the reagent. Keep the reagent away from light. A loopful of colonies from blood agar plates is smeared onto filter paper, and the reagent is dropped onto the bacterial growth. Development of a blue to purple-blue color in 2 min indicates a positive reaction.

Phenylalanine deaminase test (ferric chloride)

Ferric chloride is used to detect the action of phenylalanine deaminase. Twelve grams of $FeCl_3·6H_2O$ is added to 100.0 ml of 2% hydrochloric acid. The hydrochloric acid is prepared by adding 5.4 ml of concentrated HCl (37%) to 94.6 ml of distilled water. To perform the phenylalanine deaminase test, 4 or 5 drops of ferric chloride reagent is added to a culture grown overnight on phenylalanine agar or broth. The development of a brown color in the medium indicates a positive reaction.

Pyrrolidonyl aminopeptidase activity (PYR test)

Pyrrolidonyl aminopeptidase (pyrrolidonyl arylamidase) or PYR is a rapid colorimetric method for presumptive identification of certain groups of bacteria based on the activity of the enzyme pyrrolidonyl arylamidase. This test is used in the identification of Gram-positive cocci and nonfermentative Gram-negative bacteria. The reaction involves addition of DMACA, which can be suspended in a solution of 2.5 ml of sodium dodecyl sulfate, 2.5 ml of glacial acetic acid, 5.0 ml of 2-methoxyethanol, and 90 ml of distilled water (stored at 4°C in a dark container). There also is a commercial kit in which L-pyroglutamic acid β-naphthylamide is impregnated into the test disk and serves as the substrate for the detection of pyrrolidonyl arylamidase. Hydrolysis of the substrate yields β-naphthylamide, which combines with the PYR reagent (DMACA) to form a bright pink to cherry red color. A positive PYR tests allows for the presumptive identification of group A streptococci (*S. pyogenes*) and group D enterococci.

Urease test (phenol red)

The urease test is used to determine the ability of an organism to split urea through the production of the enzyme urease. Ammonia is produced, which causes a rise in pH that is detected by a change in color of the indicator phenol red to pink under alkaline conditions (pH 8.4). Bacteria are cultured on a medium containing urea, e.g., Christensen urea agar. While many enteric bacteria can hydrolyze urea, only a few "rapid urease-positive" organisms, e.g., *Proteus* species, can degrade urea quickly (<4 h). Urea broth is formulated to test for rapid urease-positive organisms. The restrictive amount of nutrients coupled with the use of pH buffers prevents all but rapid urease-positive organisms from producing enough ammonia to turn phenol red to pink. The rapid urease test also is used for the diagnosis of *Helicobacter pylori*. To detect *H. pylori*, this test is performed on stomach lining cells collected by biopsy. A basic broth for performing the urease test can be made by adding 10.4 ml of a 20% (wt/vol) aqueous solution of urea to a solution containing 0.1 g of KH_2PO_4, 0.1 g of K_2HPO_4, and 0.5 ml of 1:500 phenol red, adjusted to pH 6.8 in 100 ml. To make 1:500 phenol red, dissolve 0.2 g of phenol red in NaOH and add distilled water to 100 ml. Red color developing within 4 h after inoculation indicates urease activity.

Voges-Proskauer (VP) test (α-naphthol/KOH)

The VP test is used to detect acetoin (acetyl-methylcarbinol), which is produced by certain bacteria during growth in a buffered peptone-glucose broth (methyl red-VP broth). The VP test is commonly used to aid in the differentiation between genera (such as *E. coli* from the *Klebsiella* and *Enterobacter* spp.) and among species of the *Enterobacteriaceae* family. The test can be used as a differential test for other organism groups (viridans group streptococci). The test uses 5% α-naphthol, which is prepared by dissolving 5 g of α-naphthol in 100 ml of absolute ethanol, and 40% KOH, which is prepared by dissolving 40 g of potassium hydroxide in 100 ml of distilled water. To perform the test, methyl red-VP broth is inoculated and incubated until good growth is obtained. Then 0.6 ml of the α-naphthol solution and 0.2 ml of the 40% KOH are added to 2.5 ml of culture broth. A positive reaction is indicated by the formation of a pink-red product within 5 min. However, allow 15 min for color development before considering the test negative.

Buffers

Bovine albumin fraction V

A 0.2% solution of bovine albumin fraction V is used to buffer mycobacterial specimens following decontamination with *N*-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH). The solution is prepared by mixing 40.0 ml of 5% bovine albumin with 8.5 g of NaCl and 960.0 ml of distilled water. The pH is adjusted to 6.8 using 4% NaOH. The solution is filter sterilized and stored refrigerated. Before addition to the buffer, samples are decontaminated with NALC-NaOH and concentrated by centrifugation. The sedimented sample is suspended in 1 to 2 ml of the sterile 0.2% bovine albumin solution. The preserved cells can then be examined microscopically or inoculated into a culture medium.

Glycine-buffered saline

Glycine-buffered saline (0.043 M glycine, 0.15 M NaCl [pH 9.0]) is used in some serological procedures and is also used as a transport medium for enteric organisms. It is prepared by dissolving 3.22 g of glycine and 8.77 g of NaCl in 1 liter of distilled water.

Phosphate-buffered saline

Phosphate-buffered saline solutions are made by mixing varying amounts of 0.1 N mono- and dibasic phosphates, depending on the pH desired, with 0.85% NaCl. These are prepared as 10× stock solutions. For 0.1 M NaH_2PO_4 (sodium phosphate, monobasic), dissolve 13.9 g of NaH_2PO_4 in 1 liter of deionized water; for 0.1 M Na_2HPO_4 (sodium phosphate, dibasic), dissolve 26.8 g of $Na_2HPO_4 \cdot 7H_2O$ in 1 liter of deionized water; and for 8.5% NaCl (sodium chloride), dissolve 85.0 g of NaCl in 1 liter of deionized water. Sterilize by autoclaving for 20 min or by filtration. Store refrigerated. For the working phosphate-buffered saline, combine the appropriate amounts of the 10× stock solutions of the mono- and dibasic phosphate solutions with 100 ml of 8.5% NaCl and bring the volume to 1 liter with distilled or deionized water.

Sorensen pH buffers

Sorensen pH buffers are prepared by mixing appropriate amounts of 0.067 M dibasic sodium phosphate with 0.067 M monobasic potassium phosphate. To prepare 0.067 M dibasic sodium phosphate, dissolve 9.464 g of anhydrous Na_2HPO_4 in 1 liter of distilled water. To prepare 0.067 M monobasic potassium phosphate, dissolve 9.073 g of anhydrous KH_2PO_4 in 1 liter of distilled water.

Decontamination Agents

Cetylpuridium chloride-sodium chloride (CPC-NaCl)

CPC-NaCl is used for decontamination of transported sputum specimens for culturing mycobacteria. It is prepared by dissolving 1 g of CPC and 2 g of NaCl in 100 ml of distilled water. It can be stored in a sealed brown bottle at room temperature. If crystals form, the solution should be gently heated before use. An equal amount of sputum and CPC-NaCl is mixed until the specimen is liquefied, and then the specimen can be shipped to the testing site. Specimens treated with CPC-NaCl must be cultured on egg-based media or else residual CPC will inhibit mycobacterial growth.

NALC-NaOH

NALC (mucolytic agent)-NaOH (decontamination agent) is used in the processing of mycobacterial specimens. The reagent consists of 50.0 ml of sterile 4% NaOH, 50.0 ml of 2.9% sodium citrate, and 0.5 g of NALC. The sodium citrate is included to stabilize the acetylcysteine. This reagent should be used within 24 h of preparation.

Oxalic acid

Oxalic acid is used as a decontamination agent for specimens that contain *Pseudomonas* spp. when culturing for mycobacteria. The reagent is especially helpful when processing respiratory specimens from cystic fibrosis patients. To prepare the solution, 50 g of oxalic acid is added to 1.0 liter of distilled water. The solution is autoclaved at 121°C for 15 min. It can be stored at room temperature for up to a year.

Dyes and Indicators

A variety of dyes and indicators are used to detect specific reactions such as pH and oxygen production. Commonly used dyes and pH indicators are shown in Table 1.

Preservatives

Skim milk

Skim milk is used to stabilize bacterial suspensions, particularly those containing anaerobes, for freezing. The skim milk is prepared by adding 20 g of skim milk powder to 100 ml of distilled water. After the skim milk is dissolved in the water, the solution is dispensed as 0.25- to 0.5-ml aliquots into 2-ml vials. The skim milk is autoclaved

TABLE 1 Dyes and pH indicators

Indicator	pH and color
Acid fuchsin (Andrade's)	5.0, pink 8.0, pale yellow
Bromcresol green	3.8, yellow 5.4, blue
Bromcresol purple	5.2, yellow 6.8, purple
Bromphenol blue	3.0, yellow 4.6, blue
Bromthymol blue	6.0, yellow 7.6, dark blue
Chlorcresol green	4.0, yellow 5.6, blue
Chlorphenol red	5.0, yellow 6.6, red
Cresolphthalein	8.2, colorless 9.8, red
m-Cresol purple	7.4, yellow 9.0, purple
Cresol red	7.2, yellow 8.8, red
Methyl red	4.4, red 6.2, yellow
Neutral red	6.8, red 8.0, yellow
Phenolphthalein	8.3, colorless 10.0, red
Phenol red	6.8, yellow 8.4, red
Resazurin	Oxidized: blue, nonfluorescent Reduced: red, fluorescent
Thymol blue	8.0, yellow 9.6, blue
Triphenyltetrazolium chloride	Oxidized: colorless Reduced: red

at 110°C for 10 min. The vials can be refrigerated for up to 6 months.

McFarland Standards

McFarland standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range. These standards can be prepared using $BaCl_2$. They also are available using latex beads. To prepare McFarland standards, mix the designated amounts of 1% anhydrous barium chloride ($BaCl_2$) and 1% (vol/vol) cold pure sulfuric acid (H_2SO_4) in screw-cap tubes. Tightly seal the tubes. When the barium sulfate is shaken up well, the density in each tube corresponds approximately to the bacterial suspension. Store the prepared standard tubes in the dark at room temperature. The absorbance of the 0.5 McFarland standard should be 0.08 to 0.10 at 625 nm using a spectrophotometer with a 1-cm light path. Standards are checked regularly using a densitometer. No policy exists regarding the frequency of monitoring. However, when placed in the densitometer and it fails to read in the defined optical range, it should be considered unusable. Companies that produce automated identification/antibiotic susceptibility testing systems provide guidelines with the instrument. The manufacturer is responsible for the accuracy and reliability of the McFarland standards and should supply a quality control record with each production lot.

STAINS

Microscopic examination is useful in the identification of clinically important specimens. Smears can be made from relevant tissues and body fluids. If there are sufficient quantities of cells, the smear may be prepared by direct contact with a tissue sample or by applying a drop of body fluid, e.g., sputum, to a clean glass slide. Cytocentrifugation may be used to concentrate cells (8–10). Samples are fixed to the slides with either heat or methanol. Methanol fixation is preferred since heating may produce artifacts, may create aerosols, and may not adhere the specimen adequately to the slide. A variety of stains can then be used to help visualize and differentiate bacteria from the specimen. The following are some of the commonly used staining procedures.

Acid-fast stain

Acid-fast staining is useful for the identification of *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Tsukamurella*, *Gordonia*, and *Legionella micdadei*. These bacteria have long-chain fatty acids (mycolic acids) that make them difficult to stain with crystal violet and other basic dyes.

Mycobacteria often appear as slender, slightly curved rods and may show darker granules that give the impression of beading. *Mycobacterium tuberculosis* can appear as beaded rods arranged in parallel strands or "cords"; *Mycobacterium kansasii* may form long, often broad and banded cells; and *Mycobacterium avium* complex cells appear as short, uniformly staining coccobacilli. *Nocardia* spp. often branch and almost always show a speckled appearance. A number of staining procedures have been developed for acid-fast staining.

In the Ziehl-Neelsen (Z-N) procedure, the slide is heat fixed for 2 h at 70°C. The slide is then flooded with carbol fuchsin (0.3 g of basic fuchsin is dissolved in 10 ml of 95% ethanol, 5 ml of phenol, and 95 ml of water; the solution is filtered before use). The slide is slowly heated to steaming and maintained for 3 to 5 min at 60°C. After cooling, the slide is washed with water and decolorized with acid-alcohol (97 ml of 95% ethanol in 3 ml of HCl). The slide is counterstained for 20 to 30 s with methylene blue (0.3 g of dye in 100 ml of water). An acid-fast organism will stain red, and the background of cellular elements and other bacteria will be blue, the color of the counterstain.

In the Kinyoun modification of the Z-N staining procedure, heating during staining with carbol fuchsin is eliminated and a higher concentration of phenol is used in the primary stain. The primary stain consists of 4 g of basic fuchsin in 20 ml of 95% alcohol, 8 g of phenol, and 100 ml of distilled water. The Z-N and Kinyoun stains have the same sensitivity and specificity; however, the Kinyoun (cold) staining procedure is less time-consuming and is easier to perform.

Another modification of the acid-fast staining procedure has been the use of a weaker decolorizing agent (0.5 to 1.0% sulfuric acid) in place of the 3% acid-alcohol. This particular stain helps differentiate those organisms known to be partially or weakly acid-fast, particularly *Nocardia*, *Rhodococcus*, *Tsukamurella*, *Gordonia*, and *Dietzia*. These organisms do not stain well with the Z-N or Kinyoun stain.

Factors such as age, exposure to drugs, and a particular acid-fast organism itself may vary the acid-fast presentation. For example, while *M. tuberculosis* is consistently acid-fast (with the Z-N or Kinyoun stain), rapidly growing mycobacteria and *Nocardia* are not. Therefore, use of the modified

Kinyoun stain may be necessary for these organisms. Other modifications used in tissue preparations, such as the Fite-Faraco stain and Pottz stain, may be preferred for unusual isolates such as *Mycobacterium leprae*.

Detection of small numbers of acid-fast organisms in clinical specimens is generally significant. However, the use of acid-fast stains for gastric aspirates in the interpretation of pulmonary disease in adults or for stool specimens from HIV-positive patients in diagnosing *M. avium-Mycobacterium intracellulare* infection yields very poor specificity (false-positive smears with saprophytic organisms) as well as poor sensitivity. In addition, patients receiving adequate therapy may still have positive smears without positive cultures for a number of weeks.

Acridine orange stain

Acridine orange is a fluorochrome that can be intercalated into nucleic acid in both the native and the denatured states. Acridine orange is useful in a number of miscellaneous infections, such as *Acanthamoeba* infections, infectious keratitis, and *H. pylori* gastritis (11). Additionally, it can be used to confirm the presence of bacteria in blood cultures when Gram stain results are questionable or difficult to interpret, especially when the presence of bacteria is suspected but failed to be detected with light microscopy. In the acridine orange staining procedure, the slide is flooded with the acridine orange solution (stock solution, 1 g of dye in 100 ml of water; working solution, 0.5 ml of stock added to 5 ml of 0.2 M acetate buffer [pH 4.0]). The slide is then examined by UV fluorescence microscopy.

Auramine-rhodamine stain

Auramine and rhodamine are nonspecific fluorochromes that bind to mycolic acids and that are resistant to decolorization with acid-alcohol. Staining procedures with these fluorochromes are thus equivalent to the fuchsin-based acid-fast procedures (12). Acid-fast organisms fluoresce orange-yellow in a black background. If the secondary stain is not used, the organisms will fluoresce a yellow-green color. In this procedure, the slide is heat fixed at 65°C for at least 2 h. It is then stained for 15 min with auramine-rhodamine solution (1.5 g of auramine O, 0.75 g of rhodamine B, 75 ml of glycerol, 10 ml of phenol, and 50 ml of H_2O) and rinsed with water, followed by decolorization for 2 to 3 min with 0.5% HCl in 70% ethanol. After being rinsed, the slide is counterstained with 0.5% potassium permanganate for 2 to 4 min. The slide is rinsed, dried, and examined under UV fluorescence microscopy.

Gimenez stain

The Gimenez stain is used for the visualization of *Rickettsia* and *Coxiella* from cell cultures and *L. pneumophila*. Carbol fuchsin is the primary stain, and fast green and malachite green are the counterstains, allowing greater contrast with the organisms and background for easier visualization of the organisms. The stain must be heated 48 h prior to use and filtered.

Gram stain

Gram staining is the differential staining procedure most commonly used for microscopic examination of bacteria. The procedure was first described by Hans Christian Joachim Gram. Based on the staining reaction, bacteria are classified as Gram-positive bacteria, which retain the primary crystal violet dye and appear deep blue or purple; and Gram negative bacteria, which can be decolorized, thereby losing the primary stain and subsequently taking up the counterstain safranin and appearing red or pink. The staining reaction reflects underlying differences in cell wall structure that are relevant for antibiotic susceptibility as well as identification. The Gram stain reaction works well with most bacteria but is not useful for bacteria that are too small or lack a cell wall, e.g., *Treponema*, *Mycoplasma*, *Chlamydia*, and *Rickettsia* (13). Mycobacteria are generally not seen by Gram staining; however, in smears illustrating heavy infections, the organisms may give a beaded appearance that is somewhat similar to that of *Nocardia* spp. or may exhibit organism “ghosts” (13). Anaerobic bacteria, older cultures, and organisms that are exhibiting the effects of antibiotics may be especially difficult to interpret.

In the conventional Gram stain procedure used in most clinical laboratories, the slide is first flooded with a primary stain of crystal violet (10 g of 90% dye in 500 ml of absolute methanol). After at least 15 s, the slide is washed with water and flooded with the mordant Gram’s iodine (6 g of I_2 and 12 g of KI in 1,800 ml of H_2O), which increases the affinity of the primary stain to the bacterial cell. The slide is washed with water after 15 s with the decolorizing agent acetone-alcohol (400 ml of acetone in 1,200 ml of 95% ethanol). The decolorizing agent will remove the primary stain from a Gram-negative cell. Gram-positive bacterial cells retain the primary stain. The slide is washed immediately and counterstained for at least 15 s with safranin (10 g of dye in 1 liter of distilled or deionized water). This slide is then washed, blotted dry, and examined by light microscopy at ×1,000 magnification.

For organisms such as *Francisella*, *Legionella*, and *Brucella* spp. that are difficult to visualize due to their tiny size and that stain only faintly with the traditional Gram stain, alternative stains may be used to enhance visualization. Substitution of safranin with basic fuchsin as a counterstain in the Gram stain has been successfully used to visualize *Francisella tularensis*; however, the direct fluorescent-antibody assay is more sensitive, although it is not commonly used in the clinical microbiology laboratory. The Gimenez stain can be used as an alternative to the Gram stain for the visualization of *Legionella* spp. This stain is difficult to prepare, is not commercially available, requires filtration before each use, and is unstable in storage; immunofluorescent staining is more sensitive but is not routinely used in the clinical microbiology setting. As with *F. tularensis*, replacement of safranin with basic fuchsin as the primary counterstain in the Gram stain increases the visualization of *Brucella* spp.

Immunofluorescent antibody stain

Immunofluorescent staining consists of labeling antibodies with a fluorescent dye, allowing the labeled antibodies to react with their specific antigens, and observing the stained bacterial cells under a fluorescence microscope (14). These methods allow the identification of specific bacterial species and subtypes based upon the specificity of the antibody reaction, e.g., for *Legionella* spp. Their utility in bacteriology is primarily for culture confirmation, as their utility for direct specimen testing has been supplanted by other immunological methods (enzyme immunoassays and PCR-immunochromatography test hybrid assays).

Methylene blue stain

Staining with methylene blue is used to show bacterial cell shape. This is useful for revealing the morphology of fusiform bacteria and spirochetes from oral infections (Vincent's angina). It may also establish the intracellular location of microorganisms such as *Neisseria*. Methylene blue is the stain of choice for identification of the metachromatic granules of diphtheria; however, one should be careful about overstaining, because this will lessen the contrast between the bacteria and the granules. Methylene blue stains organisms or leukocytes a deep blue against a light gray background. *Corynebacterium diphtheriae* appears as a blue bacillus with prominent darker blue metachromatic granules. For methylene blue staining, a 0.5 to 1.0% aqueous solution of methylene blue is applied for 30 to 60 s and up to 10 min for possible *C. diphtheriae* granules. The slide is rinsed with water, blotted dry, and examined by light microscopy at magnifications of ×100 to ×1,000.

M'Fadyean stain

The M'Fadyean stain is a modification of the methylene blue stain developed for detecting *Bacillus anthracis* in clinical specimens. The stain is prepared by dissolving 0.05 mg of methylene blue per ml in 20 mM potassium phosphate adjusted to pH 7.3. Slides are stained for 1 min and then washed. As a safety precaution, washing of the slide is performed using a 10% hypochlorite solution. The dried slide is examined by light microscopy. Virulent *B. anthracis* rods will be surrounded by a clearly demarcated zone giving the appearance of a reddish pink capsule (M'Fadyean reaction).

Spore stain

The Wirtz-Conklin spore stain is a differential stain for detection of spores. This is very useful for the identification of *Bacillus* and *Clostridium* species. Using this procedure, spores stain green while the rest of the cell stains pink. Non-spore-forming bacteria are pink. In this procedure, the slide is flooded with 5 to 10% aqueous malachite green. The stain is left on the slide for 45 min. Alternatively, the slide can be heated gently to steaming for 3 to 6 min. Heating to steaming enhances the uptake of the stain into the spores. The slide is then rinsed with water. Aqueous safranin (0.5%) is used as a counterstain for 30 s. The slide is then washed, blotted dry, and examined by light microscopy at ×1,000 magnification.

Wayson stain

The Wayson stain can be used to demonstrate bipolar staining characteristics of *Yersinia pestis* but is not commonly used in clinical microbiology laboratories. It no longer is used for screening cerebrospinal fluid for bacteria now that the incidence of *Haemophilus influenzae* meningitis has decreased. The staining reagents are prepared by dissolving 0.2 g of basic fuchsin in 10 ml of 95% ethyl alcohol and 0.75 g of methylene blue in 10 ml of 95% ethyl alcohol. The two solutions are added together slowly into 200 ml of 5% phenol in distilled water. The stain is then filtered and stored in an opaque bottle at room temperature. The stain is applied for 1 min. The slide is then washed, blotted dry, and examined by light microscopy at ×1,000 magnification.

CULTURE MEDIA

Laboratory cultivation of pathogenic microorganisms has been central to the laboratory diagnosis of infectious diseases caused by bacteria. Louis Pasteur in 1860 was the first to use culture media for growing bacteria in the laboratory. Pasteur's first medium consisted of sugar, ammonium salts, and yeast ash. It met the basic growth requirements of many bacteria, namely, a carbon source in the form of sugar that could be metabolized for energy and growth; a nitrogen source needed by bacteria to synthesize proteins and nucleic acids; and growth factors, including vitamins and minerals from the yeast ash.

Additional media were developed by Robert Koch and his colleagues, who used animal and plant tissues as sources of nutrients to support bacterial growth. One of the major discoveries of Fanny Hesse, who was the wife of one of the workers in Koch's laboratory, was that agar could be used to form solidified culture media on which microorganisms would grow. Using solid media permits the isolation of pure cultures of bacteria. Extracts of plants and animal tissues were prepared as broths or mixed with agar to form a variety of solid culture media. Virtually any plant, animal, or animal organ was considered for use in preparing media. Infusions were prepared from beef heart, calf brains, and beef liver, as a few examples. From the earliest development of media, there was a balance between scientific design and chance findings regarding which media would support the growth of specific pathogens. By the early 1900s, many media were available to aid in diagnostic activities. These classic infusions still form the primary components of many media that are widely used today in clinical microbiology laboratories, such as brain heart infusion agar.

Each medium has a specific use. See Table 2 for media used to detect emerging antibiotic-resistant pathogens. Often there are pros and cons to the choice of a specific medium—the advantages and disadvantages of each must be considered and the results obtained in the clinical laboratory must be evaluated in light of the limitations of a given medium. Articles frequently appear comparing media (see, for example, references 15–18).

Several comprehensive volumes have been compiled describing in detail the formulation of these media for the culture of bacteria (15, 19–26). Additionally, the major commercial producers of microbiological culture media maintain websites that provide important information about the composition, preparation, and use of specific culture media they supply, e.g., Becton, Dickinson and Company (http://www.bd.com/), Gibco Life Technologies (http://www.lifetechnologies.com/us/en/home/life-science/cell-culture.html?s_kwcid=AL!3652!3!26107395508!b!!g!!cell%20culture%20products&ef_id=USImGgAAAeCR0moj:20141020135720:s), Handy Diagnostics (http://www.hardydiagnostics.com/?gclid=CMifuc62tJsCFR7yDAodZlWRQg), HiMedia (http://www.himedialabs.com/), and Oxoid Ltd. (http://www.oxoid.com/uk/blue/index.asp). The inserts available with commercial media also provide critical information about their proper use.

Composition of Media

Agar is the most common solidifying agent used in microbiological media (27). Agar is a polysaccharide extract from marine algae. It melts at 84°C and solidifies at 38°C. Agar concentrations of 15.0 g/liter typically are used to form solid

TABLE 2 Media for detection of emerging antibiotic-resistant pathogens

Target pathogen(s)	Medium	Application
ESBL-producing *Enterobacteriaceae*	Brilliance ESBL Agar	For presumptive identification of ESBL-producing *Escherichia coli* and the KESC group directly from clinical samples
	CHROMagar CTX	For the detection of bacteria with ESBL CTX-M-type resistance
	CHROMagar ESBL (RambaCHROM ESBL)	For the detection of Gram-negative bacteria producing ESBLs
	chromID ESBL	For the screening of ESBL-producing enterobacteria
	HardyCHROM ESBL Agar	For the selection and differentiation of *Enterobacteriaceae* that produce ESBLs
	VACC agar (Remel VACC Agar)	For the primary isolation, selection, and differentiation of *Enterobacteriaceae* that produce ESBLs
Carbapenem-resistant *Enterobacteriaceae*	Brilliance CRE Agar	For the presumptive identification of carbapenem-resistant *E. coli* and the KESC group directly from clinical samples
	CHROMagar KPC (RambaCHROM KPC)	For the detection of Gram-negative bacteria with a reduced susceptibility to most carbapenem antimicrobial agents due to *Klebsiella pneumoniae* carbapenemase
	chromID CARBA agar	For the screening of carbapenemase-producing *Enterobacteriaceae*
	HardyCHROM CRE Agar	For the selection and differentiation of carbapenemase-producing Gram-negative bacteria
MRSA	Brilliance MRSA Agar	For universal MRSA screening
	CHROMagar MRSA (RambaCHROM MRSA)	For the qualitative direct detection of nasal colonization by MRSA
	chromID MRSA	For the direct and definitive detection of MRSA
	HiCrome MeReSa Agar with methicillin	For the isolation and cultivation of MRSA
	HiCrome MeReSa Agar Base with methicillin	For the isolation and cultivation of MRSA
	MRSA*Select* medium	For direct detection of MRSA from nasal and wound specimens
VRE	Brilliance VRE Agar	For the presumptive identification of *Enterococcus faecium* and *Enterococcus faecalis* directly from clinical samples
	CHROMagar VRE (RambaCHROM VRE)	For the differentiation and presumptive identification of VRE (*E. faecalis*/*E. faecium*)
	chromID VRE	For the rapid and reliable qualitative detection of vancomycin-resistant *E. faecium* and *E. faecalis*
	Enterococcosel agar with vancomycin	For the detection of VRE
	VRE agar	For the isolation of VRE
	VRE*Select* medium	For use with rectal swabs and fecal specimens
Penicillinase-producing *Neisseria gonorrhoeae*	PPNG selective medium	For the differentiation and presumptive identification of penicillinase-producing strains of *N. gonorrhoeae*

media. Lower concentrations, 7.5 to 10.0 g/liter, are used to produce soft agars or semisolid media.

Many media contain peptones as the source of nitrogen. Peptones are hydrolyzed proteins formed by enzymatic or acidic digestion. Casein most often is used as the protein substrate for forming peptones, but other substances, such as soybean meal, also are commonly employed.

Meat and plant infusions are aqueous extracts that are commonly used as sources of nutrients for the cultivation of microorganisms. Such infusions contain amino acids and low-molecular-weight peptides, carbohydrates, vitamins, minerals, and trace metals. Extracts of animal tissues contain relatively high concentrations of water-soluble protein components and glycogen. Extracts of plant tissues contain relatively high concentrations of carbohydrates.

The pH is generally maintained within a few tenths of a pH unit. For this reason, buffers are a key component of many media. Some pathogens are anaerobic, and factors such as thioglycolate are included in some media to reduce the availability of molecular oxygen so that anaerobes may be cultured. The use of specialized media for the culture of anaerobes is important for clinical microbiology (28).

Many microorganisms have specific growth factor requirements that must be included in media for their successful cultivation. The incorporation of key growth factors began in the 1930s. Gradually new formulations were developed that incorporated vitamins, amino acids, fatty acids, trace metals, and blood components to meet the growth factor requirements of specific pathogens. Most often, mixtures of growth factors are used in microbiological media. Acid hydrolysates of casein commonly are used as sources of amino acids. Extracts of yeast cells also are employed as sources of amino acids and vitamins for the cultivation of microorganisms. Many of the media used in the clinical laboratory contain blood or blood components that serve as essential nutrients for fastidious microorganisms.

Many media contain selective components that inhibit the growth of nontarget bacteria. Selective media are especially useful in the isolation of specific pathogens from mixed populations. Selective toxic compounds are also frequently used to select for the cultivation of particular microbial species. The isolation of a pathogen from a stool specimen, for example, where there is a high abundance of nonpathogenic normal microbiota, requires selective media. Often, antimicrobials or other selectively toxic compounds are incorporated into media to suppress the growth of the background microbiota while permitting the cultivation of the organism of interest. Bile salts, selenite, tetrathionate, tellurite, azide, phenylethanol, sodium lauryl sulfate, high sodium chloride concentrations, and various dyes—such as eosin, crystal violet, and methylene blue—are used as selective toxic chemicals. Antimicrobial agents used to suppress specific types of microorganisms include ampicillin, chloramphenicol, colistin, cycloheximide, gentamicin, kanamycin, nalidixic acid, sulfadiazine, and vancomycin. Various combinations of antimicrobials are effective in suppressing classes of microorganisms, such as enteric bacteria.

Some media contain components that permit the differentiation of specific pathogens based on key metabolic reactions. These include production of acid from various carbohydrates and other carbon sources or the decarboxylation of amino acids. Some media include indicators that permit the visual detection of changes in pH resulting from such metabolic reactions. A number of media also include chromogenic dyes that change color when specific enzymatic reactions occur. See Table 3 for sources of chromogenic media.

Some media contain components that are toxic or carcinogenic. Appropriate safety precautions must be taken when using media with such components. Basic fuchsin and acid fuchsin are carcinogens, and caution must be used in handling media with these compounds to avoid dangerous exposures that could lead to the development of malignancies. Thallium salts, sodium azide, sodium biselenite, and cyanide are among the toxic components found in some media. These compounds are poisonous, and steps must be taken to avoid ingestion, inhalation, or skin contact. Azides also react with many metals, especially copper, to form explosive metal azides. The disposal of azides must avoid contact with copper or achieve sufficient dilution to avoid the formation of such hazardous explosive compounds. Cycloheximide is toxic. Avoid skin contact or aerosol formation and inhalation. Media with human blood or human blood components must be handled with great caution to avoid exposure to HIV and other pathogens that contaminate some blood supplies. Proper handling and disposal procedures must be followed with blood-containing media as well as other media that are used to cultivate microorganisms.

Preparation of Media

It is important to follow the manufacturers' instructions in preparing media. The ingredients in a medium are usually dissolved, and the medium is then sterilized. When agar is used as a solidifying agent, the medium must be heated gently, usually to boiling, to dissolve the agar. In some cases where interactions of components, such as metals, would cause precipitates, solutions must be prepared and occasionally sterilized separately before mixing the various solutions to prepare the complete medium. The pH often is adjusted prior to sterilization, but in some cases sterile acid or base is used to adjust the pH of the medium following sterilization. Many media are sterilized by exposure to elevated temperatures. The most common method is to autoclave the medium. Different sterilization procedures are employed when heat-labile compounds are included in the formulation of the medium.

Autoclaving uses exposure to steam, generally under pressure, to kill microorganisms. Exposure for 15 min to steam at 15 lb/in^2 at 121°C is most commonly used. Such exposure kills vegetative bacterial cells and bacterial endospores. However, some substances do not tolerate such exposures, and lower temperatures and different exposure times are sometimes employed. Media containing carbohydrates often are sterilized at 116 to 118°C in order to prevent the decomposition of the carbohydrate and the formation of toxic compounds that would inhibit microbial growth.

The proper preparation of media is critical for performance. For this reason, as well as because of personnel costs, many clinical laboratories purchase prepared media. Quality control is essential regardless of whether the medium is prepared in the laboratory or purchased as a preprepared medium (29, 30). Quality control test cultures are used to periodically check the performance of the media. For general-purpose media, sufficient, characteristic growth and typical colony morphology should be obtained with all test strains. For selective media, growth of designated organisms should be inhibited and adequate growth of desired organisms must be obtained. Differential characteristics of the medium for specific bacterial strains, e.g., color and hemolytic reactions, must be met. Media must not be used past expiration dates. The Clinical Laboratory and Standards Institute issues standards for quality assurance of commercially prepared microbiological culture media (30).

Bacteriological Media

Below are descriptions of representative media used in clinical microbiology.

A7 agar (Shepard's differential agar)

A7 agar is used for the cultivation and differentiation of *Ureaplasma urealyticum* from urine based on its ability to produce ammonia from urea. The medium contains digests of casein and soybeans plus growth factors, including cysteine, NAD, cocarboxylase, vitamins, yeast extract, and penicillin as a selective factor. Manganous sulfide is included as a nutritional factor. Urea is a key component that allows the detection of *Ureaplasma* spp. Bacteria that produce ammonia from the hydrolysis of urea appear as golden to dark brown colonies.

A8 agar

This agar is used for the cultivation and differentiation of *U. urealyticum* from urine based on its ability to produce

TABLE 3 Commercial sources of chromogenic agar media for bacteria

Organism(s)	Medium	Manufacturer
Acinetobacter	CHROMagar Acinetobacter	CHROMagar
Clostridium difficile	chromID *C. difficile*	bioMérieux
Escherichia coli	CHROMagar E. coli	CHROMagar
	CHROMagar ECC	CHROMagar
	BBL CHROMagar E. coli	BD[a]
	Chromocult coliform agar ES	Merck
	Brilliance UTI Agar	Oxoid
	Brilliance UTI Clarity Agar	Oxoid
	CHROMagar STEC	CHROMagar
	chromID CPS	bioMérieux
	HiCrome UTI Agar	Sigma-Aldrich
E. coli O157:H7	CHROMagar O157	CHROMagar
	BBL CHROMagar O157	BD
	Rainbow Agar O157	Biolog[b]
	O157:H7 ID agar	bioMérieux
Listeria	CHROMagar Listeria	CHROMagar
	BBL CHROMagar Listeria	BD
	Biosynth chromogenic medium for *Listeria monocytogenes*	Biosynth International
	HiCrome Listeria Agar Base, modified	Sigma-Aldrich
MRSA	CHROMagar MRSA	CHROMagar
	BBL CHROMagar MRSA	BD
	BBL CHROMagar Staph aureus	BD
	Brilliance MRSA Agar	Oxoid
	chromID MRSA	bioMérieux
	HiCrome MeReSa Agar with methicillin	Sigma-Aldrich
Enterococcus	CHROMagar Orientation	CHROMagar
	BBL CHROMagar Orientation	BD
	Brilliance UTI Agar	Oxoid
	Brilliance UTI Clarity Agar	Oxoid
	chromID CPS	bioMérieux
Pseudomonas	CHROMagar Pseudomonas	CHROMagar
	chromID *P. aeruginosa*	bioMérieux
Salmonella	CHROMagar Salmonella	CHROMagar
	BBL CHROMagar Salmonella	BD
	Rainbow Agar *Salmonella*	Biolog
	CHROMagar Salmonella Plus	CHROMagar
	Brilliance Salmonella Agar	Oxoid
	chromID *Salmonella*	bioMérieux
	HiCrome RajHans Medium	Sigma-Aldrich
	HiCrome Salmonella Agar	Sigma-Aldrich
Staphylococcus	CHROMagar Staphylococcus	CHROMagar
	Brilliance Staph 24 Agar	Oxoid
	chromID *S. aureus*	bioMérieux
	Staphylococcus aureus agar, HiCrome	Sigma-Aldrich
	S. aureus ID	bioMérieux
	Sa*Select* Medium	Bio-Rad
	MRSA*Select*	Bio-Rad
Streptococcus, group B	CHROMagar StrepB	CHROMagar
	chromID Strepto B	bioMérieux
Vibrio	CHROMagar Vibrio	CHROMagar
	chromID *Vibrio*	bioMérieux

(Continued on next page)

TABLE 3 Commercial sources of chromogenic agar media for bacteria *(Continued)*

Organism(s)	Medium	Manufacturer
Yersinia enterocolitica	CHROMagar Y. enterocolitica	CHROMagar
VRE	CHROMagar VRE	CHROMagar
	Brilliance VRE Agar	Oxoid
	chromID VRE	bioMérieux
	VRE*Select* Medium	Bio-Rad
ESBL-producing *Enterobacteriaceae* and some *Pseudomonas* spp.	Brilliance ESBL Agar	Oxoid
	CHROMagar CTX	CHROMagar
	CHROMagar ESBL	CHROMagar
	chromID ESBL	bioMérieux
	HardyCHROM ESBL Agar	Hardy Diagnostics
	RambaCHROM ESBL	Gibson Bioscience[c]
	VACC Agar	Remel
Carbapenem-resistant *Enterobacteriaceae*	CHROMagar KPC	CHROMagar
	chromID CARBA agar	bioMérieux
	HardyCHROM CRE Agar	Hardy Diagnostics
	RambaCHROM KPC	Gibson Bioscience

[a] Becton Dickinson (BD) licensing agreement with CHROMagar.
[b] Biolog licensing agreement with FocusBiotech Sdn, Bhd. (Petaling Jaya, Malaysia).
[c] Gibson Bioscience (Lexington, KY) licensing agreement with CHROMagar.

ammonia from urea. This medium also supports the growth of *Mycoplasma hominis*. The medium contains digests of casein and soybeans plus growth factors, including cysteine, NAD, cocarboxylase, vitamins, yeast extract, and penicillin as a selective factor. Manganous sulfide, calcium chloride, and putrecine dihydrochloride are included as nutritional factors. Amphotericin B and penicillin G are added as selective factors. Urea is a key component that allows the detection of *Ureaplasma* species. *U. urealyticum* can be differentiated from other genital mycoplasmas on this medium due to manganous sulfate in the medium, which combines with the urease to form a golden brown pigment.

Acetamide agar

Acetamide agar is used for differentiating nonfermentative Gram-negative bacteria, especially *Pseudomonas aeruginosa* (31, 32). Acetamide is the key component that supports growth and forms the basis for differentiation. Most bacteria are unable to grow on acetamide as the sole source of carbon and nitrogen. Bacteria that deamidate acetamide turn the medium blue if bromthymol blue is included as the indicator or red if the medium contains phenol red. The color change is due to the liberation of ammonia from the utilization of acetamide.

Acetate differential agar (Simmons' citrate agar, modified)

This agar is used for the differentiation of *Shigella* spp. from *E. coli* and also for the differentiation of nonfermenting Gram-negative bacteria (33). The medium contains acetate as the sole source of carbon. Bacteria that can utilize acetate as the sole carbon source turn the medium blue due to the increase in pH as the acidic acetate is utilized.

Alkaline peptone salt broth

This broth is used for the cultivation of *Vibrio cholerae* and other *Vibrio* spp. from stool specimens. Peptone is the growth substrate. A salt concentration of 3% is selective for *Vibrio* species.

Alkaline peptone water

This medium is used for the cultivation and transport of *V. cholerae* and *Aeromonas hydrophila*. Peptone is the growth substrate. The salt concentration is 1%.

American Trudeau Society (ATS) medium

ATS medium is used for the isolation and cultivation of *Mycobacterium* spp. other than *M. leprae*. It is especially useful for the detection of *M. tuberculosis* from clinical specimens such as cerebrospinal fluid, pleural fluid, and tissues. The medium contains eggs, which supply the fatty acids needed for growth by mycobacteria. Potatoes are also included as a source of carbon. Malachite green is a selective factor included in the medium.

Amies transport medium with charcoal

This medium is used for the transport of swab specimens to prolong the survival of microorganisms between collection and culturing. The phosphate buffer and calcium and magnesium ions in the medium help protect cells against lysis. The addition of charcoal to this medium neutralizes metabolic products that may be toxic to *Neisseria gonorrhoeae*.

Amies transport medium without charcoal

This medium is used for the transport of swab specimens to prolong the survival of microorganisms. This medium contains phosphate buffer and calcium and magnesium ions to protect cells against lysis.

Anaerobic colistin-nalidixic acid (CNA) agar

This agar is used for the selective isolation of anaerobic streptococci. Digests of casein and animal tissue along with yeast extract, beef extract, and glucose supply the carbon and nitrogen for growth. Dithiothreitol and cysteine help create anaerobic conditions. The medium contains colistin and nalidixic acid as selective factors. Blood is added to support the growth of fastidious strains and also to permit differentiation based on hemolytic reactions.

Ashdown medium

Ashdown medium is used for the selective isolation and characterization of *Burkholderia pseudomallei* from clinical specimens such as sputum. The medium contains crystal violet and gentamicin as selective factors. It is enriched with glycerol and contains neutral red. *B. pseudomallei* produces flat, wrinkled, purple colonies on this medium. Both Ashdown agar and Ashdown broth can be modified by the addition of antimicrobials for the selective culture of *B. pseudomallei*. Modified Ashdown broth remains the standard for isolation of *B. pseudomallei* from throat swabs in patients with suspected melioidosis (34).

Bacillus cereus medium

This medium is used for the cultivation of *Bacillus cereus*. The medium contains mannitol and eggs plus yeast extract. Bromcresol purple is included to aid in differentiation of *B. cereus*.

Bacillus cereus selective agar base

This medium is used for the selective isolation, enumeration, and presumptive identification of *B. cereus*. The medium contains mannitol and eggs plus yeast extract. Polymyxin B is included as a selective factor. Bromthymol blue is included to aid in differentiation. *B. cereus* grows as moderate-sized (5-mm) crenated colonies, which are turquoise, surrounded by a precipitate of egg yolk, which is also turquoise.

Bacteroides bile esculin agar

This agar is used for the selection and presumptive identification of the *Bacteroides fragilis* group and for the differentiation of *Bacteroides* spp. based on the hydrolysis of esculin and presence of catalase (4). The medium contains digests of casein and soybeans. Hemin, vitamin K_1, oxgall, and gentamicin are included in the medium as selective factors. Esculin is a key component for the differentiation of *Bacteroides* spp. *Bacteroides* colonies appear as gray, circular, raised colonies larger than 1.0 mm. Esculin hydrolysis is indicated by the presence of a blackened zone around the colonies.

Baird-Parker medium

This medium is used for the selective isolation and enumeration of coagulase-positive staphylococci. This medium contains glycine, pyruvate digest of casein, beef extract, lithium chloride, yeast extract, eggs, and tellurite.

Barbour-Stoenner-Kelly medium

This medium is used for the cultivation of a wide variety of microorganisms in a chemically defined medium, including *Borrelia* and *Spirochaeta* spp. This is a complex medium containing numerous vitamins and growth factors. It also contains gelatin, glucose, pyruvate, peptone, albumin, and serum.

BCM O157:H7(+) plating medium

This proprietary medium from Biosynth International, Inc. (Itasca, IL) is used for the detection of highly pathogenic enterohemorrhagic *E. coli* serovar BCM O157:H7(+).

Bile esculin agar

This medium is used for differentiation between group D streptococci and non-group D streptococci; for differentiation of members of the *Enterobacteriaceae*, particularly *Klebsiella*, *Enterobacter*, and *Serratia*, from other enteric bacteria; and for differentiation of *Listeria monocytogenes*. Bile tolerance and esculin hydrolysis (seen as a dark brown to black complex) are presumptive for enterococci (group D streptococci). Vancomycin can be added to identify vancomycin-resistant enterococci (VRE).

Bile esculin agar with kanamycin

This medium is used for the selective isolation and/or presumptive identification of bacteria of the *B. fragilis* group from specimens containing a mixed biota. The medium contains beef extract and esculin. It also contains hemin and vitamin K_1 as growth factors. When examined with long-wavelength UV light, pigmented colonies of the *Bacteroides* group fluoresce red-orange. Oxgall and kanamycin are selective factors in the medium. Growth on this medium with blackening of the medium is presumptive for members of the *B. fragilis* group.

Bile esculin azide agar

This medium is used for the isolation and presumptive identification of group D streptococci. The medium contains digest of casein, proteose peptone, iron, esculin, and yeast extract. Oxgall (bile) and sodium azide are included as selective factors. The hydrolysis of esculin in the presence of ferric citrate results in the formation of a black-brown color. *Streptococcus bovis* and *Enterococcus faecalis* produce black zones around colonies as a result of this reaction.

Biosynth chromogenic medium for *Listeria monocytogenes*

This proprietary medium from Biosynth International, Inc. is used to differentiate *L. monocytogenes* and *Listeria ivanovii* from other *Listeria* spp. Supplements render the medium selective. Differential activity for all *Listeria* species is based on a chromogenic substrate included in the medium. This is a complete test system with a fluorogenic selective enrichment broth and a chromogenic plating medium both detecting the virulence factor phosphatidylinositol-specific phospholipase C (PI-PLC). The medium contains a substrate for PI-PLC (PlcA) enzymes. The selective enrichment broth is fluorogenic. The plating medium for rapid detection and enumeration of pathogenic *Listeria* combines cleavage of the chromogenic PI-PLC substrate with the additional production of a white precipitate surrounding the target colonies. This medium is not commonly used in clinical laboratories and is most often used for food analysis for detection of pathogenic *Listeria* spp.

Bismuth sulfite agar

This medium, used for the selective isolation and identification of *Salmonella enterica* serovar Typhi and other enteric bacilli, contains digests of casein and animal tissue plus glucose, beef extract, and ferrous sulfate. Bismuth sulfite is included as a selective factor. If hydrogen sulfide is produced, a black precipitate forms as a result of the reaction with the iron. *Salmonella* Typhi produces flat, black, "rabbit-eye" colonies surrounded by a zone of black with a metallic sheen.

Blood agar

This medium is used for the isolation and detection of hemolytic activity of streptococci and other fastidious microorganisms. The base medium contains beef extract and peptone. Sheep blood is added to complete the medium, although rabbit blood and horse blood may be used in place of sheep blood for the characterization of some hemolytic organisms such as *Haemophilus* spp.

Blood-free *Campylobacter* selectivity agar

This medium is used for the selective isolation of *Campylobacter* spp., especially *C. jejuni* from human feces. The medium contains digests of casein and animal tissue plus pyruvate and ferrous sulfate. Charcoal in the medium removes toxic metabolites. Sodium deoxycholate and cefazolin are included as selective factors.

Bordet-Gengou agar

This medium is used for the detection and isolation of *Bordetella pertussis* and *Bordetella parapertussis* from clinical specimens. The medium is rendered selective by the addition of methicillin. The medium contains digests of casein and animal tissue plus glycerol, potato infusion, and rabbit blood. *B. pertussis* appears as small (<1-mm), smooth, pearl-like colonies surrounded by a narrow zone of hemolysis. *B. parapertussis* appears as brown, nonshiny colonies with a green-black coloration on the reverse side. *Bordetella bronchiseptica* appears as brown, nonshiny, moderately sized colonies with a roughly pitted surface.

Bordetella pertussis selective medium

This medium is used for the selective isolation and presumptive identification of *B. pertussis* and *B. parapertussis*. The medium contains Bordet-Gengou agar base, which includes digests of casein and animal tissue. It also contains blood and cephalexin. *B. pertussis* appears as small, nearly transparent, "bisected pearl-like" colonies.

Bordetella pertussis selective medium with charcoal agar base

This medium is used for the selective isolation and presumptive identification of *B. pertussis* and *B. parapertussis*. The medium contains charcoal agar base, which includes digests of casein and animal tissue, beef extract, nicotinic acid, starch, and charcoal. It also contains blood and cephalexin. *B. pertussis* appears as small, pale, shiny colonies.

Bovine albumin Tween 80 medium, Ellinghausen and McCullough, modified (albumin fatty acid broth, *Leptospira* medium)

This medium is used for the cultivation of *Leptospira* spp. The medium contains glycerol, pyruvate, thiamine, and an albumin fatty acid supplement.

Brain heart infusion

This medium is used for the cultivation of fastidious and nonfastidious microorganisms, including aerobic and anaerobic bacteria, from a variety of clinical specimens. The medium contains digests of gelatin and animal tissue plus glucose and brain heart infusion. This medium is particularly useful for culturing streptococci, pneumococci, and meningococci. Vancomycin can be added for the detection of VRE.

Brain heart infusion agar, 0.7%

This medium is used for the detection of staphylococcal enterotoxin. The medium contains peptone, glucose, and infusions from beef heart and calf brains.

Brilliance CRE Agar

This medium is used for the presumptive identification of carbapenem-resistant *E. coli* and the *Klebsiella*, *Enterobacter*, *Serratia*, and *Citrobacter* (KESC) group, directly from clinical samples, in 18 h. The medium contains antibiotics to suppress other bacteria that are sensitive to carbapenem antibiotics and a novel chromogen to permit rapid differentiation of the target bacteria.

Brilliance ESBL Agar

This agar is used for the detection of extended-spectrum β-lactamase (ESBL)-producing microorganisms and for the presumptive identification of ESBL-producing *E. coli* and the KESC group, directly from clinical samples, in 24 h. ESBL-producing *E. coli* grows as either blue or pink colonies. ESBL-producing members of the KESC group produce green colonies; *Proteus*, *Morganella*, and *Providencia* do not utilize either chromogen but are able to deaminate tryptophan, resulting in tan colonies with a brown halo. The antibiotic mixture suppresses growth of β-lactam antibiotic-sensitive bacteria.

Brilliance MRSA Agar

This medium is used for universal methicillin-resistant *Staphylococcus aureus* (MRSA) screening. Brilliance MRSA Agar incorporates a novel chromogen that yields a blue color as a result of phosphatase activity, indicative of many staphylococci including *S. aureus*. To allow the medium to differentiate MRSA accurately, it contains a combination of antibacterial compounds designed to inhibit the growth of a wide variety of competitor organisms. Also included are compounds to suppress the expression of phosphatase activity in other staphylococci, thus ensuring a high level of sensitivity and specificity.

Brilliance Salmonella Agar

This medium is used for the presumptive detection and identification of *Salmonella* spp. from clinical specimens.

The inhibigen contained in this medium specifically targets *E. coli*, a particular benefit when testing fecal samples. Additional compounds are added to suppress growth of other competing biotas. Differentiation of *Salmonella* from the other organisms that grow on Brilliance Salmonella Agar is achieved through the inclusion of two chromogens that target specific enzymes: caprylate esterase and β-glucosidase. The action of the enzymes on the chromogens results in a buildup of color within the colony. The color produced depends on which enzymes the organisms possess. The action of caprylate esterase present in all salmonellae results in a purple colony. Some *Enterobacteriaceae* also produce caprylate esterase, but these are differentiated from *Salmonella* by a β-glucosidase substrate. This results in blue colonies, which are easy to distinguish from the purple *Salmonella* colonies.

Brilliance Staph 24 Agar

This medium is used for the isolation and enumeration of coagulase-positive staphylococci in foods within 24 h. Coagulase-positive staphylococci grow as dark blue colonies on a clear background.

Brilliance UTI Agar

This medium is used for the presumptive identification and differentiation of all the main microorganisms that cause urinary tract infections (UTIs). Brilliance UTI Agar contains two specific chromogenic substrates that are cleaved by enzymes produced by *Enterococcus* spp., *E. coli*, and coliforms. In addition, it contains phenylalanine and tryptophan, which provide an indication of tryptophan deaminase activity, indicating the presence of *Proteus* spp., *Morganella* spp., and *Providencia* spp.

Brilliance UTI Clarity Agar

This medium is used for the detection and differentiation of coliform bacteria and for the presumptive identification of the main pathogens that cause UTIs. Brilliance UTI Clarity Agar contains two chromogenic substrates that are cleaved by enzymes produced by *E. coli*, *Enterococcus* spp., and coliforms. Of the two chromogens included in the medium, one is metabolized by β-galactosidase, an enzyme produced by *E. coli*, which grows as pink colonies. The other is cleaved by β-glucosidase enzyme activity, allowing the specific detection of enterococci, which form blue or turquoise colonies. Cleavage of both the chromogens gives dark blue or purple colonies and indicates that the organism is a coliform. The tryptophan in the medium is an indicator of tryptophan deaminase activity, resulting in colonies of *Proteus*, *Morganella*, and *Providencia* spp. with brown halos.

Brilliance VRE Agar

This chromogenic agar is used for the detection of VRE and for the presumptive identification of *Enterococcus faecium* and *E. faecalis*, directly from clinical samples, in 24 h. VRE grow as either light blue colonies (*E. faecalis*) or as indigo-purple colonies (*E. faecium*), both of which are very easy to read against the new, semiopaque background.

Brilliant green agar

This medium is used for the selective isolation of salmonellae other than *Salmonella* Typhi from feces and other clinical specimens. The medium contains lactose, sucrose, and digests of casein and animal tissue plus phenol red and brilliant green. *Salmonella* species other than *Salmonella* Typhi appear as red, pink, or white colonies surrounded by a zone of red in the agar, indicating nonfermentation of lactose and sucrose. *Proteus* or *Pseudomonas* spp. may appear as small red colonies. Lactose- or sucrose-fermenting bacteria appear as yellow-green colonies surrounded by a zone of yellow-green in the agar.

Brilliant green-phenol red agar

This medium is used for the cultivation of *Salmonella* spp., with the exception of *Salmonella* Typhi, from feces. The medium contains lactose and peptone plus the differential indicators brilliant green and phenol red. *Salmonella* spp. produce pinkish white colonies.

Bromcresol purple-deoxycholate agar

This medium is used for the isolation and differentiation of Gram-negative enteric bacilli from clinical specimens, especially from fecal specimens. The medium contains lactose, sucrose, and sodium deoxycholate, which form the basis for differentiation. The medium also contains peptones, yeast extract, and citrate. Non-lactose- and non-sucrose-fermenting microorganisms appear as colorless or blue colonies. Lactose- and sucrose-fermenting microorganisms, such as coliform bacteria, appear as yellow-opaque white colonies surrounded by a zone of precipitated deoxycholate.

Brucella agar

This medium is used for the cultivation of *Brucella* spp. and for the isolation of both nonfastidious and fastidious microorganisms from a variety of clinical specimens. The medium contains digests of casein and animal tissue plus sodium sulfite, yeast extract, glucose, and blood. The medium can be supplemented with hemin and vitamin K_1 to improve growth. Blood can also be added for the detection of hemolytic reactions of *Streptococcus* and *Haemophilus* spp.

Brucella agar base campylobacter medium

This medium is used for the selective isolation and cultivation of *C. jejuni* from fecal specimens or rectal swabs. The medium contains digests of casein and animal tissue, yeast extract, glucose, pyruvate, sodium sulfite, and ferrous sulfate. It also includes cycloheximide, sodium cefazolin, novobiocin, bacitracin, and colistin sulfate as selective factors.

Brucella agar base with blood and selective supplement

This medium is used for the cultivation and maintenance of *Brucella* spp. and for the isolation and cultivation of nonfastidious and fastidious microorganisms from a variety of clinical specimens. This medium contains hydrolysate of casein, peptone, yeast extract, glucose, sodium sulfite, and blood. Cycloheximide, nalidixic acid, vancomycin, bacitracin, and polymyxin B are included as selective supplements.

Brucella blood culture broth

This medium is used for the isolation and cultivation of microorganisms from blood. This medium is especially useful for the cultivation of anaerobes. It contains digests of casein and animal tissue plus sodium sulfite, yeast extract, sucrose, glucose, hemin, vitamin K_1, and blood. The medium can be supplemented with hemin.

Brucella broth (brucella Albimi broth)

This medium is used for the cultivation and maintenance of *Campylobacter coli*, *Campylobacter fecalis*, and *Brucella* spp. It is also used for the isolation of a wide variety of fastidious and nonfastidious microorganisms. The medium contains digests of casein and animal tissue, yeast extract, glucose, sodium sulfite, and blood.

Brucella laked sheep blood agar with kanamycin and vancomycin

This medium is used for the selective isolation of fastidious and slow-growing, obligately anaerobic bacteria from the same specimen. The medium contains peptones, dextrose, yeast extract, sheep blood, hemin, and vitamin K_1. Kanamycin and vancomycin are included as selective factors. The laked blood improves pigmentation of the *Prevotella melaninogenica-Porphyromonas asaccharolytica* group.

Buffered charcoal-yeast extract agar with cysteine (BCYE alpha base)

This medium is used for the isolation of *L. pneumophila* and other *Legionella* spp. from clinical specimens. The medium contains cysteine, α-ketoglutarate, iron, yeast extract, *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer, and activated charcoal. The iron and cysteine are specific growth factors required by *Legionella* species for growth. The charcoal removes toxic metabolites. Antibiotics such as polymyxin B, anisomycin, and either vancomycin or cefamandole are typically included as selective factors. *L. pneumophila* produces light blue colonies with a pale green tint on this medium.

Buffered charcoal-yeast extract differential agar

This medium is used for the isolation, cultivation, and maintenance of *L. pneumophila* and other *Legionella* spp. from clinical specimens. The medium contains charcoal to reduce toxicity from metabolites. It also includes cysteine and iron, which are required growth factors for *Legionella*. Vancomycin and polymyxin B are included as selective factors.

Burkholderia cepacia agar

This medium is used for the selective isolation of *B. cepacia* from the respiratory secretions of patients with cystic fibrosis. Slow-growing *B. cepacia* can be missed on conventional media such as blood or MacConkey agar due to overgrowth caused by other faster-growing organisms found in the respiratory tract of cystic fibrosis patients, such as mucoid *Klebsiella* spp., *P. aeruginosa*, and *Staphylococcus* spp. This may lead to the infection being missed or wrongly diagnosed. The medium contains bile salts, gentamicin, ticarcillin, and polymyxin B as selective factors. It also contains pyruvate, peptone, and yeast extract.

Burkholderia cepacia selective agar

This medium is used for the selective isolation of *B. cepacia*. It contains digests of casein, yeast extract, sucrose, and lactose. It also contains polymyxin B, gentamicin, vancomycin, and crystal violet as selective factors.

Burkholderia pseudomallei selective agar

This medium is used for the selective isolation of *B. pseudomallei*. This medium is an improvement over Ashdown selective agar for clinical specimens from nonsterile sites. The medium contains standard agar, maltose, and neutral red.

Campylobacter agar, Blaser's

This medium is used for the selective isolation of *C. jejuni* from fecal specimens. The medium contains cephalothin, vancomycin, trimethoprim, amphotericin B, and polymyxin B as selective factors. It also contains peptone, yeast extract, and liver digest.

Campylobacter agar, Skirrow's

This medium is used for the selective isolation of *C. jejuni* from fecal specimens. The medium contains vancomycin, trimethoprim, and polymyxin B as selective factors. It also contains peptone, yeast extract, and liver digest.

Campylobacter blood agar

This medium is used for the isolation of *C. jejuni* from clinical specimens. It contains peptone, glucose, yeast extract, and blood. It also contains cephalothin, vancomycin, trimethoprim, amphotericin B, and polymyxin B as selective factors.

Campylobacter charcoal differential agar (Preston blood-free medium)

This medium is used for the cultivation of *Campylobacter* spp. The medium contains cefoperazone and sodium deoxycholate as selective factors. It also contains charcoal to remove toxic metabolites. Peptone, beef extract, pyruvate, and ferrous sulfate also are included in the medium. This medium does not require blood to support the growth of *Campylobacter* spp.

Campylobacter selective medium, Blaser-Wang

This medium is used for the selective isolation of *Campylobacter* spp. The medium contains vancomycin, trimethoprim, and polymyxin B as selective factors. It also includes peptone, starch, and blood.

Campylobacter thioglycolate (Campy-Thio) medium

This medium is used for the maintenance—as a holding or transport medium—of *Campylobacter* spp. isolated from clinical specimens on swabs. The medium contains cephalothin, vancomycin, trimethoprim, amphotericin B, and polymyxin B as selective factors to prevent unwanted growth of contaminating organisms. It contains sodium sulfite and sodium thioglycolate to protect the cells against damage.

Cary-Blair transport medium

This medium is used primarily for the maintenance—as a holding medium or transport medium—of clinical specimens, especially enteric pathogens, following collection or shipment. The medium contains sodium thioglycolate and calcium chloride to protect cells.

CDC anaerobe 5% sheep blood agar

This medium is used for the isolation and cultivation of fastidious and slow-growing, obligately anaerobic bacteria from a variety of clinical materials. The medium employs Trypticase soy agar supplemented with additional agar, yeast extract, vitamin K_1, hemin, cystine, and 5% sheep blood. Improved growth of *P. melaninogenica*, *Fusobacterium necrophorum*, and *Clostridium haemolyticum*, as well as certain strains of *Actinomyces israelii* and *Bacteroides thetaiotaomicron*, has been demonstrated on this medium.

Cefsulodin-Irgasan-novobiocin agar

This medium is used for the selective isolation and differentiation of *Yersinia enterocolitica* based on mannitol fermentation. The medium contains strontium chloride, cefsulodin, novobiocin, crystal violet, neutral red, Irgasan, magnesium sulfate, mannitol, peptone, yeast extract, and pyruvate. *Y. enterocolitica* appears as "bull's eye" colonies with deep red centers surrounded by a transparent periphery.

Cellobiose-arginine-lysine agar

This medium is used for the isolation of fastidious bacteria from clinical specimens and for the cultivation under reduced oxygen tension of fastidious microorganisms such as *H. influenzae*, *Neisseria meningitidis*, and *N. gonorrhoeae*. This medium contains arginine, lysine, cellobiose, deoxycholate, and neutral red.

Cetrimide agar, non-USP

This medium is used for the selective isolation, cultivation, and identification of *P. aeruginosa* and other Gram-negative, nonfermentative bacteria. The medium contains tryptose, cetrimide, and infusion from beef heart.

Cetrimide agar, USP (Pseudosel agar)

This medium is used for the selective isolation, cultivation, and identification of *P. aeruginosa* and other Gram-negative, nonfermentative bacteria. The medium contains glycerol, digest of gelatin, cetrimide, potassium sulfate, and magnesium chloride.

Chocolate agar

This is an enriched medium used for the cultivation of nutritionally fastidious microorganisms. The primary components of the medium are peptone, starch, digest of beef heart, and sheep blood.

Chocolate tellurite agar (tellurite blood agar)

This medium is used for the selective isolation of *Corynebacterium* spp. The medium contains extensive growth factors, including amino acids, vitamins, iron, NAD, and cocarboxylase. It also contains digests of casein and meat cornstarch, and tellurite. *C. diphtheriae* appears as gray-black colonies.

Cholera medium base with tellurite and blood

This medium is used for the isolation of pathogenic vibrios, especially *V. cholerae*, and for the selective isolation of *Vibrio* spp. from specimens grossly contaminated with *Enterobacteriaceae*. This medium contains digest of animal tissue, beef extract, sucrose, sodium carbonate, sodium lauryl sulfate, blood, and tellurite.

Cholera medium (thiosulfate-citrate-bile salts-sucrose [TCBS] agar)

This medium is used for the isolation of pathogenic vibrios, especially *V. cholerae*. The medium is suitable for the growth of *V. cholerae*, *Vibrio parahaemolyticus*, and most other vibrios. Most of the *Enterobacteriaceae* encountered in feces are totally suppressed for at least 24 h. Slight growth of *Proteus* spp. and *E. faecalis* may occur, but the colonies are easily distinguished from vibrio colonies. While inhibiting nonvibrios, it promotes rapid growth of pathogenic vibrios after overnight incubation at 35°C. The following species form yellow colonies: *V. cholerae* El Tor biotype, *Vibrio alginolyticus*, *Vibrio metschnikovii*, *Vibrio fluvialis*, *Enterococcus* spp., and some strains of *A. hydrophila*. *V. parahaemolyticus*, *Vibrio vulnificus*, *Vibrio mimicus*, and *Pseudomonas* spp. form blue-green colonies. *Proteus* spp. form yellow-green colonies. *Plesiomonas shigelloides* does not usually grow well on this medium. This medium contains sucrose, peptone sodium thiosulfate, ox bile, ferric citrate, bromthymol blue, and thymol blue.

Chopped meat broth

This medium is used for the cultivation of various anaerobes. The medium contains digest of casein, yeast extract, and meat. It also contains cysteine to protect against oxygen exposure.

Christensen agar

See Urea agar.

CHROMagar Acinetobacter

Red colonies that appear on this medium are indicative of *Acinetobacter* spp.

CHROMagar CTX (RambaCHROM CTX)

This chromogenic agar is used for the detection of bacteria that produce ESBLs and carbapenemase. ESBL producers, such as *Klebsiella pneumoniae*, produce metallic blue colonies, while *E. coli* produces pink-red colonies and *Proteus* spp. produce brown colonies surrounded with a halo. Carbapenemase-producing organisms such as members of the KESC group produce blue-green to bluish gray colonies. *E. coli* produces pink to burgundy colonies or translucent colonies with a pink to burgundy center. *Acinetobacter* spp. produce cream, opaque colonies, and colonies of *Pseudomonas* spp. appear translucent or have a neutral pigment.

CHROMagar E. coli (RambaCHROM E. coli)

This is a proprietary medium from CHROMagar Microbiology used for the differentiation and presumptive identification of *E. coli*, which forms blue colonies. (CHROMagar is a Paris-based company; its products are available in the

United States from Becton, Dickinson and Company, which has a licensing agreement.)

CHROMagar ECC (RambaCHROM ECC)

This is a proprietary medium from CHROMagar Microbiology used for the differentiation and presumptive identification of *E. coli* and other coliform bacteria, which form red colonies.

CHROMagar ESBL (RambaCHROM ESBL)

This medium is used for the detection of Gram-negative bacteria producing ESBLs. Extended-spectrum β-lactam-resistant strains of *E. coli* form dark pink to reddish colonies. Extended-spectrum β-lactam-resistant strains of *Klebsiella*, *Enterobacter*, and *Citrobacter* form metallic blue colonies. Colonies of extended-spectrum β-lactam-resistant strains of *Proteus* have a brown halo.

CHROMagar KPC (RambaCHROM KPC)

Carbapenem-resistant strains of *E. coli* form dark pink to reddish colonies on this agar, which is used for the detection of Gram-negative bacteria with reduced susceptibility to most carbapenem antimicrobial agents. Carbapenem-resistant strains of *Klebsiella*, *Enterobacter*, and *Citrobacter* form blue colonies. Carbapenem-resistant strains of *Pseudomonas* form translucent cream colonies.

CHROMagar Listeria (RambaCHROM Listeria)

This is a proprietary medium from CHROMagar Microbiology used for the differentiation and presumptive identification of *L. monocytogenes*, which forms blue colonies surrounded by white halos.

CHROMagar MRSA (RambaCHROM MRSA)

This is a proprietary medium from CHROMagar Microbiology used for the qualitative direct detection of nasal colonization by MRSA to aid in the prevention and control of MRSA infections in health care settings.

CHROMagar O157 (RambaCHROM O157)

This is a proprietary medium from CHROMagar Microbiology used for the differentiation and presumptive identification of *E. coli* O157.

CHROMagar Orientation (RambaCHROM Orientation)

This is a proprietary medium from CHROMagar Microbiology used for the differentiation and presumptive identification of Gram-negative bacteria and *Enterococcus* spp., specifically for use in identifying these pathogens in urine. Isolates produce characteristic diagnostic colors; e.g., *E. coli* produces pinto red colonies.

CHROMagar Pseudomonas (RambaCHROM Pseudomonas)

This is a proprietary medium from CHROMagar Microbiology used for the simultaneous detection and enumeration of *P. aeruginosa* with coloring (blue colonies) that is markedly different from other microorganisms such as *S. saprophyticus*, *E. coli*, and *P. mirabilis* which are inhibited or form colorless colonies.

CHROMagar Salmonella (RambaCHROM Salmonella)

This is a proprietary medium from CHROMagar Microbiology used for the differentiation and presumptive identification of *Salmonella* spp.

CHROMagar Salmonella Plus (RambaCHROM Salmonella Plus)

This is a proprietary medium from CHROMagar Microbiology that allows for the direct detection of *Salmonella* spp., including *Salmonella* Typhi, *S. enterica* serovar Paratyphi, and lactose-positive *Salmonella*, by colony color according to ISO 6579:2003 norm.

CHROMagar Staph Aureus (RambaCHROM Staph Aureus)

This is a proprietary medium from CHROMagar Microbiology used for the differentiation and presumptive identification of *S. aureus*.

CHROMagar STEC (RambaCHROM STEC)

This agar is used for the detection of Shiga toxin-producing *E. coli* (STEC) in foods, environmental samples, and fecal specimens. Most common STEC serotypes form mauve colonies. Other *Enterobacteriaceae* form colorless or blue colonies.

CHROMagar StrepB (RambaCHROM StrepB)

This is a proprietary medium from CHROMagar Microbiology used for the differentiation and presumptive identification of GBS (*Streptococcus agalactiae*) based on color formation. GBS form mauve to pink colonies.

CHROMagar Vibrio (RambaCHROM Vibrio)

This is a proprietary medium from CHROMagar Microbiology used for the differentiation and presumptive identification of *V. parahaemolyticus*, which forms mauve colonies; *V. cholerae*, which forms turquoise blue colonies; and *V. alginolyticus*, whose colonies are colorless.

CHROMagar VRE (RambaCHROM VRE)

This is a proprietary medium from CHROMagar Microbiology used for the differentiation and presumptive identification of VRE (*E. faecalis* and *E. faecium*). Vancomycin-resistant *Enterococcus* strains form rose to mauve colonies.

CHROMagar Y. enterocolitica (RambaCHROM Y. enterocolitica)

This medium is used for the detection and direct differentiation of pathogenic *Y. enterocolitica*. Pathogenic *Y. enterocolitica* produces mauve colonies. Nonpathogenic *Y. enterocolitica* and other microbes (*Citrobacter*, *Enterobacter*, *Aeromonas*, etc.) produce metallic blue colonies.

chromID *C. difficile*

This medium is used for the identification and isolation of *Clostridium difficile*. As part of a comprehensive infection prevention program, chromID *C. difficile* can help institutions identify the reservoir and control the spread of this pathogenic organism.

chromID CARBA agar

This medium is used for the screening of carbapenemase-producing *Enterobacteriaceae*.

chromID CPS

This is an agar used for the isolation, enumeration, and direct identification of *E. coli*, *Proteus*, enterococci, and members of the KESC group in a single step using urine specimens. *E. coli* forms pink to burgundy colonies, *Proteus* forms dark brown colonies, and enterococci form turquoise colonies.

chromID ESBL

E. coli forms pink to burgundy colonies on this agar, which is used for the screening of ESBL-producing enterobacteria. KESC group members form green-blue to brown-green colonies. *Proteeae* (*Proteus*, *Providencia*, and *Morganella*) form dark to light brown colonies of deaminase-expressing strains.

chromID MRSA

This medium was developed for use in health care facilities to actively reinforce MRSA surveillance culture and control in health care-associated infections. MRSA strains are indicated by green colonies resulting from α-glucuronidase-producing colonies in the presence of the antibiotic cefoxitin.

chromID *P. aeruginosa*

This medium detects *P. aeruginosa* based on the specific violet coloration of aminopeptidase-producing colonies.

chromID *S. aureus*

This agar is used for the direct identification of *S. aureus* and the selective isolation of staphylococci. Direct identification of *S. aureus* is based on the spontaneous green coloration of β-glucosidase-producing colonies.

chromID *Salmonella*

Three chromogenic substrates contained in this medium optimize the selective isolation and differentiation of all *Salmonella* serotypes. Specific detection of the esterase enzymatic activity on a colorless background yields pale pink to mauve colonies of *Salmonella* within 18 to 24 h.

chromID Strepto B

This medium is used for the screening of all *S. agalactiae* organisms. All GBS form round, pearly, pale pink to red colonies after 18 to 24 h.

chromID *Vibrio*

This agar is used for the selective isolation of *Vibrio* spp. and the presumptive identification of *V. cholerae* and *V. parahaemolyticus*. Presumptive identification of *V. cholerae* is through the blue-green coloration of β-galactosidase-producing colonies. *V. parahaemolyticus* forms pink colonies through arabinose assimilation.

chromID VRE

This agar is used for the rapid and reliable qualitative detection of *E. faecium* and *E. faecalis* showing acquired vancomycin resistance. As part of a comprehensive infection prevention program, chromID VRE can help institutions identify the reservoir and control the spread of these pathogenic organisms. Vancomycin-resistant *E. faecium* forms violet colonies, whereas resistant *E. faecalis* forms blue to green colonies.

Clostridial agar

This medium is used for the selective isolation of pathogenic clostridia from mixed biotas. The medium contains digests of casein and soybean meal, glucose, sodium thioglycolate, sodium formaldehyde sulfoxylate, cysteine, sodium azide, and neomycin.

Coagulase-mannitol agar

This medium is used for the cultivation and differentiation of *S. aureus* from other *Staphylococcus* spp. based on coagulase production and mannitol fermentation. The medium contains digest of casein, mannitol, brain heart infusion, digest of soybean meal, plasma, and bromcresol purple.

Colistin-oxolinic acid-blood agar

This medium is used for the isolation and cultivation of streptococci from mixed biotas in clinical specimens. The medium contains colistin sulfate and oxolinic acid as selective factors. It also contains starch, beef extract, yeast extract, and digests of animal tissue and casein plus blood.

Columbia blood agar

This medium is used for the cultivation of *Corynebacterium* spp., *Actinomyces* spp., *S. pneumoniae*, *Staphylococcus* spp., and a variety of fastidious microorganisms. The medium contains starch, peptone, and blood (usually sheep blood at 5%).

Columbia CNA agar

This medium is used for the selective isolation, cultivation, and differentiation of Gram-positive cocci from clinical specimens. The medium contains starch, peptone, and blood. It also contains colistin and nalidixic acid as selective factors.

Congo red acid morpholinepropanesulfonic acid pigmentation agar

This medium is used for the cultivation of *Yersinia* spp., especially those with virulence plasmids. This medium contains morpholinepropanesulfonic acid, Casamino Acids, galactose, tricine, sodium thiosulfate, and Congo red.

Congo red-brain heart infusion-agarose medium

This medium is used for the isolation and detection of virulent strains of *Y. enterocolitica*. The medium contains digest of gelatin, agarose (instead of agar), digest of animal tissue, infusion from brain heart, glucose, magnesium chloride, and Congo red.

Cooked meat medium

This medium is used for the cultivation of anaerobes, especially pathogenic clostridia. The medium contains heart tissue, glucose, and digest of animal tissue.

Cycloserine-cefoxitin-egg yolk-fructose agar (*Clostridium difficile* agar)

This medium is used for the selective isolation and cultivation of *C. difficile* from feces. The medium contains peptone, fructose, hemin, neutral red, and egg yolk emulsion. It also contains cycloserine and cefoxitin as selective factors.

Cysteine albumin broth

This medium is used for the transport and storage of biopsy samples for the detection of *H. pylori*. The medium contains cysteine, albumin, and glycerol.

Cystine heart agar (cystine-glucose-blood agar)

This medium is used for the cultivation and maintenance of *F. tularensis*. The medium contains glucose, peptone, and infusion from beef heart. It also contains hemoglobin and cystine. Without the hemoglobin enrichment, it supports excellent growth of Gram-negative cocci and other pathogenic microorganisms.

Cystine-tellurite-blood agar

This medium is used for the isolation, differentiation, and cultivation of *C. diphtheriae*. The medium contains infusion from beef heart, tryptose, yeast extract, cystine, blood, and potassium tellurite. *C. diphtheriae* appears as dark gray to black colonies.

Cystine tryptic agar

This medium is used for the cultivation and maintenance of a variety of fastidious microorganisms, including *C. diphtheriae*. It is also used for carbohydrate fermentation tests in the differentiation of *Neisseria* spp. The medium contains sodium sulfite, cystine, and digest of casein plus phenol red indicator.

DeMan, Rogosa, Sharpe (MRS) agar (*Lactobacillus* MRS agar)

This medium is used for the isolation and cultivation of *Lactobacillus* spp. from clinical specimens. The medium contains digest of gelatin, beef extract, yeast extract, sodium acetate, ammonium citrate, and Tween 80.

Deoxycholate agar

This medium is used for the selective isolation and differentiation of Gram-negative enteric microorganisms from a variety of clinical specimens. The medium contains lactose, digests of casein and animal tissue, ferric citrate, sodium citrate, sodium deoxycholate, and neutral red. *E. coli* appears as large, flat, rose red colonies. *Enterobacter* and *Klebsiella* spp. appear as large, mucoid, pale colonies with a pink center. *Proteus* and *Salmonella* spp. appear as large, colorless to tan colonies. *Shigella* spp. appear as colorless to pink colonies. *Pseudomonas* spp. appear as irregular, colorless to brown colonies.

Deoxycholate-citrate agar

This medium is used for the selective isolation and cultivation of enteric pathogens, especially *Salmonella* and *Shigella* spp. The medium contains citrate, lactose, digest of animal tissue, infusion of meat, deoxycholate, ferric citrate, and neutral red.

Deoxycholate-citrate agar, Hynes

This medium is used for the selective isolation and differentiation of enteric pathogens, especially *Salmonella* and *Shigella* spp. The medium contains lactose, citrate, peptone, beef extract, deoxycholate, ferric citrate, and neutral red. Lactose-fermenting bacteria appear as pink colonies that may or may not be surrounded by a zone of precipitated deoxycholate. Non-lactose-fermenting bacteria appear as colorless colonies that are surrounded by a clear orange-yellow zone.

Deoxycholate-citrate-lactose-sucrose agar

This medium is used for the selective isolation of *Salmonella* spp., *Shigella* spp., and *Vibrio* spp. from fecal specimens. The medium contains citrate, lactose, sucrose, thiosulfate, beef extract, deoxycholate, digests of casein and animal tissue, and neutral red.

Deoxycholate-citrate-lactose-sucrose agar, Hajna

This medium is used for the selective isolation of *Salmonella* spp., *Shigella* spp., and *Vibrio* spp. from fecal specimens. The medium contains citrate, lactose, sucrose, thiosulfate, beef extract, deoxycholate, digests of casein and animal tissue, and bromcresol purple.

Differential agar for group D streptococci

This medium is used for the differentiation and identification of group D streptococci. The medium contains digests of casein and animal tissue, glucose, infusion from brain heart, and bromcresol purple.

Diphtheria virulence agar base with tellurite and diphtheria virulence supplement

This medium is used for the detection of diphtheria toxin-producing strains of *C. diphtheriae* and for testing the toxigenicity of *C. diphtheriae*. The reaction of antitoxin forms the actual basis for the detection of the diphtheria toxin. The medium contains peptone, tellurite, horse serum, and a filter paper strip saturated with potent diphtheria antitoxin.

DNase test agar with toluidine blue

This medium is used for the differentiation of microorganisms, especially *Staphylococcus* spp. and *Serratia marcescens*, based on their production of DNase. The medium contains digests of casein and animal tissue, DNA, and toluidine blue.

Dubos broth (Dubos Tween albumin broth)

This medium is used for the cultivation of *M. tuberculosis* and other *Mycobacterium* spp. The medium contains asparagine, digest of casein, Tween 80, ferric ammonium citrate, and serum albumin or serum.

E. coli O157:H7 MUG agar

This medium is used for the isolation and differentiation of enterohemorrhagic *E. coli* O157:H7 strains from clinical specimens. The medium contains peptone, sorbitol, meat extract, thiosulfate, deoxycholate, yeast extract, ammonium ferric citrate, MUG, and bromthymol blue.

Egg yolk agar

This medium is used for the isolation and differentiation of *Clostridium* spp. and some other anaerobic bacteria. The medium contains peptone, glucose, hemin, and egg yolk emulsion.

Ellinghausen-McCullough-Johnson-Harris medium

This medium is used for the isolation and cultivation of *Leptospira* spp. Albumin and Tween 80 are included to provide lipids. Lysed erythrocytes also are included in the medium to provide iron. The medium can be rendered selective by the addition of 5-fluorouracil.

EMB agar, Levine

This medium is used for the isolation, cultivation, and differentiation of Gram-negative enteric bacteria based on lactose fermentation. The medium contains digest of casein, lactose, sucrose, eosin, dipotassium phosphate, and methylene blue. Bacteria that ferment lactose, especially the coliform bacterium *E. coli*, appear as colonies with a green metallic sheen or blue-black to brown color. Bacteria that do not ferment lactose appear as colorless or transparent, light purple colonies.

EMB agar, modified, Holt-Harris and Teague

This medium is used for the isolation, cultivation, and differentiation of Gram-negative enteric bacteria based on lactose fermentation. The medium contains digest of casein, lactose, sucrose, eosin, dipotassium phosphate, and methylene blue. Bacteria that ferment lactose, especially the coliform bacterium *E. coli*, appear as colonies with a green metallic sheen or blue-black to brown color. Bacteria that do not ferment lactose appear as colorless or transparent, light purple colonies.

Endo agar

This medium is used for the selective isolation, cultivation, and differentiation of coliform and other enteric microorganisms based on their ability to ferment lactose. The medium contains lactose, digest of animal tissue, and basic fuchsin. Lactose-fermenting bacteria appear as dark red colonies with a gold metallic sheen. Non-lactose-fermenting bacteria appear as colorless or translucent colonies.

Enterococcosel agar

This medium is used for the rapid, selective isolation of enterococci. It is also used for the cultivation of staphylococci and *L. monocytogenes*. The medium contains digest of casein, proteose peptone, iron, esculin, and yeast extract. Oxgall (bile) and sodium azide are included as selective factors. The hydrolysis of esculin in the presence of ferric citrate results in the formation of a black-brown color.

Enterococcosel agar with vancomycin

This medium is used for the detection of VRE, particularly for primary screening of asymptomatic gastrointestinal carriage of VRE. The medium contains digest of casein, proteose peptone, iron, esculin, and yeast extract. Oxgall (bile), sodium azide, and vancomycin (8.0 mg/liter) are included as selective factors.

Enterococcosel broth

This medium is used for the differentiation of enterococci. The medium contains digest of casein, proteose peptone, iron, esculin, and yeast extract. Oxgall (bile) and sodium azide are included as selective factors.

Esculin azide broth

This medium is used for the cultivation of enterococci from feces. The medium contains digest of animal tissue, bile salts, yeast extract, esculin, sodium citrate, ferric ammonium citrate, and sodium azide.

ESP Myco medium

This medium is used with ESP Culture System II (TREK Diagnostic Systems) for the detection of mycobacterial growth. The medium is a Middlebrook 7H9 broth enriched with glycerol, Casitone, and cellulose sponge disks. Oleic acid-albumin-dextrose-catalase (OADC) enrichment is added before use.

Eugonic agar

This medium is used for the cultivation of a variety of fastidious microorganisms, e.g., *Brucella*, *Haemophilus*, *Neisseria*, *Pasteurella*, and *Lactobacillus* spp. The medium contains digests of casein and soybean meal, glucose, cystine, and sodium sulfite.

Eugonic LT100 medium base without Tween 80

This medium is used for the cultivation of fastidious microorganisms such as *Haemophilus*, *Neisseria*, *Pasteurella*, *Brucella*, and *Lactobacillus* spp.

Feeley-Gorman agar

This medium is used for the isolation and cultivation of *L. pneumophila*. The medium contains acid hydrolyzed casein, beef extract, starch, cysteine, and ferric pyrophosphate.

Fildes enrichment agar

This medium is used for the isolation of *H. influenzae*. The medium contains peptone, beef extract, pepsin, and blood.

Fletcher medium

This medium is used for the isolation of *Leptospira* spp. The medium contains peptone, beef extract, pepsin, and blood.

It can be supplemented with 5-fluorouracil to render it selective.

GC agar

This medium is used for the isolation of *Neisseria* and *Haemophilus* spp. from clinical specimens. The medium contains digest of casein and starch. It may be enriched by the addition of hemin and NAD. Cysteine and antibiotics can also be added to make the medium selective.

GC II agar

This medium is used for the isolation of fastidious microorganisms, especially *Neisseria* and *Haemophilus* spp., from clinical specimens. The medium contains digest of casein, meat peptone, and starch.

GC-Lect agar

This medium is used for the isolation and cultivation of *N. gonorrhoeae* from clinical specimens. The medium contains digest of casein, meat peptone, starch, hemoglobin, a selective supplement of numerous growth factors, and a proprietary selective supplement. This medium is described in the *Difco & BBL Manual: Dehydrated Culture Media and Reagents for Microbiology* (22). GC-Lect Agar is used in the Miles Laboratory JEMBEC system, which consists of a John E. Martin Biological Environmental Chamber-style plate, a carbon dioxide tablet, and resealable plastic bag.

GN broth, Hajna

This medium is used for the selective cultivation of *Salmonella* and *Shigella* spp. The medium contains digests of casein and animal tissue, citrate, mannitol, glucose, and deoxycholate.

Gum *Listeria* medium (gum base-nalidixic acid medium)

This medium is used for the isolation of *L. monocytogenes* from clinical specimens. This medium contains gellan gum, digests of casein and soybean meal, glucose, and nalidixic acid.

H broth

This medium is used for the preparation of the H agglutination antigen used in the differentiation and identification of *Salmonella* spp. types and subtypes. The medium contains digest of casein, peptone, beef extract, and glucose.

Haemophilus test medium

This medium is used for antibiotic susceptibility testing of *Haemophilus* spp. The medium contains digests of casein and animal tissue, yeast extract, hematin, NAD, and growth factors.

HardyCHROM CRE Agar

This medium is used for the selection and differentiation of carbapenemase-producing Gram-negative bacteria. It is intended to serve as a supplemental medium or to be combined with other media for processing specimens. It is not intended for use in the definitive identification of colonization with carbapenem-resistant bacteria in the prevention and control of such bacteria in a health care setting. It is not cleared by the U.S. Food and Drug Administration for diagnosis of infections by carbapenem-resistant bacteria. Exposure to light during storage and incubation should be minimized.

HardyCHROM ESBL Agar

This medium is used for the selection and differentiation of *Enterobacteriaceae* that produce ESBLs. It is intended to serve as a supplemental medium to be combined with other media for processing specimens. It is not intended for use in the definitive identification of colonization with extended-spectrum β-lactam-resistant bacteria in the prevention and control of such bacteria in a health care setting. It is not cleared by the U.S. Food and Drug Administration for diagnosis of infections by extended-spectrum β-lactam-resistant bacteria. Exposure to light during storage and incubation should be minimized.

Hartley's digest broth

This medium is used for the isolation and cultivation of actinomycetes. This medium contains ox heart, pancreatin, sodium carbonate, and hydrochloric acid.

Heart infusion agar

This medium is used for the isolation and cultivation of a wide variety of microorganisms, including *B. cereus*, *S. aureus*, *V. vulnificus*, and *V. cholerae*. The medium contains tryptose and infusion from beef heart. It can be used as a base for the preparation of blood agar in determining hemolytic reactions.

Hektoen enteric agar

This medium is used for the isolation and cultivation of Gram-negative enteric microorganisms from a variety of clinical specimens based on lactose or sucrose fermentation and H_2S production. Bacteria that ferment lactose or sucrose appear as yellow to orange colonies. Bacteria that produce H_2S appear as colonies with black centers. The medium contains lactose, digest of animal tissue, sucrose, bile salts, thiosulfate, yeast extract, salicin ferric ammonium citrate, acid fuchsin, and bromthymol blue.

HiCrome Aureus Agar Base with egg yolk tellurite (*Staphylococcus aureus* agar, HiCrome)

This medium is used for the isolation and enumeration of coagulase-positive *S. aureus*. Coagulase-positive *S. aureus* gives brown-black colonies, whereas *S. epidermidis* gives yellow, slightly brownish colonies. The medium contains digests of casein and gelatin, pyruvate, beef extract, lithium chloride, yeast extract, eggs, tellurite, and a chromogenic mixture.

HiCrome Listeria Agar Base, modified, with moxalactam (*Listeria* HiCrome Agar Base, modified)

This medium is used for the rapid and direct identification of *Listeria* spp., specifically *L. monocytogenes*. The medium contains peptone, rhamnose, lithium chloride, meat extract, yeast extract, phenol red, and a chromogenic mixture. It also includes moxalactam as a selective supplement.

HiCrome MeReSa Agar with methicillin

This medium is used for the isolation and cultivation of MRSA. The medium contains a chromogenic substrate that allows differentiation of *S. aureus*. It also contains methicillin.

HiCrome RajHans Medium (Salmonella Agar)

This medium is used for the identification and differentiation of *Salmonella* spp. from members of the *Enterobacteriaceae*, especially *Proteus* spp. The medium contains a chromogenic mixture that permits differentiation.

HiCrome RajHans Medium, modified (Salmonella Agar, modified)

This medium is a selective chromogenic medium used for the isolation and differentiation of *Salmonella* spp. from members of the family *Enterobacteriaceae*, especially *Proteus* spp. The medium contains hydrolysate of casein, yeast extract, digest of animal tissue, lactose, sodium deoxycholate, neutral red, and a chromogenic mixture.

HiCrome Salmonella Agar

This medium is used for the identification, differentiation, and confirmation of enteric bacteria, *E. coli*, and *Salmonella* from specimens such as urine that may contain a large number of *Proteus* spp. as well as other potentially pathogenic Gram-positive organisms. The medium contains digest of animal tissue, yeast extract, bile salts, and a chromogenic mixture.

HiCrome UTI Agar

This medium is used for the differentiation and enumeration of thermotolerant *E. coli* from water by the membrane filtration method. It is also used for the identification, differentiation, and confirmation of enteric bacteria from specimens such as urine, water, or food that may contain large number of *Proteus* spp. as well as potentially pathogenic Gram-positive organisms. The medium contains digests of casein and animal tissue, beef extract, and a chromogenic mixture.

HiFluoro Pseudomonas Agar Base

This medium is used for the selective isolation of *P. aeruginosa* from clinical specimens by a fluorogenic method. The medium contains digest of gelatin, potassium sulfate, glycerol, cetrimide, and a fluorogenic mixture.

Horie Arabinose Ethyl Violet Broth

This medium is used for the cultivation of *Vibrio* spp. The medium contains peptone, beef extract, arabinose, ethyl violet, and bromthymol blue.

Hoyle medium

This medium is used for the isolation and differentiation of *C. diphtheriae* strains. It permits very rapid growth of all types of *C. diphtheriae*, so that diagnosis is possible after 18 hours' incubation. The medium contains peptone, Lab-Lemco powder, tellurite, and laked blood.

Kanamycin-esculin-azide agar

This medium is used for the isolation of enterococci. The medium contains digest of casein, yeast extract, esculin, sodium citrate, ferric ammonium citrate, sodium azide, and kanamycin.

Kligler iron agar

The H_2S produced from thiosulfate by sulfate reductase will react with the Fe salt to produce FeS, a strong black precipitate, which for clinically relevant nonfermenters is only observed for *Shewanella* spp. The medium contains pancreatic digest of casein, ferric ammonium citrate, peptic digest of animal tissue, sodium thiosulfate, lactose, agar, glucose, phenol red, and sodium chloride.

Lim broth

This medium is a modification of Todd-Hewitt broth and is an enriched selective liquid medium used for the isolation and cultivation of *S. agalactiae*. Peptones, salts, and dextrose provide the nutritive base. Yeast extract provides B vitamins and additional enrichment. The antibiotics colistin and nalidixic acid inhibit Gram-negative bacteria. The effectiveness of this medium has been evaluated by Elsayed et al. (35).

Listeria monocytogenes confirmatory agar base

This medium is used for the selective and differential isolation of *L. monocytogenes* from clinical specimens. The medium contains peptone, lithium chloride, yeast extract, α-methyl-D-mannoside, phosphatidylinositol, polymyxin B, ceftazidime, nalidixic acid, and amphotericin B. The multiple antimicrobials make this a highly selective medium.

Listeria Oxford medium base with antibiotic inhibitor

This medium is used for the isolation and cultivation of *L. monocytogenes* from specimens containing a mixed bacterial biota, including from pathological specimens. The medium contains peptone, lithium chloride, starch, esculin, and ammonium ferric citrate. It also includes cycloheximide, colistin sulfate, fosfomycin, acriflavine, and cefotetan as selective factors.

Listeria transport enrichment medium

This medium is used for the maintenance—as a transport medium—and enrichment of *Listeria* spp. The medium contains sodium glycerophosphate, sodium thioglycolate, nalidixic acid, and acridine.

Liver infusion agar

This medium is used for the cultivation of *Brucella* spp. and other fastidious pathogenic bacteria. The medium contains potassium thiocyanate infusion from beef liver, peptone, and salt.

Loeffler medium

This medium is used for the detection of *C. diphtheriae*. The medium contains beef serum, eggs, infusion from heart muscle, glucose, and digest of animal tissue.

Lombard-Dowell agar

This medium is used for the identification of a variety of obligate anaerobic bacteria, including *Bacteroides* spp., *Fusobacterium* spp., *Clostridium* spp., and non-spore-forming Gram-positive anaerobes. The medium contains digest of casein, yeast extract, cystine, tryptophan, sodium sulfite, hemin, and vitamin K_1.

Lombard-Dowell egg yolk agar

This medium is used for the cultivation and differentiation of a wide variety of anaerobic bacteria based on lecithinase production, lipase production, and proteolytic ability. The medium contains digest of casein, yeast extract, cystine, tryptophan, sodium sulfite, egg yolk emulsion, hemin, and vitamin K_1. Bacteria that produce lecithinase appear as colonies surrounded by a zone of insoluble precipitate. Bacteria that produce lipase appear as colonies with a pearly iridescent sheen. Bacteria that produce proteolytic activity appear as colonies surrounded by a clear zone.

Lowenstein-Gruft medium

This medium is used for the cultivation and differentiation of *Mycobacterium* spp. The medium contains starch, asparagine, magnesium citrate, malachite green, nalidixic acid, RNA, eggs, glycerol, and penicillin. *M. tuberculosis* appears as granular, rough, dry colonies. *M. kansasii* appears as smooth to rough, photochromogenic colonies. *Mycobacterium gordonae* appears as smooth, yellow-orange colonies. *M. avium* appears as smooth, colorless colonies. *Mycobacterium smegmatis* appears as wrinkled, creamy white colonies.

Lowenstein-Jensen medium

This medium is used for the cultivation and differentiation of *Mycobacterium* spp. The medium contains starch, asparagine, magnesium citrate, malachite green, eggs, and glycerol. *M. tuberculosis* appears as granular, rough, dry colonies. *M. kansasii* appears as smooth to rough, photochromogenic colonies. *M. gordonae* appears as smooth, yellow-orange colonies. *M. avium* appears as smooth, colorless colonies. *M. smegmatis* appears as wrinkled, creamy white colonies.

MacConkey agar

This medium is used for the selective isolation and differentiation of coliforms and enteric pathogens based on the ability to ferment lactose. This medium contains digest of gelatin, lactose, bile salts, digests of casein and animal tissue, neutral red, and crystal violet. Lactose-fermenting organisms appear as red to pink colonies. Non-lactose-fermenting organisms appear as colorless or transparent colonies.

MacConkey agar, Fluorocult

This medium is used for the isolation of *Salmonella*, *Shigella*, and coliform bacteria, in particular *E. coli*, from various specimens. The medium contains digest of gelatin, lactose, bile salts, digests of casein and animal tissue, neutral red, crystal violet, and MUG. The bile salts and crystal violet largely inhibit the growth of Gram-positive microbial biotas. Lactose and the pH indicator neutral red are used to detect lactose-positive colonies, and *E. coli* can be seen among these because of fluorescence under UV light.

MacConkey agar with sorbitol

This medium is used for the selective isolation and differentiation of *E. coli* O157:H7. The medium contains digest of gelatin, bile salts, digests of casein and animal tissue, neutral red, crystal violet, and sorbitol. STEC strains do not ferment sorbitol and appear as colorless colonies. Sorbitol-fermenting strains appear as pink colonies.

Malachite green broth

This medium is used for the cultivation of *P. aeruginosa*. The medium contains peptone, beef extract, and malachite green.

Mannitol-egg yolk-polymyxin agar

This medium is used for the cultivation and enumeration of *B. cereus* from clinical specimens. The medium contains mannitol, peptone, beef extract, phenol red, polymyxin B, and egg yolk emulsion.

Mannitol-lysine-crystal violet-brilliant green agar

This medium is used for the selective isolation and cultivation of *Salmonella* spp. from fecal material. The medium contains peptone, yeast extract, lysine, sodium thiosulfate, mannitol, beef extract, ferric ammonium citrate, crystal violet, and brilliant green.

Mannitol salt agar

This medium is used for the selective isolation, cultivation, and enumeration of staphylococci from clinical specimens. The medium contains mannitol, digests of casein and animal tissue, beef extract, and phenol red. Mannitol-utilizing organisms turn the medium yellow.

Mannitol salt broth

This medium is used for the selective isolation of presumptive pathogenic staphylococci. The medium contains peptone, mannitol, beef extract, and phenol red.

Mannitol selenite broth (selenite mannitol broth)

This medium is used for the selective enrichment of *Salmonella* spp. from clinical specimens. The medium contains digest of animal tissue, mannitol, and sodium selenite.

Martin-Lewis agar

This medium is used for the isolation and cultivation of pathogenic *Neisseria* from specimens containing mixed biotas. The medium contains hemoglobin, digest of casein, meat peptone, and starch. It also contains a complex growth supplement solution with amino acids, nucleotides, and vitamins. Colistin, trimethoprim, lactate, vancomycin, and anisomycin are included as selective factors.

McBride *Listeria* agar

This medium is used for the selective isolation of *L. monocytogenes* from clinical specimens containing mixed biotas. The medium contains glycine, digests of casein and animal tissue, beef extract, phenylethyl alcohol, and lithium chloride.

Methyl red-VP medium

This medium is used for the differentiation of bacteria based on acid production (methyl red test) and acetoin production (VP reaction). The medium contains glucose, peptone, and phosphate buffer.

Middlebrook albumin-dextrose-catalase (ADC) enrichment

This medium is used as a supplement to other Middlebrook media for the isolation, cultivation, and maintenance of *Mycobacterium* spp. It is also used as a supplement to other Middlebrook media for determining the antimicrobial susceptibility of mycobacteria. This enrichment supplement contains bovine albumin fraction V, glucose, and catalase.

Middlebrook 7H9 broth with Middlebrook ADC enrichment

This medium is used for the isolation of *Mycobacterium* spp., including *M. tuberculosis*, and also for determining the antimicrobial susceptibility of mycobacteria. The medium contains glutamate, citrate, ferric ammonium citrate, pyridoxine, biotin, glycerol, and Middlebrook ADC enrichment.

Middlebrook 7H10 agar with Middlebrook ADC enrichment

This medium is used for the isolation, cultivation, and maintenance of *Mycobacterium* spp., including *M. tuberculosis*. It is also used for determining the antimicrobial susceptibility of mycobacteria. The medium contains glutamate, citrate, ferric ammonium citrate, pyridoxine, biotin, glycerol, malachite green, and Middlebrook ADC enrichment.

Middlebrook 7H11 agar with Middlebrook ADC enrichment (mycobacteria 7H11 agar with Middlebrook ADC enrichment)

This medium is used for the cultivation of drug-resistant (isoniazid) strains of *M. tuberculosis*, and particularly for the cultivation of fastidious strains of tubercle bacilli that occur following treatment of tuberculosis patients with secondary antitubercular drugs. Generally, these strains fail to grow on 7H10 medium. The medium contains glutamate, citrate, ferric ammonium citrate, pyridoxine, biotin, glycerol, malachite green, and Middlebrook ADC enrichment.

Middlebrook OADC enrichment

This medium is used as a supplement to other Middlebrook media for the isolation, cultivation, and maintenance of *Mycobacterium* spp. It is also used as a supplement to other Middlebrook media for determining the antimicrobial susceptibility of mycobacteria. This enrichment supplement contains bovine albumin fraction V, glucose, oleic acid, and catalase.

MRSA*Select* medium

This is a selective and differential medium (Bio-Rad Laboratories, Redmond, WA) for the detection of MRSA from nasal swabs and wound specimens.

Mueller-Hinton agar

This medium is used for the isolation of pathogenic *Neisseria* spp., including *N. meningitidis*. It is also used for antimicrobial susceptibility testing of a variety of bacterial species. The medium contains infusion from beef, acid hydrolysate of casein, and starch.

Mueller-Hinton broth

This medium is used for antimicrobial susceptibility testing. The medium contains infusion from beef, acid hydrolysate of casein, and starch.

Mueller-Hinton chocolate agar

This medium is used for the cultivation of *N. gonorrhoeae* and *N. meningitidis* and for antimicrobial susceptibility testing of fastidious microorganisms. The medium contains infusion from beef, acid hydrolysate of casein, starch, and blood.

Mueller-Hinton II agar

This medium is used for antimicrobial disk diffusion susceptibility testing of a variety of bacteria by the Bauer-Kirby method. The medium contains acid hydrolysate of casein and starch. This medium, supplemented with 5% sheep blood, is recommended for use in antimicrobial susceptibility testing of *S. pneumoniae* and *H. influenzae*.

Mueller tellurite medium

This medium is used for the isolation, cultivation, and differentiation of *C. diphtheriae*. The medium contains casein, Casamino Acids, tryptophan, serum, sodium lactate, ethyl alcohol, calcium pantothenate, and tellurite.

Mycobactosel agar

This medium is used for the selective isolation of mycobacteria from specimens containing mixed biotas. The medium contains digest of casein, albumin, glutamate, biotin, glycerol, oleic acid, catalase, and malachite green. It also contains cycloheximide, nalidixic acid, and lincomycin as selective factors.

Mycobactosel L-J medium

This medium is used for the isolation and cultivation of *Mycobacterium* spp. from clinical specimens. The medium contains potato flour, asparagine, citrate, glycerol, malachite green, and eggs. It also contains cycloheximide, nalidixic acid, and lincomycin as selective factors. Mycobactosel L-J medium is Lowenstein-Jensen medium plus cycloheximide, lincomycin, and nalidixic acid for use with specimens likely to contain many contaminating organisms.

Mycoplasma agar base (PPLO agar base)

This medium is used for the preparation of media for the cultivation of *Mycoplasma* spp. The medium contains digest of casein, beef extract, yeast extract, infusion from beef heart, horse serum, and fresh yeast extract solution.

Mycoplasma broth base without crystal violet and with ascitic fluid (PPLO broth base without crystal violet)

This medium is used for the enrichment of pleuropneumonia-like organisms (PPLOs) and *Mycoplasma* spp. from clinical specimens. The medium contains peptone, infusion from beef, and ascitic fluid.

NaCl agar

This medium is used for the differentiation of Gram-positive cocci, especially *Staphylococcus* spp., based on salt tolerance. The medium contains various concentrations of NaCl (e.g., 6.5 or 12%) and digests of casein and soybean meal. It can be supplemented with glucose and sheep blood.

Neisseria meningitidis medium

This medium is used for the selective isolation and cultivation of *N. meningitidis*. The medium contains acid hydroxylate of casein and starch. It also contains vancomycin, colistin, and nystatin as selective factors.

Neomycin blood agar

This medium is used for the isolation and cultivation of group A streptococci (*S. pyogenes*) and GBS (*S. agalactiae*) from throat cultures and other clinical specimens. The medium contains digest of casein, growth factors, blood, and neomycin.

New York City medium

This medium is used for the isolation and cultivation of pathogenic *Neisseria* spp. It is also used as a transport medium for urogenital and other clinical specimens and for the isolation and presumptive identification of *Mycoplasmatales*, including large-colony species (*Mycoplasma pneumoniae*) and T-mycoplasmas from urogenital specimens. The medium contains starch, peptone, glucose, plasma, and blood. It also contains colistin, trimethoprim, and vancomycin as selective factors.

Nitrate broth

This medium is used for the differentiation of aerobic and facultative Gram-negative microorganisms based on their ability to reduce nitrate. The medium contains digest of gelatin and potassium nitrate. The test for nitrates uses sulfanilic acid and α-naphthylamine reagents. Bacteria that reduce nitrate to nitrite turn the reagents red or pink.

Nutrient agar, 1.5%, HiVeg with ascitic fluid

This medium is used for the enrichment of PPLOs and *Mycoplasma* spp. from clinical specimens. The medium contains peptones and ascitic fluid.

N-Z-Amine A glycerol agar

This medium is used for the isolation and cultivation of *Actinomadura* spp. The medium contains N-Z-Amine A, beef extract, and glycerol.

O157:H7 ID agar

This proprietary medium from bioMérieux is a chromogenic medium for the detection of *E. coli* O157:H7.

ONE Broth-Listeria (Oxoid novel enrichment broth-*Listeria*)

This medium is used for the selective enrichment of *Listeria* spp. This proprietary medium from Oxoid contains peptone, salt mix, carbohydrate mix, and a selective supplement.

Önöz Salmonella agar

This medium is used for the isolation and cultivation of *Salmonella* from feces. The medium contains sucrose, lactose, sodium citrate, meat peptone, beef extract, phenylalanine, thiosulfate, bile salts, yeast extract, ferric citrate, metachrome yellow, aniline blue, neutral red, and brilliant green.

Oxford agar (*Listeria* selective agar, Oxford)

This medium is used for the isolation and cultivation of *L. monocytogenes* from specimens containing a mixed bacterial biota. The medium contains peptone, starch, esculin, ferric ammonium citrate, lithium chloride, and the antimicrobials cycloheximide, colistin, fosfomycin, acriflavine, and cefotetan.

Oxford agar, modified (*Listeria* selective agar, modified Oxford)

This medium is used for the isolation of *L. monocytogenes* from specimens containing a mixed bacterial biota. The medium contains peptone, lithium chloride, starch, esculin, ferric ammonium citrate, and the antimicrobials moxalactam and colistin sulfate.

Oxidation-fermentation medium, Hugh-Leifson's

This medium is used for differentiating Gram-negative bacteria, such as *Vibrio* spp., based on determining the oxidative and fermentative metabolism of carbohydrates. The medium contains peptone, a carbohydrate substrate, and bromthymol blue. Bacteria that ferment the carbohydrate turn the medium yellow.

Oxidation-fermentation medium, King's

This medium is used for differentiating bacteria based on determining the oxidative and fermentative metabolism of carbohydrates. The medium contains digest of casein, a carbohydrate substrate, and phenol red. Bacteria that ferment the carbohydrate turn the medium yellow.

Oxoid Salmonella chromogenic agar (OSCM)

This medium is used for the identification of *Salmonella* spp. and differentiation of *Salmonella* spp. from other organisms in the family *Enterobacteriaceae*. The medium combines two chromogens for the detection of *Salmonella* spp.: 5-bromo-6-chloro-3-indolyl caprylate (Magenta-caprylate) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). X-gal is a substrate for the enzyme β-D-galactosidase. Hydrolysis of the chromogen Magenta-caprylate by lactose-negative *Salmonella* spp. results in magenta colonies. The medium contains bile salts to inhibit the growth of Gram-positive organisms, and the addition of the selective supplement solution increases the selectivity of the medium. Novobiocin inhibits *Proteus* growth and cefsulodin inhibits growth of pseudomonads.

P agar

This medium is used for the cultivation of *Staphylococcus* spp. The medium contains peptone, NaCl, yeast extract, and glucose.

Pasteurella haemolytica selective medium

This medium is used for the selective cultivation of *P. haemolytica*. The medium contains digests of casein and animal tissue, glucose, peptic digest of blood, and the antimicrobials cycloheximide, novobiocin, and neomycin.

Penicillinase-producing *Neisseria gonorrhoeae* medium (PPNG selective medium)

This medium is used for the differentiation and presumptive identification of penicillinase-producing strains of *N. gonorrhoeae*. PPNG selective medium is a two-sectored plate, each containing a different Martin-Lewis agar.

Peptone iron agar

This medium is used for the cultivation and differentiation of microorganisms based on their ability to produce H_2S. The medium contains peptones, sodium glycerophosphate, ferric ammonium citrate, and sodium thiosulfate. Microorganisms that produce H_2S turn the medium black.

Perfringens agar (Shahidi-Ferguson perfringens agar)

This medium is used for the isolation and enumeration of *Clostridium perfringens* organisms, which appear as black colonies surrounded by a precipitate. The medium contains tryptose, digest of soybean meal, yeast extract, ferric ammonium citrate, sodium sulfite, egg yolk emulsion, kanamycin, and polymyxin sulfate.

Petragnani medium

This medium is used for the isolation and cultivation of *Mycobacterium* spp. from clinical specimens, particularly for the cultivation and maintenance of *M. smegmatis*. The medium contains milk, potato flour, asparagine, digest of casein, eggs, glycerol, and malachite green.

Pfizer TB medium base with glycerol, egg yolk, glucose, and malachite green

This medium is used for the cultivation of *M. tuberculosis*. The medium contains starch, digest of casein, beef extract, asparagine, citric acid, ferric ammonium citrate, egg yolks, glycerol, glucose, and malachite green.

PGT medium

This medium is used for the cultivation and maintenance of *C. diphtheriae*. The medium contains Casamino Acids, glutamic acid, maltose, cysteine, tryptophan, pantothenate, ferrous sulfate, and a complex mixture of growth factors.

Phenethyl alcohol agar (phenylethanol agar, phenylethyl alcohol agar)

This medium is used for the selective isolation of Gram-positive bacteria, particularly Gram-positive cocci, from specimens with a mixed biota. The medium should not be used for the observation of hemolytic reactions. The medium contains digests of casein and soybean meal, blood, and phenethyl alcohol. Moxalactam and lithium chloride can be added as selective factors.

Phenol red agar

This medium is used for the determination of fermentation reactions. The medium contains peptone, beef extract, a carbohydrate substrate, and phenol red. Bacteria that can ferment the added carbohydrate turn the medium yellow.

Phenol red tartrate broth

This medium is used for the differentiation of Gram-negative bacteria of the enteric groups, particularly members of the *Salmonella* (paratyphoid) group, based on their ability to ferment tartrate. The medium contains digest of casein, tartrate, and phenol red.

Pike streptococcal broth

This medium is used for the isolation and enrichment of hemolytic streptococci from throat swabs and other clinical specimens. The medium contains digest of casein, tryptose, yeast extract, glucose, sodium azide, blood, and crystal violet. After incubation of bacteria for 18 to 24 h in this medium, they may be isolated by streaking the culture onto blood agar plates.

Polymyxin B-lysozyme-EDTA-thallous acetate agar

This medium is used for the selective isolation and cultivation of *Bacillus anthracis*. The medium contains infusion from beef heart, tryptose, EDTA, thallous acetate, lysozyme, and polymyxin B.

Potassium tellurite agar

This medium is used for the cultivation and differentiation of *E. faecalis*. The medium contains infusion from beef heart, tryptose, blood, and potassium tellurite. *E. faecalis* appears as black colonies.

PPLO agar

This medium is used for the isolation and cultivation of *Mycoplasma* spp. (PPLOs). The medium contains infusion from beef heart, peptone, and serum.

Pseudomonas isolation agar base with glycerol

This medium is used for the selective isolation and identification of *P. aeruginosa* from clinical specimens. The medium contains peptone, Irgasan, and glycerol.

Rainbow Agar O157

This proprietary medium from Biolog Inc. is used for the detection and presumptive identification of verotoxin-producing strains of *E. coli*, particularly serotype O157:H7. The medium contains chromogenic substrates that are specific for two *E. coli*-associated enzymes: β-galactosidase (a blue-black chromogenic substrate) and β-glucuronidase (a red chromogenic substrate). The distinctive black or gray coloration of *E. coli* O157:H7 colonies is easily viewed by laying the petri plate against a white background. When *E. coli* O157:H7 is surrounded by pink or magenta nontoxigenic colonies, it may have a bluish hue. The addition of selective agents improves performance. *E. coli* O157:H7 colony coloration will be slightly bluer with these selective agents added. Tellurite is highly selective for *E. coli* O157:H7 and can reduce background biotas considerably. Novobiocin inhibits *Proteus* swarming and the growth of tellurite-reducing bacteria. Rare strains of O157:H7 are tellurite sensitive.

Rainbow Agar *Salmonella*

This proprietary medium from Biolog Inc. is used as a selective, chromogenic medium to aid in the detection and isolation of H_2S-producing *Salmonella* spp. Black colonies are formed even by weakly H_2S-producing strains.

RAMBACH agar (CHROMagar Rambach)

This medium is used for the detection of enteric bacteria, including coliforms and *Salmonella* spp. Sodium desoxycholate inhibits the accompanying Gram-positive biotas. The medium contains polypropylene glycol, peptone, desoxycholate, and a chromogenic mixture. This medium enables *Salmonella* spp. to be differentiated unambiguously from other bacteria. *Salmonella* spp. form a characteristic red color. To differentiate coliforms from salmonellae, the medium contains a chromogen indicating the presence of β-galactosidase splitting, a characteristic of coliforms. Coliform microorganisms grow as blue-green or blue-violet colonies. Other *Enterobacteriaceae* and Gram-negative bacteria, such as *Proteus*, *Pseudomonas*, *Shigella*, and *Salmonella* Typhi and Paratyphi A, grow as colorless to yellow colonies.

Rapid fermentation medium

This medium is used for the differentiation of *Neisseria* spp. isolated from clinical specimens. The medium contains digest of casein, cystine, sodium sulfite, and phenol red.

Regan-Lowe charcoal agar (Regan-Lowe medium)

This medium is used for the selective isolation of *B. pertussis* and *B. parapertussis* from clinical specimens. The medium contains beef extract, digest of gelatin, starch, charcoal, niacin, blood, and cephalexin.

Regan-Lowe semisolid transport medium

This medium is used for the transport of *B. pertussis* and *B. parapertussis* isolated from clinical specimens. The medium contains beef extract, digest of gelatin, starch, charcoal, niacin, blood, and cephalexin.

S. aureus ID

This proprietary medium from bioMérieux is used for the direct identification of *S. aureus* and the selective isolation of staphylococci. Direct identification of *S. aureus* is based on the spontaneous green coloration of α-glucosidase-producing colonies.

Salmonella-shigella agar

This medium is used for the selective isolation and differentiation of pathogenic enteric bacilli, especially those belonging to the genus *Salmonella*. The medium is not recommended for the primary isolation of *Shigella* spp. The medium contains lactose, bile salts, thiosulfate, citrate, beef extract, digests of casein and animal tissue, ferric citrate, neutral red, and brilliant green. Lactose-fermenting bacteria such as *E. coli* or *K. pneumoniae* appear as small, pink or red colonies. Non-lactose-fermenting bacteria—such as *Salmonella* spp., *Proteus* spp., and *Shigella* spp.—appear as colorless colonies. Production of H_2S by *Salmonella* spp. turns the center of the colonies black.

Salt meat broth

This medium is used for the isolation and cultivation of staphylococci from specimens with a mixed biota such as fecal specimens, especially during the investigation of staphylococcal food poisoning. The medium contains 10% NaCl, ox heart tissue, beef extract, and peptone.

Salt tolerance medium

This medium is used for the cultivation and differentiation of salt-tolerant *Streptococcus* spp., *Staphylococcus* spp., and other salt-tolerant Gram-positive cocci. The medium contains 6.5% NaCl, infusion from beef heart, tryptose, glucose, and bromcresol purple.

Sa*Select* Medium

This selective and differential medium (Bio-Rad Laboratories) detects *S. aureus* from various clinical specimens including suppurations, wounds, respiratory secretions, nasal swabs, and blood culture specimens.

Schaedler agar (Schaedler anaerobic agar)

This medium is used for the isolation of anaerobic and aerobic microorganisms. The medium contains glucose, digests of casein and soybean meal, peptone, yeast extract, Tris buffer, cystine, and hemin.

Schaedler CNA agar with vitamin K_1 and sheep blood

This medium is used for the selective isolation of anaerobic Gram-positive cocci. The medium contains digests of casein, animal tissue, and soybean meal; glucose; yeast extract; cystine; hemin; colistin; nalidixic acid; blood; and vitamin K_1.

Schleifer-Krämer agar

This medium is used for the isolation and cultivation of *Staphylococcus* spp. The medium contains glycerol, pyruvate, digest of casein, beef extract, yeast extract, potassium isothiocyanate, lithium chloride, glycine, and sodium azide.

Selenite broth (Selenite-F broth)

This medium is used for the isolation and enrichment of *Salmonella* spp. from clinical specimens. The medium contains digest of casein, lactose, and sodium biselenite.

Selenite broth base, mannitol

This medium is used for the isolation and cultivation of *Salmonella* Typhi and *Salmonella* Paratyphi. The medium contains peptone, mannitol, and sodium selenite.

Selenite cystine broth

This medium is used for the isolation and cultivation of *Salmonella* spp. from feces. The medium contains digest of casein, lactose, cystine, and sodium selenite.

Serum glucose agar (serum dextrose agar)

This medium is used for the maintenance of *Brucella* spp. and as a transport medium for *S. pyogenes*. The medium contains peptone, beef extract, serum, and glucose.

Serum tellurite agar

This medium is used for the isolation and cultivation of *Corynebacterium* spp., especially in the laboratory diagnosis of diphtheria. The medium contains digests of casein and animal tissue, glucose, lamb serum, and tellurite.

Simmons' citrate agar (citrate agar)

This medium is used for the differentiation of Gram-negative bacteria on the basis of citrate utilization. The medium contains citrate, phosphate buffer, and bromthymol blue. Bacteria that can utilize citrate as sole carbon source turn the medium blue. The medium is yellow at pH 6.0, green at pH 6.9, and blue at pH 7.6.

Skirrow brucella medium

This medium is used for the selective isolation and cultivation of *Campylobacter* spp. The medium contains peptone, yeast extract, liver digest, and blood. It also contains vancomycin, trimethoprim, and polymyxin B as selective factors.

Sodium hippurate broth (hippurate broth)

This medium is used for the identification and differentiation of beta-hemolytic streptococci based on hippurate hydrolysis. The medium contains infusion from beef heart, tryptose, and sodium hippurate. After inoculation and incubation, tubes are treated with $FeCl_3$ reagent. A heavy precipitate remaining after 10 to 15 min indicates that hippurate has been hydrolyzed.

Sorbitol-MacConkey agar with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG)

This agar is used as a selective and differential medium for the detection of *E. coli* O157:H7 incorporating the chromogen BCIG. The medium combines two different screening mechanisms for the detection of *E. coli* O157:H7, the failure to ferment sorbitol and the absence of β-glucuronidase activity. The non-sorbitol-fermenting and β-glucuronidase-negative *E. coli* O157:H7 will appear as straw-colored colonies. Organisms with β-glucuronidase activity will cleave the substrate, leading to a distinct blue-green coloration of the colonies.

Soybean medium with 0.1% agar (tryptone soya HiVeg broth with 0.1% agar)

This medium is used for the cultivation of anaerobes from root canals, blood, and other clinical specimens. The medium contains 0.1% agar, casein hydrolysate, and digest of soybean meal.

Special infusion broth with blood

This medium is used for the propagation of pathogenic cocci and other fastidious organisms associated with blood culture work and allied pathological investigations. The medium contains infusion from animal tissues, peptones, glucose, and blood.

Specimen preservative medium

This medium is used for the preservation and transport of viable microorganisms in stool specimens. The medium contains glycerol, citrate, yeast extract, and deoxycholate.

Standard fluid medium 10B (Shepard's M10 medium)

This medium is used for the isolation and cultivation of *U. urealyticum* from clinical specimens. The medium contains numerous growth factors, including serum yeast extract, glucose, amino acids, cocarboxylase, NAD, vitamins, and serum. It also contains infusion from beef heart, peptone, and phenol red. Penicillin is included as a selective factor.

Staphylococcus agar no. 110

This medium is used for the isolation of staphylococci from clinical specimens. The medium contains gelatin, mannitol, digest of casein, yeast extract, and lactose.

Staphylococcus-Streptococcus selective medium

This medium is used for the selective isolation of *S. aureus* and streptococci from clinical specimens. The medium contains peptone, starch, and blood. It also contains nalidixic acid and colistin sulfate as selective factors.

StrepB Carrot Broth

This is a proprietary broth that is used for detecting the presence of GBS infections in pregnant women. The medium contains peptone, starch, morpholinepropanesulfonic acid, glucose, pyruvate, growth factors, and selective factors. It is a modification of Granada medium consisting of a one-step method for screening pregnant women for the presence of GBS. Tubes show an orange-to-red color change, typical of GBS. The production of orange, red, or brick red pigment is a unique characteristic of hemolytic GBS due to reactions with substrates such as starch, proteose peptone, serum, and folate pathway inhibitors. Church et al. reported that StrepB Carrot Broth compared favorably with Lim broth and was less labor-intensive (36).

Streptococcus selective medium

This medium is used for the selective isolation of streptococci from clinical specimens. The medium contains peptone, starch, and blood. It also contains colistin sulfate and oxolinic acid as selective factors.

Streptosel agar

This medium is used for the selective isolation, cultivation, and enumeration of streptococci from specimens containing a mixed biota. The medium contains digest of casein, glucose, digest of soybean meal, sodium citrate, cystine, sodium azide, sodium sulfite, and crystal violet.

Stuart transport medium, modified

This medium is used for the preservation of *Neisseria* spp. and other fastidious organisms during their transport from clinic to laboratory. The medium contains sodium glycerophosphate, cysteine, sodium thioglycolate, and methylene blue.

Sucrose-Teepol-tellurite agar

This medium is used for the selective isolation and differentiation of *Vibrio* spp. based on their ability to ferment sucrose. The medium contains beef extract, peptone, sucrose, Teepol, tellurite, and bromthymol blue. *V. cholerae* appears as flat, yellow colonies. *V. parahaemolyticus* appears as elevated, green-yellow, mucoid colonies.

Sulfide-indole-motility medium

This medium is used for the differentiation of members of the *Enterobacteriaceae* based on H_2S production, indole production, and motility. The medium contains peptone, beef extract, peptonized iron, and sodium thiosulfate.

TCBS agar

This medium is used for the selective isolation of *V. cholerae* and *V. parahaemolyticus* from a variety of clinical specimens. The medium contains sucrose, citrate, thiosulfate, yeast extract, digests of casein and animal tissue, oxgall (bile), sodium cholate, ferric citrate, thymol blue, and bromthymol blue.

Tetrathionate broth, Hajna

This medium is used for the isolation of *Salmonella* spp., except *Salmonella* Typhi and *S. enterica* subsp. *arizonae*, from fecal specimens and urine. The medium contains thiosulfate, peptones, mannitol, yeast extract, glucose, sodium deoxycholate, brilliant green, and iodine.

Tetrazolium tolerance agar

This medium is used for the differentiation of bacteria based on the ability to tolerate and grow in the presence of tetrazolium. The medium contains digests of casein and soybean meal plus triphenyltetrazolium chloride. *E. faecalis* rapidly reduces tetrazolium.

Thayer-Martin medium

This medium is used for the isolation and cultivation of fastidious microorganisms, especially *Neisseria* spp. The medium contains peptone starch, hemoglobin, and a complex mixture of amino acids, glucose, nucleotides, iron, and vitamins.

Thayer-Martin medium, modified

This medium is used for the isolation and cultivation of fastidious microorganisms, especially *Neisseria* spp. The medium contains peptone starch, hemoglobin, and a complex mixture of amino acids, glucose, nucleotides, iron, and vitamins. The glucose and agar concentrations are lower than in the original formulation, which improves growth. The medium also contains trimethoprim.

Thioglycolate bile broth

This medium is used for the cultivation of *B. fragilis* and *C. perfringens* from clinical specimens. The medium contains digest of casein, glucose, yeast extract, cystine, sodium thioglycolate, and bile.

Thioglycolate medium, enriched (thioglycolate medium with vitamin K_1 and hermin, anaerobic thioglycolate medium)

This medium is used for the isolation, cultivation, and identification of a wide variety of obligate anaerobic bacteria. The medium contains digest of casein, glucose, yeast extract, cystine, sodium thioglycolate, bile, hemin, and vitamin K_1.

Tinsdale agar

This medium is used for the primary isolation and identification of *C. diphtheriae*. The medium contains peptone, yeast extract, cystine, sodium sulfite, and potassium tellurite.

Todd-Hewitt broth

This medium is used for the cultivation of group A streptococci used in serological typing and for the cultivation of a variety of pathogenic microorganisms. The medium contains infusion from beef heart, neopeptone, and glucose. Gentamicin and naladixic acid may be added to render the medium selective.

Transport medium, Stuart

This medium is used for the transportation of swab specimens for the recovery of a wide variety of microorganisms, including *N. gonorrhoeae*. The medium contains sodium glycerophosphate, sodium thioglycolate, and methylene blue.

Tryptic soy blood agar (tryptose blood agar, TSA blood agar)

This medium is used for the cultivation of a wide variety of fastidious microorganisms and for the observation of hemolytic reactions of a variety of bacteria. It may be used to perform the CAMP test for the presumptive identification of GBS (*S. agalactiae*). The medium contains digests of casein and soybean meal, and blood.

Trypticase soy agar (tryptic soy agar, soybean-casein digest medium)

This medium is used as a base for the general culture of numerous bacteria or, when supplemented, for the cultivation of fastidious microorganisms. The medium contains digests of casein and soybean meal. It can be supplemented with glucose, various amino acids (e.g., glutamine), and vitamins. It can also be supplemented with various antimicrobials as selective factors. When supplemented with sheep blood, this medium is useful for the observation of hemolytic reactions of a variety of bacteria.

Trypticase soy agar with sheep blood and gentamicin (TSA II with sheep blood and gentamicin)

This medium is used for the isolation of *S. pneumoniae* from a variety of clinical specimens. The medium contains digests of casein and soybean meal, growth factors, blood, and gentamicin.

Trypticase soy agar with sheep blood, sucrose, and tetracycline

This medium is used for the isolation of *S. pneumoniae* from a variety of clinical specimens. The medium contains digests of casein and soybean meal, growth factors, sucrose, blood, and tetracycline.

Trypticase soy agar with sheep blood and vancomycin

This medium is used for the isolation of VRE from a variety of clinical specimens. The medium contains digests of casein and soybean meal, blood, and vancomycin.

Trypticase tellurite agar base

This medium is used for the selective isolation of microorganisms from clinical specimens, especially from the nose, throat, and vagina. The medium contains digests of casein and soybean meal, blood, glucose, serum, and tellurite.

U9B broth

This medium is used for the isolation and identification of T-strain mycoplasmas from clinical specimens, especially *U. urealyticum*. T-mycoplasmas are the only members of the *Mycoplasma* group known to contain urease. Bacteria with urease activity turn the medium dark pink. The medium contains digests of casein and soybean meal, glucose, cysteine, penicillin, phenol red, and urea.

Urea agar (urease test agar; urea agar base, Christensen)

This medium is used for the detection of *Proteus* spp. and other members of the *Enterobacteriaceae* based on rapid urease activity. Urease-positive bacteria turn the medium pink. The medium contains urea, peptone, glucose, and phenol red.

Urinary Tract Infections Chromogenic Agar (UTIC Agar)

This medium is used for the presumptive detection and differentiation of bacteria that cause UTIs.

Urogenital *Mycoplasma* broth base

This medium is used for the selective culture of *M. hominis* and *U. urealyticum*. The medium contains heart infusion, digest of casein, yeast extract, arginine, cysteine, phenol red, serum, vitamins, and urea. It also contains penicillin and amphotericin B as selective factors.

UVM (University of Vermont) Modified Listeria Enrichment Broth

This medium is used for the selective isolation of *L. monocytogenes*. The medium contains 2% NaCl, digests of casein and animal tissue, beef extract, yeast extract, esculin, nalidixic acid, and acriflavine.

V agar

This medium is used for the isolation and differentiation of *G. vaginalis* from clinical specimens. The medium contains digests of casein and animal tissue, peptone, beef extract, yeast extract, starch, and blood. Plates are incubated under an atmosphere with 3 to 10% CO_2. *G. vaginalis* appears as small white colonies with diffuse beta-hemolysis.

VACC agar (Remel VACC Agar)

This medium is used for the primary isolation, selection, and differentiation of *Enterobacteriaceae* that produce ESBLs.

Vibrio parahaemolyticus agar

This medium is used for the isolation, cultivation, enumeration, and presumptive identification of *V. parahaemolyticus*. Sucrose-fermenting bacteria appear as yellow colonies with pale yellow peripheries. Non-sucrose-fermenting bacteria appear as mucoid, green colonies with a dark green center. The medium contains sucrose, citrate, thiosulfate, peptone, sodium taurocholate, yeast extract, sodium lauryl sulfate, bromthymol blue, and thymol blue.

Vibrio parahaemolyticus sucrose agar

This medium is used for the isolation, cultivation, and differentiation of *V. parahaemolyticus*. *V. parahaemolyticus* and *V. vulnificus* appear as blue to green colonies. Other *Vibrio* spp. appear as yellow colonies (sucrose positive). This medium contains sucrose, yeast extract, tryptose, digest of casein, bile salts, and bromthymol blue.

Vogel and Johnson agar

This medium is used for the detection of coagulase-positive *S. aureus*. The medium contains digest of casein, mannitol, glycine, yeast extract, lithium chloride, phenol red, and tellurite.

VRE agar

This medium is used for the isolation of VRE and high-level-aminoglycoside-resistant enterococci from clinical samples. Nonresistant enterococci containing the *van*C genes will not grow on this medium. The medium contains tryptone, yeast extract, citrate, esculin, ferric ammonium citrate, sodium azide, and a selective supplement of meropenem and vancomycin. The selective supplement suppresses growth of Gram-negative bacteria and *Enterococcus gallinarum*. The medium contains an indicator system to detect the growth of esculin-hydrolyzing organisms. Enterococci produce black zones around the colonies from the formation of black iron phenolic compounds derived from esculin hydrolysis products and ferrous iron.

VRE*Select* medium

This is a selective and differential medium (Bio-Rad Laboratories) for vancomycin-resistant *E. faecium* and *E. faecalis* for use with rectal swabs and fecal specimens.

Wallenstein medium

This medium is used for the isolation of *Mycobacterium* spp. other than *M. leprae*. The medium contains egg yolks, glycerol, and malachite green.

Wilkins-Chalgren anaerobe broth (anaerobe broth, MIC)

This medium is used for the cultivation and antimicrobial susceptibility (MIC) testing of anaerobic bacteria. The medium contains digest of casein, gelatin peptone, yeast extract, glucose, arginine, sodium pyruvate, hemin, and menadione.

Xylose-lactose-Tergitol 4

This medium is used for the isolation and identification of salmonellae from clinical samples. The medium contains lactose, sucrose, thioglycolate, lysine, xylose, yeast extract, peptone, phenol red, and a selective supplement containing Tergitol. The presence of the selective agent, Tergitol 4, in this medium inhibits many organisms that can be problematic on other plating media. In addition, biochemical and pH changes within the medium allow *Salmonella* spp. (black colonies) to be differentiated from organisms such as *E. coli* (yellow colonies) and *Shigella* spp. (red colonies). The enhanced selectivity of this agar reduces the need for further identification procedures, saving time and money, and results in fewer false presumptive positive colonies than do other *Salmonella* plating media.

Xylose-lysine-deoxycholate agar

This medium is used for the isolation and differentiation of enteric pathogens, especially *Shigella* and *Providencia* spp. The medium contains lactose, sucrose, thioglycolate, lysine, xylose, sodium deoxycholate, ferric ammonium citrate, and phenol red. Bacteria that do not ferment xylose, lactose, or sucrose appear as red colonies. Xylose-fermenting, lysine-decarboxylating bacteria appear as red colonies. Xylose-fermenting, non-lysine-decarboxylating bacteria appear as opaque yellow colonies. Lactose- or sucrose-fermenting bacteria appear as yellow colonies.

Xylose-sodium deoxycholate-citrate agar

This medium is used for the cultivation of *Salmonella* spp. and some *Shigella* spp. The medium contains xylose, citrate, thiosulfate, beef extract, peptone, ferric ammonium citrate, deoxycholate, and neutral red.

Yersinia selective agar base

This medium is used for the isolation and enumeration of *Y. enterocolitica* from clinical specimens. The medium contains mannitol, peptones, yeast extract, sodium pyruvate, deoxycholate, neutral red, and crystal violet. It also contains cefsulodin, Irgasan, novobiocin, and selective factors.

APPENDIX

Medium Additives

Many media contain additives that have specific functions ranging from selection to differentiation to protection of certain bacterial species. Below are descriptions of some of the commonly used medium additives.

ACES: allows optimal pH buffering capacity without inhibition of bacteria as seen with other inorganic buffers

Acriflavine: selective agent; suppresses Gram-positive organisms

ADC enrichment: a supplement added to mycobacteriology media that includes albumin, dextrose, catalase, and sodium chloride; catalase destroys peroxides that may be in the medium

Agar used in broth medium (0.05 to 0.1%): used to reduce O_2 tension

Albumin: protects against toxic by-products in medium; binds free fatty acids

Antibiotics: one or many may be added to make a medium selective; inhibitory capacity may vary depending on the concentration used

Bicarbonate-citric acid pellet: used to generate CO_2 gas within a closed environment after exposure to moisture: used in transport devices for isolating *N. gonorrhoeae*

Bismuth sulfite: heavy metal that is inhibitory to commensal organisms

Carbohydrates: energy source; used to make medium differential when combined with an indicator

Cetrimide: acts as a quaternary ammonium cationic detergent that causes nitrogen and phosphorus to be released from bacterial cells other than *P. aeruginosa*

Charcoal: detoxifying agent, surface tension modifier, scavenger of radicals and peroxides

Cornstarch: works as a detoxifying agent; may provide additional nutrients as an energy source

Dent's supplement (Oxoid): vancomycin, trimethoprim, cefsulodin, and amphotericin B added to Columbia blood agar and laked horse blood for isolation of *Helicobacter*

Dextrose (glucose): makes the medium hypertonic; energy source

Egg yolk: used to demonstrate lecithinase, lipase, and proteolytic activities and fatty acids

Ferric ammonium citrate: iron salt used in combination with other agents (esculin and sodium thiosulfate) to make medium differential by producing a black precipitate

Fildes enrichment: peptic digest of sheep blood that provides a rich source of nutrients, including X (hemin) and V (NAD) factors; X originally stood for "unknown" and V originally stood for "vitamin"

Glycerol: a purified alcohol and an abundant source of carbon; used in culture, transport, and storage medium and reagent preparation

Glycine: a selective agent that is inhibitory to organisms

Horse serum: an enrichment used in growth media for such organisms as *Mycoplasma* and *Ureaplasma*

IsoVitaleX (BBL): provides V factor (NAD) and additional nutritive ingredients, such as vitamins, amino acids, ferric ion, and dextrose, to stimulate growth of fastidious organisms

Laked blood or laked horse blood: created by freeze-thaw cycles of blood; enhances pigment production of anaerobes and used in susceptibility testing of fastidious organisms

Lithium chloride: a selective agent that inhibits organisms

Malachite green: a dye that partially inhibits bacteria

NAD (V factor): necessary for growth of some fastidious organisms

OADC enrichment: a supplement added to mycobacteriology media that includes oleic acid, albumin, dextrose, catalase, and sodium chloride; the oleic acid provides fatty acids utilized by mycobacteria, and the catalase destroys peroxides that may be in the medium

Oxgall (bile): inhibits specific organisms; allows medium to be selective

Peptones: carbohydrate-free source of nutrients

Phenylethyl alcohol: reversibly inhibits DNA synthesis; results in inhibition of facultative anaerobic Gram-negative organisms

Pyridoxal phosphate: liquid supplement added to media for isolation of fastidious organisms; also comes in the form of a disk to be used in satelliting tests

Rabbit blood: enhances pigment production of anaerobes; hemolytic reactions of streptococci are "correct"; additive to heart infusion agar for isolation of *Bartonella* spp.

Serum: albumin, fatty acids

Sheep blood and human blood: provide hemin and other nutrients; allow true hemolytic reactions of streptococci; NADase enzyme inactivates the NAD in the sheep blood and is not available for organisms

Skirrow's supplement: vancomycin, trimethoprim, and polymyxin B added to Columbia agar and laked horse blood for isolation of *Helicobacter*

Sodium azide: a selective agent that inhibits Gram-negative organisms

Sodium bicarbonate: neutralization agent used with gastric wash or lavage specimens for recovery of acid-fast organisms

Sodium bisulfite: disinfectant, antioxidant, or reducing agent

Sodium chloride: maintains osmotic equilibrium; when added at a high concentration, it may be a selective agent

Sodium citrate: a selective agent inhibitory to organisms

Sodium deoxycholate: a salt of bile acid and a selective agent that inhibits Gram-positive and spore-forming organisms

Sodium polyanethol sulfonate: a polyanionic anticoagulant that inactivates aminoglycosides and interferes with the complement cascade, lysozyme activity, and phagocytic activity inherent in blood. May be inhibitory to *Neisseria*, *Gardnerella*, *Streptobacillus*, *Peptostreptococcus*, *Francisella*, and *Moraxella* spp.

Sodium pyruvate: growth stimulant

Sodium selenite: a selective agent that inhibits coliforms

Sodium thioglycolate: a reducing agent

Starch: a polysaccharide and detoxifying agent incorporated into some media as a differential agent

Tellurite: is toxic to egg-clearing strains of bacteria; imparts black color to colony

Tween 80 (polysorbate 80): an oleic acid ester that stimulates growth and provides fatty acids as well as acts as a dispersal agent

Vitamin K: ingredient required for optimal growth of certain obligate anaerobes, such as the *Bacteroides* group

Vitox (Oxoid): provides V factor (NAD) and other essential growth factors to stimulate growth of fastidious organisms; see IsoVitaleX

Yeast extract: water-soluble product that provides B vitamins and protein

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GRAM-POSITIVE COCCI

General Approaches to Identification of Aerobic Gram-Positive Cocci

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The majority of aerobic, or facultatively anaerobic, Gram-positive cocci isolated from clinical specimens are distributed among the genera *Staphylococcus*, *Streptococcus*, and *Enterococcus*. Molecular taxonomic studies of this group of bacteria have revealed additional genera and species that are phenotypically similar to the commonly encountered organisms but are infrequently isolated from clinical specimens. Tables 1 and 2 and chapters 21 to 24 describe basic phenotypic tests that can be used to distinguish these infrequent isolates from staphylococci, streptococci, and enterococci. It should be noted that the reactions listed in Tables 1 and 2 represent those of the majority of strains in each group; isolates with variant reactions may be encountered. Each of the tables contains organisms with similar cellular morphologies, either "streptococcal," consisting of Gram-positive cocci or coccobacilli arranged primarily in pairs and/

TABLE 1 Characteristics of catalase-negative Gram-positive cocci that grow aerobically and form cells arranged in pairs and chains[a]

PYR	LAP	6.5% NaCl	BE	Motility	45°C	Probe	HIP	Satellitism	10°C	Vancomycin resistance	Organism (chapter)
+	+	+	+	+	+	NA	NA	NA	NA	NA	*Enterococcus* (23)
+	+	+	+	+	−	NA	NA	NA	NA	NA	*Vagococcus* (24)
+	+	+	+	−	NA	+	NA	NA	NA	NA	*Enterococcus* (23)
+	+	+	+	−	NA	−	NA	NA	NA	NA	*Lactococcus* (24)
+	+	+	−	NA	NA	NA	+	−	NA	NA	*Facklamia* spp.[b] (24)
+	+	+	−	NA	NA	NA	−	V	NA	NA	*Ignavigranum* (24)
+	+	−	+	+	NA	NA	NA	NA	NA	NA	*Vagococcus* (24)
+	+	−	+	−	NA	NA	NA	NA	NA	NA	*Lactococcus* (24)
+	+	−	−	NA	NA	NA	NA	+	NA	NA	*Abiotrophia*, *Granulicatella* (24)
+	+	−	−	NA	NA	NA	NA	−	NA	NA	*Gemella* spp.[c] (24)
+	−	+	NA	NA	NA	NA	NA	NA	NA	NA	*Globicatella sanguinis* (24)
+	−	−	NA	NA	NA	NA	NA	NA	NA	NA	*Dolosicoccus* (24)
−	+	−	NA	−	NA	NA	+	NA	NA	−	*Gemella asaccharolytica*[d] (24)
−	+	NA	NA	NA	NA	NA	NA	NA	+	NA	*Lactococcus* (24)
−	+	NA	NA	NA	NA	NA	NA	NA	−	NA	*Streptococcus*[e] (22)
−	−	NA	NA	NA	NA	NA	NA	NA	NA	+	*Leuconostoc*[f] (24)
−	−	NA	NA	NA	NA	NA	NA	NA	NA	−	*Globicatella sulfidifaciens* (24)

[a] See chapters 19 and 22 to 24 for methods for performing tests referred to in this table. Reactions shown are typical, but exceptions may occur. Abbreviations and symbols: PYR, production of pyrrolidonyl arylamidase; LAP, production of leucine aminopeptidase; 6.5% NaCl, growth in 6.5% NaCl; BE, hydrolysis of esculin in the presence of 40% bile; 45°C, growth at 45°C; probe, reaction with commercially available nucleic acid probe for the genus *Enterococcus*; HIP, hydrolysis of hippurate; satellitism, satelliting growth behavior; 10°C, growth at 10°C; +, most strains positive; −, most strains negative; V, variable reactions are observed; NA, not applicable.

[b] The reactions in this table are typical for *F. hominis*, *F. sourekii*, and *F. ignava*. *F. languida* cells tend to be arranged in clusters, and isolates are hippurate hydrolysis negative (Table 2).

[c] *G. asaccharolytica*, *G. bergeri*, *G. morbillorum*, and *G. sanguinis* cells tend to be arranged in pairs and chains, in contrast to the cells of *G. haemolysans*, which are arranged in pairs, tetrads, and clusters (Table 2).

[d] *G. asaccharolytica* cells are Gram variable (see chapter 24).

[e] Most streptococci are PYR negative, with the exception of *Streptococcus pyogenes* isolates and some strains of *Streptococcus pneumoniae*, which are PYR positive.

[f] *Leuconostoc* is distinguished from the other catalase-negative organisms in Table 1 by its ability to produce gas as an end product of glucose metabolism and intrinsic resistance to vancomycin. The phenotypically similar genus *Weissella* contains organisms formerly classified as leuconostocs and the species formerly named *Lactobacillus confusus* (see chapter 24).

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TABLE 2 Differentiating features of Gram-positive cocci that grow aerobically and form cells arranged in clusters or irregular groups[a]

Catalase	Obligate aerobe	Oxidase	PYR	LAP	NaCl	ESC	Hemolysis	Vancomycin	BGUR	Organism (chapter)
+	+	+	NA	NA	+[b]	NA	NA	NA	NA	*Micrococcus*[c] (21)
+	+	−	NA	NA	+[d]	NA	NA	NA	NA	*Alloiococcus* (21)
+	−	−	NA	NA	+[b]	NA	NA	NA	NA	*Staphylococcus* (21)
+	−	−	NA	NA	−[b]	NA	NA	NA	NA	*Rothia mucilaginosa*[e] (28)
−	−	NA	+	+	+[d]	+	NA	NA	−	*Dolosigranulum* (24)
−	−	NA	+	+	+[d]	+	NA	NA	+	*Aerococcus sanguinicola* (24)
−	−	NA	+	+	+[d]	−	NA	NA	−	*Facklamia languida*[f] (24)
−	−	NA	+	+	−[d]	+	NA	NA	NA	*Rothia mucilaginosa*[e] (28)
−	−	NA	+	+	−[d]	−	NA	NA	NA	*Gemella haemolysans*[g] (24)
−	−	NA	+	−	NA	NA	α	NA	NA	*Aerococcus viridans*[h] (24)
−	−	NA	+	−	NA	NA	γ	NA	NA	*Helcococcus kunzii*[h] (24)
−	−	NA	−	+	NA	NA	NA	R	NA	*Pediococcus*[i] (24)
−	−	NA	−	+	NA	NA	NA	S	+	*Aerococcus urinae* (24)
−	−	NA	−	−	NA	NA	NA	NA	+	*Aerococcus urinaehominis* (24)
−	−	NA	−	−	NA	NA	NA	NA	−	*Aerococcus christensenii* (24)

[a] See chapters 19, 21, 24, and 28 for methods for performing the phenotypic tests referred to in this table. Reactions shown are typical; exceptions may occur. Abbreviations and symbols: PYR, production of pyrrolidonyl arylamidase; LAP, production of leucine aminopeptidase; NaCl, growth in the presence of either 5 or 6.5% NaCl (see footnotes b and d); ESC, esculin hydrolysis; BGUR, production of β-glucuronidase; +, most strains positive; −, most strains negative; V, variable reactions are observed; NA, not applicable; α, alpha-hemolysis on sheep blood agar; γ, nonhemolytic reaction on sheep blood agar; S, susceptible; R, resistant.

[b] Growth in the presence of 5% sodium chloride.

[c] *Kocuria*, a related genus infrequently isolated from clinical specimens, is distinguished from *Micrococcus* by its ability to produce acid aerobically from D-glucose and glycerol (see chapter 21).

[d] Growth in the presence of 6.5% sodium chloride.

[e] *R. mucilaginosa* isolates are usually catalase negative or weakly positive but may be strongly catalase positive. *Rothia aeria* and *Rothia dentocariosa* are usually catalase positive.

[f] *Ignavigranum ruoffiae* (Table 1) exhibits reactions identical to those of *F. languida* in the PYR, ESC, and NaCl tests. However, *I. ruoffiae* cells are arranged primarily in chains, while *F. languida* cells usually form clusters. Other *Facklamia* species form cells arranged in pairs and chains (Table 1).

[g] *G. haemolysans* cells tend to be arranged in pairs, tetrads, and groups, in contrast to the cells of other *Gemella* species, which usually occur in pairs and short chains (Table 1).

[h] *H. kunzii* strains form tiny pinpoint nonhemolytic colonies on blood agar after 24 h of aerobic incubation at 35°C, while *A. viridans* isolates form larger alpha-hemolytic colonies under similar incubation conditions. In contrast to *H. kunzii*, *A. viridans* prefers aerobic incubation atmospheres. Two additional species of *Helcococcus* isolated from human sources have been described, each based on a single isolate. In contrast to *H. kunzii*, the new species *Helcococcus sueciensis* and the proposed species "*Helcococcus pyogenes*" are PYR negative (see chapter 24).

[i] The genera *Pediococcus* and *Tetragenococcus* have similar phenotypic characteristics, except that tetragenococci are vancomycin susceptible. The bile esculin test can differentiate between tetragenococci (positive) and *Aerococcus urinae* (negative) (see chapter 24).

or chains, or "staphylococcal," signifying that cells appear as cocci arranged in pairs, tetrads, clusters, and irregular groups. No taxonomic kinship is implied by division of these bacteria into two groups based on cellular morphology.

The commonly isolated aerobic Gram-positive cocci (staphylococci, streptococci, and enterococci) can usually be accurately identified by determining a few basic phenotypic traits (cellular morphology, catalase reaction, production of pyrrolidonyl arylamidase [PYR], etc. [see reference 1, Tables 1 and 2 herein, and chapters 21 to 24]). Reliance on a single or only a few phenotypic tests can, however, lead to misidentification. For example, optochin-resistant *Streptococcus pneumoniae* strains (2) might be incorrectly identified as alpha-hemolytic (viridans) streptococci (chapter 22). Clumping factor (slide coagulase)-positive *Staphylococcus lugdunensis*, a coagulase-negative species, could be misidentified as *Staphylococcus aureus* (see reference 3 and chapter 21). PYR-positive *Lactococcus* isolates might be incorrectly identified as members of the genus *Enterococcus* (see reference 4 and chapter 23).

As new genera and species of aerobic Gram-positive cocci are described and characterized, it becomes increasingly difficult to identify some of the less frequently isolated organisms solely on the basis of phenotypic traits. A variety of automated and manual systems have proven to be fairly accurate for identification of commonly encountered staphylococcal, streptococcal, and enterococcal species (see the "Identification" sections in chapters 21 to 24 and references 5–10). These systems are less effective for identification of infrequently isolated aerobic Gram-positive cocci (see chapter 24). The less commonly isolated organisms may not be identified by these systems or may be misidentified as other genera or species. Basic phenotypic tests can usually suggest a possible identity for strains of infrequently encountered aerobic Gram-positive cocci, but evaluation with a larger battery of phenotypic tests, molecular identification methods, and, more recently, matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) is often valuable, if not indispensable, for accurate identification.

Nucleic acid probe tests and amplification methods for identification of some of the commonly isolated aerobic Gram-positive cocci are commercially available and designed for use in medium- to large-volume clinical microbiology laboratories. These methods may also be useful for ruling out enterococcal, streptococcal, or staphylococcal strains when attempting to identify phenotypically similar, infrequently isolated organisms. One of the most useful methods for molecular characterization of the aerobic Gram-positive cocci of clinical interest is comparison of 16S rRNA gene sequences (11, 12), although sequence comparison of other genes may also be helpful for identification. Genes that have been examined for this purpose include the *rpoB*, *tuf*, and *sodA* genes of staphylococci (13–15); the *rpoB* gene of streptococci (16); the *atpA* gene of enterococci (17); and the *sodA* gene of lactococci (4). Other identification methods, such as multilocus sequence analysis of enterococci (*rpoA* and *pheS* genes) (18) and melting-curve analysis of restriction fragments obtained from amplified regions of the *sodA* gene and the 16S-23S intergenic spacer region (enterococci) (19), have also been reported.

MALDI-TOF MS is increasingly used for routine identification of bacterial strains, and its usefulness is steadily expanding due to new developments and improvements (20). The commercially available MS instruments and software packages have many strengths in common. However, the packages do have different strengths and weaknesses, and improvements are steadily introduced. Comparisons of MALDI-TOF MS results to those obtained with other methods, phenotypic as well as genotypic, have been very encouraging. In general, quite reliable identifications have been obtained when examining strains from the genera *Enterococcus*, *Streptococcus*, and *Staphylococcus* and the taxa of catalase-negative, Gram-positive cocci not belonging to the streptococci or enterococci, though some species may be difficult to separate with the currently available MALDI-TOF MS database and software options (21–28). More information on the use of molecular methods for identification of this group of organisms can be found in chapters 21 to 24.

Each microbiology laboratory needs to establish criteria for the extent of identification of routinely isolated aerobic Gram-positive cocci. Efforts should be made to recognize and report organisms described as pathogens in various clinical scenarios or organisms with well-known susceptibility patterns, since identification should, in these cases, play an important role in guiding patient treatment. For example, in one scenario, all staphylococcal isolates could be fully identified to the species level with an automated system; alternatively, in a different setting, staphylococci might be examined initially with the coagulase test to identify *S. aureus*. Coagulase-negative strains could then be subjected to a few simple screening tests and be identified simply as coagulase-negative staphylococci, presumptively as *Staphylococcus saprophyticus*, or presumptively as *S. lugdunensis*. An identification of "viridans streptococcus" might be sufficient when these organisms are isolated in mixed culture, but species identification of important isolates can offer clinically relevant information (e.g., "*Streptococcus bovis* group" in endocarditis cases or "*Streptococcus anginosus* group" ["*milleri*" group] in cases of brain or hepatic abscess). Procedures for identification should reflect laboratory resources, workflow, the composition of patient populations served by the laboratory, and the clinical utility of results for the laboratory's users.

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Staphylococcus, Micrococcus, and Other Catalase-Positive Cocci

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TAXONOMY

Historically, the genera *Staphylococcus* and *Micrococcus* were placed together with the genera *Stomatococcus* and *Planococcus* in the family *Micrococcaceae*, containing Gram-positive, catalase-positive cocci. However, molecular phylogenetic and chemotaxonomic analyses revealed that staphylococci and "micrococci" are not closely related (1). Staphylococci belong to the *Bacillus-Lactobacillus-Streptococcus* cluster, which consists of Gram-positive bacteria with DNA of a low G+C content. In the taxonomic outline (2004) of the 2nd edition of *Bergey's Manual of Systematic Bacteriology* (2), a classification of the *Staphylococcus* genus together with the genera *Jeotgalicoccus*, *Macrococcus*, *Salinicoccus*, and *Gemella* was outlined in a newly established family, *Staphylococcaceae*, which has since been supplemented with *Nosocomiicoccus* and diminished by *Gemella* (now *Bacillales* family XI, *incertae sedis*) (3). The *Staphylococcaceae* combined with the *Bacillaceae*, *Planococcaceae*, *Listeriaceae*, and other families are now part of the order *Bacillales* of the class *Bacilli* (3).

The *Micrococcus* genus was sustained, but certain micrococcal species previously belonging to this genus were reclassified into the newly established genera *Kocuria*, *Nesterenkonia*, *Kytococcus*, and *Dermacoccus*. These genera were rearranged into two families, the redefined *Micrococcaceae* and the newly established *Dermacoccaceae*, both consisting of Gram-positive cocci with high G+C-content DNA (4, 5). Both families are assigned to the suborder *Micrococcineae* (class *Actinobacteria*) (1). The type genus *Micrococcus* and the genera *Kocuria* and *Nesterenkonia* as well as the genera *Acaricomes*, *Arthrobacter*, *Citricoccus*, *Enteroactinococcus*, *Renibacterium*, *Rothia*, *Sinomonas*, *Yaniella*, and *Zhihengliuella* now constitute the *Micrococcaceae* family. The only species of the former genus *Stomatococcus*, *S. mucilaginosus*, was reclassified as *Rothia mucilaginosa* (6). The other family of the *Micrococcineae* also containing previous *Micrococcus* species, designated *Dermacoccaceae*, contains the type genus *Dermacoccus* as well as the genera *Branchiibius*, *Calidifontibacter*, *Demetria*, *Flexivirga*, *Kytococcus*, *Luteipulveratus*, and *Yimella*.

An unrelated species of Gram-positive cocci exhibiting a positive catalase reaction and occurring in human specimens is *Alloiococcus otitis*, the only species of this genus, which is a member of the *Carnobacteriaceae* family belonging to the order *Lactobacillales* (class *Bacilli*) (3).

For further taxonomic details and references, see the taxonomic outline of the 2nd edition of *Bergey's Manual of Systematic Bacteriology* (2) and the *List of Prokaryotic Names with Standing in Nomenclature* (http://www.bacterio.net).

DESCRIPTION OF THE FAMILIES

Staphylococcaceae

Staphylococci are characterized by Gram-positive, nonmotile, non-spore-forming, spherical cells of 0.5 to 1.5 µm in diameter, occurring as single cocci, in pairs, as tetrads, or as short chains, which characteristically divide in more than one plane, thereby forming irregular clusters like a bunch of grapes. With the exception of the anaerobic species *Staphylococcus saccharolyticus* (formerly *Peptococcus saccharolyticus*) and *Staphylococcus aureus* subsp. *anaerobius*, the staphylococci are facultative anaerobes (Table 1). Although staphylococci are usually catalase positive, rare catalase-negative strains have been reported (7). Most *Staphylococcus* species are cytochrome oxidase negative in the modified oxidase test, with the exception of *S. fleurettii*, *S. lentus*, the three subspecies of *S. sciuri*, *S. stepanovicii*, and *S. vitulinus*. Staphylococci grow in the presence of 10% NaCl between 18°C and 40°C. The metabolism is respiratory and fermentative. The cell wall contains peptidoglycan and teichoic acid. Staphylococci are susceptible to lysostaphin.

A major genotypic criterion of the members of the genus *Staphylococcus* is a G+C content of 30 to 39 mol%. Whole-genome sequencing has been performed for many staphylococcal strains, and completed genome sequences are available for *S. aureus* (e.g., COL, Mu3, Mu50, MW2, N315, NCTC8325, Newman, USA300-FPR3757, MRSA252, LGA251), *Staphylococcus pseudintermedius* (HKU10-03, ED99), and some coagulase-negative staphylococci (CoNS), such as *S. carnosus* (TM300), *S. epidermidis* (ATCC 12228, RP62A), *S. haemolyticus* (JCSC1435), and *S. lugdunensis* (N920143, HKU09-01). The *S. aureus* genome is composed of a single chromosome ranging in size from approximately 2.8 to 2.9 Mbp. In comparative genomic analyses, the species' pan-genome (supragenome) was found to comprise 3,221 genes, with 2,245 core genes (8). A wide range of mobile DNA elements have been identified. Most naturally occurring staphylococcal strains contain small multicopy and/or large, conjugative multiresistance plasmids (classes I to III). For more details and other staphylococcal species,

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TABLE 1 Differentiation of members of the genus *Staphylococcus* from other Gram-positive cocci[a]

								Growth on:										Resistance to:			
Genus and exceptional species	G+C content (molecular %) of DNA	Strict aerobe	Facultative anaerobe or microaerophile	Strict anaerobe	Tetrad cell arrangement	Strong adherence on agar	Motility	5% NaCl agar	6.5% NaCl agar	12% NaCl agar	P agar in 18 h[b]	Schleifer-Kramer agar[c]	Catalase reaction result[d]	Benzidine test result[e]	Modified oxidase test result[f]	Anaerobic acid production from glucose[g]	Aerobic acid production from glycerol	Lysostaphin (200 μg/ml)	Erythromycin (0.4 μg/ml)	Bacitracin (0.04 U)[h]	Furazolidone (100 μg)[i]
Staphylococcus spp.	30–39	−	*d*	−	*d*	−[j]	−	+	+	*d*	+	+	+	+	−	*d*	+	−	+	+	−
S. aureus subsp. *anaerobius*		−	±	±	−	−	−	+	+	*d*	−	ND	−	−	−	+	+	−	+	ND	−
S. saccharolyticus		−	±	±	+	−	−	+	+	±	−	ND	−	±	−	+	+	−	+	ND	−
S. hominis		±[k]	±	−	+	−	−	+	+	±	+	+	+	+	−	+	+	−	+	+	−
S. auricularis		−	+	−	+	−	−	+	+	±	−	ND	+	+	−	+	+	−	+	+	−
S. saprophyticus, *S. cohnii*, *S. xylosus*		*d*	*d*	−	−	−	−	+	+	±	+	+	+	+	−	−	+	−	+	+	−
S. kloosii, *S. equorum*, *S. arlettae*		±	±	−	−	−	−	+	+	±	*d*	+	+	+	−	−	+	−	+	+	−
S. intermedius		−	+	−	−	−	−	+	+	+	+	±	+	+	−	+	+	−	+	+	−
S. sciuri, *S. lentus*, *S. vitulinus*		±	±	−	*d*	−	−	+	+	*d*	*d*	+	+	+	+	−	+	−	+	+	−
Macrococcus[l]	38–45	±	±	−	*d*	−	−	+	+	±	*d*	ND	+	+	+	−	*d*	−	+	+	−
Enterococcus	34–42	−	+	−	−	−	*d*	+	+	(±)	±	(±)	−	−	−	+	*d*	+	+	+	−
Streptococcus	34–46	−	+	*d*	−	−	−	*d*	*d*	−	−	−	−	−	−	+	*d*	+	−	*d*	−
Aerococcus	35–40	−	+	−	+	−	−	+	+	+	−	ND	−	−	−	(+)	ND	+	ND	−	−
Planococcus	39–52	+	−	−	*d*	−	+	+	+	+	−	ND	+	+	ND	−	−	+	ND	ND	−
Alloiococcus	44–45	+	−	−	−	−	−	+	+	ND	ND	ND	±	−	−	ND	−	ND	ND	ND	ND
R. mucilaginosa	56–60	−	+	−	*d*	+	−	−	−	−	−	ND	±	+	−	+	*d*	+	ND	−	*d*
Micrococcus	66–75	+	−	−	+	−	−	+	+	*d*	−	−	+	+	+	−	−	+	−[m]	−	+
Kocuria kristinae	67	±	±	−	+	−	−	+	+	±	−	(±)	+	+	+	(+)	+	+	−	−	+

[a] Symbols and abbreviations: +, 90% or more species or strains positive; ±, 90% or more species or strains weakly positive; −, 90% or more species or strains negative; *d*, 11 to 89% of species or strains positive; ND, not determined. Parentheses indicate a delayed reaction.

[b] Growth on P agar is under aerobic conditions at 35 to 37°C. Positive growth is indicated for detectable formation of colonies of at least 1 mm in diameter; ± indicates detectable formation of colonies of between 0.5 and 1 mm in diameter. Growth on sheep or bovine blood agar is slightly greater but less discriminative of staphylococci and other genera.

[c] Growth is under aerobic conditions at 35 to 37°C for 24 to 48 h. Positive growth is indicated for a number of CFU on selective medium comparable to that on plate count agar and a colony of 0.5 mm in diameter; ± indicates a significant reduction in the number of CFU on the selective medium compared to that on plate count agar, and parentheses indicate a colony of pinpoint size to 0.5 mm in diameter.

[d] Sometimes a weak catalase or pseudocatalase reaction can be observed with certain strains of species designated catalase negative. In some species, catalase activity may be activated by hemin supplementation.

[e] Detects the presence of cytochromes. Some strains of benzidine test-negative species can synthesize cytochromes on aerobic media supplemented with hemin (140).

[f] Determined by the modified oxidase test using tetramethyl-*p*-phenylenediamine dihydrochloride-impregnated disks or strips to detect the presence of cytochrome *c* (140).

[g] Standard oxidation/fermentation test.

[h] A disk is used. + indicates resistance and no zone of inhibition. *Micrococcus*, *Kocuria*, *Kytococcus*, *Stomatococcus*, and *Aerococcus* spp. are susceptible and have an inhibition zone of 10 to 25 mm in diameter.

[i] A disk is used. + indicates resistance and no zone of inhibition or a zone of up to 9 mm in diameter. Susceptible species have an inhibition zone of 15 to 35 mm in diameter.

[j] Some strains of *S. epidermidis* adhere tenaciously to the surface of agar, and this property is correlated with heavy slime production.

[k] *S. hominis* does not demonstrate growth in the anaerobic portion of a thioglycolate medium within 24 h and may produce only very poor growth in this portion following 3 to 5 days of incubation. However, it grows and ferments glucose anaerobically (standard oxidation-fermentation test). Failure to grow anaerobically in thioglycolate may be due in part to inhibition by the ingredients.

[l] *Macrococcus* species can also be differentiated from *Staphylococcus* species on the basis of their generally larger Gram-stained cell size (≥2 μm) and larger number of chromosome fragments produced by digestion with NotI (12 to 36 fragments).

[m] A few *Micrococcus* strains demonstrate high-level (MIC, ≥50 μg/ml) erythromycin resistance.

see the NCBI Genome database (http://www.ncbi.nlm.nih.gov/genome).

As of January 2013, 45 species, including 21 subspecies, are recognized in the genus *Staphylococcus* (Table 2). In 1925, the differentiation into coagulase-positive streptococci ("*S. aureus* group") and CoNS based on the detection of the plasma coagulase was introduced (9). This was later followed by a further subclassification of the CoNS into novobiocin-susceptible ("*S. epidermidis* group") and novobiocin-resistant ("*S. saprophyticus* group") species (10, 11). The *S. epidermidis* group comprises, besides the eponym, historically *Staphylococcus capitis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis* subsp. *hominis*, *Staphylococcus lugdunensis*, *Staphylococcus saccharolyticus*, and *Staphylococcus* *warneri;* however, many other species are also novobiocin susceptible (Table 2). In addition to both *S. saprophyticus* subspecies, several CoNS species are novobiocin resistant (e.g., *Staphylococcus cohnii*, *Staphylococcus kloosii*, and *Staphylococcus xylosus*) (Table 2). Despite limitations, coagulase activity and novobiocin susceptibility are still used for presumptive identification of clinical isolates.

The genus *Macrococcus* was established by delimiting the genus *Staphylococcus* through description of *Macrococcus caseolyticus*, the former *Staphylococcus* (*Micrococcus*) *caseolyticus*, and the description of novel species. The macrococcal cells are similar to those of staphylococci, with the exception of their larger cell diameter, reaching 2.5 µm in some species, hence their genus name. Recently, *Macrococcus* was considered to reflect the genome of ancestral bacteria before the speciation of staphylococcal species (12). As shown for the fully sequenced strain JCSC5402, *Macrococcus caseolyticus* has a G+C content of 36.9 mol% (12). In contrast to the cell walls of other macrococci, the cell wall of *M. caseolyticus* contains teichoic acid. With the exception of *Nosocomiicoccus*, the other genera, *Salinicoccus* and *Jeotgalicoccus*, have a G+C content that is generally higher than those of staphylococci. Whereas salinicocci are strictly aerobes, the other genera comprise facultative anaerobes.

Micrococcaceae and *Dermacoccaceae*

Most species of the Gram-positive, non-spore-forming families *Micrococcaceae* and *Dermacoccaceae* are characterized by nonmotile and spherical cells that are 0.5 to 1.8 µm in diameter and arranged in tetrads and clusters. Deviating from this, rod-shaped species are found in some genera. *Arthrobacter* and *Rothia* species may display coccoid forms. Except for *Rothia mucilaginosa*, both genera are discussed in chapter 28. Members of the *Micrococcaceae* and *Dermacoccaceae* have G+C contents within the range of 52 to 75%.

The *Micrococcaceae* family covers mostly strictly aerobic and mesophilic species. The type genus *Micrococcus* contains the classical micrococcal species *M. luteus* and *M. lylae*. Other former *Micrococcus* members are now part of the *Kocuria* genus, such as *K.* (*Micrococcus*) *kristinae*, *K.* (*Micrococcus*) *varians*, and *K. rosea* (formerly *Micrococcus roseus*, now united with the former *Pelczaria aurantia*) (4). The former *Micrococcus halobius* was transferred into the novel genus *Nesterenkonia* as *N. halobia*. For all these genera, several novel species have been described. In this chapter, the term "micrococci" in quotes is used to indicate the members of the genus *Micrococcus* as understood before the emendation, reflecting most of the clinically relevant species (4).

Two of the former "micrococcal" species are now assigned to the *Dermacoccaceae* family, either to the genus *Dermacoccus* containing *D.* (*Micrococcus*) *nishinomiyaensis* and other species or to the genus *Kytococcus*, comprising, besides *K.* (*Micrococcus*) *sedentarius*, the recently described species *K. schroeteri* and *K. aerolatus*.

Alloiococcus

The name of the only species of the genus *Alloiococcus* (family *Carnobacteriaceae*) still standing in nomenclature is *A. otitis* [sic]; however, the proposed emended name, *A. otitidis*, is frequently used in the literature. This slow-growing species is characterized by Gram-positive ovoid cocci, occurring mostly in clusters and pairs. Alloiococci show aerobic atmospheric requirements, but they are able to grow sparsely in a candle jar atmosphere (13). Their G+C content is 44 to 45%.

EPIDEMIOLOGY AND TRANSMISSION

Staphylococcus

The major habitats of most staphylococcal species are the skin and mucous membranes of mammals and birds. *S. aureus* subsp. *aureus* is considered to be the most important human pathogen among staphylococci, followed by *S. epidermidis*, *S. haemolyticus*, *S. saprophyticus* subsp. *saprophyticus*, and *S. lugdunensis*. *S. auricularis*, *S. capitis* subsp. *capitis* and subsp. *urealyticus*, *S. caprae*, *S. cohnii* subsp. *cohnii* and subsp. *urealyticus*, *S. hominis* subsp. *hominis* and subsp. *novobiosepticus*, *S. pasteuri*, *S. pettenkoferi*, *S. saccharolyticus*, *S. schleiferi* subsp. *schleiferi*, *S. simiae*, *S. simulans*, *S. warneri*, and *S. xylosus* are also encountered in human specimens (14). These species are found mainly as part of the resident microbiota. For references concerning species description, see the List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.cict.fr/).

S. aureus and Other Coagulase-Positive Species

The anterior nares (*vestibulum nasi*) are the principal habitat for *S. aureus*. Approximately 50% of people are either permanently or intermittently colonized. As determined in longitudinal studies, three types of *S. aureus* nasal carriers have been historically distinguished: persistent carriers (10 to 35%, carrying one strain over a prolonged period), intermittent carriers (20 to 75%, carrying different strains), and noncarriers (5 to 50%) (15). Since intermittent carriers and noncarriers have similar *S. aureus* nasal elimination kinetics, it was recently proposed that there are only two types of nasal carriers: persistent carriers and others (16). From the *vestibulum nasi*, *S. aureus* can be transferred to skin and other body areas. Whether health care personnel, patients with insulin-dependent diabetes mellitus, patients receiving long-term hemodialysis, and users of illegal intravenous drugs may have higher-than-normal *S. aureus* nasal carriage rates (17–19) is a matter of debate because certain studies do not support earlier findings (20–22). Of particular interest, nasal colonization plays a crucial role as a source of invasive infections (23–26). The throat, the intestine, the vagina, the intertriginous skin folds, the axillae, and the perineum are also found to be regularly colonized.

The population of *S. aureus* strains presents a highly clonal structure. By multilocus sequence typing (MLST), *S. aureus* strains can be grouped in sequence types (STs) (about 2,400 [www.mlst.net]) that can be arranged in clonal complexes (CCs) (27). Common *S. aureus* lineages comprise CCs 1, 5, 8, 15, 22, 30, 45, 59, 80, 97, 121, and 398 (28, 29). The *S. aureus* populations are also divided into four distinct groups based on *agr* (accessory gene regulator system) allelic variation (30).

Within health care facilities, *S. aureus* strains are transmitted from patient to patient primarily via hand carriage of medical personnel but also by means of contaminated objects. This is of utmost importance for the transmission of methicillin-resistant *S. aureus* (MRSA) strains (MRSA and their clonal lineages are further discussed under Antimicrobial Susceptibilities below). The role of the external environment is less important, except in certain areas, such as intensive care units and burn units. Colonized or infected health care personnel may act as reservoirs (31). Until recently, direct or indirect human-to-human transmission has been the primary route for the transmission of both methicillin-susceptible *S. aureus* (MSSA) and MRSA strains, but now animals have become a significant additional reservoir. For companion animals, MRSA infection is primarily a humanosis (i.e., humans infect the animals, which then can transmit the organism back). This is in contrast to the situation with the emerging livestock-associated (LA) MRSA and MSSA strains, such as ST398, for which livestock (especially pigs but also chickens, turkeys, and veal calves) represent a genuine zoonotic risk to humans (32).

Staphylococcus aureus subsp. *anaerobius* has been recovered from subcutaneous abscesses of sheep (33). So far, it has not been isolated from human clinical specimens. *S. intermedius*, *S. pseudintermedius* (delineated in 2005), and two clusters of *S. delphini* are closely related coagulase-positive species commonly recovered from birds, carnivores, and other mammals (e.g., horses and cats). Since it is very difficult to differentiate among these species, it is very likely that previously reported *S. intermedius* isolates with variant biotypes might have included *S. delphini* and, in particular, *S. pseudintermedius* (34). *S. pseudintermedius* might be the predominant coagulase-positive *Staphylococcus* species recovered from normal and infected canine skin (35). Further coagulase-positive animal-adapted staphylococci are *S. schleiferi* subsp. *coagulans*, *S. felis*, and *S. lutrae*. *S. hyicus* is found in pigs, and *S. agnetis* has been reported from bovine milk; both species are coagulase variable.

Coagulase-Negative Staphylococci

In humans, *S. epidermidis* is the most frequently recovered staphylococcal species colonizing the body surface, where it is particularly prevalent on moist areas, such as the axillae, inguinal and perineal areas, anterior nares, and toe webs. *S. auricularis* is part of the healthy human external auditory canal microbiota exclusively colonizing this region; *S. capitis* is found surrounding the sebaceous glands on the forehead and scalp following puberty; *S. haemolyticus* and *S. hominis* are preferentially isolated from axillae and pubic areas high in apocrine glands; and *S. saprophyticus* subsp. *saprophyticus* frequently colonizes the rectum and the genitourinary tract of young women (14, 36, 37). *S. lugdunensis* is frequently found on the lower extremities and the groin (38). However, these species may be found occasionally on other body sites. Some recently described staphylococcal species, such as *S. massiliensis*, *S. pettenkoferi*, and *S. petrasii*, are also likely to be part of the human skin microbiota (39–41).

S. sciuri and *S. xylosus* are commensals of the skin and the mucous membranes of many animals and, occasionally, of humans (14, 42). Both species are also found in food; *S. xylosus* represents one of the major starter cultures used for meat fermentation. *S. sciuri* subsp. *carnaticus* is recovered mainly from bovine hosts, and subsp. *rodentium* is found mainly in rodents. *S. kloosii*, *S. equorum* subsp. *equorum*, and *S. gallinarum* are found on several mammals and food products (43, 44). *S. chromogenes* and *S. lentus* are common residents of cloven-hoofed animals and, in addition, may be isolated from their food products. *S. vitulinus* (synonym, *S. pulvereri*) is found preferentially on horses and whales. *S. arlettae* is found on mammals and birds. *S. nepalensis* has been isolated from Himalayan goats. *S. muscae* has been described on flies. *S. rostri* was isolated from the noses of pigs, *S. devriesei* from bovine udder, *S. microti* from free-living common voles, and *S. stepanovicii* from rodents and mammalian insectivores. *S. carnosus* subsp. *carnosus* and subsp. *utilis*, *S. condimenti*, *S. devriesei*, *S. equorum* subsp. *linens*, *S. fleurettii*, *S. piscifermentans*, and *S. succinus* subsp. *casei* have been associated with fermented food and dairy products, and *S. succinus* subsp. *succinus* was isolated from an amber fragment (45). However, the complete and/or true natural habitat for many of these species is still unclear.

Macrococcus, Salinicoccus, Jeotgalicoccus, and *Nosocomiicoccus*

The *Macrococcus* genus comprises seven hoofed-animal-adapted species, including *M. caseolyticus*, first described as *Staphylococcus caseolyticus* (46). This species is found on food such as sausages and meat products. The halotolerant/halophilic *Jeotgalicoccus* and *Salinicoccus* species are recovered from fermented seafood and salted fish or found in saline and desert soil or salt mines. Thus far, the only reported recovery of *Nosocomiicoccus ampullae* has been isolation from surfaces of bottles of saline solution used in wound cleansing (47).

Micrococcaceae and *Dermacoccaceae*

The skin of humans and other mammals is the primary habitat for most *Micrococcaceae* and *Dermacoccaceae* recovered from clinical specimens. Cutaneous populations of micrococci are carried by most people (ca. 96%), with *M. luteus* being the most frequent species, followed by *Kokuria varians* (48). *Rothia mucilaginosa* is probably a normal inhabitant of the mouth and upper respiratory tract. Animal and dairy products may be considered secondary sources of "micrococci." Many of the recently discovered members of the *Micrococcaceae* and *Dermacoccaceae* are associated with different environmental habitats.

Alloiococcus

A. otitis was detected in the outer ear canal of healthy persons at a high incidence, suggesting that alloiococci are part of the normal bacterial microbiota (49).

CLINICAL SIGNIFICANCE

Staphylococcus

Many staphylococcal species are classical opportunists colonizing skin and mucous membranes but may become pathogenic in a species- and strain-dependent manner following breaks in the cutaneous epithelial barrier through trauma or medical interventions. The recovery of a staphylococcal isolate always requires assessment of clinical significance to determine whether it is a contaminant, colonizer, or pathogen.

S. aureus is the clinically most important species, as it is capable of causing a wide range of human and animal diseases. *S. aureus* possesses an extensive arsenal of often redundant and overlapping virulence factors, such as adhesins, enzymes, and toxins, and has various strategies to evade the host immune response. In addition, the pathogen has become resistant to many of the therapeutic agents available. National Nosocomial Infection Surveillance and National Healthcare Safety Network data indicate that *S. aureus* is the most common cause of nosocomial pneumonia and skin and soft tissue infections (SSTIs). *S. aureus* is second only to CoNS as a cause of primary bacteremia in hospitals (50, 51).

Disease entities caused by *S. aureus* can be broadly divided into toxin-mediated diseases and suppurative infections, comprising SSTIs, organic and systemic infections, and foreign-body-related infections (FBRIs). SSTIs are the most frequent infections in community-associated *S. aureus* infections, including those caused by community-associated MRSA (CA-MRSA). The spectrum of SSTIs ranges from superficial (impetigo, folliculitis, furuncles/carbuncles, hydradenitis suppurativa, pyoderma, and wound infections) to deep entities (abscesses, mastitis, cellulitis, and pyomyositis)

TABLE 2 Differentiation of *Staphylococcus* species

Species	Characteristic[a]																																			
						Expression of:																			Acid production (aerobically) from:											
	Large colonies[b]	Colony pigmentation[c]	Anaerobic growth[d]	Aerobic growth[e]	Coagulase test result	Clumping factor[f]	Heat-stable nuclease	Hemolysins[g]	Catalase[h]	Oxidase[i]	Alkaline phosphatase	Arginine arylamidase	Pyrrolidonyl arylamidase[j]	Ornithine decarboxylase	Urease[j]	β-Glucosidase[j]	β-Glucuronidase[j]	β-Galactosidase[j]	Arginine utilization[j]	Acetoin production	Nitrate reduction	Esculin hydrolysis	Novobiocin resistance[k]	Polymyxin B resistance[l]	D-Trehalose	D-Mannitol	D-Mannose	D-Turanose	D-Xylose	D-Cellobiose	L-Arabinose	Maltose	α-Lactose	Sucrose	N-Acetylglucosamine	Raffinose
S. agnetis	+	−	+	+	*d*	−	ND	−	+	−	−	−	−	−	−	ND	*d*	−	ND	−	+	−	−	+	*d*	−	+	−	−	−	−	−	+	+	+	−
S. arlettae	*d*	+	−	+	−	−	−	−	+	−	(+)	−	−	−	−	ND	+	*d*	−	ND	−	−	+	ND	+	+	+	+	+	−	+	+	+	+	−	+
S. aureus subsp. *anaerobius*	−	−	(+)	(±)	+	−	+	+	−	−	+	ND	ND	ND	ND	−	−	−	ND	−	−	−	−	ND	−	ND	−	ND	−	−	−	+	−	+	−	−
S. aureus subsp. *aureus*	+	+	+	+	+	+	+	+	+	−	+	−	−	−	*d*	+	−	−	+	+	+	−	−	+	+	+	+	+	−	−	−	+	+	+	+	−
S. auricularis	−	−	(±)	(+)	−	−	−	−	+	−	−	+	*d*	−	−	−	−	(*d*)	*d*	−	(*d*)	−	−	−	(+)	−	−	(*d*)	−	−	−	(+)	−	*d*	−	−
S. capitis subsp. *capitis*	−	−	(+)	+	−	−	−	(*d*)	+	−	−	−	−	−	−	−	−	−	*d*	*d*	*d*	−	−	−	−	+	+	−	−	−	−	−	−	(+)	−	−
S. capitis subsp. *urealyticus*	−	(*d*)	(+)	+	−	−	−	(*d*)	+	−	−	−	(*d*)	−	+	−	−	−	+	*d*	+	−	−	ND	−	+	+	−	−	−	−	+	(*d*)	+	−	−
S. caprae	*d*	−	(+)	+	−	−	−	(*d*)	+	−	(+)	−	*d*	−	+	−	−	−	+	+	+	−	−	−	(+)	*d*	+	−	−	−	−	(*d*)	+	−	−	−
S. carnosus subsp. *carnosus*	+	−	+	+	−	−	−	−	+	−	+	−	+	−	−	−	−	+	+	+	+	−	−	−	*d*	+	+	−	−	−	−	−	*d*	−	ND	−
S. carnosus subsp. *utilis*	−	−	+	+	−	ND	ND	ND	+	ND	−	ND	ND	ND	−	ND	−	−	+	ND	*d*	−	ND	ND	*d*	−	−	−	ND	−	−	−	−	−	ND	−
S. cohnii subsp. *cohnii*	*d*	−	*d*	+	−	−	−	(*d*)	+	−	−	−	−	−	−	−	−	−	−	*d*	−	−	+	−	+	*d*	(*d*)	−	−	−	−	(*d*)	−	−	−	−
S. cohnii subsp. *urealyticus*	+	*d*	(+)	+	−	−	−	(*d*)	+	−	+	−	*d*	−	+	−	+	+	−	*d*	−	−	+	−	+	+	+	−	−	−	−	(+)	+	−	*d*	−
S. chromogenes	+	+	+	+	−	−	−	−	+	−	+	−	*d*	−	+	*d*	−	−	+	−	+	−	−	+	+	*d*	+	*d*	−	−	−	*d*	+	+	*d*	−
S. condimenti	ND	*d*	+	+	−	ND	ND	ND	+	ND	+	ND	ND	ND	+	ND	−	+	+	ND	+	−	ND	ND	+	+	+	−	ND	−	−	−	+	±	ND	−
S. delphini	+	−	(+)	+	+	−	−	+	+	−	+	ND	ND	ND	+	ND	ND	ND	+	−	+	ND	−	ND	−	(+)	+	ND	−	ND	−	+	+	+	ND	ND
S. devriesei	+	+	(+)	+	−	−	ND	+	+	−	−	ND	ND	ND	*d*	+	−	−	ND	ND	*d*	±	−	ND	+	*d*	*d*	+	−	−	−	+	+	+	*d*	−
S. epidermidis	−	−	+	+	−	−	−	(*d*)	+	−	+[m]	−	−	(*d*)	+	(*d*)	−	−	*d*	+	+	−	−	+	−	−	(+)	(*d*)	−	−	−	+	*d*	+	−	−
S. equorum subsp. *equorum*	−	−	−	(+)	−	−	−	(*d*)	+	−	(+)	−	−	−	+	ND	+	*d*	−	−	+	*d*	+	ND	+	+	+	*d*	+	(*d*)	+	*d*	*d*	+	*d*	−
S. equorum subsp. *linens*	ND	−	−	(+)	−	−	−	(*d*)	+	−	*d*	−	−	−	+	ND	+	−	ND	−	+	−	−	ND	−	ND	+	±	−	−	−	−	−	−	ND	ND
S. felis	+	−	+	+	−	−	−	(*d*)	+	−	+	ND	ND	ND	+	−	−	+	+	−	+	ND	−	ND	+	+	+	ND	−	−	−	−	+	*d*	+	−
S. fleurettii	−	−	+	+	−	−	−	ND	+	+	*d*	−	−	−	−	ND	−	−	−	*d*	+	*d*	+	ND	+	ND	+	+	*d*	−	*d*	+	−	+	ND	−
S. gallinarum	+	*d*	(+)	+	−	−	−	(*d*)	+	−	(+)	−	−	−	+	+	*d*	*d*	−	−	+	+	+	−	+	+	+	+	+	+	+	+	*d*	+	+	+
S. haemolyticus	+	*d*	(+)	+	−	−	−	(+)	+	−	−	−	+	−	−	*d*	*d*	−	+	+	+	−	−	−	+	*d*	−	(*d*)	−	−	−	+	*d*	+	+	−
S. hominis subsp. *hominis*	−	*d*	−	+	−	−	−	−	+	−	−	−	−	−	+	−	−	−	*d*	*d*	*d*	−	−	−	*d*	−	−	+	−	−	−	+	*d*	(+)	*d*	−
S. hominis subsp. *novobiosepticus*[n]	−	−	−	+	−	−	−	−	+	−	−	ND	−	−	+	−	−	−	−	*d*	*d*	−	+	ND	−	−	−	ND	−	−	−	+	*d*	(+)	−	−
S. hyicus	+	−	+	+	*d*	−	+	−	+	−	+	−	−	−	*d*	*d*	+	−	+	−	+	−	−	+	+	−	+	−	−	−	−	−	+	+	+	−
S. intermedius	+	−	(+)	+	+	*d*	+	*d*	+	−	+	−	+	−	+	*d*	−	+	*d*	−	+	−	−	−	+	(*d*)	+	*d*	−	−	−	(±)	*d*	+	+	−
S. kloosii	*d*	*d*	−	+	−	−	−	(*d*)	+	−	*d*	−	*d*	−	*d*	*d*	*d*	*d*	−	*d*	−	*d*	+	−	+	+	−	−	(*d*)	−	*d*	*d*	(*d*)	(±)	−	−
S. lentus	−	*d*	(±)	(+)	−	−	−	−	+	+	(±)	−	−	−	−	+	−	−	−	−	+	+	+	−	+	+	(+)	(±)	(±)	+	*d*	*d*	*d*	+	*d*	+
S. lugdunensis	*d*	*d*	+	+	−	(+)	−	(+)	+	−	−	−	+	+	*d*	+	−	−	−	+	+	−	−	*d*	+	−	+	(*d*)	−	−	−	+	+	+	+	−
S. lutrae	−	−	+	+	+	−	(±)	+	+	−	+	ND	ND	ND	+	ND	ND	+	−	−	+	ND	−	ND	+	*d*	+	ND	+	ND	ND	+	+	ND	ND	ND
S. massiliensis	+	−	±	+	−	−	ND	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	ND	−	−	−	−	−	−	−	−	−	−	−	−
S. microti	+	−	ND	+	−	−	ND	+	+	−	+	+	+	−	−	−	−	+	ND	±	+	+	+	−	+	−	±	−	+	−	−	−	+	+	+	−
S. muscae	*d*	−	+	+	−	−	−	(+)	+	−	+	ND	ND	−	−	ND	ND	−	−	−	ND	ND	−	ND	+	−	−	+	+	−	−	−	−	+	ND	−
S. nepalensis	−	−	+	+	−	−	−	−	+	−	+	−	+	−	+	ND	+	+	−	−	+	+	+	−	+	+	+	*d*	+	−	+	+	+	+	+	−

S. *pasteuri*	*d*	*d*	+	+	−	−	−	(*d*)	+	−	−	−	−	−	+	+	+	−	*d*	*d*	*d*	−	−	ND	+	*d*	−	(*d*)	−	−	−	(*d*)	*d*	+	ND	−
S. *pettenkoferi*	ND	*d*	+	+	−	−	ND	ND	+	−	+	−	+	−	+	ND	−	−	ND	−	+	ND	−	+	−	−	−	−	−	−	−	−	−	+	ND	−
S. *piscifermentans*	ND	*d*	+	+	−	ND	−	−	+	−	+	ND	ND	ND	+	+	−	−	+	−	+	+	−	ND	+	*d*	−	−	−	−	−	*d*	*d*	*d*	ND	−
S. *pseudintermedius*	+	−	ND	+	+	−	+	+	+	ND	+[o]	ND	+	ND	+	+	−	+	ND	+	+	ND	−	ND	+	(+)	+	(+)	−	−	−	+	+	+	+	−
S. *rostri*	+	−	+	+	−	−	−	+	+	−	+	−	+	−	−	ND	−	−	−	−	+	±	−	ND	+	−	−	−	ND	−	−	−	d	+	+	−
S. *saccharolyticus*	−	−	+	(±)	−	−	−	−	−	−	*d*	−	ND	ND	ND	ND	ND	ND	+	ND	+	ND	−	ND	−	−	(+)	ND	−	−	−	−	−	−	ND	−
S. *saprophyticus* subsp. *bovis*	−	+	+	+	−	−	−	−	+	−	−	−	+	−	+	*d*	−	*d*	−	*d*	+	−	+	ND	+	+	−	+	−	−	−	+	−	+	+	−
S. *saprophyticus* subsp. *saprophyticus*	+	*d*	(+)	+	−	−	−	−	+	−	−	−	−	−	+	*d*	−	+	−	+	−	−	+	−	+	*d*	−	+	−	−	−	+	*d*	+	*d*	−
S. *schleiferi* subsp. *coagulans*	*d*	−	+	+	+	−	+	(+)	+	−	+	ND	ND	ND	+	ND	ND	ND	+	+	+	ND	−	ND	−	*d*	+	ND	−	−	−	−	*d*	*d*	ND	−
S. *schleiferi* subsp. *schleiferi*	−	−	+	+	−	+	+	(+)	+	−	+	−	+	−	−	−	−	(+)	+	+	+	−	−	−	*d*	−	+	−	−	−	−	−	−	−	(+)	−
S. *sciuri* subsp. *carnaticus*	−	*d*	(*d*)	+	−	*d*[p]	−	(±)	+	+	*d*	−	−	−	−	+	−	−	−	−	+	+	+	−	+	+	(*d*)	ND	+	(*d*)	*d*	(*d*)	(*d*)	+	−	−
S. *sciuri* subsp. *rodentium*	*d*	*d*	(*d*)	+	−	+[p]	−	(±)	+	+	*d*	−	−	−	−	+	−	−	−	−	+	+	+	−	(+)	+	(+)	ND	(*d*)	*d*	(*d*)	(*d*)	−	+	−	−
S. *sciuri* subsp. *sciuri*	+	*d*	(+)	+	−	−	−	(±)	+	+	+	−	−	−	−	+	−	−	−	−	+	+	+	−	+	+	(*d*)	(±)	(*d*)	+	*d*	(*d*)	(*d*)	+	−	−
S. *simiae*	ND	−	+	+	−	−	−	ND	+	−	+	−	−	−	+	ND	−	−	ND	−	+	−	−	+	+	+	−	+	*d*	−	*d*	+	+	+	(+)	−
S. *simulans*	+	−	+	+	−	−	−	(*d*)	+	−	(*d*)	−	+	−	+	−	*d*	+	+	*d*	+	−	−	−	*d*	+	*d*	−	−	−	−	(±)	+	+	+	−
S. *stepanovicii*	+	−	ND	+	−	−	ND	ND	+	+	−	−	−	−	+	ND	−	−	ND	−	+	−	+	+	+	+	+	−	ND	−	−	+/±	−	+	−	−
S. *succinus* subsp. *casei*	ND	−	−	(+)	ND	ND	ND	−	+	−	±	−	−	−	+	ND	+	+	ND	−	+	+	+	ND	+	+	+	+	±	−	−	+	+	+	ND	−
S. *succinus* subsp. *succinus*	ND	−	−	(+)	ND	ND	ND	−	+	−	+	−	−	−	+	ND	+	+	ND	−	−	+	+	ND	+	+	*d*	±	−	±	−	+	+	+	ND	−
S. *vitulinus*	−	+	±	+	−	−	−	±	+	+	−	ND	−	−	−	*d*	−	−	−	−	+	*d*	+	ND	(*d*)	−	−	−	*d*	*d*	−	−	−	+	−	−
S. *warneri*	*d*	*d*	+	+	−	−	−	(*d*)	+	−	−	−	−	−	+	+	*d*	−	*d*	+	*d*	−	−	−	+	*d*	−	(*d*)	−	−	−	(+)	*d*	+	−	−
S. *xylosus*	+	*d*	*d*	+	−	−	−	−	+	−	*d*	−	*d*	−	+	+	+	+	−	*d*	*d*	*d*	+	−	+	+	+	*d*	+	−	*d*	+	*d*	+	+	−

[a]Symbols and abbreviations (unless otherwise indicated): +, 90% or more strains positive; ±, 90% or more strains weakly positive; −, 90% or more strains negative; *d*, 11 to 89% of strains positive; ND, not determined. Parentheses indicate a delayed reaction.

[b]Positive is defined as a colony diameter of ≥6 mm after incubation on P agar at 34 to 35°C for 3 days and at room temperature (ca. 25°C) for an additional 2 days; exceptions are *S. succinus* (4 to 6 mm on tryptic soy agar) and *S. fleurettii* (8 to 12 mm on tryptic soy agar).

[c]Positive is defined by the visual detection of carotenoid pigments (e.g., yellow, yellow-orange, or orange) during colony development at normal incubation or room temperatures. Pigments may be enhanced by the addition of milk, fat, glycerol monoacetate, or soaps to P agar.

[d]Growth is in a semisolid thioglycolate medium. Symbols: +, moderate or heavy growth down the tube within 18 to 24 h; ±, heavier growth in the upper portion of the tube and weaker growth in the lower, anaerobic portion of tube; −, no visible growth within 48 h but very weak diffuse growth or a few scattered, small colonies may be observed in the lower portion of the tube by 72 to 96 h. Parentheses indicate delayed growth appearing within 24 to 72 h, sometimes noted as large, discrete colonies in the lower portion of the tube.

[e]Growth is on P agar or bovine, sheep, or human blood agar at 34 to 37°C. The subspecies of *S. equorum* and *S. succinus* grow slowly at 35 to 37°C; the optimum growth temperatures of *S. equorum* subsp. *equorum* and *S. equorum* subsp. *linens* are 30°C and 32°C, respectively, and those of *S. succinus* subsp. *succinus* and of *S. succinus* subsp. *casei* are 28°C and 32°C, respectively. Anaerobic species *S. saccharolyticus* and *S. aureus* subsp. *anaerobius* grow very slowly in the presence of air. *S. aureus* subsp. *anaerobius* requires the addition of blood, serum, or egg yolk for growth on primary isolation medium. *S. auricularis*, *S. lentus*, and *S. vitulinus* produce just-detectable colonies on P agar in 24 to 36 h, and these colonies remain very small (1 to 2 mm in diameter).

[f]The slide agglutination test using rabbit or human plasma detects the expression of clumping factor. Use human plasma for *S. lugdunensis* and *S. schleiferi*. Latex agglutination is less reliable for the detection of clumping factor in *S. lugdunensis*.

[g]Hemolysis on bovine blood agar. Symbols and abbreviations: +, wide zone of hemolysis within 24 to 36 h; (+), delayed moderate to wide zone of hemolysis within 48 to 72 h; (*d*), no or delayed hemolysis; −, no or only very narrow (1-mm) zone of hemolysis within 72 h. Some strains designated negative may produce a slight greening or browning of blood agar. Analysis of hemolysis for both *S. succinus* subspecies was performed on Wilkins-Chalgren anaerobe agar plates containing 5% sheep blood.

[h]Catalase and cytochrome synthesis cannot be induced in *S. aureus* subsp. *anaerobius* by the addition of H_2O_2 or hemin to the culture medium. Catalase activity can be induced in *S. saccharolyticus* by hemin supplementation. In this species, cytochromes *a* and *b* are present in small quantities.

[i]Determined by the modified oxidase test to detect the presence of cytochrome *c* (140).

[j]Determined primarily by commercial rapid identification tests (see the text).

[k]Positive (resistant) is defined by an MIC of ≥1.6 μg/ml or a growth inhibition zone diameter of ≥16 mm with a 5-μg novobiocin disk.

[l]Positive is defined by a growth inhibition zone diameter of <10 mm with a 300-U polymyxin B disk.

[m]Approximately 6 to 15% of strains of *S. epidermidis* are negative for alkaline phosphatase activity, depending on the population sampled. A low but significant number of clinical isolates are phosphatase negative.

[n]All strains tested are also resistant to penicillin G, methicillin, oxacillin, gentamicin, and streptomycin.

[o]Positive with the STAPH-ZYM tests but negative with the API STAPH tests (35).

[p]Positive reactions occur with the Staph latex agglutination test (Remel) that detects clumping factor and/or protein A.

to acute life-threatening necrotizing fasciitis and myositis. Infection of deep sites may involve any body compartments and organ systems resulting in empyemas, osteomyelitis, arthritis, endocarditis, pneumonia, otitis media, sinusitis, mastoiditis, and parotitis. Any localized *S. aureus* infection can become invasive and lead to bacteremia. Systemic infections comprise primary and secondary bacteremia, meningitis, and endocarditis. Bacteremia may be complicated by metastatic foci (e.g., vertebral osteomyelitis). Congenital or acquired defects in host defense and the presence of foreign bodies may predispose patients to serious infections.

Besides an acute, aggressive course of infection, *S. aureus* may cause chronic, persistent, and relapsing infections often due to a phenotypic subpopulation designated small-colony variants (SCVs), which present adapted phenotypes for intracellular persistence (52, 53). Rapid switching between the normal and the SCV phenotype seems to be a highly dynamic process in both directions for adaptation to intra- and extracellular conditions, respectively; this phenotype switching also challenges accurate detection in the routine laboratory (see below) (54–56). SCVs of *S. aureus* and other staphylococcal species (e.g., *S. epidermidis* and *S. lugdunensis*) have been isolated from patients with chronic osteomyelitis, abscesses, and FBRIs as well as from cystic fibrosis patients with chronic airway infection (57–59).

Classical toxin-mediated diseases due to *S. aureus* include staphylococcal toxic shock syndrome (TSS), staphylococcal food poisoning, and staphylococcal scalded skin syndrome (SSSS). TSS is associated with colonization by or infection with an isolate of *S. aureus* that is positive for TSS toxin-1 (TSST-1) or, less frequently, for other members of the staphylococcal pyrogenic toxin superantigen (PTSAg) family (primarily staphylococcal enterotoxin B or C). TSS is diagnosed on clinical grounds and is characterized by high fever, rapid-onset hypotension, a diffuse erythematous rash that becomes desquamating 1 to 2 weeks after onset, and involvement of three or more organ systems. After its initial description in children, it was associated with menstruating women who were using highly absorbent tampons. While the incidence of menstrual TSS decreased due to changes in the tampons' absorbency and chemical composition, the frequency of nonmenstrual TSS entities has remained constant since the 1980s (60). Even though normally no source of infection is confirmed, nonmenstrual TSS is usually associated with focal postoperative wound or soft tissue infections.

Staphylococcal food poisoning is caused by consumption of food contaminated with one or more preformed, relatively heat-stable enterotoxins. Nausea, vomiting, abdominal cramps, and diarrhea occur 2 to 6 h after food ingestion. Symptoms usually subside 8 to 12 h later.

Blisters in bullous impetigo and SSSS are caused by exfoliative (epidermolytic) toxins (ETA and ETB). SSSS is typically found in neonates and young children (61). In addition to severe exfoliation affecting up to 90% or more of the entire body surface (Ritter's disease), a localized form (*pemphigus neonatorum*) with a few blisters is known. Diagnosis is made on the basis of clinical features, including Nikolsky's sign, in which the skin wrinkles on gentle pressure.

The other coagulase-positive or -variable staphylococci are members of the skin microbiotas of various animal species and occasionally cause infections in their hosts. The members of the *S. intermedius*/*S. pseudintermedius*/*S. delphini* cluster are the most common etiologic agents of the canine pyoderma. *S. hyicus* is predominantly associated with the exudative epidermitis (greasy pig syndrome) in pigs, *S. schleiferi* subsp. *coagulans* is found in dogs suffering from external otitis, and *S. aureus* subsp. *anaerobius* is the etiological agent of abscess disease, a specific lymphadenitis of sheep and goats. In humans, *S. intermedius*/*S. pseudintermedius* appears to be occasionally responsible for canine-inflicted wound infections, FBRIs, food poisoning, and invasive infections in immunocompromised patients (62, 63). Only a few human infections due to *S. schleiferi* subsp. *coagulans* and *S. hyicus* are known. For *S. agnetis*, isolates have been recovered from bovine intramammary infections (64).

Since 1980, CoNS have been increasingly recognized as nosocomial pathogens, especially *S. epidermidis*. *S. epidermidis* and *S. haemolyticus* are often referred to as "medium-pathogenicity" staphylococci, as they cause nosocomial infections mainly in patients with predisposing factors, such as immunodeficiency and/or the presence of indwelling or implanted foreign polymer bodies (51, 65–67). Also, *S. lugdunensis* and *S. saprophyticus* may be categorized into this group. CoNS are less often implicated as the cause of infections of natural tissue. Overall, the clinical appearance of infections caused by CoNS has been characterized as subtle (associated with subacute or chronic clinical courses without fulminant signs), and they are seldom life-threatening (68). CoNS are the most common cause of nosocomial bloodstream infections typically associated with central and peripheral intravascular catheters (50). Most important in the pathogenesis of FBRIs is the ability of CoNS to colonize the surface of a device by the formation of a thick, multilayered biofilm (69). *S. epidermidis* is the predominant cause of infections associated with prosthetic vascular grafts, prosthetic orthopedic devices, and cerebrospinal fluid shunts. CoNS are frequently isolated as causative agents of prosthetic-valve endocarditis; rarely (ca. 5% of the time), they are involved in infections of (previously damaged) native valves (70). Right-sided native-valve endocarditis is observed in intravenous drug abusers. Virtually any other surgically inserted materials and devices may become infected by CoNS. CoNS also account for 45 to 75% of all late-onset bloodstream infections in preterm and low-birth-weight neonates in neonatal intensive care units (71).

Of all CoNS, *S. lugdunensis* holds a special position because its infections generally resemble those caused by *S. aureus* rather than those caused by other CoNS. It is especially renowned for causing unusually fulminant cases of native-valve endocarditis in addition to prosthetic-valve endocarditis (72). Thus, patients with *S. lugdunensis* bacteremia should be carefully examined for signs of endocarditis. Besides causing other invasive infections, this organism is a common pathogen involved in FBRIs (59). In addition, *S. lugdunensis* is not a rare cause of skin abscesses and wound infections, especially below the waist (73).

Based on special urotropic and ecologic characteristics, *S. saprophyticus* subsp. *saprophyticus* is a well-documented causative agent of acute, commonly recurrent, urinary tract infections in young, otherwise healthy, sexually active women and, less frequently, in young men or boys. This pathogen is the second-most-common (after *Escherichia coli*) cause of uncomplicated cystitis among young women. While colony counts of ≥100,000 CFU/ml in two or more cultures of midstream urine usually indicate significant bacteriuria; lower counts may be significant in the symptomatic patient. Infections due to the recently described subspecies *S. saprophyticus* subsp. *bovis* have not been reported.

For various other CoNS species (e.g., *S. capitis*, *S. chromogenes* *S. cohnii*, *S. hominis*, *S. pasteuri*, *S. pettenkoferi*, *S. warneri*, and *S. xylosus*), case reports and series predominantly reporting on FBRIs have been published. In contrast, *S. carnosus*, *S. condimenti*, *S. piscifermentans*, *S. equorum* subsp. *linens*, and other staphylococcal species involved mainly in food production have been regarded as nonpathogenic staphylococci. Since human infections due to *Macrococcus*, *Jeotgalicoccus*, *Nosocomiicoccus*, and *Salinicoccus* have not been described, these genera are not discussed further in this chapter.

Micrococcaceae and *Dermacoccaceae*

While "micrococci" are generally acknowledged as harmless saprophytes, they can also act as opportunistic pathogens. *Micrococcus*, *Kocuria*, and *Kytococcus* species have been found to cause infections such as endocarditis, pneumonia, and sepsis or FBRIs predominantly in immunocompromised patients (74–76). Recovery of the more recently described "micrococcal" species associated primarily with the environment must be assessed for clinical significance, as reported for *K. rhizophila* (77).

R. mucilaginosa has been implicated in cases of bacteremia, endocarditis, endophthalmitis, intravascular catheter-related and central nervous system infections, pneumonia, peritonitis, septicemia, and cervical necrotizing fasciitis (78–80).

Since human infections due to *Acaricomes*, *Citricoccus*, *Luteipulveratus*, *Nesterenkonia*, *Renibacterium*, *Sinomonas*, *Yimella*, and *Zhihengliuella* have not been reported, these genera are not discussed further in this chapter.

Alloiococcus

A. otitis has been associated with infections of the middle ear (81). While its immunostimulatory capacity suggests that *A. otitis* has pathogenic potential (82), other studies revealed that *A. otitis* may be a commensal rather than a cause of otitis media (49).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

The general principles of collection, transport, and storage of specimens as given in chapter 18 of this *Manual* are applicable to the microorganisms listed in this chapter. No special methods or precautions are usually required for these organisms because they are easily obtained from clinical material of most infection sites and are relatively resistant to drying and to moderate temperature changes. While some staphylococcal species may require anaerobic conditions or CO_2 supplementation for satisfactory growth, they survive transport and limited storage in air.

DIRECT EXAMINATION

Microscopy

Direct microscopic examination of normally sterile fluids, such as cerebrospinal fluid and joint aspirates, may be helpful. For nonsterile fluids, microscopy may also be useful; however, the presence of inflammatory cells versus epithelial cells has to be taken into consideration.

Cells of microorganisms discussed here are Gram-positive, nonmotile, non-spore-forming cocci that are arranged mostly in pairs and tetrads but also occur singly, in irregular (grape-like) clusters, or in short chains (three or four cells). However, within the *Micrococcaceae* and *Dermacoccaceae*, some species exhibit rod-shaped cells and have been shown to be motile. Positive findings of direct microscopic examination should be reported as "Gram-positive cocci resembling staphylococci."

Antigen Detection

Direct antigen detection plays no role in the detection of catalase-positive cocci.

Nucleic Acid Detection

Rapid amplification-based approaches have been introduced for the detection of MRSA directly from surveillance swabs. The multiple-locus approach, which detects the *mecA* gene and additionally an *S. aureus*-specific target (see "Species Identification by Nucleic Acid-Based Approaches" below), may be influenced by the coexistence of MSSA and methicillin-resistant CoNS (MR-CoNS) in a patient's physiological microbiota and thus may lead to false-positive MRSA findings (83). Nevertheless, the fundamental advantage of this approach is the direct detection of the methicillin resistance-encoding *mecA* gene and, if additionally targeted, its diagnostically relevant, recently detected homolog, *mecC*. Tests applying this principle, such as hyplex *Staphylo*Resist (plus) (BAG Health Care, Lich, Germany) and the StaphPlex panel (Qiagen), are commercially available. Some *mecA*-targeting assays, such as hyplex MRSA plus (Amplex Diagnostics, Gars-Bahnhof, Germany), the BD MAX StaphSR test (BD Diagnostics, Sparks, MD), a MRSA/SA ELITe MGB kit (Nanogen Advanced Diagnostics, Italy), and the RIDA Gene MRSA system (R-Biopharm, Darmstadt, Germany) have already been refined by including *mecC* as a target (84).

The alternative single-locus amplification strategy overcomes the MSSA/MR-CoNS coexistence drawback by using oligonucleotide primers that bind the right extremity of the staphylococcal cassette chromosome (SCC*mec*) and the neighboring *orfX* region of the *S. aureus* chromosome. This approach amplifyies both a taxonomic marker and a resistance marker in one step (85). This principle is the basis for several rapid test systems (e.g., BD GeneOhm MRSA assay [BD, Franklin Lakes, NJ]; GenoType MRSA Direct, GenoQuick MRSA, and FluoroType MRSA [Hain Lifescience]; Xpert MRSA [Cepheid, Sunnyvale, CA]; and LightCycler MRSA advanced test [Roche, Basel, Switzerland]). However, the use of the SCC*mec* region as a surrogate marker instead of direct detection of *mecA* may lead to false-negative results due to the high variability of the SCC*mec* primer binding sites, a situation that is particularly problematic in the case of rare or still-unknown SCC*mec* (sub)types. On the other hand, false-positive results may occur due to the replacement of the *mecA* gene within the cassette by other genes (e.g., resistance or capsule-encoding genes) and, particularly, by partial excision of the cassette, leading to remnants that lack the *mec* gene complex but still contain the primer binding sites on the 3′ end of the SCC cassette ("drop-out " isolates) (86–89). The Detect-Ready MRSA panel (Molecular Detection Inc., Wayne, PA) combines the detection of an *S. aureus*-specific gene target, the SCC*mec*::*orfX* junction, and the *mecA* gene. The same holds true for the BD MAX StaphSR nasal kit, which also targets *mecC* and is designed to detect both MRSA and MSSA.

Overall, these amplification-based rapid MRSA screening assays, not designed to identify MRSA or MSSA infections, are characterized by very good negative predictive values (approximately 97 to 99%) but are hampered by moderate positive predictive values (approximately 65 to 95%). To date, MRSA cultures remain essential for confirming molecular results, for typing purposes, and for determination of the complete susceptibility profile.

The Xpert MRSA/SA SSTI test (Cepheid) was launched to detect the presence of *S. aureus* or MRSA in skin and soft tissue infections; however, it has been applied in a preclinical evaluation to lower respiratory tract secretions (90) and to perioperative bone and joint samples (91).

ISOLATION PROCEDURES

Considering the widespread distribution of staphylococci and "micrococci" on the skin and mucous membranes, careful procedures should be used to isolate organisms from the presumed focus of infection without collecting surrounding microbiota. The basic procedures for culture and isolation described in chapters 18 and 20 of this *Manual* should be followed.

The primary culture plate used for the isolation of staphylococci from clinical specimens is Columbia or tryptic soy blood agar containing 5% defibrinated sheep (or horse) blood (see also chapter 19). The abundant growth of most staphylococcal species occurs within 18 to 24 h. The simultaneous use of an enrichment broth (i.e., dextrose broth) streaked after 24 and 48 h on Columbia blood agar may enhance the recovery rate of *S. aureus* and other staphylococci.

The use of selective agars for *S. aureus*, such as mannitol salt agar, egg yolk-tellurite pyruvate-containing Baird-Parker medium, Columbia colistin-nalidixic acid agar, lipase-salt-mannitol agar (Remel, Lenexa, KS), and phenylethyl alcohol agar, may be appropriate for specimens from heavily contaminated sources, such as feces and respiratory cultures from patients with cystic fibrosis. It is mandatory to confirm putative *S. aureus* isolates recovered on these media.

Various selective agars using chromogenic enzyme substrates specifically for *S. aureus*, such as CHROMagar Staph aureus (CHROMagar, Paris, France), BBL CHROMagar Staph aureus (BD Diagnostics, Sparks, MD), Brilliance Staph 24 (Oxoid, Cambridge, United Kingdom), and chromID S. aureus (bioMérieux, La Balme Les Grottes, France), are commercially available with chromogen-dependent coloration of the colonies. In particular, for screening purposes, the chromogenic agars have been proven to be suitably sensitive and specific, allowing a presumptive but not final identification (92). Chromogenic agars designed for MRSA detection are discussed in Antimicrobial Susceptibilities below.

The diagnosis of catheter-related bloodstream infections by CoNS and other organisms remains a major challenge. One of the most frequently studied diagnostic techniques is represented by the semiquantitative roll plate catheter culture. With this technique, the distal segment of the central venous catheter is cut and rolled across the surface of a Columbia blood agar plate at least four times, followed by overnight incubation. A colony count of 15 CFU/ml or more may indicate catheter colonization (93). Examination of paired quantitative blood cultures drawn simultaneously from the catheter and a peripheral vein enhanced by the analysis of differential time to positivity represents an example of an approach that does not require catheter removal (94) (see also Evaluation, Interpretation, and Reporting of Results below).

Cultivation of "micrococci" and *R. mucilaginosa* should be performed as described for staphylococci on Columbia blood agar at 35°C to 37°C under aerobic conditions. However, abundant growth of *Micrococcaceae* and *Dermacoccaceae* needs consistent incubation times of 36 to 48 h.

Because of the low growth rate, it is difficult to isolate *A. otitis* by conventional nonselective culture methods (95). Blood agar plates with 6% NaCl were shown to be useful.

IDENTIFICATION

The basic criteria distinguishing catalase-positive Gram-positive cocci and their relatives from among themselves and from other microbial taxa are given in Description of the Families above and in Table 1. Misidentification is likely to occur if automated test system results are accepted without critical review by skilled lab personnel. In specialized settings, species can also be identified by chemotaxonomic procedures. Nucleic acid-based molecular methods have become standard in particular for verification of ambiguous identification results. The recent introduction of matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) instruments in clinical microbiology laboratories has the potential to accelerate and ease the routine identification of staphylococci and other catalase-positive cocci.

Staphylococcus **and Related Genera**

Staphylococcus species can be identified phenotypically on the basis of a variety of conventional characteristics (Tables 1 to 3). The most clinically significant species in human and veterinary medicine can be identified on the basis of several key characteristics (Table 3). The application of the extensive scheme originally published by Kloos and Schleifer in 1975 (96) has been mostly replaced by the use of commercial identification systems.

Colony Morphology

Most staphylococcal colonies are 1 to 3 mm in diameter within 24 h and 3 to 8 mm in diameter after 72 h of incubation in air at 34 to 37°C. Exceptions are those of *S. aureus* subsp. *anaerobius*, *S. saccharolyticus*, *S. auricularis*, *S. equorum*, *S. vitulinus*, and *S. lentus*, which grow more slowly and usually require 24 to 36 h for detectable colony development. Further incubation of agar plates for a period of up to 48 to 72 h (optimally followed by a 2-day incubation at room temperature) enhances morphologic differences.

On routine blood agar, the typical *S. aureus* colony is pigmented (gray/gray-white with a yellowish tint through yellow to orange), smooth, entire, slightly raised, and hemolytic (Fig. 1). Mucoid colonies due to highly encapsulated strains are rarely encountered. A number of isolates of *S. aureus* as well as some CoNS species (e.g., *S. haemolyticus* and *S. lugdunensis*) may have a hazy or distinct zone of beta-hemolysis around the colonies, ranging from weak to strong. SCVs of *S. aureus* or other staphylococcal species are characterized by pinpoint colonies (1/10 the size of the wild type), mostly nonpigmented and nonhemolytic after 24 to 72 h of incubation (Fig. 1) (52, 97). SCVs are often mixed with colonies displaying the normal phenotype, thus giving the appearance of a mixed culture. Upon subculture, they may remain stable or revert to the wild type. Depending on their auxotrophy, normal growth may be restored if the isolate is grown in the presence of hemin, menadione, or thymidine and/or CO_2 supplementation (52).

The typical colony appearance of most CoNS species is nonpigmented, smooth, entire, glistening, and opaque. Rare strong slime producers display mucoid colony morphology. Colony diameters reach 3 to 6 mm after 3 days of incubation. Colonies of *S. chromogenes*, *S. devriesei*, *S. lugdunensis*, *S. sciuri*, *S. vitulinus*, *S. warneri*, and *S. xylosus* are found to be more or less regularly gray to yellow, yellow, or yellow to orange. Other CoNS species may show pigmentation that is usually yellowish (Table 2).

Coagulase Production

A widely used criterion for the identification of *S. aureus* in the clinical laboratory is the clotting of plasma proven by two different tests: (i) detection of the extracellular free coagulase by the tube test due to staphylococcal coagulase,

TABLE 3 Key tests for identification of the most clinically significant *Staphylococcus* species

Species	Result of test for[a]:																			
	Colony pigmentation[b]	Staphylocoagulase	Clumping factor[b]	Heat-stable nuclease	Alkaline phosphatase	Pyrrolidonyl arylamidase[b]	Ornithine decarboxylase	Urease[b]	β-Galactosidase[b]	Acetoin production	Novobiocin resistance[b]	Polymyxin B resistance[b]	Acid production (aerobically) from:							
													D-Trehalose	D-Mannitol	D-Mannose	D-Turanose	D-Xylose	D-Cellobiose	Maltose	Sucrose
S. *aureus* subsp. *aureus*	+	+	+	+	+	−	−	*d*	−	+	−	+	+	+	+	+	−	−	+	+
S. *epidermidis*	−	−	−	−	+	−	(*d*)	+	−	+	−	+	−	−	(+)	(*d*)	−	−	+	+
S. *haemolyticus*	*d*	−	−	−	−	+	−	−	−	+	−	−	+	*d*	−	(*d*)	−	−	+	+
S. *hyicus* (veterinary)	−	*d*	−	+	+	−	−	*d*	−	−	−	+	+	−	+	−	−	−	−	+
S. *intermedius* (veterinary)	−	+	*d*	+	+	+	−	+	+	−	−	−	+	(*d*)	+	*d*	−	−	(±)	+
S. *lugdunensis*	*d*	−	(+)	−	−	+	+	*d*	−	+	−	*d*	+	−	+	(*d*)	−	−	+	+
S. *pseudintermedius* (veterinary)	−	+	−	ND	+[c]	+	ND	+	+	+	−	+	+	(±)	+	(±)	−	−	+	+
S. *schleiferi* subsp. *schleiferi*	−	−	+	+	+	+	−	−	(+)	+	−	−	*d*	−	+	−	−	−	−	−
S. *saprophyticus* subsp. *saprophyticus*	*d*	−	−	−	−	−	−	+	+	+	+	−	+	*d*	−	+	−	−	+	+

[a]Symbols and abbreviations (unless otherwise indicated): +, 90% or more strains positive; ±, 90% or more strains weakly positive; −, 90% or more strains negative; *d*, 11 to 89% of strains positive; ND, not determined. Parentheses indicate a delayed reaction.

[b]Descriptions are the same as those in Table 2.

[c]Alkaline phosphatase reactions tested positive in the STAPH-ZYM gallery but negative in the API STAPH gallery.

which converts fibrinogen to fibrin, and (ii) detection of the cell wall-bound coagulase (i.e., the clumping factor) by the slide agglutination test (see below).

The tube coagulase test is performed by transferring a large, well-isolated colony from a noninhibitory agar into 0.5 ml of reconstituted rabbit plasma. It is crucial to incubate the tube at 37°C for 4 h and to observe the tube for clot formation by slowly tilting the tube 90° from the vertical. Any degree of clotting represents a positive test. A flocculent or fibrous precipitate is not a true clot and should be recorded as negative. If no clot is formed by 4 h, the tube should be read again after 18 h of incubation at 37°C. False-negative results may occur for some strains producing staphylokinase, which may lyse the clot after formation (usually after prolonged incubation) and if SCVs are tested (often with no or delayed clotting). Inaccurate results may occur if nonsterile plasma is used or the colony tested is not pure.

Particularly in veterinary microbiological laboratories, the other coagulase-positive or -variable species (Table 1) must not be disregarded. The detection of free coagulase in staphylococci obtained from human specimens is usually equated with the species identification of S. *aureus*. However, for animal-inflicted wounds, additional testing should be performed to provide identification beyond "coagulase-positive staphylococci."

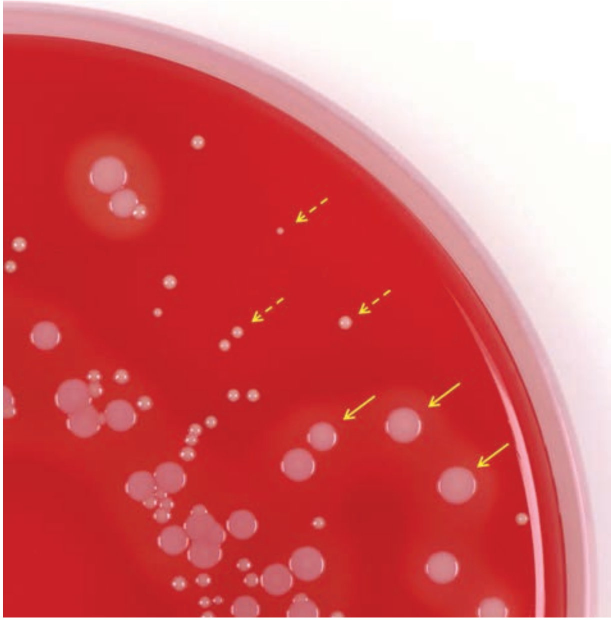

FIGURE 1 Columbia blood agar plate with 5% sheep blood showing S. *aureus* subsp. *aureus* after 24 h of incubation. The normal phenotype is grayish colonies with a yellowish tint surrounded by a hemolysis zone (arrows), and the small-colony phenotype is characterized by tiny, nonhemolytic, and nonpigmented colonies (dashed arrows).
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Agglutination Assays

To overcome the low sensitivity and specificity of the classical slide agglutination test (see previous editions of this *Manual*) and of the long incubation time of the tube coagulase test, rapid latex and hemagglutination assays allowing presumptive identification of S. *aureus* have been developed. Besides detecting protein A and clumping factor A, third-generation assays include monoclonal antibodies recognizing the clinically most prevalent capsular polysaccharide serotypes, 5 and 8, or other structures (e.g., Pastorex Staph-Plus [Bio-Rad Laboratories, Hercules, CA]; Slidex Staph Plus [bioMérieux, Marcy l'Etoile, France]; and Staphaurex Plus and Staphytect Plus [Oxoid]). The higher sensitivity (>98 to 100%) of the third-generation tests has reduced their specificity (72 to 99%). False-positive reactions occur with some CoNS strains (S. *haemolyticus* and S. *hominis*) possessing type 8 capsular polysaccharide or a cell wall hemagglutinin (S. *saprophyticus*). When an isolate is suspected of being *S. aureus*, negative slide tests should be confirmed either by the tube coagulase test or a nucleic acid-based assay for identification of *S. aureus*.

Identification of Species by Susceptibility Tests

Resistance to novobiocin is intrinsic to *S. saprophyticus* and other CoNS species (Table 2) but is uncommon in the other clinically important CoNS species of the "*S. epidermidis* group." A disk diffusion test for estimating novobiocin susceptibility can be performed using a 5-µg novobiocin disk on Mueller-Hinton agar or tryptic soy sheep blood agar. With an inoculum suspension equivalent in turbidity to a 0.5 McFarland opacity standard and incubation at 35 to 37°C overnight or for up to 24 h, novobiocin resistance is indicated by an inhibition zone diameter of ≤16 mm with any of these media.

Resistance to polymyxin B is usually observed for isolates of *S. aureus*, *S. epidermidis*, *S. hyicus*, *S. chromogenes*, and, to a lesser extent, for some strains of *S. lugdunensis*. A disk diffusion test may be performed using a 300-U polymyxin B disk on tryptic soy sheep blood agar with the same test conditions as those described for novobiocin. Polymyxin B resistance is indicated by an inhibition zone diameter of <10 mm.

Identification of Species by Biochemical Procedures

For speed, standardization, cost reduction, and convenience, the classical tests for fermentation, oxidation, degradation, and hydrolysis of various substrates (details are available in chapter 19) have been incorporated into commercial manual and automated biochemical test systems (see below and chapter 4). They are often complemented by simultaneously performed antimicrobial susceptibility testing using an advanced expert system and an interface with the laboratory informatics software. Results from conventional tests (Tables 2 and 3) may be slightly different from those obtained with rapid biochemical test systems due to the use of alternative, more sensitive indicators. Commercial identification systems identify the staphylococci (and some other aerobic Gram-positive cocci) of clinical importance with an accuracy of 70 to >90%. For some systems, reliability depends on additional testing as suggested by the manufacturer. Uncommon strains or phenotypic variants (e.g., SCVs) may have altered patterns of biochemical reactions requiring molecular testing for identification.

The API Staph (bioMérieux) strip represents an overnight method for manual identification of (primarily) staphylococci for health care and product safety applications. Necessitating the same incubation time as the API Staph and also fashioned in the strip format, the ID32 Staph (bioMérieux) may be read manually as well as automatically by the bioMérieux ATB system. The databases of both systems comprise more than 20 staphylococcal species with clinical significance, some "micrococcal" species, and *R. mucilaginosa*. A rapid version allowing 2-h identification of *S. aureus*, *S. epidermidis*, and *S. saprophyticus* is provided by the same manufacturer (Rapidec Staph). The Vitek 2 (bioMérieux) system is a fully automated platform that performs bacterial identification and antibiotic susceptibility testing. The VITEK 2 Gram-positive identification card encompasses a total of more than 100 species, including 26 staphylococcal species, a small spectrum of "micrococci," and *R. mucilaginosa*; identification of CoNS usually requires 10 h.

The MicroScan product Pos ID family (Siemens Healthcare Diagnostics, Deerfield, IL) includes a conventional panel (overnight identification time), a rapid panel (2.5-h identification time), and a Synergies plus panel (2- to 2.5-h identification time, with key antimicrobial results in as little as 4.5 h) in a microtiter format. Identification of 19 clinically important staphylococcal species, some "micrococcal" species, and *R. mucilaginosa* is available with either manual or automated processing on the autoSCAN-4 and WalkAway systems.

The BD BBL Crystal rapid Gram-positive identification kit (BD Diagnostic Systems, Sparks, MD) is a three-row panel that may be read manually or with the BBL Crystal AutoReader, requiring a 4-h incubation period. The database encompasses, besides 14 staphylococcal species, a total of 88 taxa, including *M. caseolyticus*, *R. mucilaginosa*, and several "micrococci." The BBL Crystal Gram-positive identification system represents the overnight incubation (18-h) version with an extended taxon profile of 121 Gram-positive organisms. A further enhanced profile (covering about 200 taxa) is covered by the automated nephelometry-based BD Phoenix automated microbiology system, which uses one combination panel with the identification substrates on one side and the antimicrobial agents on the other side of the panel. For staphylococci, about 10 to 15.5 h is required for complete results (identification and antimicrobial susceptibility testing) (98).

The Sensititre GPID identification plate (TREK Diagnostic Systems, Oakwood Village, OH) includes biochemical media reformulated for fluorometric reading and has been designed for the automated identification of Gram-positive organisms after 18 or 24 h of incubation. Overall, a limited number of staphylococcal species ($n = 8$) and a few "micrococcal" species are part of the database. The differentiation of *S. hominis*, *S. saprophyticus*, and *S. warneri* may need additional tests.

The Gram-positive aerobic-bacterium database (339 taxa) of the Biolog Systems family (Biolog, Hayward, CA) comprises 34 staphylococcal and 4 macrococcal (sub)species and many members of the *Micrococcaceae* and *Dermacoccaceae* families not found in other commercial phenotype-based systems. The system's redox chemistry, based on the utilization of a wide variety of carbon sources, is used to generate a "metabolic fingerprint," providing results in 4 h or less, and is available with different automation levels. This system requires subculture of the isolate to be tested with a proprietary medium called Biolog Universal Growth (BUG) or Bug+blood (BUG+B) agar.

The Sherlock microbial identification system (MIDI, Newark, DE) automates microbial identification by combining cellular fatty acid analysis with computerized high-resolution gas chromatography. In addition to a multitude of other species, 30 staphylococcal species, *M. caseolyticus*, and several "micrococci" are listed in the database of fatty acid profiles. A new sample preparation method (Instant FAME) allows for rapid identification from pure cultures in less than 15 min.

Most systems are fairly successful in differentiating *S. aureus*, *S. epidermidis*, and *S. saprophyticus*, while the accurate identification of less common species is more variable (99). Systems may fail in distinguishing commonly encountered staphylococcal species, in particular if phenotypic variants or isolates recovered from livestock and food are tested (100, 101). A verification of the identification result by a second, independent approach is recommended for isolates presumptively identified as *S. aureus*, particularly for oxacillin-resistant strains. Additional tests should be performed on clinically significant isolates with questionable identification results that impact patient management.

Species Identification by Nucleic Acid-Based Approaches

Extraction of staphylococcal nucleic acids may be challenging due to the Gram-positive nature of staphylococci, which necessitates special conditions for lysis of the cell wall. For this purpose, lysostaphin, lysozyme, proteinase K, and achromopeptidase have been used.

PCR fingerprinting techniques based on DNA sequence polymorphisms may be used, and species-specific variable regions of universal genes or genes unique to *S. aureus* or other staphylococcal species may serve as targets for identification and differentiation of staphylococcal isolates. Assays based on the specific amplification of fragments of the universal 16S and 23S rRNA genes and of their spacer sequences have been published (101–107). The 16S rRNA gene is also used as a target for the *in situ* detection and identification of *S. aureus* and *S. epidermidis* (108). Other universal DNA targets shown to be useful for identification of staphylococci include the elongation factor gene (*tuf*), the gyrase gene (*gyrA*), the manganese-dependent superoxide dismutase gene (*sodA*), the glyceraldehyde-3-phosphate dehydrogenase-encoding gene (*gap*), and a 60-kDa heat shock protein (HSP60/GroE) (109–113).

Sequencing of selected universal phylogenetic marker genes represents the ultimate approach to identify known and not-yet-described staphylococcal species. For 16S rRNA gene sequencing, the regions between bp 70 to 300, 420 to 500, 1000 to 1050, and 1250 to 1290 (corresponding to nucleotides of the *Escherichia coli* 16S rRNA gene) are useful to determine sequence differences among the staphylococcal species. For differentiation of staphylococcal subspecies, sequencing of partial *rpoB* gene sequences seems to be superior to partial 16S rRNA gene sequencing (114). The recognized limitations of currently available public sequence databases apply to sequencing of staphylococcal isolates (101).

The most popular and well-studied specific target for *S. aureus* identification is the *nuc* gene, which encodes thermostable nuclease (thermonuclease or TNase) (115, 116). PCR methods targeting *nuc* are highly specific for *S. aureus*. A specific PCR for the *S. intermedius nuc* gene has also been described (117). Further specific targets used for identification of *S. aureus* include the genes encoding clumping factor (*clfA*), coagulase (*coa*), manganese-dependent superoxide dismutase (*sodM*, absent in CoNS), the factors essential for the expression of methicillin resistance (*femA* and *-B*), and (for MRSA only) the staphylococcal insertion element *431* (112, 118–121). Misidentification using the *fem* factors may occur due to *fem*-negative *S. aureus* strains and CoNS with a gene structurally related to *femA*.

For molecular identification of *S. aureus* and some other staphylococcal species isolated from culture, several commercial tests (e.g., GenoType Staphylococcus and GenoType MRSA [Hain Lifescience, Nehren, Germany], StaphPlex panel [Qiagen, Germantown, MD], and AccuProbe *S. aureus* culture [Gen-Probe, San Diego, CA]) are available. Some assays also detect resistance genes and/or toxin genes. The GeneXpert (Xpert MRSA/SA BC; Cepheid) and BD GeneOhm (StaphSR assay; BD Diagnostics) instruments offer assays to detect MSSA and MRSA directly from positive blood cultures. Other assays, such as the BioFire FilmArray blood culture identification (BCID) assay (BioFire Diagnostics, Salt Lake City, UT) and the Verigene Gram-positive blood culture (BC-GP) assay (Nanosphere, Northbrook, IL), include MSSA/MRSA detection as part of a broader panel that identifies bacteria directly from positive blood culture bottles. The RiboPrinter microbial characterization system (DuPont Qualicon, Wilmington, DE) utilizes ribotype pattern analysis for the differentiation of staphylococcal species with patterns in the database.

The *S. aureus*/CNS PNA FISH and the faster *Staphylococcus Quick*FISH BC kit (AdvanDx, Woburn, MA) are qualitative nucleic acid hybridization assays targeting rRNA sequences based on peptide nucleic acid (PNA) fluorescence *in situ* hybridization (FISH). These assays are intended for rapid identification of *S. aureus* and several CoNS species in a smear prepared from a positive blood culture (122).

Diagnostic DNA oligonucleotide microarrays that identify the genus *Staphylococcus*, clinically important staphylococcal species, other pathogens, drug resistance genes, and toxin genes have been designed and successfully tested on clinical isolates, indicating their potential for array-based high-throughput screening (123, 124).

Species Identification by Spectroscopic Approaches

An alternative high-throughput approach is represented by identification of staphylococci based on peptidic spectra obtained by MALDI-TOF MS. In the past few years, commercially available mass spectrometers adapted to routine diagnostic requirements have become available (Microflex LT [Bruker Daltonics, Billerica, MA] and VITEK MS [bioMérieux]), and the usefulness of this approach has been evaluated for routine identification of staphylococcal isolates (125–129). Most studies reported on identification of staphylococci at the species level of >97%. Investigating a large collection of clinical, molecularly defined strains of *S. aureus* and CoNS, the MALDI-TOF MS approach (Microflex LT, Biotyper 2.0-based identification) was shown to be faster than and equivalent to the molecularly defined reference methods irrespective of the methicillin resistance of the included strains (129). Nevertheless, for non-human-associated and/or newly described CoNS species, expansion of the MALDI-TOF MS databases is needed (128). There are few or incomplete data for MALDI-TOF MS identification of other catalase-positive cocci. Some "micrococci" and *A. otitis* are part of the databases of both Microflex LT and VITEK MS. Of note, the content and quality of the database and the standardization of variable parameters, such as culture conditions and length of incubation, are crucial to achieve reproducible results.

By compiling universal and target gene-specific primer sets, PCR coupled to electrospray ionization-mass spectrometry (ESI-MS) gives the opportunity for high-throughput species identification and further molecular characterization (e.g., toxin gene detection) in parallel (130).

Nondestructive techniques, such as Fourier transform infrared (FTIR) and Raman spectroscopy, are currently being developed as alternative methods for the rapid identification of staphylococci (131). This approach also allows discrimination between the SCV and the normal *S. aureus* phenotype (55).

Diagnosis of Toxin-Mediated Staphylococcal Syndromes

The diagnosis of TSS and SSSS is based on clinical signs (supplemented by serologic tests) and the detection of toxin production by staphylococcal isolates (*S. aureus* but rarely other species). While the skin manifestations are mostly culture negative, isolates are usually recovered from the suspected site of infection. Blood cultures are positive in fewer than 5% of cases of staphylococcal TSS. In patients with TSS, protective antibodies against causative PTSAgs are absent or present at very low levels. However, seroconversion after onset of the disease and during convalescence may be observed. The same phenomenon holds true for exfoliatin antibodies in patients with SSSS. Currently available immunoassays for antibody detection are for research use only. Beyond the recognition of the characteristic rapid onset and the clinical signs, staphylococcal food poisoning is difficult to verify because the incriminated food source may not contain cultivable staphylococcal cells and detection of staphylococcal enterotoxin is required.

Traditional immunological procedures may be used to measure the toxin in culture supernatants of isolated strains, in contaminated food extracts, or in patient specimens. Kits for the detection of strains producing TSST-1 (TST-RPLA [Oxoid], TST-RPLA Seiken [Denka Seiken, Tokyo, Japan], and TSST-1 Evigene [AdvanDx, Woburn, MA]), staphylococcal enterotoxins (Ridascreen sets A, B, C, D, and E [R-Biopharm, Darmstadt, Germany], SET-RPLA Seiken [Denka Seiken], and SET-RPLA kit toxin detection kit [Oxoid]), and ETA/ETB (EXT-RPLA Seiken [Denka Seiken]) are offered. A quantitative real-time immuno-PCR was recently described for the detection of small amounts of enterotoxins (132). An adaptation of a flow cytometry-assisted multiplex immunoassay (Bio-Plex system; Bio-Rad, Hercules, CA) for the detection of ETA and ETB has been recently reported (133). Since phenotypic methods may be hampered by low sensitivity and specificity (cross-reactivity between PTSAgs) and *in vitro* expression of PTSAgs might be negatively influenced by various factors, detection of PTSAg/ET-encoding sequences by nucleic acid-based methods has come into favor (134–137).

Due to renewed interest in Panton-Valentine leukocidin (PVL), PCR procedures targeting leukocidal (synergohymenotropic) pore-forming toxins produced by *S. aureus* were established (138). The detection of PVL-encoding genes is included in the GenoType Staphylococcus and MRSA test systems (Hain Lifescience) and offered by the PVL Evigene kit (AdvanDx, Woburn, MA). A rapid phenotypic assay for PVL toxin detection in *S. aureus* cultures using recombinant monoclonal antibodies was developed as an immunochromatographic membrane-based lateral-flow assay (139).

Micrococcaceae and *Dermacoccaceae*

"Micrococci" and staphylococci might be easily confused with one another on the basis of similar cellular morphologies, Gram stain appearance, and positive catalase activities. The exact species affiliation of "micrococci" may frequently be misjudged. The frequent pigmentation of "micrococcal" colonies with a high convex profile leads to their presumptive identification as members of the *Micrococcaceae* and *Dermacoccaceae* families (Fig. 2). Colonies of *M. luteus*, *K. varians*, and the kytococcal species are characterized by yellowish tints. *K. kristinae* and *D. nishinomiyaensis* appear orange-like, and *K. rosea* shows pink-to-red colonies. Some species (e.g., *M. lylae*) or strains of the usually pigmented species are nonpigmented. On routine blood agar, *R. mucilaginosa* colonies are mucoid or sticky, transparent to white, and nonhemolytic and in the majority of cases adhere to the agar (differentiation from streptococci). This organism may be distinguished from other similar organisms by its weak catalase reaction and its inability to grow in the presence of 5% NaCl.

In the clinical laboratory, "micrococcal" species can be preliminarily distinguished from staphylococci by their resistance to furazolidone (100 µg/disk [Oxoid, United

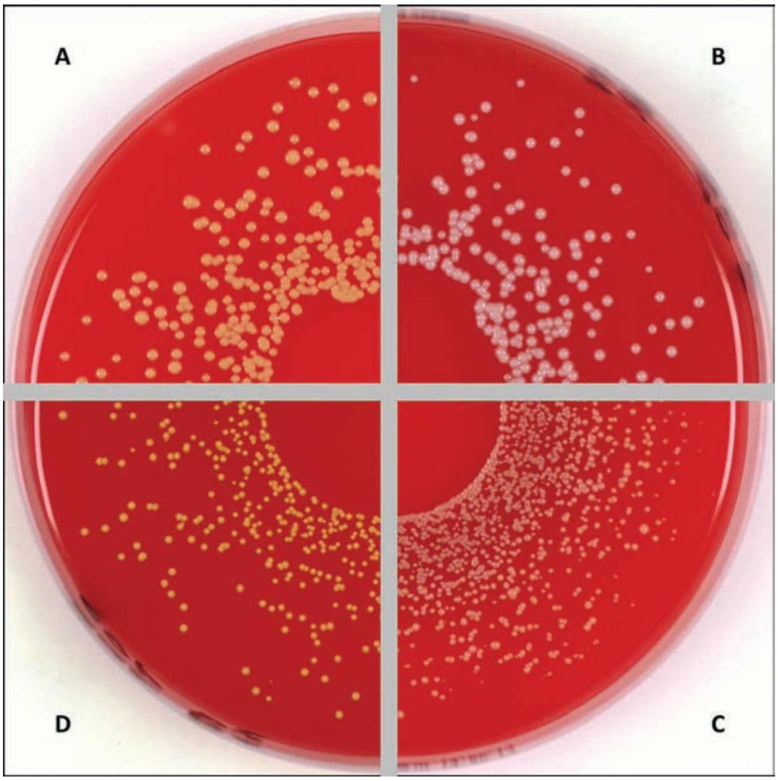

FIGURE 2 Sections of Columbia blood agar plates after 48 h of incubation showing yellow colonies of *M. luteus* (A), cream-white colonies of *M. lylae* (B), pastel-pink colonies of *K. rosea* (C), and orange colonies of *D. nishinomiyaensis* (D). doi:10.1128/9781555817381.ch21.f2

Kingdom]; resistance is indicated by a ≤9-mm zone diameter) and lysostaphin (200 μg/disk [Mast, Bootle, United Kingdom]; resistance, no zone) and susceptibility to bacitracin (0.04 U/disk [Oxoid]; susceptibility, ≥10-mm zone diameter), in contrast to members of the *Staphylococcus* genus, which show inverse susceptibility patterns (zone diameters as follows: furazolidone, ≥15 mm; lysostaphin, 10 to 16 mm; bacitracin, no zone) (Table 1). Details are available in previous editions of this *Manual*. In contrast to most staphylococci, "micrococci" are positive by the modified oxidase test (140). Regarded as a reference method to distinguish "micrococcal" species from staphylococci, the fermentation of glucose in a manner similar to that of the oxidation-fermentation test for nonfermenters requires a specific oxidation-fermentation medium and prolonged incubation. In contrast to staphylococci, "micrococci" are characterized by the lack of acid production from glucose under anaerobic conditions.

Key features for differentiation of species reported to occur in human specimens are given in Table 4. Data concerning the applicability of manual and automated identification systems for members of *Micrococcaceae* and *Dermacoccaceae* are given in the respective *Staphylococcus* sections; however, their use is limited to a small spectrum of the clinical "micrococcal" species. In cases of doubt and extraordinary clinical relevance, the use of sequencing-based approaches is recommended for definite species recognition.

Alloiococcus

After 48 h of incubation at 37°C, alloiococci form small alpha-hemolytic colonies on blood agar. Colonies formed on brain heart infusion agar with 5% rabbit blood are small, moist, and slightly yellow at 72 h, and the blood is partially hemolyzed. Growth occurs in the presence of 6.5% NaCl and on bile esculin agar. No growth occurs at 10°C or 45°C. *A. otitis* can be distinguished from similar organisms by its positive catalase and negative oxidase activities, its obligate aerobic nature, and its inability to produce acid from glucose or other carbohydrates (Table 1). Arginine dihydrolase is not produced. Pyrrolidonyl arylamidase, leucine aminopeptidase, and P-galactose are produced. Starch and esculin are not hydrolyzed; hippurate is mostly hydrolyzed (95). *A. otitis* is included in the database of the API Strep gallery (bioMérieux).

As the bacterium is quite inert biochemically, molecular methods are often necessary to confirm the identification. For the molecular verification of suspected colonies, an *A. otitis*-specific PCR assay has been described (141). A PCR assay for direct detection of this microorganism in clinical specimens has been reported as part of a multiplex approach targeting pathogens that cause otitis media with effusion (142).

TYPING SYSTEMS

Traditional phenotyping techniques, such as phage typing, capsule serotyping, antibiotic susceptibility pattern analysis, and other biotyping methods, have been replaced by molecular-band-based and sequence-based typing methods (143).

For local MRSA outbreak investigations as well as for long-term MRSA surveillance, SmaI macrorestriction pattern analysis by PFGE still represents a highly discriminatory "gold standard" tool with detailed performance and interpretation guidelines (144, 145). However, PFGE is technically demanding, with low throughput, limited portability, and problems with intercenter reproducibility. In addition, the

TABLE 4 Key tests for the identification of *Micrococcaceae* and *Dermacoccaceae* found in clinical specimens

Species	Result of test for[a]:														
	Colony pigmentation	Nitrate reduction	β-Galactosidase	β-Glucuronidase	α-D-Glucosidase	Arginine dihydrolase	Urease	Gelatin hydrolysis	Tween 80 hydrolysis	Acid production (aerobically) from:					
										D-Mannose	Lactose	Sucrose	D-Trehalose	D-Xylose	D-Mannitol
Kocuria carniphila	Yellow	+	+	+	ND	−	−	−	−	−	+	±	−	−	−
Kocuria kristinae	Pale cream to pale orange	*d*	−	−	ND	−	*d*	±	−	+	*d*	+	+	−	−
Kocuria rhizophila	Yellow	−	−	−	ND	−	−	+	+	+	−	−	−	−	−
Kocuria rosea	Pastel or orange-red	+	−	−	ND	−	−	−	−	−	−	−	−	+	+
Kocuria varians	Different shades of (dark) yellow	+	+	−	−	−	+	±	−	−	*d*	*d*	−	+	−
Micrococcus luteus	Different shades of yellow, yellowish green, or cream white	−	ND	ND	+	−	*d*	+	−	*d*	−	*d*	−	−	−
Micrococcus lylae	Unpigmented or cream white	−	ND	ND	ND	−	−	+	*d*	−	−	−	+	−	−
Dermacoccus nishinomiyaensis	Bright orange pigment	*d*	ND	ND	ND	−	+	+	*d*	−	−	*d*	ND	−	ND
Kytococcus schroeteri	Muddy yellow	−	−	ND	−	+	−	+	+	−	ND	*d*	+	−	−
Kytococcus sedentarius	Cream white to deep buttercup yellow	−	ND	ND	+	+	−	+	−	*d*	−	*d*	+	−	ND
Rothia mucilaginosa	Transparent or whitish	+	ND	ND	+	−	−	+	−	+	−	+	+	−	−

[a] Symbols and abbreviations: +, 90% or more strains positive; ±, 90% or more strains weakly positive; −, 90% or more strains negative; *d*, 11 to 89% of strains positive; ND, not determined. Parentheses indicate a delayed reaction.

use of different national nomenclatures and attribution of PFGE clusters to genetic lineages may be problematic (143). Since no digestion occurs due to methylation of the respective restriction endonuclease site, the emerging livestock-associated CC398 isolates are mostly nontypeable by standard PFGE protocols applying SmaI (NT_{SmaI}) (146–148). Those NT_{SmaI} MRSA can be PFGE typed by neoschizomers, such as XmaI or Cfr9I, that are partially or fully insensitive for the DNA methylation (148–150).

The reference method for defining the core genetic population structure of *S. aureus* is MLST (151). In a standardized manner, the allelic polymorphisms of seven housekeeping genes are indexed. A Web-based database is available (http://saureus.mlst.net/). MLST has limited discriminatory power and a low-throughput capacity in the context of MRSA outbreak investigation and surveillance. In the case of common ancestry but assumed distinct epidemiological origins of MRSA isolates, subtyping of SCC*mec* may provide additional information (http://www.sccmec.org/) (152).

spa typing, based on the number of polymorphisms and the sequence of tandem repeat elements of the hypervariable X region of the *S. aureus* protein A (*spa*) gene, is the basis of a single-locus sequence typing approach that has become one of the primary genotyping methods for MRSA surveillance (153, 154). Beside excellent reproducibility, other advantages include low costs, high throughput, a standardized nomenclature, and complete portability of data transferable into an international database (http://spaserver.ridom.de/) curated by SeqNet.org (http://www.seqnet.org/) (155). For particular genetic lineages, misclassification may occur, necessitating the use of additional tests for reliable inference.

Other molecular typing techniques used for typing of staphylococci include several other band-based molecular-fingerprinting approaches, ribotyping, and, more recently, multiple-locus variable-number tandem-repeat analysis (MLVA) and microarray-based approaches (156–158).

In the near future, whole-genome sequencing may supersede traditional molecular typing methods, serving as a universal approach that may provide information useful for outbreak investigation, surveillance purposes, detection of unknown and/or emerging strains, and prediction of the clinical importance of a given isolate (159). However, costs (although rapidly declining), bioinformatic processing issues, missing universal nomenclature, and challenging interpretation of sequence data still hamper its use.

Modern approaches based on phenotype structures applied to staphylococci include the MALDI-TOF MS and whole-cell fingerprinting techniques, such as FTIR spectroscopy (160).

While many typing systems have been developed and evaluated for *S. aureus*, fewer applications are available for CoNS. These include antibiotic resistance analysis, phage typing, slime production, and some modern genotyping procedures (PFGE and ribotyping) (161, 162).

For "micrococcal" species, phage typing and PFGE approaches applying phage sets and restriction enzymes, respectively, different from those used for staphylococci (163) have been described. PFGE and restriction fragment length polymorphism approaches have been described for *Alloiococcus* (95, 164).

SEROLOGIC TESTS

Because serological testing for antistaphylococcal antibodies lacks specificity and predictive accuracy, it plays no role in the diagnosis of most staphylococcal diseases. The one exception is the determination of protective antibodies in the case of toxin-mediated syndromes such as TSS and SSSS (see above). For the other microorganisms discussed in this chapter, detection of antibodies is in fact not clinically useful.

ANTIMICROBIAL SUSCEPTIBILITIES

Staphylococcus and Related Genera

Genetic Basis and Prevalence of Antimicrobial Susceptibilities

A number of studies and reviews describing the antimicrobial and biocide susceptibilities of clinically important staphylococcal species have been published (165–167). Networks that provide online or published resistance data for staphylococci have also been established, namely, the National Healthcare Safety Network (NHSN) (http://www.cdc.gov/hai/), the European Antimicrobial Resistance Surveillance System (EARS) (http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net/Pages/index.aspx), the Asian Network for Surveillance of Resistant Pathogens (ANSORP) (http://www.ansorp.org/), and the International Nosocomial Infection Control Consortium (INICC) (http://www.inicc.org/espanol/esp_index.php).

Methicillin resistance mediated by the *mecA* gene or the newly described *mecC* gene is the most important resistance mechanism, as it has a profound impact on patient management by excluding all traditional β-lactam antibiotics (penicillins, group 1 to 4 cephalosporins, and carbapenems) from the antibiotic armamentarium. Since the early 1980s, the prevalence of health care-associated MRSA (HA-MRSA) has increased in many regions. In some areas of the United States, the prevalence of MRSA is >50% (168, 169). In Europe, the prevalance of MRSA ranges from 10 to 50%, except in The Netherlands and the Scandinavian countries, where MRSA rates are very low (170). In Asia, Australia, and Africa, high MRSA rates (approximately 20 to 80%) have also been noted (171–173). The worldwide burden of infections caused by MRSA has further increased due to the advent of CA-MRSA in the past decade and recently by livestock-associated *S. aureus* infections (174–176). However, in some parts of the world (the United States and several European countries), there are signs of stabilization or even a decrease in the incidence of MRSA infections (177, 178).

The *mecA* gene and its homologs acquired by *S. aureus* and other staphylococcal species on a foreign, mobile DNA element (SCC*mec*) encode an additional penicillin-binding protein (PBP), PBP2a or PBP2c, responsible for the reduced activities of traditional β-lactam antibiotics. Homologs of the *mecA* gene have been described for *S. sciuri* (*mecA1*) and *S. vitulinus* (*mecA2*), *Macrococcus caseolyticus* (*mecB*), *S. aureus* (*mecC*), and *S. xylosus* (*mecC1*) (179). *mecC* possession, so far associated with SCC*mec* type XI, may result in the expression of a functional PBP2a homolog, likewise leading to methicillin resistance (180–182). Eleven major variants of SCC*mec*, types I to XI, and many subtypes have been recognized (178, 181–189). SCC*mec* types I, II, III, VI, and VIII are predominantly associated with HA-MRSA, and types IX and X are associated with LA-MRSA (190).

Multidrug resistance is regularly observed in HA-MRSA and usually includes resistance to aminoglycosides, fosfomycin, fusidic acid, ketolides, lincosamides, macrolides, quinolones, rifampin, tetracyclines, and trimethoprim-sulfamethoxazole (166, 167, 191). While reports are still rare, an increasing number of reports of *S. aureus* and CoNS isolates resistant to newer agents, such as daptomycin, tigecycline, and linezolid, has to be noted, including plasmid-mediated resistance to linezolid based on the acquisition of the *cfr* gene (192–194). The transferable multiresistance gene *cfr* confers resistance not only to oxazolidinones but also to streptogramin A, lincosamides, phenicols, and pleuromutilins (195).

In contrast to HA-MRSA, CA-MRSA strains are mostly susceptible to non-β-lactam antibiotic classes. They harbor different SCC*mec* types (mostly types IVa, V, and VII) (185–187, 196). CA-MRSA isolates are more likely to carry PVL, a pore-forming toxin with potent cytolytic and inflammatory activities (197). Whereas in Europe, an array of diverse CA-MRSA clones (e.g., the "European" ST-80, *spa* type t044) have been reported, in the United States, CA-MRSA infections are dominated by a single clone, i.e., the PFGE type USA300 (ST-8, *spa* type t008); PFGE type USA400 (ST-1) was most prominent before 2001 (190, 198–200). However, also in Europe and other parts of the world, an increase in USA300 strains has been reported (201–204). Outside North America, other CA-MRSA clones are prevalent, e.g., ST80 in Europe, ST59, ST30 (Southwest Pacific clone), and ST5 in the Asia and Pacific regions, ST88 and ST1797 in Africa, and ST772 (Bengal Bay clone) in South Asia (199, 201, 205–207). Of note, PVL possession is not restricted to CA-MRSA. Isolates of many pandemic MSSA lineages may carry PVL genes. Particularly in African populations, a high prevalence of PVL-positive MSSA has been reported (208–210). Populations at increased risk for CA-MRSA colonization and/or infections include children in day care centers, athletes, military recruits, jailed inmates, and men who have sex with men (211–215). CA-MRSA causes superficial skin and soft tissue infections and can also be associated with necrotizing fasciitis and myositis, necrotizing pneumonia, and other severe entities (138). Infections by PVL-positive MRSA and MSSA in young, otherwise healthy children following a respiratory viral infection (most frequently influenza) can be a devastating disease (197). However, some of the distinctions between HA-MRSA and CA-MRSA strains may disappear, since CA-MRSA is now becoming endemic in hospitals and acquiring additional resistance phenotypes (212, 216, 217).

For LA-MRSA, the possession of various SCC*mec* types has been reported. Most LA-MRSA belong to CC398, as defined by MLST, with t011 and t034 as the most frequent *spa* types (218). LA-MRSA isolates may exhibit resistance to antibiotic classes other than the tetracyclines, resistance to which is found in almost all isolates, but so far, LA-MRSA isolates exhibit this trait to a less frequent extent than HA-MRSA isolates (175).

Vancomycin-intermediate *S. aureus* (VISA) isolates (MIC of 4 to 8 µg/ml) (219) and their putative precursors, termed heterogeneous VISA (hVISA) strains, were identified first (1996) in Japan and then in the United States and other regions (220–222). The mechanism of resistance for hVISA and VISA strains is not due to a single gene but is complex and can be obtained by several pathways, all of which, however, include cell wall alterations resulting in reorganization and thickening in addition to reduced autolytic activity (223–225). Moreover, current data may suggest that hVISA and VISA strains represent a bacterial evolutionary state favoring persistence in the host environment (226). In 2002, the first of multiple vancomycin-resistant *S. aureus* (VRSA) strains containing the *vanA* gene were reported in the United States (227). The MICs for *S. aureus* isolates currently defined as vancomycin resistant according to the CLSI are ≥16 µg/ml (228). Since most VISA strains are also resistant to teicoplanin, the acronym GISA (glycopeptide-intermediate *S. aureus*) is preferred by some authors. A few clinical isolates that are resistant to teicoplanin but fully susceptible to vancomycin have been reported (229). For hVISA and VISA isolates, heteroresistance to daptomycin has been rarely reported (230).

For *S. pseudintermedius*/*S. intermedius*, a significant emergence of methicillin resistance has been observed (231).

More than 90% of all of nosocomial CoNS (mainly *S. epidermidis* and *S. haemolyticus*) are resistant to penicillin due to β-lactamase production, and resistance to methicillin and other agents can be as high as 60 to 80% for nosocomial isolates (167, 232, 233). In terms of glycopeptides, resistant CoNS isolates have been described primarily as resistant to teicoplanin.

Determination of Antimicrobial Susceptibilities

Antimicrobial susceptibility testing of staphylococci may be performed conventionally by Clinical and Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) reference methods or commercial systems as described in chapters 70 to 73 of this *Manual*. Direct detection of MRSA is discussed in Direct Examination above. For SCVs, no approved method has been developed to determine the susceptibility (97, 234).

Detection of MRSA represents the most important task in determining the antimicrobial susceptibilities of staphylococci. Of note, traditional methods have reduced sensitivity and specificity in distinguishing MRSA from MSSA due to heteroresistance and the borderline oxacillin resistance of *S. aureus* strains characterized by β-lactamase hyperproduction. With the enhanced discriminatory power of applying cefoxitin as the agent for detecting MRSA (both *mecA* and *mecC* mediated), most of these problems have disappeared. The use of cefoxitin obviates the need for specific assay conditions (see previous editions of this *Manual*). However, oxacillin MIC determination continues to require the addition of 2% NaCl to the media, a maximum incubation temperature of 35°C, and a full 24 h of incubation. Even when these optimal expression conditions are present, MICs below the breakpoint, indicating false susceptibility, can still be obtained for some *mecA*- or *mecC*-positive isolates. This phenomenon is due to heterogeneous PBP2a expression of some MRSA isolates, where only a small fraction of the bacterial cell population (10^{-8} to 10^{-4}) expresses the resistance phenotype under *in vitro* test conditions. The resistant subpopulation may thus be overlooked because it usually grows more slowly than the susceptible population. The cefoxitin disk (30 µg) is today the disk diffusion method recommended by the CLSI for testing of *S. aureus* and *S. lugdunensis* (resistance is indicated by a zone diameter of ≤21 mm) and can be used for other CoNS (resistance, ≤24 mm) for *mecA*-mediated resistance. According to EUCAST (http://www.eucast.org), *S. saprophyticus* should be tested like *S. aureus* and *S. lugdunensis*. Isolates of these species are categorized as resistant if cefoxitin disk zone diameters are less than 22 mm (235, 236). For other CoNS, resistance is indicated by a zone diameter of <25 mm. It should be noted that these cefoxitin breakpoints are inappropriate for *S. pseudintermedius* in cases where, e.g., oxacillin is recommended (237). For this species, EUCAST now recommends a cefoxitin screen zone diameter breakpoint of <35 mm as resistant.

For broth microdilution testing, oxacillin or cefoxitin may be used to detect *mecA*-mediated resistance in *S. aureus* and *S. lugdunensis* (resistance to cefoxitin, MIC ≥ 8 μg/ml; oxacillin, MIC ≥ 4 μg/ml). For other CoNS isolates, the presence of *mecA* is predicted by applying lower oxacillin MIC breakpoints (resistance to oxacillin, MIC ≥ 0.5 μg/ml). Cefoxitin has also improved the detection of MRSA by automated susceptibility testing systems (238). The low growth rate of SCVs prevents the use of disk diffusion and automated methods to determine the susceptibilities of these strains (52).

An alternative method for the detection of methicillin resistance is the use of anti-PBP2a monoclonal antibodies available in a latex agglutination assay (MRSA screen; Denka Seiken, Tokyo, Japan, and other suppliers) or immunochromatographic assay (PBP2a culture colony test; Alere, Waltham, MA) that may be performed on isolated colonies from a pure culture (239). These assys, however, do not detect *mecC* isolates. If the latex test is used for SCVs, then the number of colonies must be increased 100-fold (240).

For a definite verification of a given MRSA isolate, the occurrence of both the oxacillin resistance-determining gene and an *S. aureus* species-specific marker (see above) has to be proven as a matter of principle, representing the gold standard. The use of pure colony material is a vital premise for this approach. In particular, for heterogeneous methicillin-resistant staphylococci, strains displaying growth-impaired phenotypes (SCVs), or *S. aureus* strains with borderline oxacillin resistance, the methicillin resistance of cultivated staphylococcal isolates may be determined by detection of the *mecA* gene (241). At least in the case of unexplainable discrepancies between a positive MRSA result obtained by culture-based approaches and a negative result obtained by molecular approaches targeting *mecA* and/or the SCC*mec*/*orfX* junction, testing of *mecA* homologues should be included. The same holds true for CoNS if molecular verification of oxacillin resistance is desired. For the detection of *mecC* and other *mecA* alleles, specific oligonucleotide primers have been described and a microarray-based approach has been published (180, 182, 242).

For MRSA screening to detect colonization (preferentially nasal but also pharyngeal), selective agar media (e.g., mannitol salt agar) supplemented with oxacillin or, preferably, cefoxitin are widely used. Inclusion of a broth enrichment step prior to plating enhances sensitivity but delays results. A commercial MRSA screening broth is supplied by Oxoid (Contrast MRSA broth). Agar media containing chromogenic enzyme substrates (e.g., chromID MRSA [bioMérieux], BBL CHROMagar MRSA II [BD], and Brilliance MRSA 2 agar [Oxoid]) have better specificity; however, confirmation of MRSA with a coagulase test is recommended for some products (243–245). A semiselective tryptic soy broth with 2.5% salt containing cefoxitin and aztreonam (TSB-SSI; SSI Diagnostica, Hillerød, Denmark) was shown to be superior to both nonselective enrichment broth and direct plating on a chromogenic agar or Columbia blood agar (246).

Regarding glycopeptide susceptibility testing, differences between CLSI and EUCAST interpretative criteria have to be taken into account. Applying EUCAST criteria, the different underlying mechanisms leading to glycopeptide nonsusceptibility are not addressed. The disk diffusion test is unreliable and not recommended for glycopeptide susceptibility testing either by the CLSI or by EUCAST.

According to CLSI guidelines, VRSA strains (MIC ≥ 16 μg/ml) are reliably detected by screening tests such as the broth microdilution reference method (for screening, the MIC is ≥8 μg/ml), most FDA-cleared automated systems, and a brain heart infusion vancomycin (6 μg/ml) agar screen plate (219). Detection of the VISA phenotype is unreliable and underreported by routine susceptibility testing methods, including automated methods (247). According to current CLSI recommendations, *S. aureus* isolates for which vancomycin MICs are 4 to 8 μg/ml are classified as VISA (219). These vancomycin MIC breakpoints were lowered for *S. aureus* (the intermediate category remained defined as 8 to 16 μg/ml for CoNS) in order to better detect VISA strains (248). The CLSI recommends sending *S. aureus* isolates for which the vancomycin MIC is ≥8 μg/ml to a reference laboratory (219). The glycopeptide MIC breakpoints have been considerably lowered by EUCAST (resistance to vancomycin and teicoplanin, >2 μg/ml for *S. aureus* and >4 μg/ml for CoNS [http://www.eucast.org]) to avoid reporting VISA/GISA isolates as "intermediate" (249) and to address the finding that strains of the hVISA/VISA phenotypes appear to be associated with impaired clinical responses to vancomycin (250). The poor clinical outcome of infections with *S. aureus* isolates for which the vancomycin MICs are 1.5 or 2.0 (studies listed in reference 249) as well as the "vancomycin MIC creep" phenomenon are matters of debate (251).

Detection of hVISA requires a modified population analysis profile-area under the curve method (252, 253). As this method is laborious, complicated, and not suited for routine use, a number of screening assays including antibiotic-incorporated agar media and specialized gradient tests (macroEtest) have been developed (225). Recently, FTIR spectroscopy was successfully applied for rapid and accurate identification of VISA and hVISA among isolates of MRSA (254).

Additional information on the determination of antimicrobial susceptibilities is contained in chapters 70 to 73 of this *Manual*. There are no CLSI methods for susceptibility testing of "micrococci" and alloiococci.

Treatment

For additional and annually updated information regarding treatment, the reader should consult the current edition of *The Sanford Guide to Antimicrobial Therapy* (255) or other guidelines, such as the IDSA practical guideline for the treatment of MRSA infections (256). Effective treatment of focal infections, such as abscesses, requires incision and drainage. Penicillin G is the most effective compound for the treatment of the uncommon penicillin-susceptible *S. aureus* strain. In general, penicillin-resistant, oxacillin-susceptible staphylococcal strains should be treated with penicillinase-stable penicillins, β-lactam/β-lactamase inhibitor combinations, and cephalosporins (255, 257). For patients with penicillin allergy or chronic renal failure, clindamycin or vancomycin may be an option in the case of MSSA. However, the use of vancomycin, known to be poorly bactericidal against staphylococci, is not recommended for severe infections due to MSSA, as it is inferior to β-lactams in terms of mortality and bacteriological outcome.

Strains that are oxacillin and cefoxitin resistant (MRSA) should be considered resistant to all β-lactams, including penicillins, carbapenems, and cephalosporins (except for the new "fifth-generation" cephalosporins with anti-MRSA activity) (258). Vancomycin and the newer agents, such as linezolid, daptomycin, telavancin, tigecycline, and the anti-MRSA cephalosporins (ceftobiprole and ceftaroline), are suitable options for empirical therapy of MRSA infections (note the different spectra and the approved indications of these compounds) (256, 259–265). For the treatment of MRSA nosocomial pneumonia, it was shown in a prospective multicenter trial that clinical responses at the end of the study in the per-protocol population were significantly higher with linezolid than with vancomycin, while all-cause 60-day mortalities were similar (266). Occasionally, trimethoprim-sulfamethoxazole may be helpful, but it should be used with caution (267). In particular, in terms of "collateral damage" (referring to ecological adverse effects, such as subsequent MRSA colonization/infection after receipt), quinolones should be avoided for the therapy of staphylococcal infections (268).

While there are only a few, partly uncorroborated studies and case reports available supporting combination therapy for treatment of severe staphylococcal infections, aminoglycosides, rifampin, fosfomycin, co-trimoxazole, and fusidic acid in combination with glycopeptides and β-lactams have been recommended (269). A careful risk-benefit assessment concerning drug-drug interactions and side effects should be performed. Since resistance to rifampin, fusidic acid, and fosfomycin develops rapidly, these compounds must not be administered alone.

Despite the challenges for *S. aureus* universal vaccines, they are urgently needed and being actively pursued in health care settings (270). Within the past several years, the *S. aureus* vaccine field has witnessed vaccine failures in clinical trials designed to prevent infections in either patients undergoing surgery or patients with end-stage renal disease undergoing hemodialysis. For patients undergoing cardiothoracic surgery with median sternotomy, the use of a vaccine against *S. aureus* compared with a placebo did not reduce the rate of serious postoperative *S. aureus* infections and was associated with increased mortality among patients who developed *S. aureus* infections (271).

Since the majority of clinically recovered CoNS strains are methicillin resistant, most infections by CoNS require treatment with vancomycin or, where appropriate, the new agents described above. Replacement of these by β-lactamase-resistant penicillins is advisable for methicillin-susceptible isolates. When used simultaneously, antibiotics with cell wall activity (β-lactams and vancomycin) combined with rifampin were shown to act synergistically; however, this combination is not recommended for catheter-related bloodstream infections (272). FBRIs remain a therapeutic challenge and frequently require removal of the device (65, 273).

Prospective studies on the most appropriate treatment for patients infected with staphylococcal SCVs are unavailable. A reduced susceptibility to aminoglycosides can be expected (274). Resistance to trimethoprim-sulfamethoxazole is observed in thymidine-auxotrophic SCVs (58). Due to the fact that SCVs may persist intracellularly, a combined treatment regimen of rifampin (intracellular activity) with either β-lactam antibiotics or vancomycin (for methicillin-resistant SCVs) may be effective.

Micrococcaceae and *Dermacoccaceae*

Systematic data on susceptibilities of the two families *Micrococcaceae* and *Dermacoccaceae* are rare, and the real species affiliation in older reports is often unclear. Members of the genera *Micrococcus* and *Kocuria* appear to be susceptible to β-lactams, macrolides, tetracycline, linezolid, rifampin, and the glycopeptides; however, clinical isolates resistant to these agents have been reported (74, 275, 276). While most kytococcal isolates have been susceptible to carbapenems, gentamicin, ciprofloxacin, tetracycline, rifampin, and glycopeptides, kytococci are usually resistant to penicillin G, cephalosporins, and oxacillin (not *mecA* based) (4, 74, 275). An antibiotic regimen that has been suggested for the treatment of infection by members of both families is a combination of vancomycin with rifampin and gentamicin (277). *R. mucilaginosa* appears to be variable in its antimicrobial susceptibilities (276, 278). The observation that *R. mucilaginosa* exhibits poor to no growth on Mueller-Hinton agar makes susceptibility testing a challenge for clinical laboratories. Supplementation with 5% sheep blood and incubation in 6% CO_2 may enhance susceptibility testing (276, 278).

Alloiococcus

A. otitis has been reported as susceptible to ampicillin, cefotaxime, tetracycline, and vancomycin but resistant to macrolides, azithromycin, and co-trimoxazole (95, 279, 280). In addition to gentamicin-resistant isolates, some *A. otitis* isolates with intermediate levels of resistance to β-lactams have been reported, although they are β-lactamase negative.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Distinguishing contaminants and colonizers from staphylococcal and "micrococcal" isolates causing infection continues to be an important challenge for laboratorians and clinicians. It is imperative to have an appreciation of the quality of the specimen under consideration. Clinical features and the results of other investigations should be taken into account during the interpretative process. There is no replacement for good communication between laboratory staff and primary physicians.

Cultivation and identification of the causative pathogen to the species level represent the gold standard for the diagnosis of staphylococcal infections. The most critical step when interpreting a culture that is suspect for staphylococci is to distinguish between *S. aureus* and other species. Due to the importance of a report of "*S. aureus*" and, in particular, of "MRSA" for prognosis, therapy, hospital hygiene, and infection control, any uncertainty regarding the species identification or the susceptibility to oxacillin (cefoxitin) should be investigated via a second independent method. Considering costs and rapidity, applicable routine proceedings may comprise the use of respective latex agglutination assays combined with the use of automated systems for identification and susceptibility testing. In case of doubt, further tests, preferentially nucleic acid-based approaches, should be applied. However, the need for additional procedures must be weighed against delay in informing the physician concerning a preliminary *S. aureus* identification as the putative causative agent.

Species-level identification of CoNS associated with infection, especially in the case of a pure culture, has become customary. In particular, in the case of *S. lugdunensis*, species identification is mandatory for interpretation of the results of susceptibility testing. The same holds true for coagulase-positive *S. pseudintermedius*, which is often misidentified as CoNS due to negative results in latex agglutination assays. To rule out *S. saprophyticus* for urine isolates, the novobiocin test may be used. Isolates from deep-tissue infections and blood cultures of patients with suspected endocarditis should be differentiated to the species level, since the identification of *S. lugdunensis* raises the index of suspicion for aggressive disease. To identify *S. lugdunensis* from cultures of invasive and sterile sites or from abscesses and wound infections, its positivity for pyrrolidonase and ornithine decarboxylase may be considered (Tables 2 and 3). Other CoNS have also been increasingly described as causative pathogens of severe infection. Contaminants and colonizing CoNS do not require susceptibility testing or identification. Several reports suggest that cultures of colonially indistinguishable CoNS may contain multiple different strains (281). Extending the incubation period of the initial cultures to 72 h may enable different colony types to be more readily identified but also delays the time to the final culture result.

Unless there is strong evidence to the contrary, isolation of *S. aureus* from a sterile-site culture (such as aspirated pus, blood, or cerebrospinal fluid) should be considered clinically significant. Contamination of high-quality samples by *S. aureus* is rare, and further samples should be taken if there is clinical doubt. Interpreting the isolation of *S. aureus* from specimens contaminated with elements of a normal microbiota requires consideration of setting, clinical features, and recent interventions. Quantitative culture may be helpful, for example, when interpreting a bronchoalveolar lavage or urine sample. In patients with *S. aureus* endocarditis, bacteremia is continuous and associated with higher loads, while in cases with transient bacteremia (e.g., manipulations with mucous membrane trauma), bacteremia is associated with lower loads and typically has a short duration. Because quantitative blood cultures are not routinely performed, the time between incubation onset and growth detection (defined as the time to positivity) may provide additional information in continuous blood culture monitoring systems. Rapid growth of *S. aureus* (within 14 h after the initiation of incubation) has been associated with a high likelihood of endovascular infection, delayed clearance, and complications (282). Isolation of *S. aureus* from surgical wounds and other sites, such as ulcers, may represent infection or colonization, and the clinician's response to the culture report should be guided by a bedside assessment of signs and symptoms of infection. Colonization alone is an insufficient reason to treat, unless the patient is colonized by MRSA and decolonization is undertaken as part of a specific infection control policy.

Interpreting the significance of cultures that are positive for CoNS is more challenging than for *S. aureus*. CoNS are an important cause of nosocomial bloodstream infections, but they are also the most common contaminants of blood cultures (283). It is obvious that samples taken from colonized sites will contain members of these species. Blood samples or biopsy specimens taken without careful skin cleaning and disinfection will become contaminated with the microbiota of the skin or mucous membranes. However, even careful attention to collection techniques will not prevent all episodes of contamination. Interpretation of blood cultures positive for CoNS requires knowledge of the presence of prosthetic material in the intravascular compartment, risk factors for true CoNS sepsis, such as prematurity or the presence of an impaired immune system, and clinical features of sepsis. Factors helpful in distinguishing between true-positive cultures with clinical impact and therapeutic consequences and contaminated specimens taken from a patient with clinical features of infection include (i) isolation of a strain in pure culture from the infected site or body fluid and (ii) the repeated isolation of the same strain or combination of strains over the course of the infection (284, 285). An algorithm to reduce misclassification of nosocomial bloodstream infections due to CoNS was defined as at least two blood cultures positive for CoNS within 5 days or one positive blood culture plus clinical evidence of infection (286).

The presence of the same CoNS strain on an intravenous catheter tip and in a blood culture is supportive evidence for intravenous catheter-associated bacteremia. Measurement of differential times to positivity between blood cultures drawn through the central venous catheter and those drawn from the peripheral vein was reported as highly sensitive and specific for the *in situ* diagnosis of catheter-related bloodstream infection in patients with short- and long-term catheters; however, other studies did not show that this method was of major diagnostic value (287–289). Isolation of CoNS from peritoneal dialysis fluid or cerebrospinal fluid taken from ventricular shunts in a patient suspected of having infection is usually significant. While most contaminated clinical specimens produce mixed cultures of different strains and/or species, some infections may be attributable to more than one strain or species (281).

For patients with complicated cases who probably have a true CoNS infection, it is advisable to develop a sampling strategy. This is particularly pertinent when dealing with patients with low-grade infection associated with implanted prosthetic material, such as from a joint replacement or vascular graft. Samples should include those from each anatomical layer or region, and fresh instruments should be used to gather deep-site samples (290).

Susceptibility testing, and in particular the detection of methicillin resistance, should always be carried out following the identification of *S. aureus*. For surveillance cultures to detect MRSA colonization, susceptibility testing beyond the determination of methicillin resistance may be needed only for patients undergoing decolonization to predict the success of mupirocin therapy. All CoNS associated with true infection require susceptibility testing.

The considerations of clinical significance discussed for CoNS are also appropriate for members of the *Micrococcaceae* and *Dermacoccaceae* families; however, the criteria used for distinguishing etiologically relevant isolates from contaminants and colonizers, respectively, should be applied much more strictly. Since species of the *Kytococcus* genus are resistant to some β-lactams, they should be (if clinically relevant) carefully distinguished from other "micrococci" that are usually susceptible.

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Streptococcus

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22

TAXONOMY

The genus *Streptococcus* comprises currently more than 100 recognized species—a number that can certainly be expected to rise with the increasing availability of next-generation sequencing technologies. More than 10 novel species have been validly published in the last 5 years alone. Most of these were found in the oral cavity and gastrointestinal tract of various mammals and have so far not been shown to play a role in human infections. This chapter focuses on well-known and novel streptococcal species found in human specimens and covers changes that were applied to the taxonomy of streptococci during recent years. Streptococci are firmicutes of the order *Lactobacillales* and belong to the family of *Streptococcaceae*. Among the currently established 17 different genera of catalase-negative Gram-positive cocci are several genera that were split off from the genus *Streptococcus* some time ago, such as *Enterococcus* and *Lactococcus*, or more recently *Abiotrophia*, *Granulicatella*, *Facklamia*, and *Globicatella*. For excellent reviews on the topic, see references 1 and 2. While streptococcal species designation based solely on the hemolysis reaction, colony size, and the presence of Lancefield antigens has several limitations for correct species identification, this traditional streptococcal classification system is well established and still of value to the clinical microbiologist and health care provider. It correlates with clinical syndromes caused by different species and enables a first distinction of broad categories of streptococci that is useful for the choice of further tests and guidance of empirical treatments. The information and the identification schemes presented in this chapter therefore adhere in many aspects to the phenotypic classification system.

The classical differentiation of streptococci separates the group of beta-hemolytic streptococci from the group of non-beta-hemolytic streptococcal species. Beta-hemolytic streptococci, also referred to as pyogenic streptococci, include the human-pathogenic species *S. pyogenes*, *S. agalactiae*, *S. dysgalactiae* subsp. *equisimilis*, and a number of primarily veterinary pathogens. The designation "pyogenic streptococci" is more precise, since the group includes species that are non-beta-hemolytic like *S. dysgalactiae* subsp. *dysgalactiae* and the term excludes beta-hemolytic strains of the *S. anginosus* group, which belong to the viridans streptococcal group. The small colony size of streptococci from the anginosus group (≤0.5 mm) helps to distinguish them from the large-colony-forming (>0.5-mm colonies) streptococci of the pyogenic group. Species from the pyogenic or beta-hemolytic group are further characterized by the presence of Lancefield antigens, which correlate to some extent with the proper streptococcal species designations. While the B antigen is limited to *S. agalactiae*, the Lancefield group A antigen has been detected not only in *S. pyogenes* but also in *S. dysgalactiae* subsp. *equisimilis* isolates (3) and in species from the *S. anginosus* group (Table 1). Correlation with other Lancefield antigens is even more complicated, and molecular taxonomic studies led to novel species designations, presented below and in Table 1.

The group of nonpyogenic streptococci includes mostly alpha-hemolytic as well as nonhemolytic and even beta-hemolytic streptococcal species from the large category of viridans group streptococci. In a study of the genus *Streptococcus* based on sequence comparisons of small-subunit (16S) rRNA genes, five species groups of viridans group streptococci were demonstrated (4) in addition to the pyogenic group (beta-hemolytic, large-colony formers). These nonpyogenic groups were designated the *S. mitis* group, the *S. anginosus* group, the *S. mutans* group, the *S. salivarius* group, and the *S. bovis* group. Several streptococcal species were not unequivocally assigned and remain ungrouped (1, 2). Among alpha-hemolytic streptococci, *S. pneumoniae* can be separated from other streptococci of the viridans group through bile solubility and optochin susceptibility. However, phenotypic characterization and taxonomic considerations place *S. pneumoniae* into the *S. mitis* group (4). The relationship of *S. pneumoniae* to other species of the *S. mitis* group is so close that 16S rRNA gene analysis reveals greater than 99% identity to the nucleotide sequences of *S. mitis* and *S. oralis* and the current concept of separate species in this group has been challenged (5). A closely related species, *S. pseudopneumoniae*, was split off from *S. pneumoniae* several years ago (6). Strains are nonencapsulated, insoluble in bile, and optochin susceptible only when incubated in ambient air. Strains are often isolated from respiratory tract infections in patients with predisposing conditions such as chronic obstructive pulmonary disease (COPD) (7).

S. mitis, *S. sanguinis*, *S. parasanguinis*, *S. gordonii*, *S. cristatus*, *S. oralis*, *S. infantis*, *S. peroris* (8), *S. australis* (9), *S. oligofermentans* (10), *S. massiliensis* (11), *S. sinensis*, *S. oris-ratti*, *S. pseudopneumoniae*, *S. pneumoniae*, and the novel species *S. tigurinus* (12) and *S. lactarius* (13) are members of the *S. mitis* group. These species form a group whose classification and nomenclature have been a source of considerable confusion in the past. Among the changes that were made are the reclassification of the original *S. mitis*

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TABLE 1 Phenotypic characteristics of beta-hemolytic streptococci[a]

Species	Lancefield group(s)	Colony size[e]	Hosts[i]	Bacitracin susceptibility	PYR[f]	CAMP[g]	VP[h]	Hippurate hydrolysis	Trehalose	Sorbitol
S. pyogenes	A	Large	Humans	+	+	−	−	−	+	−
S. agalactiae	B	Large	Humans, cows	−	−	+	−	+	v	−
S. dysgalactiae subsp. *dysgalactiae*[b]	C	Large	Animals	−	−	−	−	−	+	v
S. dysgalactiae subsp. *equisimilis*	A, C, G, L	Large	Humans (animals)	−	−	−	−	−	+	−
S. equi subsp. *equi*	C	Large	Animals	−	−	−	−	−	−	−
S. equi subsp. *zooepidemicus*[c]	C	Large	Animals (humans)	−	−	−	−	−	−	+
S. canis[c]	G	Large	Dogs (humans)	−	−	+	−	−	v	−
S. anginosus group[d]	A, C, G, F, none	Small	Humans	−	−	−	+	−	+	−
S. porcinus[c]	E, P, U, V, none	Large	Swine (humans)	−	+	+	+	v	+	+

[a] Symbols and abbreviations: +, positive; −, negative; v, variable.

[b] *S. dysgalactiae* subsp. *dysgalactiae* is alpha-hemolytic on sheep blood agar plates.

[c] *S. equi* subsp. *zooepidemicus*, *S. canis*, and *S. porcinus* are primarily animal pathogens that are only rarely isolated from humans.

[d] Species included in the *S. anginosus* group can be beta-hemolytic, alpha-hemolytic, or nonhemolytic on sheep blood agar plates.

[e] Size: large, >0.5 mm after 24-h incubation; small, <0.5 mm.

[f] Presence of the enzyme pyrrolidonyl aminopeptidase.

[g] CAMP factor reaction (cohemolysis in the presence of the *Staphylococcus aureus* beta-hemolysin).

[h] Voges-Proskauer test (formation of acetoin from glucose fermentation).

[i] Parenthetical entries represent hosts from which the organism is only rarely isolated.

type strain as an *S. gordonii* strain and the subsequent replacement by a new *S. mitis* type strain (NCTC12261^{T}) (14).

The small-colony-forming *S. anginosus* group consists of the three distinct species *S. anginosus*, *S. constellatus*, and *S. intermedius* (15) and the two recently described species *S. anginosus* subsp. *whileyi* and *S. constellatus* subsp. *viborgensis* (16). It includes streptococcal species previously referred to as Lancefield group F streptococci, "*S. milleri*" group or "*S. milleri*," but "*S. milleri*" has no standing taxonomically. The *S. mutans* group comprises the species *S. mutans*, *S. sobrinus*, *S. criceti*, *S. ratti*, *S. downei*, and numerous species that have been identified only from animals thus far (*S. ferus*, *S. macacae*, *S. hyovaginalis*, and *S. devriesei*).

The human species *S. salivarius*, *S. vestibularis*, and *S. thermophilus*, which is found in dairy products, belong to the *S. salivarius* group. The whole *S. salivarius* group is closely related to the *S. bovis* group. Some streptococcal species that are currently part of the *S. bovis* group (*S. infantarius* and *S. alactolyticus*) (17) were formerly part of the *S. salivarius* group (1).

The *S. bovis* group has experienced extensive taxonomic changes (17–19) in the past. These changes were made because DNA-DNA reassociation studies revealed considerable heterogeneity among the human isolates described as *S. bovis* biotypes. Four DNA clusters are currently recognized. DNA cluster I consists of animal strains of *S. bovis* and *S. equinus*, which were shown to belong to a single species. The earlier species name *S. equinus* has been formally adopted. DNA cluster II consists of *S. gallolyticus*, with three subspecies: subsp. *gallolyticus* (formerly *S. bovis* biotype I), subsp. *pasteurianus* (formerly *S. bovis* biotype II.2), and subsp. *macedonicus*. DNA cluster III consists of *S. infantarius* (formerly *S. bovis* biotype II.1), with two subspecies: subsp. *infantarius* and subsp. *coli* (formerly called *S. lutetiensis*). DNA cluster IV consists of *S. alactolyticus*.

DESCRIPTION OF THE GENUS

Bacterial species belonging to the genus *Streptococcus* are catalase-negative, Gram-positive cocci of less than 2 μm that tend to grow in chains in liquid medium. Most species of the genus *Streptococcus* have a low G+C content of DNA ranging between 34 and 46%. The cell wall composition is typical for Gram-positive bacteria and consists mainly of peptidoglycan with glucosamine and muramic acid as amino sugars and galactosamine as a variable component. A variety of carbohydrates, surface protein antigens, and teichoic acid are attached to the cell wall and are, among other characteristics, responsible for intra- and interspecies differences among streptococci. Streptococci are facultative anaerobic bacteria. Due to a lack of heme compounds, streptococci are incapable of respiratory metabolism. Some species of the viridans streptococcal group and *S. pneumoniae* require 5% CO_2 levels for adequate growth, and the growth of many streptococcal species is enhanced in the presence of 5% CO_2. The optimum temperature for growth of most streptococci is around 37°C, while some species, like *S. uberis*, also grow at temperatures as low as 10°C. The complex nutritional requirements of streptococci are usually provided by the addition of blood or serum to the growth medium. Glucose and other carbohydrates are metabolized fermentatively with lactic acid as the major metabolic end product. The addition of glucose or other carbohydrates to liquid medium enhances growth but lowers the pH, resulting in growth inhibition unless the medium is highly buffered (e.g., Todd-Hewitt broth [THB]). Leucine aminopeptidase (LAP) is produced by all streptococci and enterococci but can also be found in lactococci, pediococci, and other catalase-negative Gram-positive cocci. LAP production helps to distinguish these species from the LAP-negative *Aerococcus* species and *Leuconostoc*. All streptococci are catalase negative upon exposure to 3% hydrogen peroxide with the exception of *S. didelphis*, a veterinary pathogen (20). False-positive catalase reactions may occur if bacteria are grown on blood-containing medium.

EPIDEMIOLOGY AND TRANSMISSION

Streptococci can cause infections in humans and in many different animal species, including mammals and fish. Different species of streptococci are frequently found as commensal bacteria on mucous membranes. Occasionally, streptococci are present as transient skin microbiota. Several species exhibit a high virulence potential, but even the highly pathogenic streptococcal species are frequently found as colonizing strains. *S. pneumoniae* was responsible for approximately 31,600 invasive infections in the United States in 2012, leading to an estimated 3,300 deaths (http://www.cdc.gov/abcs/reports-findings/surv-reports.html) and was found as a colonizing bacterial species in many asymptomatic carriers. The asymptomatic carriage rate for *S. pneumoniae* differs considerably between children and adults. Detection rates of 30 to 70% have been reported for young children, depending on the sampling method, while carriage rates among healthy adults are often reported to be below 5% (21, 22). Significantly higher colonization rates for adults living in households with preschool children suggests the occurrence of household transmission between parents and their children (23).

Due to active bacterial surveillance in the emerging infections program network (24), reliable epidemiologic data on invasive infections due to *S. pneumoniae* (described above), *S. pyogenes* (group A), and *S. agalactiae* (group B) have been obtained for a population of more than 30 million people in the United States during 2012. National estimates are that *S. pyogenes* caused 10,700 cases of invasive disease and 1,100 deaths with the peak of infections in people older than 65 years. Invasive infections due to *S. agalactiae* were second to those due to *S. pneumoniae*, with an estimated 28,150 cases and 1,865 deaths in 2012. Reflecting the ongoing changes in the epidemiology of group B streptococcal (GBS) disease, the highest attack rates were observed in patients less than 1 year and adults greater than 65 years of age. Apart from causing invasive infections, pyogenic streptococci are frequently encountered as colonizing strains. While asymptomatic pharyngeal colonization with *S. pyogenes* occurs in less than 5% of the adult population, *S. agalactiae* colonization rates of the urogenital and gastrointestinal tracts can be demonstrated in 10 to 30% of the female as well as the male population. No significant differences are observed in the colonization rates of pregnant and nonpregnant women.

Transmission of streptococcal infections can occur by different routes. Pathogenic species like *S. pyogenes* and *S. pneumoniae* are primarily transmitted through droplets or direct contact. Transmission can first lead to colonization with the potential for the development of a subsequent infection. Transmission from mother to child is typical for neonatal invasive *S. agalactiae* infections. Newborns acquire the bacteria usually during delivery, although prenatal transmissions leading to stillbirths occur. Postnatal transmissions, from the mother or health care personnel to infants, are probably responsible for the majority of late-onset neonatal infections. Another streptococcal species transmitted from mother to child is the tooth decay-causing species *S. mutans*; transmission occurs during early infancy most probably through oral secretions. Infections due to viridans group streptococci, which are part of the oral microbiota, do not require a prior transmission; they most often occur as endogenous infections.

Streptococcal infections do not represent classical zoonoses. Most species have a preferred host, while occasional animal-to-human transmissions do occur, as in the case of *Streptococcus suis* (25). Genotypic and phenotypic analyses of animal and human strains show distinct differences between strains causing human infections and veterinary isolates. For large-colony-forming group C and G streptococci, such an analysis led to an important change in species designations (26, 27). Currently, all beta-hemolytic group C and L and human group G streptococci are defined as *S. dysgalactiae* subsp. *equisimilis*, while alpha-hemolytic group C streptococcal animal isolates are classified as *S. dysgalactiae* subsp. *dysgalactiae* and animal group G streptococcal strains as *S. canis* (28). Other closely related veterinary species are *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*. The predominant reservoir for *S. dysgalactiae* subsp. *equisimilis* strains is the human host, and transmission usually occurs among humans.

CLINICAL SIGNIFICANCE

Streptococcus pyogenes (Group A Streptococci)

S. pyogenes colonizes the human throat and skin and has developed complex virulence mechanisms to avoid host defenses (29, 30). The upper respiratory tract and skin lesions serve as primary focal sites of infections and principal reservoirs of transmission. *S. pyogenes* can cause superficial or deep infections due to toxin-mediated and immunologically mediated mechanisms of disease. *S. pyogenes* is the most common cause of bacterial pharyngitis and impetigo. In the past, *S. pyogenes* was a common cause of childbed fever or puerperal sepsis. *S. pyogenes* is responsible for deep or invasive infections, especially bacteremia and sepsis, and deep soft tissue infections, such as erysipelas, cellulitis, and necrotizing fasciitis. Less common presentations include myositis, osteomyelitis, septic arthritis, pneumonia, meningitis, endocarditis, pericarditis, and severe neonatal infections following intrapartum transmission. One or more erythrogenic exotoxins produced by *S. pyogenes* may cause a confluent erythematous sandpaper-like rash characteristic of scarlet fever. While systemic toxic effects occur rarely with scarlet fever, severe clinical manifestations in streptococcal toxic shock syndrome (STSS) may result from massive superantigen-induced cytokine and lymphokine production. Nonsuppurative complications include poststreptococcal glomerulonephritis and acute rheumatic fever. While either of these conditions may follow pharyngitis, only glomerulonephritis is linked with skin infections due to *S. pyogenes*. *S. pyogenes* has also been associated with pediatric autoimmune neuropsychiatric disorders (31).

The causes of the emergence of STSS, frequently accompanied by necrotizing fasciitis, and the resurgence of invasive *S. pyogenes* infections since the mid-1980s are mostly unexplained (32). *S. pyogenes* remains exquisitely susceptible to penicillin. Efforts to find highly virulent clones include subtyping of *S. pyogenes* by serological determination of the surface M protein or genetic detection of the *emm* genes, encoding the M protein. However, despite the continuous exposure and subsequent type-specific immunity, the most prevalent M types associated with STSS continue to be M1 and M3, together accounting for approximately 50% of invasive infections. Since identical strains have accounted for less serious infections (33), host factors and comorbid conditions account for different diseases. The incidence of STSS seems highest among young children, particularly those with varicella, and the elderly. Other persons at risk include those with diabetes mellitus, chronic cardiac or pulmonary diseases, HIV infection, and intravenous drug or alcohol abuse. The risk for severe invasive infection in contacts has been estimated to be 200 times greater than for the general population, but most contacts are asymptomatically colonized (34).

Streptococcus agalactiae (Group B Streptococci)

S. agalactiae was first identified as the cause of bovine mastitis at the end of the 19th century. Since the 1970s, it has been reported as the cause of invasive neonatal infections. Neonatal infections present as two different clinical entities: early-onset neonatal disease, characterized by sepsis and pneumonia within the first 7 days of life; and late-onset disease with meningitis and sepsis between 7 days and 3 months of age. The most important risk factor for the development of invasive neonatal disease is the colonization of the maternal urogenital or gastrointestinal tract by *S. agalactiae*, which is found in 10 to 30% of pregnant women. Prevention of early-onset neonatal infections can be achieved in the majority of cases by administration of intrapartum antibiotic prophylaxis starting at least 4 hours before delivery. Official CDC recommendations for the prevention of neonatal *S. agalactiae* infections were first issued in 1996 and then revised in 2002 and 2010 (35). These guidelines resulted in a substantial decline of early-onset neonatal GBS disease. Invasive *S. agalactiae* infections of adult patients may be observed as postpartum infections or in immunocompromised adult patients with alcoholism, diabetes mellitus, cancer, or HIV infection (36). The spectrum of infections in adult patients includes pneumonia, bacteremia, meningitis, endocarditis, urinary tract infections, skin and soft tissue infections, and osteomyelitis.

Streptococcus dysgalactiae subsp. *equisimilis* (Human Group C and G Streptococci)

Human isolates of large-colony-forming beta-hemolytic streptococci harboring the Lancefield group C or group G antigens belong to this novel species (26, 27). While most isolates of this species possess either the Lancefield group C or G antigen, strains harboring the Lancefield group L as well as the group A antigen (3) have been described. The clinical spectrum of disease caused by *S. dysgalactiae* subsp. *equisimilis* resembles infections caused by *S. pyogenes* (28). The responsible strains harbor genes similar to virulence factor genes of *S. pyogenes*, such as *emm*-like genes, and can be isolated from upper respiratory tract infections, skin infections, soft tissue infections, and invasive infections, such as necrotizing fasciitis, STSS, bacteremia, and endocarditis. However, convincing reports about scarlet fever due to *S. dysgalactiae* subsp. *equisimilis* have so far not been published. Similar to what has been seen with *S. pyogenes*, cases of glomerulonephritis (GN) and acute rheumatic fever (ARF) have been reported (37, 38) following *S. equi* subsp. *zooepidemicus* (GN) and *S. dysgalactiae* subsp. *equisimilis* (GN and ARF) infections.

Streptococcus pneumoniae

S. pneumoniae is described separately in this section due to its clinical features that distinguish it from other species of the *S. mitis* group. *S. pneumoniae* is the most frequently isolated respiratory pathogen in community-acquired pneumonia (CAP). In as many as 30% of CAP cases, *S. pneumoniae* can be found in blood cultures of patients. *S. pneumoniae* is also a major cause of meningitis, leading to high morbidity and mortality in pediatric and adult patients. The most frequently observed infection due to *S. pneumoniae* is otitis media, with an estimate of one infection for every child up to the age of 6 in the United States. Other infections due to *S. pneumoniae* include sinusitis, peritonitis, and rare cases of endocarditis. *S. pneumoniae* colonizes the upper respiratory tract especially in children, without evidence of infection. Prevention of pneumococcal infections can be achieved by immunization with a 23-valent capsular polysaccharide vaccine in adults or the 13-valent conjugate vaccine in children. Widespread use of vaccines has resulted in a reduction of invasive pneumococcal infections during the past several years but also in changes of the serotypes responsible for invasive and noninvasive infections (39–41). The recent introduction of the 13-valent conjugate vaccine resulted in a general decrease of nasopharyngeal colonization rates with the vaccine-included serotypes in vaccinated children (42, 43). First data on the impact of the 13-valent vaccine on the burden of invasive disease in children are also promising (44, 45).

Streptococcus mitis Group

S. mitis, *S. sanguinis*, *S. parasanguinis*, *S. gordonii*, *S. cristatus*, *S. oralis*, *S. infantis*, *S. peroris*, *S. australis*, *S. sinensis*, *S. orisratti*, *S. oligofermentans*, *S. massiliensis*, *S. pseudopneumoniae*, and *S. pneumoniae* are members of this group. Members of the *S. mitis* group are regular commensals of the oral cavity, the gastrointestinal tract, and the female genital tract. The *S. mitis* group organisms can be found as members of the transient microbiota of the normal skin and may represent contaminants when isolated from blood cultures. At the same time, these species are the most frequently isolated bacteria in bacterial endocarditis in native valve and, less frequently, in prosthetic valve infections. Careful evaluation of the clinical situation is therefore crucial to correctly interpret the clinical significance of blood culture isolates from the *S. mitis* group. In neutropenic patients, streptococcal species from the *S. mitis* group are often responsible for life-threatening sepsis and pneumonia cases following immunosuppression by chemotherapy (46). Treatment of these infections is further complicated by high penicillin resistance rates.

Streptococcus anginosus Group

Species from this group (*S. anginosus*, *S. constellatus*, and *S. intermedius*) are commensals of the oropharyngeal, urogenital, and gastrointestinal microbiota. While these organisms are strongly associated with abscess formation in the brain, oropharynx, or the peritoneal cavity, their role as invasive pathogens may be underrecognized (47). In addition, the *S. anginosus* group has recently been implicated as an emerging pathogen in the respiratory tract of cystic fibrosis patients (48). All species of this group are small-colony-forming bacteria (colony size, ≤0.5 mm) that can display variable patterns of hemolysis (alpha, beta, or gamma). Since they can also harbor the Lancefield group antigens A, C, F, or G (or none at all), it is especially important to reliably distinguish them from large-colony-forming (>0.5-mm colony) beta-hemolytic streptococci of the pyogenic group. Association of certain species with specific isolation sites has been reported. While *S. anginosus* is frequently found in specimens from the urogenital or gastrointestinal tracts, *S. constellatus* is commonly isolated from the respiratory tract, and *S. intermedius* is most often identified in abscesses of the brain or liver.

Streptococcus salivarius Group

Streptococcal species that belong to the *S. salivarius* group include *S. salivarius* and *S. vestibularis*. They have been primarily isolated from the oral cavity and blood. Another species of this group, *S. thermophilus*, is found only in dairy products. *S. salivarius* has been repeatedly reported as a cause of bacteremia, endocarditis, and meningitis (sometimes iatrogenic), while *S. vestibularis* has not been clearly associated with human infection. Isolation of *S. salivarius* from blood cultures does correlate to some extent with neoplasia (49, 50).

Streptococcus mutans Group

S. mutans and *S. sobrinus* belong to the *S. mutans* group. They are the most commonly isolated species of the group that originate from human clinical specimens, usually obtained from the oral cavity. *S. criceti*, *S. ratti*, and *S. downei* have occasionally been identified from human sources, while the other streptococcal species of the *S. mutans* group (*S. ferus*, *S. macacae*, *S. hyovaginalis*, and *S. devriesei*) have been identified only in animals. *S. mutans* is the primary etiologic agent of dental caries, and infection is transmissible. By 18 years of age, 85% of the population have at least one carious lesion (51). Permanent colonization with *S. mutans* occurs under normal living conditions in the Western world between the second and the end of the third year of life (51). Molecular analysis of mother and infant isolates reveals that strains are usually acquired from the mother and that the colonization rate of infants depends on the bacterial load of the mother (52). Analyses of streptococcal blood culture isolates show that *S. mutans* is the most frequently isolated species of this group in cases of bacteremia (1).

Streptococcus bovis Group

Extensive taxonomic changes have occurred in this group, and strains formerly known as human *S. bovis* isolates are designated as different species (see "Taxonomy" above). The group now includes *S. equinus*, *S. gallolyticus*, *S. infantarius*, and *S. alactolyticus*. Species from this group are frequently encountered in blood cultures of patients with bacteremia, sepsis, and endocarditis. The clinical significance of blood cultures growing streptococci from the *S. bovis* group lies in the association of (i) *S. gallolyticus* subsp. *gallolyticus* with gastrointestinal disorders, including colon cancer and chronic liver disease, and (ii) *S. gallolyticus* subsp. *pasteurianus* with meningitis (53–55).

Other Streptococci Infrequently Isolated from Human Specimens

Streptococcal species that are primarily animal pathogens are sometimes isolated from human hosts, in most cases from humans that are in close contact with animals. *S. suis*, *S. porcinus*, and *S. iniae* belong to this category. *S. suis* is a swine pathogen that has occasionally been isolated from cases of human meningitis and bacteremia. *S. suis* is encapsulated and appears to be alpha-hemolytic on sheep blood agar plates, although some strains are beta-hemolytic on horse blood agar. *S. suis* strains are positive for the Lancefield group antigen R, S, or T, which helps to distinguish them from the phenotypically similar species *S. gordonii*, *S. sanguinis*, and *S. parasanguinis*. Similar to *S. suis*, *S. porcinus* (Lancefield groups E, P, U, and V) is primarily a swine pathogen. Beta-hemolytic *S. porcinus* strains have rarely been isolated from human sources such as peripheral blood, wounds, and the female genital tract (56). Molecular studies of *S. porcinus* isolates from the female genital tract, however, indicate that these isolates belong to a novel species designated *S. pseudoporcinus* (57). *S. pseudoporcinus* may be more prevalent in the female genital tract than previously assumed (58). Strains can easily be misidentified as *S. agalactiae* due to isolation from the female genital tract, false-positive reactions with commercially available group B antisera, and a positive CAMP test reaction. *S. porcinus* and *S. pseudoporcinus* can be L-pyrrolidonyl-beta-naphthylamide (PYR) positive and do not hydrolyze hippurate, in contrast to *S. agalactiae*. *S. iniae* is a fish pathogen that is beta-hemolytic but does not possess any Lancefield group antigens. It has been isolated from soft tissue infections, bacteremia, endocarditis, and meningitis in people handling fish (59, 60). *S. iniae* isolates resemble *S. pyogenes* strains due to the fact that both are PYR positive. Beta-hemolysis of the species can be observed only around agar stabs or under anaerobic culture conditions. Commercial identification systems do not correctly identify the species; the failure to react with Lancefield group antisera is important to notice, since it is rare among beta-hemolytic streptococci. Very recently, a novel streptococcal species associated with the handling of fish has been reported as a human pathogen. The species has been designated *S. hongkongensis*; it is closely related to *S. iniae* and *S. parauberis* (61).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Specimens suspected of harboring streptococci should be collected by the methods outlined elsewhere in this *Manual* (chapter 18). Since many streptococcal species lose viability fairly quickly, it is best to place swabs in an appropriate moist transport medium and process specimens rapidly. If transport time is below 1 to 2 h, a special transport system is not absolutely necessary. *S. pyogenes* can safely be transported on dry swabs; desiccation enhances recovery from mixed cultures by suppression of the accompanying microbiota (62). Detailed recommendations for collection and storage of swabs from pregnant women to detect *S. agalactiae* colonization have been issued by the U.S. Centers for Disease Control and Prevention (35). These recommendations are summarized below under "Special Procedures for *Streptococcus agalactiae* Screening."

DIRECT EXAMINATION

Microscopy

Microscopic examination shows streptococci as Gram-positive bacteria growing in chains of various lengths. *S. pneumoniae* isolates most often present as Gram-positive diplococci with an elongated appearance, but a reliable microscopic distinction of *S. pneumoniae* from enterococci and other streptococci is not possible. In blood culture specimens, *S. pneumoniae* tends to form chains of varying lengths, similar to other streptococci. Direct identification of streptococci by microscopic methods is most helpful in the case of clinical specimens from sterile body sites, such as cerebrospinal fluid. Tiny, irregular cocci in clumps of chains seen in abscess- or peritonitis-associated aspirates are suggestive of the *S. anginosus* group. Interpretation of Gram stain results from nonsterile body sites is difficult due to the residential microbiota, which frequently includes streptococci. Thus, for example, throat swabs should not be examined by Gram stain for diagnosis of "strep" throat.

Direct Antigen Detection of *S. pyogenes* from Throat Specimens

S. pyogenes is the most common cause of acute pharyngitis and accounts for 15 to 30% of cases of acute pharyngitis in children and 5 to 10% of cases in adults. If the diagnosis can be provided rapidly, antibiotic therapy can be initiated promptly to relieve symptoms, to avoid sequelae, and to reduce transmission. Numerous assays for direct detection of the group A-specific carbohydrate antigen in throat swabs by agglutination methods or immunoassays (enzyme, liposome, or optical), also referred to as "rapid antigen assays," have become commercially available during the past 2 decades. A list of FDA-cleared tests is accessible via the Internet (http://www.accessdata.fda.gov/scripts/cdrh/devicesatfda/index.cfm?Search_Term=866.3740). Although these tests provide rapid results and allow early treatment decisions, the throat culture remains the gold standard. Sensitivities of rapid antigen tests range from 70% to 96% and have never equaled that of culture (63, 64). Negative rapid antigen test results should therefore be confirmed by culture in children and adolescents when typical clinical signs are present (65). In adults, the confirmation of negative rapid antigen test results has not been regarded as necessary for many years, but the most recent Infectious Diseases Society of America (IDSA) guidelines challenge this concept (66). The specificity, however, is generally high, even though false-positive antigen results are seen from patients previously diagnosed and/or treated for *S. pyogenes* (67). Moreover, the low positive predictive value of rapid group A antigen tests in the adult population frequently results in prescribing unnecessary antimicrobial therapy (68).

Antigen Detection of *S. agalactiae* in Urogenital Tract Samples

Several different commercially available antigen detection tests have been developed for the identification of *S. agalactiae* in samples from the urogenital tract. Independent from the technique involved (latex agglutination, enzyme immunoassay, or optical immunoassay), without a prior cultural enrichment step, all of the currently available tests lack sufficient sensitivities to detect bacterial colonization with *S. agalactiae* (69). They are not recommended for screening of pregnant women by the CDC (35).

Antigen Detection of *S. pneumoniae* in Urine Samples

An immunochromatographic membrane test relying on the detection of the cell wall-associated polysaccharide that is common to all *S. pneumoniae* serotypes (C-polysaccharide antigen) (Binax NOW; Binax Inc., Portland, ME) has proven helpful for the identification of *S. pneumoniae* infections in adult patients, especially in patients that already received antibiotic treatment. In contrast to conventional diagnostic methods, reported sensitivities of antigen detection in urine samples range between 50 and 80%, and specificities are higher than 90% (70, 71). Following pneumococcal infection, the test can remain positive for about 1 to 6 months (72). Due to the fact that the test is also positive in *S. pneumoniae* carriage without infection, as is often observed among infants (73), it is of limited value in pediatric patients. The test should not be used in children below the age of 6 (73), and comprehensive studies on schoolchildren with lower colonization rates have not been performed. It can currently be recommended only in adults as an addition to conventional diagnostic culture techniques for *S. pneumoniae* (74) and is probably most helpful in patients who received antimicrobial treatment before cultures were obtained.

Streptococcal Antigen Detection in CSF

Commercially available antigen detection tests for the diagnosis of pathogenic microorganisms in cerebrospinal fluid (CSF) samples include reagents for the detection of *S. agalactiae* and *S. pneumoniae*. These tests have also been used on positive blood culture specimens. The tests are not recommended for routine use, as the results should not be used to change decisions about empiric therapy based on clinical and laboratory criteria (75). It has also been shown that the sensitivity of direct antigen detection in CSF is low (<30%) and offers no advantage over a conventional Cytospin Gram stain (76). However, very promising results have been published for the use of the *S. pneumoniae* urinary antigen test on CSF samples (77), an application for which the test received FDA clearance.

Nucleic Acid Detection Techniques

S. pyogenes

A rapid method for the detection of *S. pyogenes* in pharyngeal specimens is based on a single-stranded chemiluminescent nucleic acid probe assay to identify specific rRNA sequences (Group A Streptococcus Direct Test [GP-ST]; Gen-Probe, Inc., San Diego, CA). This test performed well in comparative studies with the culture technique. Sensitivity and specificity for the probe test ranged from 89% to 95% and from 98% to 100%, respectively, in contrast to the results of the culture technique with a sensitivity of 98% to 99% (67, 78). These data suggest that the probe test may be suitable as a primary test or as a backup test for negative antigen tests, particularly for batch screening of throat cultures. Recently, a commercial PCR for the direct detection of *S. pyogenes* using the Illumigene system (Meridian Bioscience Inc., Cincinnati, OH) received FDA clearance. A multicenter evaluation study revealed an excellent sensitivity (99%) and specificity (99.6%) for this test (79).

S. agalactiae

Several nucleic acid-based methods for the detection of *S. agalactiae* colonization in pregnant women have been developed and evaluated (80–82). Currently, 6 nucleic acid amplification tests (NAATs) received FDA clearance, of which 3 are performed following cultural enrichment while the 3 other tests are designed to be used directly on clinical samples. Molecular platforms used in these assays include the BD max system (Becton, Dickinson), the Smartcycler and Xpert technology (Cepheid, Sunnyvale, CA), and the Illumigene system (Meridian Bioscience). A current list of the approved tests can be accessed at the following website: http://www.fda.gov/medicaldevices/productsandmedicalprocedures/invitrodiagnostics/ucm330711.htm. While the costs are exceedingly higher than for selective culture, these tests were designed to provide results within a short time, in order to be able to assess vaginal and/or rectal colonization status at the time of delivery. Although PCR tests performed at the time of delivery provide rapid results, this is often not sufficient to allow effective administration of peripartum antibiotics, since many women deliver within a few hours after hospital admission. Therefore, the CDC does not recommend performing routine screening by NAATs at delivery. The most recent CDC recommendations for antenatal *S. agalactiae* screening include detection by NAAT, but only after prior cultural enrichment in selective broth (35). Enrichment procedures considerably increase the sensitivity of NAAT to detect *S. agalactiae* colonization.

S. pneumoniae

Several different laboratory-developed NAATs have been developed for the identification of *S. pneumoniae* from culture isolates. Tests are based on the detection of the genes for autolysin *lyt*A, the pneumococcal surface antigen *psa*A, and the pneumolysin gene *ply*. Comparison of the ability to distinguish difficult-to-identify *S. pneumoniae* strains and closely related atypical streptococci revealed that the *lyt*A-based PCR was the most specific method (83, 84). While results based on the detection of *psa*A are also acceptable, the different pneumolysin-targeted methods appear to be relatively nonspecific. So far, none of these assays are commercially available, and they have to be established as laboratory-developed tests. A quantitative real-time PCR based on the detection of the *lyt*A gene directly from nasopharyngeal swabs has recently been published (85). The study showed promising results for the identification of patients presenting with pneumococcal pneumonia. Nucleic acid probes for the confirmation of cultured isolates as *S. pneumoniae* are commercially available (AccuProbe; GenProbe, San Diego, CA) (86). Detection relies on hybridization of a specific probe to 16S rRNA sequences, but the test fails to distinguish *S. pseudopneumoniae* from *S. pneumoniae*. These tests are not routinely performed for standard identification procedures but can aid in the identification of atypical *S. pneumoniae* isolates with unusual patterns of bile solubility and optochin susceptibility.

ISOLATION PROCEDURES

General Procedures

Streptococci are usually grown on blood agar medium because the assessment of the hemolytic reaction is important for identification. Growth of streptococci is often enhanced in the presence of an exogenous catalase source. Streptococcal species with low or absent hydrogen peroxide production, such as *S. agalactiae*, can be grown on other commonly used nonselective media without blood.

Agar media selective for Gram-positive bacteria (e.g., phenylethyl alcohol-containing agar or Columbia agar with colistin and nalidixic acid) support the growth of streptococci. The optimal incubation temperature range for most streptococcal species lies between 35°C and 37°C. Supplemental carbon dioxide (5% CO_2) or anaerobic conditions enhance the growth of many streptococcal species since streptococci are facultative anaerobes. Although some streptococci grow well in ambient air, incubation in 5% CO_2 is recommended for the culture of *S. pneumoniae* and other streptococcal species of the viridans group.

Special Procedures for *Streptococcus pyogenes* Throat Cultures

A properly performed and interpreted throat culture on a 5% sheep agar with Trypticase soy base incubated in air remains the gold standard for the diagnosis of *S. pyogenes* acute pharyngitis (87). The isolation of only a few colonies of *S. pyogenes* does not allow the differentiation between a carrier and an acutely infected individual and may reflect inadequate specimen collection (88). Lack of hemolysis, overgrowth and production of toxic bacterial metabolites by the normal upper respiratory tract microbiota, or depletion of substrates often leads to false-negative results or delays caused by labor-intensive reisolation steps. In order to enhance *S. pyogenes* isolation, numerous studies analyzed incubation conditions in anaerobic or CO_2-enriched atmosphere as well as different media selective for beta-hemolytic streptococci (89–91). Due to cost restraints and an uncertain benefit, these additional efforts are not generally recommended for *S. pyogenes* but may increase the yield of other beta-hemolytic streptococci. After 18 to 24 h of incubation, culture plates should be examined for growth of beta-hemolytic colonies. Negative cultures should be reexamined after an additional 24-h incubation period. Presumptive identification of *S. pyogenes* can be achieved by susceptibility to bacitracin or testing for PYR activity. Other beta-hemolytic streptococci are occasionally positive in one of these tests but not in both. Definitive diagnosis includes the demonstration of the Lancefield group A antigen by immunoassay. Although other species may rarely possess the group A antigen (Table 1), they lack PYR activity (1).

Special Procedures for *Streptococcus agalactiae* Screening

Early-onset neonatal group B streptococcal (*S. agalactiae*) infections can be prevented by administration of antibiotic prophylaxis during delivery (92). An essential requirement for efficient prophylaxis is the reliable detection of colonization with *S. agalactiae* before delivery. Screening should be performed between weeks 35 and 37 of pregnancy. A lower vaginal and rectal swab (i.e., inserted through the anal sphincter) should be obtained with either one swab or two different swabs and placed in an appropriate transport medium (Amies or Stuart's with or without charcoal; see chapter 18). While culture counts decline to some extent, the viability of *S. agalactiae* is preserved in transport medium kept at room temperature or 4°C for up to 4 days. To reduce costs, vaginal and rectal swabs can be placed in a single transport medium tube and cultured together. Swabs should be cultured in selective broth medium for 18 to 24 hours at 35 to 37°C in ambient air or 5% CO_2 and subsequently plated on tryptic soy agar (TSA) blood agar plates or *S. agalactiae* selective agar medium. Selective broth medium is commercially available (Trans-Vag broth supplemented with 5% sheep blood from Thermo Fisher Scientific Remel Products, Lenexa, KS; or LIM broth from BD BBL, Franklin Lakes, NJ). Selective broth can also be prepared by supplementation of Todd-Hewitt Broth with nalidixic acid (15 μg/ml) and colistin (10 μg/ml) or supplementation of THB with nalidixic acid (15 μg/ml) and gentamicin (8 μg/ml). TSA blood agar plates should be checked for typical colonies (narrow zone of beta-hemolysis) of *S. agalactiae* after 24 and 48 h of incubation at 35 to 37°C. Identification of *S. agalactiae* is then achieved by standard techniques as described below. Selective media relying on the detection of the orange *S. agalactiae* pigment (Granada medium, StrepB Carrot broth from Hardy Diagnostics, Santa Maria, CA; or GBS broth from Northeast Laboratory Services, Winslow, ME) are highly specific and sensitive (93, 94). Subculture of enrichment broth on Granada medium enhances sensitivity and obviates the need for further identification steps due to excellent specificity, but nonhemolytic strains cannot be detected with pigment-dependent selective medium. NAAT-based detection of *S. agalactiae* following overnight enrichment broth increases sensitivity and reduces detection time in comparison to conventional selective culture (95). It is recommended as an alternative screening method by the most recent CDC guidelines (35). For the identification of questionable cultured strains as *S. agalactiae*, a 16S RNA-based nucleic acid detection test (AccuProbe; GenProbe, San Diego, CA) or matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF MS) can be helpful.

IDENTIFICATION

Description of Colonies

Colonies of streptococci usually appear gray or almost white with moist or glistening features. Dry colonies are rarely encountered. Colony size varies between the different beta-hemolytic species and helps to distinguish groups of streptococci. Beta-hemolytic streptococci of the pyogenic group (*S. pyogenes*, *S. agalactiae*, and *S. dysgalactiae* subsp. *equisimilis*) form colonies of >0.5 mm after 24 h of incubation, in contrast to beta-hemolytic strains of the *S. anginosus* group (formerly called the "*S. milleri*" group), which present with pinpoint colonies of ≤0.5 mm after the same incubation time (Fig. 1). Members of the *S. anginosus* group emit a distinct odor resembling butterscotch or caramel, presumably due to the production of diacetyl by the species belonging to this group. Among the beta-hemolytic species of the pyogenic group, *S. agalactiae* produces the largest colonies with a relatively small zone of hemolysis. Nonhemolytic *S. agalactiae* strains do occur and resemble enterococci.

Within the group of alpha-hemolytic streptococci, *S. pneumoniae* has a colony morphology that helps to distinguish pneumococcal isolates from other streptococci of the viridans group. Due to the production of capsular polysaccharide, colonies glisten and appear moist. Colonies may be large and mucoid if large amounts of capsular polysaccharide are made, a feature often encountered in serotype 3

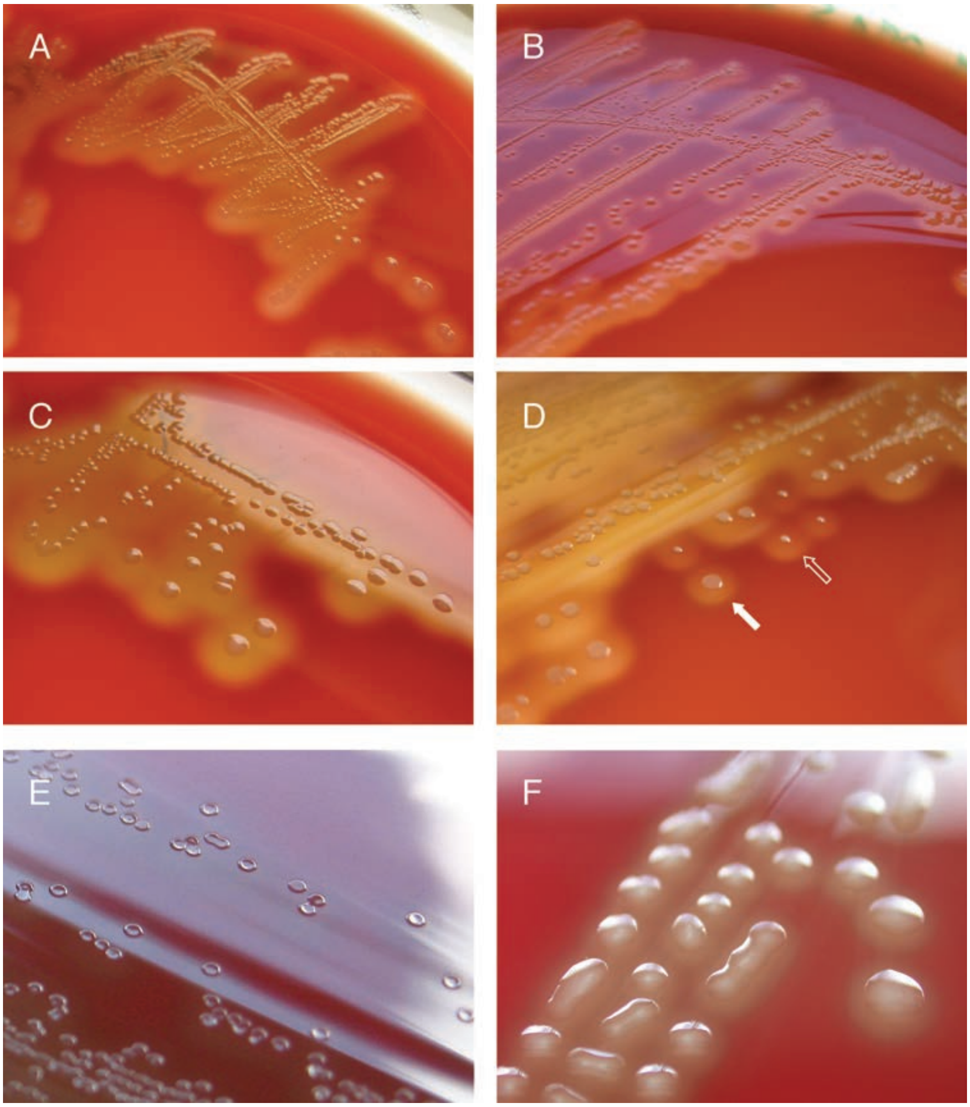

FIGURE 1 Colony morphology of selected streptococci. (A) *S. pyogenes* strain ATCC12344; (B) clinical isolate of *S. agalactiae*; (C) clinical isolate of *S. dysgalactiae* subsp. *equisimilis* (Lancefield group G); (D) mixed culture of *S. anginosus* (ATCC12395, open arrow) and clinical isolate of *S. dysgalactiae* subsp. *equisimilis* (closed arrow) (note the difference in colony size); (E) clinical isolate of *S. pneumoniae* (note the central depression of the colonies); (F) clinical isolate of *S. pneumoniae* (note the mucoid appearance of colonies). doi:10.1128/9781555817381.ch22.f1

strains. This phenotype is usually typical for *S. pneumoniae* but can also occasionally be observed in *S. pyogenes*. Another characteristic feature of *S. pneumoniae* is the central navel-like depression of the colonies that is caused by the pneumococcal autolysin. Other viridans group streptococci lack this feature and have a dome-like appearance; however, up to 20% of *S. pneumoniae* strains display a phenotype that is indistinguishable from that of viridans group streptococci (96). Nonhemolytic gray colonies are typical for species of the *S. bovis* and *S. salivarius* groups. Typical streptococcal colony morphologies are presented in Fig. 1.

Identification of Beta-Hemolytic Streptococci by Lancefield Antigen Immunoassays

Commercially available Lancefield antigen grouping sera are primarily used for the differentiation of beta-hemolytic streptococci. Products for rapid antigen extraction and subsequent agglutination can be obtained from many different suppliers. The presence of the Lancefield group B antigen in beta-hemolytic isolates from human clinical specimens correlates with the species *S. agalactiae*, but cross-reactivity of the group B antigen with the newly described species *S. pseudoporcinus* has been reported (58). Similarly, the detection of the Lancefield group F antigen in small-colony-forming streptococci from human clinical material allows a fairly reliable identification of a strain as a member of the *S. anginosus* group. The presence of Lancefield group A, C, or G antigens necessitates further testing (Table 1). Beta-hemolytic streptococcal strains not reacting with any of the Lancefield antisera are rare and should be further identified by phenotypic tests or nucleic acid detection techniques.

Identification of Beta-Hemolytic Streptococci with Phenotypic Tests and MALDI-TOF MS

A number of streptococcal identification products incorporating batteries of physiologic tests are commercially available (see chapters 4 and 19). In general, these products perform well with commonly isolated pathogenic streptococci but may lack accuracy for identifying streptococci of the viridans group. The MALDI-TOF (Bruker Daltonics, Billerica, MA)-based bacterial identification also has limitations concerning several streptococcal species. While among beta-hemolytic streptococci, identification of *S. pyogenes* and *S. agalactiae* corresponds well to conventional tests, the correct identification of *S. dysgalactiae* to the species level cannot be achieved for the majority of isolates (97). Further problems include the misidentification of *S. mitis* or *S. oralis* as *S. pneumoniae* and continuing difficulties in the correct species identification of viridans group streptococci. For the bulk of pathogenic streptococci isolated in clinical laboratories (e.g., *S. pyogenes*, *S. agalactiae*, and *S. pneumoniae*), serologic or presumptive physiologic tests (as described below) still offer an acceptable alternative to commercially available identification systems.

PYR Test

The presence of the enzyme pyrrolidonyl aminopeptidase is often tested to distinguish *S. pyogenes* from other beta-hemolytic streptococci. Hydrolysis of L-pyrrolidonyl-beta-naphthylamide by the enzyme to β-naphthylamide produces a red color with the addition of cinnamaldehyde reagent (see chapter 19). The beta-hemolytic streptococcal species *S. iniae*, *S. porcinus*, and *S. pseudoporcinus* can be PYR positive but are only rarely identified in human clinical specimens. PYR spot tests are commercially available. It is important to distinguish *Streptococcus* from *Enterococcus* prior to PYR testing, and strains of other related genera may be PYR positive (including the genera *Abiotrophia*, *Aerococcus*, *Enterococcus*, *Gemella*, and *Lactococcus*). However, PYR-positive beta-hemolytic enterococcal isolates typically present with a different colonial morphology (smaller zone of beta-hemolysis and bigger colony size) and, when combined with other phenotypic characteristics (see chapter 23), may be distinguished from streptococci. To avoid false-positive reactions caused by other PYR-positive bacterial species (for example, staphylococci), the test should be performed only on pure cultures.

Bacitracin Susceptibility

With rare exceptions, *S. pyogenes* displays bacitracin susceptibility, in contrast to other human beta-hemolytic streptococci. Together with Lancefield antigen determination, it can be used for the identification of *S. pyogenes*, since beta-hemolytic strains of other streptococcal species that may contain the group A antigen are bacitracin resistant. The test can also be used to distinguish *S. pyogenes* from other PYR-positive beta-hemolytic streptococci (*S. iniae* and *S. porcinus*). A bacitracin disk (0.04 U) is applied to a sheep blood agar (SBA) plate that has been heavily inoculated with 3 or 4 colonies of a pure culture of the strain to be tested. It is important to perform the test from a subculture on SBA, since placement of bacitracin disks on primary plates is not sensitive enough. After overnight incubation at 35°C in 5% CO_2, any zone of inhibition around the disk is interpreted as indicating susceptibility. Importantly, bacitracin-resistant *S. pyogenes* isolates have been reported and clusters of bacitracin-resistant strains were observed in several European countries (98–100).

VP Test

The Voges-Proskauer (VP) test detects the formation of acetoin from glucose fermentation. It is performed on streptococci as a modification of the classical VP reaction that is used for the differentiation of enteric bacteria. Small-colony-forming beta-hemolytic streptococci of the *S. anginosus* group that are VP positive may be distinguished from large-colony-forming beta-hemolytic streptococci harboring identical Lancefield antigens (A, C, or G). Streptococci of the *S. mitis* group are VP negative. For the modified VP reaction as described by Facklam and Washington in the 5th edition of this *Manual* (160), the culture growth of an entire agar plate is used to inoculate 2 ml of VP broth and incubated at 35°C for 6 h. Following the addition of 5% α-naphthol and 40% KOH, the tube is shaken vigorously for a few seconds and incubated at room temperature for 30 min. A positive test yields a pink-red color that results from the reaction of diacetyl with guanidine.

BGUR Test

Detection of β-glucuronidase (BGUR) activity distinguishes *S. dysgalactiae* subsp. *equisimilis* strains containing Lancefield group antigen C or G from BGUR-negative, small-colony-forming streptococci of the *S. anginosus* group with the same Lancefield group antigens. Rapid methods for the BGUR test are commercially available. Alternatively, a rapid fluorogenic assay with methylumbelliferyl-β-D-glucuronide (MUG)-containing MacConkey agar, often used for *Escherichia coli*, has been described (101).

CAMP Test

The CAMP factor reaction was first described in 1944 by Christie, Atkins, and Munch-Petersen and refers to the synergistic lysis of erythrocytes by the beta-hemolysin of

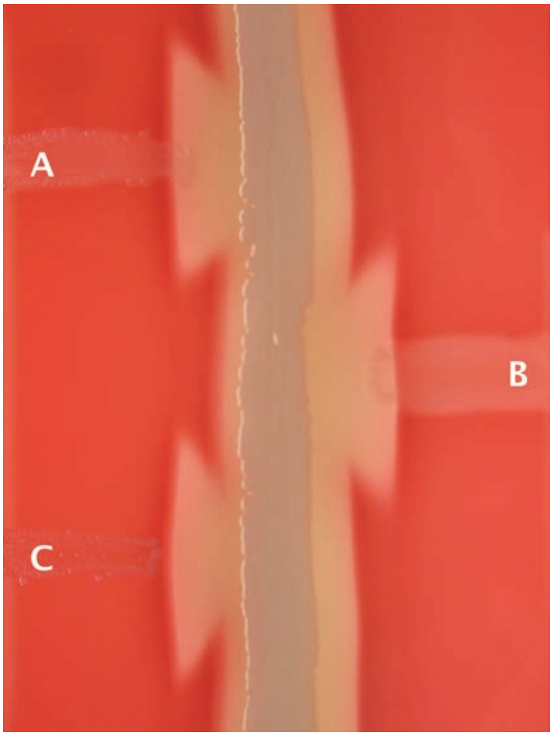

FIGURE 2 CAMP factor test. Arrowhead-shaped zone of hemolysis in the zone of the *S. aureus* beta-hemolysin. (A) Clinical isolate of a weakly beta-hemolytic *S. agalactiae* strain. (B) Beta-hemolytic *S. agalactiae* strain O90R. (C) Nonhemolytic *S. agalactiae* strain R268.
doi:10.1128/9781555817381.ch22.f2

Staphylococcus aureus and the extracellular CAMP factor of *S. agalactiae*. The gene (*cfb*) and its expression can be demonstrated in the vast majority (>98%) of *S. agalactiae* isolates, but CAMP-negative mutants do occur. The strain to be tested and a *Staphylococcus aureus* strain (ATCC 25923) are streaked onto a sheep blood agar plate at a 90° angle. Plates are incubated in ambient air overnight at 36 ± 1°C. A positive reaction can be detected by the presence of a triangular zone of enhanced beta-hemolysis in the diffusion zone of the beta-hemolysin of *S. aureus* and the CAMP factor (Fig. 2). CAMP factor-positive strains can also be detected by a method using β-lysin-containing disks (Remel Inc., Lenexa, KS) or by a rapid CAMP factor spot method (102). Despite the fact that close homologs of the CAMP factor gene are present in many *S. pyogenes* strains, most beta-hemolytic streptococci other than *S. agalactiae* are negative in the above-described CAMP factor test, except for the rare human isolates of *S. iniae*, *S. porcinus*, and *S. pseudoporcinus*. Several Gram-positive rods including corynebacteria and *Listeria monocytogenes* strains may also be CAMP factor positive.

Hippurate Hydrolysis Test

The ability to hydrolyze hippurate is an alternative test for the presumptive identification of *S. agalactiae*. A rapid version of the test, as it is used for campylobacters, can be performed by incubating a turbid suspension of bacterial cells in 0.5 ml of 1% aqueous sodium hippurate for 2 h at 35°C. Glycine formed as an end product of hippurate hydrolysis is detected by adding ninhydrin reagent and observing the development of a deep purple color, signifying a positive test (103). Streptococci other than *S. agalactiae* may also be hippurate hydrolysis positive, especially viridans group streptococci.

Identification of *S. pneumoniae* and Viridans Group Streptococci

The correct species identification of viridans group streptococci other than *S. pneumoniae* is challenging. Recent taxonomic changes and identification of novel streptococcal species have further complicated matters. The number of recognized species in this group is now greater than 30. The viridans group includes alpha-hemolytic, nonhemolytic (*S. salivarius* group and *S. bovis* group), and beta-hemolytic (*S. anginosus* group) streptococcal strains. All of the viridans group streptococci are leucine aminopeptidase positive and pyrrolidonyl arylamidase negative. Conventional microbiologic tests are limited with respect to species identification but are helpful in placing isolates into the correct streptococcal groups (Table 2). Beighton et al. described an identification scheme based on phenotypic tests that allowed the differentiation and correct species identification of the majority of viridans group species (104). The scheme requires the evaluation of enzymatic reactions performed by in-house fluorogenic tests that are not commercially available. Importantly, most clinical laboratories must strive for group, instead of species, classifications with current phenotypic test panels.

The API tests (bioMérieux, Marcy l'Etoile, France) offer species identification of viridans group streptococci. While many species from this group are identified with acceptable accuracy, several species have not been included in the database. Comparisons of molecular species identification by DNA reassociation studies with the results of the API Rapid ID 32 Strep system showed that more than 85% of 156 strains from streptococcal species included in the database were correctly identified (105). However, for species not included in the database, more than 50% were incorrectly identified by the test (105). Evaluation studies performed under routine clinical conditions appear to yield less favorable results (106). Evaluations of the Vitek 2 (bioMérieux) automated phenotypic identification system showed that streptococcal group assignment for 75% of isolates were concordant with 16S rRNA gene sequencing data (107). In another study, correct identification at the species level was accomplished for only 55% of the tested isolates (108). Similarly, some publications on MALDI-TOF MS technology report major problems with the correct species identification of streptococci from the viridans group and a frequent misidentification of *S. pneumoniae* (97, 109). However, very recent publications indicate that a different system (Vitek MS; bioMérieux) has solved this problem for *S. pneumoniae* (110, 111). Novel commercial identification systems for streptococci include the FDA-cleared Verigene Gram-positive blood culture (BC-GP) nucleic acid test (Nanosphere, Inc., Northbrook, IL) and the FilmArray platform (FA; BioFire, Salt Lake City, UT) for the identification of bacterial pathogens directly from blood culture bottles (112, 113). While very promising results are published for major bacterial pathogens, including *S. pyogenes* and *S. agalactiae*, a reliable species identification for *S. pneumoniae* and streptococci from the viridans group is currently not possible with these tests. In conclusion, phenotypic and automated species identification of viridans group streptococci remains challenging, and acceptable results can currently be achieved only at the group level.

TABLE 2 Phenotypic characteristics of major streptococcal groups[a]

Streptococcal group	Arginine hydrolysis	Esculin	Mannitol	Sorbitol	Urea hydrolysis	VP
S. mitis[b]	v	v	−	v	−	−
S. anginosus[c]	+	+	−	−	−	+
S. mutans[d]	−	+	+	+	−	+
S. salivarius[e]	−	v	−	−	v	+
S. bovis[f]	−	v	v	−	−	+

[a] Symbols and abbreviations: +, positive; −, negative; v, variable.

[b] The *S. mitis* group comprises the species *S. mitis*, *S. sanguinis*, *S. parasanguinis*, *S. gordonii*, *S. cristatus*, *S. oralis*, *S. infantis*, *S. peroris*, *S. australis*, *S. sinensis*, *S. orisratti*, *S. oligofermentans*, and *S. massiliensis*. *S. sanguinis*, *S. parasanguinis*, *S. gordonii*, and *S. cristatus* are arginine hydrolysis positive; other species from the *S. mitis* group are arginine hydrolysis negative.

[c] *S. anginosus*, *S. constellatus*, and *S. intermedius* belong to the *S. anginosus* group.

[d] The *S. mutans* group includes *S. mutans*, *S. sobrinus*, and the following species rarely isolated from humans: *S. criceti*, *S. ratti*, and *S. downei*.

[e] The *S. salivarius* group contains *S. salivarius*, *S. vestibularis*, and *S. thermophilus*. *S. salivarius* is variable, *S. vestibularis* is positive, and *S. thermophilus* is negative for urea hydrolysis.

[f] The *S. bovis* group now includes *S. equinus*, *S. gallolyticus*, *S. infantarius*, and *S. alactolyticus*. *S. gallolyticus* subsp. *gallolyticus* is positive for the acidification of mannitol, and the other species from the *S. bovis* group are negative.

Molecular methods may offer alternative approaches to conventional phenotypic identification schemes. The most common molecular identification method, 16S rRNA gene sequencing, does not yield reliable species identification for several species including *S. mitis*, *S. oralis*, and *S. pneumoniae*. The 16S rRNA gene sequences are more than 99% identical (4). Sequence determination of the manganese-dependent superoxide dismutase gene *sodA* appears to be more reliable (108, 114). In contrast to 16S rRNA sequencing, it allows the differentiation of *S. mitis*, *S. oralis*, and *S. pneumoniae* and the correct identification of streptococcal species from the viridans group.

Descriptions of the species belonging to the viridans group streptococci are given below, and physiological traits of the groups are shown in Table 2.

S. mitis Group

The large number of different streptococcal species belonging to the *S. mitis* group has been mentioned earlier. This group of predominantly alpha-hemolytic streptococci includes several species of known clinical significance together with others for which few or no clinical data have been collected. Among the phenotypic characteristics of the species in this group, extracellular polysaccharide production is negative for *S. mitis* strains but is a variable characteristic of *S. oralis* isolates. This feature correlates with the smooth colony surface of many *S. oralis* strains and the rough and dry appearance of *S. mitis* colonies.

S. anginosus Group

The small-colony-forming species *S. anginosus*, *S. constellatus*, and *S. intermedius* belong to the *S. anginosus* group. Strains of the *S. anginosus* group may be non-, alpha-, or beta-hemolytic on blood agar plates with some variations between the species. While *S. constellatus* is frequently beta-hemolytic, most isolates of *S. intermedius* are nonhemolytic. For many strains, growth is enhanced in the presence of CO_2 with some strains requiring anaerobic conditions. *S. anginosus* and *S. constellatus* strains may possess Lancefield group antigen A, C, F, or G. Most *S. constellatus* or *S. intermedius* strains react with antisera against Lancefield group F antigen or are nongroupable.

The species *S. constellatus* has been further subdivided into two subspecies, *S. constellatus* subsp. *constellatus* and *S. constellatus* subsp. *pharyngis* (115). *S. constellatus* subsp. *constellatus* is phenotypically different from *S. constellatus* subsp. *pharyngis*, which usually possesses the Lancefield group antigen C, is beta-hemolytic, and has been associated with pharyngitis. Detailed phenotypic characteristics of the *S. anginosus* group are shown in Table 3 (1, 116, 117).

S. mutans Group

The *S. mutans* group includes *S. mutans*, *S. sobrinus*, *S. criceti*, *S. ratti*, *S. downei*, *S. ferus*, *S. hyovaginalis*, *S. devriesei*, and *S. macacae*. *S. mutans* and *S. sobrinus* are frequently found in human hosts, while the other species are only rarely encountered in humans or represent animal pathogens. The species of the *S. mutans* group are characterized by the production of extracellular polysaccharides from sucrose, which can be tested by culturing the bacteria on sucrose-containing agar and by the ability to produce acid from a relatively wide range of carbohydrates. *S. mutans* strains may present with an atypical morphology for streptococci, forming short rods on solid medium or in broth culture under acidic conditions. On blood agar, colonies are often hard and adherent and usually alpha-hemolytic. Under anaerobic growth conditions, some strains are beta-hemolytic. *S. sobrinus* strains are mostly nonhemolytic or occasionally alpha-hemolytic. On sucrose-containing agar, species from this group form colonies that are rough (frosted-glass appearance), heaped, and surrounded by liquid-containing glucan.

S. salivarius Group

Streptococcal species in the *S. salivarius* group are *S. salivarius*, *S. vestibularis*, and *S. thermophilus*. *S. salivarius* strains are usually non- or alpha-hemolytic on blood agar. On sucrose-containing agar, strains form large mucoid or hard colonies due to the production of extracellular polysaccharides. A high proportion of *S. salivarius* strains react with the Lancefield group K antiserum. Species in this group may also react with the streptococcal group D antiserum. It is unclear if these strains truly possess the group D antigen or yield a nonspecific cross-reaction. *S. vestibularis* is alpha-hemolytic, and the failure of this species to produce extracellular polysaccharides on sucrose-containing agar is helpful in distinguishing *S. vestibularis* from *S. salivarius* strains. *S. thermophilus* is found in dairy products but has not been isolated from clinical specimens.

S. bovis Group

The species belonging to the *S. bovis* group (*S. equinus*, *S. gallolyticus*, *S. infantarius*, and *S. alactolyticus*) are nonentero-

TABLE 3 Phenotypic characteristics of streptococcal species from the *S. anginosus* group[a]

Test	*S. anginosus*	*S. constellatus*[b]	*S. intermedius*
β-D-Fucosidase	−	−	+
β-*N*-Acetylglucosaminidase	−	−	+
β-*N*-Acetylgalatosaminidase	−	−	+
Neuraminidase	−	−	+
α-D-Glucosidase	v	+	+
β-D-Glucosidase	+	−	v
β-D-Galactosidase	v	−	+
Amygdalin (acidification)	+	v	v
Mannitol (acidification)	v	−	−
Sorbitol (acidification)	−	−	−
Lactose (acidification)	+	v	+
Arginine hydrolysis	+	+	+
Esculin hydrolysis	+	v	+
VP[c]	+	+	+
Urease	−	−	−

[a] Symbols and abbreviations: +, positive; −, negative; v, variable; data from Summanen et al. (116), Facklam (1), and Whiley (117).

[b] The species *S. constellatus* subsp. *pharyngis* is β-D-fucosidase, β-D-acetylglucosaminidase, β-D-acetylgalactosaminidase, and β-D-glucosidase positive, in contrast to *S. constellatus* subsp. *constellatus*.

[c] Voges-Proskauer test (formation of acetoin from glucose fermentation).

coccal group D streptococci that are PYR negative. Most strains grow on bile esculin agar and are unable to grow in 6.5% NaCl. On blood agar, strains are either nonhemolytic or alpha-hemolytic. Strains of the *S. bovis* group share phenotypic characteristics with *S. mutans* strains, such as production of glucan, fermentation of mannitol, and growth on bile esculin agar. However, the *S. bovis* group does not ferment sorbitol. *S. gallolyticus* subsp. *gallolyticus* and *S. infantarius* subsp. *coli* typically ferment starch or glycogen and give a Lancefield group D reaction, in contrast to *S. gallolyticus* subsp. *pasteurianus*. A detailed phenotypic characterization and emended description of the different subspecies has recently been published (53). For the identification of species in this group, testing of β-glucuronidase, α- and β-galactosidase, β-mannosidase, acid production from starch, glycogen, inulin, and mannitol is helpful. As described, strains formerly known as *S. bovis* currently belong to several species of the *S. bovis* group.

Physiologic Tests

Optochin Test

Most *S. pneumoniae* isolates are optochin susceptible. Before application of the optochin disk, several colonies of a pure culture are streaked on a sheep blood agar plate. Optochin testing should be performed on plates that are incubated at 35 to 36°C overnight in 5% CO_2 because up to 8% of strains do not grow under ambient conditions. *S. pneumoniae* isolates show zones of inhibition of ≥14 mm with a 6-mm disk (containing 5 μg of optochin) and zones of inhibition of ≥16 mm with a 10-mm disk. Incubation in 5% CO_2 yielded increased specificity (6, 96). Optochin-resistant *S. pneumoniae* strains have been reported as well as optochin-susceptible *S. mitis* isolates (especially when tested under ambient conditions). Since optochin testing may miss between 4% and 11% of bile-soluble *S. pneumoniae* isolates (6, 96), strains displaying a smaller zone of inhibition (9 to 13 mm for the 6-mm disk) should be subjected to additional testing (e.g., bile solubility or genetic testing) to confirm species identification.

Application of an optochin disk onto the primary culture medium may facilitate a rapid presumptive identification but may miss pneumococcal isolates in a mixed culture. The optochin susceptibility test should be repeated with a pure culture in cases of mixed cultures, or additional tests should be performed (e.g., bile solubility).

Bile Solubility Test

The bile solubility test can be performed either in a test tube or by direct application of the reagent to an agar plate. For the test tube method, a saline suspension of a pure culture is adjusted to a McFarland standard of 0.5 to 1.0, and 0.5 ml of the suspension is added to a small tube. The bacterial suspension is mixed with 0.5 ml of 10% sodium deoxycholate (bile) and incubated at 35°C. A control containing 0.5 ml of bacterial suspension with 0.5 ml of saline should be prepared for each strain tested. A positive result is characterized by clearing of the bile suspension within 3 h; clearing can start as early as 5 to 15 min after inoculation and allows the identification of a strain as *S. pneumoniae*. For the plate method, 1 drop of 10% sodium deoxycholate is placed directly on a colony of the strain in question, and the plate is incubated at 35°C for 15 to 30 min in ambient air. It is important to keep the plates in a horizontal position in order to prevent the reagent from washing away the colony. Colonies of *S. pneumoniae* disappear or demonstrate a flattened colony morphology, while other viridans group streptococci appear unchanged. In contrast to optochin susceptibility testing, bile solubility testing demonstrated excellent sensitivity and specificity in a recent comprehensive evaluation (96).

Bile Esculin Test

For the bile esculin test, bile esculin medium (available from commercial sources) in either plates or slants should be inoculated with one to three colonies of the organism to be tested and incubated at 35°C in ambient air for up to 48 h. Optimal results can be achieved by using medium supplemented with 4% oxgall (the equivalent of 40% bile) (Remel, Lenexa, KS) and a standardized inoculum of 10^6 CFU (118). A definitive blackening of plated medium or blackening of at least one-half of an agar slant is considered a positive result, indicative of species belonging to the *S. bovis* group or enterococci. Occasional other viridans group streptococci are positive with this test or display weakly positive reactions that are difficult to interpret. Isolates from patients with serious infections (e.g., endocarditis) should be more completely characterized.

Arginine Hydrolysis

Arginine hydrolysis is a key reaction for the identification of viridans group streptococci. Discrepancies can occur among test methods (119). Two commonly used methods are detailed here. Moeller's decarboxylase broth containing arginine (Becton Dickinson, Franklin Lakes, NJ, and other sources) should be inoculated with the test organism, overlaid with mineral oil, and incubated at 35 to 37°C for up to 7 days. Degradation of arginine results in elevated pH, indicated by development of a purple color. Negative results are indicated by a yellow color, which is due to acid accumulation from metabolism of glucose only. For the microtiter plate method (104), 3 drops of the arginine-containing reagent are inoculated with 1 drop of an overnight Todd-Hewitt broth culture and incubated for 24 h at 37°C anaerobically. Production of ammonia is detected by the appearance of an orange color following addition of 1 drop of Nessler's reagent.

Urea Hydrolysis

Christensen's urea agar (Becton Dickinson and other sources) is inoculated and incubated aerobically at 35°C for up to 7 days. Development of a pink color indicates a positive reaction. An alternative format is to dispense Christensen's medium without agar into a microtiter tray well and, after inoculation, overlay it with mineral oil prior to incubation.

VP Test

The VP test can be performed as described above for the identification of beta-hemolytic streptococci. A standard method for performing the VP test, requiring extended incubation, is described in chapter 19.

Esculin Hydrolysis

Esculin agar slants (Becton Dickinson and other sources) are inoculated and incubated for up to 1 week. A positive reaction appears as a blackening of the medium; no change in color indicates a negative esculin hydrolysis test result.

Hyaluronidase Production

Hyaluronidase activity can be detected on brain heart infusion agar plates supplemented with 2 mg/liter of sodium hyaluronate (Sigma-Aldrich, St. Louis, MO). The strains to be tested are inoculated by stabbing into the agar, and plates are incubated anaerobically at 37°C overnight. After the plate is flooded with 2 M acetic acid, hyaluronidase activity is indicated by the appearance of a clear zone around the stab. A quantitative method for determining hyaluronidase activity can be performed in microtiter trays (120).

Production of Extracellular Polysaccharide

Strains may be isolated as single colonies on sucrose-containing agar. The two most commonly used media are (i) mitis-salivarius agar containing 0.001% (wt/vol) potassium tellurite (Becton Dickinson) and (ii) tryptone-yeast-cystine agar (Lab M, Bury, United Kingdom). Incubation may require up to 5 days at 37°C under anaerobic incubation conditions.

TYPING SYSTEMS

In the majority of cases, typing of streptococci has no immediate clinical or therapeutic consequences. It is most often performed by reference laboratories for the purposes of epidemiologic studies and the evaluation of vaccine efficacy. Although classical antibody-dependent typing systems for capsular serotypes and surface proteins have been used for years, molecular methods are increasingly applied, since they do not require special techniques or the maintenance of rarely used reagents such as a large antibody panel. Another advantage is the independence of DNA sequences from culture conditions and gene expression. For the differentiation of distinct clones, pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) systems have been established for many streptococcal species (121).

S. pneumoniae comprises more than 90 antigenically distinct capsular serotypes that can be detected by the Neufeld test (Quellung reaction), which is still regarded as the gold standard for epidemiologic studies. Pure cultures of pneumococci are grown on a freshly prepared 5% sheep blood agar plate or a 10% horse blood agar plate at 35°C to 37°C and 5% CO_2 for 18 to 24 h. A small amount of bacterial growth (less than a 10-μl loopful) is resuspended in a droplet of phosphate-buffered or physiological saline (McFarland standard of ~0.5). A few microliters of the saline suspension are mixed with an equal amount of specific pneumococcal rabbit antisera on a glass slide. The specimen is subsequently evaluated for capsular swelling (a clear area surrounding the bacterial cells) by phase-contrast microscopy (oil immersion; magnification, ×1,000) (122). Following the same principle, commercially available kits (Pneumotest Statens Serum Institut, Copenhagen, Denmark) allow rapid testing of *S. pneumoniae* serotypes with pooled antisera by a checkerboard method. A rapid antigen detection test using pooled antisera coupled to latex beads (Statens Serum Institut) has been developed (123). Due to strain discrepancies, confirmation by the Neufeld Quellung reaction is recommended. In addition, PCR methods for pneumococcal serotype determination have been developed during the last years (124–126). For the distinction of single clones, PFGE (127) or

MLST typing schemes (128) have been used in pneumococcal investigations.

Ten different antibody-defined capsular polysaccharides have been described for *S. agalactiae* (Ia, Ib, and II to IX). The percentage of nontypeable strains can be minimized by optimization of capsular expression (129). In addition to antibody detection of capsular serotypes, PCR- and DNA sequencing-based techniques allow the detection of capsular serotypes (130, 131). Individual clones of *S. agalactiae* have been detected either by MLST (132) or by PFGE (133).

Conventional typing of *S. pyogenes* is based upon the antigenic specificity of the surface-expressed T and M proteins (134). The trypsin-resistant T protein is part of the pilus structures (135). The T type can be identified by agglutination with commercially available serologic assays utilizing approximately 20 accepted anti-T sera. M proteins are major antiphagocytic virulence factors of *S. pyogenes* (136). N-terminal sequence variation in genes encoding these highly protective antigens is the basis of the *S. pyogenes* precipitation typing system. At present, 83 M serotypes are unequivocally validated and internationally recognized to be serologically unique and are designated M1 to M93 in the Lancefield classification (137). M serotypes that are not included are from non-*S. pyogenes* organisms or correspond to an existing M serotype.

A molecular typing system is based on the nucleotide sequences encoding the amino termini of M proteins. The *emm* gene sequences encode M proteins and have been correlated with Lancefield M serotypes. This methodology allows assignment to a validated M protein gene sequence (*emm1* through *emm124*) and the identification of new *emm* sequence types and has evolved into the gold standard of *S. pyogenes* typing (137). A large database of approximately 350 *emm* gene sequences from strains originally used for Lancefield serotyping and including *emm* sequences from beta-hemolytic streptococci of groups C, G, and L is maintained at the CDC (http://www.cdc.gov/ncidod/biotech/strep/strepblast.htm). An MLST scheme is also available for *S. pyogenes*. Population genetic studies demonstrated stable associations between *emm* type and MLST among isolates obtained decades apart and/or from different continents (138).

In outbreak situations that include *S. pyogenes*, restriction enzyme-mediated digestion of *emm* amplicons is a valuable tool for rapid identification of isolates containing similar *emm* genes (139). For clusters of isolates sharing the same *emm* type, PFGE profiles may be helpful for distinguishing similar strains (140).

SEROLOGIC TESTS

Determination of streptococcal antibodies is indicated for the diagnosis of poststreptococcal disease, such as acute rheumatic fever or glomerulonephritis (141). A 4-fold rise in antibody titer is regarded as definitive proof of an antecedent streptococcal infection. Multiple variables, including site of infection, time since the onset of infection, age, background prevalence of streptococcal infections (142), antimicrobial therapy, and other comorbidities, influence antibody levels. The most widely used antibodies are anti-streptolysin O (anti-ASO) and anti-DNase B.

Antibodies against ASO reach a maximum at 3 to 6 weeks after infection. While ASO responses following streptococcal upper respiratory tract infections are usually elevated, pyoderma caused by *S. pyogenes* does not elicit a strong ASO response. *Streptococcus dysgalactiae* subsp. *equisimilis* can also produce streptolysin O, and thus elevated ASO titers are not specific for *S. pyogenes* infections.

Among the four streptococcal DNases produced, the host response is most consistent against DNase B. Anti-DNase B titers may not reach maximum titers for 6 to 8 weeks but remain elevated longer than ASO titers and are more reliable than ASO for the confirmation of a preceding streptococcal skin infection. Moreover, since only 80 to 85% of patients with rheumatic fever have elevated ASO titers, additional anti-DNase B titers may be helpful.

Due to frequent exposure to *S. pyogenes*, ASO and anti-DNase B titers are higher in children in the United States from 2 to 12 years of age. Geometric mean values are 89 Todd Units for ASO and 112 Units for anti-DNase B, while the upper limits of normal values are 240 Todd Units (ASO) and 640 Units (anti DNAse B) (143). Prompt antibiotic therapy of streptococcal infections can reduce the titer but does not abolish antibody production. Streptococcal carriers do not experience a rise in streptococcal antibody titers.

The hemagglutination-based Streptozyme test (Carter-Wallace, Inc., Cranbury, NJ) was developed to detect antibodies against multiple extracellular streptococcal products. However, variabilities in test standardization and inconsistent specificities have been reported (144). Antibody detection against other *S. pyogenes* proteins (hyluronidase, streptokinase, and NAD glycohydrolase [NADase]) are technically difficult to perform and not commercially available.

ANTIMICROBIAL SUSCEPTIBILITIES

Beta-Hemolytic Streptococci

Penicillin remains the drug of choice for the empirical treatment of streptococcal infections due to *S. pyogenes*, because in contrast to *S. pneumoniae* and other alpha-hemolytic streptococci, *S. pyogenes* remains uniformly susceptible to penicillin. Reports about reduced penicillin susceptibility in strains of *S. pyogenes* have not been confirmed by reference laboratories. This is, however, no longer true for *S. agalactiae*. The emergence of diminished susceptibility to penicillin G caused by a mutation of the penicillin binding proteins (PBPs) Pbp2x in isolated strains in Asia and the United States has been reported (145, 146). Due to suspected or confirmed penicillin allergies in more than 10% of patients, macrolides are often given as an alternative treatment. Macrolide resistance rates among isolates of *S. pyogenes* and *S. agalactiae* have been increasing in North America as well as in Europe (147). Resistance rates correlate with the use of macrolides in clinical practice, and geographic differences in resistance rates are often due to differences in macrolide use. In the United States, macrolide resistance among *S. agalactiae* strains rose from 12 to 38% from 1990 to 2006 (148, 149) but has substantially declined to about 5% in one recent investigation (150). While isolates with a reduced susceptibility to glycopeptides have not been found in *S. pyogenes*, a very recent publication reports two independent *S. agalactiae* isolates from invasive infections, harboring *vanG* resistance genes (151). Due to the uniform susceptibility of *S. pyogenes* to penicillin, resistance testing for penicillins or other β-lactams approved for treatment of *S. pyogenes* and *S. agalactiae* is not necessary for clinical purposes. So far, the rare isolates of *S. agalactiae* with reduced susceptibility to penicillin have not resulted in a change of this recommendation, which may of course change if increasing numbers of such strains are encountered. Susceptibility testing for macrolides should be performed using erythromycin, since resistance and susceptibility of azithromycin, clarithromycin, and dirithromycin can be predicted by testing erythromycin. As specified in the CDC guidelines (35), *S. agalactiae* isolates from pregnant women with severe penicillin allergy should be tested for erythromycin and clindamycin resistance. Testing should include constitutive as well as inducible clindamycin resistance, preferentially with the double-disk diffusion method (D-zone test). In accordance with changes in the most recent CLSI recommendations, however, only results for clindamycin should be reported.

S. pneumoniae and Viridans Group Streptococci

In view of the development of penicillin resistance in *S. pneumoniae* and other alpha-hemolytic streptococci, penicillin can no longer be recommended as the empirical treatment of choice in many countries. Penicillin is considered a preferred antimicrobial agent only for *S. pneumoniae* and other alpha-hemolytic streptococci with demonstrated susceptibilities to penicillin. Penicillin resistance in *S. pneumoniae* is caused by altered PBPs. Approximately 25% of *S. pneumoniae* isolates from the United States were not fully susceptible to penicillin in 2007 (http://www.cdc.gov/abcs/reports-findings/surv-reports.html). But the changes of *S. pneumoniae* breakpoints in nonmeningeal isolates (≤2 µg/ml, susceptible [S]; 4 to 8 µg/ml, intermediate [I]; ≥8 µg/ml, resistant [R]) for penicillin in CLSI definitions caused this value to drop to less than 10% (152). Currently, 5.9% of strains are reported as penicillin resistant (http://www.cdc.gov/abcs/reports-findings/survreports/spneu12.pdf). Susceptibility to penicillin can be determined by a disk diffusion test with 1 µg of oxacillin. According to the current CLSI guidelines, in all cases where oxacillin zone sizes (≤19 mm) indicate a reduced penicillin susceptibility, MIC determinations for penicillin should be performed. For susceptibility testing of all other β-lactams in *S. pneumoniae*, MIC determinations are recommended. *S. pneumoniae* infections should be treated according to current guidelines (153). Depending on the clinical situation, treatment options include penicillin, extended-spectrum cephalosporins, macrolides, fluoroquinolones, and vancomycin. In addition, more than one-third of blood culture isolates of the viridans group streptococci collected in the late 1990s in the United States were not susceptible to penicillin (154). Elevated percentages of penicillin-resistant strains can be found among *S. mitis* and *S. salivarius* isolates. *S. pneumoniae* was uniformly susceptible to macrolides until the late 1980s in the United States, but macrolide resistance rates as high as 29% for *S. pneumoniae* strains have been reported in more-recent investigations (41, 155).

The increased use of fluoroquinolones to treat *S. pneumoniae* infections has been accompanied by a rise in fluoroquinolone-resistant *S. pneumoniae* strains. Resistance occurs in a stepwise fashion and is due to mutations in DNA topoisomerase IV (ParC) and/or a subunit of DNA gyrase (GyrA). While the overall prevalence of fluoroquinolone resistance remains below 1% according to the most recent Active Bacterial Core Surveillance (ABCs) data (http://www.cdc.gov/abcs/reports-findings/survreports/spneu12.pdf), the increase in resistant strains during recent years emphasizes the need for close monitoring. Clinical failures of levofloxacin therapy due to resistance have been reported (156). Vancomycin-resistant *S. pneumoniae* isolates have not been described. However, the isolation of a vancomycin-resistant *S. bovis* isolate has been reported (157).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Streptococci from the pyogenic group are important human pathogens. Timely identification of these species in clinical specimens is therefore crucial to treat infections adequately and to reduce transmission. Tonsillopharyngitis caused by *S. pyogenes* remains a substantial health problem in childhood and adolescence. Diagnosis by either rapid antigen tests, NAATs, or bacteriological culture minimize unjustified antibiotic treatment of viral pharyngitis. Serologic tests are usually applied in cases of suspected poststreptococcal sequelae. Proper identification and reporting should, however, not be limited to *S. pyogenes*, since *S. dysgalactiae* subsp. *equisimilis* (human group C and G streptococci) has been documented as an agent of pharyngitis, including cases complicated by nonsuppurative sequelae. In this context, it is important to correctly differentiate these pathogens from the small-colony-forming beta-hemolytic species of the *S. anginosus* group that make up part of the oropharyngeal microbiota. While invasive neonatal *S. agalactiae* infections are declining due to improved prenatal screening and peripartal antibiotic prophylaxis, increased detection of *S. agalactiae* from adult patients has been reported (158). Thorough identification and reporting of this organism should therefore not be confined to screening swabs during pregnancy or in newborns.

Despite the fact that *S. pneumoniae* is often found as a colonizer in respiratory samples, it should always be clearly distinguished from viridans group streptococci and reported. Cultural methods remain the mainstay in pneumococcal pneumonia and sepsis as well as meningitis. To enable the initiation of adequate antibiotic treatment, resistance testing should be performed for all isolates. Special care should be taken to ensure the reporting of the correct β-lactam breakpoints for non-CSF and CSF *S. pneumoniae* isolates. If antibiotic treatment was started before microbiological samples were obtained, the urinary antigen test or nucleic acid detection techniques may help to properly identify the causative agent, especially in invasive infections.

The correct identification of viridans group streptococci and the distinction of strains causing infections from isolates of the physiological microbiota remains a major challenge. Identification to the group or species level should be confined to strains causing abscesses, endocarditis, and serious infections in neutropenic patients. Many *S. mitis* isolates are no longer penicillin susceptible, and special attention has to be paid to susceptibility testing. Due to the association of *S. gallolyticus* subsp. *gallolyticus* with malignancies of the gastrointestinal tract, and in view of the taxonomic changes within the *S. bovis* group, reports of novel species designations should include the information that the species belongs to the *S. bovis* group (159).

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Enterococcus

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23

TAXONOMY

The genus *Enterococcus* includes microorganisms that have a historical connection with the genus *Streptococcus*, and their initial documentation is related to the "streptococci of fecal origin" or "enterococci" (see reference 1 for a brief historical overview). They were initially considered a distinct category within the genus *Streptococcus*, distinguished by their higher resistance to chemical and physical agents and accommodating most of the serological group D streptococci. After the introduction of molecular methods for studying these microorganisms, however, the enterococci have undergone considerable changes in taxonomy, which started with the splitting of the genus *Streptococcus* and the recognition of *Enterococcus* as a separate genus in 1984 (2). *Streptococcus faecalis* and *Streptococcus faecium* were the first species to be transferred to the new genus as *Enterococcus faecalis* (the type species) and *Enterococcus faecium*, respectively. Subsequently, other earlier streptococcal species and subspecies were transferred and received new denominations as members of the genus *Enterococcus* (3). The continuous use of molecular approaches has allowed major developments in the classification of the enterococci, resulting in the recognition of 49 enterococcal species as of April 2014 (for further details, see references 1 and 4, as well as the *List of Prokaryotic Names with Standing in Nomenclature* [http://www.bacterio.net]).

The enterococci belong to the low DNA guanine-plus-cytosine (G+C <50 mol%) content branch of the phylum *Firmicutes*. Phylogenetic analyses based on the comparison of the 16S rRNA gene sequences have revealed that the members of the genus *Enterococcus* are more closely related to those included in the genera *Vagococcus* and *Tetragenococcus* than they are to *Streptococcus* and *Lactococcus*, genera to which they have been phenotypically associated (1, 4, 5). Such observations culminated in the recent proposal to allocate the genus *Enterococcus* to a newly designated family (*Enterococcaceae* fam. nov.) together with the genera *Melissococcus*, *Tetragenococcus*, and *Vagococcus* (4, 6, 7).

Current criteria for inclusion in the genus *Enterococcus* and for the description of new enterococcal species encompass a polyphasic approach resulting from a combination of different molecular techniques (frequently involving DNA-DNA reassociation experiments, 16S rRNA gene sequencing, and whole-cell protein [WCP] profiling analysis) and phenotypic tests. Partial or nearly entire sequencing of the 16S rRNA gene is a practical and powerful tool in aiding the identification of enterococcal species, and it has been performed for all recognized species of *Enterococcus*. Figure 1 shows the phylogenetic relationships among the species of *Enterococcus* based on the analysis of 16S rRNA gene sequences, which are available from the GenBank database. Several other molecular methods, mostly nucleic acid-based assays, have been used as additional tools to assess the phylogenetic relationships among enterococcal species and to formulate the description of new species, but their use is still limited (for further details, see references 1 and 4 and "Identification" below).

DESCRIPTION OF THE GENUS

The members of the genus Enterococcus are Gram-positive, catalase-negative cocci that occur singly or are arranged in pairs or as short chains. Cells are sometimes coccobacillary when Gram stains are prepared from growth on solid medium but tend to be ovoid and in chains when grown in liquid medium, such as thioglycolate broth. After growth on blood agar medium for 24 h, colonies are usually between 1 and 2 mm in diameter, although some variants may appear smaller. Some (about one-third) of the *E. faecalis* isolates may be beta-hemolytic on agar containing rabbit, horse, or human blood but nonhemolytic on agar containing sheep blood. Some isolates of *E. faecalis* and *Enterococcus durans* may be beta-hemolytic regardless of the type of blood used. All other species are usually alpha-hemolytic or nonhemolytic. Enterococci are facultative anaerobes with a homofermentative metabolism that results in the production of L-(+)-lactic acid as the major end product of glucose fermentation. Because of their ability to ferment a wide range of carbohydrates to lactic acid, the enterococci are referred to as typical lactic acid bacteria (LAB). Gas is not produced. These microorganisms are usually able to grow at temperatures ranging from 10 to 45°C with optimum growth at 35 to 37°C. The majority of the species grow in broth containing 6.5% NaCl, and they hydrolyze esculin in the presence of bile salts (bile-esculin [BE] test). They also hydrolyze leucine-β-naphthylamide by producing leucine aminopeptidase (LAPase). Apart from *Enterococcus cecorum*, *Enterococcus columbae*, *Enterococcus pallens*, *Enterococcus saccharolyticus*, and some isolates of the more recently described species *Enterococcus canintestini*, *Enterococcus devriesei*, *Enterococcus*

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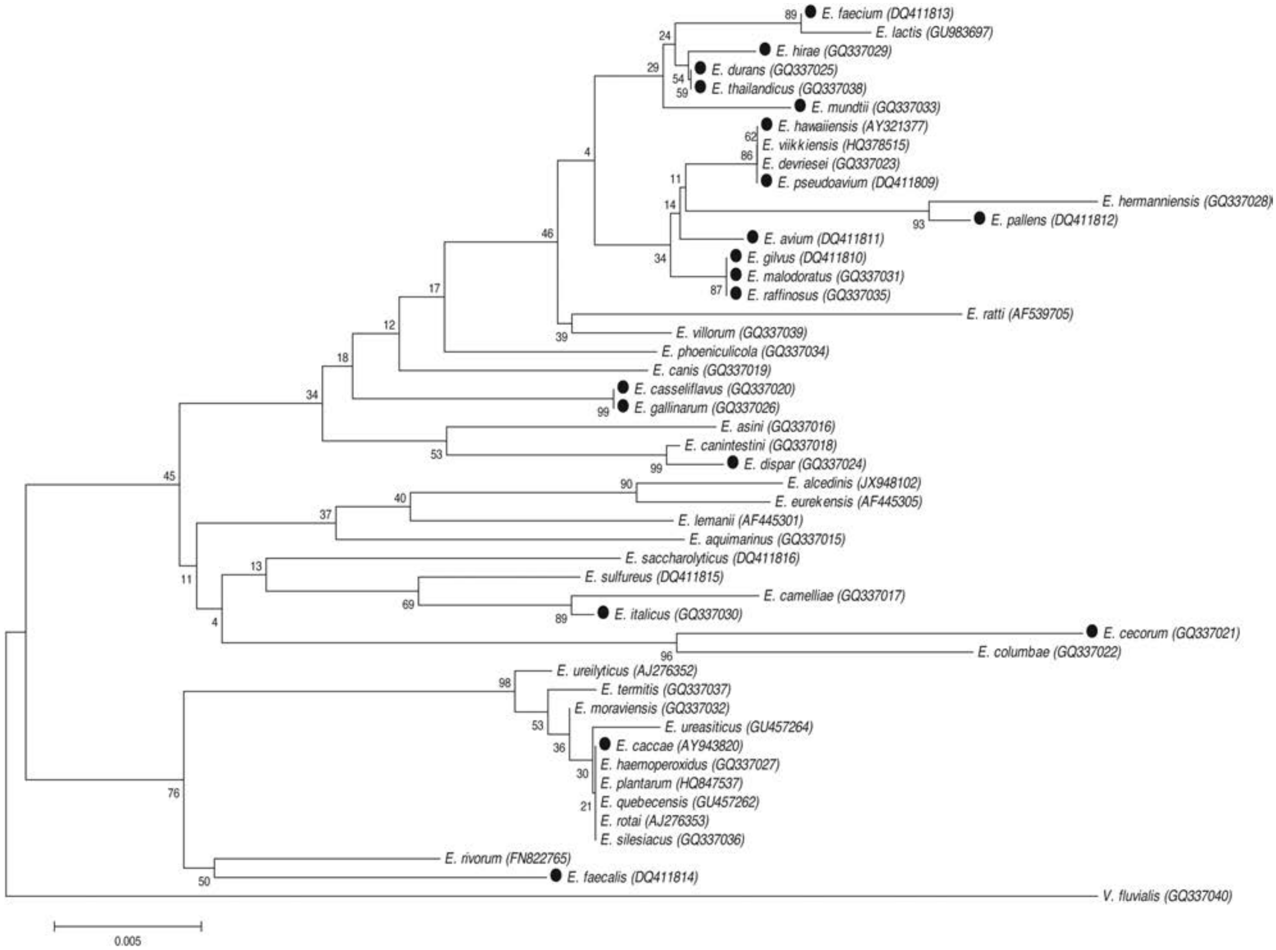
89
E. faecium (DQ411813)
E. lactis (GU983697)
24
E. hirae (GQ337029)
29
54
E. durans (GQ337025)
59
E. thailandicus (GQ337038)
E. mundtii (GQ337033)
E. hawaiiensis (AY321377)
62
E. viikkiensis (HQ378515)
86
E. devriesei (GQ337023)
E. pseudoavium (DQ411809)
4
11
14
E. hermanniensis (GQ337028)
93
E. pallens (DQ411812)
46
E. avium (DQ411811)
34
E. gilvus (DQ411810)
E. malodoratus (GQ337031)
87
E. raffinosus (GQ337035)
17
E. ratti (AF539705)
12
39
E. villorum (GQ337039)
E. phoeniculicola (GQ337034)
18
E. canis (GQ337019)
E. casseliflavus (GQ337020)
99
E. gallinarum (GQ337026)
34
E. asini (GQ337016)
53
E. canintestini (GQ337018)
99
E. dispar (GQ337024)
45
90
E. alcedinis (JX948102)
40
E. eurekensis (AF445305)
37
E. lemanii (AF445301)
E. aquimarinus (GQ337015)
E. saccharolyticus (DQ411816)
13
E. sulfureus (DQ411815)
11
69
E. camelliae (GQ337017)
89
E. italicus (GQ337030)
4
E. cecorum (GQ337021)
96
E. columbae (GQ337022)
E. ureilyticus (AJ276352)
98
E. termitis (GQ337037)
E. moraviensis (GQ337032)
53
E. ureasiticus (GU457264)
36
E. caccae (AY943820)
30
E. haemoperoxidus (GQ337027)
E. plantarum (HQ847537)
21
E. quebecensis (GU457262)
E. rotai (AJ276353)
E. silesiacus (GQ337036)
76
E. rivorum (FN822765)
50
E. faecalis (DQ411814)
V. fluvialis (GQ337040)
0.005

FIGURE 1 Phylogenetic tree based on comparative analysis of the 16S rRNA gene sequences showing the relationship among the type strains of the species included in the genus *Enterococcus*. *Vagococcus fluvialis* was used as an outgroup, and bootstrap values at the nodes were displayed as percentages. Black dots indicate the species that have been isolated from human sources. doi:10.1128/9781555817381.ch23.f1

moraviensis, and *Enterococcus termitis*, most enterococci hydrolyze L-pyrrolidonyl-β-naphthylamide (PYR) by producing pyrrolidonyl arylamidase (pyrrolidonase [PYRase]). Results for both LAP and PYR testing, especially for some of the more recently described species, may vary according to the methodology, including the test format and media used to grow the bacterial cells. A few species are motile (*Enterococcus casseliflavus* and *Enterococcus gallinarum*), and some are pigmented (*E. casseliflavus*, *Enterococcus gilvus*, *Enterococcus mundtii*, *E. pallens*, *Enterococcus plantarum*, *Enterococcus rotai*, *Enterococcus sulfureus*, and *Enterococcus ureilyticus*) (1, 4, 5, 8). Methods used for detection of enterococcal motility have to be selected carefully, as differences in motility due to the composition of the medium have been demonstrated (9). Enterococci are not able to synthesize porphyrins and therefore do not produce cytochrome enzymes (4, 8). However, cytochrome activity is sometimes expressed when strains of *E. faecalis* are grown on blood-containing medium, and a weak effervescence is observed in the catalase test. Positive catalase testing has also been reported for strains of *Enterococcus haemoperoxidus* (10), *Enterococcus silesiacus* (11), and *E. plantarum* (12) when cultivated on blood-containing agar media. Most enterococcal strains produce a cell wall-associated glycerol teichoic acid that is identified as Lancefield's serological group D antigen. The DNA G+C contents range from 32 to 45 mol%. The genome sizes are in the range of approximately 2.3 to 5.4 Mb (13, 14, 15). Genome sequencing of *E. faecalis* V583 (16), the first vancomycin-resistant clinical isolate in the United States, has opened many lines of investigation to improve our understanding about the genus *Enterococcus*. A variety of *Enterococcus* genomes are at different stages of analysis, and some have been completely sequenced, according to information available at specialized sites and databases, such as the NCBI (http://0-www.ncbi.nlm.nih.gov.elis.tmu.edu.tw/genome/?term=enterococcus), the Broad Institute (https://olive.broadinstitute.org/projects/enterogenome), and the American Society for Microbiology (http://genomea.asm.org/).

The other genera of catalase-negative Gram-positive cocci and the characteristics that distinguish them from the enterococci are discussed in chapters 22 and 24. No phenotypic criteria are available for clearly distinguishing the genus *Enterococcus* unequivocally from other genera, since there are no particular characteristics that are common to all enterococci. However, certain characteristics are usually found in the majority of the strains belonging to the most frequently isolated enterococcal species. Presumptive identification of a Gram-positive, catalase-negative coccus as an *Enterococcus* can be accomplished by demonstrating that the strain is positive for the BE, PYR, and LAP tests and grows in the presence of 6.5% NaCl and at 45°C. Because strains of *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Vagococcus* with phenotypic similarities have been isolated from human infections (17, 18), the presumptive identification of enterococci based only on the BE reaction and growth in 6.5% NaCl broth can be erroneous. Demonstrating the presence of group D antigen by a serological reaction may be helpful in the identification, although this antigen is detected in only about 80% of the enterococcal strains. On the other hand, pediococci and leuconostocs (17), as well as some vagococcal strains (18), can also react with anti-group D serum. Taking into account that certain characteristics traditionally considered to be typical for the genus do not apply to some atypical strains and to several of the more recently described species, the above-mentioned criteria may exclude several isolates or species. The use of molecular identification methods, such as a PCR-based test having the *tuf* gene as a genus-specific target, may be helpful in circumventing these difficulties by correctly identifying both typical and unusual *Enterococcus* isolates at the genus level (19).

EPIDEMIOLOGY AND TRANSMISSION

Several intrinsic characteristics of the enterococci allow them to grow and survive under harsh conditions and persist almost everywhere, colonizing several ecological niches. These microorganisms are widespread in nature and can be found in soil, plants, water, food, and animals, including mammals, birds, insects, and reptiles (4, 5, 20). In humans, they are predominantly inhabitants of the gastrointestinal tract and are less commonly found in other sites, such as the genitourinary tract, the oral cavity, and skin, especially in the perineal area. The prevalence of the different enterococcal species appears to vary according to the host and is also influenced by age, diet, and other factors that may be related to changes in physiologic conditions, such as underlying diseases and prior antimicrobial therapy. Enterococci are considered among the most abundant Gram-positive cocci colonizing the intestine, with *E. faecalis* being one of the most common bacterial species recovered from this site (5, 20, 21). Other species, such as *E. faecium*, *E. casseliflavus*, *E. durans*, and *E. gallinarum*, are also found in variable proportions in the gastrointestinal tract of humans. Since the enterococci are opportunistic pathogens, the incidence of each species found in human infections probably reflects the distribution of the different species of *Enterococcus* in the human gastrointestinal tract. This site is believed to represent an important reservoir for strains associated with disease; from this location, they may migrate to cause infections and can also disseminate to other hosts and to the environment (22, 23, 24). On the other hand, the occurrence of high numbers of enterococci in the feces and their ability to resist different chemical and physical conditions and to survive in the environment imply that the enterococci can be used as indicators of fecal contamination and of the hygienic quality of food, milk, and drinking water (25, 26). The occurrence of enterococci as members of the intestinal microbiota of humans (20) and the relationship between the presence of enterococci in foods and human safety (25) have been extensively reviewed.

CLINICAL SIGNIFICANCE

The enterococci are commensal microorganisms that act as opportunistic agents causing a variety of infections in humans. Many of these infections have been suggested to arise from translocation of the enterococcal cells from their major site of colonization in the gastrointestinal tract. They most commonly infect the urinary tract, bloodstream, endocardium, burn and surgical site wounds, abdomen, biliary tract, and catheters and other implanted medical devices (24, 27, 28, 29, 30). The ubiquitous presence of enterococci, however, requires caution in establishing the clinical significance of a particular isolate. Unnecessary work and potentially misleading laboratory reports should be avoided whenever possible, especially with respect to *in vitro* susceptibility testing decisions (see "Antimicrobial Susceptibilities" below). Over the last decades, they have emerged from being considered virtually harmless bacteria to medically important multiple-antibiotic-resistant health care-associated pathogens that contribute significantly to patient morbidity and mortality as well as health care costs. Changes in the dynamics of the commensal host-bacterial relationship, such as those promoted by the use of broad-spectrum antibiotics, host injury, or diminished host immunity, could allow these bacteria to gain access to extraintestinal host sites and cause infection. Therefore, elderly patients with serious underlying diseases and other severely ill immunocompromised patients who have been hospitalized for prolonged periods, have been treated with invasive devices, and/or have received broad-spectrum antimicrobial therapy are at higher risk to acquire enterococcal infections (22, 24, 27, 28, 29).

The pathogenesis of enterococcal infections is still poorly understood. Although the debate continues over whether serious enterococcal infections arise from one's own indigenous microbiota or from exogenously acquired strains, epidemiological studies show the existence of clonal relationships among outbreak isolates and support the notion that a subset of virulent lineages with greater propensity to cause disease exist and are often responsible for infections of epidemic proportions (24, 31, 32, 33, 34, 35). Several potential virulence factors that may play a role in the pathogenesis of enterococcal infections have been identified in enterococcal isolates, including the surface adhesins Esp (Enterococcal surface protein) and aggregation substance (AS), secreted toxin cytolysin/hemolysin, secreted proteases gelatinase and serine protease, MSCRAMM Ace (Adhesin to collagen of *E. faecalis*), *E. faecalis* antigen A (EfaA), enterococcal capsule, cell wall polysaccharides, and extracellular superoxide (24, 31, 36, 37). Nevertheless, none has been established as making a major contribution to enterococcal virulence in humans. One mechanism by which the enterococci can deviate from their commensal behavior is through the acquisition of new traits that allow the bacterium to overcome host defenses and colonize new niches, as suggested by the identification of the *E. faecalis* pathogenicity island (PAI), which highlights genetic differences between infection-derived and commensal strains (31, 38, 39). In this context, acquired antimicrobial resistance is considered one of the many traits that virulent enterococci possess, in contrast to commensal isolates, and allow members of this genus to survive for extended periods of time in the host or environment, leading to their persistence and role as prominent health care-associated pathogens (24, 31, 36, 38). In addition, enterococci can transfer resistance determinants to other bacteria, for example, staphylococci, which further increases the clinical importance of the enterococci. The ability to form biofilms has recently been listed among the most prominent virulence properties of these microorganisms, allowing colonization of inert and biological surfaces while protecting against antimicrobial substances and mediating adhesion and invasion of host cells (27, 30, 40, 41, 42). Biofilm formation may be of particular importance in the development of endocarditis and endodontic and urinary infections, as well as implant and other medical-device-associated infections.

The variety of infections associated with the enterococci have been thoroughly reviewed and summarized (28, 29). Although the spectrum of infections has remained relatively unchanged since the extensive review by Murray (29), trends toward an increasing prevalence of these organisms as health care-associated pathogens have been frequently observed. Enterococci have become the second or third leading cause of nosocomial urinary tract infections (UTIs), wound infections (mostly surgical site wounds, decubitus ulcers, and burn wounds), and bacteremia in the United States (28, 29, 43, 44, 45, 46). UTIs are the most common of the enterococcal infections: enterococci have been implicated in approximately 10% of all UTIs and in up to approximately 16% of health care-associated UTIs. Enterococcal bacteremia is frequently associated with metastatic abscesses in multiple organs and high mortality rates. Enterococci have also been considered an important cause of infective endocarditis and are estimated to account for about 20% of the cases of native valve bacterial endocarditis and for about 6 to 7% of prosthetic valve endocarditis cases. Endocarditis remains one of the most difficult enterococcal infections to treat because of limitations on bactericidal antimicrobial therapy for enterococcal infections, especially when caused by vancomycin-resistant enterococci (VRE). Intra-abdominal and pelvic infections are also commonly associated with enterococci. However, cultures from patients with peritonitis, intra-abdominal or pelvic abscesses, biliary tract infections, surgical site infections, and endomyometritis are frequently polymicrobial, and the role of enterococci in these settings remains controversial. The significance of isolates from some of these sites should then be carefully evaluated before clinical decisions are made. There is also a growing concern regarding the role of the enterococci in endodontic and implanted and medical device-associated infections (27, 41). Infections of the respiratory tract or the central nervous system, as well as otitis, sinusitis, septic arthritis, and endophthalmitis, may occur but are rare.

The ratios of isolation of the different enterococcal species can vary according to each setting and can be affected by a number of aspects, including the increasing dissemination of outbreak-related strains such as vancomycin-resistant *E. faecium*. *E. faecalis* and *E. faecium* are usually the most frequent enterococcal species isolated from human clinical specimens. Historically, *E. faecalis* represented about 80 to 90% of the clinically significant enterococcal isolates, while *E. faecium* was found in 5 to 10% of enterococcal infections (47, 48, 49). However, a trend for a progressive decline in the ratio of *E. faecalis* to *E. faecium* is notable, particularly among isolates from bloodstream infections (24, 33, 44, 45, 46, 50, 51). The other enterococcal species are identified less frequently, even though clusters of infections associated with *E. casseliflavus* (52), *E. gallinarum* (53, 54, 55), and *E. raffinosus* (56, 57) have been reported. Several other enterococcal species, including *E. avium*, *E. caccae*, *E. canintestini*, *E. cecorum*, *E. dispar*, *E. durans*, *E. gilvus*, "*E. hawaiiensis*," *E. hirae*, *E. italicus*, *E. malodoratus*, *E. mundtii*, *E. pallens*, *E. pseudoavium*, and *E. thailandicus*, have also been isolated from human sources.

Although the enterococci can cause human infections in the community and in the hospital, these microorganisms began to be recognized with increasing frequency as common causes of health care-associated infections (HAIs) in the late 1970s, paralleling the increasing resistance to most currently used antimicrobial agents. A major impact on the incidence and epidemiology of enterococcal infections was noted after the first reports on the occurrence and epidemic increase of VRE in hospitals in the United States. From 1989 to 1999, the percentage of VRE isolates reported by U.S. hospitals increased from 0.3% to over 25% of all isolates (43). Since then, the enterococci have been usually listed as the second or third most frequent health care-associated pathogen isolated from intensive care unit (ICU) patients in the United States, depending on the type of infection (45, 46, 58). In the 2009-2010 report from the National Healthcare Safety Network (NHSN) at the Centers for Disease Control and Prevention (CDC), the enterococci were listed as the second most common antimicrobial-resistant pathogen associated with HAIs, accounting for about 14% of the total cases of infection (46). In this report, 9.5% of *E. faecalis* and 82.6% of *E. faecium* isolates recovered from the bloodstream infections were found to be resistant to vancomycin (46). The occurrence and spread of VRE have now reached a more global dimension. According to the 2011-2012 surveillance report from the European Centre for Disease Prevention and Control (ECDC), the prevalence of health care-associated VRE ranges from 3.6% to 31% in several European countries (51). The occurrence of VRE has also been reported from several other parts of the world, including South America, Asia, and Australia, illustrating the pandemic spread of health care-associated VRE (24, 34).

Hospitalized patients with gastrointestinal carriage of VRE appear to be the major reservoir of the organism, and once colonized, the patients remain so for weeks or months. Thus, as colonized patients leave the hospital, the possibility that transmission might occur in the community cannot be discounted. VRE can be disseminated by direct patient-to-patient contact or indirectly via transient carriage on health care personnel hands, contaminated medical instruments, such as glucose meters, blood pressure cuffs, electronic thermometers, electrocardiogram (ECG) monitors and wires, and environmental surfaces, such as patient gowns and linens, beds, bedside rails, overbed tables, floors, toilets, door knobs, and wash basins (22, 59). Besides surviving for long periods on environmental surfaces, these microorganisms are tolerant to heat, chlorine, and some alcohol preparations, enhancing the possibilities for transmission.

As a response to the rising rates of VRE colonization and infection in U.S. hospitals, the CDC's Hospital Infection Control Practices Advisory Committee (HICPAC) established guidelines with recommendations for preventing the spread of VRE (60). These include prudent vancomycin use and implementation of surveillance procedures for early detection of VRE, as well as infection control procedures to limit cross-contamination, such as isolation precautions, hand washing, and education about transmission of VRE.

Although only a small percentage of colonized patients develop serious systemic enterococcal infections, intestinal colonization with VRE has been clearly associated with subsequent VRE infections. However, in certain specific clinical situations (i.e., for liver transplant recipients, patients on chronic hemodialysis, and patients with hematological malignancies), VRE-colonized patients appear to be at increased risk for developing serious enterococcal infections (22, 24, 27). This underscores the importance of active surveillance in high-risk patient groups to prevent transmission and outbreaks.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

The standard methods for collecting blood, urine, wound secretions, and other secretions or swab specimens suspected of harboring enterococci are adequate (see chapter 18). No special methods or procedures are usually necessary for transporting and storage of clinical specimens containing enterococci because these microorganisms are easily recovered and are relatively resistant to environmental changes and adverse conditions. Transport can be performed on almost any transport medium or on swabs that are kept dry. Like most clinical samples, the material should be cultured as soon as possible. In a recent study, VRE strains were shown to survive for 14 days in Amies medium when stored at 4°C or room temperature (61).

Enterococcal strains can be stored indefinitely when lyophilized. In our experience, cultures frozen at −70°C or less can be stored for several years as heavy cell suspensions made directly in defibrinated sheep or rabbit blood or in a skim milk (10%) solution containing glycerol (10%). These are the preferable methods for preservation of enterococcal strains. They can also be preserved for many years at −20°C in other cryopreservative media commonly used for maintenance of bacteria. Most strains of enterococci can survive for several months at 4°C on agar slants prepared with ordinary agar bases, such as brain heart infusion (BHI) agar and Trypticase soy agar. Certain of the less well known species, however, are not as resistant to adverse conditions and may not survive long if more-adequate preservation procedures are not used.

DIRECT EXAMINATION

Microscopy

The direct microscopic examination of Gram-stained smears of normally sterile clinical specimens, such as blood, may be useful for the diagnosis of enterococcal infections. Direct examination of certain nonsterile specimens may also be informative but should not be overemphasized. In any case, only a presumptive report of the "presence of Gram-positive-cocci" should be made, as microscopy by itself cannot differentiate the enterococci from most of the other Gram positive-cocci. Culture and appropriate identification techniques should be performed for confirmation.

Nucleic Acid Detection

As the occurrence of VRE continues to represent an important problem worldwide, hospitals are encouraged to implement surveillance programs for VRE detection. In an attempt to overcome the inherent limitations of the culture-based methods of detection (discussed in "Isolation Procedures" below), PCR-based methods have been evaluated for direct detection of these microorganisms in clinical and surveillance specimens. Three major systems are commercially available. They are the LightCycler VRE assay (Roche Molecular Diagnostics, Indianapolis, IN), the GenOhm VanR assay (Becton-Dickinson Microbiology Systems, Cockeysville, MD), and the Cepheid Xpert *vanA*/*vanB* assay (Cepheid, Sunnyvale, CA) (62–73). The GenOhm VanR and the Xpert *vanA*/*vanB* assays have been approved by the U.S. Food and Drug Administration (FDA), while the LightCycler VRE assay has not been licensed for use in diagnostic procedures in the United States yet. An updated version of the GenOhm VanR assay has also been evaluated, and improved specificity was reported (71). Recently, the Cepheid Xpert *vanA* assay for identification of VRE (*vanA* only) was evaluated, and excellent results were reported (74). Nearly all of these studies have reported improved detection of VRE in rectal swabs and fecal specimens over that obtained with conventional culture techniques. It is important to note that these systems use proprietary sequences for detection of the *van* genes and sensitivities may vary. Specificity is also a potential problem unless PCR controls for genus and/or species identification are included, because *van* genes, especially *vanB*, may be found in bacteria other than enterococci (64, 67, 74, 75). The type of sample tested may influence the outcome of testing for VRE. Improved specificities were reported in some studies to be achieved by testing perianal swabs in place of rectal swabs or stool specimens (68, 71). Also, some investigators have used the *vanA*/*vanB* assays to test selective enrichment broths (62, 69) or colonies taken from selective agar plates (66). It may be advisable, if time is not a concern, to detect only *vanA*/*vanB* from an enrichment broth like the Enterococcosel broth (EB), which turns black if enterococci are present.

Assays for detection and identification of enterococci directly in blood samples have been reported. A laboratory-developed real-time PCR assay for quantitative detection of *E. faecalis* DNA in blood samples without prior cultivation has been proposed for the diagnosis of bacteremia (76). The LightCycler SeptiFast Test (Roche Molecular Systems) is a commercially available multiplex real-time PCR assay for the rapid detection and identification of major pathogens involved in nosocomial bacteremia, including *E. faecalis* and *E. faecium*, directly from whole blood (65). As the technology evolves, these molecular methods may become widely available for the rapid and precise detection of enterococci directly in clinical samples. However, further evaluation is needed to determine the real impact of their use on laboratory diagnosis of invasive enterococcal infections and on patient management.

ISOLATION PROCEDURES

The source of clinical specimens to be tested for the presence of enterococci influences the type of medium needed for primary isolation. Clinical specimens from normally sterile body sites can be plated directly onto a nonselective medium such as Trypticase soy agar, brain heart infusion agar, or other blood agar base containing 5% sheep, horse, or rabbit blood. In general, strains of the most clinically relevant species grow well at 35 to 37°C and do not require an atmosphere containing increased levels of CO_2, although some strains grow better in this atmosphere. Samples for blood culture can be inoculated into conventional blood culture systems. For clinical specimens obtained from nonsterile sites, especially those heavily contaminated with Gram-negative bacteria, use of selective media is a good option for primary isolation. In these cases, and particularly when enterococci may be present in low numbers, consideration must also be given to whether or not an enrichment broth should be employed. The use of a broth enrichment step in the primary isolation delays the identification but increases the recovery rates of enterococci. Many different media have been devised for selective isolation of enterococci. Most of them contain bile salts, sodium azide, and/or antibiotics as selective components and esculin or tetrazolium as indicator substances. Not all enterococcal species grow on these selective media, although most *E. faecalis* and *E. faecium* isolates should do so. The diversity of media used for the isolation of enterococci from various sources has been reviewed (27).

The increasing incidence of vancomycin resistance among the enterococci has raised the importance of selective isolation of VRE. Early identification of infection or colonization by VRE is recommended to prevent the spread of these microorganisms (60, 77). Current recommendations for hospital infection control may include VRE fecal surveillance cultures, but the optimal methods for these cultures are still unclear. Different selective agar and/or broth medium formulations and several procedures have been employed for the isolation of VRE from sources containing a normal microbiota, such as stool samples and rectal or perianal swab specimens. Most of them are variations of selective media, differing with regard to the antimicrobial agents or the antimicrobial concentrations used. Although no consensus for a VRE isolation protocol has been established, the use of a selective-enrichment broth seems to be a highly effective procedure to enhance the recovery of VRE. For this purpose, BHI broth has been used with vancomycin concentrations varying from 3 μg/ml (67, 78) and 6 μg/ml (79) to 30 μg/ml (80). Bile esculin azide (BEA) broth has been supplemented with vancomycin at concentrations of 4 μg/ml (69), 6 μg/ml (68), 8 μg/ml (63, 66), and 15 μg/ml (81). Enterococcosel broth (EB; a BEA medium supplied by Becton Dickinson Microbiology Systems) has also been used in a number of studies as the base medium supplemented with concentrations of vancomycin varying from 4 μg/ml (72), 6 μg/ml (68, 82–84), and 8 μg/ml (62) to 64 μg/ml (85). One advantage that the bile-esculin-based broth media (BEA and EB) have over BHI-based media is that those media containing esculin turn black as the enterococci grow, and if the esculin-containing medium does not turn black, it can be assumed that no enterococci are present. Several studies report improved recovery of VRE from selective-enrichment broth over that obtained with direct plating (78, 80, 81, 83). Among the different concentrations of vancomycin used, 6 μg/ml is the most commonly used. In some of the protocols, various concentrations of other antimicrobial agents, such as aztreonam, meropenem, and clindamycin, have been used in the enrichment broth (67, 69, 85). In a recent work, a 30-μg vancomycin disk was simply added to BHI broth (73). Overall, there are no studies reporting extensive comparison of recovery rates of VRE from different selective-enrichment broths.

Media containing chromogenic substrates have also been proposed for the isolation and presumptive identification of enterococci. Table 1 contains a list of various chromogenic media that are commercially available, as well as the concentration of vancomycin in each, the distributors, and references reporting the results of studies performed with each of these media. Some of these media have not yet been fully evaluated. CHROMagar orientation and CPS ID3 were promoted as isolation and identification media for enterococci from urine (86, 87, 88). Additionally, some chromogenic media that are selective for VRE have the advantage over the bile esculin-based selective media in that the colors of the colonies can help in the presumptive identification of VRE species (87, 89, 90). In some surveillance studies, selective enrichment was used prior to inoculation of the chromogenic agar (67, 78, 81), while in others the perianal, rectal, or stool samples were directly inoculated onto the surface of the agar. In some circumstances, it may be necessary to recover VRE from environmental surfaces for epidemiological studies. Isolation of the organisms from these surfaces can be accomplished by swabbing the surfaces with premoistened swabs and placing them either into a selective-enrichment broth or onto selective agar plates.

TABLE 1 List of commercially available agar-based media for isolation of *Enterococcus*

Medium	Distributor	Vancomycin concn (μg/ml)	Organism: colony color	Reference(s)
Bile-esculin agar	Many	None	*Enterococcus* spp.: black	68, 72
Bile-esculin-azide agar	Many	None; 6/8	*Enterococcus* spp.: black	63, 90
Enterococcosel agar	Many	6/8	*Enterococcus* spp.: black	71, 83, 86, 88
Enterococcus selective agar (VSA)	Oxoid	6	*Enterococcus* spp.: brown with black zones around colonies	82
CHROMagar orientation	bioMérieux; Becton Dickinson	None	*Enterococcus* spp.: blue	87
CPS-ID-3	bioMérieux	None	*Enterococcus* spp.: turquoise	87
Brilliance VRE agar	Oxoid/Thermo Fisher Scientific	Not reported	*E. faecalis*: blue to light blue; *E. faecium*: navy blue (indigo), pink-purple	89, 90
ChromID VRE	bioMérieux	8	*E. faecalis*: green to blue; *E. faecium*: purple	67, 69, 73, 78, 79, 80, 84, 89
Spectra VRE	Remel/Thermo Fisher Scientific	6	*E. faecalis*: blue to light blue; *E. faecium*: pink to light purple or navy blue	84
HardyChrom VRE agar	Hardy Diagnostics	10	*E. faecalis*: dark red; *E. faecium*: blue	Package insert
VRESelect	Bio-Rad	8	*E. faecalis*: blue; *E. faecium*: pink	Package insert
Campy agar	Becton Dickinson; Remel/Thermo Fisher	10	*Enterococcus* spp.: similar to colonies on regular blood agar media	84

Culture-based screening methods for VRE may be especially demanding, can take several days to complete, and have variable degrees of sensitivity, all of which can impact the timely implementation of infection control procedures. Therefore, some microbiology laboratories have considered the introduction of molecular techniques to detect VRE to facilitate the rapid and accurate identification of these organisms and to improve sensitivity for detecting this pathogen in comparison with culture. However, many of the current approaches still require bacterial growth in culture prior to detection, requiring 24 h or more to complete. Consequently, the application of methods for a more rapid detection of VRE directly from clinical samples is still an area of significant interest (see "Direct Examination" above).

Furthermore, because a laboratory report of VRE can initiate a cascade of infection control events that are time-consuming and costly, laboratories must be certain of the epidemiological importance of any suspected VRE isolate. The transferable and high-level vancomycin resistance, especially that encoded by the *van*A or the *van*B genes, most frequently associated with *E. faecalis* and *E. faecium* isolates, is the major focus of infection control efforts (see "Antimicrobial Susceptibilities" below). In contrast, the intrinsic low-level *van*C resistance, which is not associated with wide dissemination of resistant strains, is less likely to be important for infection control surveillance efforts. While the isolation of VRE from clinical and surveillance specimens is important, the need for establishing protocols to rapidly determine the likely underlying mechanism of resistance (see "Antimicrobial Susceptibilities" below) is equally important.

IDENTIFICATION

Identification by Conventional Physiological Testing

Once it is established that an unknown catalase-negative Gram-positive coccus is an *Enterococcus* or closely related genus (see "Description of the Genus" above), the appropriate tests (see reference 17 and chapters 4, 19, and 20) listed in Table 2 can be used to identify the species. The data presented in Table 2 for the phenotypic characteristics are derived from conventional testing (except for β-galactosidase) as performed at the CDC *Streptococcus* Laboratory (see http://www.cdc.gov/ncidod/biotech/strep/strep-doc/index.htm). Table 2 presents the phenotypic characteristics of isolates most frequently recovered from humans. Isolates from nonhuman sources, even those belonging to well-known species, may have different results for some tests. Species that have not been recovered from humans to date are also included in Table 2, as the possibility exists that they may be isolated from human sources in the future. Two recently described species (*Enterococcus diestrammenae* and *Enterococcus xiangfangensis* (see http://www.bacterio.net for further details) were not included in Table 2 because their respective type strains were not available for testing by the time this chapter was prepared. Identification of enterococcal species by conventional tests can be time-consuming and may require incubation of the tests for up to 10 days before a definitive identification can be reached. On the other hand, the majority of the isolates recovered from human sources can be identified after 2 days of incubation.

Enterococcal species can be initially separated into five physiological groups of species based on acid formation from mannitol and sorbose and hydrolysis of arginine (see Table 2 for details). Group I includes 10 species (Table 2). "*E. hawaiiensis*" is a denomination that has been proposed for the new species previously provisionally designated as *Enterococcus* sp. nov. CDC PNS-E3 (91). *E. avium* and *E. raffinosus* are possibly the most relevant species in this group, considering their association with human clinical sources.

Group II comprises 9 species. The majority of the isolates recovered from human sources belong to species included in this group. Atypical strains that do not hydrolyze arginine or do not form acid from mannitol have been documented. *Lactococcus* sp. is also listed in this group because the phenotypic characteristics of some strains can lead to their misidentification as *Enterococcus* isolates. If nonmotile variants of *E. casseliflavus* and *E. gallinarum* are encountered, production of acid from methyl-α-D-glucopyranoside can be used to help in the identification of these species.

Group III consists of 7 species. Three of these species (*E. durans*, *E. ratti*, and *E. villorum*) have very similar phenotypic profiles in the tests listed in Table 2. Reactions in litmus milk and hydrolysis of hippurate may also be used to help differentiate the species in addition to those reactions listed in Table 2. In litmus milk, *E. durans* forms acid and clot, *E. villorum* forms acid but no clot, and *E. ratti* does not form acid or clot. *E. durans* hydrolyzes hippurate, while *E. villorum* does not. *E. ratti* is variable in the hippurate hydrolysis test. Uncommon mannitol-negative variant strains of *E. faecalis* and *E. faecium* resemble species in this group. However, *E. faecalis* strains are positive in the pyruvate test but not for acid formation from arabinose, raffinose, or sucrose, and *E. faecium* variant strains form acid from arabinose.

Group IV includes 8 species. *E. caccae* and *E. cecorum* are the only species in this group that have been isolated from human sources to date.

Group V comprises 13 species. They correspond to isolates from a variety of environmental and animal sources. Only *E. italicus* has already been recovered from human sources (91, 92). Variant strains of *E. casseliflavus*, *E. gallinarum*, and *E. faecalis* that fail to hydrolyze arginine resemble the microorganisms included in this group. However, these variant strains have characteristics similar to the strains that hydrolyze arginine and can be differentiated by these same phenotypic tests. *Vagococcus fluvialis* is listed here because the phenotypic characteristics of this species are very similar to those of the genus *Enterococcus* and some strains may be identified as enterococci (18).

Identification by Commercial Systems

Several miniaturized, manual, semiautomated, and automated systems are commercially available and represent alternatives to conventional testing for the phenotypic identification of enterococcal species in routine diagnostic laboratories. Since their introduction, these systems have been updated to improve their performance characteristics and expand their identification capabilities, as investigators have become more aware of inaccuracies (19, 57, 93, 94). In general, these systems are reliable for the identification of the most common species: *E. faecalis*, and to a lesser extent, *E. faecium*. Precise identification of other species, by most systems, depends on additional testing, although improvements have been observed with updated formats and databases. Commercial systems available for the identification of enterococcal species include the API 20S and the API Rapid ID32 STREP systems (bioMérieux, Durham, NC), the Crystal Gram-Positive and the Crystal Rapid Gram-Positive identification systems (Becton Dickinson Microbiology Systems, Sparks, MD), and the commercial

TABLE 2 Phenotypic characteristics used for the identification of *Enterococcus* species and some physiologically related species of other Gram-positive cocci

Group and species[a]	Phenotypic characteristic[b]														
	MAN	SOR	ARG	ARA	SBL	RAF	TEL	MOT	PIG	SUC	PYU	MGP	TRE	XYL	GAL
Group I															
E. avium	+	+	−	+	+	−	−	−	−	+	+	V	+	−	V
E. raffinosus	+	+	−	+	+	+	−	−	−	+	+	V	+	−	−
E. gilvus	+	+	−	−	+	+	−	−	+	+	+	V	+	−	−
E. pallens	+	+	−	+	+	+	−	−	+	+	+	+	+	−	−
E. saccharolyticus[c]	+	+	−	−	+	+	−	−	−	+	−	+	+	−	+
E. malodoratus	+	+	−	−	+	+	−	−	−	+	+	V	+	V	+
E. ureilyticus[c]	+	+	−	−	+	−	−	−	+	+	+	+	+	−	−
E. pseudoavium	+	+	−	−	+	−	−	−	−	V	V	+	+	V	V
E. devriesei[c]	+	+	−	−	+	−	−	−	−	+	−	−	+	−	−
“*E. hawaiiensis*”[c]	+	+	−	−	+	−	−	−	−	−	+	−	+	−	−
Group II															
E. faecium	+[d]	−	+	+	V	V	−	−	−	+[d]	−	−	+	−[d]	V
E. casseliflavus	+	−	+[d]	+	V	+	−[d]	+[d]	+[d]	+	V	+	+	+	+
E. gallinarum	+	−	+[d]	+	−	+	−	+[d]	−	+	V	+	+	+	+
E. mundtii	+	−	+	+	V	+	−	−	+	+	V	−	+	+	V
E. lactis[c]	+	−	+	+	−	−	−	−	−	+	−	−	+	−	+
E. faecalis	+[d]	−	+[d]	−	+	−	+	−	−	+[d]	+	−	+	−[d]	−
E. haemoperoxidus[c]	+[e]	−	+[e]	−	−	−	−	−	−	+	−	+	+	−	−
E. rotai[c]	+	−	+	−	−	−	−	−	+	+	−	+	+	−	−
E. thailandicus[c]	+	−	+	−	−	−	−	−	−	+	−	−	+	−	−
Lactococcus sp.	+	−	+	−	−	−	−	−	−	V	−	−	+	−	−
Group III															
E. dispar	−	−	+	−	−	+	−	−	−	+	+	+	+[f]	−	+
E. canintestini[b]	−	−	+	−	−	+	−	−	−	+	+	+	+	−	−
E. hirae	−	−	+	−	−	+	−	−	−	+	−	−	+	−	V
E. quebecensis[c]	−	−	+	−	−	−	−	−	−	+	−	−	+	−	+
E. durans	−	−	+	−	−	−	−	−	−	−	−	−	−[d]	−	V
E. ratti	−	−	+	−	−	−	−	−	−	−	−	−	V	−	−
E. villorum	−	−	+	−	−	−	−	−	−	−	−	−	+	+	+
Group IV															
E. aquimarinus[c]	−	−	−	+	−	+	−	−	−	+	−	+	+	+	+
E. phoeniculicola[c]	−	−	−	+	−	+	−	−	−	+	V	+	+	+	+
E. cecorum[c]	−	−	−	−	−	+	−	−	−	+	+	−	+	−	+
E. sulfureus[c]	−	−	−	−	−	+	−	−	+	+	V	+	+	−	+
E. asini[c]	−	−	−	−	−	−	−	−	−	+	−	+[e]	+	+	+
E. caccae	−	−	−	−	−	−	−	−	−	+	+	+[e]	+	−	−
E. silesiacus[c]	−	−	−	−	−	−	−	−	−	+	−	+[e]	+	+	+
E. termitis[c]	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−
Group V															
E. canis[c]	+	−	−	+	−	−	−	−	−	+	+	+	+	+	+
E. columbae[c]	+	−	−	+	+	+	−	−	−	+	+	V	+	+	−
E. lemanii[c]	+	−	−	+	−	+	−	−	−	+	V	+	+	+	+
E. moraviensis[c]	+	−	−	+	−	−	−	−	−	+	+	+	+	V	+
E. ureasiticus[c]	+	−	−	+	−	−	−	−	−	+	−	+	+	−	−
E. camelliae[c]	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−
E. hermanniensis[c]	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−
E. italicus	V	−	−	−	V	−	−	−	−	+	+	+	+	−	−
E. alcedinis[c]	+	−	−	−	−	−	−	−	−	+	−	+	+	+	+
E. eurekensis[c]	+	−	−	−	−	+	−	−	−	+	−	+	+[c]	+	+
E. plantarum[c]	+	−	−	−	−	−	−	−	+	+	−	+[f]	+	−	−
E. rivorum[c]	+	−	−	−	+	−	−	−	−	+	−	+	+	+	−
E. viikkiensis[c]	+	−	−	−	−	−	−	−	−	−	V	−	+	−	−
Vagococcus fluvialis	+	−	−	−	+	−	−	+	−	+	−	+	+	−	−

[a] Names in boldface represent species that have been isolated from human sources.

[b] Abbreviations and symbols: MAN, mannitol; SOR, sorbose; ARG, arginine; ARA, arabinose; SBL, sorbitol; RAF, raffinose; TEL, 0.04% tellurite; MOT, motility; PIG, pigment; SUC, sucrose; PYU, pyruvate; MGP, methyl-α-D-glucopyranoside; TRE, trehalose; XYL, xylose; GAL, 2-naphthyl-β-D-galactopyranoside; +, 90% or more of the strains are positive; −, 90% or more of the strains are negative; V, variable (11 to 89% of the strains are positive).

[c] Phenotypic characteristics based on data from type strains.

[d] Occasional exceptions occur (<3% of strains show atypical reactions).

[e] Late positive (3 days of incubation or longer).

[f] Weak reaction.

identification panels on the Vitek (bioMérieux), MicroScan (Dade MicroScan, West Sacramento, CA), and BD Phoenix (Becton Dickinson Microbiology Systems) systems. Overall, a large proportion of enterococcal isolates recovered from human sources are accurately identified by most of the commercial systems now available; however, the accuracy is dependent on the distribution of species found in each specific setting. Difficulties in the identification of a variety of enterococcal species, sometimes including *E. faecalis* and *E. faecium*, have been reported (19, 95). Strict adherence to the instructions provided by the manufacturer, including the base of the medium used to grow strains for testing, is of paramount importance. Our own unpublished experimental results indicate that differences in growth conditions can lead to variation in the results of some tests, interfering with the accuracy of the identification. Identification of an unusual enterococcal species by a commercial system should be confirmed by a reference method before being reported.

Identification by MALDI-TOF MS

Currently, bacterial identification using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is being considered a revolutionary tool for bacterial identification in the clinical microbiology laboratory (96, 97). Compared with other methods, this technology can readily and conveniently identify a wide range of microorganisms, including enterococcal species (98–102). Two major MALDI-TOF MS platforms are available at this time: the Microflex LT Biotyper (Bruker Daltonics, Bremen, Germany) and the Vitek MS system (bioMérieux, Marcy l'Etoile, France). Results of a comparative evaluation of these platforms indicated that they both were able to identify correctly all the enterococcal isolates tested (98). However only *E. faecalis*, *E. faecium*, *E. casseliflavus*, and *E. gallinarum* were identified in this study. At this point, an extensive evaluation of either system including the different enterococcal species is still not available. Although the numbers of studies and species tested are still limited, the results indicate that the method constitutes an important tool to rapidly identify the major enterococcal species recovered from human sources and certainly deserves to be further evaluated for the identification of the diverse members of the genus *Enterococcus*, including isolates with a variety of biochemical profiles and obtained from different sources. In addition, MALDI-TOF MS has also been applied directly to positive blood cultures without the need for additional culturing (103, 104).

Identification by Molecular Methods

Molecular methods, such as DNA-DNA hybridization and sequencing of the 16S rRNA genes, have been primarily used for taxonomic purposes in reference or research laboratories. In the past decades, however, the application of molecular techniques for the rapid identification of *Enterococcus* species has also been expanded dramatically for use in clinical microbiology laboratories.

A variety of molecular procedures have been proposed for the identification of enterococcal species as previously reviewed and summarized (1, 105). Many of these molecular procedures have been performed in only a few laboratories and have not been evaluated for all the species of *Enterococcus*. Most of them are potentially applicable to all enterococcal species, and others are species specific. Several of these methods deserve consideration for expanded testing and future improvements, as they represent promising adjunct tools for a more rapid and precise identification of enterococcal species.

Among the molecular techniques proposed to identify the different enterococcal species, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole-cell protein (WCP) profiles and sequencing of the 16S rRNA genes have been more extensively evaluated in reference laboratories. SDS-PAGE analysis of WCP profiles was shown to be a reliable tool for the differentiation and identification of typical and atypical *Enterococcus* strains, since WCP profiles are species specific (1, 12, 91, 106, 107). After the introduction of DNA-based methods, however, the use of this molecular phenotypic approach has decreased and is now basically limited to taxonomic studies.

Table 2, which describes the phenotypic characteristics of the *Enterococcus* species discussed in this chapter, is based on correlations between the WCP profiles and the phenotypic tests, in conjunction with DNA-DNA reassociation experiments and 16S RNA gene sequencing. Sequencing of the 16S rRNA gene is currently the most frequently used among the nucleic acid-based methods for identification of enterococcal species. It has been performed for all species of enterococci, and the sequences are available for comparison purposes via public databases of nucleotide sequences, such as GenBank. Comparisons can be made by using one of the several sequence-comparing software packages, many of which are available for public access. Figure 1 depicts a dendrogram generated by comparison of 16S rRNA gene sequences of the type strains of the species included in the genus *Enterococcus*. Nevertheless, clear-cut differentiation is not always obtained for all enterococcal species, since some of them differ by only 2 or 3 bases over the approximately 1,500-base span of the 16S rRNA gene. Therefore, this procedure should not be used alone, but together with phenotypic characterization and other alternative molecular methods, it can be an important tool to establish enterococcal species identity. Several other housekeeping genes have been investigated as alternative tools to develop sequencing-based methods to identify enterococci, either alone or in association. They include the gene sequences encoding the ATP synthase β subunit (*atpD*), the phenylalanyl-tRNA synthase (*pheS*), the RNA polymerase α subunit (*rpoA*), the RNA polymerase β subunit (*rpoB*), the manganese-dependent superoxide dismutase (*sodA*), and the elongation factor Tu (*tufA*) (108–113). Their use has been restricted to taxonomic purposes mostly, and none has been applied to all the enterococcal species described to date.

PCR-based techniques have also been a focus of major interest for rapid and accurate enterococcal identification. Tests based on the amplification of the gene encoding the D-ala D-ala ligase (*ddl*) (19, 98, 114) or the *sodA* gene (19) have already been designed for several enterococcal species and constitute convenient alternative approaches for rapid routine identification. PCR methods to identify all the enterococcal species, however, are not available yet.

The use of molecular methods for the rapid identification of enterococci directly from positive blood culture bottles has also been investigated. Fluorescence *in situ* hybridization (FISH) techniques have been developed and evaluated for the identification of enterococci from positive blood culture bottles (115–117). One of these assays, the *E. faecalis*/Other *Enterococcus* species (EF/OE) PNA FISH (AdvanDx, Woburn, MA) is a peptide nucleic acid (PNA) FISH test that is FDA cleared. Recent multicenter studies have indicated that the EF/OE PNA FISH procedure can offer a time to reliable results of 1.5 h, thus further reducing the reporting turnaround time when compared to the original 2.5-h assay procedure (117). Other commercially available diagnostic systems based on nucleic acid technologies that have been cleared by the FDA include the Verigene Gram-positive blood culture (BC-GP) nucleic acid test (Nanosphere, Inc., Northbrook, IL) (118, 119) and the FilmArray Blood Culture ID Panel (Biofire Diagnostics, Inc., Salt Lake City, UT) (120). These multiplex tests are capable of detecting most leading causes of Gram-positive bacterial bloodstream infections, including enterococci, as well as the genetic markers of vancomycin resistance *vanA* and *vanB*. These assays have been considered relatively easy to implement, leading to earlier identification of invasive infections caused by *Enterococcus* species than that obtained with conventional microbiological methods. As recently reported, these technologies can also have a significant impact on the clinical and economical outcome of patients with enterococcal bacteremia by decreasing the time to antimicrobial therapy, the hospital length of stay, and health care costs (121).

TYPING SYSTEMS

The increasing documentation of *Enterococcus* as a leading nosocomial pathogen frequently resistant to several antimicrobial agents, as well as the evidence supporting the concept of exogenous acquisition of enterococcal infections, has generated demand for strain typing and epidemiological studies. Classic phenotypic methods used to investigate the diversity among isolates of a given enterococcal species have frequently failed to adequately discriminate among strains, and they have limited value in epidemiological studies. However, phenotypic information in association with molecular data can constitute valuable information (1, 105).

The introduction of molecular techniques has substantially improved the ability to discriminate enterococcal isolates and has provided critical insights into the epidemiology of the enterococci. By using molecular typing approaches, it was possible to demonstrate the exogenous acquisition of enterococcal strains by direct and indirect contact among patients, breaking the traditional conception that enterococcal infections were endogenous in nature. Intrahospital transmission and interhospital spread have been extensively documented for antimicrobial-resistant enterococci, especially VRE (1, 24, 28, 122, 123). In addition to epidemiological investigations, some of the molecular typing techniques are now used to trace the dissemination of enterococci in different environments and hosts and the evolution of multidrug-resistant strains, greatly expanding our understanding of enterococcal epidemiology, population structure, antimicrobial resistance, and virulence. Emergence and global dispersion of certain epidemic enterococcal clonal complexes have been identified (32–35, 39, 124).

Several molecular methods have been proposed to type enterococcal isolates, as previously reviewed (1, 105). In addition to differences in complexity and costs, these methods vary in their reproducibility and discriminatory power. Overall, there is not a single definitive typing technique for enterococci, so a strong match among the results of different typing techniques, particularly those based on different genomic polymorphisms, should be used as indicative of high relatedness. Among these techniques, analysis of chromosomal DNA restriction profiles by pulsed-field gel electrophoresis (PFGE) has been extensively evaluated for epidemiological characterization of enterococcal outbreaks, showing improved discrimination and allowing the identification of clonal complexes that predominate among multidrug-resistant enterococci, mainly isolates with high-level resistance (HLR) to aminoglycosides and VRE (1, 48, 122, 125). SmaI is the restriction enzyme most frequently used to digest enterococcal DNA, although the usefulness of others, such as ApaI and SfiI, has also been reported (1). PFGE is possibly the typing method most commonly used in clinical microbiology settings, and it is considered by many investigators to be the gold standard for the epidemiological analysis of enterococcal outbreaks. Several protocols for performing PFGE typing of enterococcal strains have been published. However, the development of standardized protocols for execution, interpretation, and nomenclature, as a result of collaborative studies, is still needed in order to allow for interlaboratory data exchange and comparisons. On the other hand, although PFGE is quite discriminatory, epidemiological interpretation of PFGE profiles is not always clear-cut. The occurrence of genetic events can be associated with substantial changes in the PFGE profiles, leading to problems in clonality assessment (126). Due to the possibility of such inconsistencies in DNA banding patterns of enterococci, PFGE is recommended mostly for the purpose of evaluating the genetic relatedness and tracing transmission of strains that are associated in time and location, as its usefulness for long-term epidemiological studies may be limited. The use of PFGE in conjunction with at least one additional typing technique or an independent PFGE analysis using different restriction enzymes is highly recommended to help in clarifying epidemiological interpretation. General principles proposed for the interpretation of molecular typing data based on fragment differences are usually applied to interpret PFGE profiles obtained for enterococcal strains (1, 127). Well-characterized control strains should be evaluated along with unknown isolates. For that purpose, two reference strains, *E. faecalis* OG1RF (ATCC 47077) and *E. faecium* GE1 (ATCC 51558), have been proposed (127).

Two other robust molecular techniques are multilocus sequence typing (MLST) and multiple-locus variable-number tandem repeat analysis (MLVA). These techniques circumvent the difficulties in data exchange between different laboratories by generating information that is suitable for the development of Web-based databases. MLST is based on identifying alleles after sequencing of internal fragments of a number of selected housekeeping genes, resulting in a numeric allelic profile. Each profile is assigned a sequence type (ST). Internet sites with the possibility for data exchange (www.mlst.net and www.pubMLST.org), which contain MLST schemes for *E. faecium* (128) and *E. faecalis* (129), have been developed. Application of MLST has revealed the occurrence of host-specific genogroups of *E. faecium* and allowed the recognition of a hospital-adapted *E. faecium* polyclonal subpopulation (particularly MLST sequence type 17 [ST17], ST18, ST78, and ST192) that predominates in several geographic areas and is associated with the increased frequency of isolation of *E. faecium* worldwide (24, 32, 33, 39, 123, 124, 130). This hospital-adapted lineage was initially named “C1 lineage” and later renamed “clonal complex-17” (CC17), and it is classified as an example of the so-called high-risk enterococcal complexes (HiRECC). Recent comparisons of available genome sequences support the concept of a hospital-associated clade that is genetically distinct from most commensal isolates (34, 131). Two major clonal complexes (named CC2 and CC9) have also been identified among hospital-derived *E. faecalis* isolates (35, 39, 122, 129) by using MLST.

Two simultaneously published studies described the development of MLVA typing schemes for *E. faecalis* (132) and *E. faecium* (133). MLVA is based on variation in variable-number tandem repeats (VNTR) loci dispersed over the enterococcal genome. For each VNTR locus, the number of repeats is determined by PCR using primers based on the conserved flanking regions of the tandem repeats. PCR products are separated on agarose gels, and the band size determines the number of repeats. These numbers together result in an MLVA profile, and each profile is assigned an MLVA type (MT). Comparative studies indicate that both techniques can achieve high degrees of discrimination between isolates and have discriminatory power (134) comparable to that of PFGE-based typing (122, 129, 133). In contrast to the overt advantages (being reproducible, portable, highly discriminatory, and unambiguous), MLST is time-consuming, expensive, and still limited to laboratories that have facilities for both PCR and sequencing, while MLVA requires PCR and basic electrophoresis facilities.

More recently, whole-genome sequencing (WGS) using next-generation instruments has emerged as a cost-effective and convenient approach for several microbiological applications, including typing of bacterial isolates (135, 136). The use of this technology is still limited, but the advantages of using it for genotyping enterococcal isolates and the consequent impact on our understanding on the epidemiology of enterococcal infections have already been reported (136, 137).

SEROLOGIC TESTS

Serologic tests for detecting antibody responses to different enterococcal antigens have been proposed (138, 139). However, their usefulness in the clinical laboratory setting for the diagnosis of enterococcal infections has not been demonstrated.

ANTIMICROBIAL SUSCEPTIBILITIES

Resistance to several commonly used antimicrobial agents is a remarkable characteristic of most enterococcal species. Moreover, most of this information is based on studies with *E. faecalis* and *E. faecium*, the two species that are frequently associated with human infections. Antimicrobial resistance can be classified as either intrinsic or acquired. Intrinsic resistance is related to inherent or natural chromosomally encoded characteristics present in all or most of the enterococci. Furthermore, certain specific mechanisms of intrinsic resistance to some antimicrobial agents are typically associated with a particular enterococcal species or groups of species. In contrast, the occurrence of acquired resistance is more variable, resulting from either mutations in existing DNA or acquisition of new genetic determinants carried in plasmids or transposons (24, 29, 36, 140, 141, 142). Enterococcal intrinsic resistance involves several antimicrobial agents, particularly two major groups: the aminoglycosides and the β-lactams. Because of the poor activity of several antimicrobial agents against enterococci due to intrinsic resistance, the recommended therapy for serious infections (i.e., endocarditis, meningitis, and other systemic infections), especially in immunocompromised patients, includes a combination of a cell wall-active agent, such as a β-lactam (usually penicillin or ampicillin) or vancomycin, and an aminoglycoside (usually gentamicin or streptomycin). These combinations overcome the intrinsic resistance exhibited by the enterococci, and a synergistic bactericidal effect is generally achieved since the intracellular penetration of the aminoglycoside is facilitated by the cell wall-active agent.

In addition to the intrinsic resistance traits, enterococci have acquired different genetic determinants that confer resistance to several classes of antimicrobial agents, including chloramphenicol, tetracyclines, macrolides, lincosamides and streptogramins, aminoglycosides, β-lactams, glycopeptides, quinolones, and even some of the more recently available drugs such as linezolid, daptomycin, and quinupristin-dalfopristin (22, 23, 140, 142, 143). During the past several decades, the occurrence of acquired antimicrobial resistance among enterococci, especially HLR to aminoglycosides, β-lactams, and resistance to glycopeptides (especially vancomycin), has been increasingly reported. Isolates that are resistant to the cell wall-active agent or have HLR to aminoglycosides are resistant to the synergistic effects of combination therapy and constitute a problem of peculiar importance. Therefore, the detection of resistance to these groups of antimicrobial agents is critical in order to predict the likelihood of synergy by using antimicrobial combinations as a therapeutic strategy. Enterococcal isolates exhibiting HLR to aminoglycosides are now present in large proportions in several geographic areas (24, 36, 48, 140, 142, 144). Such strains frequently have MICs of >2,000 μg/ml and cannot be detected by diffusion tests with conventional disks. Special tests using high-content gentamicin and streptomycin disks as well as a single-dilution method were developed to screen for this type of resistance (see references 1 and 145 and chapters 71 and 73). Strains exhibiting high-level resistance to penicillin and ampicillin due to altered penicillin-binding proteins have also disseminated widely (24, 29, 36, 140, 142), while strains producing β-lactamase have been rarely identified (29, 48). Results of susceptibility testing with ampicillin may be used to predict susceptibility to amoxicillin, amoxicillin-clavulanate, ampicillin-sulbactam, piperacillin, and piperacillin-tazobactam among non-β-lactamase-producing enterococci. Ampicillin susceptibility can also be used to predict imipenem susceptibility, provided that the species is *E. faecalis* (145, 146). However, enterococcal susceptibility to penicillin cannot be predicted on the basis of ampicillin testing results. If penicillin results are needed, testing of penicillin is required. On the other hand, enterococci susceptible to penicillin are predictably susceptible to ampicillin and the other β-lactams mentioned above. Penicillin and ampicillin resistance due to β-lactamase production is not reliably detected by routine disk or dilution methods but is detected by using a nitrocefin-based β-lactamase test (1, 145).

The emergence of acquired vancomycin resistance as a therapeutic problem among enterococcal isolates was first documented in Western Europe (147, 148). Thereafter, the isolation of VRE has been continuously reported, showing epidemic proportions in diverse geographic locations (22, 24, 36, 140, 142). VRE strains have been classified according to phenotypic and genotypic features (Table 3). Nine types of glycopeptide resistance have been described to date among enterococci. Each type is associated with different genetic elements, some of which, in turn, can be divided into subtypes. Three of them are the most common: the VanA phenotype, with inducible high-level resistance to vancomycin as well as to teicoplanin, encoded by the *vanA* gene; the VanB phenotype, with variable (moderate to high) levels of inducible resistance to vancomycin only, encoded by the *vanB* genes; and the VanC phenotype, encoded by the *vanC* genes, conferring constitutive low-level resistance to vancomycin. VanA and VanB are considered the most clinically relevant phenotypes and are usually associated with *E. faecium* and *E. faecalis* isolates, while the VanC resistance is an intrinsic characteristic of *E. gallinarum* (*vanC1* genotype) and *E. casseliflavus* (*vanC2* to *vanC4* genotypes) (36, 141, 149, 150). The additional types of glycopeptide resistance, encoded by the *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN* genes (141, 151, 152, 153) seem to

TABLE 3 Types of resistance to glycopeptides among members of the genus *Enterococcus*[a]

Characteristic	Characterization according to the type of resistance to glycopeptides								
	VanA	VanB	VanC	VanD	VanE	VanG	VanL	VanM	VanN
MIC (in μg/ml) and resistance level									
Vancomycin	64–1,000 (R)	8–1,000 (I/R)	2–32 (S/R)	64–128 (R)	8–32 (I)	16 (I)	8 (I)	>256 (R)	12–16 (I)
Teicoplanin	16–512 (R)	0.5–1 (S)	0.5–1 (S)	4–64 (S/R)	0.5 (S)	0.5 (S)	0.5 (S)	64 to >256 (R)	0.5 (S)
Classification (level)	High	Variable	Low	Moderate	Low	Low	Low	Variable	Low
Genotype	*vanA*	*vanB*[b]	*vanC*[b]	*vanD*[b]	*vanE*	*vanG*[b]	*vanL*	*vanM*	*vanN*
Mobile element	Tn*1546*	Tn*1547* or Tn*1549* to Tn*5382*	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
Occurrence of conjugation	+	+	−	−	−	+	−	+	+
Location of *van* genes	Plasmid chromosome	Plasmid chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Unknown	Unknown	Plasmid
Type of expression	Inducible	Inducible	Constitutive	Constitutive	Inducible	Inducible	Unknown	Unknown	Constitutive
Gene product[c] (modified target)	D-Ala-D-Lac	D-Ala-D-Lac	D-Ala-D-Ser	D-Ala-D-Lac	D-Ala-D-Ser	D-Ala-D-Ser	D-Ala-D-Ser[d]	D-Ala-D-Lac[d]	D-Ala-D-Ser[d]
Distribution among species	*E. faecalis*, *E. faecium*, *E. avium*, *E. casseliflavus*, *E. durans*, *E. gallinarum*, *E. hirae*, *E. mundtii*, *E. raffinosus*, *E. thailandicus*	*E. faecalis*, *E. faecium*, *E. durans*, *E. gallinarum*	*E.casseliflavus*, *E. gallinarum*	*E. faecium*, *E. faecalis*, *E. avium*, *E. gallinarum*, *E. raffinosus*	*E. faecalis*	*E. faecalis*	*E. faecalis*	*E. faecium*	*E. faecium*

[a] R, resistant; S, susceptible; I, intermediate.
[b] Subtypes exist: *vanB1* to *vanB3*; *vanC1* to *vanC4*; *vanD1* to *vanD5*; *vanG1* to *vanG2*.
[c] Ala, alanine; Lac, lactate; Ser, serine.
[d] Product deduced but not confirmed.

occur rarely among enterococci. Furthermore, the isolation of vancomycin-dependent (154) and vancomycin-heteroresistant (155) enterococcal strains from clinically significant infections, although sporadically reported, may also represent additional serious threats for the treatment and control of enterococcal infections.

While *in vitro* methods for detecting vancomycin resistance are discussed in detail in references 1 and 145 as well as in chapters 71 and 73, some aspects regarding *van*C-containing species (e.g., *E. gallinarum* and *E. casseliflavus*) need to be emphasized. Resistance associated with *van*C genotypes is not usually detected by disk diffusion, but VanC strains frequently grow on vancomycin agar screen tests. Because of the low clinical significance of the VanC resistance, the implications of susceptibility testing for patient management may be unclear. However, the need to differentiate VanA or VanB strains, as well as strains displaying the other less commonly found types of acquired vancomycin resistance, from VanC strains is quite evident for therapeutic, infection control, and surveillance reasons. Because growth on vancomycin screening agar fails to help with this important distinction, species identification is necessary. For practical purposes, growth on the vancomycin screening agar test by *E. faecalis* or *E. faecium* is likely due to the presence of *vanA* or *vanB*. Although rare, the occurrence of the other kinds of vancomycin resistance may also be considered. Additionally, simultaneous occurrence of the *vanA* gene has been increasingly reported in *van*C-carrying species *E. casseliflavus* and especially *E. gallinarum*, so that identification of a species that usually harbors only VanC resistance does not completely rule out moderate to high levels of vancomycin resistance (28, 49, 55). In this regard, determining vancomycin MICs is useful, as VanC resistance frequently results in MICs of <32 µg/ml, whereas VanA and VanB usually result in MICs of >32 µg/ml. In such cases, determination of the genetic elements associated with vancomycin resistance has important epidemiological and infection control implications. Also, resistance to other agents such as ampicillin and aminoglycosides is uncommon among VanC isolates. Moreover, the unexpected finding of simultaneous occurrence of the *vanA* and *vanC1* genes in an *E. faecalis* isolate from ewe bulk tank milk was recently reported (156), illustrating the increasing complexity for precise determination of glycopeptide resistance and its association with the different enterococcal species. Because of limited alternatives, chloramphenicol, erythromycin, tetracycline (or doxycycline or minocycline), and rifampin may be tested for VRE. Selective testing of quinupristin-dalfopristin, linezolid, and daptomycin, based on the site of infection, is recommended when reporting vancomycin-resistant *E. faecium* isolates (144, 145).

Molecular methods (see chapter 77) have been used to detect specific antimicrobial resistance genes and have substantially contributed to the understanding of the spread of acquired resistance among enterococci, especially resistance to vancomycin. However, because of their high specificity, molecular methods do not detect antimicrobial resistance due to mechanisms not targeted by the testing, including emerging resistance mechanisms.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The diversity and species specificity of acquired antimicrobial resistance traits among enterococcal isolates created an additional need for accurate identification at the species level and for *in vitro* evaluation of susceptibility to antimicrobial agents. The significance of a particular enterococcal isolate is a major factor in determining when antimicrobial testing should be done. Once the need to test a particular isolate has been established, selection of the appropriate antimicrobial agents for testing must be considered on the basis of the site of infection. Testing of antimicrobial agents to which enterococci are intrinsically resistant is contraindicated. The drugs that should not be reported include aminoglycosides at standard concentrations, cephalosporins, clindamycin, and trimethoprim-sulfamethoxazole. They may appear active for enterococci *in vitro* but are not effective clinically, and as such they should not be reported as drugs to which enterococci are susceptible. Updated guidelines (145) for the selection of antimicrobial agents should be followed for routine testing and reporting. The *in vitro* methods for detecting antimicrobial resistance in enterococcal isolates were reviewed and summarized in reference 1 and are also discussed in chapters 71 and 73.

As already mentioned, testing for high-level aminoglycoside resistance as a predictor of synergy should be done with any enterococcal isolate implicated in infections for which combination therapy is indicated, e.g., from systemic infections. Enterococci are also frequently encountered in polymicrobial infections associated with the gastrointestinal tract or superficial wounds of hospitalized patients. Their pathogenic significance in such settings is uncertain, but susceptibility testing is warranted when predominant or heavy growth is observed. Testing of *E. faecalis* isolates from lower urinary tract infections is optional, as these infections usually respond to therapy with ampicillin or nitrofurantoin. However, many hospital infection control programs require routine testing as part of surveillance programs for VRE. For those instances when testing a urinary tract isolate is appropriate, ciprofloxacin, fosfomycin, levofloxacin, norfloxacin, and tetracycline could be selected, in addition to nitrofurantoin and ampicillin (28, 36, 142, 143, 145). In cases of treatment failure, testing is always warranted.

The findings and conclusions in this chapter are those of the authors and do not necessarily represent the views of the CDC. The use of product names in the manuscript does not imply their endorsement by the U.S. Department of Health and Human Services.

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Aerococcus, *Abiotrophia*, and Other Aerobic Catalase-Negative, Gram-Positive Cocci

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24

TAXONOMY

The catalase-negative, Gram-positive cocci included in this chapter form a taxonomically diverse group of bacteria that are isolated infrequently as opportunistic agents of infection. Most of these organisms resemble other more well-known clinical isolates (i.e., streptococci and enterococci) and consequently may be mistaken for members of those genera. Although probably misidentified or overlooked in clinical cultures in the past, these organisms may represent emerging pathogens in immunocompromised patient populations. Table 1 lists the organisms included here along with some of their basic characteristics. The bacteria discussed in this chapter are members of the phylum *Firmicutes* (low-G+C, Gram-positive bacteria). *Helcococcus* is the only genus in the group to reside in the class "*Clostridia*," while the remaining genera are classified in the class "*Bacilli*" (1). The reader is referred to chapter 21 for information on *Rothia mucilaginosa*, another infrequently isolated Gram-positive coccus that may be catalase negative.

The genus *Lactococcus* is composed of organisms formerly classified as Lancefield group N streptococci (2). The species *Lactococcus lactis* and *Lactococcus garvieae* have been documented in human infections. Motile *Lactococcus*-like organisms with Lancefield's group N antigen (a teichoic acid antigen) are classified in the genus *Vagococcus* (3, 4). The vagococci also resemble the enterococci, and Facklam and Elliott (5) reported that *Vagococcus fluvialis* (the principal species described in human clinical specimens to date) isolates examined at the Centers for Disease Control and Prevention gave positive reactions in a commercially available nucleic acid probe test for enterococci.

The genera *Abiotrophia* and *Granulicatella* accommodate organisms previously known as nutritionally variant or satelliting streptococci (6, 7). These bacteria were originally thought to be nutritional mutants of viridans streptococcal strains, most notably of the species *Streptococcus mitis*. Bouvet and colleagues (8) suggested that this group of organisms were really members of two novel streptococcal species given the names *Streptococcus defectivus* and *Streptococcus adjacens*. A comparative analysis of 16S rRNA gene sequences led Kawamura and coworkers to propose the creation of a new genus, *Abiotrophia*, containing two species, *Abiotrophia defectiva* and *Abiotrophia adiacens*, to accommodate these bacteria (7). A third species from human sources, *Abiotrophia elegans*, was described in 1998 (9). Kanamoto et al. noted the heterogeneity among *Abiotrophia* strains and proposed a fourth species, *Abiotrophia para-adiacens* (10). In 2000, Collins and Lawson proposed a new genus, *Granulicatella*, with *Granulicatella adiacens* and *Granulicatella elegans* representing strains formerly called *A. adiacens* and *A. elegans*. *A. defectiva* remains as the sole *Abiotrophia* species (6).

Among the intrinsically vancomycin-resistant, catalase-negative, Gram-positive cocci, a number of *Leuconostoc* species have been noted in human infection (*Leuconostoc mesenteroides*, *Leuconostoc lactis*, *Leuconostoc pseudomesenteroides*, and *Leuconostoc citreum* [11]). In 1993, the former *Leuconostoc paramesenteroides* and related species were placed into a novel genus, *Weissella* (12). *Pediococcus acidilactici* and *Pediococcus pentosaceus* are the most common clinical isolates of pediococci (13). The vancomycin-susceptible species formerly named *Pediococcus halophilus* was reclassified in the genus *Tetragenococcus* (14). The organism formerly called *Enterococcus solitarius* has also been transferred to the *Tetragenococcus* genus as *Tetragenococcus solitarius* (15). Little is known about the role of the tetragenococci in human infection.

The organism we now know as *Gemella morbillorum* was described in 1917 by Tunnicliff (16) as an isolate from the blood of patients with measles. *G. morbillorum* was originally named *Diplococcus rubeolae* and was also called *Diplococcus morbillorum*, *Peptostreptococcus morbillorum*, and *Streptococcus morbillorum* until a proposal to include it in the genus *Gemella* as *G. morbillorum* was made in 1988 (17). A second species, *Gemella haemolysans*, was originally classified as a *Neisseria* species, due to its Gram-variable or even Gram-negative nature and its cellular morphology (diplococci with flattened adjacent sides). Collins and coworkers described two additional *Gemella* species isolated from human sources, *Gemella bergeri* (originally named *Gemella bergeriae* [18]) and *Gemella sanguinis* (19). *Gemella asaccharolytica* was recently described by Ulger-Toprak and colleagues (20). The genus *Dolosigranulum* shows phenotypic similarities to *Gemella*, although it is not phylogenetically closely related to *Gemella* strains (21, 22).

Aerococcus urinae, described in 1992, is negative for pyrrolidonyl arylamidase production (PYR) and positive for leucine aminopeptidase production (LAP), showing opposite reactions of *Aerococcus viridans* in these important identification tests (23). In spite of these phenotypic differences,

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TABLE 1 Possible identities of catalase-negative, Gram-positive cocci based on certain phenotypic reactions and cellular morphology[a]

Phenotypic reaction			Cellular morphology	
PYR	LAP	NaCl	Pairs, chains	Clusters, tetrads, irregular groups
+	+	+	*Enterococcus*,[b,c,d] *Vagococcus*,[c,e] *Lactococcus*,[d] *Facklamia* spp. other than *F. languida*, *Ignavigranum*[f]	*F. languida*, *Dolosigranulum*, *Aerococcus sanguinicola*
+	+	−	*Abiotrophia*,[g] *Granulicatella*,[g] *Gemella* spp. other than *G. haemolysans* and *G. asaccharolytica*	*G. haemolysans* (cells arranged primarily in pairs, with adjacent sides flattened)
+	−	+	*Globicatella*	*Aerococcus viridans*,[h] *Helcococcus kunzii*[h,i]
+	−	−	*Dolosicoccus*	
−	+	+		*Aerococcus urinae*, *Pediococcus*,[j] *Tetragenococcus*
−	+	−	Viridans group streptococci,[k] *G. asaccharolytica* (Gram variable)	
−	−	+	*Leuconostoc*,[j] *Weissella*[j]	

[a] Abbreviations and symbols: NaCl, growth in 6.5% NaCl; +, ≥90% of strains positive; −, ≤10% of strains positive.

[b] Some strains may display vancomycin resistance, and some strains are motile.

[c] Most enterococcal strains are capable of growth at 45°C, differentiating them from vagococci, which may be phenotypically similar. Strains of vagococci have been reported as testing positive with a commercially available nucleic acid probe for members of the genus *Enterococcus*.

[d] Phenotypically similar strains of enterococci and lactococci can be differentiated with a commercially available nucleic acid probe for members of the genus *Enterococcus*.

[e] Motile.

[f] Some strains display satelliting growth; some strains are urease positive.

[g] Members of this genus display satelliting growth.

[h] Although *H. kunzii* shares some phenotypic traits with *A. viridans*, it is facultative and usually nonhemolytic, in contrast to *A. viridans*, which prefers an aerobic growth atmosphere and is alpha-hemolytic.

[i] Two additional species of *Helcococcus* (*H. sueciensis* and "*H. pyogenes*") have been proposed (36–38), both based on the isolation of a single strain. These new species display negative reactions in the PYR test, in contrast to *H. kunzii*.

[j] Vancomycin resistant.

[k] Viridans group streptococci include streptococci of the anginosus, mitis, mutans, salivarius, and bovis species groups. Some strains of *Streptococcus pneumoniae* (a member of the mitis species group) may produce positive reactions in the PYR test.

molecular taxonomic studies suggest that *A. urinae* should remain in the *Aerococcus* genus. Organisms currently included in the *A. urinae* species are fairly heterogeneous and can probably be subdivided into at least two subspecies (24). *Aerococcus christensenii*, isolated from the human genitourinary tract, was described by Collins and coworkers in 1999 (25) and was joined by the species *Aerococcus sanguinicola* (originally named *Aerococcus sanguicola* [26, 27]) and *Aerococcus urinaehominis* (28) in 2001.

Globicatella, *Facklamia*, *Ignavigranum*, and *Dolosicoccus* are related genera that are isolated infrequently from clinical specimens. *Globicatella sanguinis*, initially named *Globicatella sanguis*, was described in 1992 (29). *Facklamia* currently contains four species isolated from human sources: *Facklamia hominis* (30), *Facklamia sourekii* (31), *Facklamia ignava* (32), and *Facklamia languida* (33). The genus *Ignavigranum*, currently consisting of a single species, *Ignavigranum ruoffiae*, was described by Collins and coworkers (34), along with the genus *Dolosicoccus* and its single species, *Dolosicoccus paucivorans* (35).

The genus *Helcococcus*, originally composed of the single species *Helcococcus kunzii* (36), came to include a new species isolated from humans, *Helcococcus sueciensis*, in 2004 (37). A third human species, "*Helcococcus pyogenes*," has been proposed, but to date has not received official taxonomic standing (38, 39). *Helcococcus ovis*, isolated from infections in animals, displays satelliting growth, unlike the human *Helcococcus* species (40).

DESCRIPTION OF THE GENERA

The organisms included in this chapter form Gram-positive coccoid cells, but *G. haemolysans* may appear Gram variable or Gram negative due to the ease with which its cells are decolorized. Cell shape and arrangement can be used to divide these organisms into two broad groups: those with a "streptococcal-like" Gram stain (coccobacilli in pairs and chains) and those with a "staphylococcal-like" Gram stain (more spherical cocci in pairs, tetrads, clusters, or irregular groups). *Abiotrophia* and *Granulicatella* isolates (formerly the nutritionally variant streptococci) form coccobacilli arranged in pairs and chains, but these organisms may also appear pleomorphic, especially when grown under suboptimal nutritional conditions (41). Dividing these diverse bacteria into two groups based on cellular shape and arrangement serves only as an aid in identification; no relatedness of organisms is implied by this grouping. With the exception of the infrequently isolated vagococci, these bacteria are all nonmotile.

Most of the genera described here are catalase-negative facultative anaerobes, but *A. viridans* is classified as a microaerophile that grows poorly, if at all, under anaerobic conditions. Some strains of *Aerococcus* may exhibit weakly positive catalase reactions due to nonheme catalase activity. None of the genera are beta-hemolytic on routinely employed blood agars, but strains of *G. haemolysans*, *G. bergeri*, and *Gemella sanguinis* have been described as beta-hemolytic on agars supplemented with horse blood (18, 19, 42).

EPIDEMIOLOGY AND TRANSMISSION

The organisms discussed in this chapter are opportunistic pathogens. Some of the genera have been characterized as constituents of the normal microbiota of the human oral cavity or upper respiratory tract (*Gemella*, *Abiotrophia*, and *Granulicatella*) and skin (*Helcococcus*). Lactococci, pediococci, and leuconostocs can be isolated from foods and vegetation (5, 43, 44) and may also be found as part of the normal microbiota of the alimentary tract. Aerococci are environmental isolates that can also be found on human skin. Although they have been isolated from human clinical cultures, the natural habitats of many of the organisms mentioned here are not well characterized.

The bacteria examined here seem to be of low virulence and are usually pathogenic only in immunocompromised hosts. Infection often occurs in previously damaged tissues (e.g., heart valves) or may be nosocomial and associated with prolonged hospitalization, antibiotic treatment, invasive procedures, and the presence of foreign bodies.

CLINICAL SIGNIFICANCE

The bacteria described in this chapter may be present as contaminants in clinical cultures, but they are also isolated infrequently as opportunistic pathogens. Blood, cerebrospinal fluid, urine, and wound specimens are likely to yield significant isolates of these bacteria. Details on reported infections due to each of the genera follow.

Lactococcus

Due to their phenotypic similarities with streptococci and enterococci, clinical isolates of lactococci have probably been misidentified in the past, accounting at least in part for the paucity of reports concerning the clinical role of these bacteria. Elliott and coworkers (45) studied the phenotypic characteristics of a number of lactococcal strains isolated from blood, urinary tract infections, and an eye wound culture. Lactococci have been associated with native valve and prosthetic valve endocarditis (46–49), septicemia in an immunosuppressed patient, osteomyelitis, spondylodiscitis (46), peritonitis (50), liver abscess (51, 52), acalculous cholecystitis (53), and prosthetic joint infection (54). *L. garvieae* is a known pathogen of aquacultured fish, and human infections have been linked to consumption of or contact with fish (46, 54).

Vagococcus

To date, only a handful of *Vagococcus* isolates from human sources have been reported in the literature. Teixeira and coworkers (55) described strains isolated from blood, peritoneal fluid, and a wound. Al-Ahmad and colleagues reported isolation of *V. fluvialis* from an infected root canal system (56). Vagococci are motile organisms that, like lactococci, elaborate Lancefield's group N antigen (5). Difficulties encountered in identifying vagococci may partially account for their infrequent recognition in clinical cultures.

Abiotrophia and *Granulicatella*

Organisms in the genera *Abiotrophia* and *Granulicatella* (formerly known as nutritionally variant streptococci) are normal residents of the oral cavity and are recognized as agents of endocarditis involving both native and prosthetic valves (57–60). These organisms have also been isolated from other types of infections, including ophthalmic infections (61, 62), central nervous system infections (63, 64), peritonitis in patients undergoing continuous ambulatory peritoneal dialysis (65), musculoskeletal infection (66), septic arthritis (67), and a breast implant-associated infection (68).

Leuconostoc, *Pediococcus*, and *Weissella*

The vancomycin-resistant genera *Leuconostoc* and *Pediococcus* were first recognized in clinical specimens in the mid-1980s. Handwerger and colleagues (69) observed that host defense impairment, invasive procedures breaching the integument, gastrointestinal symptoms, and prior antibiotic treatment were common features among adult patients with *Leuconostoc* infection. They also noted a predisposition to *Leuconostoc* bacteremia among neonates, suggesting that infants may become colonized during delivery by leuconostocs inhabiting the maternal genital tract. Leuconostocs have been isolated from blood, cerebrospinal fluid, peritoneal dialysate fluid, and wounds. Case reports have implicated leuconostocs as agents of infection in osteomyelitis (70), ventriculitis (71), brain abscess (72), postsurgical endophthalmitis (73), and bacteremia in the setting of short gut syndrome. Short gut syndrome favors a microbiota with a high prevalence of *Lactobacillus* and *Leuconostoc* bacteria (74). Several reports have related *Leuconostoc* bacteremic episodes to the presence of short gut syndrome, central venous catheters, and disrupted bowel mucosa (75).

Pediococcus strains have been isolated from bacteremia and cases of sepsis and hepatic abscess in compromised patients (13, 76–79). Barros and coworkers (13) noted that *P. acidilactici* was isolated from clinical specimens more frequently than *P. pentosaceus* and was also more commonly isolated from cases of bacteremia. Barton and coworkers noted the role of *Pediococcus* in bacteremia in infants with gastrointestinal malformations requiring surgical correction (76).

Weissella confusa, formerly classified as *Lactobacillus confusus*, has been reported infrequently as an agent of bacteremia and endocarditis (80, 81).

Gemella

G. haemolysans has been isolated from cases of endocarditis (82), meningitis (83), brain abscess (84), a total knee arthroplasty (85), and ocular infection (86–88). *G. morbillorum* has been implicated in cases of endocarditis (89, 90), empyema and lung abscess (91), septic shock (92), brain abscess (93), osteomyelitis (94), septic arthritis (95), and peritonitis (96). Information on the clinical significance of the other *Gemella* species continues to accumulate. *G. bergeri* and *G. sanguinis* have been isolated from blood cultures, and they may also be causative agents of endocarditis (18, 19, 97). A strain of *G. sanguinis* was isolated from an infected prosthetic hip joint (98), and *G. asaccharolytica* has been isolated from wound cultures (20).

Dolosigranulum

Dolosigranulum, a genus phenotypically similar, but not closely related, to *Gemella* (21), has been documented in blood, eye, and respiratory specimens (22). The single species of the genus, *Dolosigranulum pigrum*, has been associated with nosocomial pneumonia and septicemia (99), synovitis (100), acute cholecystitis accompanied by acute pancreatitis (101), and biomaterial-associated arthritis (102).

Aerococcus

A. viridans has been noted as a contaminant in clinical cultures and infrequently as a clinically significant isolate from cases of endocarditis and bacteremia and a case of spondylodiscitis (103–105). Four additional *Aerococcus* species isolated from humans have been described since the early 1990s. *A. urinae* (23, 106) has been implicated as a urinary tract pathogen in patients predisposed to infection (107–109) and as an agent of endocarditis (110, 111), lymphadenitis (112), and peritonitis (113). *A. sanguinicola* has been isolated from blood and urine specimens (26, 27) and cases of urosepsis and endocarditis (114). Little is currently known about the clinical significance of *A. christensenii* (isolated from vaginal specimens [25]) and *A. urinaehominis* (isolated from urine [28]).

Globicatella

G. sanguinis, isolated from human clinical specimens, has been implicated in cases of bacteremia, urinary tract infection, and meningitis (29, 115, 116). A second species in the genus, *Globicatella sulfidifaciens*, has been isolated from purulent infections in domestic mammals (117).

Facklamia

The *Facklamia* genus is closely related to, but phenotypically and phylogenetically distinct from, *Globicatella* (30). Strains of the four *Facklamia* species isolated from humans have been recovered from blood, wound, and genitourinary sites (30–33) and a case of chorioamnionitis (118).

Ignavigranum

A limited number of isolates of *I. ruoffiae*, the sole species of *Ignavigranum*, have been described to date. Sites of isolation include a wound and an ear abscess (34).

Dolosicoccus

The single species of the genus *Dolosicoccus*, *D. paucivorans*, has been isolated from blood cultures (35, 119).

Helcococcus

H. kunzii can be isolated from intact skin of the lower extremities (120) as well as from mixed cultures of wounds, notably foot infections (36, 121). In such scenarios the clinical significance of this organism is difficult to interpret, since it may be present merely as a colonizer of the wound site. The ability of *H. kunzii* to function as an opportunist is, however, suggested by its isolation as the sole or predominant organism from an infected sebaceous cyst (122), a breast abscess (123), a postsurgical foot abscess (124), an infected prosthetic joint (125), and cases of bacteremia and empyema in intravenous drug users (126). Two additional species isolated from humans, *H. sueciensis* and "*H. pyogenes*," are based on single isolates from a wound and a prosthetic joint infection, respectively (37–39).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

No special requirements for collection and transport of specimens for isolation of the organisms discussed in this chapter have been described. Routine procedures for collection, transport, and storage of specimens for aerobic culture allow for the isolation of these bacteria, since the majority are facultative anaerobes or microaerophiles. These organisms should also be recovered from specimens that have been collected and transported under anaerobic conditions (see chapter 18).

DIRECT EXAMINATION

The organisms described in this chapter can be visualized in direct Gram stains of clinical material but have no outstanding morphological characteristics that distinguish them from commonly isolated Gram-positive cocci (streptococci and staphylococci). Although *Abiotrophia* and *Granulicatella* isolates may appear pleomorphic in direct Gram stains, they form Gram-positive cocci in pairs and chains when grown on nutritionally adequate media. Direct detection of these genera by antigenic methods has not been described, but some authors have employed amplification of 16S rRNA genes for direct detection in clinical specimens (59).

ISOLATION PROCEDURES

Generally, there are no special requirements for isolation of the group of bacteria discussed here; general recommendations for the culture of blood, body fluids, and other specimens should be followed (see chapter 18). These organisms are likely to be isolated on rich, nonselective media (e.g., blood or chocolate agar and thioglycolate broth) since they are nutritionally fastidious. If selective isolation of the vancomycin-resistant genera *Leuconostoc* and *Pediococcus* is desired, Thayer-Martin medium may be used to inhibit normal microbiota or other contaminating microorganisms (127). Some of the genera (e.g., *Helcococcus*) grow slowly, forming tiny colonies that may not be visible unless extended incubation (48 to 72 h) is employed. The recovery of many of the genera included in this chapter may be enhanced by CO_2 enrichment of the incubation atmosphere.

Members of the genera *Abiotrophia* and *Granulicatella* usually grow on chocolate agar, on brucella agar with 5% horse blood, and in thioglycolate broth, but not on Trypticase soy agar with 5% sheep blood. These organisms can be cultured on nonsupportive media that have been appropriately supplemented (see "Procedures for Phenotypic Differentiation, *Abiotrophia* and *Granulicatella*" below).

IDENTIFICATION

Procedures for Phenotypic Differentiation

While molecular characterization may be required for accurate species-level identification of the aerobic catalase-negative, Gram-positive cocci encountered infrequently in clinical laboratories, phenotypic methods can be helpful in characterization of these bacteria to the genus level. Gram stain morphology has been employed as a major decision point in the identification protocols in Fig. 1 and 2 and Table 1, with two general categories: morphology resembling that of streptococci, meaning cocci or coccobacilli in pairs and chains, versus staphylococcal morphology, consisting of coccoid cells arranged in pairs, clusters, tetrads, or irregular groups. Broth-grown cells (thioglycolate broth is suitable) should be used for making accurate morphological determinations. Note that *Gemella* and *Facklamia* strains may display either type of cellular morphology, depending on the species. Figures 1 and 2 display phenotypic tests used to differentiate the genera of bacteria discussed in this chapter. Descriptions of tests for catalase, PYR, LAP, β-glucuronidase, and hippurate hydrolysis, as well as bile esculin agar and *Lactobacillus* MRS (DeMan, Rogosa, Sharpe) broth media, can be found in chapter 19 and reference 5. Additional phenotypic tests are described below in the discussion of identification criteria for each genus.

Lactococcus and *Vagococcus*

The members of the genera *Lactococcus* and *Vagococcus* are usually PYR and LAP positive, grow in the presence of 6.5% NaCl, and can be confused with enterococci or streptococci. For the salt tolerance test, heart infusion broth supplemented with 6.0% NaCl (producing a final NaCl concentration of 6.5%), with or without the acid-base indicator bromcresol purple, is inoculated with two or three colonies and incubated at 35°C for up to 72 h. Turbidity with or without a color change from purple to yellow indicates growth (5, 128). Facklam and colleagues (5, 128) recommended growth temperature tests for distinguishing lactococci from streptococci and enterococci. Consult Fig. 1 for growth temperature characteristics of each of the genera. For growth temperature tests, broths (heart infusion broth containing 1% glucose

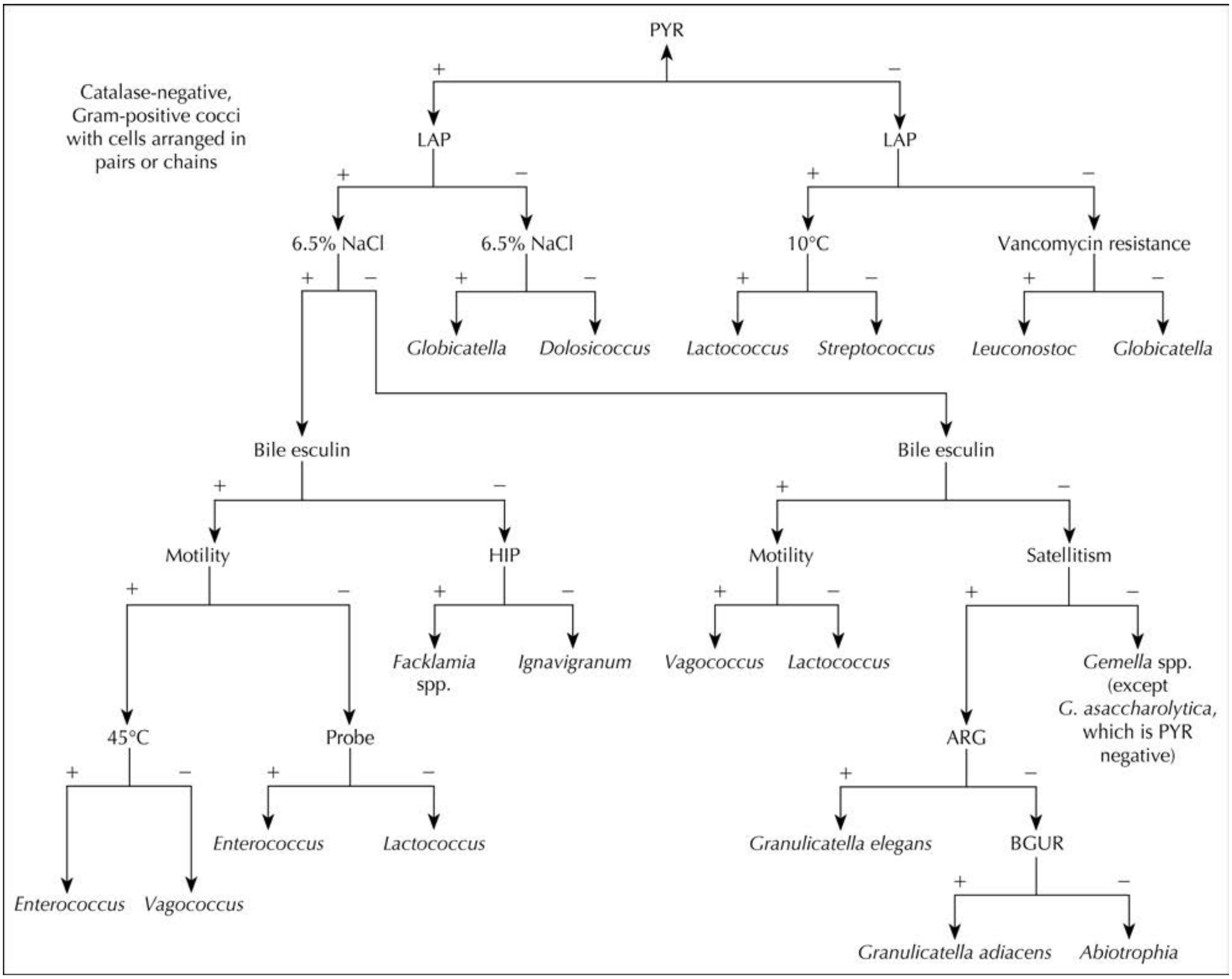
PYR
+
−
Catalase-negative, Gram-positive cocci with cells arranged in pairs or chains
LAP
LAP
+
−
+
−
6.5% NaCl
6.5% NaCl
10°C
Vancomycin resistance
+
−
+
−
+
−
+
−
Globicatella
Dolosicoccus
Lactococcus
Streptococcus
Leuconostoc
Globicatella
Bile esculin
Bile esculin
+
−
+
−
Motility
HIP
Motility
Satellitism
+
−
+
−
+
−
+
−
Facklamia spp.
Ignavigranum
Vagococcus
Lactococcus
Gemella spp. (except G. asaccharolytica, which is PYR negative)
45°C
Probe
ARG
+
−
+
−
+
−
Enterococcus
Lactococcus
Granulicatella elegans
BGUR
+
−
Enterococcus
Vagococcus
Granulicatella adiacens
Abiotrophia

FIGURE 1 Identification of catalase-negative, Gram-positive cocci that grow aerobically with cells arranged in pairs and chains. Abbreviations: 6.5% NaCl, growth in broth containing 6.5% NaCl; bile esculin, hydrolysis of esculin in the presence of 40% bile; motility, motility in motility test medium; 45°C, growth at 45°C; 10°C, growth at 10°C; probe, reaction with commercially available nucleic acid probe for the genus *Enterococcus*; HIP, hydrolysis of hippurate; satellitism, satelliting growth behavior; ARG, arginine hydrolysis activity; BGUR, β-glucuronidase activity. doi:10.1128/9781555817381.ch24.f1

and bromcresol purple indicator) are inoculated with a single colony or drop of broth culture of the test strain and incubated at 35°C for up to 7 days. A water bath is recommended for incubation of cultures at 45°C. Turbidity with or without a change in the broth's indicator to yellow indicates a positive test. The motile vagococci can be distinguished from lactococci with modified motility test medium, stab-inoculated and incubated at 30°C for up to 48 h, according to the method of Facklam and Elliott (5). Further information on the phenotypic traits of *Lactococcus* and *Vagococcus* isolates may be found in references 2, 45, 55, and 129.

Abiotrophia and *Granulicatella*

A test for satelliting behavior is important for identification of these two genera. The strain to be examined is streaked for confluent growth on a medium that does not support growth or supports only weak growth (e.g., sheep blood agar). A single cross streak of *Staphylococcus aureus* (ATCC 25923 or another suitable strain) is applied to the inoculated area. After incubation at 35°C in an atmosphere containing elevated CO_2, strains of *Abiotrophia* or *Granulicatella* grow only in the vicinity of the staphylococcal growth. Some strains of *Ignavigranum* may also show satelliting behavior (34). Alternatively, media can be supplemented with pyridoxal. An aqueous stock solution of filter-sterilized 0.01% pyridoxal hydrochloride (which can be stored frozen) should be added to media to achieve a final concentration of 0.001%. Pyridoxal disks (Remel, Lenexa, KS) may also be used in the satelliting test.

Detailed phenotypic information for the PYR- and LAP-positive *Abiotrophia* and *Granulicatella* species can be found in references 6, 41, 130, and 131. Davis and Peel (132) reported that the API 20 Strep system (bioMérieux, Durham, NC) was superior to the Rapid ID32 Strep system (bioMérieux) for identification of these organisms.

Leuconostoc, Pediococcus, and *Weissella*

Members of the PYR-negative, vancomycin-resistant genera *Leuconostoc*, *Pediococcus*, and *Weissella* produce small, alpha-

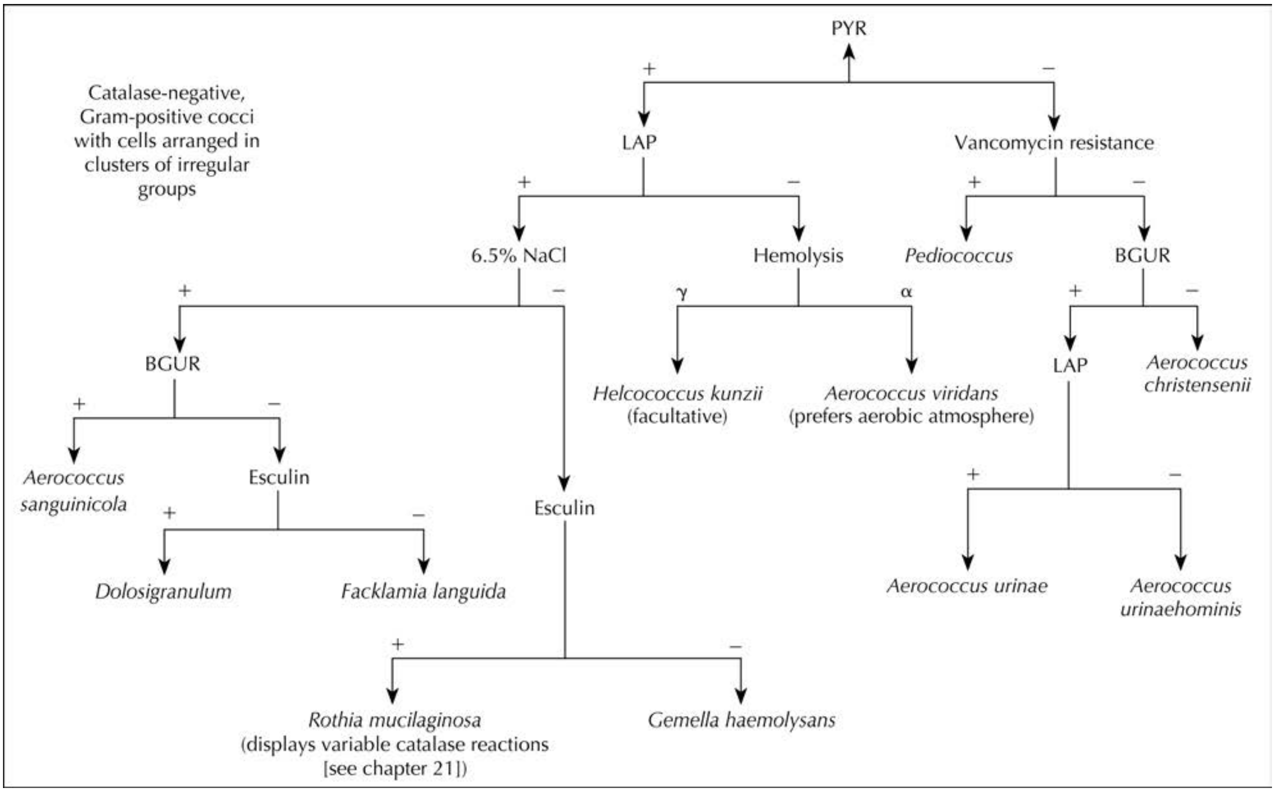
Catalase-negative, Gram-positive cocci with cells arranged in clusters of irregular groups
PYR
+
−
LAP
Vancomycin resistance
+
−
+
−
6.5% NaCl
Hemolysis
Pediococcus
BGUR
+
−
γ
α
+
−
BGUR
Helcococcus kunzii (facultative)
Aerococcus viridans (prefers aerobic atmosphere)
LAP
Aerococcus christensenii
+
−
Aerococcus sanguinicola
Esculin
Esculin
+
−
+
−
Dolosigranulum
Facklamia languida
Aerococcus urinae
Aerococcus urinaehominis
+
−
Rothia mucilaginosa (displays variable catalase reactions [see chapter 21])
Gemella haemolysans

FIGURE 2 Identification of catalase-negative, Gram-positive cocci that grow aerobically with cells arranged in pairs, tetrads, clusters, or irregular groups. Abbreviations: 6.5% NaCl, growth in broth containing 6.5% NaCl; esculin, hydrolysis of esculin; BGUR, β-glucuronidase activity. doi:10.1128/9781555817381.ch24.f2

hemolytic or nonhemolytic colonies on blood agar. Vancomycin resistance can be tested by streaking several colonies over half of a Trypticase soy agar with 5% sheep blood plate. After placing a 30-μg vancomycin disk in the center of the inoculated area, the plate is incubated overnight in a CO_2-enriched atmosphere at 35°C. Any zone of inhibition indicates susceptibility, while resistant strains exhibit no inhibition zone (5, 128). In addition to differing cellular morphologies (Table 1), these vancomycin-resistant genera, along with vancomycin-resistant strains of lactobacilli that form short coccoid cells, can be differentiated by tests for gas production from glucose and arginine hydrolysis. Leuconostocs produce gas and are always arginine negative. Lactobacilli are variable in both tests, but a positive arginine test for a gas-producing strain would rule out identity of the organism as a leuconostoc. Pediococci are gas production negative and show variable reactions in the arginine test, although *P. acidilactici* and *P. pentosaceus*, the two species commonly found in clinical material, are arginine positive. *Weissella* strains may be misidentified as leuconostocs or lactobacilli. These organisms produce gas from glucose. The few clinical isolates reported in the literature have been described as positive for hydrolysis of arginine (12, 81).

MRS broth (BD Diagnostic Systems, Franklin Lakes, NJ; Hardy Diagnostics, Santa Maria, CA; see chapter 19), sealed with melted petrolatum and incubated for up to 7 days at 35°C, is used to test for gas production, indicated by displacement of the petrolatum plug (5, 128). The arginine hydrolysis test can be performed with Moeller decarboxylase broth containing arginine (5). Lancefield group D antigen can be detected in pediococci (128). References 5, 11, 13, 128, and 131 should be consulted for further information on identification of *Leuconostoc* and *Pediococcus* to the species level.

Gemella

On sheep blood agar media, members of the *Gemella* genus (usually PYR positive) form small colonies that are similar in appearance to those of viridans group streptococci. Slow growth of some *Gemella* strains may lead to confusion of these organisms with *Abiotrophia* or *Granulicatella* (formerly called nutritionally variant streptococci). A test for satelliting behavior should separate these two groups of bacteria (128), but Leung and coworkers (98) reported on a single *G. sanguinis* strain that exhibited "pseudosatelliting" behavior (satelliting growth after 24 h, but widespread growth after 48 h of incubation). Cells of *G. haemolysans* are easily decolorized and resemble those of neisserias, since they occur in pairs with the adjacent sides flattened. *G. haemolysans* prefers an aerobic growth atmosphere. The esculin hydrolysis test for differentiation of *G. haemolysans* and *R. mucilaginosa* in Fig. 2 is performed with esculin agar slants (heart infusion agar containing 0.1% esculin and 0.5% ferric citrate) that are inoculated and incubated at 35°C for up to 7 days. Partial or complete blackening of the agar indicates a positive reaction (5). *G. morbillorum* cells are Gram positive and arranged in pairs and short chains; individual cells in a given pair may be of unequal sizes. Only a small number of strains of *G. bergeri* and *G. sanguinis* have been reported on to date. Information on phenotypic characteristics of these *Gemella* species can be found in references 18 and 19. The recently described *G. asaccharolytica* is PYR negative, unlike other *Gemella* species (20).

Aerococcus

The PYR-positive, LAP-negative member of the genus, *A. viridans*, is characterized by displaying weak or no growth when incubated in an anaerobic atmosphere (134). This trait can be tested by incubating duplicate blood agar plate cultures of the organism in question in anaerobic and aerobic atmospheres and comparing growth after 24 to 48 h. *A. viridans* forms alpha-hemolytic colonies that could be confused with those of either viridans group streptococci or enterococci. *A. sanguinicola* is positive in the PYR and LAP tests, while *A. urinaehominis* is negative in both. The PYR-negative, LAP-positive species, *A. urinae* and *A. christensenii*, are differentiated by production of β-glucuronidase (*A. christensenii* is negative and *A. urinae* is positive). *A. urinae* forms small (0.5 mm in diameter after 24 h of incubation), alpha-hemolytic, convex, shiny, transparent colonies on blood agar media. Additional information on the identifying characteristics of *A. urinae* can be found in reference 107, and a second biotype (esculin hydrolysis positive) of this species is described in reference 24. Additional information on phenotypic traits of the species *A. christensenii*, *A. sanguinicola*, and *A. urinaehominis* can be found in Table 1, Fig. 2, and references 25–28.

Dolosigranulum

D. pigrum, the sole species of *Dolosigranulum* described to date, displays positive PYR and LAP reactions and was initially described as phenotypically similar, though not closely related, to members of the genus *Gemella* (21). *D. pigrum* is distinguished from *Gemella* spp. by its abilities to hydrolyze arginine and to grow in the presence of 6.5% NaCl.

Globicatella and Related Genera (*Facklamia, Dolosicoccus,* and *Ignavigranum*)

Globicatella and the related genera *Facklamia*, *Dolosicoccus*, and *Ignavigranum* are all, with the exception of the species *G. sulfidifaciens*, PYR positive. *Facklamia* and *Ignavigranum* are also LAP positive and salt tolerant. *Globicatella* is LAP negative and salt tolerant, while *Dolosicoccus* is LAP negative and salt intolerant. *Dolosicoccus* strains are also hippurate hydrolysis negative, which further distinguishes them from strains of *Facklamia* and *Globicatella* (hippurate hydrolysis positive). Strains of *F. hominis* and *Ignavigranum* may produce urease. *Ignavigranum* strains may exhibit satelliting behavior. Further details of phenotypic traits of these organisms can be found in references 30–35 and 135.

Helcococcus

Colonial morphology (tiny, gray, usually slightly alpha-hemolytic colonies), good growth under anaerobic conditions, and stimulation of growth by addition of 1% horse serum or 0.1% Tween 80 to the medium differentiate *H. kunzii* from aerococci (36). Isolates of *H. kunzii* are PYR positive, and most produce an API 20 Strep profile of 4100413. Additional *Helcococcus* species isolated from humans (*H. sueciensis* and the proposed "*H. pyogenes*") are negative in the PYR test. Detailed phenotypic data on these organisms can be found in references 36–39.

Commercially Available Kits and Automated Methods Based on Phenotypic Traits

There have been no comprehensive evaluations of the ability of commercially available products to identify the diverse and infrequently isolated bacteria described in this chapter. Phenotypic variation among isolates classified in the same species, the relative metabolic inactivity of some organisms, and a relatively small number of strains available for inclusion in databases have challenged the capabilities of these products for accurate identification. Manual methods for performance of some of the basic differentiation tests (e.g., PYR and LAP) are available (e.g., BactiCard Strep; Remel). Commercially available identification kits or systems offering a more comprehensive array of phenotypic tests are improving in their ability to identify many of the organisms discussed in this chapter (26, 135–139). These products include manual methods, e.g., API 20 Strep and RapID Strep (Remel), and automated systems, e.g., VITEK 2 (bioMérieux), MicroScan (Siemens Healthcare Diagnostics, Inc., Deerfield, IL), and Phoenix (BD Diagnostics, Sparks, MD). In the absence of an accurate genus- or species-level identification, these systems will at least provide additional phenotypic information that can be used to augment results of the basic tests mentioned above.

Molecular Methods

Sequence-Based Techniques

16S rRNA gene sequencing-based identifications have proven to be more accurate than phenotypic methods for identifying many of the infrequently isolated aerobic catalase-negative, Gram-positive cocci (136, 138). Bosshard and colleagues (136) analyzed 171 clinical strains of the genera *Streptococcus*, *Enterococcus*, *Abiotrophia*, *Aerococcus*, *Granulicatella*, and *Gemella* and observed that more species- or genus-level identifications were achieved based on 16S rRNA gene sequence analysis than based on a commercially available phenotypic method (API 20 Strep), and identifications based on phenotypic traits often disagreed with those determined by 16S rRNA gene sequencing. Woo and coworkers (138) examined strains of *Abiotrophia*, *Granulicatella*, *Gemella*, and *Helcococcus* in their evaluation of a commercially available 16S rRNA gene sequence-based identification system that analyzes a 527-bp fragment of the 16S rRNA gene (MicroSeq 500; Life Technologies, Foster City, CA). They noted disagreement in identifications obtained with commercially available phenotypic test systems (API 20 Strep and VITEK) and MicroSeq 500 compared with conventional sequencing of the entire 16S rRNA gene. The authors stressed the importance of adequate databases for accurate rRNA gene sequence-based identification.

Different regions of the 16S rRNA gene have been used for identification. The first 500 bp is the most applied target for identification. The entire 16S rRNA gene (1.5 kb) has also been sequenced when making more detailed descriptions of taxa or strains (139). 16S rRNA gene sequencing is an effective method for identifying the genera included in this chapter, many of which are difficult to identify with conventional phenotypic methods (139, 140). Accumulating data on the clinical significance of species make it possible to link an accurate species identification to clinically relevant information (139). Other reports on identification of strains of the genera *Aerococcus*, *Granulicatella*, and *Globicatella* support the benefits of using 16S rRNA gene sequencing for these genera (41, 114, 141–143).

Broad-range amplification of the 16S rRNA gene directly from clinical samples has made it possible to detect taxa described in this chapter from various clinical samples giving no growth by culture (for instance, *G. elegans* and *A. urinae* from cardiac valvular tissue [144, 145]). The sensitivity of direct 16S rRNA gene sequencing on valve material is reported to be between 72 and 93% when looking at definite infective endocarditis cases. A major challenge for this methodology is the difficulty in analyzing sequence data when multiple bacterial species are present in the same specimen. Kommedal and coworkers used a set of group-specific, broad-range primers targeting the 16S rRNA gene followed by DNA sequencing and RipSeq analysis (iSentio AS, Paradis, Norway) and identified *Gemella* species from abscess material also containing bacterial DNA from other taxa (146). When examining clinical samples, cross-reactivity with human DNA is a major pitfall that can result in mixed electropherograms complicating subsequent sequence analysis and species identification. Kommedal and colleagues employed the principle of dual-priming oligonucleotides to circumvent cross-reactivity even in specimens with a high ratio of human to bacterial DNA (147).

Alternative sequencing targets for identification of the organisms discussed in this chapter have also been explored. PCR amplification of a 740-bp *rpoB* gene fragment followed by sequence analysis was shown to be a suitable molecular approach for the species identification of *Streptococcus*, *Enterococcus*, *Gemella*, *Abiotrophia*, and *Granulicatella* isolates by Drancourt and colleagues (148). Hung et al. determined the *groESL* sequences of three species of nutritionally variant streptococci and three *Gemella* species and developed a multiplex PCR enabling identification of strains of *Abiotrophia*, *Granulicatella*, and *Gemella* at the genus level. To a large extent, intraspecies sequences were well circumscribed, though higher intraspecies heterogeneity was observed in *G. haemolysans*, with the six isolates examined being separated into two subgroups (149).

Tung and coworkers (150) evaluated the feasibility of sequence analysis of the ribosomal 16S-23S intergenic spacer region (ITS) for identification of 24 species of *Streptococcus*, 1 species of *Abiotrophia*, 18 species of *Enterococcus*, and 3 species of *Granulicatella*. The correct species identification rate by ITS sequence analysis was 98.2%. However, all of the genera except *Streptococcus* produced more than one PCR amplicon, necessitating agarose gel separation and in some cases cloning to purify the amplicons before sequencing. Tung and colleagues (151) further developed an array-based identification setup based on PCR amplification of the ITS regions and found it to be a useful and reliable alternative to phenotypic identification methods. The approach of having a single molecular platform for strains resembling nonhemolytic streptococci, enterococci, and the taxa considered in this chapter based on ITS analysis has also been examined by Nielsen and colleagues (152). Clinical strains of the genera *Aerococcus* (*n* = 37), *Abiotrophia* (*n* = 2), *Granulicatella* (*n* = 9), *Gemella* (*n* = 22), and *Leuconostoc* (*n* = 5) were examined. All 75 clinical strains, irrespective of obtained maximum score value, were allocated to the expected species except for 2 strains of *Gemella*.

MALDI-TOF MS Techniques

Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) (see chapter 4) for analysis of bacterial proteins is a promising technique for identification of the bacteria mentioned in this chapter. The MALDI Biotyper (Bruker Daltonics, Billerica, MA) and the VITEK MS (bioMérieux) are two commercially available identification systems employing this technology. Christensen and coworkers (153) examined 51 culture collection strains and 90 well-characterized strains with the Bruker Biotyper system. A mass spectrum profile (MSP) based on 24 separate determinations was created for each strain and stored in a separate library that could be combined with the standard database (Biotyper version 2.0.43.1), which did not include all the taxa examined. The following genera were represented: *Aerococcus* (*n* = 42), *Gemella* (*n* = 30), *Granulicatella* (*n* = 11), *Abiotrophia* (*n* = 3), *Lactococcus* (*n* = 9), *Globicatella* (*n* = 7), *Leuconostoc* (*n* = 9), *Rothia* (*n* = 9), *Facklamia* (*n* = 7), *Vagococcus* (*n* = 2), *Helcococcus* (*n* = 4), *Alloiococcus* (*n* = 2), *Pediococcus* (*n* = 3), *Ignavigranum* (*n* = 1), *Dolosicoccus* (*n* = 1), and *Dolosigranulum* (*n* = 1). The protocol for creating one's own MSPs in a separate library, though somewhat labor-intensive, makes it possible to extend the database. After extension of the database, all challenge strains were correctly identified to the species level except for a few difficult-to-identify *Gemella* strains. The obtained mean score values illustrated diversity within the different species and also an effect on mean score values of different conditions for creating MSPs. The MSP dendrograms for *Aerococcus*, *Gemella*, *Granulicatella*, and *Abiotrophia* convincingly delineated the included species. Thus, for routine use, MALDI-TOF MS was shown to be robust. In a study by Rychert et al., 1,146 strains of Gram-positive cocci were examined with the VITEK MS system (154). Fifteen of these strains belonged to taxa described in this chapter: *A. defectiva* (*n* = 2), *A. viridans* (*n* = 6), *G. haemolysans* (*n* = 3), *G. adiacens* (*n* = 1), *L. garvieae* (*n* = 1), *L. lactis* (*n* = 1), and *L. mesenteroides* (*n* = 1). All strains were correctly identified except the *L. mesenteroides* strain, which gave no identification. Other studies have corroborated the usefulness of MALDI-TOF MS for identification of aerococci (155) and *Lactococcus* (156, 157) and *Leuconostoc* (157) species and have investigated using this technique for identification of *Weissella* and *Pediococcus* strains (158).

TYPING SYSTEMS

Little information exists on typing methods for the genera of infrequently isolated Gram-positive cocci included in this chapter. Typing is not routinely used for characterizing these organisms.

SEROLOGIC TESTS

Serologic response to the organisms described in this chapter has not been extensively investigated. No clinically useful tests have been described.

ANTIMICROBIAL SUSCEPTIBILITIES

Antimicrobial susceptibility studies on the organisms mentioned in this chapter have generally employed dilution testing methods. Little or no data exist on the utility of disk diffusion or the correlation of Etest results with those of broth or agar dilution methods. Standardized dilution methods and interpretive criteria for observed MICs have been described for only four of the genera (*Abiotrophia*, *Granulicatella*, *Leuconostoc*, and *Pediococcus* [reference 159 and chapter 74]. The lack of standardized methods and interpretive criteria and the relatively small collections of isolates for some of the genera discussed in this chapter make it difficult to accurately assess antimicrobial susceptibility patterns. With the exception of *Leuconostoc*, *Pediococcus*, and *Weissella*, all of the genera display susceptibility to vancomycin. While many of the genera are susceptible to β-lactams and other antimicrobials, observed strain variations suggest that MICs of antimicrobials used for treatment should be determined for individual isolates. When susceptibility testing is requested for isolates for which no guidelines exist, dilution methods may be used to generate MICs that can be reported without interpretation. Since many of the bacteria discussed here are fairly fastidious, investigators have often employed blood-supplemented Mueller-Hinton media and, if necessary for good growth, incubation in a CO_2-enriched atmosphere for susceptibility testing. Pyridoxal hydrochloride (final concentration of 0.001%) should also be added to blood-supplemented media for testing strains of *Abiotrophia* and *Granulicatella* (159). Details of published susceptibility testing studies for each of the genera appear below.

Information on the *in vitro* antimicrobial susceptibility of *L. lactis* and *L. garvieae* strains isolated from humans suggests that *L. garvieae* isolates are less susceptible to penicillin and cephalothin than are strains of *L. lactis*. The uniform resistance of *L. garvieae* versus the uniform susceptibility to clindamycin of the *L. lactis* strains examined by Elliott and Facklam (129) led them to propose a test for clindamycin susceptibility as an aid in differentiation of these two species. In clinical practice, cases of lactococcal endocarditis have been successfully treated with either penicillin alone or with penicillin and gentamicin (47, 48).

Teixeira and colleagues observed that a collection of *Vagococcus* isolates were all susceptible to ampicillin, cefotaxime, and trimethoprim-sulfamethoxazole. All strains were resistant to clindamycin, lomefloxacin, and ofloxacin. Variable results were observed with other antimicrobial agents (55).

The vancomycin-resistant genera *Leuconostoc* and *Pediococcus* are penicillin susceptible when MICs are interpreted using criteria adapted from those for *Enterococcus* spp. (159, 160). They are usually susceptible to chloramphenicol, tetracyclines, and aminoglycosides. Carbapenem and cephalosporin resistance has been noted in some strains of *Leuconostoc* (159). Huang and colleagues (161) noted MIC ranges of 0.5 to 8 μg/ml for linezolid and 0.06 to 2 μg/ml for daptomycin in 68 strains of *Leuconostoc* tested and ranges of 1 to 4 μg/ml for linezolid and 0.06 to 0.5 μg/ml for daptomycin in 13 *Pediococcus* isolates. Iwen and colleagues reported on the successful treatment of a case of *P. acidilactici* endocarditis with daptomycin (162).

Abiotrophia and *Granulicatella* isolates display a range of penicillin MICs, with authors reporting reduced penicillin susceptibility in 33 to 65% of isolates (163–165). Susceptibility to aminoglycosides is also variable, but no cases of high-level resistance have been reported. A synergistic effect between β-lactam agents and aminoglycosides has been demonstrated for isolates of *Abiotrophia*, and combination therapy with penicillin and gentamicin is the currently recommended treatment for endocarditis caused by *Abiotrophia* and *Granulicatella*. High relapse rates have been reported, even with appropriate therapy (163). Tuohy and colleagues (165) examined a collection of 27 *G. adiacens* and 12 *A. defectiva* strains, noting susceptibility of all isolates to clindamycin, rifampin, levofloxacin, ofloxacin, and quinupristin-dalfopristin. These authors noted that susceptibilities of *G. adiacens* and *A. defectiva*, respectively, to other agents tested were as follows: penicillin, 55 and 8%; amoxicillin, 81 and 92%; ceftriaxone, 63 and 83%; and meropenem, 96 and 100% (165). Zheng and coworkers reported high rates of β-lactam and macrolide resistance in a collection of pediatric *Abiotrophia* and *Granulicatella* isolates (166). A daptomycin MIC range of ≤0.125 to 2 μg/ml was observed for 10 strains of this group of bacteria (167).

A. viridans and *G. haemolysans* appear to be susceptible to penicillin and display a low level of resistance to aminoglycosides (168, 169). Resistance to tetracycline and macrolides has been described in *Gemella* isolates (170), as well as a synergistic effect for penicillin and gentamicin (169). Piper and colleagues (167) noted daptomycin MICs of ≤0.125 μg/ml for four strains of *G. morbillorum*. Buu-Hoi and colleagues (168) noted that while *A. viridans* seems to be naturally susceptible to macrolides, tetracyclines, and chloramphenicol, resistance to these agents has been observed. *A. urinae* has been described as susceptible to penicillin, amoxicillin, piperacillin, cefepime, rifampin, and nitrofurantoin but resistant to sulfonamides and netilmicin (107, 171, 172). Humphries and coworkers (173) noted that *A. urinae*'s resistance to trimethoprim-sulfamethoxazole is test medium dependent. Strains of this organism are susceptible to trimethoprim-sulfamethoxazole when tested on lysed horse blood-containing media that display low levels of thymidine (173). *A. sanguinicola* isolates display susceptibility to penicillin, amoxicillin, cefotaxime, cefuroxime, erythromycin, chloramphenicol, quinupristin-dalfopristin, rifampin, linezolid, and tetracycline (26).

Clinical isolates of *D. pigrum* studied by LaClaire and Facklam (22) were all susceptible to penicillin, amoxicillin, cefotaxime, cefuroxime, clindamycin, levofloxacin, meropenem, quinupristin-dalfopristin, rifampin, and tetracycline. Variable susceptibility to erythromycin was noted, and 1 of the 27 strains examined was resistant to trimethoprim-sulfamethoxazole. The small number of *Helcococcus* isolates examined displayed susceptibility to penicillin and clindamycin, and most strains were resistant to erythromycin (122, 123). Woo and coworkers described an *H. kunzii* strain with *ermA*-mediated erythromycin and clindamycin resistance (126). Strains of *Facklamia* exhibit variable MICs for a variety of antibiotics (174). A study of 27 strains of *Globicatella sanguinis* reported susceptibility of all isolates to amoxicillin but varying levels of resistance to other antimicrobials tested (175).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Efforts to identify the Gram-positive cocci included in this chapter should be made only when isolates are considered to be clinically significant (i.e., isolated repeatedly, in pure culture, or from normally sterile sites), since these organisms may also appear in clinical cultures as contaminants or constituents of the normal microbiota. Communication with clinicians should guide the microbiology laboratory in evaluating the significance of these infrequently isolated organisms. The phenotypic tests mentioned in Table 1 and Fig. 1 and 2 facilitate presumptive identification of the infrequently isolated catalase-negative, Gram-positive cocci. More extensive phenotypic testing using commercially available identification systems and molecular methods should be employed for definitive identification. Currently there are susceptibility testing guidelines for only four of the genera mentioned in this chapter. The MICs generated with dilution methods can be reported without interpretation when susceptibility testing is requested for significant isolates for which no guidelines exist.

Abiotrophia, *Granulicatella*, and *Gemella* species are well-documented agents of endocarditis. The satelliting behavior of *Abiotrophia* and *Granulicatella* and the positive PYR reactions of all three genera are useful for distinguishing them from viridans group streptococci. CLSI guidelines (159) should be employed for susceptibility testing and interpretation of results for *Abiotrophia* and *Granulicatella*. The vancomycin-resistant genera *Leuconostoc*, *Pediococcus*, and *Weissella* are infrequent clinical isolates, but they have been described as agents of bacteremia and central nervous system and other infections in compromised hosts. Phenotypic testing for vancomycin resistance (see "Identification" above) is important for identifying these genera and also helps guide antimicrobial therapy. Guidelines for antimicrobial susceptibility testing and interpretation of results are available for *Leuconostoc* and *Pediococcus* (159). Among the aerococci, *A. urinae* is a well-documented urinary tract pathogen and should be reported when isolated in significant amounts as the predominant organism in urine cultures. Phenotypic tests mentioned in this chapter presumptively identify *A. urinae*, which has been described as susceptible to β-lactam agents and nitrofurantoin (107, 171, 172).

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General Approaches to the Identification of Aerobic Gram-Positive Rods

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The purpose of this algorithm for the identification of aerobic Gram-positive rods is to assist the reader in finding the appropriate chapter in this *Manual* for further information. The algorithm emphasizes that the Gram stain (performed on 24- to 48-h-old colonies from rich media) and macroscopic morphologies are initial key features for the differentiation of aerobic Gram-positive rods. All strains of aerobic Gram-positive rods (except the non-rapidly growing mycobacteria) are initially grown on blood agar plates.

Gram-positive organisms demonstrating "regular" rods are those with cells whose longitudinal edges are usually not curved but are parallel. If spore formation is not observed initially, it can be tested for on a nutritionally depleted medium. Catalase activity should be tested with colonies grown on media lacking heme groups. Type of metabolism can be evaluated using oxidative-fermentative media or in cystine Trypticase agar medium. "Irregular" rods are those organisms with cells whose longitudinal edges are curved and not parallel. Slight beta-hemolysis is best observed when cells are incubated in a CO_2-enriched atmosphere. Organisms that have yellow- or orange-pigmented colonies are usually composed of irregular rods. Some genera that stain partially acid-fast (e.g., *Gordonia* and *Rhodococcus*) may also show a yellow-orange pigment (see chapter 29). Rods exhibiting vegetative substrate filaments may show branched-type hyphae, which either form spores or reproduce by fragmentation. Vegetative substrate filaments might not be present initially (i.e., within 48 h), and so these organisms are prone to being misidentified. For yellow-orange-pigmented genera (e.g., *Microbacterium*, *Curtobacterium*, and *Leifsonia*; see chapter 28), as well as for those rods exhibiting vegetative substrate filaments, chemotaxonomic or molecular genetic methods may be required for definitive identification to the genus level; partially acid-fast bacteria may be identified to the genus level by genetic means.

Genera that contain strictly anaerobic Gram-positive rods may also contain species, or strains within a species, that grow reasonably well aerotolerantly or aerobically. This is particularly true for the genera *Actinomyces* and *Actinobaculum*, some *Propionibacterium* spp. (see chapter 52), and *Clostridium tertium* (a strong gas producer) as well as other aerotolerant *Clostridium* spp. (see chapter 53). Some aerobic Gram-positive cocci, e.g., *Leuconostoc* spp. (see chapter 24) and *Streptococcus mutans* (see chapter 22), might initially be misidentified as Gram-positive rods because of their initial Gram stain appearance after growth on plate agar rather than from a broth. Some Gram-positive rods (e.g., *Rhodococcus* spp. [see chapter 29] or *Dermabacter* [see chapter 28]) might be misidentified as Gram-positive cocci because of their initial Gram stain appearance. After preliminary examination of the pathogen, the microbiologist should refer to Table 1, where a wide variety of genera of Gram-positive rods are cross-referenced, with respect to relevant chapters in this *Manual*.

Molecular approaches to characterize or subtype pathogens described in this section, as adjuncts to conventional phenotypic or chemotaxonomic assays, are described briefly in each chapter of this *Manual*, as well as in overview in chapters 6, 10, and 16. Use of 16S rRNA gene sequencing as a means to characterize an isolate has become an established tool in many microbiology laboratories; use of this approach can positively affect patient treatment and facilitate earlier hospital discharge (1). However, interpretation of this work must be done by personnel with a good working knowledge of current approaches for taxonomy and systematics (see chapter 17). Instances exist where 16S rRNA gene sequence analysis cannot be used as the sole characterization method, particularly where two or more validly named taxon groups have >98.7% identity to each other, a cutoff value described as being suggestive of members of the same species (2). In such cases, sequencing of secondary or additional gene targets is recommended (3). Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry is being used increasingly for identification of a wide variety of pathogens (4). The application and efficacy of this technology are outlined for individual taxa within each chapter. Additional reviews describing some of these pathogens are found in references 5–7.

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TABLE 1 Algorithm for identification of Gram-positive rods

Cellular morphology	Pigmented	Vegetative substrate filaments	Spore-former	Catalase	Metabolism	UnusualGram stain features	Acid-fast	Partially or weakly acid-fast	Aerial vegetative filaments	Motility	Genus or genera (chapter)	Additional comment
Regular	−	−	+	V	O or F	Often large cells	−	−	−	V	*Bacillus*, *Paenibacillus*, *Aneurinibacillus*, *Virgibacillus*, occasionally other genera in family *Bacillaceae* (26)	*Bacillus anthracis*, also chapter 14
	−	−	−	+	F	−	−	−	−	+	*Listeria* (27)	Motility better observed at 20–25°C
	−	−	−	−	F	−	−	−	−	−	*Erysipelothrix* (27)	H_2S produced in TSI
	−	−	−	−	F	−	−	−	−	−	*Lactobacillus* (52)	Some strains weakly catalase +
Irregular	−, also Y or B-G	−	−	+	O or F	Club shaped; rarely, some unusual forms, e.g., “whip handles” or “bulges”	−	−	−	−	*Corynebacterium* (28)	
	−	−	−	+	O	Long slim rods	−	−	−	−	*Turicella* (28)	
	−	−	−	+	F	Coccoid	−	−	−	−	*Dermabacter* (28)	
				+	F	Short rods	−	−	−	−	*Helcobacillus* (28)	
	−, also Y	−	−	+	O	Shorter rods	−	−	−	−	*Brevibacterium* (28)	
	−	−	−	+	F	May show branching or short rods	−	−	−	−	*Actinomyces*, *Propionibacterium* (52 for both)	*Actinomyces radicidentis*, coccoid
	−, also B-G	−	−	+	F	Pleomorphic	−	−	−	−	*Rothia* (21 and 28)	Some strains catalase −
	−	−	−	−	O	Coccoidal or rod forms	−	−	−	+	*Auritidibacter* (28)	May demonstrate rod-coccus cycle
	−	−	−	−	F	Gram variable, coccoidal	−	−	−	−	*Gardnerella* (28)	Beta-hemolysis on vaginalis agar
	−	−	−	−	F	Some *Actinomyces* spp. branching	−	−	−	−	*Arcanobacterium* and *Trueperella* (28 for both), *Actinomyces* and *Actinobaculum* (52 for both)	Some species with slight beta-hemolysis
	−	−	−	−	F	Pleomorphic, “bifidoforms”	−	−	−	−	*Bifidobacterium* and genera formerly *Bifidobacterium*[b] (52)	
	Y, Y-O	+	−	+	F		−	−	−	V	*Oerskovia* (28)	
					F						*Cellulosimicrobium* (28)	

Y, Y-O	−	−	+	O		−	−	−	V	*Curtobacterium* (28)	
Y, Y-O	−	−	+	O or F		−	−	−	V	*Microbacterium* (28)	Some *Microbacterium* spp. catalase −
				O					+	*Leifsonia* (28)	
Y, Y-O	−	−	+	F		−	−	−	V+	*Cellulomonas*, *Exiguobacterium* (28 for both)	
−	+	−	+	O		+	−	−	−	*Mycobacterium* (30–32)	
−	+	−	+	O		+	−	−	−	*Segniliparus* (29)	No growth on MacConkey agar
−	+	−	+	O		−	+	+	−	*Nocardia* (29)	
−, Y, P-C	+ or V	−	+	O		−	+ or −	−	−	*Tsukamurella*, *Gordonia*, *Rhodococcus*, *Dietzia*, *Williamsia* (29 for all)	*Dietzia* generally not considered acid-fast
−	+	−	+	O	Dense aggregates of rounded cells	−	−	−	+	*Dermatophilus* (29)	Beta-hemolysis
−, Y	+	−	+	O		−	−	+	−	*Actinomadura*, *Streptomyces*, *Amycolatopsis*, *Nocardiopsis*, occasionally other genera among *Pseudonocardiaceae* (29 for all)	Some strains lack aerial filaments
−	+	−	+	O		−	−	+	−	*Saccharomonospora*, *Saccharopolyspora*, *Thermoactinomyces* (29 for all)	Can grow at 50°C

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[a] Abbreviations and symbols: +, all or nearly all strains positive; −, all or nearly all strains negative; V, feature variable; O, oxidative metabolism; F, fermentative metabolism; TSI, triple sugar iron slant. “Pigment” implies that colonies have pigment other than gray-white or white colony; yellow or yellowish (Y) or yellow-orange (Y-O) pigment is typical, blackish gray (B-G) pigment is occasionally seen, and pinkish coral (P-C) is seen for some *Rhodococcus* and *Williamsia* spp. Table excludes extremely infrequently isolated genera described in chapter 28, e.g., *Brachybacterium*, *Knoellia*, and *Janibacter*.

[b] Strains of the genus *Alloscardovia* in particular can be aerotolerant.

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Bacillus and Other Aerobic Endospore-Forming Bacteria*

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26

TAXONOMY

Historically, most aerobic endospore-forming bacteria were classified as species of *Bacillus*. However, the application of phylogenetic methods to prokaryotic systematics has triggered a taxonomic transformation. This includes the valid publication of a new class, *Bacilli*, within the phylum *Firmicutes*. *Bacilli*, as defined by 16S rRNA gene sequence analyses, comprises two orders, *Bacillales* and *Lactobacillales*. Currently, the aerobic endospore-forming bacteria are distributed among >60 genera and seven families within the order *Bacillales*.

Minimal standards for describing new taxa of aerobic endospore-forming bacteria have been published (1). These standards acknowledge the role of genetic features but highlight the importance of detailed phenotypic characterization. For any new taxon, it is recommended that descriptions are based on at least five strains and ideally 10 or more. Unfortunately, many new species, and some genera of aerobic endospore-forming bacteria, are represented only by single isolates. The absence of multiple specimens precludes any assessment of intragenus or intraspecies variability.

For the clinical microbiologist, the reallocation of taxa and proliferation of new species are of limited practical concern. The vast majority of aerobic endospore-forming bacteria are nonpathogenic, environmental organisms. The medically important and commonly encountered genera, primarily *Bacillus*, *Lysinibacillus*, *Paenibacillus*, and *Brevibacillus*, are found within the two largest families, *Bacillaceae* and *Paenibacillaceae*. The families *Alicyclobacillaceae*, *Planococcaceae*, and *Sporolactobacillaceae* are not known to contain clinically relevant species, and *Pasteuria*, the only genus in the family *Pasteuriaceae*, comprises obligate bacterial parasites of invertebrates that have never been grown in axenic culture. In contrast to other *Bacillales*, organisms in the family *Thermoactinomycetaceae* appear filamentous and produce mycelia, aerial hyphae, and sporophores. Although some are true aerobic endospore-forming bacteria, for the purposes of this *Manual*, the *Thermoactinomycetaceae* are grouped with the aerobic actinomycetes (see chapter 29).

DESCRIPTION OF THE GENERA

Bacillus is the type genus of the *Bacillaceae*. The typical *Bacillus* isolate is a rod-shaped, endospore-forming organism that is aerobic, mesophilic, catalase positive, and motile by means of peritrichous flagella. Young cultures usually stain Gram positive, whereas older cultures are more likely to be Gram variable or Gram negative. However, the genus is phenotypically diverse such that some species are asporogenous, facultatively anaerobic or strictly anaerobic, and thermophilic or psychrophilic.

Many *Bacillus* species have been renamed and reallocated to new monophyletic and phenotypically coherent taxa, which is consistent with a recent proposal that the designation *Bacillus* be reserved exclusively for organisms belonging to the *Bacillus subtilis* and *Bacillus cereus* clades (2). Even so, the genus *Bacillus* remains polyphyletic, phenotypically diverse, and large. Frequently encountered species include *B. subtilis* (the type species), *B. licheniformis*, *B. megaterium*, *B. pumilus*, and the *B. cereus* group. Also called *B. cereus* sensu lato, this clade includes *B. cereus* (sensu stricto), *B. anthracis*, and *B. thuringiensis* as well as the less common but phylogenetically related *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, *B. cytotoxicus* (143), and *B. toyonensis*.

Other clinically important genera of aerobic endospore-forming organisms are phenotypically similar to the *Bacillus*, but some differences do exist. *Geobacillus* species are obligately thermophilic and *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*) is commonly employed as a biological indicator for laboratory quality control activities (e.g., for monitoring autoclave efficacy and the sporicidal activity of disinfectants). *Lysinibacillus* species are distinguished by the composition of the cell wall peptidoglycan, which is rich in lysine and aspartic acid. *Lysinibacillus sphaericus* (formerly *Bacillus sphaericus*) occasionally causes opportunistic infections in human hosts but is best known for its insecticidal toxin, which has been commercialized for use in mosquito control programs (3, 4).

Paenibacillus and *Brevibacillus* cultures may stain Gram positive but are more likely to be Gram negative or Gram variable, and sporangia usually have a swollen appearance. *Paenibacillus* is the type genus of the family *Paenibacillaceae* and includes species originally defined by 16S rRNA gene sequencing as *Bacillus* "rRNA group 3" (5). The majority of species are of environmental origin. These include nitrogen-fixing organisms associated with plant roots and insect pathogens used for biocontrol applications. *Paenibacillus* species have been isolated from a variety of clinical sources, including blood, cerebrospinal fluid (CSF), wounds, and urine (6–8).

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Brevibacillus, also in the family *Paenibacillaceae*, includes species originally defined by 16S rRNA gene sequencing as *Bacillus* "rRNA group 4" (9). *Brevibacillus brevis* (the type species), *Brevibacillus agri*, *Brevibacillus centrosporus*, and *Brevibacillus parabrevis* have been isolated from clinical sources (10, 11). *Brevibacillus laterosporus* is primarily an insect pathogen (12), but human infection has been reported (13).

Additional species of aerobic endospore-forming bacteria may be encountered in the microbiology laboratory. Environmental organisms may act as opportunistic pathogens, but most isolates, especially those obtained from nonsterile sites and wound tracts, are likely to be contaminants of unknown clinical significance.

EPIDEMIOLOGY AND TRANSMISSION

Aerobic endospore-forming bacteria are ubiquitous in nature. They are found in terrestrial and aquatic habitats of all kinds, ranging from acid to alkaline, nonsaline to highly saline, and hot to cold. Because endospores are resistant to heat, desiccation, radiation, and disinfectants, they persist in places where most other organisms cannot. These exotic and extreme environments include air at high altitude, subterranean waters, glacial ice cores, permafrost, hot springs, and volcanic soils. Dissemination of spores, via wind, dust, and aerosols, contributes to contamination of man-made environments, including hospital operating rooms and industrial clean rooms. Because they contribute to food spoilage and food poisoning, aerobic endospore-forming organisms are unwanted contaminants in food production and preparation environments. However, these bacteria are also essential to the production of certain fermented traditional foods (e.g., natto, cheonggukjang, and gergoush) and the probiotic properties of animal feed and horticultural supplements. Similarly, some of the same species encountered in the clinical laboratory (e.g., *B. subtilis*, *B megaterium*, *B. licheniformis*) are also employed by industry for the commercial production of enzymes (e.g., amylases, proteases, cellulases), antibiotics (e.g., polymyxins, colistins, gramicidins), and pesticides (e.g., Bt toxins).

The majority of aerobic endospore-forming bacteria are nonpathogenic saprophytes. Although they are commonly encountered in the microbiology laboratory, only a small minority (~5%) of isolates are clinically relevant, and most of the clinically significant species are best described as opportunistic human pathogens. Transmission is restricted to ingestion, injection, injury, inhalation, or other contact with material that has been contaminated with spores or vegetative cells. Several environmental species (e.g., *B. thuringiensis*, *Paenibacillus larvae*, *Paenibacillus lentimorbus*, *Paenibacillus popilliae*, and *Pasteuria* spp.) are recognized as professional pathogens of insects and other invertebrates, but only *B. anthracis* has been described as an obligate pathogen of animals. Infection of wild and domesticated mammals occurs through ingestion of spores from contaminated pastures. *B. anthracis* spores remain viable for many years and are exceedingly difficult to eradicate. Some authorities contend that, in certain soils, self-maintenance can occur (14), and it has recently been demonstrated that soil-dwelling amoebae can support the germination and amplification of *B. anthracis* spores (15). However, the environmental presence of *B. anthracis* is generally associated with contamination from animal sources, such as anthrax-infected carcasses. Human cases are almost always due to direct contact with infected animals or animal products. Person-to-person spread has been reported but is rare, as are cases of anthrax due to laboratory accidents, biological warfare, and bioterror events.

CLINICAL SIGNIFICANCE

The majority of aerobic endospore-forming species appear to have little or no pathogenic potential and are rarely associated with disease. The principal exceptions to this are species of the *B. cereus* group, which includes *B. anthracis*. However, due to the medical and historical importance of anthrax, *B. cereus* and *B. anthracis* are discussed separately below.

Aerobic Endospore-Forming Bacteria (Other Than the *B. cereus* Group and *B. anthracis*)

Reports of infections due to aerobic endospore-forming bacteria are relatively rare. Cases typically result from contamination of wounds, surgical sites, or medical devices with bacterial spores. Systemic disease most often occurs in patients who are immunocompromised or afflicted with a comorbid condition, but some cases do involve immunocompetent individuals. Mixed infections involving multiple aerobic endospore-forming species have also been reported (16). *B. subtilis* is the type species of the genus *Bacillus*. Due to taxonomic changes, some clinical isolates originally identified as *B. subtilis* have been reallocated to other taxa, but cases involving authentic isolates of *B. subtilis* do occur. Reports include pneumonia, bacteremia, and septicemia in patients with leukemia or other neoplastic diseases, isolations from surgical wound drainage sites, breast prosthesis and ventriculo-atrial shunt infections, endocarditis following drug abuse (17, 18), and meningitis following a head injury (19). *B. subtilis* has also been implicated as an agent of food-borne illness and isolated in high numbers (10^5 to 10^9 CFU/g) from food. Typical symptoms include vomiting, sometimes accompanied by diarrhea, and reported onset periods have been short (range, 10 min to 14 h; median, 2.5 h) (20). Two cases of severe hepatotoxicity have been attributed to ingestion of *B. subtilis*-contaminated nutritional supplements (21). A similar case, contamination of an oral probiotic preparation, with an organism ultimately identified as *Bacillus clausii*, led to a fatal septicemia in an immunocompromised patient. *B. clausii* was also associated with a case of cholangitis in a renal transplant patient (22).

B. licheniformis is another species commonly associated with clinical illness. Its occasional virulence is attributed, at least in part, to the production of lichenilysin, a heat-stable and cytotoxic cyclic lipopeptide. *B. licheniformis* cases include prosthetic valve endocarditis, pacemaker wire infection (23), ventriculitis following the removal of a meningioma, brain abscesses, septicemia following arteriography, bacteremia associated with indwelling central venous catheters, and peritonitis in patients undergoing continuous ambulatory peritoneal dialysis. *B. licheniformis* contamination of nonsterile cotton wool led to a nosocomial outbreak of bacteremia among patients with blood malignancies (24). Sepsis in immunocompetent patients has also been reported (25). Self-harming behavior was the cause of two unusual cases. In one, *B. licheniformis* infection followed self-inoculation with drain cleaner (26). In the other, self-inoculation with soil resulted in a polymicrobial infection from which *B. licheniformis*, *B. pumilus*, and *Paenibacillus polymyxa* were isolated (27). *B. licheniformis* is also an agent of food-borne diarrheal illness, which has led to at least one fatality (28). *B. pumilus* isolates are known to produce heat-stable, cytotoxic cyclic lipopeptides called pumilacidins. *B. pumilus* has been implicated in cases of cutaneous, pustule, and rectal fistula infections, bacteremias in immunosuppressed patients (24), a central venous catheter infection in an immunocompetent child, and sepsis in neonates (29), and at least one fatality has been reported (30). Toxigenic strains of *B. pumilus* have been isolated from cases of food-borne illness and implicated in a rice-associated food poisoning outbreak (31). *B. megaterium* is occasionally recovered from blood, wounds, and urine. Skin lesions due to *B. megaterium* can resemble cutaneous anthrax, and phenotypically, the organism can mimic *B. anthracis* (32, 33). Organisms identified as *Bacillus circulans* have been isolated from cases of bacteremia in cancer patients, meningitis, CSF shunt infections, endocarditis, wound infections, and peritonitis in a patient undergoing dialysis (34). However, it should be noted that, due to taxonomic changes, some of these isolates might now be classified as species of *Paenibacillus*.

Paenibacillus alvei (formerly *Bacillus alvei*) has been isolated from cases of meningitis, a prosthetic hip infection in a patient with sickle cell anemia, wound infections, and in association with *Clostridium perfringens*, a case of gas gangrene. *Paenibacillus macerans* has been isolated from a wound infection following removal of a malignant melanoma, from a brain abscess following penetrating periorbital injury, and from a catheter-associated infection in a leukemic patient, whereas *P. polymyxa* has been isolated from patients with bacteremia (27). *P. popilliae* has been reported from a case of endocarditis, and *P. larvae* has been reported from infection of a CSF shunt system. *Paenibacillus glucanolyticus* was involved in cardiac device-related endocarditis (35). *Cohnella hongkongensis*, a species historically grouped with *Paenibacillus* (36), was isolated from a case of pseudobacteremia in a boy with neutropenic fever. *Lysinibacillus sphaericus* has been implicated in a fatal lung pseudotumor, bacteremia, and meningitis (3).

Additional clinical cases involve a variety of recently described *Bacillus* and *Paenibacillus* species, many of which were proposed on the basis of single isolates of unknown significance from clinical sources: *B. idriensis* and *B. infantis* from neonatal sepsis (37); *P. konsidensis*, *P. macerans*, *P. sanguinis*, *P. thiaminolyticus*, and *P. timonensis* from blood cultures (6, 8, 30, 38); *P. provencensis* isolated from CSF (7, 39); *P. turicensis* from a CSF shunt (40); *P. pasadenensis* and *P. vulneris* from wounds (41, 42); and *P. urinalis* from urine (7). Several *Brevibacillus* species have also been recovered from clinical sources, including *B. centrosporus* from bronchoalveolar lavage fluid, *B. parabrevis* from a breast abscess, and both species from human blood (10).

Eye infections due to aerobic endospore-forming bacteria can be quite severe. Many species have been associated with endophthalmitis, including *B. circulans*, *B. licheniformis*, *P. alvei*, and *B. laterosporus* (43). *Bacillus* species, including *B. megaterium*, can cause keratitis. It has also been suggested that *B. oleronius*, a species found in the gut of *Demodex* mites, may act as a copathogen in cases of severe or chronic blepharitis (44).

Bacillus cereus Group

Bacillus cereus was initially described in an 1887 survey of environmental bacteria (45). The species name is derived from "cera," the Latin term for wax, and refers to the wax-like morphology of colonies grown on agar media. *B. cereus* has been isolated from diverse ecological niches and is widely distributed in nature. The original isolate was obtained from the air in a cow shed, and additional strains have been isolated from soil, plant leaves, insects, water, and sewage (46). Psychrotolerant (*B. weihenstephanensis*), thermotolerant (*B. cytotoxicus*), crystal-forming (*B. thuringiensis*), and morphological (*B. mycoides*, *B. pseudomycoides*) variants and a probiotic strain (*B. toyonensis*) have been classified as distinct species. *B. anthracis* also belongs to the *B. cereus* group, but due to the medical and historical importance of anthrax, it will be considered separately.

Clinically, *B. cereus* group organisms are opportunistic pathogens, and cases involving local (e.g., eyes, skin, and wounds) and systemic (e.g., bacteremia, septicemia, meningitis, peritonitis, endocarditis, and respiratory and urinary tract) infections have all been reported (47, 48). Systemic infections are most common in patients who are immunocompromised due to some comorbid condition (e.g., cancer and diabetes). *B. cereus* spores are resistant to many disinfectants, and postoperative and hospital-acquired infections have been traced to contaminated gloves, gowns, linens, dressings, medical devices (e.g., catheters, shunts and implants, bronchoscopy equipment, and ventilators), and even alcohol-based prep pads used for disinfection purposes (49). A recent nosocomial outbreak, involving 171 patients, was attributed to insufficient air filtration, cleaning, and laundry practices, which failed to eliminate spores that had been introduced to the hospital environment by nearby construction work (50). *B. cereus* infections secondary to trauma, including cuts and scrapes, surgery, and burns, occur when, during the initial injury, spores from soil, water, or other sources, are introduced to the wound tract. *B. cereus* contamination of drugs, both legal and illegal, has also been reported (51, 52). Eye injuries and medical procedures (e.g., cataract surgery and intravitreous injections) can result in *B. cereus* endophthalmitis. Without rapid therapeutic intervention, this serious condition can lead to vision loss and even eye evisceration (43, 53). Keratitis has been associated with contact lens wear, and in at least two cases, *B. cereus* was cultured from the eyes as well as the contact lens cases of the infected patients (54, 55).

However, *B. cereus* isolates are most commonly associated with food-borne illness. Between 1998 and 2008, *B. cereus* was suspected or confirmed as the cause in 235 (1.75%) of the 13,405 food-borne outbreaks reported to the Foodborne Diseases Active Surveillance Network (FoodNet) of the Centers for Disease Control and Prevention's Emerging Infections Program (56). However, that statistic likely underrepresents the true burden of *B. cereus* disease because these organisms are not always considered in clinical, epidemiological, and laboratory investigations of food-borne illness. Because they survive normal cooking temperatures and many cleaning procedures, *B. cereus* spores can be widespread in food preparation environments. Food poisoning typically follows the germination of spores and multiplication of toxigenic vegetative cells in improperly stored food. *B. cereus* group organisms cause two distinct food poisoning syndromes. The emetic type is characterized by nausea and vomiting, with symptoms appearing 1 to 5 h after ingestion of the contaminated food. The diarrheal type is characterized by abdominal pain and diarrhea, and onset is later, 8 to 16 h after food consumption (20, 46). *B. cereus* has been isolated from diverse foods, but rice dishes, especially Asian-style fried rice, are frequently implicated in outbreaks of emetic type illness, whereas meat dishes are more often associated with diarrheal type illness (56). *B. cereus* is of increasing concern to the dairy industry. It causes mastitis in goats and dairy cattle, spores persist in barns and processing facilities, and cold-tolerant strains, especially *B. weihenstephanensis*, can survive both pasteurization and refrigeration (57). Outbreaks of food-borne illness have been linked to milk, cheese, pudding, and other dairy products

(20). Contamination also contributes to food spoilage and shortens the shelf life of dairy products (58).

Bacillus anthracis

Robert Koch's landmark work on anthrax helped establish the germ theory of disease, and an 1877 report on *B. anthracis* included the first published photomicrographs of any bacteria (59). Anthrax is primarily a disease of wild and domesticated animals that, historically, was a leading cause of mortality among cattle, sheep, goats, and horses. The use of veterinary and human vaccines, improvements in factory hygiene, effective sterilization procedures for imported animal products, and the increased use of synthetic alternatives to animal hides and hair have all contributed to a marked decline in the incidence of disease. Nevertheless, anthrax remains endemic to many countries, particularly those that lack effective vaccination policies. In countries with national vaccination programs, the reduced incidence of anthrax contributes to public ignorance of disease symptoms as well as diminishing veterinary experience. Delays in case recognition can undermine control measures, prolong outbreaks, and lead to the sale and slaughter of affected animals. Because of these challenges, anthrax remains common, especially in agricultural regions of Central and South America, southern and eastern Europe, central and southwestern Asia, sub-Saharan Africa, the United States, and Canada (60).

Animals are usually infected through ingestion of *B. anthracis* spores from contaminated pastures. Direct animal-to-animal transmission is rare, although scavengers (e.g., vultures and hyenas) can become infected by feeding on anthrax-infected carcasses (61). Human cases usually involve direct contact with infected animals or products from infected animals. Cases resulting from close contact with infected animals or their carcasses are traditionally classified as nonindustrial, whereas those acquired while processing animal products, such as wool, hair, hides, or bones, are classified as industrial. Although anthrax is not a contagious disease, person-to-person transmission has occasionally been reported, including cases of mother-to-child spread from an infected finger, brother-to-brother spread from an abdominal lesion (62), and nosocomial spread from an umbilical infection (63). Regrettably, *B. anthracis* has been developed, and effectively deployed, for biological warfare and bioterror purposes. Few reports of laboratory-acquired infections exist, but the accidental release of spores from a military production facility has been implicated as the cause of a major 1979 outbreak in the Ural city of Yekaterinburg (formerly Sverdlovsk), Russia, which claimed at least 66 lives (64).

In humans, the major clinical forms of the disease—cutaneous anthrax, gastrointestinal anthrax, inhalational anthrax, and injectional anthrax—are linked to the route of infection (65). Adult males account for the majority of anthrax cases, but disease may be more severe in pregnant women and children (66, 67). Initial symptoms may be nonspecific and mild (e.g., fatigue, malaise, fever, and/or gastrointestinal symptoms), but following lymphohematogenous dissemination from a primary lesion, fulminant disease can rapidly develop. Symptoms can include dyspnea, cyanosis, severe pyrexia, and disorientation, followed by circulatory failure, shock, coma, and death. The bacteria can multiply rapidly in the blood, and depending on the host, reach terminal levels of 10^7 to 10^9 CFU/ml over the final few hours. Enhanced clinical and laboratory expertise is a critical component of rapid anthrax diagnosis, and due to the biothreat potential of *B. anthracis*, there is a need for prospective surveillance and response preparedness. Similarly, case investigations should strive to establish the origin of any infection so as to differentiate between naturally occurring events and those of malicious origin.

Cutaneous anthrax accounts for about 99% of naturally acquired human anthrax cases. In the United States, the incidence of anthrax is extremely low, with 0 to 2 cases reported annually for the last 3 decades (68). The global incidence is difficult to determine and varies between countries depending on control strategies and the strength of health systems. Rates are highest in Africa and central and southern Asia (69) and, in some regions, are increasing due to funding cuts (70). Infection typically occurs when spores are inoculated through a break in the skin, although case reports and animal studies suggest that preexisting lesions are not necessary for infection. Rarely, infection has been attributed to insect bites (71, 72). Following an incubation period of usually 2 to 6 days (range, a few hours to 3 weeks), a small papule appears. Over the next 24 h, this progresses to a ring of vesicles, which subsequently ulcerates to form a blackened eschar, the characteristic lesion of cutaneous anthrax. Once formed, the eschar may become thick and surrounded by extensive edema. Fever, pus, and pain at the site are normally absent, and the presence of such symptoms may be indicative of a secondary bacterial infection. Before the availability of antimicrobial therapy, 10 to 20% of untreated cutaneous anthrax cases were fatal. Today, less than 1% of cases are fatal. Fatalities are mainly due to obstruction of the airways by the edema that accompanies lesions that form on the face or neck but can also occur when cutaneous disease progresses to systemic infection. Eschars take several days to evolve, but even with effective antimicrobial therapy, they may take several weeks to resolve.

Gastrointestinal anthrax results from the consumption of anthrax-infected animals, especially meat that is raw or undercooked. Asymptomatic cases can occur, and may not be uncommon, but disease usually presents in one of two forms. The oral, or oropharyngeal, form features lesions on the buccal cavity, tongue, tonsils, or posterior pharyngeal wall. Symptoms include sore throat, dysphagia, and regional lymphadenopathy, followed by severe edema of the neck and chest. The intestinal form can develop anywhere within the gastrointestinal tract, but ulcerations typically appear in the mucosa of the terminal ileum or cecum. Symptoms include nausea, vomiting, anorexia, abdominal pain, fever, mild diarrhea (which can progress to bloody diarrhea), hematemesis, and massive ascites. Owing to the nonspecific nature of the early symptoms, antimicrobial therapy may be initiated late, and mortality rates range widely (73, 74).

Inhalational anthrax is a rare but serious form of the disease. During the 20th century, only 18 cases of naturally acquired inhalational anthrax were recorded in the United States. Of these, 16 (89%) were fatal (75). Figures from the United Kingdom show a similar picture. Previously known as pulmonary anthrax, the preferred designation, "inhalational anthrax," more accurately reflects the fact that active infection occurs in the lymph nodes rather than the lungs themselves. In late 2001, the malicious dissemination of *B. anthracis* spores in mailed letters was responsible for an outbreak of 22 cases in the United States. Laboratory-confirmed inhalational anthrax accounted for 11 cases, including all 5 of the deaths attributed to the outbreak (76). All of these patients developed severe illness and were hospitalized, but thanks to early recognition and treatment, the case survival rate was 55% (6/11 patients). Analysis revealed a median incubation period of 4 days (range, 4 to 6 days). Clinical presentation included fever or chills, fatigue or malaise, minimal or nonproductive cough, dyspnea, and nausea or vomiting. Some patients experienced chest pain and sweats. All patients had abnormal chest radiographic images, with pleural effusion, infiltrates, or mediastinal widening (77).

Injectional anthrax refers to cases associated with injection drug use (78). Although the route of infection is through the skin, symptoms of injectional anthrax differ from those of cutaneous anthrax. Skin around the injection site may be bruised or discolored, but the characteristic papules and eschar are absent. Cases usually present as severe soft tissue infections with significant edema, and progression to septic shock can be rapid (79). Contaminated heroin has been implicated in cases from Norway, Denmark, France, Germany, and the United Kingdom. In Scotland, a large outbreak (47 confirmed, 35 probable, and 37 possible cases) occurred between December 2009 and December 2010 (80). Genotyping methods have been used to analyze *B. anthracis* isolates associated with cases of injectional anthrax. The European isolates collected between 2000 and 2012 are highly related, share two distinctive single nucleotide polymorphisms, and likely originate from a single source (81, 82). Genotypically, the drug-associated strain is most similar to veterinary isolates from Turkey and not *B. anthracis* strains from opium (i.e., heroin)-producing countries. Drug traffickers are known to use animal skins for smuggling contraband. As such, it has been hypothesized that the heroin associated with the European cases could have become contaminated with *B. anthracis* spores if transported in skins from an anthrax-infected animal (80).

During the past decade, there have also been several cases of naturally occurring infection. In 2006, a maker of African drums became the first naturally occurring case of inhalational anthrax in the United States since 1976. Infection was traced to imported hides from West Africa which were contaminated with *B. anthracis* spores. In 2007, two cases of cutaneous anthrax, in a drum maker and a family member, occurred in the United States (83). In 2008, a fatal case of inhalation anthrax occurred in a drum maker in the United Kingdom (84). The handling or playing of goatskin drums contaminated with spores of *B. anthracis* has been associated with additional cases of cutaneous, inhalational, and gastrointestinal anthrax (83, 85).

In rare instances, cases have occurred for which no source has been found. In 2011, a man acquired inhalational anthrax while traveling through four U.S. states, including midwestern states where *B. anthracis* is enzootic. Despite thorough sampling and the involvement of a dedicated anthrax investigative team, no exposure source was definitively identified (86). A 2012 case from the United Kingdom involved a member of the armed forces who acquired inhalational anthrax despite being vaccinated. An exhaustive inquiry was conducted, but no exposure history to anthrax was identified (87).

Toxins and Other Virulence Factors of the *B. cereus* Group

The pathogenicity of *B. cereus* group organisms (including *B. anthracis*) is attributed to the production of numerous exotoxins, enterotoxins, hemolysins, and cytolysins. Ongoing studies aim to define roles for individual toxins, including their distribution, structures, mechanisms of action, and specific contribution to *B. cereus* disease (88–97). Expression of these toxins is controlled by multiple environmental cues, including nutrient status, oxygen concentration (e.g., redox potential), pH, and cell density. The PlcR-PapR quorum-sensing system regulates expression of at least 45 genes, including those that encode Nhe, HBL, and CytK (see below) (98). Remarkably, this "master" virulence regulator is inactive in *B. anthracis* strains, due to a lineage-specific *plcR* mutation.

Two toxins, lethal toxin and edema toxin, are associated with the virulence of *B. anthracis*. Each of these binary toxins contains two components: a protective antigen (PA) plus an enzymatic factor, lethal factor (LF) for lethal toxin and edema factor (EF) for edema toxin. PA facilitates the delivery of the toxin to target cells, where LF or EF then acts to disrupt cell signaling pathways. The *pag*C, *lef*, and *cya* genes that encode PA, LF, and EF, respectively, are found on a virulence plasmid, pXO1. Loss of pXO1 is associated with attenuation of *B. anthracis*, but even plasmid-cured strains retain some pathogenic potential (88, 89). Conversely, experimental acquisition of pXO1, or natural acquisition of pXO1-like plasmids, can enhance pathogenicity of other *B. cereus* group organisms.

Multiple toxins contribute to the symptoms of *B. cereus* food poisoning. Ingestion of a heat-stable toxin, cereulide, triggers the vomiting that is characteristic of emetic food poisoning. Cereulide is produced by the plasmid-borne *ces* gene cluster (90) and is only found in a subset of *B. cereus* sensu stricto and *B. thuringiensis* strains (91). Diarrheal symptoms of *B. cereus* food poisoning are attributed to multiple toxins, including the nonhemolytic enterotoxin (Nhe), hemolysin BL (HBL), and cytotoxin K (CytK; also known as hemolysin IV) (92). The genes responsible for Nhe production are chromosomally encoded (93) and present in most *B. cereus* group isolates (91). A plasmid-borne variant has been described for *B. weihenstephanensis* (94). The HBL toxin is also widely distributed. At least two variants have been described (92), but *hbl* genes appear to be absent from *Bacillus cytotoxicus*. CytK was first isolated from an enterotoxigenic strain that is now recognized as the type strain of *B. cytotoxicus*. Despite the absence of cereulide or HBL production, this strain was responsible for an outbreak that included three fatalities. Two variants of the CytK toxin are now recognized: CytK-1 is produced by *B. cytotoxicus*, whereas CytK-2 is found in other *B. cereus* group species and is most prevalent among *B. cereus* sensu stricto and *B. thuringiensis*. In vitro, the cytotoxic activity of CytK-1 appears to be greater than that of CytK-2 (95).

Traditionally, *B. thuringiensis* strains are defined by their production of parasporal crystals comprised of insecticidal proteins. The pesticidal potential of Bt toxin was recognized in the 1920s and first commercialized in the 1930s. To date, more than 200 variants have been isolated. Bt toxins are used extensively for the control of agricultural pests, and targets include moths, butterflies, beetles, flies, and nematode worms. *B. thuringiensis* spores can be sprayed onto fields and more recently, crops have been genetically engineered to express the *cry* and *cyt* toxin genes (99). Remarkably, these applications do not pose a threat to human health. *B. thuringiensis* can be isolated from agricultural workers exposed to commercial Bt strains, but reports of illness are rare and no deaths have occurred (100).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Bacillus Species Other than *B. anthracis*

Clinical specimens for the isolation of *Bacillus* species other than *B. anthracis* can be handled using the standard methods (see chapter 18 of this *Manual* and laboratory biosafety references, such as the CDC's *Biosafety in Microbiological and Biomedical Laboratories*) (101, 102). If the transportation time will be less than a few hours, most specimens, including serum, can be shipped at either room temperature or 2°C to 8°C. If transportation will be overnight or longer, specimens such as stool, sputum, pleural fluid, blood, and material on swabs should be sent at 2°C to 8°C, while fresh tissue and serum samples should be shipped frozen. While blood culture contamination from skin flora may occur despite best practices, rates should not exceed 3% (103). If collecting blood for nucleic acid-based testing (e.g., nucleic acid amplification test [NAAT], PCR), collection tubes containing EDTA or citrate as an anticoagulant are preferable to those containing heparin. Formalin-fixed tissues can be sent at room temperature. In cases of suspected endophthalmitis, vitreous aspirate and biopsy tissue are the optimal specimens. These should be processed as quickly as possible, ideally, at the bedside.

Clinical specimens for isolation of *Bacillus* species other than *B. anthracis* can be handled safely on the open bench using standard precautions. Efforts should be made to avoid methods that produce aerosols. Any procedures that have the potential to generate aerosols should be performed in a biological safety cabinet. Biosafety level 2 (BSL-2) practices, containment equipment, and facilities are recommended for all activities involving clinical materials and diagnostic quantities of infectious cultures (101).

The clinically significant isolates reported to date are of species that grow, and often sporulate, on routine laboratory media at 37°C. Maintenance is simple if spores can be obtained, but it is a mistake to assume that a primary culture or subculture on blood agar will automatically yield spores if it is stored on the bench or in the incubator. It is best to grow the organism for a few days on nutrient agar or Trypticase soy agar containing 5-mg/liter manganese sulfate and refrigerate when microscopy shows that most cells have sporulated. For most species, sporulated cultures on slants of this medium, sealed after incubation, can survive in a refrigerator for years. Alternatively, cultures (preferably sporulated) can be frozen or lyophilized.

B. anthracis

In many countries, possession of *B. anthracis* is regulated by legislation. The U.S. Department of Health and Human Services/CDC and the U.S. Department of Agriculture/APHIS define *B. anthracis* as a tier I, category A select agent. The Canadian Human Pathogen and Toxins Act (http://lois-laws.justice.gc.ca/eng/acts/H-5.67/FullText.html) defines *B. anthracis* and anthrax toxin as schedule 3 and schedule 1 agents, respectively. Human infectious doses have not been established, but using data based largely on nonhuman primate studies, the U.S. Department of Defense estimates that a 50% lethal dose for humans is 8,000 to 10,000 *B. anthracis* spores. When collecting clinical specimens for suspected anthrax, appropriate personal protective equipment should be used, including disposable gloves, disposable apron or overalls, and boots which can be disinfected after use. The use of a face shield and/or a respirator should be considered, especially for dusty samples that might contain many spores. Full details of personal protective equipment and of disinfection and decontamination are given in annexes 1 and 3 of the WHO *Anthrax in Humans and Animals* guidelines (69). It should be noted that waterless rubs containing ethanol are not effective at removing endospores. Hand washing with soap and water or with chlorhexidine gluconate, and the use of hypochlorite-releasing towels, may reduce endospore contamination of the skin. Preexposure vaccination recommendations from the Advisory Committee on Immunization Practices (ACIP) are summarized below (104).

Bioterrorism-Related Specimens and the LRN

In 1999, the United States Department of Health and Human Services/CDC, in partnership with the Association of Public Health Laboratories and Federal Bureau of Investigation, established a Laboratory Response Network (LRN) to integrate laboratory responses to public health emergencies, including acts of bioterrorism. The LRN links local laboratories (sentinel level) with state (reference level) and federal (national level) laboratories that provide specialized testing and increased biosafety capacity. In all 50 states, there are reference level laboratories able to rapidly detect and confirm the identity of select agents, including *B. anthracis*. The LRN also provides guidance for testing and transportation of suspicious or challenging specimens. Guidelines for sentinel level laboratories and contact information for state and territorial public health laboratories are available through the CDC (http://www.bt.cdc.gov/lrn/) and American Society for Microbiology (http://www.asm.org) websites. For general questions, there is a 24-h hotline number [(800) CDC-INFO/ (800) 232-4636] and an e-mail address (cdcinfo@cdc.gov). LRN consultations may be also requested by calling the CDC Emergency Operations Center [(770) 488-7100]. Although initially limited to the United States, there are now over 150 national and international locations, including laboratories within Canada, Australia, Germany (U.S. military base), Japan (U.S. military base), South Korea (U.S. military base), and the United Kingdom, capable of providing a rapid response to acts of biological terrorism, chemical terrorism, emerging infectious diseases, and other public health threats. Similar programs exist in other countries. In Canada, the National Microbiology Laboratory (NML; Winnipeg, Manitoba, Canada) serves as the federal center and helps coordinate training and testing activities at Canadian LRN (CLRN) sites located in five provinces.

Specimens from Patients Suspected To Have Anthrax

The CDC has published guidelines for clinical evaluation of persons with possible anthrax (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5043a1.htm). In all cases, specimens from potential sources of infection (e.g., animal hides, hair, and carcasses, etc.) should be sought in addition to patient specimens. Due to the hazardous nature of *B. anthracis*, it is recommended that sentinel level laboratories refer testing to a LRN reference level, or higher, laboratory. The preferred diagnostic specimen depends on the form of the disease (69). Whenever possible, patient specimens should be collected prior to the initiation of antimicrobial therapy. If cutaneous anthrax is suspected, the edge of an eschar should be lifted and two specimens of vesicular fluid collected by rotating swabs beneath it. One swab is used for Gram stain and culture and the other for NAAT. Immunohistochemical analyses are rarely performed any more, but if such testing is available, a full-thickness punch biopsy specimen from a papule or vesicle lesion that includes adjacent skin should be taken and fixed in 10% buffered formalin. If vesicle and eschar are present, biopsy specimens should be taken from both (105). Inhalational anthrax will be suspected only if the patient's history suggests it. Chest radiographs and chest computed tomography scans are recommended. Blood should be collected for culture. Serology is useful for the diagnosis of cases where culture fails owing to previous treatment. Acute-phase serum (obtained within 7 days of onset) and convalescent-phase samples (obtained

2 to 3 weeks later) should be collected for serologic testing at a federal center (e.g., CDC, NML). Pleural fluid, if present, should be obtained for Gram stain, culture, and PCR. Pleural and/or bronchial biopsy samples can also be tested using immunohistochemical methods. Gastrointestinal anthrax will only be suspected if an adequate history of the patient is known. Specimens from oral lesions may be collected in the same way as those for cutaneous disease and processed for Gram stain, culture, and NAAT. Blood should be obtained for culture, whereas acute- and convalescent-phase serum samples for serological testing should be collected 2 to 4 weeks apart. A stool or rectal swab and, if present, ascites fluid should be collected for Gram stain, culture, and NAAT. Any hemorrhagic fluid from the nose, mouth, or anus should be cultured. Anthrax meningitis may occur on its own or, more frequently, subsequent to other forms of the disease. CSF and blood specimens should be collected for culture, Gram stain, and NAAT. Specimens should also be subjected to antigen detection testing, if available. Postmortem samples are also of diagnostic value. A characteristic of anthrax is nonclotting blood at death. As such, blood for culture, stains, and NAAT may be collected by venipuncture. If these are positive, no further specimens are needed. If negative, additional postmortem specimens should be collected. For example, in cases of suspected gastrointestinal anthrax, peritoneal or ascites fluid, spleen, and/or mesenteric lymph node specimens may be informative. Care should be taken to use techniques that avoid spillage of fluids.

Additional information regarding the collection and transport of specimens that may contain *B. anthracis* is described in chapter 14 of this *Manual*, the LRN Sentinel Level Clinical Laboratory Protocols website (http://www.asm.org/index.php/guidelines/sentinel-guidelines), the Canadian Transportation of Dangerous Goods regulations website (http://www.tc.gc.ca/eng/tdg/clear-menu-497.htm), and the *Clinical Microbiology Procedures Handbook* (106). In the United States, shipment of *B. anthracis* requires completion of the APHIS/CDC form 2 (Request to Transfer Select Agents and Toxins; http://www.selectagents.gov/TransferForm.html) plus prior approval from either the CDC or APHIS (see http://www.selectagents.gov/ for additional information about regulation of select agents and toxins in the United States). Nonregistered clinical laboratories, sentinel level, must adhere to current packing and shipping guidelines (LRN Packing and Shipping Guidelines; www.asm.org) for mailing or transferring infectious substances (107). Leakproof containers must be used, then placed in secondary containers for "double-bagging," and sealed in secure, outer containers for carriage.

Specimens from Animals Suspected To Have Anthrax

Anthrax should be considered as the possible cause of death in herbivorous animals that have died suddenly and unexpectedly, especially if hemorrhage from the nose, mouth, or anus has occurred, or if death has taken place at a site with any history of anthrax (e.g., even from several decades before). Specimen collection is influenced by the type of animal involved and the time since death (61, 69). With animals that have died of anthrax, *B. anthracis* is rapidly destroyed by putrefaction in the intact carcass. However, sporulation will still occur in some tissues, so a carcass generally yields positive cultures. For fresh carcasses (1 to 2 days old), appropriate specimens include peripheral blood and tissue biopsy specimens, such as snips from the tip of an ear. For direct demonstration of *B. anthracis* using Gram, Giemsa, or polychrome methylene blue (M'Fadyean) staining, hemorrhagic exudate is preferred to aspirated blood or tissue specimens. In pigs, the enormous terminal bacteremia seen in herbivores may not develop, so blood may not be as informative as other bodily fluids. When cervical edema is present, smears and cultures should be made of fluid aspirated from the enlarged mandibular and suprapharyngeal lymph nodes. Intestinal anthrax of pigs may be obvious only at necropsy, but *B. anthracis* is usually visible in stained smears made from mesenteric lymph nodes. Because *B. anthracis* competes poorly with putrefactive organisms, it may not be visible in smears prepared from older carcasses (2 to 3 days old). Instead, sections of tissue, or any blood-stained material, should be collected for culture. If the animal has been opened, spleen or lymph node specimens should also be taken. With putrefied and very old carcasses, swabs of the nostrils, nasal turbinates, and eye sockets are likely to yield *B. anthracis*, but the best specimens may be samples of contaminated soil taken from beneath the head and tail.

B. anthracis Vaccines and Vaccination

Anthrax Vaccine Adsorbed (AVA, also called BioThrax; see http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ucm061751.htm) is the current human vaccine in the United States. It is a cell-free filtrate (formalin treated) in an aluminum hydroxide-adsorbed gel prepared from a noncapsulated, nonproteolytic derivative of strain V770-NP1-R grown under microaerobic conditions. The FDA has approved a new schedule for preexposure immunization with AVA such that the five-dose primary schedule has been replaced by a three-dose primary schedule (intramuscular injections at 0, 1, and 6 months) with boosters at 12 and 18 months (http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm304758.htm). To maintain immunity, an annual booster injection is recommended. Anthrax Vaccine Precipitated (AVP) is the current human vaccine in the United Kingdom. It is an alum-precipitated cell-free filtrate of the Sterne strain (34F2) cultured under static batch conditions, with activated charcoal, to increase PA production. Both AVA and AVP contain PA as well as trace amounts of LF, EF, and cell wall proteins. In 2010, the updated ACIP recommendations for pre-event vaccination and postexposure prophylaxis were published (104). In addition to previously approved recommendations for occupational and laboratory populations, the updated recommendations included new language to address emergency responders. The ACIP recommends routine preexposure vaccination with AVA for persons engaged in work (i) with high concentrations or pure cultures of *B. anthracis* spores, (ii) with environmental samples associated with anthrax investigations, or (iii) in spore-contaminated areas or other settings with exposure to aerosolized *B. anthracis* spores. Immunization is not routinely recommended for emergency and other responders but may be offered on a voluntary basis as part of a comprehensive occupational health and safety program to persons who perform site investigations, respond to suspicious substance reports (e.g., white powder incidents), or perform other activities that might lead to exposure to aerosolized *B. anthracis* spores. Because workers in the general diagnostic laboratories, using standard BSL-2 practices in the routine processing of clinical or environmental specimens, are not at increased risk for exposure to *B. anthracis* spores, immunization with AVA is not recommended (104). New anthrax vaccines, including second- and third-generation products, as well as several human monoclonal and polyclonal antibody products, are currently in their early development and testing (108).

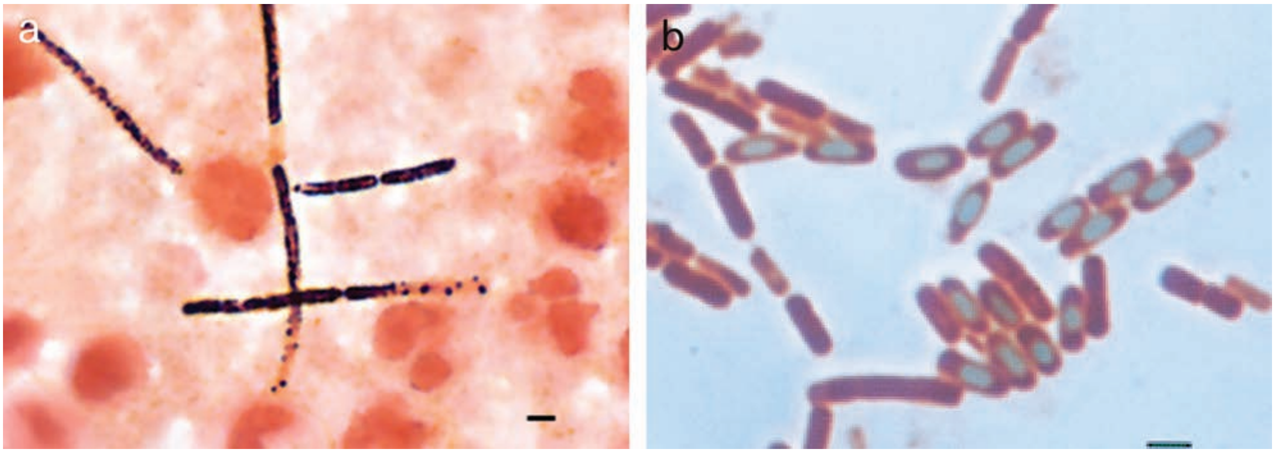

FIGURE 1 (a) Gram stain of *B. anthracis*, associated with a bioterrorism attack, showing Gram-positive rods in peripheral blood buffy coat following admission of patient. Bar, 3 µm. (Courtesy of H. Masur.) (b) Spore-stained preparation of *Bacillus cereus* sporangia, viewed by bright-field microscopy. Spores are stained green, and vegetative cells are counterstained red. Bar, 2 µm. (Photograph kindly provided by M. Rodríguez-Díaz.) doi:10.1128/9781555817381.ch26.f1

DIRECT EXAMINATION

Microscopic examination of Gram-stained smears remains a primary tool of the microbiology laboratory (Fig. 1a). Clinically significant aerobic, Gram-positive bacilli include various species of *Corynebacterium*, *Listeria*, *Lactobacillus*, and *Nocardia*, among others, but *Bacillus* species are readily distinguished by their microscopic and phenotypic morphology. Aerobic endospore-forming organisms do not always stain Gram positive, but the presence of unstained areas within the cell may be indicative of spores. Phase-contrast microscopy (at a magnification of ×1,000) allows spores to be distinguished from other kinds of inclusions, such as polyhydroxybutyrate (PHB) granules. Spores are larger, more phase bright, and more regular in shape, size, and position. Sporangial appearance is also valuable for species-level identification. Although less convenient, a Gram-stained smear can be stripped of oil with acetone-alcohol, washed, and then stained for spores. Based on the Schaeffer-Fulton endospore stain (109), a heat-fixed smear is flooded with 10% aqueous malachite green for up to 45 min (without heating), then washed and counterstained with 0.5% aqueous safranin for 30 s. When visualized at 1,000× magnification, the cells are pink-red and spores, if present, are green (Fig. 1b).

The polychrome methylene blue (M'Fadyean) staining test allows visualization of the capsule, a characteristic feature of *B. anthracis* (Fig. 2a). Direct examination of blood smears can reveal capsulated rods or, if blood was collected after treatment was initiated, capsule "ghosts." Notably, capsule visualization is not recommended in the LRN sentinel level clinical laboratory guidelines, and staining of *B. anthracis* should only be performed by reference level laboratories. For putrefied specimens, Giemsa stain may be more effective than the M'Fadyean test. The genes required for capsule biosynthesis (*capB*, *-C*, *-A*) are located on the pXO2 virulence plasmid (110). Loss of pXO2 gives rise to nonencapsulated *B. anthracis* strains which are frequently mistaken for *B. cereus*. Similarly, species other than *B. anthracis* occasionally acquire genes for capsule biosynthesis, which may confound staining methods as well as NAAT assays that target *capBCA* (see below).

Molecular and antigen-based methods for direct examination of specimens exist, but most have been developed for the detection of *B. cereus* or *B. anthracis*. The symptoms associated with *B. cereus* food poisoning are attributed to bacterial toxins. The enterotoxin complex responsible for the diarrheal type of *B. cereus* food poisoning has been increasingly well characterized (92). Tissue culture-based assays have been developed, and two commercial kits are available for the detection of enterotoxin in foods and feces. The Oxoid BCET-RPLA (Oxoid Ltd., Basingstoke, United Kingdom) and TECRA VIA (TECRA Diagnostics, Roseville, New South Wales, Australia) detect different antigens, and there is some controversy about their reliabilities. Bioassays using human (HEp-2, HepG2) and mouse (Hepa-1) cell lines, boar spermatozoa, and bacterial cultures have been developed for the nonspecific detection of the *B. cereus* emetic toxin in food extracts and culture filtrates (111). Specific detection of cereulide requires high-performance liquid chromatography–mass spectrometry methods (112). Toxin profiling can also be performed using real-time PCR assays that target toxin biosynthesis genes (113).

Because of its pathogenicity and biothreat potential, it is critical for laboratory staff to be able to distinguish *B. anthracis* from morphologically similar bacteria. Whenever large, nonmotile, Gram-positive rods are observed, and especially if large numbers of these bacilli are observed in a patient's blood at death, then *B. anthracis* should be suspected. The LRN provides protocols for ruling out *B. anthracis* and referring out potential isolates. Several methods, including a *B. anthracis*-specific LRN PCR assay (114) and serology (115), were effectively used during investigation of the 2001 bioterrorism-associated outbreak as well as for confirmation of more recent cases associated with drum making (83, 105).

The most widely used and efficacious detection method in the U.S. public health system is the LRN PCR (114). This test targets several distinct loci on the *B. anthracis* chromosome and pXO1 and pXO2 virulence plasmids. The use of multiple loci increases specificity and allows for the detection of avirulent or noncapsulated *B. anthracis* strains (e.g., lacking pXO1 or pXO2), that might otherwise be mistaken for some other organism (e.g., *B. cereus*). At the

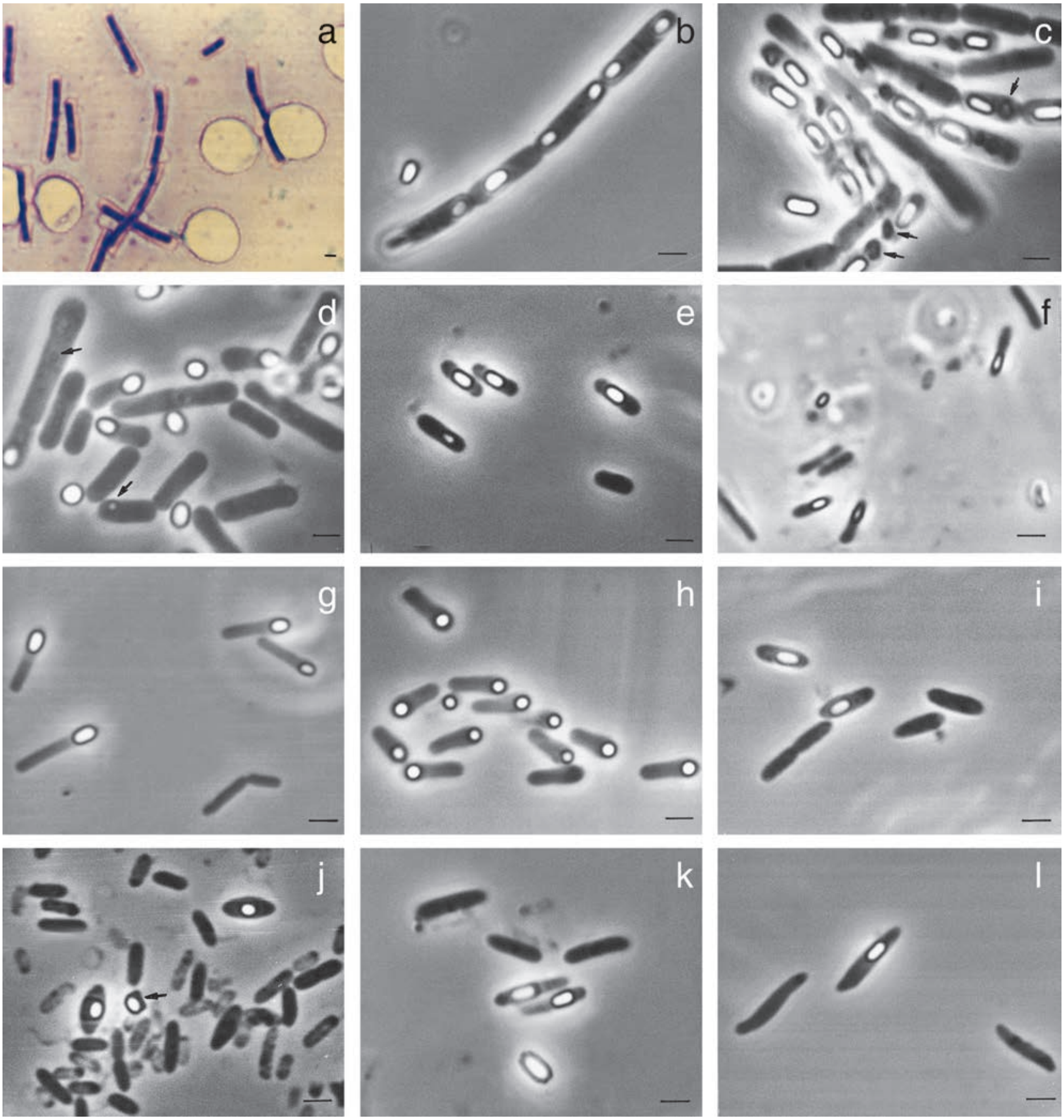

FIGURE 2 Photomicrographs of endospore-forming bacteria viewed by bright-field microscopy (a) and phase-contrast microscopy (b to l). Bars, 2 μm. (a) *B. anthracis*, M'Fadyean stain showing capsulate rods in guinea pig blood smear; (b) *B. cereus*, broad cells with ellipsoidal, subterminal spores, not swelling the sporangia; (c) *B. thuringiensis*, broad cells with ellipsoidal, subterminal spores, not swelling the sporangia, and showing parasporal crystals of insecticidal toxin (arrows); (d) *B. megaterium*, broad cells with ellipsoidal and spherical, subterminal and terminal spores, not swelling the sporangia, and showing PHB inclusions (arrows); (e) *B. subtilis*, ellipsoidal, central and subterminal spores, not swelling the sporangia; (f) *B. pumilus*, slender cells with cylindrical, subterminal spores, not swelling the sporangia; (g) *B. circulans*, ellipsoidal, subterminal spores, swelling the sporangia; (h) *Lysinibacillus sphaericus*, spherical, terminal spores, swelling the sporangia; (i) *Brevibacillus brevis*, ellipsoidal, subterminal spores, one swelling its sporangium slightly; (j) *Brevibacillus laterosporus*, ellipsoidal, central spores with thickened rims on one side (arrow), swelling the sporangia; (k) *Paenibacillus polymyxa*, ellipsoidal, paracentral to subterminal spores, swelling the sporangia slightly; (l) *Paenibacillus alvei*, cells with tapered ends, ellipsoidal, paracentral to subterminal spores, not swelling the sporangium. doi:10.1128/9781555817381.ch26.f2

CDC, a positive PCR result on any clinical specimen from a patient collected from a normally sterile site (such as blood or CSF) or a lesion of other affected tissue (e.g., skin, pulmonary, reticuloendothelial, or gastrointestinal) is regarded as a supportive or presumptive diagnostic test. It is considered sufficient to provide a probable diagnosis but is not confirmatory in itself. The principal reason for such stringent guidelines on the use of NAAT approaches and the value of their results towards providing a confirmatory diagnosis is based on the possibility that environmental contamination of a non-anthrax-related lesion could result in a positive result. This is especially the case with the use of some previously published PCR primers for capsule and chromosomal genes that can produce false positives with reactions to soil microbiota. This is quite similar to the recommendations that are included in the 2008 WHO guidelines (69), in which PCR can be used for identification of an isolate but is not recommended for testing of specimens.

A two-component direct fluorescent-antibody assay was used to identify encapsulated vegetative cells of *B. anthracis* (116), but NAAT-based detection methods are now preferred.

An antibody specific for one of the *B. anthracis* S-layer proteins is the basis of RedLine Alert (Tetracore, Inc., Gaithersburg, MD), a rapid test that can provide a result within 15 min. This assay has been approved by the FDA for use on nonhemolytic *Bacillus* species colonies cultured on sheep blood agar plates. Manufacturer's data suggest that the test was 98.6% sensitive when tested on 145 *B. anthracis* isolates and 45 nonhemolytic, non-*B. anthracis* isolates. However, such identification of *B. anthracis* is only considered presumptive, and this test should not be used as a stand-alone test.

The *Bacillus anthracis* immunochromatographic field assay, developed by the U.S. Naval Medical Research Center (Silver Spring, MD), is an immunochromatographic assay for detection of PA in blood samples and tissue exudates. The highly sensitive and specific assay has been used to detect *B. anthracis* in animals, even several days after death (117).

Numerous reports describe the use of alternative technologies, such as flow cytometry, differential pulse voltammetry, fluorescence resonance energy transfer, surface-enhanced Raman scattering, locked nucleic acid probes, and atomic force microscopy for the detection of *B. anthracis* (106, 118, 119). Because anthrax toxins are highly expressed during infection, methods for direct detection of PA and LF in specimens (i.e., instead of bacilli) have also been explored. These include immunoassays that target PA (120) as well as bioassays that exploit the metalloprotease activity of LF and the adenylyl cyclase activity of EF (121). Direct detection of spores in environmental samples has been achieved using diagnostic electron microscopy (122), whereas a technique developed by the Norwegian Defence Research Establishment uses matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) for examination of suspicious powders (123). The MALDI-TOF method can accurately distinguish spores from common hoax materials and differentiate *B. anthracis* from other *Bacillus* species. Inactivation of BSL-3 organisms, an essential step prior to performing MALDI-TOF (MS) in a BSL-2 space, has been successful for *B. anthracis* using trifluoroacetic acid (124) and gamma irradiation (125). A genetically engineered *B. anthracis*-specific bioluminescent reporter phage was capable of detecting *B. anthracis*, in clinically relevant concentrations, from simulated blood specimens in as little as 5 h (126) and could also be used for rapid susceptibility testing. These, and other novel approaches, may allow for detection of *B. anthracis* infection earlier in the course of the disease and thus allow for more successful treatment.

ISOLATION PROCEDURES

Isolation of Aerobic Endospore-Forming Bacteria

Specimens from patients should be inoculated onto plates of blood agar according to standard methods. Though not commonly used in clinical laboratories, heat treatment (e.g., 70°C for 30 min or 80°C for 10 min) may be used as an enrichment procedure for the isolation of aerobic endospore-forming bacteria. The heat-resistant spores survive temperatures that kill other organisms (e.g., non-spore-forming contaminants), and spores may also be heat shocked into subsequent germination. In general, enrichment procedures are not appropriate for fresh clinical specimens because spores are usually sparse or absent, and heat treatment will kill any vegetative cells. However, for episodes of food poisoning where *B. cereus* is suspected, nutrient or tryptic soy broth with polymyxin (100,000 U/liter) may be added to a heat-treated stool specimen.

For the majority of aerobic endospore-forming species, selective media are not available. However, such media have been designed for the isolation, identification, and enumeration of *B. cereus* group organisms. These exploit the phenotypic features of the organism, including the positive egg yolk reaction (i.e., phospholipase C activity) and the negative acid-from-mannitol reaction. Addition of pyruvate and polymyxin can enhance the selectivity of these media. Effective formulations include BCM (*Bacillus cereus* medium; LabM, Heywood, United Kingdom), PEMBA (polymyxin B, egg yolk, mannitol, bromthymol blue agar; Oxoid), and MEYP (mannitol, egg yolk, polymyxin B agar; Oxoid), which is also called MYP (Difco, BD, Franklin Lakes, NJ). Newer formulations, including *Bacillus cereus* group plating medium (Biosynth Chemistry and Biology, Staad, Switzerland, also available as Cereus-Ident-Agar from Heipha, Eppelheim, Germany) and *Bacillus cereus*/*Bacillus thuringiensis* chromogenic plating medium (R&F Laboratories, Downers Grove, IL), use specific chromogenic substrates, rather than natural egg yolk, to reveal phospholipase C activity. Selective media for *B. anthracis* include PLET agar (polymyxin-lysozyme EDTA-thallous acetate) and the differential/selective chromogenic medium, R&F *Anthracis* chromogenic agar (R&F Laboratories). Independent studies indicate that both formulations are effective for isolation of *B. anthracis*, but PLET agar may be more sensitive and selective.

Isolation of *B. anthracis*

Provided that standard good laboratory practice is observed, isolation and procedures to rule out *B. anthracis* can be performed safely in the routine clinical microbiology laboratory (e.g., by sentinel level laboratories). In the case of a suspect identification of *B. anthracis*, the isolate must be referred to an LRN reference center equipped for confirmatory identification of *B. anthracis*. Due to the infectious nature of this pathogen, all manipulations of specimens should be handled in a BSL-2 facility using BSL-3 precautions (see chapter 14 of this *Manual* and *Biosafety in Microbiological and Biomedical Laboratories*) (101). BSL-3 facilities are recommended for laboratory personnel doing routine processing of clinical or environmental specimens, but preexposure vaccination is not (104). When working with pure cultures of *B. anthracis*, the primary hazards to laboratory personnel are direct and indirect contact of broken skin with cultures and contaminated laboratory surfaces, accidental parenteral inoculation, and, rarely, exposure to infectious clinical specimens. Laboratories that frequently centrifuge *B. anthracis* suspensions should use an aerosol-tight rotor that can be repeatedly autoclaved (101). In the United States, occupational exposure or release of the organism outside the primary barriers of the biocontainment area (e.g., on the open bench) requires the completion of APHIS/CDC form 3 ("Information Document for Report of Theft, Loss, or Release of Select Agents and Toxins"; http://www.selectagents.gov/TLRForm.html).

Possession of *B. anthracis* requires registration of a laboratory within the United States with either the CDC or APHIS. When *B. anthracis* is identified by a laboratory, the identification of this agent must be reported to the CDC or APHIS immediately and an APHIS/CDC form 4 ("Report of the Identification of a Select Agent or Toxin"; http://www.selectagents.gov/CDForm.html) submitted within 7 days. Other authorities should be notified as required by federal, state, or local laws. When *B. anthracis* is isolated in an unregistered laboratory (e.g., diagnostic sentinel level clinical laboratory), the organism must either be destroyed on-site by a recognized sterilization or inactivation process or be transferred to a registered laboratory within 7 days following notification of the isolate having been confirmed as *B. anthracis*. In Canada, all materials containing or suspected to contain anthrax must be referred to a CLRN laboratory for further characterization in order to comply with the Human Pathogens and Toxin Act (http://www.phac-aspc.gc.ca/lab-bio/regul/hpta-lapht-eng.php). Similar protocols exist in other countries.

Tests for the presence of *B. anthracis* may be requested for diverse specimens, such as animal products (e.g., wool, hides, hair, and bone meal) from regions of endemicity, soil or other materials from old burial sites or tannery or laboratory sites due for redevelopment, or other environmental materials associated with outbreaks (e.g., sewage sludge). Detection in such specimens may mean searching for rather few spores of *B. anthracis* among those of many other species, especially other members of the *B. cereus* group. Some environmental specimens may contain substances that inhibit germination and growth of *B. anthracis* (69). At present, there is no enrichment method specific for *B. anthracis*, and culture by the selective agar techniques described above is the best approach.

IDENTIFICATION

Species level identification of an aerobic spore-forming organism usually requires examination of microscopic features, colonial morphology, biochemical tests, and increasingly, analysis of nucleic acid or protein profiles. As a first step, it is important to establish that the isolate really is an aerobic endospore-forming organism. *Bacillus* species are typically described as Gram-positive rods, but some are Gram variable, and Gram positivity is readily lost in older cultures. Some species or strains simply appear Gram negative. *Paenibacillus* and *Brevibacillus* are more likely to stain Gram variable or Gram negative. Phase-contrast microscopy and spore staining can be used to distinguish PHB granules and other storage inclusions from true spores. For some species, the storage granules can actually assist with identification. When grown on carbohydrate-rich media, such as glucose nutrient agar, the large cells of *B. megaterium* may accumulate PHB (Fig. 2d) and appear vacuolated or foamy. However, the microscopic morphology of the sporangia are much more helpful for distinguishing between species (Fig. 2). Spore shapes vary from cylindrical through ellipsoidal to spherical. Occasionally, bean- or kidney-shaped, curved-cylindrical, and pear-shaped spores are also seen. Spores may be positioned terminally, subterminally, or centrally; the sporangia may appear swollen or distended. Despite within-species and within-strain variation, sporangial morphologies can be sufficiently characteristic to allow an experienced worker to make a tentative identification. For example, *B. laterosporus* produces very distinctive ellipsoidal spores that have thickened rims on one side, such that they appear to be laterally displaced in the sporangia (Fig. 2j). Recognition of *B. thuringiensis* is largely dependent on observation of its cuboid or diamond-shaped parasporal crystals in sporulated cultures (Fig. 2c).

In general, morphological features of the vegetative cell tend to be less informative, but they can still assist in species identification. Vegetative cells vary in width from about 0.5 to 1.5 µm and in length from 1.5 to 8 µm. They may occur singly or in chains. Usually, cells are round-ended, but some are square or, occasionally, tapered (e.g., *P. alvei*) (Fig. 2l). Many species are motile.

Growth characteristics are often diagnostic. *B. subtilis* and *B. licheniformis* exhibit similar colonial and microscopic morphologies but different growth characteristics, being strictly aerobic and facultatively anaerobic, respectively. *B. megaterium* and *B. cereus* are both large-celled species, but the former is strictly aerobic while the latter is facultatively anaerobic.

Species of aerobic endospore-forming bacteria show a very wide range of colonial morphologies, both within and between species (Fig. 3). After 24 to 48 h of growth, sizes range from 1 to 5 mm. Color commonly ranges from buff or creamy gray to off-white, but some strains may produce orange pigment. Morphology can vary from moist and glossy, through granular, to wrinkled. Shapes can vary from round to irregular, sometimes spreading, with entire, through undulate or crenate, to fimbriate edges. Elevations range from effuse, through raised, to convex. Consistency is usually butyrous, but mucoid and dry, adherent colonies are not uncommon. On blood agar, hemolysis may be absent, slight, marked, partial, or complete. Despite this diversity, *Bacillus* colonies are not generally difficult to recognize. *B. subtilis* and *B. licheniformis* produce similar colonies which are exceptionally variable and often appear to be mixed cultures (Fig. 3j). These colonies are of moderate (2 to 4 mm) diameter, irregular in shape, and range from moist and butyrous or mucoid, with margins varying from undulate to fimbriate through membranous with an underlying mucoid matrix, with or without mucoid beading at the surface, to rough and crusty as they dry. The "licheniform" colonies of *B. licheniformis* tend to be quite adherent. *B. circulans* is another heterogeneous species, and in about 13% of strains, rotating and migrating microcolonies, which may show spreading growth, have been observed (Fig. 3k). However, closer examination of the spreading strains has revealed some of them to be genotypically distinct, such that they are now classified as species of *Paenibacillus*.

Despite their variability, colonies of *B. cereus* are readily recognized. They are characteristically large (2 to 7 mm in diameter) and vary in shape from circular to irregular, with entire to undulate, crenate, or fimbriate edges (Fig. 3b). They usually have matte or granular textures, but smooth and moist colonies are not uncommon. When grown on egg yolk agar, strains of the *B. cereus* group, including albeit

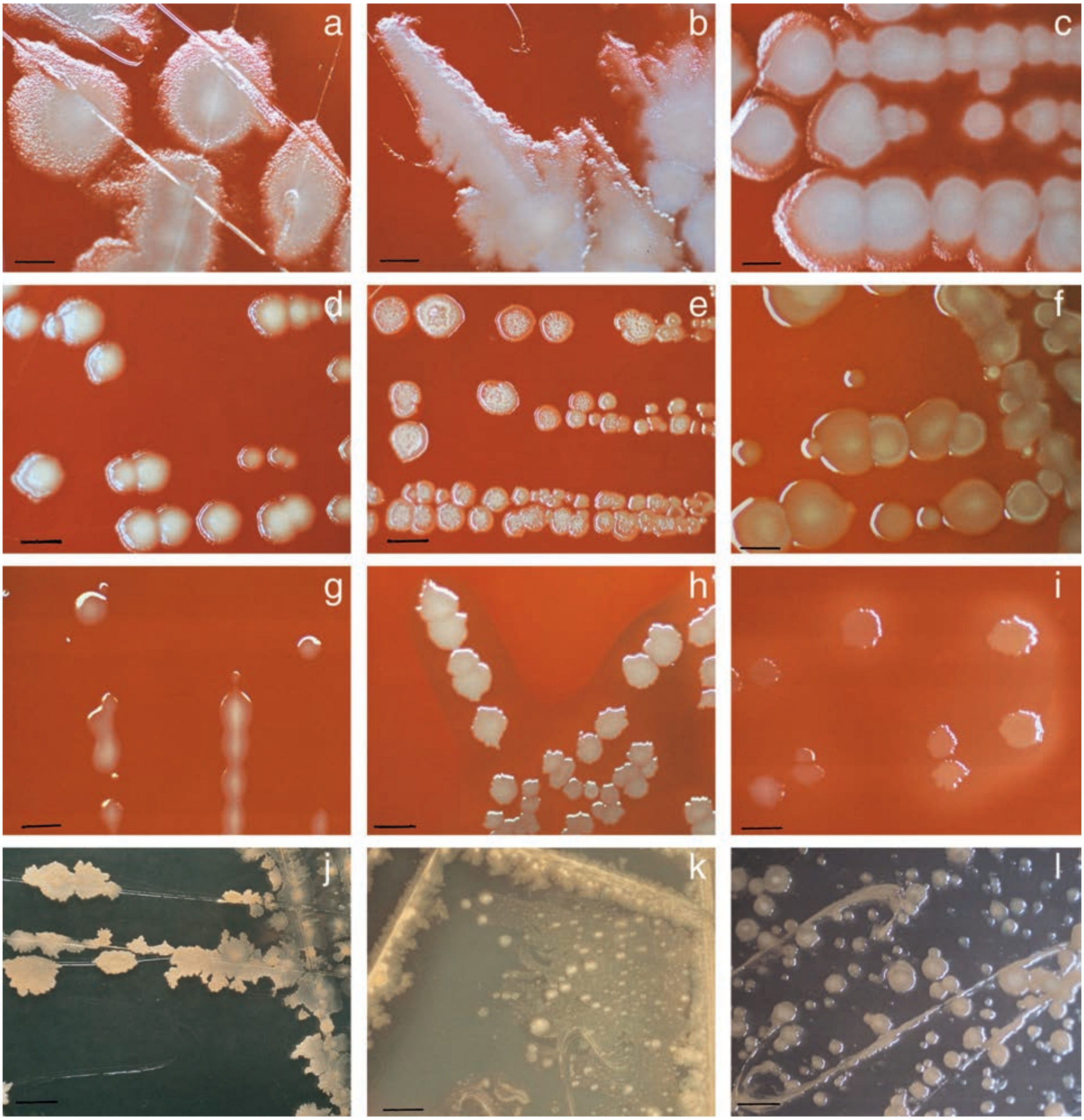

FIGURE 3 Colonies of endospore-forming bacteria on blood agar (a to i) and nutrient agar (j to l) after 24 to 36 h at 37°C. Bars, 2 mm. (a) *B. anthracis*; (b) *B. cereus*; (c) *B. thuringiensis*; (d) *B. megaterium*; (e) *B. pumilus*; (f) *Lysinibacillus sphaericus*; (g) *Brevibacillus brevis*; (h) *Brevibacillus laterosporus*; (i) *Paenibacillus polymyxa*; (j) *B. subtilis*; (k) *B. circulans*; (l) *Paenibacillus alvei*. doi:10.1128/9781555817381.ch26.f3

to a lesser extent, *B. anthracis*, synthesize lecithinases, which produce opaque zones of precipitation around colonies (i.e., usually after overnight or perhaps 24 h of incubation). The optimum growth temperature is about 37°C, with minima and maxima of 15 to 20°C and 40 to 45°C, respectively. However, routine, reliable differentiation of individual species within the *B. cereus* group can be challenging. Features considered characteristics of individual species, including the anthrax toxin and capsule of *B. anthracis*, and the parasporal crystals of *B. thuringiensis*, are encoded by plasmids. The acquisition or loss of a plasmid may alter the features of a particular strain and can undermine identification. Colonies of *B. anthracis* and *B. cereus* can be similar in appearance, although those of the former are generally smaller and nonhemolytic and may show more spiking or tailing along the lines of inoculation streaks. Compared with the usually butyrous consistency of *B. cereus* or *B. thuringiensis*, colonies of *B. anthracis* are very tenacious and may be pulled into standing peaks with a loop. *B. anthracis* colonies are also susceptible to diagnostic gamma phage.

B. mycoides produces characteristic rhizoid or hairy-looking, adherent colonies which readily cover the whole agar surface. *B. anthracis* capsule production is stimulated by plating on nutrient agar containing 0.7% sodium bicarbonate followed by overnight incubation under 5 to 7% CO_2 (candle jars perform well). Colonies of the capsulated organism appear mucoid, and the capsule can be visualized by M'Fadyean or India ink staining of smears.

Phenotypic tests are widely used for species-level identification. For diagnostic purposes, the aerobic endospore formers comprise two groups: the reactive ones, which give positive results in various routine biochemical tests and which are therefore easier to identify (Table 1), and the nonreactive ones, which give few, if any, positive results in such tests. Nonreactive isolates tend to dominate the identification requests sent to reference laboratories. In addition to the traditional phenotypic testing methods, convenient miniaturized formats such as API (bioMérieux SA, Marcy-l'Etoile, France) and automated identification systems such as VITEK (bioMérieux, Inc., Durham, NC) and Biolog (Hayward, CA) also exist. With such systems, it is important to keep in mind that, if a species is not included in the database, accurate identification is not possible. These systems also have limited value in distinguishing between some members of the *B. cereus* group (127), with the exception of the presumptive identification of *B. anthracis* (128). Both the API 50 CH and API 20 E strips together or the API 50 CH alone, along with API 50 CHB/E medium, can be used for the identification of 38 taxa of *Bacillus* species and related genera and requires up to 48 h of incubation. A large number of *Bacillus* and related genera are listed in the Biolog database using the GEN III (63 taxa) or GP2 (53 taxa) microplates. Set-up is minimal, and bacteria can be identified in as little as 2 h. BioMérieux has designed the BCL card for the automated identification of aerobic endospore-forming bacteria of the *Bacillaceae* family using the VITEK 2 system. A total of 42 species can be identified, and final results are obtained after 14 h (129). The BD Phoenix automated microbiology system (BD Diagnostics, Sparks, MD) lists 12 *Bacillus* species and related taxa in its Gram-positive panel identification database. The commercially available databases are expanding, and continue to

TABLE 1 Characters for differentiating some species of *Bacillus*, *Geobacillus*, and *Paenibacillus*[a]

	Bacillus														*Geobacillus*		*Paenibacillus*			
	B. subtilis group				*B. cereus* group						*B. circulans* group									
Character[b]	*B. subtilis*	*B. amyloliquefaciens*	*B. licheniformis*	*B. pumilus*	*B. cereus*[c]	*B. anthracis*	*B. thuringiensis*	*B. mycoides*	*B. cytotoxicus*	*B. megaterium*	*B. circulans*	*B. firmus*	*B. lentus*	*B. coagulans*	*G. stearothermophilus*	*G. thermodenitrificans*	*P. polymyxa*	*P. alvei*	*P. macerans*	*P. validus*
Rod mean diam (μm)	0.8	0.8	0.8	0.7	1.2	1.2	1.2	1.2	≥1.0	1.4	0.8	0.8	0.8	0.8	0.9	0.8	0.9	0.8	0.7	0.8
Chains of cells	(−)	(+)	(+)	−	+	+	+	+	(+)	+	−	(+)	(+)	v	v	v	−	(−)	−	−
Motility	+	+	+	+	+	−	+	−	−	+	+	+	+	+	+	+	+	+	+	+
Sporangia[d]																				
Spore shape	E	E	E (C)	C, E	E (C) [E]	E	E (C)	E	E	E, S	E	E (C)	E	E (S)	E	E	E	E	E	E
Spore position	S, C	S, C	S, C	S, C	S, C	S	S	S (C)	S, C	S, C	S, T	S, C	S, C	S, C, T	S, T	S, T	S (C)	S, C	S (T)	S, T
Sporangium swollen	−	−	−	−	−	−	−	−	−	−	+	v	v	+	v	−	+	+	+	+
Parasporal crystals	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Anaerobic growth	−	−	+	−	+	+	+	+	+/w	−	+	+	−	+	−/w	+	+	+	+	−
Growth at:																				
50°C	v	v	+	v	−	−	−	−	+	v	+	v	−	+	+	+	−	−	v	+
65°C	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
Egg yolk reaction	−	−	−	−	+	+	+	+	+/w	−	−	−	−	−	−	−	−	−	−	−
Casein hydrolysis	+	+	+	+	+	+	+	+		+	w	w	−	−	+/w	−	+	+	−	−
Starch hydrolysis	+	+	+	−	+	+	+	+	−	+	+	+	+	+	+	+/w	+	+	+	+
Arginine dihydrolase	−	−	(+)	−	v [(−)]	−	+	v	+	−	−	−	−	v	−	−	−	−	−	−
Indole production	−	−	−	−	−	−	−	−		−	−	−	−	−	−	−	−	+	−	−
Gelatin hydrolysis	+	+	+	+	+	+	+	+	+	+	v	+	−	v	+	v	+	+	v	−
Nitrate reduction	+	+	+	−	(+) [+]	+	+	(+)	+	v	v	+	+	v	v	v	+	−	v	v
Gas from carbohydrates	−	−	−	−	−	−	−	−		−	−	−	−	−	−	−	+	−	+	−
Voges-Proskauer	+	+	+	+	+	+	+	v	+/w	−	−	−	−	v	−	−	+	+	−	v
Acid from:																				
D-Arabinose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Glycerol	+	+	+	+	+ [v]	−	+	+	−	+	+	v	−	+	w	−	+	+	+	+
Glycogen	+	+	+	−	+ [−]	+	+	+	−	+	+	v	−	−	+	−	+	v	+	v
Inulin	(+)	−	v	−	−	−	−	−	−	+	+	−	−	−	v	−	+	−	+	v
Mannitol	+	+	+	+	−	−	−	−	−	+	+	+	+/w	−	−	w	+	−	+	+
Salicin	+	+	+	+	+ [−]	−	(+)	(+)	+	+	+	−	+/w	v	−	v	+	v	+	−
D-Trehalose	+	+	+	+	+	+	+	+	−	+	+	v	+	+	+	+	+	v	+	+

[a] Symbols and abbreviations: +, ≥85% positive; +/w, positive or weakly positive; w, weakly positive; (+), 75 to 84% positive; v, variable (26 to 74% positive); (−), 16 to 25% positive; −, 0 to 15% positive; −/w, negative or weakly positive.

[b] Arginine dihydrolase, indole production, gelatin hydrolysis, and nitrate reduction reactions were determined using tests in the API 20E strip (bioMérieux). Acid from carbohydrate reactions was determined using the API 50CHB System (bioMérieux).

[c] Results shown in brackets are for the biotype isolated particularly in connection with outbreaks of emetic-type food poisoning and for strains of serovars 1, 3, 5, and 8, which are commonly associated with such outbreaks.

[d] Spore shape: C, cylindrical; E, ellipsoidal; S, spherical. Spore position: C, central or paracentral; S, subterminal; T, terminal. The most common shapes and positions are listed first, and those shown in parentheses are infrequently observed.

improve, but keeping up with the changing taxonomy and deluge of new aerobic, endospore-forming species is a significant challenge. To be effective in identifying a particular species, a diagnostic database must account for intraspecies variability. Creation of a robust profile requires multiple authentic strains from a range of sources, yet many new species are proposed on the basis of single isolates. As such, commercial kits should always be used in conjunction with other methods.

Nucleic acid-based methods, especially 16S rRNA gene sequencing, may effectively identify strains that cannot be definitively characterized by morphological or biochemical methods. Guidelines for sequencing-based identification of bacteria are outlined in Clinical Laboratory Standards Institute (CLSI) document MM-18A (130). Some species, notably *B. cereus*, *B. thuringiensis*, and *B. anthracis*, are not effectively differentiated by 16S rRNA gene sequencing. *B. anthracis*-specific single-nucleotide polymorphisms have been identified using the hypervariable 16S-23S internal transcribed spacer region (131), but sequencing of this target for the definitive identification of *B. anthracis* is not recommended. In the past, there were concerns about the accuracy and availability of reference sequences. However, these have largely been resolved and, for type and reference strains of most species, high quality 16S rRNA gene sequences can be found in public databases (e.g. GenBank).

In recent years, there has been growing interest in the use of MALDI-TOF (MS) platforms for bacterial identification. Three commercial systems, the VITEK-MS (bioMérieux), the MALDI Biotyper (Bruker Corp., Billerica, MA), and the Andromas (Andromas SAS, Paris, France), are currently available. Systems are rapid, easy to use, and facilitate identification of isolates cultured on solid media. Initial instrument cost is high; however, reagent costs are minimal. Methods compatible with liquid media (e.g., blood culture bottles) have been described. As with the phenotypic methods, the key limitation to accurate MS-based identification is the availability of robust databases for interpretation of the MALDI-TOF profiles. Results at the genus level only or misidentifications are still common for this group of organisms (132). These databases may not include recent or uncommon species, and profiles for closely related species (e.g., *B. subtilis* group or *B. cereus* sensu lato) may be too similar to differentiate (133). However, analytical methods continue to improve, and effective differentiation of *B. subtilis* from *B. amyloliquefaciens* and *B. cereus* (sensu stricto) from *B. thuringiensis* has been reported (134). Supplementing commercial databases with locally produced profiles using in-house strains may help improve identification (125).

Other approaches to identification include chemotaxonomic fingerprinting by fatty acid methyl ester profiling, pyrolysis MS, and Fourier transform infrared spectroscopy (135). All of these approaches have been successfully applied either across genera or to small groups, but as with other profiling methods, large databases of authentic strains are necessary for accurate identification. Some databases, such as the Microbial Identification System software (Microbial ID, Inc., Newark, DE) for fatty acid methyl ester analysis, are commercially available.

TYPING

Typing schemes have been developed only for a few species of aerobic endospore-forming bacteria, most notably the *B. cereus* group (including *B. anthracis*). In the past, this group was differentiated into serovars based on flagellar antigen variations, but serotyping is not commonly used today. Similarly, protein-based multilocus enzyme electrophoresis has been replaced by nucleic acid-based genotyping methods. Many PCR- and DNA sequencing-based typing methods have been described, but only a few have gained widespread acceptance, including multiple-locus variable-number tandem repeat analysis (MLVA), multilocus sequence typing (MLST), and increasingly, whole-genome sequencing.

MLVA is primarily used for typing of *B. anthracis*. It is a PCR-based fragment analysis method that targets copy number variations among DNA elements located on the *B. anthracis* chromosome and virulence plasmids. The differences can be resolved by agarose electrophoresis, capillary electrophoresis, or MS (136–139). The MLVA-8 scheme targets eight loci: six chromosomal plus one on each of pXO1 and pXO2 (139). It was the first typing method that could reliably differentiate *B. anthracis* strains, and during the 2001 biothreat event, MLVA-8 implicated the Ames strains as the cause of the outbreak (114). The resolution of MLVA increases with the number of targets, and schemes that use 15, 25, and 31 loci have been developed (81, 137). MLVAbank (mlva.u-psud.fr/mlvav4/genotyping/index.php) is an online repository of MLVA profiles and a valuable resource for strain comparison.

MLST is a DNA sequencing-based method that exploits nucleotide polymorphisms found in sets of “housekeeping” genes. Because DNA sequences are unequivocal and portable, many view MLST as the gold standard method for *B. cereus* genotyping. Several MLST schemes have been described, and each targets a different set of six to seven genes. Online databases are available for several of these, including the Priest (pubmlst.org/bcereus) and Tourasse-Helgason (mlstoslo.uio.no) schemes (140, 141), the latter of which provides data on any of five published MLTS schemes for *B. cereus*. The HyperCat database (also at mlstoslo.uio.no) includes typing data for >2,200 strains and allows comparison of MLST, multilocus enzyme electrophoresis, and amplified fragment length polymorphism data (142). Despite some differences among gene targets, the MLST methods provide a consistent view of the *B. cereus* group. From the genotypic perspective, *B. cereus* sensu lato is heterogeneous, and correlation between the phylogenetic clusters and traditional, phenotypic divisions is poor. Some analyses indicate that *B. pseudomycoides*, *B. cytotoxicus* (143), and *B. toyonensis* do comprise distinct clades, *B. anthracis* is recognized as a pathogenic clone, but *B. cereus* and *B. thuringiensis* strains are largely indistinguishable. Although reports have suggested that the *B. cereus* strains associated with emetic food poisoning exhibit distinct characteristics, the emetic toxin can actually be produced by multiple discrete phylogenetic lineages (144–146).

Whole-genome sequencing has become an increasingly affordable and popular tool for strain typing and molecular epidemiology. Whereas MLST may involve sequencing a few thousand nucleotides, the average genomic dataset includes millions of nucleotides. This provides extraordinary resolution and can reveal single nucleotide polymorphisms among strains that, by other typing methods, appear to be identical (147). This approach has been used to examine the population structure of *B. cereus* sensu lato (148) and the evolution of *B. anthracis* (149–151). Increasingly, genomic approaches are also being used to examine phylogenetic relationships among other aerobic endospore-forming bacteria (152).

SEROLOGIC TESTS

Serologic tests for anthrax have been developed, but such assays are not available for infections due to other aerobic endospore-forming bacteria. In outbreak situations, serologic tests for anthrax are of limited utility because seroconversion takes time, whereas effective treatment and public health response require rapid diagnostics. Moreover, in human anthrax, early treatment sometimes prevents development of a detectable rise in antibody titer (115). However, serologic assays are valuable for retrospective surveillance and epidemiological investigations and can also be used to monitor vaccination effectiveness. After the 2001 bioterror event, serologic assays aided in the effort to confirm cases (153).

The Ascoli test, which dates from 1911, is a precipitin test that uses hyperimmune serum raised to *B. anthracis* whole-cell antigen to provide rapid, retrospective evidence of infection. Despite its age, the Ascoli test is still used by veterinarians in Eastern Europe and central Asia (154). Anthraxin, a heat-stable extract from a noncapsulated strain of *B. anthracis*, is the basis for a skin test that has been licensed for human and animal use since 1962. It is widely acclaimed and remains in use in countries of the former USSR (155). If a delayed-type hypersensitivity reaction develops after injection of anthraxin, it is interpreted as indicating cell-mediated immunity to *B. anthracis*. The test can be used to monitor vaccine-induced immune status after periods of several years or to diagnose anthrax retrospectively. Anthraxin does not contain highly specific antigens and relies on the fact that the only *Bacillus* species likely to proliferate within and throughout an animal is *B. anthracis*.

The three protein components of the anthrax toxin (PA, LF, and EF), and antibodies to them, can be used in enzyme immunoassay systems. For routine confirmation of anthrax infection or for monitoring response to anthrax vaccines, antibodies against PA alone appear to be satisfactory and have proved useful for epidemiological investigations with humans and animals. PA, LF, and EF are available commercially (List Biological Laboratories, Inc., Campbell, CA; http://www.listlabs.com). In cases where culture has failed, serologic assays for the detection of antibody response against PA have been used, in combination with NAAT or immunohistochemistry test results, to confirm the diagnosis of anthrax. During the 2001 outbreak, a quantitative human anti-PA immunoglobulin G (IgG) enzyme-linked immunosorbent assay, performed at the CDC, was positive only with sera from individuals with anthrax or vaccinated with AVA vaccine (153). A qualitative kit (QuickELISA Anthrax-PA kit) for the detection of anti-PA IgG and IgM antibodies in human serum has been developed by Immunetics (Boston, MA). It is FDA cleared and CE marked in Europe.

ANTIMICROBIAL SUSCEPTIBILITIES

The Clinical Laboratory Standards Institute (CLSI) has published approved guidelines describing susceptibility testing methods and suggested agents for primary testing in the case of *B. anthracis*, as well as interpretive criteria for other *Bacillus* spp. (156). Comparable information is also included in chapter 74 of this *Manual*. The recommended procedure is broth microdilution. Agar dilution and Etest may also be performed (157–160). LRN sentinel level laboratories do not and should not perform antimicrobial susceptibility testing of *B. anthracis*. If *B. anthracis* cannot be ruled out, the sentinel level laboratory submits the isolate to its designated LRN reference laboratory for confirmatory identification; the latter laboratory will forward the isolate to the federal level (CDC) for antimicrobial susceptibility testing, sequencing, and archiving. The same is true for sentinel level food and veterinary laboratories.

Bacillus anthracis

Most strains of *B. anthracis* are susceptible to penicillin, although exceptions have been reported (157–159). However, the presence of inducible β-lactamases in some isolates precludes the use of penicillin as a single agent in the treatment of systemic anthrax (161). Strains are also susceptible to ciprofloxacin, clindamycin, chloramphenicol, doxycycline, levofloxacin, gentamicin, tetracycline, tobramycin, and vancomycin (157–160, 162, 163). In contrast, the majority of *B. anthracis* isolates exhibit reduced susceptibility (or "nonsusceptibility") to some extended- and broad-spectrum cephalosporins, specifically cefuroxime (157), ceftriaxone (158), and cefotaxime (159), rendering these a poor choice for treatment (158), and in vitro results, even if susceptible, may not predict clinical efficacy. Resistance or reduced susceptibility has also been shown for trimethoprim-sulfamethoxazole (TMP-SMX), erythromycin, and azithromycin (158–160). In general, susceptibility testing of *B. anthracis* is often not required, nor are interpretive breakpoints established (other than susceptible) by the CLSI, with the exception of penicillin (156, 164). Agents for primary testing may include penicillin, doxycycline, tetracycline, and ciprofloxacin and must be performed in a reference laboratory with BSL-3 capacity.

Postexposure prophylaxis can prevent inhalational anthrax following exposure to aerosols containing *B. anthracis* spores. The recommended regimen is 60 days of oral antimicrobial therapy and three doses of AVA (165). When selecting antimicrobial agents, consideration for the possibility of antimicrobial resistance should be given. For adult and pediatric patients, the recommended first-line antimicrobial agent is ciprofloxacin or doxycycline (166). Levofloxacin is recommended as a second-line antimicrobial for adults (165), although there is limited safety data regarding use beyond 28 days. For pregnant women, ciprofloxacin is recommended over doxycycline, though doxycycline may be used if ciprofloxacin is unavailable (167). Amoxicillin may be used if the isolate at issue is susceptible to penicillin. Amoxicillin is also recommended in the treatment of children and lactating women and when other antimicrobial agents are not considered safe. Clindamycin, chloramphenicol, rifampin, vancomycin, and other fluoroquinolones are also suitable for the treatment of patients unable to tolerate recommended antibiotics (165).

For severe cases of anthrax (e.g., fulminant bacteremia, inhalational, gastrointestinal, or injectional anthrax), there is often meningeal involvement and the recommended treatment is intravenous ciprofloxacin for 7 to 10 days plus one or two additional drugs (65, 165). In the presence of inflammation, central nervous system penetration of ciprofloxacin is much higher than that of doxycycline. For cases of uncomplicated, naturally acquired, cutaneous anthrax, a 7- to 10-day course of oral ciprofloxacin or doxycycline is recommended, but penicillin V or amoxicillin may also be used if susceptibility is confirmed. Bioterrorism-related cutaneous anthrax should be subject to treatment as described for postexposure prophylaxis due to the risk of aerosol exposure.

Bacillus cereus Group (Not *B. anthracis*)

There have been rather few studies of the antimicrobial susceptibility of *B. cereus*, and most information has to be gleaned from reports of individual cases or outbreaks. *B. cereus* and *B. thuringiensis* produce penicillinases and a broad-spectrum β-lactamase and are thus resistant to penicillins, cephalosporins, and β-lactamase inhibitor combinations (160, 168). Moderate or intermediate susceptibility has been demonstrated for TMP-SMX, clindamycin, and tetracycline in a portion of isolates (160, 168). All strains are susceptible to imipenem and vancomycin, and most are susceptible to chloramphenicol, erythromycin, gentamicin, ciprofloxacin, and daptomycin (160, 168, 169). Despite this, treatment failures have been reported, even for regimens containing vancomycin. Such cases were summarized in the previous version of this chapter (170).

Oral ciprofloxacin has been used successfully in the treatment of *B. cereus* wound infections, bacteremia, and pulmonary infection. Clindamycin with gentamicin, given early, appears to be the best treatment for ophthalmic infections caused by *B. cereus* (171). Recommended agents for primary susceptibility testing of *Bacillus* spp. (not *B. anthracis*) include vancomycin, fluoroquinolones, and clindamycin, and interpretive criteria are available for many antibiotics (156).

Other Species

There is a paucity of antimicrobial susceptibility data for other *Bacillus* species and related genera, due to the low frequency at which these are found to be clinically significant. Most of the available information has been derived from individual clinical case studies, where antibiotics were administered empirically and treatment may or may not have been guided by *in vitro* susceptibility results. As environmental organisms, *Bacillus* spp. may exhibit a wide array of resistance and susceptibility patterns against commonly used antibiotics (169). When isolated from normally sterile sites and clinically warranted, the laboratory may use the published CLSI approved guidelines (156). The antimicrobial agents suggested for primary testing are vancomycin, clindamycin, and fluoroquinolones. When tested, clinical isolates of non-*B. cereus*, non-*B. anthracis* species are typically susceptible to vancomycin, daptomycin, gentamicin, ciprofloxacin, imipenem, erythromycin, and TMP-SMX (168). Variable susceptibility exists for penicillins, cephalosporins, chloramphenicol, clindamycin, and tetracycline (168). Vancomycin resistance is rare, but it has been reported (172, 173).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The isolation of *B. anthracis* is always significant and requires urgent reporting, but the majority of aerobic endospore-forming bacteria are innocuous environmental organisms, not professional pathogens. Although frequently encountered in the microbiology laboratory, most isolates are of no clinical relevance. However, opportunistic infections have been reported since the late 19th century, and over the past 30 years, the clinical importance of aerobic endospore formers (most, but not all, of them *Bacillus* species) has become widely accepted. One must be wary of dismissing any organism as a mere contaminant before considering the clinical context of its isolation. If it is obtained in pure culture (or at least appears to dominate the microbiota), if it is isolated in large numbers, or if it is isolated from more than one clinical specimen, then the organism should be considered of potential clinical significance. Moderate or heavy growth of bacilli from wounds is usually significant, and *B. cereus* infections of the eye are serious emergencies that should always be reported immediately to the physician. In food-borne illness investigations, qualitative isolation tests are insufficient because low-level contamination of foodstuffs by aerobic endospore-forming bacteria is common, as is transient and asymptomatic fecal carriage. Ideally, to establish that a putative pathogen is the etiological agent of a food poisoning event, the organism should be isolated from the epidemiologically incriminated food in significant numbers ($>10^5$ CFU/g) and the same strain (genotype, biovar, or plasmid type, etc.) should also be isolated in significant numbers from acute-phase specimens (feces or vomitus) obtained from patients but not from healthy controls. For events associated with *B. cereus*, it should also be possible to detect emetic toxin and/or enterotoxin in the food.

In cases where clinical significance is suspected or established, but the isolate does not represent a "usual suspect," it is preferable that the identification be confirmed by DNA sequencing. Ideally, nucleotide sequences associated with rare and potentially novel species should be submitted to public databases, where they will be accessible to other laboratories searching for information about the same aerobic endospore-forming organism.

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Listeria and *Erysipelothrix*

NELE WELLINGHAUSEN

27

LISTERIA

Taxonomy

The genus *Listeria* consists of Gram-positive, non-spore-forming, facultative anaerobic, regular rod-shaped bacteria with a low G+C content of 36 to 42 mol%. While early phylogenetic studies suggested a close relation between *Listeria* and the *Lactobacillaceae*, comparisons of 16S rRNA gene sequences have shown that *Listeria* is most closely related to *Staphylococcus* and *Bacillus*. *Listeria* belongs to the newly assigned family *Listeriaceae* within the order *Bacillales* (1). Synthesis of menaquinones and major amounts of branched-chain fatty acids confirms the taxonomic separation of *Listeria* from the *Lactobacillaceae* (2).

Until recently, the genus *Listeria* comprised six validated species including *Listeria monocytogenes* as the type species in the genus, *L. grayi*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, and *L. welshimeri*. Within *L. ivanovii*, two subspecies, *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis*, are differentiated. In recent years, further *Listeria* species, i.e., *L. marthii*, *L. rocourtiae*, *L. fleischmannii*, including a recent subspecies *L. fleischmannii* subsp. *coloradonensis* (3), and *L. weihenstephanensis*, have been described from the natural environment and from food items (4–7).

Based on the results of multilocus enzyme electrophoresis, DNA-DNA hybridization, and 16S rRNA gene sequencing, a group of species closely related to *L. monocytogenes*, including *L. innocua*, *L. ivanovii*, *L. marthii*, *L. monocytogenes*, *L. seeligeri*, and *L. welshimeri*, can be differentiated from *L. grayi* and the remaining recently described species (4–7). Only *L. monocytogenes* and *L. ivanovii* are pathogenic for humans and animals.

Description of the Agent

Members of the genus *Listeria* are Gram-positive, facultative anaerobic, non-spore-forming, nonbranching, regular, short (0.5 to 2 μm by 0.4 to 0.5 μm) rods that occur singly or in short chains. Filaments of 6 to 20 μm in length may occur in older or rough cultures. Temperature-regulated expression of flagellin results in a characteristic tumbling motility at 20 to 28°C by means of one to six peritrichous flagella. At 37°C, the organisms are much less motile. Colonies are small (1 to 2 mm in diameter after 1 or 2 days of incubation at 37°C), smooth, and blue-gray on nutrient agar when examined with obliquely transmitted light and typically show a narrow zone of beta-hemolysis. *Listeria* spp. show an exceptionally large growth temperature range from 0 to 50°C. The optimum growth temperature is between 30 and 37°C, but at 4°C, growth is also observed within a few days. Catalase is typically produced, but catalase-negative strains causing disease in humans have been described (8, 9). The oxidase test is negative. Acid is produced from D-glucose and other sugars. The Voges-Proskauer and methyl red tests are positive. Esculin is hydrolyzed in a few hours. Urea and gelatin are not hydrolyzed. Neither indole nor H_2S is produced. The cell wall contains a directly cross-linked peptidoglycan based on *meso*-diaminopimelic acid, as well as lipoteichoic acid, but no mycolic acids. The two predominant cellular fatty acids are $Cai_{15:0}$ and $Cai_{17:0}$ (branched-chain type) (10).

Epidemiology and Transmission

The primary habitat of *Listeria* species is the environment, where they exhibit a saprophytic lifestyle. *L. monocytogenes* has been isolated from various animals, like mammals, birds, fish, and crustaceans. Infected animals can asymptomatically pass the organism or develop clinical disease. Due to its widespread distribution, *L. monocytogenes* has many opportunities to enter human food production, resulting in contamination of fresh and processed poultry, meat, and vegetables; raw milk; cheese; smoked salmon; etc. Numbers of organisms exceeding 10^3 CFU/g were detected in food products (11). Infection of humans ingesting colonized food is potentiated by the ability of the organism to multiply at 4°C. The intestinal tract of adults is consistently colonized with nonpathogenic *Listeria* species and, to a lesser extent (1 to 5%), with pathogenic *L. monocytogenes*. Cervicovaginal carriage in women has not been reported. Apart from food-related infections, nosocomial outbreaks, mainly in neonatal wards, have been described (12, 13).

The number of sporadic cases of listeriosis in countries that report the illness is typically in the range of 0.1 to 0.9 cases per 100,000 persons. While the number of cases and the mortality in the United States have decreased until the end of the last millennium, the incidence remained quite stable in the last decade around 0.27 cases per 100,000 persons (14–17). In contrast, in several European countries, the incidence of sporadic cases of listeriosis has increased, reaching numbers from 0.4 up to 1.0 per 100,000 per year (18–20). People with an underlying condition, like chronic lymphocytic leukemia, myeloproliferative disorder, cancer, organ transplantation, alcoholism, and hepatic disorders;

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pregnant women; and individuals ≥60 years have an up to 100 to >1,000-fold increased risk of acquiring listeriosis (15, 17, 18, 20).

Clinical Significance

In adults, *L. monocytogenes* causes primarily septicemia, meningitis, and encephalitis with a mortality reaching up to 50%. According to a recent study, the rate of unfavorable outcome of *Listeria* meningitis has increased significantly during the last 15 years and could be linked to infection with *L. monocytogenes* serotype 6 (21). Focal infections with *Listeria* spp. have been infrequently described and include endocarditis, pericarditis, arthritis, osteomyelitis, intra-abdominal and brain abscesses, endophthalmitis, (sclero-)keratitis, peritonitis, cholecystitis, and intravenous catheter and pleuropulmonary infections (22–24). Among veterinarians and abattoir workers, but recently also in a gardener, primary cutaneous listeriosis with or without bacteremia has been reported (25, 26).

In pregnant women, *L. monocytogenes* often causes a mild, self-limited influenza-like illness. Transient bacteremia can result in placentitis and/or amnionitis, and since *Listeria* is able to cross the placenta, it can infect the fetus, causing abortion, stillbirth, or most commonly, preterm labor (27, 28). In neonates, an early-onset form and a late-onset form of listeriosis occur. The early form is presumably caused by intrauterine infection and manifests as granulomatosis infantisepticum. The organism is widely disseminated in the body, including the central nervous system. The source of the organism in the late-onset cases, which manifest at a mean of 14 days after birth, is unclear and may comprise the mother's genital tract or environmental sources.

The incubation period for human listeriosis varies between 1 day and 2 to 3 months and is significantly longer for pregnancy-associated cases (median, 28 days) than for central nervous system and bacteremia cases (median of 9 and 2 days, respectively) (29). The infectious dose has not been firmly established, but 10^5 CFU or greater have been reported to cause gastroenteritis in outbreak situations (30). A dose-response model using rhesus monkeys as a surrogate for pregnant women recently indicated that oral exposure to 10^7 CFU of *L. monocytogenes* results in about 50% stillbirths (31). In a pregnant guinea pig model, doses of 10^4 to 10^8 CFU have been shown to invade fetal liver and brain tissue in up to 75%, and fetal infection occurred as early as day 2 after maternal infection (32). Thus, the infectious dose may be much less than the extrapolated estimate of 10^{13} CFU from the FDA-U.S. Department of Agriculture-CDC risk assessment based on mouse data (33).

Most cases of *Listeria* gastroenteritis are linked to foodborne outbreaks (34). Typically, patients with *Listeria* gastroenteritis have no known predisposing risk factors for listeriosis, illness occurs about 24 h after ingestion of a food item that is contaminated with a large number of bacteria (10^5 to 10^9 CFU/g or ml), and illness lasts about 2 days. Apart from gastroenteritis, fever, headache, and pain in joints and muscles are frequently seen.

After ingestion of *L. monocytogenes*, pathogen and host factors as well as the number of pathogens ingested determine whether invasive infection develops. Immunity to listeriosis is effected primarily via the cell-mediated immune system. Penetration of the epithelial barrier in the gut by *L. monocytogenes* is facilitated by its ability to escape from the host cell vacuole, intracytoplasmic multiplication, movement via bacterially induced polymerization of host cell actin, and spread to neighboring cells through pseudopod-like extensions of the host cell membrane. By *N*-deacetylation of peptidoglycan of the cell wall, *Listeria* evades innate immune defenses (35). Virulence genes are clustered on an 8.2-kb pathogenicity island and include genes coding for internalin A and B and listeriolysin, a hemolysin (36). Interaction between internalin and E-cadherin, a receptor of the trophoblast, facilitates the spread of the organism to the fetus.

L. ivanovii is primarily a pathogen of ruminants, but systemic infections in humans with underlying conditions and a case of stillbirth in a pregnant woman have been described (37).

Collection, Transport, and Storage of Specimens

Suitable specimens for detection of listeriosis include blood and cerebrospinal fluid (CSF). In neonates with suspicion of listeriosis, investigation of blood, CSF, amniotic fluid, respiratory secretions, placental or cutaneous swabs, gastric aspirates, or meconium can facilitate detection of the organism. For epidemiologic purposes or rare causes of gastroenteritis, stool specimens are preferred to rectal swabs. In general, specimens for detection of *Listeria* do not need special handling during collection.

Clinical specimens for culture of *L. monocytogenes* should be processed as soon as possible or stored and transported at room temperature or 4°C for up to 48 h. At 4°C, even longer storage times may be tolerated due to the specific cold resistance of the organism, but multiplication of *Listeria* has to be regarded. Stool samples (1 g each) can be inoculated into 100 ml of a selective broth, e.g., University of Vermont, polymyxin-acriflavine-lithium chloride-ceftazidime esculin-mannitol (PALCAM), or *Listeria* enrichment broth (38–41), analogous to the preparation of food samples (see below), and then shipped overnight at room temperature. Nevertheless, the value of enrichment of clinical samples has not been investigated yet. To avoid overgrowth of *L. monocytogenes* by contaminating microbiota during longer periods of storage, nonsterile-site specimens should be stored at 4°C for 24 to 48 h or frozen at −20°C.

Food samples should include a minimum of 100 g of a sample and should be collected aseptically in sterile containers. Food packaged in original containers should always be preferred. Samples should be shipped overnight frozen. Although *L. monocytogenes* is relatively resistant to freezing, repeated freezing and thawing should be avoided.

Cultures of *Listeria* spp. should be frozen at −20 to −70°C for long-term storage.

They can be shipped on a non-glucose-containing agar slant and packaged and declared according to the respective national and international requirements.

Because *L. monocytogenes* can infect the fetus, pregnant women should be particularly careful when working in a laboratory where *L. monocytogenes* is propagated or handled.

Direct Examination

Direct microscopy should be performed on CSF, positive blood cultures, and if available, tissue samples. Detection of Gram-positive, regular short rods in CSF or blood cultures should lead to the suspicion of listeriosis. Nevertheless, *L. monocytogenes* may be confused with members of the coryneform rods (especially in direct slides from positive blood cultures), since the cells may be arranged in V forms or palisades. Commercial tests licensed for antigen detection in clinical specimens are not available.

Sensitive and specific in-house PCR assays have been described for detection of *L. monocytogenes* in CSF, stool, or lung tissue (39, 42–46) and may be particularly useful for specimens from patients with prior antimicrobial therapy. Regarding commercial assays, the Probelia *Listeria monocytogenes* assay (Bio-Rad, Hercules, CA) has been evaluated in clinical stool specimens, while other commercial assays (e.g., LightCycler PCR, Roche Diagnostics, Indianapolis, IN; TaqMan *Listeria monocytogenes* detection kit, Applied Biosystems, Carlsbad, CA; BAX System PCR, DuPont, Wilmington, DE) have been validated only for food specimens.

Recently, a fluorescent *in situ* hybridization assay has been described that allows detection of *L. monocytogenes* in wastewater samples with a sensitivity of 10^4 cells/ml (47).

Isolation Procedures

Clinical specimens from normally sterile sites should be plated onto tryptic soy agar containing 5% sheep, horse, or rabbit blood. Plates should be incubated at 35 to 37°C in an atmosphere enriched with 5% CO_2 for a minimum of 48 h. *Listeria* colonies typically show a narrow zone of beta-hemolysis on blood agar. Blood samples should be inoculated into conventional blood culture media. Clinical specimens obtained from nonsterile sites, like stool samples, as well as food and environmental specimens should be plated on *Listeria* spp. selective agars. In addition, enrichment by inoculation into selective broth for *Listeria* spp. should be done before plating.

Selective agars for culture of *Listeria* spp. include lithium chloride-phenylethanol-moxalactam (LPM) (48), Oxford, modified Oxford, and PALCAM agars (39). On LPM agar, colonies have to be examined under a stereomicroscope with Henry illumination (magnification, ×15 to ×25, with oblique lighting directed to the microscope stage by a concave mirror positioned at a 45° angle to the incident light). *Listeria* colonies appear blue, and colonies of other bacteria appear yellowish or orange. Oxford and PALCAM agars contain selective substances that eliminate the need for examination under oblique lighting. On Oxford and modified Oxford agars, *Listeria* colonies appear black due to esculin hydrolysis, are 1 to 3 mm in diameter, and are surrounded by a black halo after 24 to 48 h of incubation at 35 to 37°C. On PALCAM agar, *Listeria* colonies appear gray-green, are approximately 2 mm in diameter, and have black sunken centers.

For the detection of *Listeria* spp. in food samples, enrichment methods have to be used. The most widely used reference methods for food and environmental samples are the Food and Drug Administration (FDA) *Bacteriological and Analytical Manual* (41) and the U.S. Department of Agriculture (USDA) method (40) in the United States and the International Organization for Standards (ISO) 11290 method in Europe (49). All methods require enrichment of the samples in a selective broth (buffered *Listeria* enrichment broth in the FDA *Bacteriological and Analytical Manual* method, University of Vermont broth in the USDA method, and Fraser broth in the ISO method) prior to plating onto selective agar (see above; also agar *Listeria* according to Ottaviani and Agosti [ALOA]) and biochemical identification of typical colonies (40, 41). Confirmation of species identification is necessary, since growth of *Bacillus circulans* strains on ALOA has been described (50). A detailed comparison of methods is given by Dever et al. (51).

Chromogenic media allow selective isolation of *Listeria* species (39, 52, 53). Chromogenic media are mainly based on ALOA and include ALOAgar (Biolife, Milan, Italy), BCM *L. monocytogenes* (Biosynth, Staad, Switzerland), Li-mono-Ident-Agar (Heipha, Eppelheim, Germany), Chromoplate *Listeria* (Merck KGaA, Darmstadt, Germany), Oxoid Chromogenic *Listeria* agar (Oxoid, Basingstoke, United Kingdom), and BBL Chromagar (BD, Sparks, MD) (53–55). However, none of these agars differentiate between *L. monocytogenes* and *L. ivanovii*. Specific detection of *L. monocytogenes* is facilitated on Rapid *L.mono* agar (Bio-Rad, Hercules, CA) (56). Chromogenic media showed sensitivities comparable to those of Oxford and PALCAM agar, but they appeared to be less specific (52, 53, 55, 56).

Identification

A simplified identification is based on the following tests: Gram staining, observation of tumbling motility in a wet mount, and tests for a positive catalase reaction and esculin hydrolysis. Acid production from D-glucose and a positive Voges-Proskauer test are confirmatory results.

Listeria spp. may be confused with other Gram-positive bacteria due to similar morphologic or biochemical characteristics. *Streptococcus* and *Enterococcus* spp. can be differentiated from *Listeria* spp. on the basis of Gram stain morphology, motility, and catalase reaction. *Erysipelothrix* spp. differ from *Listeria* spp. in motility, catalase reaction, and ability to grow at 4°C (*Erysipelothrix* spp. do not grow at that temperature). *Lactobacillus* spp. are usually nonmotile and catalase negative.

Identification of *Listeria* isolates to the species level is crucial, because all species can contaminate foods but only *L. monocytogenes* is of public health concern. A scheme for identification of medically relevant *Listeria* species based on morphological and biochemical characteristics is shown in Table 1. Among these markers, hemolysis is essential for differentiating between *L. monocytogenes* and the most frequently isolated nonpathogenic species, *L. innocua*.

Production of hemolysin is regarded as a key virulence factor of *L. monocytogenes* and is visible on sheep blood agar plates as a narrow zone of beta-hemolysis that frequently does not extend much beyond the edges of the colonies (Fig. 1). Like *L. monocytogenes*, *L. ivanovii* is also hemolytic, but hemolysis alone cannot be used to discriminate pathogenic and nonpathogenic species, since *L. seeligeri* is, besides rare exceptions (57), hemolytic and since hemolytic strains of *L. innocua* have been described as well (58).

The CAMP (Christie, Atkins, Munch-Petersen) test can be used to differentiate among hemolytic *Listeria* species. The test is carried out by streaking a beta-hemolysis-producing *Staphylococcus aureus* strain and *Rhodococcus equi* parallel to each other on a blood agar plate. Suspect cultures are streaked at right angles in between (but not touching) the two streaks. Hemolysis by *L. monocytogenes* and, to a lesser degree, *L. seeligeri* is enhanced in the vicinity of *S. aureus*, and hemolysis by *L. ivanovii* is enhanced in the vicinity of the *R. equi* streak (Fig. 2). However, the reliability of the CAMP test is limited. As recommended in the USDA method (40), commercially available β-lysis disks (Remel, Lenexa, KS) may be used instead.

L. monocytogenes is, apart from rare atypical strains, L-rhamnose and α-methyl-D-mannoside positive and D-xylose negative. Incubation of test tubes for up to 7 days (37°C, aerobically) may be necessary.

Commercially available miniaturized tests considerably speed up biochemical identification of *Listeria* spp. The API *Coryne* and *Listeria* (bioMérieux, Durham, NC), Micro-ID *Listeria* (Remel), BBL Crystal Gram-Pos ID (BD, Franklin Lakes, NJ), and Microbact *Listeria* 12L (Oxoid) reliably identify *Listeria* isolates to the genus and species level (59).

TABLE 1 Biochemical differentiation of medically relevant and selected environmental species in the genus *Listeria*[a]

Characteristic	*L. grayi*	*L. innocua*	*L. ivanovii* subsp. *ivanovii*	*L. ivanovii* subsp. *londoniensis*	*L. monocytogenes*	*L. seeligeri*	*L. welshimeri*
Beta-hemolysis	–	–	++[b]	++	+	+	–
CAMP[c] test reaction							
S. aureus	–	–	–	–	+	+	–
R. equi	–	–	+	+	V	–	–
Acid production from:							
Mannitol	+	–	–	–	–	–	–
α-Methyl-D-mannoside	+	–	–	–	+	–	+
L-Rhamnose	V	V	–	–	+	–	V
Soluble starch	+	–	–	–	–	ND	ND
D-Xylose	–	–	+	+	–	+	+
Ribose	V	–	+	–	–	–	–
N-Acetyl-β-D-mannosamine	ND	ND	V	+	ND	ND	ND
Hippurate hydrolysis	–	+	+	+	+	ND	ND
Reduction of nitrate	V	–	–	–	–	ND	ND
Associated serovar(s)	S	4ab, 6a, 6b	5	5	1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 7	1/2a, 1/2b, 1/2c, 4a, 4b, 4d, 6b	1/2b, 4c, 6a, 6b

[a] Symbols and abbreviations: +, ≥90% of strains are positive; –, ≥90% of strains are negative; ND, not determined; V, variable; US, undesignated serotype; S, specific.

[b] ++, usually a wide zone or multiple zones.

[c] See text and Fig. 2.

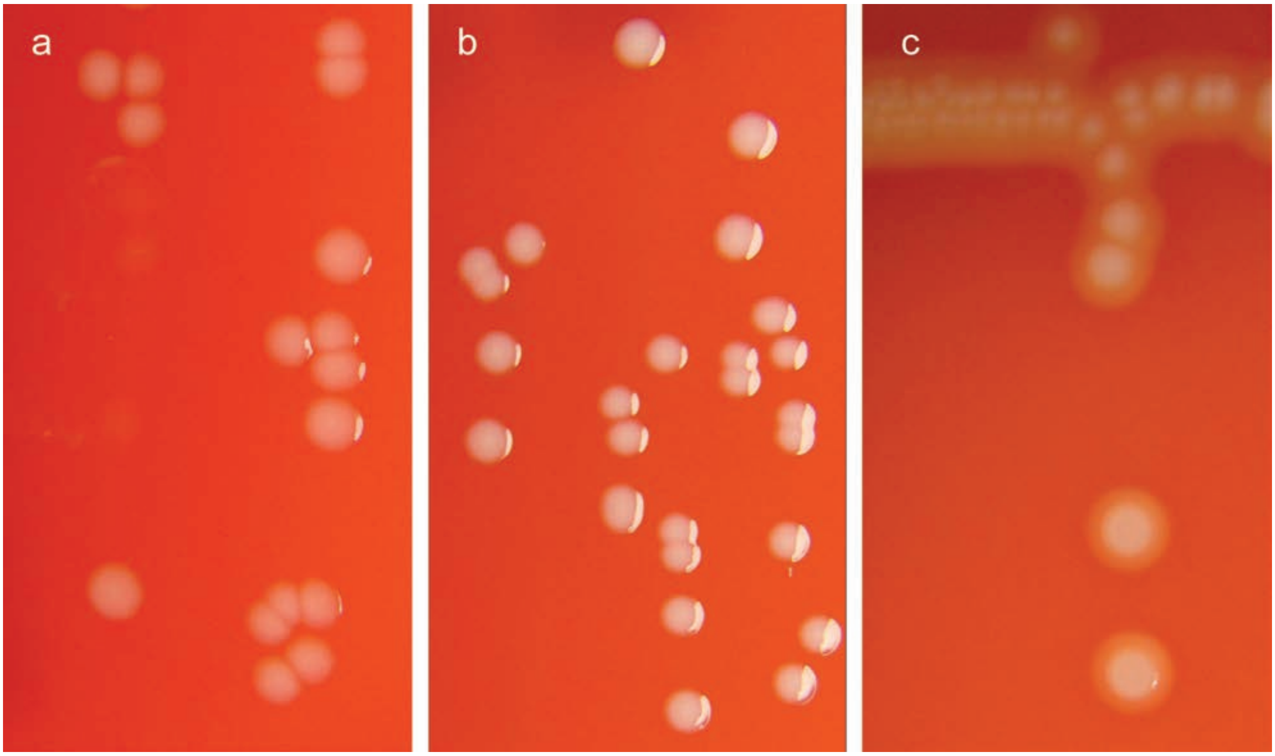

FIGURE 1 Macroscopic view of colonies on 5% human blood agar plates after 24 h of incubation. (A) *L. monocytogenes*: discrete zone of beta-hemolysis under the removed colonies. (B) *L. innocua*: no hemolysis. (C) *L. ivanovii*: wide zone of beta-hemolysis around the colonies. doi:10.1128/9781555817381.ch27.f1

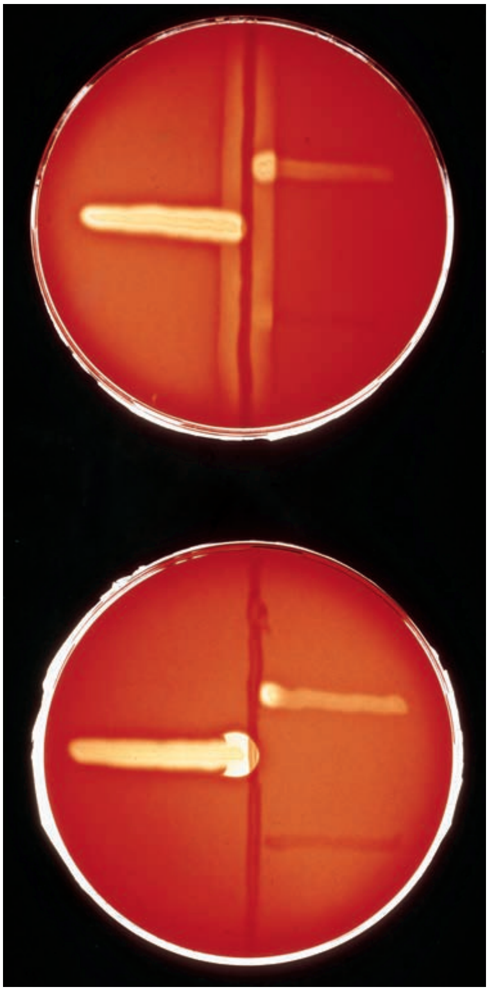

FIGURE 2 CAMP test done with *S. aureus* CIP 5710 (top plate) and *R. equi* CIP 5869 (bottom plate) after 24 h of incubation. Upper right, *L. monocytogenes*; lower right, *L. innocua;* middle left, *L. ivanovii.*
doi:10.1128/9781555817381.ch27.f2

The RapID CB Plus system (Oxoid) allows reliable identification of *Listeria* species to the genus level only (60, 61).

Identification of *L. monocytogenes* is possible with the Vitek2 (bioMérieux), MicroScan WalkAway (Siemens Healthcare, Malvern, PA), Micronaut (Merlin, Bornheim-Hesel, Germany), Phoenix (BD), and Biolog (Biolog, Inc., Hayward, CA) systems. All systems allow reliable species identification, while Vitek1 (bioMérieux) facilitates identification of *Listeria* isolates to the genus level only.

A chemiluminescence DNA probe assay (AccuProbe, Hologic Gen-Probe, Bedford, MA) (62) is available for rapid identification of *L. monocytogenes* from primary isolation plates. However, false-positive results were observed with *L. marthii* (5). Recently described peptide nucleic acid fluorescent *in situ* hybridization probes also allow rapid and specific identification of *L. monocytogenes* and *L. ivanovii* (63).

The matrix-assisted laser desorption ionization–time of flight (mass spectrometry) (MALDI-TOF [MS]) technique is increasingly used, especially in European laboratories, and allows discrimination of the *Listeria* species by use of the respective software (MALDI Biotyper, Bruker Daltonics, Billerica, MA; Vitek MS, bioMérieux; in-house databases) and an optional pretreatment protocol for colonies (64–66). The use of MALDI-TOF (MS) on the Andromas system directly from colony material facilitated species identification of *L. grayi*, while *L. monocytogenes* clustered with all other *Listeria* species and was, thus, identifiable to the genus level only. By use of the Vitek MS v2.0 system, 75% of the tested *L. monocytogenes* isolates could be identified to the species level and 9% to the genus level, while 15% failed to be identified (66). Differentiation of *L. grayi* from a cluster including *L. monocytogenes* and all other *Listeria* species by MALDI-TOF (MS) corroborates molecular and phylogenetic studies of the genus *Listeria* (67).

Typing Systems

Subtyping of *L. monocytogenes* is crucial for the workup of disease acquired from food-borne agents. Based on somatic "O" and flagellar "H" antigens, 13 serovars of *L. monocytogenes* are known (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b/4bX, 4c, 4d, 4e, and 7). Since the vast majority of *L. monocytogenes* strains that cause sporadic infections or outbreaks belong to the same serotypes, i.e., 1/2a, 1/2b, and 4b, and since serotyping antigens are shared among *L. monocytogenes*, *L. innocua*, *L. seeligeri*, and *L. welshimeri*, reliable discrimination below the level of serotype is necessary. Thus, serotyping is only useful as a first-level discriminator or for the selection of further typing methods in suspected outbreaks. Antisera are commercially available from Difco (Difco Laboratories/BD, Sparks, MD) and Denka Seiken (Tokyo, Japan). Multiplex PCR assays, also validated by interlaboratory comparison, have been described for identification of the four major serovars of *L. monocytogenes* (1/2a, 1/2b, 1/2c, and 4b) (68–72).

Pulsed-field gel electrophoresis (PFGE) is considered the standard typing method for *L. monocytogenes* and is particularly useful for subtyping of serovar 4b isolates. Its discriminatory power and reproducibility of results have been confirmed in a World Health Organization multicenter international typing study (73) as well as in a large number of other studies. To optimize quality and interlaboratory comparability of PFGE, standardized laboratory protocols should be used. PulseNet USA has published a 1-day protocol (74) (available at http://www.cdc.gov/pulsenet/PDF/listeria-pfge-protocol-508c.pdf) that has recently been optimized and re-evaluated by a multilaboratory study (75). According to the protocol, restriction endonucleases *Apa*I and *Asc*I are used. A proficiency testing trial of the PulseNet Europe involving 29 national reference laboratories showed a high level of agreement between typing results but also stressed the necessity of adherence to the standardized protocol and revealed failures in DNA extraction (72).

Faster and simpler molecular subtyping methods, like multilocus variable-number tandem-repeat analysis and multilocus sequence typing, have evolved, and their application for subtyping of *L. monocytogenes* is supported by PulseNet. Both methods showed a discriminatory power comparable to that of PFGE (76–79). By multilocus sequence typing and multi-virulence locus sequence typing, the predominant PFGE clone of *L. monocytogenes* involved in recent listeriosis outbreaks in Canada could be further characterized and traced back to 1988 (80). Other molecular typing methods, like single-nucleotide polymorphism-based multilocus genotyping and a mixed-genome DNA microarray, have been developed and also showed results comparable to those of PFGE (81–84). High-throughput genome pyrosequencing allowed rapid molecular characterization of the outbreak strain of *L. monocytogenes* serotype 1/2a in a large listeriosis outbreak in Canada in 2008, confirming differences in *Asc*I PFGE patterns at the molecular level (85). Altogether, sequence-based molecular methods may further improve subtyping of *L. monocytogenes*. They may replace typing methods with high discriminatory power but lacking interlaboratory standardization, like random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism, multilocus single-strand conformation polymorphism, and ribotyping, and may also add relevant molecular information about outbreak strains to the knowledge obtained by PFGE.

As a nonmolecular technique, MALDI-TOF (MS) has been used successfully for rapid typing of *L. monocytogenes*. It allowed clear discrimination of all lineages and serotypes of *L. monocytogenes* in one study so far (64).

An overview of the methods used for typing of *L. monocytogenes* is given by Jadhav et al. (44).

Serologic Tests

Antibodies directed against listeriolysin O have been detected in listeriosis patients by blotting techniques with sensitivities from 50 to 96%, but the sensitivity was markedly lower with complement fixation or O-agglutination tests (86, 87). A test based on the detection of antibodies against recombinant truncated forms of listeriolysin O may be more specific (88). Serologic tests cannot be recommended for the detection of past or acute listeriosis.

Antimicrobial Susceptibilities

Treatment with an aminopenicillin (ampicillin or amoxicillin) is still regarded as the most effective therapeutic regimen for listeriosis. *In vitro* resistance of *L. monocytogenes* isolates to ampicillin has been reported sporadically (89–91), but the methods used in these studies appear to be inadequate for susceptibility testing of *Listeria*. Therefore, these reports have to be interpreted with great caution. In other studies and reviews, *in vitro* resistance against ampicillin was not detected (92–94). Since aminoglycosides exhibit a synergistic effect on aminopenicillins, they are usually added for therapy of listeriosis. However, in a retrospective cohort study, addition of gentamicin did not decrease early or late mortality in listeriosis (95). Trimethoprim-sulfamethoxazole is recommended for patients who are allergic to penicillin, and moxifloxacin may be a valuable alternative, since it shows bactericidal efficacy comparable to that of amoxicillin *in vitro* (96). *L. monocytogenes* is intrinsically resistant to cephalosporins, fosfomycin, and fusidic acid, and even when *in vitro* susceptibility may be determined, cephalosporins should not be used for therapy. Isolated resistance against tetracycline has been noted (97) as well as multidrug resistance to chloramphenicol, macrolides, and tetracyclines due to the presence of resistance plasmids. Newer substances against Gram-positive pathogens, like linezolid, tigecycline, and daptomycin, elicited high susceptibility *in vitro*, and linezolid also showed clinical efficacy (98–102). In addition, *L. monocytogenes* is generally susceptible *in vitro* to erythromycin and vancomycin (97, 103). Antimicrobial susceptibility testing should be performed in cases of suspected treatment failures, severe disease, and patients with penicillin allergy. A CLSI guideline (M45-A2) for broth microdilution antimicrobial susceptibility testing of *L. monocytogenes* including interpretive breakpoints for penicillin, ampicillin, and trimethoprim-sulfamethoxazole is available (104).

Evaluation, Interpretation, and Reporting of Results

The diagnosis of listeriosis can be made by isolation of *L. monocytogenes* from blood, CSF, or specimens from other normally sterile sites. Species identification is necessary to differentiate *L. monocytogenes* from nonpathogenic *Listeria* species. Especially for patients with underlying immunosuppression and for individuals older than 60 years and younger than 1 month, direct microscopic detection of Gram-positive, regular, short rods in the above-mentioned specimens should raise suspicion of listeriosis and should promptly be communicated to the clinician to ensure eradication of *L. monocytogenes* by antimicrobial therapy. Detection of *Listeria* species in stool samples likely represents colonization. Routine screening of stool samples for *Listeria* remains unwarranted, although sporadic cases of *L. monocytogenes* gastroenteritis have been reported.

Standard antimicrobial therapy of meningitis with cefotaxime or ceftriaxone is not active against *L. monocytogenes*. Antimicrobial susceptibility testing should be performed in cases of suspected treatment failures, severe disease, and patients with penicillin allergy. Cultures from blood and CSF that were obtained after the initiation of antimicrobial therapy may be negative. In these cases, detection of *Listeria* DNA may be useful. Commercial kits for PCR-based detection of *L. monocytogenes* in CSF specimens are not available, but in-house protocols and multiplex PCR formats facilitate rapid and specific detection of *L. monocytogenes* DNA.

ERYSIPELOTHRIX

Taxonomy

Erysipelothrix is taxonomically classified within the class *Erysipelotrichia* (105) in the phylum *Firmicutes*. The genus *Erysipelothrix* has three validly published species, *E. rhusiopathiae*, *E. tonsillarum*, and *E. inopinata*. Based on peptidoglycan antigens of the cell wall, several serovars can be distinguished in *E. rhusiopathiae* (serovars 1a, 1b, 2a, 2b, 3, 4, 5, 6, 8, 9, 11, 12, 15, 16, 17, 19, 21) and *E. tonsillarum* (serovars 3, 7, 10, 14, 20, 22, and 23) (106). Only *E. rhusiopathiae* has been detected as a pathogen of humans. The vast majority of infections are caused by serovars 1 and 2.

Description of the Agent

Erysipelothrix organisms are facultatively anaerobic, non-spore-forming, non-acid-fast, Gram-positive bacteria that appear microscopically as short rods (0.2 to 0.5 µm by 0.8 to 2.5 µm) with rounded ends and occur singly, in short chains, or in long, nonbranching filaments (60 µm or more in length). Some cells stain unevenly. They are nonmotile and grow in complex media at a wide range of temperatures (5 to 42°C; optimum, 30 to 37°C) and at alkaline pH (pH 6.7 to 9.2; optimum, pH 7.2 to 7.6). Like *Listeria* organisms, they can grow in the presence of high concentrations of sodium chloride (up to 8.5%). *Erysipelothrix* organisms are catalase negative and oxidase negative, do not hydrolyze esculin, and weakly ferment glucose without the production of gas. They are methyl red and Voges-Proskauer negative and do not produce indole or hydrolyze urea but distinctively produce H_2S in triple sugar iron agar. Key fatty acids are $C_{16:0}$ and $C_{18:cis9}$ (10).

Epidemiology and Transmission

E. rhusiopathiae is distributed worldwide in nature and is remarkably stable under varying environmental conditions. The organism is carried by a variety of animals, like mammals, birds, and fish, in their digestive tracts or tonsils but is most frequently associated with pigs. Other domestic animals that are frequently infected include sheep, rabbits, cattle, and turkeys. Infected animals, both sick and asymptomatic, pass the organism by urine and feces, leading to contamination of water and soil.

Infection in animals is most likely acquired by ingestion of contaminated matter. Human infection with *E. rhusiopathiae* is a zoonosis. Most cases are related to occupational exposure, occurring most frequently among fish handlers, veterinarians, and butchers. The disease is contracted through direct contact via skin abrasions, injuries, or animal bites (107).

E. tonsillarum has been recovered from tonsils of healthy pigs and cattle, water, and seafood. *E. inopinata* has been isolated once from a vegetable-based peptone broth (108).

Clinical Significance

E. rhusiopathiae has been recognized for more than 100 years as the agent of swine erysipelas, an acute or chronic disease. In humans, *E. rhusiopathiae* causes erysipeloid, a localized cellulitis developing within 2 to 7 days around the inoculation site. The infected area is swollen, and the mostly painful lesion consists of a well-defined, slightly elevated, violaceous zone which spreads peripherally as discoloration of the central area fades. Vesicles may be present, but suppuration does not occur. Regional lymphangitis is present in one-third of patients, and low-grade fever and arthralgias occur in about 10% of patients. Healing of erysipeloids usually takes 2 to 4 weeks and sometimes months, and relapses are frequently seen. Dissemination of the organism can occur and manifests in most of the cases as endocarditis with a poor prognosis (107). Uncommon manifestations of infection with *E. rhusiopathiae* include peritonitis, endophthalmitis, osteomyelitis, intracranial and spinal abscesses, prosthetic joint arthritis, pneumonia, and meningitis (109–111).

Data about *E. rhusiopathiae* pathogenesis are still scarce. *E. rhusiopathiae* has a capsule consisting of polysaccharide antigen that confers increased resistance to phagocytosis. Neuraminidase plays a significant role in bacterial attachment and subsequent invasion into host cells. The 69-kDa surface antigens SpaA, SpaB, and SpaC appear to be the major protective antigens of *E. rhusiopathiae*, and recombinant SpaA and SpaC elicit a protective immune response in pigs and mice, making them potential vaccine candidates (112, 113). A multiplex real-time PCR for detection of Spa types has been described (114).

Collection, Transport, and Storage of Specimens

Biopsy specimens from erysipeloid lesions are the best source of *E. rhusiopathiae*. Care should be taken to cleanse and disinfect the skin before sampling. The organisms typically are located deep in the subcutaneous layer of the leading edge of the lesion; hence, a biopsy of the entire thickness of the dermis at the periphery of the lesion should be taken for Gram staining and culture. Swabs from the surface of the skin are not useful. In disseminated disease, the organism can be cultured in standard blood cultures or from aspirates of the respective infected location. For transport and storage of specimens, standard procedures should be followed.

Direct Examination

Direct microscopy should be performed with aspirates, biopsy specimens, and positive blood cultures. Gram stain morphology of *E. rhusiopathiae* includes short rods and very long filaments and, thus, is not distinctive. However, the presence of long, slender, Gram-positive rods in tissue from an individual with a known exposure is suggestive of erysipeloid. It has to be noted that the organism may appear Gram negative in stains from cultures (see below).

Conventional and real-time PCR assays for specific detection of *E. rhusiopathiae* in animal tissue as well as for discrimination of *E. rhusiopathiae* from *E. tonsillarum* have been described, but their application to human samples has not been evaluated yet (115–117).

Isolation Procedures

Tissue or biopsy specimens should be processed as described in chapter 18 and plated onto blood agar or chocolate blood agar, placed in tryptic soy, Schaedler, or thioglycolate broth, and incubated at 35 to 37°C aerobically or in 5% CO_2 for 7 days. Special pretreatment of samples is not necessary, but inoculation of an enrichment broth significantly increases the detection rate. Blood from patients with septicemia or endocarditis can be inoculated into commercial blood culture systems. *E. rhusiopathiae* colonies generally develop in 1 to 3 days, appearing as pinpoints (<0.1 to 0.5 mm in diameter) on blood agar plates after 24 h of incubation; at 48 h, two distinct colony types can be observed. The smaller, smooth colonies are 0.3 to 1.5 mm in diameter, transparent, convex, and circular with entire edges. Larger, rough colonies are flatter and more opaque and have a matte surface and an irregular, fimbriated edge. While a temperature of 37°C favors rough colonies, smooth colonies are favored at 30°C. A zone of greenish discoloration frequently develops underneath the colonies on blood agar plates after 2 days of incubation (105).

Identification

Cells stain Gram positive but, especially those from rough colonies, can decolorize and appear Gram negative, sometimes with a beaded morphology. Cells from smooth colonies appear as rods or coccobacilli, sometimes in short chains. Cells from rough colonies appear as long filaments, often more than 60 μm in length.

E. rhusiopathiae is catalase negative; it also tests negative for nitrate, urease, esculin, gelatin, xylose, mannose, maltose, and sucrose but positive for glucose, lactose, and H_2S. The extent of H_2S production is influenced by the culture medium, and the strongest reaction is found on triple sugar iron agar. Vitek2 and Phoenix automated systems, as well as the API system (API Coryne, API ID 32 Strep), identify *E. rhusiopathiae* reliably. *E. tonsillarum* differs biochemically from *E. rhusiopathiae* by being sucrose and alkaline phosphatase positive.

Identification of *E. rhusiopathiae* is also facilitated by MALDI-TOF (MS), as recently shown for the Andromas (Paris, France) system (67).

Human-pathogenic genera that have morphological and physiological characteristics in common with *Erysipelothrix* include mainly *Lactobacillus* and *Listeria* (118). They are regular nonpigmented, non-spore-forming, Gram-positive rods. A major discriminatory characteristic is that *E. rhusiopathiae* produces H_2S in triple sugar iron, whereas species of the other genera do not. Exceptions include some *Bacillus* strains, but they are easily differentiated from *E. rhusiopathiae* by cellular morphology, spore formation, and catalase reaction. *Listeria* species are catalase positive, motile, esculin positive, and not alpha-hemolytic. Corynebacteria and streptococci also can be confused with *E. rhusiopathiae*, but careful examination of cell morphology and catalase test should facilitate the distinction. An additional trait highly characteristic of *E. rhusiopathiae* is its "pipe cleaner" pattern of growth in gelatin stab cultures incubated at 22°C (105).

Typing Systems

Serotyping schemes are available for routine use in clinical laboratories but are of limited value, since most clinical isolates belong to serovar 1 or 2. RAPD, amplified fragment length polymorphism, and ribotyping methods have proved useful for epidemiological analysis of *Erysipelothrix* strains (119–121). PFGE using *Sma*I was superior to RAPD and ribotyping in discriminating *E. rhusiopathiae* isolates (121). Nucleotide sequence analysis of a hypervariable region in the *spa*A gene also allows discrimination of certain serovars of *E. rhusiopathiae* (113, 122).

Serologic Tests

Serologic tests for detection of antibodies to *E. rhusiopathiae* in humans are not available. Vaccines for active immunization of animals are available, and protective antibodies can be measured by enzyme immunoassay.

Antimicrobial Susceptibilities

Penicillin or ampicillin is the treatment of choice for both localized and systemic infections. Broad-spectrum cephalosporins or fluoroquinolones are suitable alternatives, since no resistance has been described yet. *E. rhusiopathiae* is also usually *in vitro* susceptible to clindamycin, erythromycin, daptomycin, imipenem, and tetracycline (123, 124). Of note, *E. rhusiopathiae* is intrinsically resistant to vancomycin and usually also to aminoglycosides and sulfonamides. Although antimicrobial susceptibility testing of isolates is not routinely required, testing of erythromycin and clindamycin, or further substances, may be warranted for patients with penicillin allergy. A CLSI guideline (M45-A2) for broth microdilution antimicrobial susceptibility testing of *Erysipelothrix*, including interpretative breakpoints for penicillin, ampicillin, cefepime, cefotaxime, ceftriaxone, imipenem, meropenem, erythromycin, ciprofloxacin, gatifloxacin, levofloxacin, and clindamycin, has been published (104).

Evaluation, Interpretation, and Reporting of Results

Since human infection is rare and clinical knowledge about the disease is scarce, diagnosis of erysipeloid is usually made accidentally by culture of *E. rhusiopathiae* from tissue biopsy specimens or blood. If there is no clinical suspicion, identification of *E. rhusiopathiae* in the clinical laboratory may be challenging. Detection of Gram-positive and Gram-variable rods, including decolorized, beaded cells and the presence of coccobacilli and very long filaments in direct microscopy of the specimens, gives a hint to this organism. A major discriminatory biochemical characteristic of *E. rhusiopathiae* is the production of H_2S.

Detection of *E. rhusiopathiae* in clinical samples should always be reported. Occurrence of this species in wound or tissue specimen indicates erysipeloid rather than contamination. Species identification is essential to ensure adequate antimicrobial therapy. While penicillin and ampicillin are generally active and recommended as first-line therapy, intrinsic resistance to vancomycin has to be noted.

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Coryneform Gram-Positive Rods

GUIDO FUNKE AND KATHRYN A. BERNARD

28

This chapter deals with aerobically growing, asporogenous, irregularly shaped, non-partially acid-fast, Gram-positive rods generally called "coryneforms." The term "coryneform" is actually somewhat misleading, since only true *Corynebacterium* spp. exhibit a typical club-shaped ("coryne," meaning "club" in ancient Greek) morphology, whereas all the other bacteria discussed in this chapter show an irregular morphology. However, in our experience, the term "coryneforms" is a common and convenient expression used by many clinical microbiologists, and therefore, the term will be used in this chapter.

The coryneform bacteria which were, for didactical reasons, not included in this chapter comprise *Actinomyces* spp. (in particular, the most frequently encountered species on aerobic plates, *A. europaeus*, *A. neuii*, *A. radingae*, and *A. turicensis*), *Actinobaculum* spp., *Propionibacterium* spp., and *Propioniferax* sp. (see chapter 52), whereas the genera *Arcanobacterium* and *Trueperella* are included. *Gardnerella vaginalis* is included in this chapter but is discussed separately. Regularly shaped aerobically growing or aerotolerant Gram-positive rods (*Bacillus*, *Listeria* and *Erysipelothrix*, *Lactobacillus*, and *Clostridium tertium*) are covered in chapters 26, 27, 52, and 53, respectively. Taxa which might be initially misidentified as coryneform bacteria also include partially acid-fast bacteria and other aerobic actinomycetes (chapter 29) as well as rapidly growing mycobacteria (chapter 32).

Features and infections caused by many of the taxa described in this chapter have recently been reviewed (1, 2).

TAXONOMY

The bacteria discussed in this chapter all belong to the class *Actinobacteria*, the genera of which are characterized by specific 16S rRNA gene signature nucleotides (3, 4) belonging to the lineage of Gram-positive bacteria with high guanine-plus-cytosine (G+C) content, with the exception of the genus *Exiguobacterium*, which belongs to the class *Firmicutes*, with a low G+C content. The coryneform bacteria are most diverse and are differentiated by chemotaxonomic features (Table 1). Phylogenetic investigations, in particular 16S rRNA gene sequencing, have, in general, confirmed the framework set by chemotaxonomic investigations. The 16S rRNA gene sequencing data demonstrate that the genera *Corynebacterium* and *Turicella* are more closely related to the partially acid-fast bacteria and to the genus *Mycobacterium* than to the other coryneform organisms covered in this chapter (5–7). The genus *Arthrobacter*, which contains rods, is phylogenetically intermixed with the genus *Micrococcus* (and genera formerly called *Micrococcus*), which contains cocci (chapter 21) (8, 9). The genus *Rothia* contains both rod-forming organisms, represented by *Rothia dentocariosa*, and coccoidal or cocci-forming species, i.e., *Rothia mucilaginosa* (formerly *Stomatococcus mucilaginosus* [10]) and *Rothia aeria* (11). The genus *Auritidibacter*, in the same family (*Micrococcaceae*) as *Arthrobacter* and *Rothia*, contains rod-shaped bacteria (12). Other genera which are phylogenetically closely related include *Oerskovia*, *Cellulosimicrobium*, and *Cellulomonas* (13–15) as well as *Arcanobacterium*, *Trueperella*, *Actinobaculum*, and *Actinomyces* (16).

DESCRIPTIONS OF THE GENERA

Genus *Corynebacterium*

The number of species belonging to the genus *Corynebacterium* has dramatically increased from 22 in 1990 to 89 species (and one taxon group) at the time of writing, about 54 of which (and one taxon group) are medically relevant. However, clinically relevant *Corynebacterium* species validly described since the last edition of this *Manual* comprise only *C. aquatimens* (17).

The cell wall of corynebacteria contains *meso*-diaminopimelic acid (*m*-DAP) as the diamino acid as well as short-chain mycolic acids with 22 to 36 carbon atoms (1). The medically relevant species *C. amycolatum*, *C. kroppenstedtii*, and *C. atypicum* lack mycolates (1, 18–20). Palmitic ($C_{16:0}$), oleic ($C_{18:1\omega9c}$), and stearic ($C_{18:0}$) acids are the main cellular fatty acids (CFAs) in all corynebacteria, and tuberculostearic acid (TBSA) can be detected in some medically relevant species (e.g., *C. urealyticum*, *C. kroppenstedtii*, *C. confusum*, *C. appendicis*, *C. minutissimum)* (19, 21–23). The G+C content of *Corynebacterium* spp. varies from 46 to 74 mol%, indicating the enormous diversity within this genus (1). The phylogenetic relationships within the genus *Corynebacterium* have been outlined (5, 6), creating an extensive and reliable database for future comparative 16S rRNA gene studies, e.g., for the delineation of new species. Complete genome sequences of 16 *Corynebacterium* species, including the medically relevant species *C. diphtheriae*, *C. aurimucosum*, *C. jeikeium*, *C. kroppenstedtii*, *C. pseudotuberculosis*, *C. resistens*, *C. striatum*, *C. tuberculostearicum*, *C. ulcerans*, and *C. urealyticum*, have been determined at the time of writing.

Gram staining of corynebacteria shows slightly curved, Gram-positive rods with sides not parallel and sometimes

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TABLE 1 Some chemotaxonomic features of the bacteria covered in this chapter

Genus	Major CFAs	Mycolic acids	Peptidoglycan diamino acid[a]	Acyl type
Corynebacterium	18:1ω9c, 16:0, 18:0	+[b]	*m*-DAP	Acetyl
Turicella	18:1ω9c, 16:0, 18:0	–	*m*-DAP	Glycolyl
Arthrobacter	15:0*ai*, 17:0*ai*, 15:0*i*	–	LYS	Acetyl
Brevibacterium	15:0*ai*, 17:0*ai*, 15:0*i*	–	*m*-DAP	Acetyl
Dermabacter	17:0*ai*, 15:0*ai*, 16:0*i*	–	*m*-DAP	ND[c]
Helcobacillus	17:0*ai*, 15:0*ai*, 16:0*i*	–	*m*-DAP	ND
Rothia	15:0*ai*, 17:0*ai*, 16:0*i*	–	LYS	ND
Exiguobacterium	17:0*ai*, 15:0*ai*, 16:0, 13:0*i*	–	LYS	ND
Oerskovia	15:0*ai*, 15:0*i*, 17:0*ai*	–	LYS	Acetyl
Cellulomonas	15:0*ai*, 16:0, 17:0*ai*	–	ORN	Acetyl
Cellulosimicrobium	15:0*ai*, 15:0*i*, 17:0*ai*	–	LYS	Acetyl
Microbacterium	15:0*ai*, 17:0*ai*, 16:0*i*	–	LYS, ORN	Glycolyl
Curtobacterium	15:0*ai*, 17:0*ai*, 16:0*i*	–	ORN	Acetyl
Leifsonia	17:0*ai*, 15:0*ai*, 16:0*i*	–	DAB	ND
Janibacter	17:1, 16:0*i*, 17:0	–	*m*-DAP	Acetyl
Pseudoclavibacter	15:0*ai*, 17:0*ai*, 16:0*i*, 16:0	–	DAB	Acetyl
Brachybacterium	15:0*ai*, 16:0*i*, 17:0*ai*	–	*m*-DAP	Acetyl
Knoellia	17:1*i*, 15:0*i*, 16:0*i*, 17:0*i*	–	*m*-DAP	Acetyl
Auritidibacter	17:0*ai*, 15:0*i*, 16:0*i*, 15:0*ai*	–	LYS	ND
Arcanobacterium	18:1ω9c, 16:0, 18:0	–	LYS	Acetyl
Trueperella	18:1ω9c, 16:0, 18:0, 14:0	–	LYS	Acetyl
Gardnerella	16:0, 18:1ω9c, 14:0	–	LYS	ND

[a] *m*-DAP, *meso*-diaminopimelic acid; LYS, lysine; ORN, ornithine; DAB, diaminobutyric acid.
[b] Exceptions: *C. amycolatum*, *C. atypicum*, and *C. kroppenstedtii*.
[c] ND, no data.

slightly wider ends, giving some of the bacteria a typical club shape (Fig. 1a, 1e, 1g). Corynebacteria whose morphologies differ from this morphology include *C. canis*, *C. durum*, *C. matruchotii* (Fig. 1f), and *C. sundsvallense* (see below under each species). Cells generally stain evenly. If *Corynebacterium* cells are taken from fluid media, they are arranged as single cells, in pairs, in V forms, in palisades, or in clusters with a so-called Chinese letter appearance. It is again emphasized that the club-shaped form of the rods is observed only for true *Corynebacterium* spp. All medically relevant taxa in the genus *Corynebacterium* are catalase positive and nonmotile, and the genus *Corynebacterium* includes both fermenting and nonfermenting species.

Genus *Turicella*

The genus *Turicella* is phylogenetically closely related to genus *Corynebacterium* but contains *T. otitidis* as the only species. The cell wall contains *m*-DAP, but mycolic acids are not present (7). The main CFAs for *T. otitidis* are the same as those for *Corynebacterium* spp., but all *T. otitidis* strains also contain significant amounts of TBSA (2 to 10% of all CFAs) (7). The G+C content varies between 65 and 72 mol%.

Gram staining shows relatively long Gram-positive rods (Fig. 1b). *T. otitidis* is catalase positive, nonmotile, and an oxidizer.

Genus *Arthrobacter*

The genera *Arthrobacter* and *Micrococcus* are so closely related phylogenetically that it has been stated that micrococci are, in fact, arthrobacters which are unable to express rod forms (8). Presently, the genus *Arthrobacter* contains at least 70 species, of which only a few have been recovered from human clinical specimens (24). Lysine is the diamino acid of the cell wall, and $C_{15:0ai}$ is the overall dominating CFA which represents more than 50% of all CFAs in most *Arthrobacter* species. The G+C content varies between 59 and 70 mol%, indicating the diversity within this genus.

Gram staining may demonstrate a rod-coccus cycle (i.e., rod forms in younger cultures and cocci in older colonies) when cells are grown on rich media (e.g., Columbia base agar). Jointed rods (i.e., rods in a rectangular form, which contributed to the designation of this genus as “arthros” meaning “joint” in ancient Greek) may also be observed in younger cultures (i.e., after 24 h) but may not be demonstrable for every species. Arthrobacters are catalase positive, motility is variable, and they are always oxidizers.

Genus *Brevibacterium*

The genus *Brevibacterium* presently comprises 30 species, of which 9 species are medically relevant. *m*-DAP is the diamino acid type found in the cell wall. $C_{15:0ai}$ and $C_{17:0ai}$ usually represent more than 75% of all CFAs (25). The G+C content varies between 60 and 70 mol%.

Gram staining demonstrates relatively short rods which may develop into cocci when cultures are getting older (after 3 days). Brevibacteria are catalase positive, nonmotile, and oxidizers.

Genus *Dermabacter*

The genus *Dermabacter* presently comprises only one species, *D. hominis*. *m*-DAP is the diamino acid of the cell wall, and

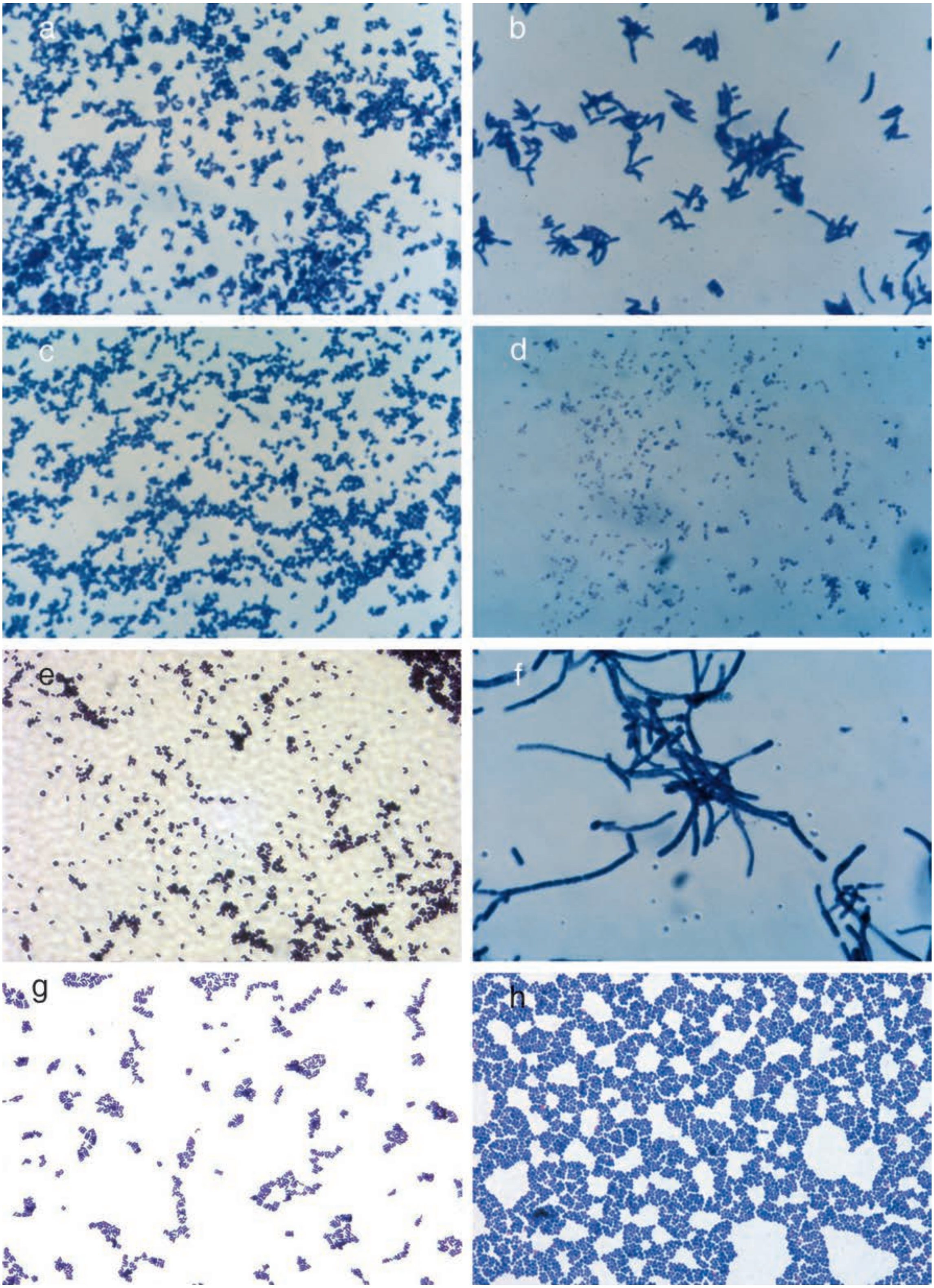

FIGURE 1 Gram stain morphologies of *Corynebacterium diphtheriae* ATCC 14779 after 48 h of incubation (a), *Turicella otitidis* DSM 8821 (48 h) (b), *Dermabacter hominis* ATCC 51325 (48 h) (c), *Gardnerella vaginalis* ATCC 14018 (48 h) (d), *Corynebacterium amycolatum* HC-NML 92-0507 (24 h) (e), *Corynebacterium matruchotii* ATCC 14266 (24 h) (f), *Corynebacterium coyleae* HC-NML 98-0068 (24 h) (g), and a black-pigmented *Rothia dentocariosa* HC-NML 77-0298 (24 h) (h). doi:10.1128/9781555817381.ch28.f1

$C_{15:0ai}$ and $C_{17:0ai}$ usually account for 40 to 60% of all CFAs. The G+C content range is between 60 and 62 mol% (26). Gram staining shows very short rods (Fig. 1c) which are often initially misinterpreted as cocci. *D. hominis* strains are catalase positive, nonmotile, and glucose fermenters.

Genus *Helcobacillus*

The genus *Helcobacillus*, part of the family *Dermabacteraceae*, has been described for an isolate recovered from a patient with a cutaneous discharge presenting with an erythrasma. Interestingly, phenotypic features of the bacterium were initially thought to be suggestive of *C. minutissimum* but later, using a polyphasic approach, of *D. hominis*. The Gram stain shows Gram-positive, straight, short (0.7 to 1.0 µm in length by 0.4 to 0.7 µm in diameter), irregular rods. Colonies are apigmented. The type strain is catalase positive, nonmotile, and a glucose fermenter. This taxon is amycolated, with a G+C content of 68.6 mol%, *m*-DAP as the cell wall diamino acid, and with CFAs $C_{15:0ai}$, $C_{17:0ai}$, and $C_{16:0i}$ (together ~70% of the total) predominating (27).

Genus *Rothia*

For didactical reasons, the genus *Rothia* is also included in this chapter because some species are rod-like. The genus *Rothia* belongs to the family *Micrococcaceae*. Collins and colleagues reclassified *Stomatococcus mucilaginosus* as *Rothia mucilaginosa* (10). Since *R. mucilaginosa* exhibits coccoid forms in the Gram stain, the genus *Rothia* is also covered in chapter 21 (on the catalase-positive, Gram-positive cocci). However, the species *R. dentocariosa* exhibits mainly but not solely (Fig. 1h) rod forms and is, therefore, covered in this chapter on coryneform bacteria. The occasional pathogen *R. aeria* has mainly coccoidal or coccobacillary forms (11).

Lysine is the diamino acid of the cell wall, and $C_{15:0ai}$ and $C_{17:0ai}$ usually represent 40 to 60% of all CFAs. The G+C content ranges between 47 and 56 mol%. *Rothia* strains can be quite pleomorphic by Gram staining, but filamentous forms are normally not observed. They have a variable catalase reaction, are nonmotile, and exhibit a fermentative metabolism.

Genus *Auritidibacter*

The genus *Auritidibacter* contains one species, *A. ignavus*, recovered from the ear, which can be observed to have a rod-coccus cycle. Lysine is the diamino acid of the cell wall, with CFAs $C_{15:0ai}$ and $C_{17:0ai}$ predominating, and the G+C content is about 60% (12). A novel second species within this genus has been observed which is close but distinguishable from *A. ignavus* (28). Both taxa are minimally reactive biochemically, are motile only if the hanging drop method is used, and are catalase positive but oxidase negative.

Genus *Exiguobacterium*

Presently, 14 species are included in the genus *Exiguobacterium*, of which only *E. acetylicum* and *E. aurantiacum* have been mentioned in publications as being isolated from human clinical material. Lysine is the diamino acid of the cell wall, and $C_{15:0ai}$ and $C_{17:0ai}$ comprise only about 30 to 40% of the total CFAs. *E. acetylicum* contains significant amounts of $C_{13:0}$ and $C_{13:0ai}$, which are not found in any other coryneform taxon (21). The G+C content is about 47 mol%.

Exiguobacteria present as relatively short rods in young cultures. Strains are catalase positive and motile and have a fermentative metabolism.

Genus *Oerskovia*

In older textbooks, oerskoviae were assigned to the nocardioform group of organisms due to their morphological features. This includes branching vegetative substrate hyphae, penetration into agar, but no aerial hyphae. Current phylogenetic and chemotaxonomic evidence suggests that *Oerskovia*, including the reclassified *Promicromonospora*, is more closely related to genera like *Cellulomonas* than to the mycolic acid-containing genera like *Nocardia*. Representatives of the type species, *O. turbata*, were recovered from soil, but human pathogens originally identified as *O. turbata* have now been placed in *Cellulosimicrobium funkei* (15). Lysine is the diamino acid of the cell wall, and $C_{15:0ai}$ is the main CFA in oerskoviae. The G+C content is 70 to 75 mol%.

Gram staining shows coccoid to rod-shaped bacteria which originate from the breaking up of mycelia. Oerskoviae strains are catalase positive, motility is variable, and they are fermentative.

Genus *Cellulomonas*

The genus *Cellulomonas* presently comprises 23 species of which only *C. hominis* and *C. denverensis* have been described as being isolated from humans (13, 29). Ornithine is the diamino acid of the cell wall, and $C_{15:0ai}$ and $C_{16:0}$ are the main CFAs. The G+C content is 71 to 76 mol%.

Gram staining shows small, thin rods. All *Cellulomonas* spp., except *C. fermentans* and *C. humilata*, are catalase positive, their motility is variable, the environmental *Cellulomonas* strains are cellulolytic (whereas *C. hominis* did not hydrolyze cellulose in the test system used) (13), and they have a fermentative metabolism.

Genus *Cellulosimicrobium*

The genus *Cellulosimicrobium* presently comprises three species. The medically relevant species, *C. funkei* and *C. cellulans*, had been designated *Cellulomonas cellulans* or *Oerskovia xanthineolytica* in the past (14). The reason for removing *C. cellulans* from the genus *Cellulomonas* was that the topology of the 16S rRNA gene dendrogram indicated that the branching point of this taxon was outside *Cellulomonas* proper. In addition, the chemotaxonomic characteristic of lysine as diamino acid supported reclassification. Predominant CFAs include $C_{15:0ai}$, $C_{15:0i}$, $C_{16:0i}$, and $C_{16:0}$. The major menaquinone (MK) is MK-9(H_4), and the G+C content is 74 mol%. It should be noted that the genus *Cellulosimicrobium* is related to the genus *Oerskovia* but nevertheless distinct.

In young cultures, a mycelium is produced that fragments later into irregular, curved and club-shaped rods. Catalase activity is detected, and strains are motile or nonmotile. All strains have a fermentative metabolism.

Genus *Microbacterium*

The genera *Microbacterium* and *Aureobacterium* were found to be phylogenetically intermixed and were unified in a redefined genus *Microbacterium* (30).

Now, over 80 *Microbacterium* species have been validly named, but only a minority of them has been demonstrated to be of clinical importance (31). Microbacteria are most frequently encountered in environmental specimens (e.g., soil). $C_{15:0ai}$ and $C_{17:0ai}$ are the two main CFAs, often representing up to 75% of the total CFAs (30, 32, 33). The G+C content of *Microbacterium* spp. is 65 to 76 mol%, indicating the diversity within the genus.

Gram staining often shows thin or short rods with no branching. Catalase activity and motility are variable. Microbacteria can be either fermenters or oxidizers.

Genus *Curtobacterium*

Curtobacterium spp., like microbacteria, belong to the peptidoglycan type B actinomycetes (i.e., cross-linkage between positions 2 and 4 of the two peptide subunits). Ornithine is the diamino acid and the only amino acid composing the interpeptide bridge. Curtobacteria have an acetyl peptidoglycan acyl type and MK-9 as the major MK, whereas microbacteria possess a glycolyl type and MK-11 and -12 (Table 1). For most curtobacteria, $C_{15:0ai}$ and $C_{17:0ai}$ represent more than 75% of all CFAs (34). The G+C contents range from 68 to 75 mol%. Presently, nine *Curtobacterium* species are validly described.

Gram staining shows small and short rods with no branching. Catalase activity is positive, motility is observed in most strains, and all strains show a respiratory metabolism which proceeds slowly in oxidizing carbohydrates.

Genus *Leifsonia*

The former "*Corynebacterium aquaticum*" had been transferred into the genus *Leifsonia* as *L. aquatica* (35) and is the only medically relevant species in this genus. *L. aquatica* strains belong to the peptidoglycan B type actinomycetes and, therefore, cannot be true corynebacteria, which actually possess an A type of peptidoglycan (i.e., cross-linkage between positions 3 and 4 of the two peptide subunits). Diaminobutyric acid is the diamino acid of the cell wall peptidoglycan, and $C_{15:0ai}$ and $C_{17:0ai}$ are the main CFAs, similar to microbacteria, but represent <75% of all CFAs (32). The G+C content is about 70 mol%.

Gram staining shows thin rods. The strains are catalase and oxidase positive (the latter is an untypical feature for coryneform bacteria), always motile, and oxidizers.

Other Unusual Coryneforms

Examination of coryneform bacteria using primarily sequence-based identification approaches has shown that additional genera could be recovered from human clinical materials.

Genus *Janibacter*

Strains of the genus *Janibacter* (36) were found to be associated with bacteremia (37, 38; K. A. Bernard, unpublished data), the first medically relevant coryneforms reported from the family *Intrasporangiaceae*. *Janibacter* strains can have Gram-variable or Gram-positive coccoidal to rod forms in singles, pairs, or irregular clumps. DNA base composition is 69 to 73 mol% G+C, with an unusual CFA profile consisting of significant volumes of CFAs $C_{16:0i}$, $C_{17:1}$, and $C_{17:0}$. These bacteria are described as oxidizers, with white, creamy, or yellowish pigments. They are nonmotile, and optimal growth may occur at 25 to 30°C.

Genus *Pseudoclavibacter*

The genus *Pseudoclavibacter* was first described in 2004 to accommodate the misidentified type strain of "*Brevibacterium helvolum*" (39). *P. alba* strains were recovered from urine and *P. bifida* from blood cultures and wounds. By Gram staining, these species were short or medium Gram-positive rods, with *P. bifida* demonstrating some rudimentary branching. The DNA base composition is 62 to 68 mol% G+C. Major CFAs are $C_{15:0ai}$, $C_{16:0}$, $C_{16:0i}$, and $C_{17:0ai}$. These strains are oxidizers, with white or yellowish colonies. Optimal growth occurs at 30°C.

Genera *Brachybacterium* and *Knoellia*

Isolates from the genus *Brachybacterium* and one strain from the genus *Knoellia*, all recovered from blood cultures, have been characterized (Bernard, unpublished). There are currently 14 species in the genus *Brachybacterium*, which is part of the family *Dermabacteraceae*, and so they are most closely related to the genus *Dermabacter*. Members of this genus grow at 37°C, exhibit Gram-positive coccoidal and rod-like forms, and have a G+C content of 68 to 73 mol%. The *Brachybacterium* blood culture isolates were metabolically fermentative and had branched-chained type CFAs. The genus *Knoellia*, like that of the genus *Janibacter*, is a member of the family *Intrasporangiaceae*. Cells are irregular Gram-positive rods or cocci, with major CFAs of the branched-chain type, and the G+C content is 68 to 69 mol%. The single *Knoellia* blood culture isolate was capnophilic, growing best in 5% CO_2 at 37°C.

Genera *Arcanobacterium* and *Trueperella*

The genus *Arcanobacterium* presently contains six species, of which only *A. haemolyticum* is recovered from human clinical specimens. The main CFAs of arcanobacteria are $C_{16:0}$, $C_{18:1\omega9c}$, and $C_{18:0}$ (as in *Corynebacterium* spp. and *T. otitidis*), but in contrast to corynebacteria, significant amounts of $C_{10:0}$, $C_{12:0}$, and $C_{14:0}$ may also be detected (21). The genus *Trueperella* comprises five species, of which *T. bernardiae* (formerly *A. bernardiae*) and *T. pyogenes* (formerly *A. pyogenes*) can be detected in human clinical specimens (40).

Gram staining of *A. haemolyticum* and *Trueperella* spp. shows irregular Gram-positive rods. They are all catalase negative, nonmotile, and fermenters. *Arcanobacterium* species are reverse CAMP or CAMP test positive, whereas *Trueperella* species are not (40).

EPIDEMIOLOGY AND TRANSMISSION

Many species of the corynebacteria are part of the microbiome of the skin and mucous membranes in humans and mammals. The habitat for some medically irrelevant corynebacteria is the environment. It is noteworthy that not all corynebacteria are equally distributed over skin and mucous membranes, but many of them occupy a specific niche. *C. diphtheriae* can be isolated from the nasopharynx as well as from skin lesions, which actually represent a reservoir for the spread of diphtheria. Important opportunistic pathogens like *C. amycolatum*, *C. striatum*, and *D. hominis* are part of the human skin microbiome but have thus far not been recovered from throat swabs from healthy individuals (41). Coryneform bacteria prominent in the oropharynx include *C. durum* and *R. dentocariosa* (41). *Corynebacterium auris* and *T. otitidis* seem to have an almost exclusive preference for the external auditory canal (42). In nearly every instance that *Corynebacterium macginleyi* has been isolated, it has been recovered from eye specimens (43). Another *Corynebacterium* species with a distinctive niche is *C. glucuronolyticum*, which is almost exclusively isolated from genitourinary specimens from males (44) and also from animals (45). *C. urealyticum*, another genitourinary pathogen, has, like *Corynebacterium jeikeium*, also been cultured from the inanimate hospital environment.

The natural habitat of arcanobacteria is not fully understood, but *A. haemolyticum* is recovered from throat as well as from wound swabs, whereas *Trueperella bernardiae* has mainly been found in abscesses adjacent to skin (G. Funke and K. Bernard, unpublished data). It is unclear whether the two species are part of the skin and/or the gastrointestinal microbiome. *Trueperella pyogenes* is found on mucous membranes of cattle, sheep, and swine. Brevibacteria can be found on dairy products (e.g., cheese) but are also inhabitants of the human skin (25). Arthrobacters are some of the most frequently isolated bacteria when soil samples are cultured, but at least *Arthrobacter cumminsii* also seems to be present on human skin (24, 46). Auritidibacters have been recovered from ear specimens (12, 28). Members of the genera *Exiguobacterium*, *Oerskovia*, *Cellulomonas*, *Cellulosimicrobium*, and *Microbacterium* have their habitats in the inanimate environment (e.g., soil and activated sludge). *Microbacterium* spp. have also been recovered from hospital environments (33). Curtobacteria are primarily plant pathogens (34).

CLINICAL SIGNIFICANCE

Estimating the clinical significance of coryneform bacteria isolated from clinical specimens is often confusing for clinical microbiologists. This is in part due to the natural habitat of coryneform bacteria, which may lead to their recovery if specimens were not taken correctly. The reader is referred to the guidelines on minimal microbiological requirements in publications on disease associations of coryneform bacteria (47).

Coryneform bacteria should be identified to the species level if they are isolated (i) from normally sterile body sites, e.g., blood (except if only one of multiple specimens became positive), (ii) from adequately collected clinical material if they are the predominant organisms, and (iii) from urine specimens if they are the only bacteria encountered and the bacterial count is $>10^4$/ml or if they are the predominant organisms and the total bacterial count is $>10^5$/ml.

The clinical significance of coryneform bacteria is strengthened by the following findings: (i) multiple specimens are positive for the same coryneform bacteria, (ii) coryneform bacteria are seen in the direct Gram stain and a strong leukocyte reaction is also observed, and (iii) other organisms recovered from the same material are of low pathogenicity.

For a comprehensive summary of case reports on individual coryneform bacteria, the reader is referred to review articles (2, 47, 48). The most frequently reported coryneforms as well as their established disease associations are listed in Table 2.

Historically, diphtheria caused by *C. diphtheriae* (or *C. ulcerans*) is the most prominent infectious disease for which coryneform bacteria are responsible. Therefore, special attention is given to that disease in this chapter; a comprehensive review on diphtheria has been recently published (49). Due to immunization programs, the disease has nearly disappeared in countries with high socioeconomic standards. However, the disease is still endemic in some subtropical and tropical countries as well as among individuals of certain

TABLE 2 Most frequently reported disease associations of coryneform bacteria in humans

Taxon	Disease or disease association
C. amycolatum	Wound infections, foreign body infections, bacteremia, sepsis, urinary tract infections
C. aurimucosum	Genitourinary tract infections (mainly females)
CDC group F-1	Urinary tract infections
C. diphtheriae (toxigenic)	Throat diphtheria, cutaneous diphtheria
C. diphtheriae (nontoxigenic)	Endocarditis, foreign body infections, pharyngitis
C. glucuronolyticum	Genitourinary tract infections (mainly males)
C. jeikeium	Endocarditis, bacteremia, foreign body infections, wound infections
C. kroppenstedtii	Granulomatous lobular mastitis
C. macginleyi	Eye infections
C. minutissimum	Wound infections, urinary tract infections, respiratory tract infections
C. pseudodiphtheriticum	Respiratory tract infections, endocarditis
C. pseudotuberculosis	Lymphadenitis (occupational)
C. resistens	Bacteremia
C. riegelii	Urinary tract infections (females)
C. striatum	Wound infections, respiratory tract infections, foreign body infections, respiratory tract infections
C. tuberculostearicum	Catheter infections, bacteremia, endocarditis, wound infections
C. ulcerans (toxigenic)	Respiratory diphtheria, cutaneous infections
C. urealyticum	Urinary tract infections, bacteremia, wound infections
Arthrobacter spp.	Bacteremia, foreign body infections, urinary tract infections
Brevibacterium spp.	Bacteremia, foreign body infections, malodorous feet
Dermabacter hominis	Wound infections, bacteremia
Helcobacillus sp.	Cutaneous infection with erythrasma
Rothia spp.	Endocarditis, bacteremia, respiratory tract infections
Cellulomonas spp.	Bacteremia, wound infections, cholecystitis
Cellulosimicrobium sp.	Foreign body infections, bacteremia
Microbacterium spp.	Bacteremia, foreign body infections, wound infections
A. haemolyticum	Pharyngitis in older children/young adults, wound and tissue infections
T. bernardiae	Abscess formation (together with mixed anaerobic flora)
T. pyogenes	Abscess formation, wound and soft tissue infections
G. vaginalis	Bacterial vaginosis, endometritis, postpartum sepsis

ethnic groups (e.g., indigenous peoples in the Americas and Australia). In the 1990s, diphtheria reemerged in the states of the former Soviet Union with a decline in the next decade. However, despite increased global travel activities, only a few imported cases have been reported by countries with well-developed health care systems.

The main manifestation of diphtheria is an upper respiratory tract illness with a sore throat, dysphagia, lymphadenitis, low-grade fever, malaise, and headache. A nasopharyngeal adherent membrane which may occasionally lead to obstruction is characteristic. The severe systemic effects of diphtheria include myocarditis, neuritis, and kidney damage caused by the *C. diphtheriae* exotoxin, which is encoded by a bacteriophage carrying the *tox* gene. *C. diphtheriae* may also cause cutaneous diphtheria or endocarditis (with either toxin-positive or toxin-negative strains). Some people with poor hygienic standards (e.g., drug and alcohol abusers) are prone to colonization (on the skin more often than in the pharynx) or, rarely, significant infection by *C. diphtheriae* strains, which are nontoxigenic (50).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

In general, coryneform bacteria do not need special handling when samples are collected.

C. diphtheriae

The diagnosis of diphtheria is primarily a clinical one. The physician should notify the receiving laboratory immediately of suspected diphtheria. In case of respiratory diphtheria, material for culture should be obtained on a swab (either a cotton- or a polyester-tipped swab) from the inflamed areas in the nasopharynx. Multisite sampling (nasopharynx) is thought to increase sensitivity. If membranes are present and can be removed (swabs from beneath the membrane are most valuable), they should also be sent to the microbiology laboratory (although *C. diphtheriae* might not be culturable from those in every instance). Nasopharyngeal swabs should be obtained from suspected carriers. It is preferable that the swabs are immediately transferred to the microbiology laboratory for culturing. If the swabs must be sent to the laboratory, semisolid transport media (e.g., Amies) ensure the maintenance of the bacteria. All coryneform bacteria are relatively resistant to drying and moderate temperature changes. Material from patients with suspected cases of wound diphtheria can be obtained by swab or aspiration.

Long-term preservation in skim milk at −70°C is applicable to all coryneform bacteria. The same skim milk tube, except for those containing lipophilic corynebacteria, can be thawed and put into the freezer again, and this can be done several times (G. Funke, unpublished data). For both nonlipophilic and lipophilic coryneforms, good results were observed with Microbank tubes (Pro Lab Diagnostics, Austin, TX) (Funke, unpublished). The advantage of using these tubes is that individual beads can be taken out of the tube. Coryneform bacteria can also be stored for decades when they are kept lyophilized in an appropriate medium (e.g., 0.9% NaCl containing 2% bovine serum albumin).

DIRECT EXAMINATION

After the appropriate isolation media have been inoculated (see below under "Isolation Procedures"), the swabs taken from diphtheritic membranes may be subjected to Neisser or Loeffler methylene blue staining (positive if metachromatic granules [polar bodies] are seen). However, it is noteworthy that the sensitivity of the microscopic examination is limited.

Antigen assays for the direct detection of coryneform bacteria are not recommended.

As described previously, *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* are the only species able to harbor the bacteriophage which carries the diphtheria *tox* gene and potentially produce diphtheria toxin. PCR-based, direct detection systems for the diphtheria *tox* gene have been described, using conventional methods to detect fragment A and/or the entire *tox* gene (51, 52) or fragment A and B subunits of the *tox* gene (53). The system described by Nakao et al. had the highest sensitivity when Dacron polyester-tipped swabs were used and when silica gel packages were stored at 4°C rather than at room temperature. Conventional PCR detection of the regulatory *dtxR* gene has been evaluated (54). Detection of the *tox* gene using a real-time platform has been outlined (55–57). Real-time detection using primers targeting the *C. diphtheriae tox* gene for *C. ulcerans* strains has required modifications (56–58). Direct detection of the diphtheria *tox* or *dtxR* as the sole test of clinical specimens has not been recommended, as expression of diphtheria toxin must be demonstrated, so microbiological culture is essential for confirming diphtheria (59).

ISOLATION PROCEDURES

Coryneform bacteria including *C. diphtheriae* can be readily isolated from a 5% sheep blood agar (SBA)-based selective medium containing 100 μg of fosfomycin per ml (plus 12.5 μg of glucose-6-phosphate per ml), since nearly all coryneforms (except *Actinomyces* spp. and *D. hominis*) are highly resistant to this compound (60). It is also possible to put disks containing 50 μg of fosfomycin (plus 50 μg of glucose-6-phosphate [already incorporated in the disk]) (BD Diagnostics, Sparks, MD) on an SBA or a colistin-nalidixic acid (CNA) plate and then examine the colonies which grow around the disk. Selective media for coryneform bacteria containing 50 to 100 μg of furazolidone (Sigma, St. Louis, MO)/ml have also been described. If lipophilic corynebacteria like *C. jeikeium* or *C. urealyticum* are sought, then 0.1 to 1.0% Tween 80 (Merck, Darmstadt, Germany) could be added to an SBA plate (add Tween 80 before pouring the medium). Additional methods to demonstrate lipophilia are described below. Medically relevant coryneforms described to date do not grow on MacConkey agar. However, if "coryneform" bacteria are recovered from this medium, they should be examined carefully to rule out rapidly growing mycobacteria.

With very few exceptions (some arthrobacters, microbacteria, and curtobacteria, which have optimal growth temperatures of between 30 and 35°C), the medically relevant coryneform bacteria all grow at 37°C. It is desirable to culture specimens for coryneform bacteria in a CO_2-enriched atmosphere, since some taxa, e.g., *Rothia*, *Arcanobacterium*, and *Trueperella*, grow much better under those conditions. Nearly all medically relevant coryneform bacteria grow within 48 h, so primary culture plates should not be incubated longer than that. However, if liquid media are used (e.g., for specimens from normally sterile body sites), these should be checked after 5 days by Gram staining for the presence of coryneform bacteria (only if growth is observed with the naked eye) before they are discarded.

It is recommended that urine specimens be incubated for longer than 24 h to check for the presence of *C. urealyti-*

cum but only when patients are symptomatic or have alkaline urine or struvite crystals in their urine sediment.

C. diphtheriae

The primary plating media for the cultivation of *C. diphtheriae* should be SBA plus one selective medium (e.g., cystine-tellurite blood agar [CTBA] or freshly prepared Tinsdale medium) (59, 61). If silica gel is used as a transport medium, the desiccated swabs need to be additionally incubated overnight in broth (supplemented with either plasma or blood), which should then be streaked onto the primary plating medium. The plates are read after 18 to 24 h of incubation at 37°C, preferably in a 5% CO_2-enriched atmosphere. Tellurite inhibits the growth of many noncoryneform bacteria, but even a few *C. diphtheriae* strains are sensitive to potassium tellurite and will therefore not grow on CTBA but may grow on SBA. It is noteworthy that growth on CTBA and tellurite reduction are not specific for *C. diphtheriae*, since many other coryneforms may also produce black (albeit smaller) colonies. The best medium for direct culturing of *C. diphtheriae* is probably Tinsdale medium (61). However, the limitations of Tinsdale medium are its relatively short shelf life (<4 weeks) and the necessity to add horse serum to it. On Tinsdale plates, both tellurite reductase activity (as shown by black colonies) and cystinase activity (as shown by a brown halo around the colonies) can be observed. If neither CTBA nor Tinsdale medium is available, CNA plates are recommended for the isolation of *C. diphtheriae* or any other coryneform bacterium. It is necessary to pick multiple colonies from CNA plates to rule out *C. diphtheriae* (first Gram staining, then subculturing, and subsequent biochemical testing or direct application of matrix-assisted laser desorption ionization–time of flight [MALDI-TOF] mass spectrometry on colonies). Nonselective Loeffler serum slants are no longer recommended for the primary isolation of *C. diphtheriae* because of overgrowth by other bacteria.

IDENTIFICATION

Basic tests available in every microbiology laboratory are of great value for the identification of coryneform bacteria. The Gram staining morphology of the cells can exclude the assignment to many genera and may even lead to the assignment to the correct genus (e.g., to the genus *Corynebacterium*, *Turicella*, or *Dermabacter*) (Fig. 1). Morphology, size, pigment, odor, and hemolysis of colonies are also valuable criteria in the differential diagnosis of coryneform bacteria.

von Graevenitz and Funke (62) had outlined a biochemical identification system for coryneform bacteria which was based on previous results from the Centers for Disease Control's Special Bacteriology Reference Laboratory (63). This system includes the following reactions: catalase; test for fermentation or oxidation (in our experience, this is best observed in semisolid cystine Trypticase agar medium [rather than on triple sugar iron or oxidation-fermentation media], with fermentation indicated by acid or alkali production in the entire tube and oxidation found at the surface of the tube); motility; nitrate reduction (24-h incubation); urea hydrolysis (24-h incubation); esculin hydrolysis (up to 48 h of incubation); acid production from glucose, maltose, sucrose, mannitol, and xylose (48-h incubation); CAMP reaction (24-h incubation) with a β-hemolysin-producing strain of *Staphylococcus aureus* (e.g., strain ATCC 25923), i.e., positive reaction indicated by an augmentation of the effect of *S. aureus* β-hemolysin on erythrocytes, resulting in a complete hemolysis in an arrowhead configuration (Fig. 2); and a test for lipophilia (24-h incubation), which is performed only for catalase-positive colonies <0.5 mm in diameter. For the test for lipophilia, colonies are subcultured onto ordinary SBA and onto a 0.1 to 1% Tween 80-containing SBA plate. Lipophilic corynebacteria develop colonies up to 2 mm in diameter after 24 h on Tween-supplemented agar. It has also been suggested that growth in brain heart infusion broth with and without supplementation of 1% vol/vol of sterile Tween 80 be compared, and strains which grow only in the supplemented broth can be called lipophilic. The identification protocols given in this chapter are, in principle, based on the identification system of von Graevenitz and Funke (62) (Tables 3 and 4).

Manually performed identification panels include the API (formerly RAPID) Coryne system (bioMérieux, Marcy l'Etoile, France) and the RapID CB Plus system (Remel, Lenexa, KS) which are widely used. The API Coryne system contains 50 taxa in its present database (version 3.0), and comparison to an online database, APIWEB, is available (https://apiweb.biomerieux.com). In a 1996 study, it was found that 90.5% of the strains belonging to the taxa included were correctly identified, with additional tests needed for correct identification for 55.1% of all strains tested (64). Similar results were

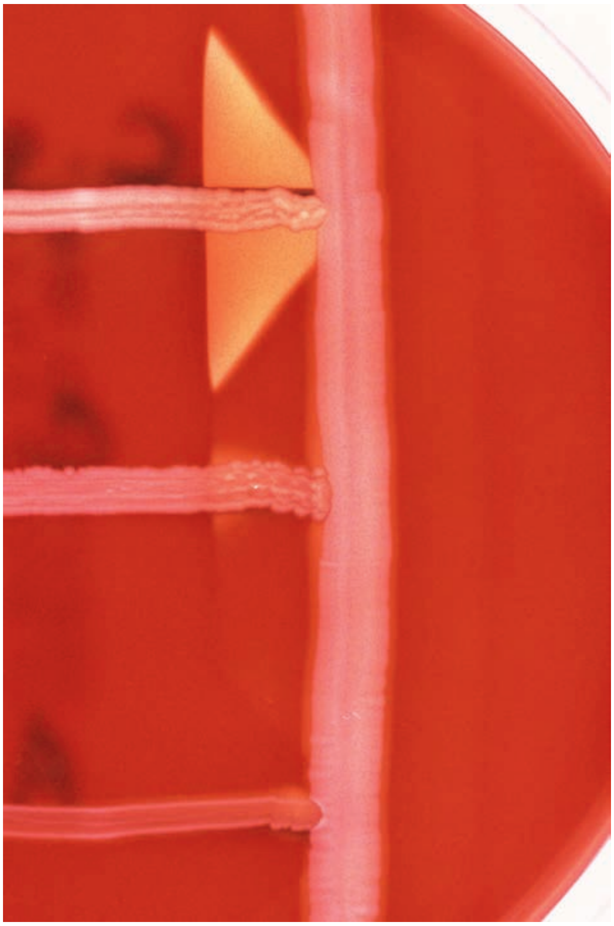

FIGURE 2 CAMP reactions of different coryneform bacteria after 24 h. (Top) *C. glucuronolyticum* DMMZ 891 (positive reaction); (Middle) *C. diphtheriae* ATCC 14779 (negative reaction); (Bottom) *A. haemolyticum* ATCC 9345 (CAMP inhibition reaction). The vertical streak is *S. aureus* ATCC 25923. doi:10.1128/9781555817381.ch28.f2

TABLE 3 Identification of medically relevant *Corynebacterium* spp.[a]

Species	Fermentation/ oxidation	Lipophilism	Nitrate reduction	Urease	Esculin hydrolysis	Pyrazinamidase	Alkaline phosphatase	Acid production from:					CAMP reaction	Other traits
								Glucose	Maltose	Sucrose	Mannitol	Xylose		
C. *accolens*	F	+	+	−	−	V	−	+	−	V	V	−	−	
C. *afermentans* subsp. *afermentans*	O	−	−	−	−	+	+	−	−	−	−	−	V	
C. *afermentans* subsp. *lipophilum*	O	+	−	−	−	+	+	−	−	−	−	−	V	
C. *amycolatum*	F	−	V	V	−	+	+	+	V	V	−	−	−	Most O/129 resistant, propionic detected
C. *appendicis*	F	+	−	+	−	+	+	+	+	−	−	−	ND	Large volume of TBSA present
C. *aquatimens*	O	+	−	−	−	+	+	+	−	−	−	−	−	
C. *argentoratense*	F	−	−	−	−	+	V	+	−	−	−	−	−	Chymotrypsin may be positive, propionic detected[b]
C. *atypicum*	F	−	−	−	−	−	−	+	+	+	−	−	ND	Pinpoint colonies, β-glucuronidase positive
C. *aurimucosum*	F	−	−	−	V	+	+	+	+	+	−	−	ND	Most strains yellowish, some exhibit blackish-gray pigment or pit agar
C. *auris*	O	−	−	−	−	+	+	−	−	−	−	−	+	Slight adherence to agar, cleaved mycolics
C. *bovis*[c]	F	+	−	−	−	−	+	+	−	−	−	−	−	TBSA positive, fructose positive
C. *canis*	F	−	+	−	+	+	+	+	+	+	−	−	−	Long rods, α-glucosidase positive, trypsin positive
C. *confusum*	F	−	+	−	−	+	+	(+)	−	−	−	−	−	Tyrosine negative, TBSA positive, propionic detected
C. *coyleae*	F	−	−	−	−	+	+	(+)	−	−	−	−	+	
CDC group F−1	F	+	V	+	−	+	−	+	+	+	−	−	−	
C. *diphtheriae* biotype gravis	F	−	+	−	−	−	−	+	+	−	−	−	−	Glycogen positive, propionic detected
C. *diphtheriae* biotype intermedius	F	+	+	−	−	−	−	+	+	−	−	−	−	Propionic detected
C. *diphtheriae* biotypes mitis and belfanti	F	−	+/−[d]	−	−	−	−	+	+	−	−	−	−	Glycogen negative, propionic detected
C. *durum*	F	−	+	(V)	(V)	+	−	+	+	+	V	−	−	Adherent to agar, propionic detected
C. *falsenii*	F	−	−	(+)	−	(+)	+	(+)	V	−	−	−	−	Yellowish
C. *freiburgense*	F	−	+	−	+	−	ND	+	+	+	−	−	−	β-Galactosidase positive, "spoke-wheel" colonies, strong adherence to agar
C. *freneyi*	F	−	V	−	−	+	+	+	+	+	−	−	ND	α-Glucosidase positive, grows at 20 and 42°C
C. *glucuronolyticum*	F	−	V	V	V	+	V	+	V	+	−	V	+	β-Glucuronidase positive, propionic detected
C. *hansenii*	F	−	−	−	−	+	−	+	+	+	−	−	ND	Yellow, dry
C. *imitans*	F	−	−	−	−	(+)	+	+	+	(+)	−	−	+	Tyrosine negative, O/129 resistant
C. *jeikeium*	O	+	−	−	−	+	+	+	V	−	−	−	−	Fructose negative, anaerobic growth negative
C. *kroppenstedtii*	F	+	−	−	+	+	−	+	V	+	−	−	−	Lacks mycolates, propionic detected
C. *lipophiloflavum*	(F)	+/−	−	(+)	−	+	+	(+)	−	−	−	−	V	Yellow, cleaved mycolics
C. *macginleyi*	F	+	+	−	−	−	+	+	−	+	V	−	−	
C. *massiliense*	F	−	−	−	−	(+)	(+)	−	−	−	−	−	ND	TBSA positive
C. *matruchotii*	F	−	+	−	V	+	−	+	+	+	−	−	−	"Whip handle" (upon Gram staining), propionic detected
C. *minutissimum*	F	−	−	−	−	+	+	+	+	V	V	−	−	Tyrosine positive
C. *mucifaciens*	O	−	−	−	−	+	+	+	−	V	−	−	−	Very mucoid yellowish colonies

(Continued on next page)

TABLE 3 *(Continued)*

Species	Fermentation/ oxidation	Lipophilism	Nitrate reduction	Urease	Esculin hydrolysis	Pyrazinamidase	Alkaline phosphatase	Acid production from:					CAMP reaction	Other traits
								Glucose	Maltose	Sucrose	Mannitol	Xylose		
C. *pilbarense*	F	−	−	−	−	+	+	+	−	+	−	−	ND	
C. *propinquum*	O	−	+	V	−	V	V	−	−	−	−	−	−	Tyrosine positive
C. *pseudodiphtheriticum*	O	−	+	+	−	+	V	−	−	−	−	−	−	
C. *pseudotuberculosis*	F	−	V	+	−	−	V	+	+	V	−	−	REV	Propionic detected
C. *pyruviciproducens*	F	+	−	−	−	+	+	+	+	+	−	−	+	β-Glucuronidase positive, pyruvic detected
C. *resistens*	F	+	−	−	−	−	+	+	−	−	−	−	−	Slow growth in anaerobic atmosphere
C. *riegelii*	F	−	−	+	−	V	V	−	(+)	−	−	−	−	
C. *simulans*[e]	F	−	+	−	−	V	+	+	−	+	−	−	−	Reduces nitrite
C. *singulare*	F	−	−	+	−	+	+	+	+	+	−	−	−	Tyrosine positive
C. *sputi*	F	−	−	+	−	+	−	+	−	−	−	−	ND	α-Glucosidase positive, TBSA positive
C. *stationis*	F	−	+	+	−	(+)	−	(+)	−	−	−	−	−	Citrate alkalinized; ribose, fructose positive
C. *striatum*	F	−	+	−	−	+	+	+	−	V	−	−	V	Tyrosine positive
C. *sundsvallense*	F	−	−	+	−	V	V	+	+	+	−	−	−	Sticky colonies
C. *thomssenii*	F	−	−	+	−	+	+	+	+	+	−	−	−	*N*-Acetyl-β-glucosaminidase positive, sticky colonies
C. *timonense*	F	−	−	−	(+)	+	+	+	−	−	−	−	ND	Yellow
C. *tuberculostearicum*	F	+	V	−	−	+	V	+	V	V	−	−	−	
C. *tuscaniense*	O	−	−	−	−	+	+	+	+	−	−	−	−	Hippurate positive, tyrosine negative
C. *ulcerans*	F	−	−	+	−	−	+	+	+	−	−	−	REV	Glycogen positive, propionic detected
C. *urealyticum*	O	+	−	+	−	+	V	−	−	−	−	−	−	
C. *ureicelerivorans*	F	+	−	+	−	+	+	+	−	−	−	(+)	ND	Very strong, rapid urease reaction; TBSA detected, hippurate positive
C. *xerosis*	F	−	V	−	−	+	+	+	+	+	−	−	−	O/129 susceptible, propionic not detected

[a] Abbreviations and symbols: F, fermentation; O, oxidation; +, positive; −, negative; V, variable; ( ), delayed or weak reaction; ND, no data; REV, CAMP inhibition reaction.

[b] Propionic acid as a glucose fermentation product.

[c] Blood culture isolate (84) was also ONPG positive, oxidase positive, weakly maltose positive but negative by API Coryne, propionic acid was not detected. β-Galactosidase was not observed using two methods (API Coryne, API Zym). API Coryne code obtained 0101104.

[d] C. *diphtheriae* biotype mitis is nitrate reductase positive, and C. *diphtheriae* biotype belfanti is nitrate reductase negative.

[e] C. *simulans* (123) is a strong nitrite reducer at low and high concentrations; nitrate reduction may appear to be negative unless further tested using zinc dust; one strain was catalase negative (84).

reported in 2006 (65). Reproducible results are best obtained if the manufacturer's recommendations for use were rigorously followed. The RapID CB Plus system is also used and correctly identified 80.9% of the strains to the genus and the species levels and an additional 12.2% to the genus level but with less accurate species designations (66). An updated version of a panel for the automated Vitek system (bioMérieux) has been described (67); however, this system is not recommended because of its limited database. It is always important to question critically the identifications provided by any commercial identification system and to correlate the results with simple basic characteristics such as macroscopic morphology and Gram staining results. Furthermore, it is important to note that, for both commercially available manual identification systems, the databases have not been updated since the end of the1990s and, therefore, the recently described taxa are not covered. Limitations found based on the use of the commercial identification systems have been recently reviewed (2).

A reference laboratory may also use chromatographic techniques for further characterization of coryneform bacteria. The analysis of CFAs by means of gas-liquid chromatography with the Sherlock system (MIDI, Inc., Newark, DE) can be a useful method for the identification of coryneform bacteria but has been replaced in many laboratories by other methods such as molecular genetic investigations (see below). This system is, in general, able to correctly identify coryneform bacteria to the genus level, but identification to the species level is, in most cases, impossible, although the commercial database suggests that it is possible. This is due to the very closely similar CFA profiles being obtained for coryneform bacteria belonging to the same genus (21) and because the quantitative profiles observed strongly depend on the incubation conditions. When a laboratory creates an individual data-

TABLE 4 Identification of medically relevant coryneform bacteria other than *Corynebacterium* spp.[a]

							Acid production from:					
Taxon	Catalase	Fermentation/ oxidation	Motility	Nitrate reduction	Urease	Esculin hydrolysis	Glucose	Maltose	Sucrose	Mannitol	Xylose	Other traits
Turicella otitidis	+	O	–	–	–	–	–	–	–	–	–	CAMP reaction positive, long rods
Arthrobacter spp.	+	O	v	v	v	v	v	v	v	–	–	
Brevibacterium spp.	+	O	–	v	–	–	v	v	v	–	–	Odor cheese-like
Dermabacter hominis	+	F	–	–	–	+	+	+	+	–	v	Small rods
Helcobacillus sp.	+	F	–	+	–	–	+	+	+	+	+	Gelatin, starch hydrolyzed
Rothia dentocariosa	v	F	–	+	–	+	+	+	+	–	–	Some strains adherent, grayish-black pigmented strains exist
Exiguobacterium acetylicum	+	F	+	v	–	+	+	+	+	+	–	Golden-yellow pigment
Oerskovia turbata	+	F	v	+	–	+	+	+	+	–	+	Xanthine not hydrolyzed
Cellulomonas spp.	+	F	v	+	–	+	+	+	+	v	+	
Cellulosimicrobium spp.	+	F	V	V	V	+	+	+	+	–	+	Hydrolysis of xanthine
Microbacterium spp.	v	F/O	v	v	v	v	+	+	v	v	v	
Curtobacterium spp.	+	O	v	–	–	+	+	v	v	v	+	
Leifsonia aquatica	+	O	+	v	–	v	+	v	v	+	+	
Auritidibacter ignavus	+	O	+	–	–	–	–	–	–	–	–	
Arcanobacterium haemolyticum	–	F	–	–	–	–	+	+	v	–	–	CAMP inhibition reaction
Trueperella bernardiae	–	F	–	–	–	–	+	+	–	–	–	Glycogen positive
Trueperella pyogenes	–	F	–	–	–	v	+	v	v	v	+	
Gardnerella vaginalis	–	F	–	–	–	–	+	+	v	–	–	Decolorized cells in Gram stain

[a]Abbreviations and symbols: +, positive reaction; −, negative reaction; v, variable reaction; O, oxidation; F, fermentation. See also references 12, 27, 47, and 48.

base based on its own entries, species identification becomes more likely (Bernard, unpublished).

MALDI-TOF mass spectrometry reliably identifies the potentially toxigenic *Corynebacterium* strains (68). Two studies including only a limited number of coryneform strains and species showed that about 90% of the isolates were correctly identified to the species level using the MALDI-TOF Bruker system (Bruker Daltonics, Bremen, Germany) applying a score of ≥1.700 as the cutoff (69, 70). Our own MALDI-TOF data using the Bruker system are as follows (Funke, unpublished): of a set of 957 strains of coryneform bacteria representing 10 genera and 53 species, 83.6% were correctly identified to the species level, 90.5% were identified to the correct genus level, a wrong species was given in 7.4%, a wrong genus was given in 0.7%, and for 8.8% of the strains, no reliable identification (i.e., no match or a score of <1.700) was observed. A challenge set consisting of 215 strains representing, again, 10 genera and 53 species was cultured on either Columbia blood agar, CNA agar, or chocolate agar, and the strains were correctly identified on the species level in 83.7%, 82.8%, or 82.8% of cases, respectively. The percentage of correct species level identification increased to 87.0% for strains grown on Columbia blood agar if the colonies were treated with 70% formic acid on the MALDI-TOF target plate. Of 509 consecutively isolated (in the author's routine clinical microbiology laboratory), clinically relevant coryneform bacteria, 86.0% of strains were correctly identified on the species level (compared to 16S rRNA gene and *rpoB* gene sequencing as the gold standard) and 91.7% were correctly identified on the genus level. In general, MALDI-TOF is a useful tool for identifying the huge variety of coryneform bacteria encountered in clinical specimens and might even be more valuable if the databases are improved by the manufacturers.

Molecular genetics-based identification systems for coryneform bacteria have been outlined. Restriction fragment length polymorphism analysis of the partly amplified and digested 16S rRNA gene has been demonstrated to be of use for the identification of species, e.g., within the genus *Corynebacterium* (71). Some corynebacteria may also be identified to the species level by examination of the length of the 16S–23S rRNA intergenic spacer region (72). A very useful approach for the identification of true corynebacteria is the sequencing of a 434- to 452-bp fragment of the *rpoB* gene (using primers designated C2700F and C3130R), since this particular region of the gene displays a high degree of polymorphism within the genus *Corynebacterium* (73, 74). A divergence of >5% within this particular part of the *rpoB* gene of two strains compared suggests that they belong to two different species. For more definitive taxonomic investigations of coryneforms, and in cases of growth of coryneform bacteria from difficult to obtain clinical material (75), full-length 16S rRNA gene sequencing might be indicated. Determination of the complete 16S rRNA gene sequence is a rational approach for identifying corynebacteria, since most established species exhibit 3% or greater divergence, except for *C. afermentans*, *C. coyleae*, and *C. mucifaciens* (<2%); *C. aurimucosum*, *C. minutissimum*, and *C. singulare* (<2%); *C. sundsvallense* and *C. thomssenii* (<1.5%); *C. ulcerans* and *C. pseudotuberculosis* (<1% to each other, both <2% to *C. diphtheriae*); *C. propinquum* and *C. pseudodiphtheriticum* (<2%); *C. xerosis*, *C. freneyi*, and *C. hansenii* (<2%); *C. macginleyi* and *C. accolens* (<2%) (1, 2).

In extremely rare cases (i.e., if the 16S rRNA gene divergence is ≤1.3%), quantitative DNA-DNA hybridizations might be necessary, with sequencing of the complete *rpoB* gene recently being suggested as a substitute for that approach (76). Because of the ever growing number of coryneform taxa encountered in clinical specimens, it has become difficult to readily differentiate these taxa by biochemical means alone, so sequencing studies are likely to replace some of the biochemical testing in the near future. It is emphasized that unidentifiable, clinically significant coryneform bacteria should be sent to an established reference laboratory experienced in corynebacterial identification for characterization which includes sequence-based analyses.

IDENTIFICATION: DESCRIPTIONS OF GENERA AND SPECIES

Genus *Corynebacterium*

C. accolens

C. *accolens* (77) is found in specimens from eyes, ears, nose, and the oropharynx. Endocarditis of native aortic and mitral valves due to this agent has been described. Colonies are, as for all other lipophilic corynebacteria, convex, smooth, and <0.5 mm in diameter on SBA. C. *accolens* has a variable pyrazinamidase reaction but is negative for alkaline phosphatase, thus differentiating it from the morphologically and biochemically closely related C. *tuberculostearicum* (Table 3). The API Coryne and RapID CB Plus systems correctly identify C. *accolens*. C. *accolens* strains are susceptible to a broad spectrum of antibiotics.

C. afermentans subsp. afermentans

C. *afermentans* subsp. *afermentans* (78) is part of the human skin microbiome and has so far been isolated mainly from blood cultures. Colonies are whitish, convex with regular edges, creamy, and about 1 to 1.5 mm in diameter after 24 h of incubation. C. *afermentans* subsp. *afermentans* has an oxidative metabolism. The API Coryne system provides the numerical code of 2100004 for this species. About 60% of all strains of this taxon are CAMP reaction positive. C. *afermentans* subsp. *afermentans* can be differentiated from C. *auris* and T. *otitidis* (both of which give the same API Coryne numerical code) by the consistency of its colonies (C. *auris* is slightly adherent to agar) and morphology on Gram staining (T. *otitidis* has longer cells). By chemotaxonomic means, both C. *afermentans* subspecies and C. *auris* contain mycolates, whereas T. *otitidis* lacks them, but these techniques are not applicable in a routine clinical laboratory. C. *afermentans* subsp. *afermentans* is generally susceptible to β-lactam antibiotics, but resistance to erythromycin, clarithromycin, azithromycin, and clindamycin have been reported (79).

C. afermentans subsp. lipophilum

Strains belonging to the species C. *afermentans* subsp. *lipophilum* (78) have mainly been isolated from blood cultures as well as from superficial wounds. Colonies are, typically for lipophilic corynebacteria, convex, smooth, and <0.5 mm in diameter after 24 h. C. *afermentans* subsp. *lipophilum* has an oxidative metabolism and does not produce acid from any of the carbohydrates usually tested (Table 3). It is the only species of nonfermenting, lipophilic corynebacteria which may exhibit a positive CAMP reaction. C. *afermentans* subsp. *lipophilum* is not included in the API Coryne database. The numerical profile observed for the species is 2100004, so by that method, it cannot be discerned from more-robustly growing C. *afermentans* subsp. *afermentans*, C. *auris*, or T. *otitidis*. Strains are usually susceptible to β-lactam antibiotics.

C. amycolatum

C. *amycolatum* is part of the human skin microbiome but was not recovered from throat swabs from healthy persons (41). C. *amycolatum* is the most frequently encountered *Corynebacterium* species in human clinical material (80). It is also the most frequently isolated nonlipophilic *Corynebacterium* species in dairy cows with mastitis. C. *amycolatum* strains are nearly always multidrug resistant (81). Colonies are very typically dry, waxy, and grayish white with irregular edges and are 1 to 2 mm in diameter after 24 h of incubation (Fig. 3a). C. *amycolatum* actually has a fermentative metabolism, but when cystine Trypticase agar media are used for the observation of acid production from carbohydrates, C. *amycolatum* appears to resemble an oxidizer (i.e., main acid production at the surface of the medium). Strains of C. *amycolatum* are remarkable for their variability in basic biochemical reactions (Table 3) and had been often misidentified in the past as the biochemically similar species C. *xerosis*, C. *striatum*, or C. *minutissimum* (80, 82). These species can be differentiated by the following reactions: C. *amycolatum* and C. *minutissimum* do not grow at 20°C but C. *xerosis* and C. *striatum* do; in addition, C. *xerosis* does not ferment glucose at 42°C, whereas the other three species do; and C. *minutissimum* and C. *striatum* produce alkali from formate but C. *amycolatum* and C. *xerosis* do not (82). When tested on Mueller-Hinton agar supplemented with 5% sheep blood, nearly all C. *amycolatum* strains were resistant to the vibriocidal compound O/129 (150-µg disks) (Oxoid, Basingstoke, UK), as indicated by no zone of inhibition around the disk (80). In contrast, only 4% of all C. *amycolatum* strains were resistant to O/129 when tested on Mueller-Hinton agar with 5% horse blood. The API Coryne system identifies this species well, but in every case, additional reactions must be carried out to confirm the identification of C. *amycolatum* (64). All C. *amycolatum* strains produce propionic acid as the major end product of glucose metabolism. In contrast to many other corynebacteria, C. *amycolatum* exhibits only weak or no leucine arylamidase activity. The identification may also be suggested by the absence of mycolic acids.

C. appendicis

The one strain of C. *appendicis* described in the literature was isolated from a patient with appendicitis accompanied by abscess formation (23). This lipophilic species contains large amounts of TBSA (up to 50% of all CFAs) not seen in any other *Corynebacterium* species. It is differentiated from CDC coryneform group F-1 bacteria by a positive alkaline phosphatase reaction but negative reactions for nitrate reduction and sucrose fermentation.

C. aquatimens

The lipophilic species C. *aquatimens* was isolated from blood cultures of a patient with bacteremia (17). Its closest phylogenetic neighbor is C. *tuscaniense*, but phenotypically, it resembles C. *argentoratense* from which it can be differentiated by negative α-chymotrypsin and cysteine arylamidase tests. C. *aquatimens* also biochemically resembles C. *mucifaciens*, from which it can be easily separated by its lipophilic colonies and the negative reaction for sucrose fermentation.

C. argentoratense

C. *argentoratense* (83) has been isolated from the human throat as well as from blood cultures (84). Colonies are

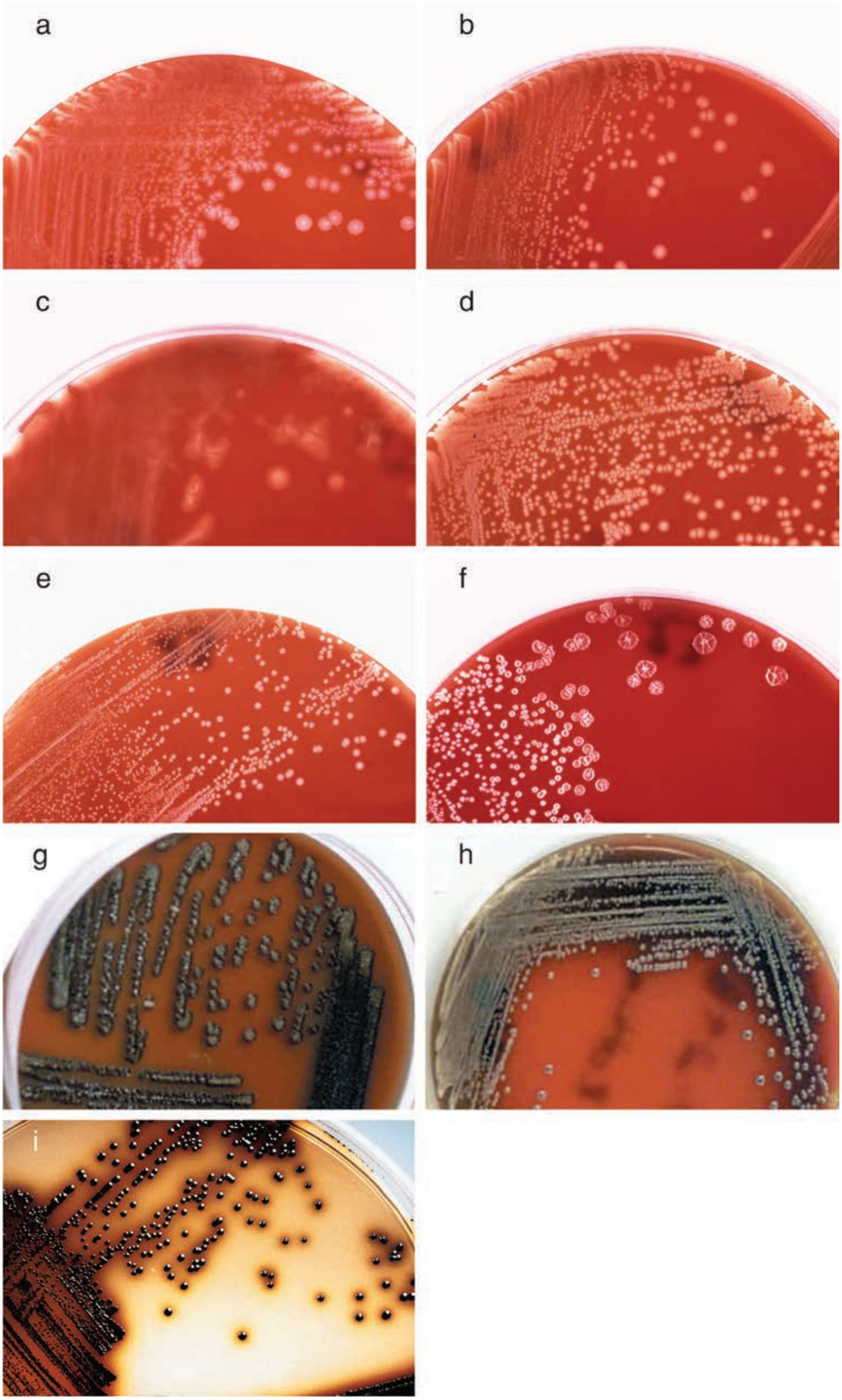

FIGURE 3 Colony morphologies of different coryneform bacteria after 48 h of incubation on SBA. (a) *C. amycolatum* LCDC 91-0077; (b) *C. diphtheriae* ATCC 14779; (c) *C. mucifaciens* LCDC 97-0202; (d) *C. striatum* ATCC 6940; (e) *D. hominis* ATCC 51325; (f) *R. dentocariosa* LCDC 95-0154; (g) *C. aurimucosum* HC-NML 91-0032 (after 96 h); (h) black-pigmented *Rothia dentocariosa* HC-NML 77-0298 (after 96 h); (i) *C. diphtheriae* biotype *gravis* colonies on a Tinsdale agar plate. The latter photo was kindly provided by C. Hinnebusch and M. Cohen, UCLA School of Medicine, Los Angeles. doi:10.1128/9781555817381.ch28.f3

cream colored, nonhemolytic, slightly rough, and 2 mm in diameter after 48 h of incubation. Phenotypically, *C. argentoratense* may appear to be very similar to (rare) ribose-negative strains of *C. coyleae*. However, glucose fermentation by *C. argentoratense* is quite rapid, compared to the slowly fermenting species *C. coyleae*. As well, CAMP-negative *C. argentoratense* produces propionic acid as a fermentation product, but CAMP-positive *C. coyleae* does not (84). *C. argentoratense* is the only medically relevant *Corynebacterium* species expressing α-chymotrypsin activity, which can be observed in the API ZYM (bioMérieux) system. Although *C. argentoratense* is phylogenetically closely related to *C. diphtheriae*, it does not harbor the *tox* gene coding for the diphtheria toxin.

C. atypicum

Corynomycolic acids, like in *C. amycolatum* and *C. kroppenstedtii*, are not detected (20). According to the initial description, *C. atypicum* is not lipophilic but shows only pinpoint colonies after 48 h of incubation. It is the only medically relevant *Corynebacterium* not expressing pyrazinamidase but β-glucuronidase activity.

C. aurimucosum

The initial description of *C. aurimucosum* was based on a single strain which exhibited slightly yellow and sticky colonies on 5% SBA plates but, on Trypticase soy agar without blood, had colorless and slimy colonies (85). Biochemically, this particular *C. aurimucosum* strain was similar to *C. minutissimum*. The number of *C. aurimucosum* strains was enhanced when it was demonstrated that some former CDC coryneform group 4 bacteria actually belong to *C. aurimucosum* (86). It is important to note that strains of *C. aurimucosum* exhibit a grayish-black pigment which is not seen in any other *Corynebacterium* species and that these strains are usually recovered from the female urogenital tract. Synthesis of this pigment, linked to genes on plasmid pET44827, is thought to assist with survival in the high hydrogen peroxide environment of the vagina (87). Strains originally designated "*C. nigricans*" were shown to be a later synonym of *C. aurimucosum* (86). Some strains of *R. dentocariosa* can also exhibit a charcoal-black pigment (86); these strains are differentiated from *C. aurimucosum* by being constantly nitrate reductase positive, a possible negative catalase reaction, and having branched-chained CFAs as opposed to straight-chained type CFAs for *C. aurimucosum*. API Coryne codes for pigmented *C. aurimucosum* strains include 0000125, 2000125, and 2100327. Phylogenetically, *C. aurimucosum* is closely related (>98.8% identity) to both *C. singulare* and *C. minutissimum* by 16S rRNA gene sequencing but can be readily discerned by partial *rpoB* gene sequence analysis.

C. auris

C. auris (88) has almost exclusively been isolated from the ear region. Colonies are dryish, slightly adherent to but do not penetrate agar, become slightly yellowish with time, and have diameters ranging from 1 to 2 mm after 48 h of incubation. *C. auris* does not produce acid from any carbohydrates usually tested. All *C. auris* strains are strongly CAMP test positive. The API Coryne system provides the numerical code 2100004 for this species. It is noteworthy that the MICs of β-lactam antibiotics for *C. auris* strains are elevated, but the molecular mechanism for this is not known at present (81).

C. bovis

Occasionally, human infections have been attributed to the lipophilic bovine species *C. bovis*. Characterization of lipophilic-like corynebacteria based solely on the use of phenotypic tests was probably incorrect, in the absence of modern polyphasic methods or identification schemes (Table 3). An oxidase-positive human blood culture isolate of *C. bovis* was identified based on a polyphasic approach with an API Coryne code of 0101104 (84) as was an isolate from a prosthetic joint infection.

C. canis

The nonlipophilic *C. canis* was isolated from a wound infection after a dog bite (89). It has some unusual microscopic features: it exhibits very filamentous rods (>15 µm in length) and some cells even show branching. *C. canis* is esculinase and α-glucosidase positive and is the only *Corynebacterium* expressing trypsin activity.

C. confusum

C. confusum has been isolated from patients with foot infections, a blood culture (22), and breast abscess (84). Colonies are whitish, glistening, convex, creamy, and up to 1.5 mm in diameter after 48 h. Acid from glucose is produced only very weakly, becoming visible in the API Coryne or the API 50CH gallery only after 48 to 72 h. Weak growth under anaerobic conditions corresponds to slow fermentative acid production. It is advisable to incubate the API Coryne system after 24 h for another day in those cases in which the results for acid production are ambiguous (i.e., only a slight change in the color of the indicator). After 48 h of incubation, the API Coryne system provides the numerical code 3100304 for this species; the breast abscess strain had a code of 3100104. Interestingly, the breast abscess strain was also CAMP positive, making it potentially more difficult to discern from *C. coyleae* isolates (84). If glucose fermentation is judged to be negative, *C. confusum* strains can be misidentified as *C. propinquum*. However, in contrast to that species, *C. confusum* does not hydrolyze tyrosine and contains small amounts of TBSA (1 to 3%), whereas *C. propinquum* hydrolyzes tyrosine but does not contain TBSA. *C. confusum* is differentiated from *C. coyleae* and *C. argentoratense* by its ability to reduce nitrate.

C. coyleae

C. coyleae (90) has mainly been isolated from cultures of blood and other normally sterile body fluids, but it may also be recovered from a variety of sterile body fluids, abscesses, and urogenital specimens (84, 91). Colonies are whitish and slightly glistening, have entire edges, and are about 1 mm in diameter after 24 h. The consistency of the colonies is either creamy or sticky. A slow fermentative acid production from glucose and a strongly positive CAMP reaction are the most significant phenotypic characteristics. *C. coyleae* is positive for cystine arylamidase, which is not observed for many other corynebacteria. Various API Coryne numerical codes have been observed, especially 2100304 and 6100304. *C. coyleae* is always positive for ribose fermentation, whereas the biochemically similar species *C. argentoratense* is variable for this reaction. The API Coryne database lists only 6% glucose-fermenting *C. coyleae* strains, and therefore, when applying this commercial identification system, the clinical microbiologist may not receive a correct identification (64). However, the two numerical profiles given above combined with a positive CAMP reaction are highly indicative of *C. coyleae*. Macrolide resistance has been reported (79).

CDC Group F-1 Bacteria

The lipophilic CDC group F-1 bacteria (92) have not been given a species name. Although genetically distinct, no distinguishing phenotypic markers which clearly allow their separation from other defined *Corynebacterium* spp. have been found. The characteristics of the CDC group F-1 bacteria are consistent with the definition of the genus *Corynebacterium* in all respects. Of note is the negative alkaline phosphatase reaction (Table 3). CDC group F-1 strains are usually susceptible to penicillin but are often resistant to macrolides.

C. diphtheriae

The complete genome sequence of a *Corynebacterium diphtheriae* strain representative for the diphtheria outbreak in the former Soviet Union states in the 1990s was the first *Corynebacterium* genome determined (93). The genome consists of a single circular chromosome of 2,488,635 bp with no plasmids. A complete set of enzymes for the glycolysis, gluconeogenesis, and pentose-phosphate pathways is present, as are all of the de novo amino acid biosynthesis pathways. Fimbrial and fimbria-related genes, sialidase (neuraminidase) genes, and iron-uptake systems have been detected as pathogenicity factors.

C. diphtheriae is commonly divided into four biotypes: gravis, mitis, belfanti, and intermedius. Biotype differentiation is recommended by WHO (59, 61), although biotypes cannot be assigned separate subspecies status, nor is biotyping satisfactory for epidemiologic tracking. Initially, these biotypes were defined by differences in colony morphology and biochemical reactions (Table 3). However, only *C. diphtheriae* biotype intermedius can be identified on the basis of colonial morphology (small, gray, or translucent lipophilic colonies) as well as positive dextrin fermentation. Other *C. diphtheriae* biotypes produce larger (up to 2 mm after 24 h) white or opaque colonies (Fig. 3b) which are indistinguishable from each other. The lipophilic *C. diphtheriae* biotype intermedius occurs only rarely in clinical infections, and *C. diphtheriae* biotype belfanti strains rarely express diphtheria toxin.

Presumptive identification of *C. diphtheriae* (as well as of *C. pseudotuberculosis* and *C. ulcerans*) may be made by testing suspicious Gram-positive rods for the presence of cystinase (as detected by using freshly prepared Tinsdale medium or diagnostic tablets [Rosco, Taastrup, Denmark]) and the absence of pyrazinamidase (diagnostic tablets are available from Key Scientific Products, Stamford, TX). The API Coryne system identifies *C. diphtheriae* strains, with additional tests needed for the differentiation of *C. diphtheriae* biotype mitis, *C. diphtheriae* biotype belfanti, and *C. diphtheriae* biotype intermedius (64). Usually, *C. diphtheriae* strains do not ferment sucrose, but in Brazil, sucrose-positive strains have been described. Large amounts of propionic acid are produced as the end product of glucose metabolism. *C. diphtheriae* strains are distinct from all other coryneform bacteria (except *C. pseudotuberculosis* and *C. ulcerans*) in their CFA patterns by the presence of a large volume of $C_{16:1\omega7c}$ (21).

Diphtheria Toxin Testing

It is recommended that at least 10 colonies of *C. diphtheriae* and related species be tested for diphtheria toxin by the Elek method, modified as described by Engler et al. (94), in a laboratory with skill in performing the test and in interpreting the test results. The modified Elek method as described by the WHO Diphtheria Reference Unit was initially used to characterize strains from the 1990s epidemic in Russia and Ukraine and was found to be faster and less technically problematic than the original version. Antitoxins from various suppliers (Berna Biotech AG, Switzerland; Mikrogen, Russia; Biomed, Russia; Pasteur Merieux/Aventis Pasteur, France; BulBio-NCIPD, Bulgaria; Instituto Butantan, Brazil; Refik Saydam National Hygiene Centre, Turkey), applied to blank filter disks at 10 IU/disk, have been successfully used with the modified Elek test.

PCR-based methods for the detection of the diphtheria toxin gene (*tox*) in isolated bacteria have been developed and validated (51, 52, 95). Conventional PCR detection of the regulatory *dtxR* gene has been evaluated (54). Detection of the *tox* gene using a real-time platform has been described (55–57). Primers targeting the *C. diphtheriae tox* gene for *C. ulcerans* strains perform better with modifications (56–58). A LightCycler PCR assay recognizing *tox* genes from both *C. diphtheriae* and *C. ulcerans* and differentiating the respective target genes by fluorescence resonance energy transfer hybridization probe melting curve analysis has been developed (96). *tox* PCR assays applied directly to clinical specimens are acceptable, particularly because isolation is not always possible for patients already receiving antibiotics. However, a PCR-positive patient from whom bacteria are not isolated or without a histopathologic diagnosis and without an epidemiologic linkage to a patient with a laboratory-confirmed case of diphtheria should be classified as a "probable case" of diphtheria, since to date, there are insufficient data to conclude that a PCR-positive result always infers diphtheria. Also, detection of the toxin gene in samples by PCR cannot automatically be attributed to one species because *C. diphtheriae*, as well as *C. ulcerans* and *C. pseudotuberculosis*, may harbor the bacteriophage which carries the diphtheria toxin gene. Furthermore, *tox*-containing, nontoxigenic isolates have been described and characterized further. Difficulties in identifying *C. diphtheriae* and in correctly performing toxigenicity tests have been demonstrated by an external quality control among 23 National Diphtheria Reference Centers in Europe in which 21% misidentifications and 13% unacceptable toxigenicity reports were observed (97).

Nontoxigenic strains of *C. diphtheriae*, i.e., those which do not express toxin in the Elek test or those which lack a detectable diphtheria toxin gene by PCR, have also caused serious disease, such as cases or outbreaks of skin disease, endocarditis, and occasional mortality among homeless people, alcoholics, and intravenous drug abusers (50, 98). For nontoxigenic *C. diphtheriae* strains circulating in the United Kingdom, it has been shown that the diphtheria toxin repressor (*dtxR*) genes are functional so that, if these strains are lysogenized by a bacteriophage, they could represent a reservoir for toxigenic *C. diphtheriae* (99).

Antibiotic treatment is required to eliminate *C. diphtheriae* and prevent its spread; however, it is not a substitute for antitoxin prevention, with antibiotics of choice being penicillins or macrolides. Sporadic isolates of *C. diphtheriae* resistant to erythromycin or rifampin have been reported, and multidrug resistance, although extremely rare, has also been reported (100).

C. durum

C. durum (101) was originally described as being exclusively isolated from respiratory tract specimens. Well-characterized isolates have now been recovered from additional sites, including the gingiva, blood cultures, and abscesses (102). *C. durum* strains were originally isolated after 2 to 3 days from nonselective charcoal-buffered yeast extract plates inoculated with sputa or bronchial washings, but it grows well on other standard laboratory media. C. *durum* is the most frequent *Corynebacterium* isolated from throat swabs of healthy persons (41). Its pathogenic potential remains unclear. C. *durum* is a peculiar nonlipophilic organism that forms colonies of only 0.5 to 1 mm in diameter after aerobic incubation for 72 h. The original description of this bacterium cited beige and rough colonies with convolutions, an irregular margin, and a strong adherence to agar if grown under aerobic conditions (101). However, strains were later described to be sometimes smoother and not necessarily adherent to agar (102). Gram staining of aerobic cultures shows long and filamentous rods, with occasional "bulges," but true C. *durum* isolates do not have C. *matruchotii*-like "whip handles." Long forms are not otherwise found among other *Corynebacterium* species (except C. *canis*) nor are they observed for C. *durum* when cells are grown in a 10% CO_2-enriched atmosphere (101). Strains grow only weakly under anaerobic conditions. They always reduce nitrate, and some may exhibit weak and delayed urease and esculinase activities. The majority (but not all) of C. *durum* strains ferment mannitol, which is another very unusual feature for true corynebacteria (41, 102) (Table 3). API Coryne codes observed for C. *durum* include 3000135, 3001135, 3040135, 3400115, 3400135, 3400305, 3400325, and 3400335 (101) as well as 3040325, 3040335, 3440335, and 3441335 (102). This suggests that most strains are negative for alkaline phosphatase and all appear to be negative for pyrrolidonyl arylamidase. It is most likely that some strains identified as C. *matruchotii* in the past may actually have been C. *durum* strains and that differentiation can be difficult if phenotypic methods alone are used. Both species produce propionic acid as a fermentation product (48). C. *durum* usually ferments galactose and very often mannitol, whereas C. *matruchotii* is usually negative for those sugars. The C. *matruchotii* type strain exhibits α-glucosidase activity, which is not observed in C. *durum* (101). It has been shown that some C. *durum* strains also express β-galactosidase activity and ferment ribose (41).

C. falsenii

C. *falsenii* strains (103) have mainly been isolated from sterile body fluids. Colonies are whitish, glistening, and smooth with entire edges, and they are 1 to 2 mm in diameter after 24 h. After 72 h, most strains described to date exhibit a yellowish pigment which becomes even more intense after 120 h. The most characteristic biochemical features of C. *falsenii* are a slow but fermentative acid production from glucose, a weak pyrazinamidase reaction, and a weak urease activity which becomes visible in either Christensen's urea broth or the API Coryne system after overnight incubation only. API Coryne codes observed for C. *falsenii* have been 2101104 and 2101304 (84, 103).

C. freiburgense

The nonlipophilic species C. *freiburgense* has probably been transmitted to a human by a dog bite (104). The 5-day-old colonies exhibit a very peculiar "spoke-wheel" macroscopic morphology not observed in other true corynebacteria but in some R. *dentocariosa* strains. Colonies are also strongly adherent to blood agar. Distinct biochemical features are the lack of pyrazinamidase activity and a positive β-galactosidase reaction.

C. freneyi

The species C. *freneyi* has initially been outlined based on the study of three strains (105) which came from skin-related material. There is now evidence that C. *freneyi* is also isolated from genitourinary specimens (106) and bacteremia. C. *freneyi* is phylogenetically closely related to C. *xerosis*. Colonies are typically wrinkled, whitish, dry, and rough; have irregular edges; and are 0.5 to 1 mm in diameter after 48 h incubation. C. *freneyi* strains are nonlipophilic. The basic biochemical profile (Table 3) is similar to that of C. *xerosis*. All C. *freneyi* strains studied so far exhibit α-glucosidase activity which is not frequently observed in other *Corynebacterium* species (very few C. *amycolatum* and all C. *xerosis* strains express this enzyme). C. *freneyi* can be further differentiated from C. *xerosis* by glucose fermentation at 42°C and growth at 20°C, whereas C. *xerosis* is negative for the latter two reactions. This species is also closely related to C. *hansenii*.

C. glucuronolyticum

C. *seminale* is a later synonym of C. *glucuronolyticum* (44, 45). This species is probably part of the genitourinary microbiome of males, while its presence in females is uncertain. Recoveries from blood cultures have been documented (84). Colonies are whitish-yellowish, convex, creamy, and 1 to 1.5 mm in diameter after 24 h. The fermentative species C. *glucuronolyticum* is remarkable for its variability in basic biochemical reactions (Table 3). It is the only medically relevant, large-colony *Corynebacterium* species exhibiting β-glucuronidase activity. When urease activity is present, it is abundant in Christensen's urea broth, becoming positive after only 5 min of incubation at room temperature (44). C. *glucuronolyticum* is also one of the very few corynebacteria which are able to hydrolyze esculin. All C. *glucuronolyticum* strains are CAMP reaction positive (Fig. 2). With the exception of strains which are alkaline phosphatase positive, the API Coryne strip identifies C. *glucuronolyticum* well (64), although profiles obtained from human strains may differ from animal isolates (45). Propionic acid is one of the major end products of glucose metabolism. C. *glucuronolyticum* strains are often tetracycline resistant and may also exhibit resistance to macrolides and lincosamides (81). 16S rRNA gene sequences derived from fluids of patients with prostatitis have been found to be homologous with sequences derived for this species, indicating that C. *glucuronolyticum* might be involved in selected cases of prostatitis (107).

C. hansenii

C. *hansenii*, a nonlipophilic species, exhibits yellow and dry colonies (108). Biochemical identification reactions are similar to those of C. *freneyi* and C. *xerosis*. However, C. *hansenii* can be distinguished from these species by being negative for alkaline phosphatase and α-glucosidase reactions. These three species are not well discerned by either 16S rRNA or partial *rpoB* gene sequencing but were found to represent different taxa by DNA-DNA hybridization (108).

C. imitans

C. *imitans* was originally isolated from a nasopharyngeal specimen of a child suspected of having throat diphtheria as well as from three adult contacts (109). This was the first well-documented case of the person-to-person transmission of a *Corynebacterium* other than C. *diphtheriae* in a nonhospital setting. Additional strains of C. *imitans* have been recovered from blood cultures (84). Colonies are whitish-grayish, glistening with entire edges, creamy, and 1 to 2 mm in diameter. The strain did not produce a brown halo on Tinsdale medium but was tellurite reductase positive. Interestingly, Neisser staining was positive for polar bodies.

Pyrazinamidase activity was weak only, as was fermentation of sucrose, which may lead to the initial misidentification as an atypical *C. diphtheriae* strain. It is not unlikely that *C. imitans* may have been misidentified as *C. minutissimum* in the past, since biochemical reactions of both taxa are similar (Table 3). However, *C. imitans* is CAMP reaction positive and does not hydrolyze tyrosine, whereas the opposite reactions are observed for *C. minutissimum*. The API Coryne system provided the numerical codes 1100325, 2100324, and 3100325 for *C. imitans*, indicating a negative α-glucosidase reaction, whereas all *C. diphtheriae* strains express this enzyme. *C. imitans* strains do not produce propionic acid as a fermentation product, unlike *C. diphtheriae* (84). Diphtheria toxin assay using the Elek test and assaying for *tox* gene by PCR were all negative for *C. imitans* strains (84, 109). *C. imitans* is resistant to O/129, while *C. diphtheriae* is not.

C. jeikeium

C. jeikeium is a frequently encountered *Corynebacterium* in clinical specimens (47). Nosocomial transmission has been described. The complete genome sequence of a *C. jeikeium* strain has been determined, indicating that the lipophilic phenotype of *C. jeikeium* originates from the absence of fatty acid synthase. *C. jeikeium* is often resistant to multiple antibiotics (including penicillin and gentamicin) (79, 110), but this cannot be used as a taxonomic characteristic because the phenotypically closely related *C. tuberculostearicum* may also demonstrate multidrug resistance. Quantitative DNA-DNA hybridization experiments had shown that *C. jeikeium* includes two genomospecies for which penicillin and gentamicin MICs are low, but as they could otherwise not be differentiated phenotypically from the resistant *C. jeikeium* strains, they were not proposed as independent species (111). Colonies of *C. jeikeium* are tiny, low, entire, and grayish white. *C. jeikeium* is a strict aerobe which may oxidatively produce acid from glucose and sometimes from maltose but not from fructose (*C. tuberculostearicum* is positive for acid production from fructose). The RapID CB Plus system correctly identifies *C. jeikeium*, as does the API Coryne system if ancillary tests are used (64, 66).

C. kroppenstedtii

C. kroppenstedtii (19), a rarely recovered species, was originally recovered from the sputum of a patient with pulmonary disease. Additional strains have been isolated from lung biopsy specimen, sputum, breast abscess, and patients with granulomatous lobular mastitis (84, 112). Colonies are grayish translucent, slightly dry, and less than 0.5 mm in diameter after 24 h of incubation at 37°C. *C. kroppenstedtii* is lipophilic and is one of the few medically relevant *Corynebacterium* species exhibiting esculinase activity. Other biochemical characteristics are given in Table 3. API codes of *C. kroppenstedtii* include 0101104, 2040104, and 2040105 (84). It can be separated from *C. durum*, *C. matruchotii*, and *C. glucuronolyticum* by colony and Gram stain morphologies and from *C. glucuronolyticum* by its negative CAMP reaction. The determination of the whole-genome sequence revealed that lipophilism is the dominant feature involved in the pathogenicity of *C. kroppenstedtii*.

C. lipophiloflavum

Initially, *C. lipophiloflavum* (113) was represented by only a single strain which had been isolated from vaginal discharge from a patient with bacterial vaginosis. Additional strains have now been described from blood cultures (Funke, unpublished). This species contains lipophilic strains, but the majority of strains are nonlipophilic (Funke, unpublished). The lipophilic strains have the same biochemical screening pattern as *C. urealyticum* except that they exhibit a strong yellow pigment and weaker urease activity and slowly produce acid from glucose (Table 3). In contrast to most *C. urealyticum* strains, the *C. lipophiloflavum* strains isolated were not multidrug resistant. The nonlipophilic strains have a biochemical profile similar to that of *C. falsenii* but do not ferment galactose and trehalose.

C. macginleyi

C. macginleyi (92) has almost exclusively been isolated from eye specimens, whether from diseased (43) or healthy conjunctiva, although extraocular infections have been occasionally reported. Colonies are typical for lipophilic corynebacteria (see above). *C. macginleyi* is one of the very few *Corynebacterium* species not expressing pyrazinamidase activity (Table 3). Most strains ferment mannitol, while the majority of other corynebacteria are unable to do so. The API Coryne system can correctly identify *C. macginleyi* (64). Strains belonging to this species are susceptible to a broad spectrum of antibiotics (43), but high-level fluoroquinolone resistance has been reported (114).

C. massiliense

C. massiliense was isolated from synovial fluid (115). It does not produce acid from any of the carbohydrates tested in the differentiation of true corynebacteria (Table 3), but its cell wall contained tuberculostearic acid.

C. mastitidis-like organisms

Bacteria recovered from ocular specimens (patients with cataracts, diabetic retinopathy, or dry eyes) were, by 16S rRNA gene sequencing, found to be closest to (98.2% identity) *C. mastitidis*, otherwise found to date to cause sheep mastitis. In contrast to *C. macginleyi*, these strains were sensitive to the fluoroquinolones (114). Description of two *species nova* involving these strains is under way (Bernard, unpublished).

C. matruchotii

C. matruchotii is thought to be a natural inhabitant of the oral cavity, particularly on calculus and plaque deposits, so it has been much studied by oral microbiologists (102). Otherwise, it is a very rare human pathogen. Microcolonies appear flat, filamentous, and spider-like, but macrocolonies have a variable appearance. *C. matruchotii* demonstrates a very unusual appearance by Gram staining in that so-called whip handles (i.e., filamentous bacteria with a single short bacillus adjacent to the end of the filament, creating the illusion of a whip) are observed (Fig. 1e). This microscopic presentation is consistent even when isolates which had been preserved for many years in a culture collection are stained. It has been demonstrated that heterogeneity existed among *C. matruchotii* strains obtained from international culture collections and that some strains represented were misidentified *C. durum* isolates (102). *C. matruchotii* strains are consistently negative for galactose, whereas *C. durum* strains can be positive. The API Coryne system database does not contain *C. matruchotii*; the numerical codes observed for *C. matruchotii* include 7000325, 7010325, and 7050325.

C. matruchotii-like strain

This taxon is represented by a single strain, ATCC 43833 (102). It had been deposited in ATCC as *C. matruchotii*, but it is a distinct species, as revealed by dot-blot hybridization and 16S rRNA gene sequencing data (Genbank accession no. AF260434). Colonies are pinpoint to 0.1 mm in diameter, grayish-white, with a smooth, nonadherent texture. Biochemical screening reactions are similar to those of C. *minutissimum*, except that strain ATCC 43833 exhibits esculinase activity in the API Coryne system, with an API Coryne code of 2140325.

C. minutissimum

C. *minutissimum* is part of human skin microbiome, and its historical association with erythrasma is highly questionable (47). Colonies of C. *minutissimum* are whitish-grayish, shiny, moist, convex, and circular, have entire edges, and are about 1 to 1.5 mm in diameter after 24 h. Most of the colonies are creamy, but some may also have a sticky consistency. C. *minutissimum* strains have a fermentative metabolism and produce acid from sucrose variably. Very few C. *minutissimum* strains are also able to produce acid from mannitol. The API Coryne system identifies C. *minutissimum*, with additional tests being necessary for most strains (64). Many C. *minutissimum* strains are pyrrolidonyl arylamidase positive. C. *minutissimum* strains exhibit DNase activity, nearly all strains hydrolyze tyrosine, and a very few strains exhibit a positive CAMP reaction. Lactic and succinic acids are major end products of glucose metabolism. Some isolates possess TBSA in their cell membranes. Nearly all C. *minutissimum* strains are susceptible to O/129 (150 μg disk); i.e., they exhibit an inhibition zone around the disk (usually between 20 and 35 mm in diameter). Phylogenetically, C. *minutissimum* is closely related (>98.8% identity) to both C. *singulare* and C. *aurimucosum* by 16S rRNA gene sequencing, but these are readily discerned by partial *rpoB* gene sequence analysis.

C. mucifaciens

C. *mucifaciens* (116) has mainly been isolated from blood cultures and other sterile body fluids, but it has also been recovered from abscesses, soft tissue, and dialysate (84). Colonies are very distinct because they are slightly to overtly yellow and very mucoid (Fig. 3c) (with very few strains not being mucoid [Funke, unpublished]). C. *mucifaciens* is the only presently known *Corynebacterium* species exhibiting such mucoid colonies; this characteristic strongly reminds the bacteriologist of *Rhodococcus equi* colonies. An extracellular substance (probably polysaccharides) causing connective filaments between the cells has been demonstrated as the ultrastructural correlate of the mucoid colonies. Colonies are about 1 to 1.5 mm after 24 h of incubation and have entire edges. They appear less mucoid after extended incubation for 96 h. C. *mucifaciens* has an oxidative metabolism. It consistently produces acid from glucose, but acid production from sucrose is variable. The API Coryne numerical codes 2000004, 2000104, 2000105, 2100104, 2100105, 6000004, 6100104, and 6100105 have been observed for C. *mucifaciens*, suggesting that occasionally glucose oxidation may be too slow to be observed by that method. C. *mucifaciens* is enzymatically less active than *R. equi* which exhibits α- and β-glucosidase activities not observed for C. *mucifaciens*. In addition, C. *mucifaciens* produces acid from fructose and may produce acid from glycerol and mannose, but acid production from these sugars is not seen in *R. equi* strains. Tuberculostearic acid can be detected in amounts of 1 to 2% of the total CFAs. β-Lactam antibiotics and aminoglycosides show very good activities against C. *mucifaciens*.

C. pilbarense

The nonlipophilic species C. *pilbarense* was isolated from an ankle aspirate of a male thought to be suffering from gout (117). It can be differentiated from C. *striatum* and C. *simulans* by being nitrate reduction negative and from C. *minutissimum* by not fermenting maltose. Phylogenetically, it is closest to (98.7 to 99.0% identity) C. *ureicelerivorans*, C. *coyleae*, C *afermentans*, and C. *mucifaciens* but was discerned from those species by DNA-DNA hybridization and biochemically (Table 3) (117).

C. propinquum

C. *propinquum* is the closest phylogenetic relative of C. *pseudodiphtheriticum* (5, 6) and shares the same niche (i.e., the oropharynx) as C. *pseudodiphtheriticum*. Colonies are whitish and somewhat dryish with entire edges and are 1 to 2 mm in diameter after 24 h of incubation. This species reduces nitrate and hydrolyzes tyrosine and was historically described as being unable to hydrolyze urea. However, a recent study demonstrated that some C. *propinquum* strains are able to produce urease (118). C. *propinquum* and C. *pseudodiphtheriticum* can be differentiated by either MALDI-TOF analysis or *rpoB* sequencing (118). The API Coryne system and the RapID CB Plus system may correctly identify C. *propinquum* strains if they do not hydrolyze urea.

C. pseudodiphtheriticum

C. *pseudodiphtheriticum* is part of the oropharyngeal microbiome. As described in Table 2, this species has been well documented to cause pneumonia in various patient populations, including an outbreak in cystic fibrosis patients (119). Colonies are whitish, slightly dry with entire edges, and 1 to 2 mm in diameter after 48 h of incubation. This nonfermenting species reduces nitrate and hydrolyzes urea but does not produce acid from any of the commonly tested carbohydrates (Table 3). Some strains hydrolyze tyrosine. C. *pseudodiphtheriticum* strains are susceptible to β-lactam antibiotics, but resistance to macrolides and lincosamides has been observed.

C. pseudotuberculosis

C. *pseudotuberculosis* is phylogenetically closest to C. *ulcerans* as well as to C. *diphtheriae* (5, 6) and so, like those species, may harbor the diphtheria toxin gene. It produces propionic acid as a fermentation product and its cell wall contains large amounts of the CFA $C_{16:1\omega7c}$ (21). Colonies are yellowish white, opaque, convex, and about 1 mm in diameter after 24 h. Like C. *ulcerans*, C. *pseudotuberculosis* is positive for urease and the CAMP inhibition test (complete inhibition of the effect of *S. aureus* β-hemolysin on sheep erythrocytes is achieved by streaking the presumed C. *pseudotuberculosis* strain in a right angle toward *S. aureus* and incubating overnight; a β-hemolysin inhibition zone in the form of a triangle is observed, as is the case for *A. haemolyticum* [Fig. 2]). C. *pseudotuberculosis* is not susceptible to O/129 whereas C. *ulcerans* strains are. C. *pseudotuberculosis* is variable for both nitrate reduction and sucrose fermentation. The API Coryne system and the RapID CB Plus panel correctly identify this species (64, 66). Human disease to date has been acquired by handling of infected sheep.

C. pyruviciproducens

C. *pyruviciproducens* was independently isolated from a groin abscess (120) and a urethral swab (Funke, unpublished) as well as from urine, a bone biopsy specimen, synovial fluid, and a blood culture (K. Bernard, unpublished data). It was initially believed to be a lipophilic variant of C. *glucuronolyticum*, which was β-glucuronidase and CAMP test positive. However, 16S rRNA gene sequence analysis, quantitative DNA-DNA hybridization, and partial *rpoB* gene sequence analysis clearly demonstrated that this species is distinct from C. *glucuronolyticum* (120).

C. resistens

C. *resistens* has entire, grayish-whitish, and glistening colonies and is lipophilic. It is unusual in having a negative pyrazinamidase reaction which separates it from the phenotypically related C. *jeikeium* or C. *tuberculostearicum*. In addition, C. *resistens* grows slowly under anaerobic conditions, whereas C. *jeikeium* is unable to do so. The C. *resistens* strains reported in the literature were resistant to penicillin, cephalosporins, aminoglycosides, clindamycin, and ciprofloxacin but remained susceptible to glycopeptides (121). It is presently unknown whether true C. *resistens* strains have been misidentified as C. *jeikeium* in the routine laboratory.

C. riegelii

C. *riegelii* strains were originally described as being isolated from females with urinary tract infections (122), but additional strains have been recovered from blood cultures, including cord blood (84). Colonies are whitish, glistening, and convex with entire margins and are up to 1.5 mm in diameter after 48 h of incubation. Some colonies are of a creamy consistency, whereas others are sticky. C. *riegelii* strains exhibit a very strong urease activity with Christensen's urea broth, becoming positive within 5 min at room temperature after inoculation. A very peculiar characteristic of C. *riegelii* is the slow fermentation of maltose but not glucose. No other defined *Corynebacterium* exhibits this feature (Table 3). The weak anaerobic growth of C. *riegelii* corresponds to the weak fermentative metabolism. API Coryne system codes observed for C. *riegelii* include 0101224, 2001224, and 2101224.

C. simulans

C. *simulans* was originally delineated from some C. *striatum*-like strains (123). The three strains described in the original publication came from skin-related specimens (foot abscess, lymph node biopsy specimen, and boil). Additional strains have been characterized (84). Colonies of C. *simulans* (greyish-white, glistening, creamy, 1 to 2 mm in diameter) are very similar to C. *minutissimum*, C. *singulare*, and C. *striatum*, its closest phylogenetic neighbors. C. *simulans* is the only valid *Corynebacterium* species described to date which reduces nitrite. Further characteristics which separate C. *simulans* from the closely related nonlipophilic, fermentative corynebacteria are the inability to acidify ethylene glycol and to grow at 20°C (in contrast to C. *striatum*). API Coryne profiles include 0100305, 2100105, 2100301, 2100305, and 3000125 (including the falsely negative nitrate reduction reaction because of the strong nitrite reduction). Nitrate reductase-negative "C. *striatum*" strains should always raise the suspicion of C. *simulans*.

C. singulare

C. *singulare* colonies are circular and slightly convex with entire margins and are of a creamy consistency, as observed for C. *minutissimum* and C. *striatum* (124). Key biochemical reactions are like those for C. *minutissimum* except that urease activity is observed (Table 3). The numerical API Coryne system profile is 6101125, indicative that pyrrolidonyl arylamidase activity is observed. Like C. *minutissimum* and C. *striatum*, C. *singulare* also hydrolyzes tyrosine. C. *singulare* does not produce propionic acid as a fermentation product, differentiating it from C. *amycolatum*. Phylogenetically, this species is closely related (>98.8% identity) to both C. *aurimucosum* and C. *minutissimum* by 16S rRNA gene sequencing but can be readily discerned by partial *rpoB* gene sequence analysis.

C. sputi

C. *sputi* is the only true *Corynebacterium* expressing α-glucosidase activity and being positive for tuberculostearic acid (125). It can be differentiated from C. *ulcerans* by being positive for pyrazinamidase but negative for alkaline phosphatase and maltose fermentation. Unlike C. *ulcerans*, this species cannot harbor the bacteriophage which bears the diphtheria *tox* gene.

C. stationis

C. *stationis*, originally called *Brevibacterium stationis*, has recently been reassigned to the genus *Corynebacterium* (126). B. *stationis* ATCC 14403^T was originally recovered from seawater, but subsequent studies found two human blood culture isolates plus "C. *ammoniagenes*" ATCC 6872, recovered from an infant's stools, had formed a single taxon group now designated C. *stationis* comb. nov. Strains of C. *stationis* and C. *ammoniagenes* sensu stricto both demonstrate the ability to alkalinize citrate, using either Simmon's citrate or a heavy (not light) inoculum in the citrate cupule found in an API 20E strip, a feature not previously associated with members of the genus *Corynebacterium*. Colonies of C. *stationis* grow well in 24 h, that is, they are not lipophilic, are yellow or yellowish, and are fermentative, being reactive with glucose, fructose, ribose, and mannose. All are positive for alkalinization of citrate, produce urease, reduce nitrate, and hydrolyze tyrosine, with other features shown in Table 3. API Coryne codes generated were 1001304 and 3001304 (that is, the enzyme PYZ is variably detected). Strains of this species were susceptible to all antimicrobials, except one blood culture isolate was resistant to erythromycin.

C. striatum

C. *striatum* is part of the human skin microbiome. Nosocomial transmission or outbreaks of C. *striatum* have been documented (127, 128). Colonies are convex, circular, shiny, moist, and creamy, have entire edges, and are about 1 to 1.5 mm in diameter after 24 h of incubation. Some investigators have described C. *striatum* colonies as being somewhat like those of small coagulase-negative staphylococci. C. *striatum* has a fermentative metabolism, and acid production from sucrose is variable. The API Coryne system identifies C. *striatum*, but additional tests are needed in most of the cases (64). All C. *striatum* strains hydrolyze tyrosine, and some strains are CAMP reaction positive; however, the CAMP reaction of C. *striatum* strains is usually not as strong as that of other CAMP test-positive species (e.g., C. *auris* or C. *glucuronolyticum*). Lactic and succinic acids are the major end products of glucose metabolism. All C. *striatum* strains are susceptible to O/129. Resistance to macrolides and lincosamides due to the presence of an rRNA methylase has been described. C. *striatum* may also be resistant to quinolones and tetracyclines (79, 110), which has led to a renewed interest in this agent as an emerging pathogen.

C. sundsvallense

C. *sundsvallense* (84, 129) has been isolated from blood cultures, a vaginal swab, and sinus drainage from an infected groin. Colonies of this nonlipophilic species are buff or slightly yellowish, are adherent to agar, and have a sticky consistency. Gram staining shows bulges or knobs at the ends of some rods, and these are not seen in any other corynebacteria. Fermentation of glucose, lactose, and sucrose is slow (Table 3). C. *sundsvallense* can be separated from C. *durum* by its positive α-glucosidase reaction and its inability to ferment galactose. It is further differentiated from C. *matruchotii* by expressing urease but not nitrate reductase activity and by not producing propionic acid as an end product of glucose metabolism (84, 129).

C. *thomssenii*

C. *thomssenii* (130) is a rarely found species, originally repeatedly isolated from a patient with pleural effusion and a second strain recovered from the environment in Canada (84). This species is fastidious and slowly growing, resulting in colonies <0.5 mm after 48 h, but it is not lipophilic. After 96 h, colonies are molar tooth-like, very sticky, and slightly adherent to agar. The clinical strain of C. *thomssenii* is the only *Corynebacterium* species expressing *N*-acetyl-β-glucosaminidase activity, which can be observed either in the API Coryne or API ZYM systems. Acid is slowly and fermentatively produced from glucose, maltose, and sucrose, and the resulting API Coryne code for C. *thomssenii* is 2121125.

C. *timonense*

C. *timonense* was isolated from blood cultures of a patient with endocarditis. Its most peculiar feature is a weakly positive esculinase reaction. It can be differentiated from other esculinase-positive corynebacteria by having negative reactions for maltose and sucrose fermentation as well as a negative CAMP reaction (115).

C. *tuberculostearicum*

C. *tuberculostearicum* was revived for a never validly published taxon and also included a strain of the unvalidated species "C. *pseudogenitalium*" (131). Nearly all "CDC group G" bacteria can be assigned to C. *tuberculostearicum* (K. A. Bernard and G. Funke, unpublished data). Unfortunately, the effective publication on C. *tuberculostearicum* did not include any strains of CDC group G bacteria (131). Recently, a comprehensive study on 18 C. *tuberculostearicum* strains has been published (132). C. *tuberculostearicum* has some variable biochemical key reactions and can be differentiated from C. *accolens* if TBSA is detected as one of its CFAs. It can be separated from C. *jeikeium* by anaerobic growth and fermentative acid production from fructose. C. *tuberculostearicum* can be multidrug resistant, but the most frequently observed resistance is to macrolides and lincosamides.

C. *tuscaniense*

C. *tuscaniense* has been isolated from blood cultures of a patient suffering from endocarditis. C. *tuscaniense* does not grow under anaerobic conditions, which distinguishes this species from the phenotypically similar C. *minutissimum*. In addition, C. *tuscaniense* hydrolyzes hippurate but not tyrosine, whereas C. *minutissimum* has opposite reactions (Table 3). C. *tuscaniense* colonies are rounded and regular, in contrast to the biochemically similar C. *amycolatum* colonies, which exhibit irregular edges (133).

C. *ulcerans*

Phylogenetically, C. *ulcerans* (134) is closely related to C. *pseudotuberculosis*, and both species are the closest relatives to C. *diphtheriae* (5, 6). As described previously, C. *ulcerans* can harbor the diphtheria toxin gene, but differences in the receptor-binding and translocation domains have been described, so C. *ulcerans* diphtheria toxin-specific PCR has been developed for conventional (135) or real-time PCR. Pharyngitis associated with this bacterium is rare but, if recovered from pseudomembranous material, must be treated like a case of diphtheria (59, 61). Recent reports have described the transmission of C. *ulcerans* from companion pets to humans (49), so expanded national case definitions for diphtheria-like disease, which includes cases involving toxigenic strains of C. *ulcerans* or C. *pseudotuberculosis*, have formally occurred in some countries. C. *ulcerans* colonies are somewhat dry, waxy, and gray-white, with light hemolysis, and are 1 to 2 mm in diameter after 24 h. C. *ulcerans* may be differentiated from C. *diphtheriae* by urease activity and a CAMP inhibition reaction and from C. *pseudotuberculosis* by source, biochemically, and by partial *rpoB* gene but not 16S rRNA gene sequencing. Strains of C. *ulcerans* are positive for glycogen, starch, and trehalose fermentation. The API Coryne system and the RapID CB Plus identification strip correctly identify C. *ulcerans* (64, 66).

C. *urealyticum*

C. *urealyticum* is strongly associated with urinary tract infections. Recovery of this bacterium is often associated with urine with an alkaline pH, resulting in struvite crystals. As for all other lipophilic corynebacteria, colonies are pinpoint, convex, smooth, and whitish-grayish on regular SBA. C. *urealyticum* is a strict aerobe and has very strong urease activity (Table 3). Commercial identification systems correctly identify C. *urealyticum*. C. *urealyticum* is almost always multidrug resistant (47, 79, 136). Sequencing of the whole C. *urealyticum* genome indicated that the multidrug resistance is mediated by transposable elements.

C. *ureicelerivorans*

The lipophilic species C. *ureicelerivorans* first described from a blood culture has very strong and rapidly detected (~60 s) urease activity as its most prominent feature (137). Subsequently, recovery of this organism from ascites and multiple blood cultures from immunocompromised patients or those with digestive disorders has been described (138). It can be differentiated from CDC group F-1 bacteria by a positive alkaline phosphatase reaction and having negative reactions for acid production from maltose and sucrose. By 16S rRNA and partial *rpoB* gene sequencing, this species is closest to C. *mucifaciens*; C. *ureicelerivorans* can be readily discerned from that species by having a smooth rather than mucoid yellowish colony as well as by observation of rapid urease activity.

C. *xerosis*

Colonies of C. *xerosis* are dry, granular, and yellowish with irregular edges and are of 1 to 1.5 mm in diameter after 24 h. It must be emphasized that nearly all "C. *xerosis*" strains which have been described in the literature before the mid-1990s may have been misidentified C. *amycolatum* strains (80). C. *striatum* strains were also misidentified as C. *xerosis* in the past. C. *xerosis* has a fermentative metabolism, is variable for the presence of nitrate reductase, but always expresses α-glucosidase as well as leucine arylamidase activities. Because C. *xerosis* was thought to be rarely encountered in clinical specimens, it was not included in the API Coryne system version 3.0 database. The numerical profiles observed for C. *xerosis* strains, such as 2110325 and 3110325, provide, using API Web, responses including "*C. striatum/C. amycolatum*." Lactic acid is the major end product of glucose metabolism, and strains are susceptible to O/129. As described previously, *C. xerosis* is phylogenetically closely related to *C. freneyi* and *C. hansenii* (108), so if definitive identification is required, characterization must include genetic testing.

Genus *Turicella*

T. otitidis is almost exclusively isolated from clinical specimens from the ear region, but it does not cause otitis media with effusion in children (42). Colonies are whitish, convex, and creamy with entire edges and are 1 to 1.5 mm in diameter after 48 h of incubation. Some young colonies show a greenish appearance when taken away from the plates with a swab. The distinctive Gram stain morphology of *T. otitidis* is given in Fig. 1b. Differentiation from *C. auris* and *C. afermentans* subsp. *afermentans* is readily achieved by morphologic features, but utilization reactions may also assist in the differentiation of these taxa (Tables 3 and 4) (88). All *T. otitidis* strains are strongly CAMP reaction positive and give the numerical code 2100004 in the API Coryne system. The MICs of β-lactam antibiotics for many strains are very low; some strains might be resistant to macrolides and clindamycin (81). The mechanism of resistance to macrolides appears to be due in part to mutations in the 23S rRNA (*rrl*) gene (139).

Genus *Arthrobacter*

Arthrobacter spp. might be part of the indigenous human microbiome, but their main habitat is soil. *A. cumminsii* seems to be a normal commensal in humans and appears to be the most frequently isolated *Arthrobacter* species in human clinical specimens (24, 46), and *A. oxydans* is the second most frequently encountered species (24). *Arthrobacter* colonies are usually whitish-grayish, slightly glistening, creamy, and 2 mm or greater in diameter after 24 h. *A. cumminsii* may also exhibit a sticky consistency (46). *Arthrobacter* spp. usually do not oxidize any of the carbohydrates routinely tested and do not express a cheese-like smell, as often found for the phenotypically closely related brevibacteria. Some arthrobacters are motile, whereas brevibacteria are always nonmotile. Like brevibacteria, *Arthrobacter* spp. express DNase and have gelatinase activity (9). The identification of arthrobacters on the species level might be achieved by carbohydrate utilization tests, but this is recommended for the reference laboratory only. *A. albus* (140) is phylogenetically most closely related to *A. cumminsii* but might be differentiated phenotypically by being resistant to desferrioxamine, whereas *A. cumminsii* is susceptible. *A. cumminsii* has a distinctive CFA pattern with $C_{14:0i}$ and $C_{14:0}$ each representing 2 to 4% of all CFAs (9). Penicillin MICs for most *Arthrobacter* strains are low, with quinolones showing only weak activities against *Arthrobacter* spp. (9, 24).

Genus *Brevibacterium*

Some *Brevibacterium* spp. are part of the human skin microbiome. Colonies are whitish-grayish (or yellowish like *B. luteolum*), convex, mostly creamy, and 2 mm or greater in diameter after 24 h. *B. mcbrellneri* colonies have a more granular appearance and are drier than those of other brevibacteria. Some brevibacteria may develop a yellowish or greenish pigment after prolonged incubation. Many *Brevibacterium* strains isolated from human clinical material give off a distinctive cheese-like odor. Brevibacteria are nonmotile, are halotolerant (6.5% NaCl), and form methanethiol from methionine, but this test is specific for brevibacteria only when it is read within 2 h (25). Brevibacteria can be identified to the species level by carbohydrate utilization tests. More than 80% of all clinical *Brevibacterium* isolates are *B. casei* (25). *B. sanguinis* is very similar to *B. casei* and can be differentiated from this species by susceptibility to thallium acetate. The MICs of β-lactam antibiotics for brevibacteria are often elevated (81).

Genus *Dermabacter*

D. hominis strains are part of the human skin microbiome. Colonies are whitish, convex, of a creamy or sticky consistency, and 1 to 1.5 mm in diameter after 48 h (Fig. 3e). *D. hominis* strains are sometimes mistaken for small-colony coagulase-negative staphylococci. The Gram staining result is distinctive, with coccobacillary or coccoidal forms (Fig. 1c). The key biochemical reactions are given in Table 4. *D. hominis* is one of the few coryneform bacteria with a variable reaction for xylose fermentation. It is the only catalase-positive coryneform bacterium (except *Actinomyces neuii*) that is able to decarboxylate lysine and ornithine (141). The API Coryne system and the RapID CB Plus panel correctly identify this species (64, 66). *D. hominis* strains may be resistant to aminoglycosides (60, 81).

Genus *Helcobacillus*

The genus *Helcobacillus* contains one species, *H. massiliensis*, which by phenotypic testing, resembles *D. hominis*. However, when using substrates found in the API Coryne or API 50CH panel, *H. massiliensis* can be differentiated from *D. hominis* by several key reactions, including being able to ferment erythritol, arabinose, xylose, inositol, and mannitol and reduce nitrate (27).

Genus *Rothia*

The genus *Rothia* presently comprises six validly named species, three of which are deemed to be clinically relevant: *R. mucilaginosa* (formerly designated *Stomatococcus mucilaginosus*) (10); *R. dentocariosa*, as emended by Daneshvar et al. (86); and *R. aeria*, initially recovered from the environment (11), which has been recovered from a variety of clinical materials (142). Some strains formerly designated as CDC coryneform group 4 (Fig. 3h) have been shown to be representatives of *R. dentocariosa* (86). These grayish-black pigmented strains were isolated primarily from respiratory materials, pus, or blood cultures (Fig. 3h), in contrast to blackish pigmented *C. aurimucosum* strains derived from the female urogenital tract (Fig. 3g).

Colonies of *R. dentocariosa* are typically whitish (or more rarely grayish-black), raised, smooth or rough, or have a "spoke-wheel" form (Fig. 3f), and they are up to 2 mm in diameter after 48 h. *Rothia* strains usually grow slightly better in a CO_2-enriched atmosphere. The biochemical features of *R. dentocariosa* are given in Table 4. The API Coryne system correctly identifies *R. dentocariosa* (64). Its CFA composition is of the branched-chain type (21), which allows differentiation from the biochemically similar species *C. durum*, *C. matruchotii*, and *Actinomyces viscosus* (chapter 52), all of which also occupy the oropharynx. *R. dentocariosa* may also be confused with *D. hominis* and *Propionibacterium avidum* (chapter 52) (143), both of which, in contrast, always exhibit smooth colonies. As shown in a study on the pharyngeal bacterial flora of healthy adults, one-third of all *R. dentocariosa* strains isolated were negative for the key biochemical reaction, catalase (41). The MICs of aminoglycosides for some *R. dentocariosa* strains are elevated, whereas penicillins usually show good *in vitro* activities against *Rothia* strains. It may be difficult to achieve acceptable growth while performing antimicrobial susceptibility testing on *R. dentocariosa* strains which are overly sticky or grow poorly in air.

Genus *Exiguobacterium*

It is not known whether exiguobacteria are part of the indigenous bacterial flora of humans. Colonies of *E. acetylicum* are smooth, golden-yellow to orange, and up to 2 mm in diameter after 24 h of incubation. Oxidase is positive, and acid from carbohydrates, except for xylose, is rapidly produced by fermentative metabolism. Exiguobacteria are motile. They might be confused with microbacteria, but CFA and phylogenetic analyses provide a clear-cut distinction between the two genera (Table 1). The pathogenic potential of *E. acetylicum* seems to be rather low; this species has been isolated from different sources (e.g., skin, wounds, and cerebrospinal fluid) (63). Cases of pseudobacteremia due to *E. acetylicum* have been observed. Six *E. aurantiacum* strains isolated from blood cultures had been received by a National Reference Center over a 10-year period, which, although biochemically similar to *E. acetylicum*, are oxidase negative and positive or variably positive for DNase and xylose, with strains being susceptible to all drug classes as tested by E-test (bioMérieux) (144).

Genus *Oerskovia*

O. turbata, a very rare human pathogen, is usually acquired from the environment (e.g., soil). Colonies are pale yellow to phosphorous yellow, convex, and creamy; they penetrate into the agar ("substrate hyphae"); and they are approximately 1 to 2 mm in diameter after 24 h. *O. turbata* rapidly produces acid from sugars by fermentation; it also exhibits a very strong esculin reaction. The genus is identified by the API Coryne system (64). *O. turbata* liquefies gelatin but does not hydrolyze xanthine or hypoxanthine. In contrast, the related *Cellulomonas cellulans* (below) does not liquefy gelatin but is able to hydrolyze xanthine and hypoxanthine (15).

Genus *Cellulomonas*

Cellulomonas strains are usually acquired from the environment. Colonies are first whitish or pale or bright yellow, but after 7 days, nearly all *Cellulomonas* strains are somewhat yellow. Colonies vary between 0.5 and 1.5 mm in diameter after 24 h, are convex and creamy, and have entire edges but do not demonstrate substrate hyphae (29). *Cellulomonas* spp. are variable for the fermentation of mannitol. Other key biochemical reactions are given in Table 4. The majority of *Cellulomonas* strains express cellulase activity, as demonstrated by incubating a heavy bacterial suspension (McFarland no. 6 standard) with a piece of sterile copy paper in a 0.9% NaCl solution for 10 days, resulting in dissolution of the paper (13). Medically relevant species *C. denverensis* can be differentiated from *C. hominis* by its positive reaction for D-sorbitol fermentation (29).

Genus *Cellulosimicrobium*

Colonies of *C. cellulans* are similar to *O. turbata* (see above) and also pit the agar. In addition, *C. cellulans* exhibits a biochemical screening profile which is very similar to that of *O. turbata* (Table 4). However, *C. cellulans* hydrolyzes either xanthine or hypoxanthine, whereas *O. turbata* does not, and *O. turbata* strains might be motile, whereas *C. cellulans* strains are not (63). *C. funkei* strains can be differentiated from *C. cellulans* (formerly *O. xanthineolytica*) by their negative inulin and raffinose fermentation reactions (15); in addition, *C. funkei* strains are motile but *C. cellulans* strains are not.

Genus *Microbacterium*

Microbacteria account for the majority of yellow-pigmented coryneform bacteria isolated from clinical specimens. All shades of yellow pigment are observed, ranging from pale to bright yellow and orange. Most of the strains are catalase positive, but catalase-negative strains might be observed. Some microbacteria grow under anaerobic conditions but only weakly. Motility is variable. Some microbacteria are nitrate reductase negative, which separates them from the phenotypically closely related genus *Cellulomonas*, where all presently defined species are nitrate reductase positive (Table 4). Microbacteria may ferment mannitol and xylose. In contrast, no *Cellulomonas* strain was observed to ferment mannitol and all fermented xylose.

Species identification is almost impossible by phenotypic analysis alone since the type strain is the only representative for many defined *Microbacterium* species, preventing the creation of a comprehensive database. Final identification to the species level is best achieved by molecular investigations (e.g., using 16S rRNA gene sequencing). The most frequently isolated, validated microbacteria in clinical specimens include *M. oxydans*, *M. paraoxydans*, and *M. foliorum* (31).

Microbacteria are usually susceptible to meropenem, linezolid, doxycycline, and vancomycin (except *M. resistens*, which is resistant to vancomycin but susceptible to teicoplanin) (145), but susceptibility to other antimicrobial agents is unpredictable and, therefore, every individual clinically significant strain must be tested (31, 146).

Genus *Curtobacterium*

Curtobacteria are very infrequently isolated yellow- or yellow-orange-pigmented oxidative coryneform bacteria. In contrast to most microbacteria, they produce acid from carbohydrates very slowly (within 4 to 7 days) (34). Curtobacteria are usually nitrate reductase negative but strongly hydrolyze esculin (Table 4). Again, the differentiation of curtobacteria is very difficult and should only be performed in a reference laboratory using primarily molecular methods. The MICs of macrolides and rifampin for curtobacteria are very low.

Genus *Leifsonia*

Leifsonia aquatica is very rarely encountered in clinical specimens. It is always motile, does not hydrolyze either gelatin or casein, and has a stronger DNase activity than most microbacteria (32). *L. aquatica* is the only species within the genus *Leifsonia* which is able to grow in broth enriched with 5% NaCl (35). Its yellow pigment develops relatively slowly within 3 to 4 days. The MICs of vancomycin for some *L. aquatica* strains were shown to be elevated (8 μg/ml) (32), but the precise mechanism of this resistance is not known.

Genera *Janibacter*, *Pseudoclavibacter* (*Zimmermannella*), *Brachybacterium*, and *Knoellia*

Strains belonging to the genera *Janibacter*, *Pseudoclavibacter* (*Zimmermannella*), *Brachybacterium*, and *Knoellia* have been derived from the environment, foods, or animals but also have been recovered recently from clinical materials and characterized using 16S rRNA gene sequencing. These taxa were described as having white, creamy, or yellowish pigment and all were nonmotile. *Janibacter*, *Pseudoclavibacter* (later synonym *Zimmermannella)*, and *Knoellia* were oxidative, and *Brachybacterium* strains were fermentative. All strains tested so far were susceptible to vancomycin. Since no comprehensive biochemical data based on a large number of strains are available at present, these genera were not included in Table 4.

Genera *Arcanobacterium* and *Trueperella*

The three medically relevant species *A. haemolyticum*, *T. pyogenes*, and *T. bernardiae* are all catalase negative and exhibit beta-hemolysis on SBA. All species show a fermentative glucose metabolism, with succinic and lactic acids as their major end products. All grow and express hemolysis best in a CO_2-enriched atmosphere.

The colonies of the type species, *A. haemolyticum*, are 0.5 mm in diameter after 48 h of incubation at 37°C. The biochemical reactions of *A. haemolyticum* are given in Table 4. Of major value for the identification of *A. haemolyticum* is the so-called CAMP inhibition test (see the description of the CAMP inhibition test in the section on *C. pseudotuberculosis*) (Fig. 2). The protein responsible for this phenomenon is a phospholipase D excreted by *A. haemolyticum*, and this protein is genetically and functionally similar to the ones expressed by *C. ulcerans* and *C. pseudotuberculosis*. *A. haemolyticum* and the two other medically relevant arcanobacteria are correctly identified by the API Coryne system (64).

T. pyogenes colonies are the largest of all former arcanobacteria, with diameters of up to 1 mm after 48 h of incubation. Of all the previous arcanobacteria, this species also shows the sharpest zone of beta-hemolysis on SBA. The protein responsible for hemolysis, named pyolysin, is also an important virulence factor *in vivo*. Gram stains may show some branching rods. *T. pyogenes* is the only *Arcanobacterium/Trueperella* species of medical relevance that expresses β-glucuronidase activity and that is capable of fermenting xylose.

T. bernardiae (40, 147) shows glassy, whitish colonies of <0.5 mm in diameter after 48 h. Some colonies have a creamy consistency, whereas others are sticky. Gram staining shows relatively short rods without branching. Most *T. bernardiae* strains belong to the very few coryneform bacteria that are able to ferment glycogen. Another peculiar feature of *T. bernardiae* strains is their ability to produce acid faster from maltose than from glucose.

The MICs of all β-lactams, rifampin, and tetracycline for *A. haemolyticum* and *Trueperella* spp. are very low, whereas aminoglycosides and quinolones have reduced activities (Funke, unpublished). Macrolides also exhibit excellent activities and are an alternative to β-lactam antibiotics for the treatment of infections. Treatment failures due to β-lactam antibiotics because of the inability of β-lactam antibiotics to act intracellularly have been reported.

TYPING SYSTEMS

Outbreaks of *C. diphtheriae* in the states of the former Soviet Union and other locations have been studied by whole-cell peptide analysis, whole-genome restriction fragment length polymorphism analysis, ribotyping, pulsed-field gel electrophoresis, PCR–single-strand conformation polymorphism, analysis of *tox* and *dtxR* as well as of the 16S-23S rRNA spacer region, amplified fragment length polymorphisms, random amplification of polymorphic DNA, and multilocus enzyme electrophoresis (148, 149). An international database for *C. diphtheriae* ribotypes using endonuclease BstEII has been established (150). Ribotyping is regarded as being highly discriminatory and, based on a comprehensive comparison of methods, was found to be a preferred typing method for *C. diphtheriae* (149). Other methods, including a spoligotyping system (similar to the spacer oligonucleotide typing for *Mycobacterium tuberculosis*), have been described (151). Sequencing studies with *C. diphtheriae* strains from the epidemic in the former Soviet Union have shown that point mutations within the *tox* gene were silent mutations, whereas multiple-point mutations (which even led to amino acid substitutions) were observed for the *dtxR* gene, corresponding to the heterogeneity of outbreak strains as revealed by PCR–single-strand conformation polymorphism, analysis (152). Isolates derived from specific populations in the United States and Canada and characterized using multilocus enzyme electrophoresis, ribotyping, and random amplification of polymorphic DNA were found to be members of persistent endemic strains, rather than being imported from other countries where diphtheria is endemic. A standardized multilocus sequence typing method based on analysis of short sequences derived from seven housekeeping alleles has been developed (153). The technical simplicity of this approach and the ease of comparison of strain data internationally have made this a useful typing method (50). The multilocus sequence typing technique has also been effectively applied to an outbreak investigation involving *C. macginleyi* and *C. mastitidis*-like isolates (114).

Typing methods have occasionally been developed for taxa other than *Corynebacterium* species. Pulsed-field gel electrophoresis has been used to study clinical isolates of *A. haemolyticum* (154).

SEROLOGIC TESTS

Detection of antibodies directed against diphtheria toxin is the only established serologic test for coryneform bacteria. Toxin neutralization assays using Vero cell culture system have mainly been replaced by enzyme immune assays. Levels of ≥0.1 IU/ml serum are thought to confer protection, whereas levels of <0.01 IU/ml indicate a susceptible host and levels of 0.01 to 0.1 IU/ml indicate partially immune individuals. It is believed that between 20 and 60% of adults in the United States lack protective antibodies to diphtheria toxin due to declining antibody titers in immunized persons and due to failure to complete primary immunization. This could pose a potentially significant public health risk and could result in the reemergence of this disease. This caution has been reiterated in recent seroepidemiological studies (155). Booster doses of toxoid should be administered at 10-year intervals.

ANTIMICROBIAL SUSCEPTIBILITIES

The susceptibility patterns for each taxon were given with the descriptions of each taxon (see above). Since the antimicrobial susceptibility of coryneform bacteria is not predictable in every case, susceptibility testing should always be performed with clinically significant isolates (see "Clinical Significance" above). Due to the emergence of vancomycin-resistant Gram-positive organisms, it has become inappropriate to recommend glycopeptides as first-line drugs for the treatment of infections caused by coryneform bacteria. It is also noteworthy that some coryneform bacteria (e.g., *Microbacterium resistens*) are intrinsically vancomycin resistant.

The Clinical Laboratory Standards Institute has published testing conditions and interpretive criteria for susceptibility testing of coryneform bacteria using a broth microdilution method (156). Direct colony suspensions equivalent to a 0.5 McFarland standard are prepared, and strains are incubated in cation-adjusted Mueller-Hinton broth with 2 to 5% vol/vol lysed horse blood at 35°C in ambient air for up to 48 h. Interpretative categories for the MICs obtained are presently available for 16 antimicrobial agents. In summary, using the broth microdilution method, it has been recently established that multidrug resistance was found for four or more drug classes (out of 16 classes tested) for some or most strains of *C. amycolatum*, *C. jeikeium*, *C. urealyticum*, *C. resistens*, *C. tuberculostearicum*, and *C. striatum* as well as representatives of *C. afermentans* and *C. aurimucosum* found in one national culture collection (C. Singh, T. Burdz, and K. Bernard, unpublished data). Very few studies have been performed comparing broth microdilution and disk diffusion results for susceptibility testing of coryneform bacteria (157).

In the past, MICs have been determined by either the E-test or the agar dilution or broth microdilution method. The results of the E-test have been shown to correlate reasonably well with those of both the broth microdilution and the agar dilution methods for *Corynebacterium* spp. (158, 159). The E-test should be carried out on Mueller-Hinton agar supplemented with 5% sheep blood.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The guidelines related to when coryneform bacteria should be identified to the species level (see "Clinical Significance") are also applicable for evaluating and interpreting culture results; i.e., whenever coryneform bacteria are identified to the species level, the results should be reported.

In the rare case of microscopically suspected *C. diphtheriae* (i.e., a positive Neisser staining result), the physician in charge of the patient should be notified immediately, although culture results and toxin testing results become available only later.

It is evident that repeated isolation of a predominant strain of a coryneform bacterium or a coryneform bacterium growing in pure culture suggests an etiological relationship to the patient's disease. If coryneform bacteria are present in blood cultures, the physician in charge should be notified immediately, and it should be emphasized when reporting that the clinical significance of the coryneform bacteria must be carefully examined by cooperation between the microbiology laboratory and the physician. In our experience, one positive blood culture out of two or three aerobically and anaerobically incubated pairs of blood cultures is hardly ever clinically significant (except in cases of treated endocarditis). Care must be taken in the interpretation of the results for those patients for whom half or more of the blood specimens taken for culture become positive for coryneform bacteria, in particular when lipophilic corynebacteria are cultured, since not all blood samples taken from patients with endocarditis due to lipophilic corynebacteria may eventually become positive.

On the other hand, coryneform bacteria should be reported as "normal microbiome" when they are grown in equal or smaller numbers from nonsterile sites together with other resident flora. It is suggested that the primary isolation plates be retained for at least 72 h before they are discarded in order to have the opportunity to assess the bacterial population retrospectively.

APPENDIX

Genus *Gardnerella*

The genus *Gardnerella* does not have a particular phylogenetic relationship to any of the established genera described in this chapter. It is remotely related to the genus *Bifidobacterium*, and these genera share some important features, such as production of acetic and lactic acids as fermentation products. *G. vaginalis* is the only species belonging to the genus *Gardnerella*. Studies on the ultrastructure of the cell wall of *G. vaginalis* have demonstrated that it has a cell wall similar to but much thinner than the cell walls of other Gram-positive bacteria (i.e., there is a smaller peptidoglycan layer) (160). Lysine is the diamino acid of the cell wall, and CFAs are similar to those detected in *Actinomyces* spp., *Arcanobacterium* spp., *Trueperella* spp., and *Corynebacterium* spp., with $C_{16:0}$ and $C_{18:1\omega9c}$ predominating. The G+C content of 42 to 44 mol% is lower than that of every other genus described in this chapter.

Gram stains show thin Gram-variable rods or coccobacilli (Fig. 1d). Catalase is not produced, and cells are nonmotile and have a slow fermentative metabolism.

Epidemiology and Transmission

G. vaginalis can be found in the anorectal microbiome of healthy adults of both sexes as well as in that of children (161). It is also part of the endogenous vaginal flora in women of reproductive age. The optimal pH for the growth of *G. vaginalis* is between 6 and 7, i.e., at elevated pH in the vagina. The organism can also be recovered from the urethras of the male partners of women with bacterial vaginosis (BV) (161).

Clinical Significance

G. vaginalis is associated with BV. Its causative role in the syndrome is controversial (162, 163) as it is certainly not the sole cause; other bacteria like *Atopobium vaginae*, *Leptotrichia*/*Sneathia*, and *Megasphaera*-like bacterium are also involved in BV (164, 165). Recurrent BV is due to reinfection rather than to relapse (i.e., overgrowth of the previously colonizing biotype). In pregnant women, BV may lead to preterm birth, premature rupture of membranes, and chorioamnionitis (161). *G. vaginalis* may also be recovered from cultures of blood from patients with postpartum or postabortal fevers and may also cause infections in newborns. Although it might be recovered from the urethras of males, its disease association in males is usually questionable. Cases involving serious infections (septicemia, wound infections) in sites other than those associated with the genital tract or obstetrics are very rare but have been reported, including in men (166).

Collection, Transport, and Storage of Specimens

Vaginal and extravaginal specimens can be collected with cotton-tipped swabs. It is best to take one swab for direct examination and to take another swab for culture if necessary, such as for epidemiologic studies. If culture media cannot be directly inoculated, then the swab should be placed in a transport medium (e.g., Amies) and culture should be done within 24 h. It is noteworthy that *G. vaginalis* is susceptible to sodium polyanethol sulfonate (SPS), so an SPS-free medium (or an SPS medium supplemented with gelatin) should be used to achieve optimal recovery of *G. vaginalis* from blood culture systems whenever *G. vaginalis* is suspected.

Direct Examination, Isolation, and Identification

The gold standard for the diagnosis of BV is direct examination of vaginal secretions and not the culture of *G. vaginalis*, since *G. vaginalis* can also be recovered from healthy women. A bedside test for BV is examination of the vaginal discharge to detect the typical "fishy" trimethylamine odor which is enhanced after alkalinization with 10% KOH (but *G. vaginalis* is not responsible for the amine production). The typical smear of vaginal discharge from BV patients shows "clue cells" (bacteria covering epithelial cell margins) together with mixed flora consisting of large numbers of small Gram-negative (predominantly *Prevotella* and *Porphyromonas* spp.) and Gram-variable (*G. vaginalis*) rods and coccobacilli, whereas lactobacilli are almost always absent. It is recommended that a standardized Gram staining interpretative scheme be used to improve the reproducibility of this method (163, 167). Although not recommended for routine laboratory procedures, the isolation of G. *vaginalis* can support the diagnosis of BV. Vaginal swabs are cultured on Vaginalis agar (see chapter 19 for the preparation) and should be semiquantitatively streaked out with a loop. Incubation is at 35 to 37°C in a 5% CO_2-enriched atmosphere or in a candle jar. Slight beta-hemolysis is observed on human or rabbit blood-containing media but not on SBA (on which G. *vaginalis* can also grow, exhibiting alpha-hemolysis). Plates may be checked for the growth of diffuse beta-hemolytic colonies of <0.5 mm in diameter after 24 h, but very often, G. *vaginalis* is best observed after 48 h. Gram staining of the suspected colonies confirms the diagnosis of G. *vaginalis*.

G. *vaginalis* strains are consistently α-glucosidase, starch hydrolysis, and proline aminopeptidase positive, but only 90% of all G. *vaginalis* strains hydrolyze hippurate. Both the API Strep and API Coryne systems identify G. *vaginalis* well (64). Confirmation of the identification of G. *vaginalis* can also be achieved by antimicrobial agent disk inhibition tests with 50 µg of metronidazole (inhibition present), 5 µg of trimethoprim (inhibition present), and 1 mg of sulfonamide (inhibition absent).

Antimicrobial Susceptibilities

Metronidazole is the drug of choice both for local therapy of BV and for systemic therapy of extravaginal infections caused by BV-associated flora. Systemic infections due to G. *vaginalis* alone can be treated with ampicillin or amoxicillin, since β-lactamase-producing G. *vaginalis* strains have not been observed so far. Susceptibility testing for G. *vaginalis* is not recommended.

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Nocardia, Rhodococcus, Gordonia, Actinomadura, Streptomyces, and Other Aerobic Actinomycetes

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29

TAXONOMY

The word "actinomycete" is derived from two Greek roots (*actino-* and *-mycete*) meaning "ray" (and hence also "rod") and "fungus," respectively. The anaerobic organisms now in the genus *Actinomyces* and the aerobic organisms grouped together as the "aerobic actinomycetes" were previously presumed to be related to one another on the basis of shared features of microscopic and colonial morphology. The aerobic actinomycetes, a group for which no agreed-upon operational definition currently exists, are now known to be an evolutionarily heterogeneous assemblage of genera. At some stage, they all form Gram-positive rods, and most of the more commonly isolated species exhibit at least rudimentary branching under certain growth conditions; all grow better under aerobic than anaerobic conditions, a feature distinguishing them from most organisms in the genus *Actinomyces*. Figure 1 shows the current classification of genera included in this chapter according to the List of Prokaryotic Names with Standing in Nomenclature (LPSN http://www.bacterio.net/).

The organisms containing mycolic acids in their cell walls (included in the genera *Dietzia*, *Gordonia*, *Nocardia*, *Rhodococcus*, *Segniliparus*, *Tsukamurella*, and *Williamsia*) are rather closely related on the basis of molecular genetic studies (1, 2); these mycolic-acid-containing genera appear phylogenetically more closely related to the genera *Corynebacterium* and *Mycobacterium* (both of which are sometimes considered aerobic actinomycetes) than to the other non-mycolic-acid-containing genera usually also included with the aerobic actinomycetes. According to a recent classification scheme, these seven genera of actinomycetes, along with the genera *Corynebacterium* and *Mycobacterium*, are classified together in the suborder *Corynebacterineae* (LPSN). Note in Fig. 1 that the genera in the suborder *Corynebacterineae* are at least weakly acid fast, with the exception of *Corynebacterium*, *Williamsia*, and possibly *Dietzia*.

The number of recognized pathogenic species of aerobic actinomycetes has been rising rapidly. With the exception of the genus *Kroppenstedtia* (see below), only genera containing species of documented clinical significance are dealt with in this chapter. Unfortunately, the increasingly fine discrimination of species has made it difficult to delineate important species-specific differences in geographic distributions, pathogenic and other biological mechanisms, disease associations, and antimicrobial susceptibility patterns. This enormous proliferation of distinct species for which association with human disease has been claimed presents several problems for the clinical microbiologist. First, phenotypic testing has been rendered virtually useless for accurate discrimination among species. Particularly for the aerobic actinomycetes, the number of phenotypic tests available in clinical laboratories (and in most research laboratories) is far too small for accurate differentiation among so many species, and often, information on the percent positivity for a specific reaction in a given species is not known. Furthermore, precisely the same biochemical testing format has generally not been employed with isolates of all the species, making the usefulness of multistudy comparisons uncertain. Second, some types of testing, such as analysis of cell wall constituents and of whole-cell sugars, are available in only a few research settings and are rarely of use for species-level identification. Some general information regarding these features is provided herein, but the references should be consulted for performance procedures and additional details. Third, only gene sequencing is currently adequately discriminatory, reproducible, and sufficiently available to be useful for precise species identifications in clinical laboratories. Fourth, because of the growing problems with accurate species determinations, the clinical literature is rife with erroneous identifications; nowhere is this problem more apparent than with organisms in the genus *Nocardia*. Additionally, as some species have been described on the basis of only a single isolate, and for others only a few reports exist, little if any meaningful clinical information can be associated with many species names. Particularly when molecular methods are not available, precise identification of isolates may be impossible and is also frequently not of immediate clinical utility.

There are a few terminology issues that, while they pertain to all bacteria, seem to cause confusion particularly frequently with regard to the aerobic actinomycetes. First, for every bacterial species, there is only one "type strain," the strain on which the original description of the species was based. Other strains contained in culture collections that are thought to belong to the same species as a given type strain can be referred to as "reference strains," but they are not type strains. Second, the term "*sensu stricto*" means

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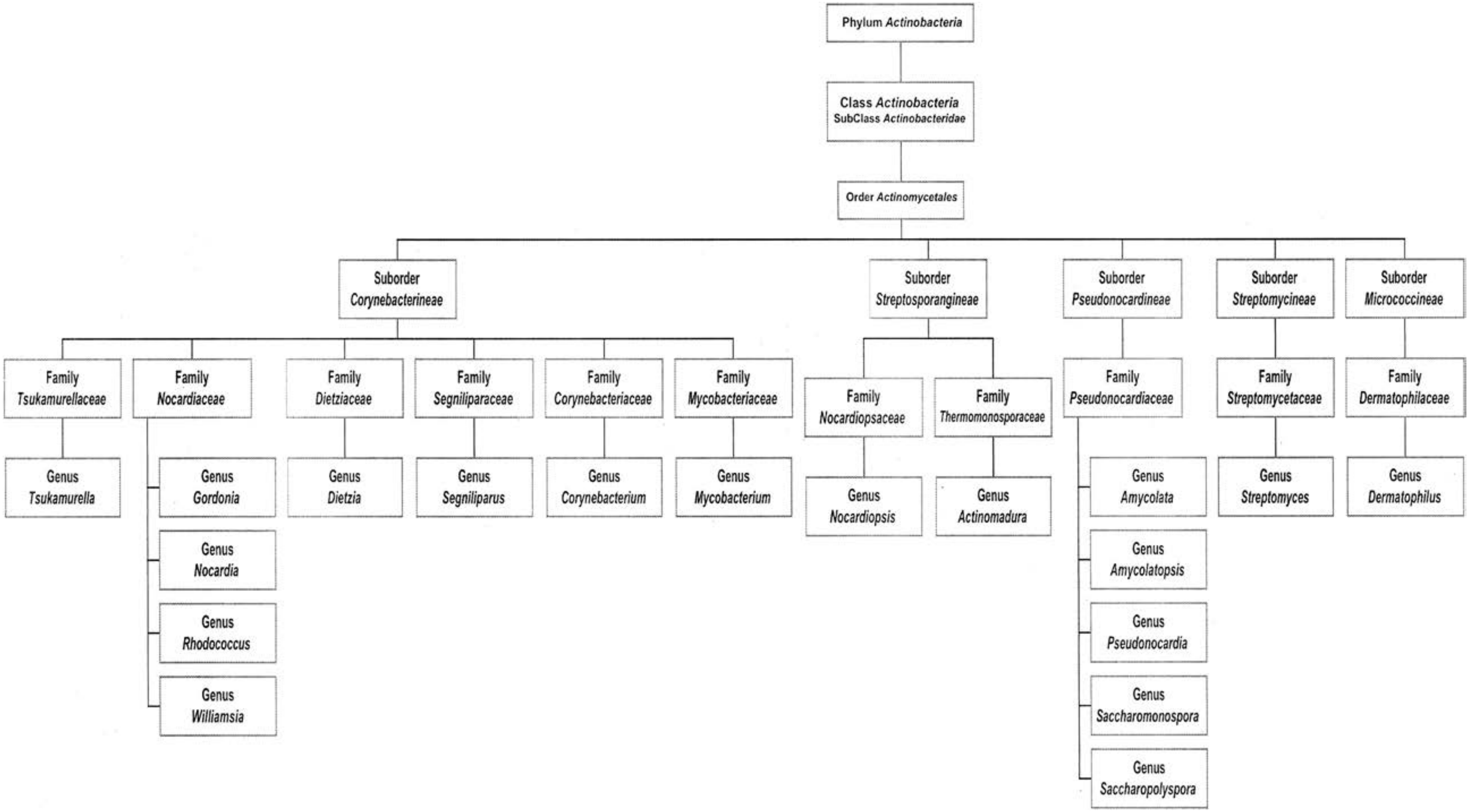

Phylum Actinobacteria
Class Actinobacteria
SubClass Actinobacteridae
Order Actinomycetales
Suborder Corynebacterineae
Suborder Streptosporangineae
Suborder Pseudonocardineae
Suborder Streptomycineae
Suborder Micrococcineae
Family Tsukamurellaceae
Family Nocardiaceae
Family Dietziaceae
Family Segniliparaceae
Family Corynebacteriaceae
Family Mycobacteriaceae
Family Nocardiopsaceae
Family Thermomonosporaceae
Family Pseudonocardiaceae
Family Streptomycetaceae
Family Dermatophilaceae
Genus Tsukamurella
Genus Gordonia
Genus Nocardia
Genus Rhodococcus
Genus Williamsia
Genus Dietzia
Genus Segniliparus
Genus Corynebacterium
Genus Mycobacterium
Genus Nocardiopsis
Genus Actinomadura
Genus Amycolata
Genus Amycolatopsis
Genus Pseudonocardia
Genus Saccharomonospora
Genus Saccharopolyspora
Genus Streptomyces
Genus Dermatophilus

FIGURE 1 Classification of genera of aerobic actinomycetes considered to be human pathogens, not including members of the family *Thermoactinomycetaceae*. Information from LPSN (http://www.bacterio.net/). doi:1128/9781555817381.ch29.f1

"in the strict sense." The term, when used with a species name, should be restricted to mean organisms belonging to that particular species, as determined by the best available methods. So, to refer to isolates that are "*Nocardia asteroides sensu stricto*" should mean isolates that by gene sequencing are identical to the type strain of *N. asteroides* (ATCC 19247).

DESCRIPTION OF THE GENERA

Basic information about specific microscopic and colonial morphologies of the following genera is presented in Table 1; additional information is included in the text below for some genera. See Table 2 for a comparison of the chemotaxonomic characteristics and lysozyme resistance (a phenotypic test, the results of which may vary among the genera) that distinguish these genera. In the listings that follow, enumeration of species within a genus is taken from the LPSN.

Actinomadura

The microscopic and colonial morphologies of organisms in the genus *Actinomadura*, which contains approximately 52 validly named species or subspecies, are very similar to those in the genus *Streptomyces* (Fig. 2A and B and 3A). The colors of the colonies may vary considerably among different species. Cell walls contain the sugar madurose, a feature shared only with organisms in the genus *Dermatophilus*.

Amycolata and *Amycolatopsis*

The genera *Amycolata* and *Amycolatopsis* were proposed by Lechevalier et al. for *Nocardia*-like organisms that were Gram positive and modified acid-fast stain negative but lacked mycolic acids (3) (Fig. 2F and 3B). Among the species transferred to the genus *Amycolatopsis* was *Nocardia orientalis*, two isolates of which were reported from clinical specimens (4). A study based on 16S rRNA gene sequences recommended combining the genera *Amycolata* and *Pseudonocardia* into an emended genus, *Pseudonocardia* (5). Currently, there are 4 validly named species in the genus *Amycolata* and approximately 52 validly named species or subspecies in the genus *Amycolatopsis*.

Dermatophilus

The two species currently making up the genus *Dermatophilus* are probably not closely related to most of the other organisms considered in this chapter. Aerial mycelia may be produced by colonies if the organism is grown in an atmosphere of increased CO_2; the organisms are facultatively anaerobic (6). The microscopic morphology is unusual and striking; this branching organism develops both longitudinal and transverse septa (Fig. 3D). The resulting chains of coccoid cells may occur in as many as eight parallel rows (7). The coccoid cells may develop into motile zoospores under favorable environmental conditions.

Desmospora

Desmospora activa is currently the only species in the recently described genus *Desmospora*. It is related to species in the genus *Thermoactinomyces*. This species is a filamentous, thermotolerant bacterium that was isolated from the sputum of a patient suspected of having tuberculosis, but it is unclear if it was the cause of the patient's pulmonary disease (8).

Dietzia

The species *Dietzia maris* was removed from the genus *Rhodococcus* because of chemotaxonomic and 16S rRNA gene sequence differences from *Rhodococcus* species (9). *Dietzia* isolates apparently rarely, if ever, branch but may show coccal and rod forms. There are approximately 13 species included in the genus *Dietzia*; however, discrimination among these species may be extremely difficult, even with gene sequencing methods.

Gordonia

Upon Gram staining, *Gordonia* species appear coryneform and could easily be mistaken for a component of the normal oral biota in sputum specimens (Fig. 3C). The genus "*Gordona*" (now *Gordonia*), originally described by Tsukamura, was revived in 1988 by Stackebrandt et al. (10) to contain species with mycolic acids of approximately 46 to 66 carbon atoms in length (11) and a predominant menaquinone of nine isoprene units. *Gordonia* and *Rhodococcus* were also found to be separable on the basis of their 16S rRNA gene sequences. The biology of the genus *Gordonia* has been reviewed (12). There are approximately 35 validly named species in the genus (Fig. 2I and 3C).

Kroppenstedtia

Kroppenstedtia was defined on the basis of an isolate found during environmental screening of a plastic surface in a manufacturing organization, but *Kroppenstedtia eburnea* has since been recovered from a variety of clinical specimens. There are two validly described species in the genus. *K. eburnea* has chemotaxonomic and molecular features typical of members of the family *Thermoactinomycetaceae*, except for containing LL-diaminopimelic acid in its cell wall peptidoglycan. (Diaminopimelic acid is in the *meso* conformation in the other members of the family [13].)

Nocardia

The most commonly isolated aerobic-actinomycete human pathogens belong to the genus *Nocardia*; there are approximately 87 validly named species included in this genus. In direct Gram smears, organisms generally appear as very long, obviously branching, thin, and finely beaded Gram-positive rods (Fig. 3E and K). Unlike the individual cells composing a streptococcal chain, the beads generally do not abut one another (Fig. 3H and O). Particularly when prepared from cultures, smears may show streptococcus-like chains or small branching filaments, probably as a result of the fragile nocardial mycelium breaking during smear preparation (14, 15) (Fig. 3E, F, G, K, and L).

Colonial morphology varies from species to species and frequently varies from isolate to isolate within a species (Fig. 2D, E, G, and L). Colony color may best be seen on the reverse when colonies are grown on translucent media (such as Sabouraud agar), as color may become obscured on the surface by the powdery aerial hyphae typically produced by members of this genus.

A brief account of the nomenclatural history of the designation "*Nocardia asteroides*" is necessary to clarify a confusing and widely unappreciated taxonomic issue. In 1888, Edmond Nocard obtained an isolate of an organism thought to be the causative agent of bovine farcy. This organism was given the name *Nocardia farcinica* by Trevisan in 1889, and thereupon, it became the type strain for both the genus and species. Gordon and Mihm (16) found, using their battery of phenotypic tests, that *N. farcinica* could not be distinguished from isolates to

TABLE 1 Morphologic characteristics of genera of aerobic actinomycetes[a]

Genus (reference[s])	Aerial hyphae	Modified acid-fast stain	Microscopic morphology	Figure(s)	Colonial morphology[b]	Figure(s)
Actinomadura (235)	V	Neg	Thin, short branching filaments	3A	Powdery aerial hyphae may be blue, brown, cream, gray, green, pink, white, violet, or yellow; colonies are wrinkled and may have a leathery or cartilaginous appearance when the aerial mycelium is absent	2A and B
Amycolata (3)	V	Neg	Best distinguished by slide culture	3B	When present, aerial hyphae may be white, yellow, or cream; colonies can be off-white, yellow, gold, or brown	
Amycolatopsis (3)	V	Neg	Best distinguished by slide culture		White, beige, yellow, olive, or brown; may have soluble brown pigment	2F
Corynebacterium[c] (236, 237)	Neg	Neg	Straight or slightly curved rods arranged singly or in pairs, often in V formation or in palisades of several parallel cells; may be coccobacillary; may be beaded or club shaped		Varies by species (see chapter 28)	
Dermatophilus (6, 7)	Pos[d]	Neg	Branching, septate hyphae, 0.6–1.0 µm in diam; mycelial elements consist of coccoid cells arranged longitudinally in a single row or in up to 8 parallel rows (see the text)	3D	Colonies gray-white to yellow or orange on prolonged incubation; rough, heaped, opaque, granular colonies with beta hemolysis; colonies may adhere to the agar	
Desmospora (8)	Pos	NF[e]	Branching		Leathery colonies, yellow aerial mycelia	
Dietzia (9)	Neg	Neg[f]	Coccobacilli to rods, often in V formation		Smooth, yellow	
Gordonia (10, 238)	Neg[g]	W	Short coryneform rods; no branching	3C	Rough, wrinkled, brownish, pink, or orange-to-red colonies	2I
Kroppenstedtia (13)	Sparse	NF[e]	Branching, straight to curved rods		Flat, irregular, colonies with undulate margins, dull surface, radial wrinkles, ivory colonies	
Mycobacterium[c] (239)	V	Pos	Straight or slightly curved rods, may appear as thin, beaded rods or as negative images by Gram staining		Varies by species (see chapters 30, 31, and 32)	
Nocardia (240)	Pos[h]	W	Thin, filamentous branching rods, 0.5–1.0 µm in diam; beading generally apparent (see the text)	3E to G, K, and L	Chalky, matte or velvety, powdery (usually), irregular, wrinkled, heaped, or smooth on the surface; may be brown, tan, pink, orange, red, purple, gray, yellow, peach, or white on the reverse; smooth or granular; soluble brown or yellow pigments may be produced (see the text)	2D, E, G, and L
Nocardiopsis (17, 241)	Pos	Neg	Best distinguished by slide culture (see the text)		Aerial mycelium can be sparse to abundant and blue, white, cream, yellow, gray, or green; zigzag arrangement of developing spores on aerial hyphae; colonies are coarsely wrinkled or folded; greenish-yellow or brown soluble pigment may be present	

(Continued on next page)

TABLE 1 Morphologic characteristics of genera of aerobic actinomycetes[a] *(Continued)*

Genus (reference[s])	Aerial hyphae	Modified acid-fast stain	Microscopic morphology	Figure(s)	Colonial morphology[b]	Figure(s)
Pseudonocardia (18)	V	Neg	Best distinguished by slide culture		White aerial hyphae; yellow colonies	
Rhodococcus (21)	Neg[i]	W	Coccoid to bacillary forms (see the text)	3I and J	Rough, smooth, or mucoid; buff, cream, yellow, orange, or red; colorless variants occur; may become increasingly pigmented and rough with age	2H
Saccharomonospora (26)	Pos	Neg	Best distinguished by slide culture		Leathery; aerial mycelium is initially white, becoming gray-green, dark green, or bluish; yellow, green, or brown soluble pigment	
Saccharopolyspora (27)	Pos	Neg	Best distinguished by slide culture		Thin, raised or convex colorless colonies, slightly wrinkled, and mucoid or gelatinous with sparse aerial mycelium; may have yellow soluble pigment	
Segniliparus (234)	Neg	Strongly Pos	Rod shaped with occasional V forms; no branching; size varies by species	3M and N	Smooth and domed to wrinkled and rough, varies by species; nonpigmented colonies; may produce a soluble pigment	2M
Streptomyces (242)	Pos	Neg	Filamentous branching rods, 0.5–2.0 µm in diam; filaments may fragment into short rods; may stain more solidly Gram positive than *Nocardia*; may show beading		Colonies are discrete and lichenoid, leathery, or butyrous; variety of pigments; aerial hyphae may appear floccose, granular, powdery, or velvety; may produce a colored soluble pigment	2C
Thermoactinomyces (31, 243)	V	NF	May become Gram negative with age		Slowly growing, white, yellow, or colorless colonies; aerial hyphae may be sparse	
Tsukamurella (32)	Neg	W	Straight to slightly curved long rods; occur singly, in pairs, or in masses; very short rods may be seen; no apparent branching	3J	White/creamy to orange; small with convex elevation; dry	2J and K
Williamsia (33)	Neg	Neg	Short rods or coccobacilli (see the text)	3O	Smooth, yellow, orange to orange-red colonies	2N and O

[a] Abbreviations: V, variable; Neg, negative; Pos, positive; NF, test result not found; W, weakly positive.
[b] Colonial morphology descriptions are from various references, the authors of which used a variety of different media; see the reference(s) for more information.
[c] Included for completeness; some consider *Corynebacterium* and *Mycobacterium* to be aerobic actinomycetes.
[d] Aerial hyphae may be produced in an increased-CO_2 atmosphere.
[e] This organism is noted to be non-acid fast; its modified acid-fast reaction is unknown.
[f] The original description was positive.
[g] Sparse aerial hyphae may be produced by *Godona amarae*.
[h] Occasionally no aerial hyphae are produced.
[i] Occasionally rudimentary aerial hyphae are seen.

which the name *Nocardia asteroides* had been applied. Because of some uncertainty relating to the isolate obtained by Nocard and what was presumed to be the conspecificity of the organisms then known as *N. farcinica* and *N. asteroides*, an appeal was made to the Judicial Commission to have the type species of the genus changed to *N. asteroides*, with strain ATCC 19247 selected as the type strain of the species. The appeal was accepted, but the isolate of *N. farcinica* was retained as the type strain for that particular species, as not all were convinced that the two species were truly identical.

Nocardiopsis

The genus *Nocardiopsis* was originally described by Meyer to accommodate an organism that at the time was called *Actinomadura dassonvillei* but that differed chemotaxonomically and in certain colonial morphologic features from other organisms in the genus *Actinomadura* (17). The cell wall of this organism does not contain madurose. The substrate mycelium fragments into coccal forms, and the aerial hyphae fragment into variously sized spores (17). Currently, there are approximately 39 named species or subspecies in the genus.

Pseudonocardia

Pseudonocardia spp. are characterized by the microscopic morphology of their aerial hyphae, which are segmented as a result of elongation by budding. Aerial and substrate hyphae are often zigzag shaped (18). Currently, there are approximately 48 published species in the genus.

TABLE 2 Chemotaxonomic and lysozyme growth characteristics of genera of aerobic actinomycetes[a]

Genus (reference[s])	Cell wall type[b]	Mycolic acids	No. of carbon atoms in mycolic acid	Growth in lysozyme[c]	Menaquinone
Actinomadura (235)	III	Neg	NA	V	MK-9($H_4H_6H_8$)
Amycolatopsis (3)	IV	Neg	NA	V	MK-9(H_2H_4)
Corynebacterium[d] (236)	IV	V	22–38	NT	MK-8(H_2) or MK-9(H_2)
Dermatophilus (6)	III	Neg	NA	NF	NF
Desmospora (8)	III	Neg	NA	NF	MK-7
Dietzia (9)	IV	Pos	34–38	NF	MK-8(H_2)
Gordonia (10, 238)	IV	Pos	48–66	V	MK-9(H_2)
Kroppenstedtia[e] (13)	I[d]	NF	NF	NF	MK-7
Mycobacterium[d] (239)	IV	Pos	60–90	Pos	MK-9(H_2)
Nocardia (240)	IV	Pos	44–64	Pos	MK-8(H_4) or MK-9(H_2)
Nocardiopsis (17, 241)	III	Neg	NA	Neg	MK-10($H_2H_4H_6$)
Pseudonocardia (18)	IV	Neg	NA	NF	MK-9(H_4)
Rhodococcus (21)	IV	Pos	34–64	Neg	MK-8(H_2) or MK-9(H_2)
Saccharomonospora (26)	IV	Neg	NA	Neg	MK-9(H_4)
Saccharopolyspora (27)	IV	Neg	NA	Neg	MK-9(H_4)
Segniliparus (234)	Meso-DAP[f]	Pos	70–90	V	NF
Streptomyces (242)	I	Neg	NA	V	MK-9(H_6H_8)
Thermoactinomyces (31, 243)	III	NF	NF	Pos	MK-7, MK-9
Tsukamurella (32)	IV	Pos	62–78	Pos	MK-9
Williamsia (33)	IV	Pos	50–56	NF	MK-9

[a] Abbreviations: V, variable; Neg, negative; NA, not applicable; Pos, positive; NF, test result not found.
[b] Cell wall types are as follows: I, L-DAP, no sugars; III, *meso*-DAP, madurose or no sugars; IV, *meso*-DAP, arabinose, and galactose.
[c] The lysozyme test may assist in initial genus assignment.
[d] Included for completeness; some consider *Corynebacterium* and *Mycobacterium* to be aerobic actinomycetes.
[e] Cell wall chemotype I is implied, as sugars were not detected in whole-cell hydrolysates.
[f] The cell wall contains *meso*-DAP; no data regarding cell wall sugars were found.

Rhodococcus

The microscopic morphology of rhodococci can range from coccoid to bacillary depending on species and specimen type and on the stage of growth of the organism (19). The organisms exhibit a rod-to-coccus growth cycle. Rod forms are best visualized by growing isolates in a liquid medium, and under such conditions, some branching of individual cells may be found. Generally, however, the organisms appear as Gram-positive coccobacilli which may be solidly stained or have a beaded appearance (Fig. 3H). Modified acid-fast staining must be performed and interpreted with particular care when dealing with isolates that may belong to this genus, as only a tiny fraction of the cells may retain the stain (Fig. 3I). Modified acid-fast smears prepared from isolates growing on Trypticase soy agar with 5% sheep blood or chocolate agar may appear to be acid-fast stain negative (20). *Rhodococcus* species can easily be dismissed as "diphtheroids" because of their Gram stain morphology (Fig. 3H) and their frequent failure to develop obvious pigmentation during the first few days of growth. At 37°C, colonies may be only about 1 mm in diameter after 24 h of incubation. An aerial mycelium is generally not macroscopically visible but may occasionally be seen microscopically (21). *Rhodococcus* has been the subject of several reviews (22, 23). Some data suggest that it may be justifiable to separate the genus into several additional genera (23). There are approximately 35 named species in the genus. There has been a recent proposal that *Rhodococcus equi* be transferred to a new genus, *Prescottella*, which to date has been effectively but not validly described in the LPSN. The new genus contains only the species *P. equi* (24, 25).

Saccharomonospora

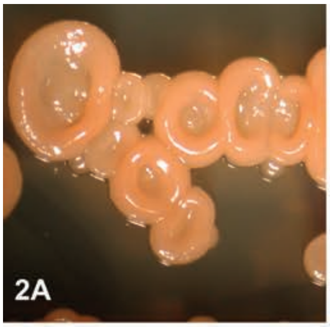
The approximately nine described species of the genus *Saccharomonospora* are characterized by the presence of single spores tightly packed on aerial hyphae. The organisms are commonly found in soil, lake sediment, peat, compost, and manure. They are moderately thermophilic, with optimum growth occurring at 35 to 50°C (26).

Saccharopolyspora

Species in the genus *Saccharopolyspora* were initially thought to be related to *Nocardia* and *Streptomyces* by biochemical characteristics but are phylogenetically distinct from those genera. The organism was originally isolated from sugar cane and has a microscopic morphology similar to that of species in the genus *Nocardiopsis*. It is so named because of the presence of beadlike chains of sheath-enclosed spores formed by segmentation of the aerial hyphae. The organism grows at temperatures between 25 and 50°C, with optimum growth at 37 to 40°C (27). There are approximately 21 described species and subspecies in the genus.

Segniliparus

The presence of unique high-pressure liquid chromatography mycolic acid patterns in four clinical isolates thought to belong to the genus *Mycobacterium* initiated an investigation into the characteristics of this recently described genus. By 16S rRNA gene sequencing, the genus is most closely related to members of the genus *Rhodococcus*, and cell wall chemistry places it in the suborder *Corynebacterineae* with *Rhodococcus* and other related genera (28). *Segniliparus* is the only aerobic actinomycete (besides *Mycobacterium*) that is strongly acid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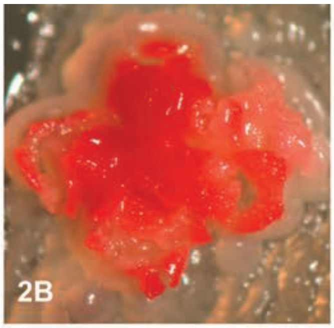
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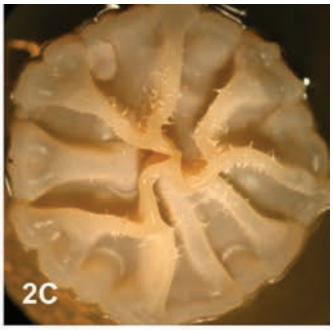
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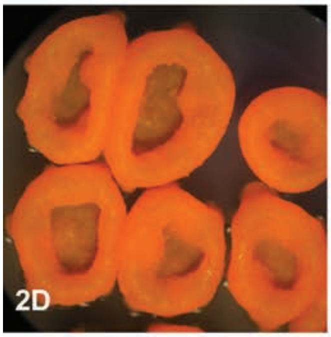
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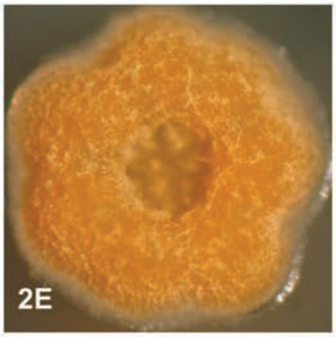
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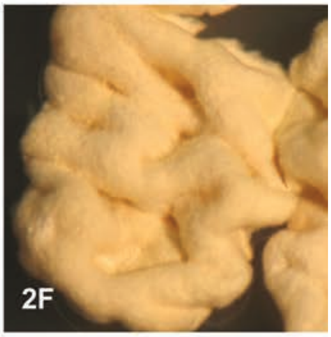
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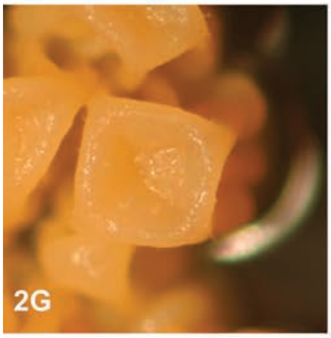
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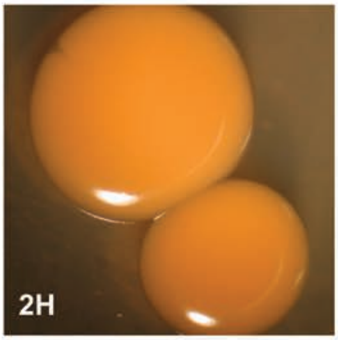
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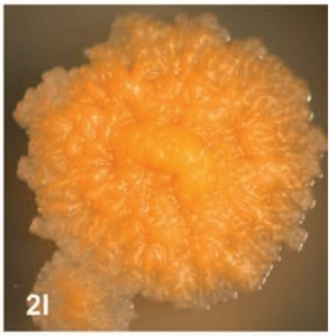
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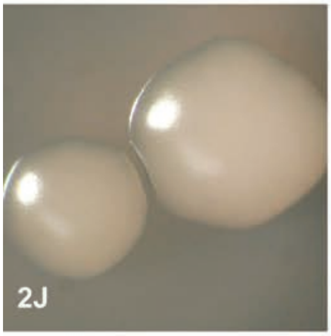
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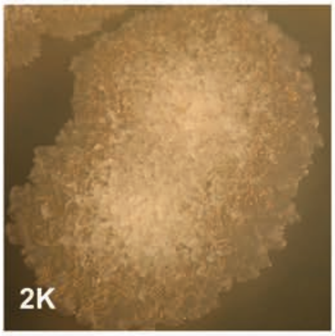
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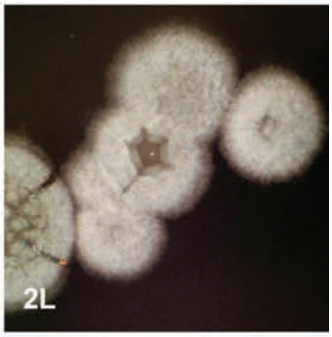
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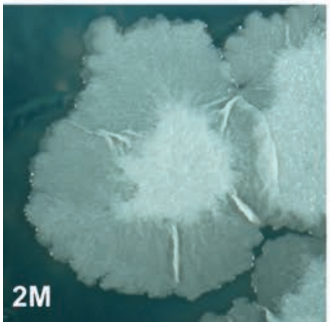
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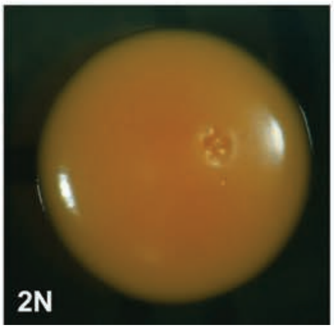
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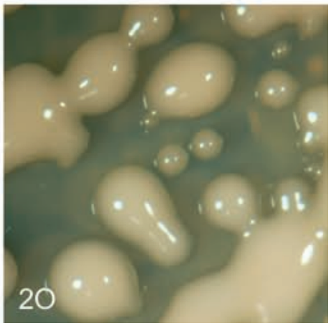
2O

fast (Fig. 2M and 3M and N). There are two validly described species in this genus.

Streptomyces

The genus- and species-level taxonomy of this huge group, which contains approximately 525 named species or subspecies, remains problematic (29, 30). Many of these species have been patented because of the commercially useful products that they synthesize (29) (Fig. 2C).

Thermoactinomyces

The approximately three species of the genus *Thermoactinomyces* are characterized by their production of abundant endospores that are resistant to heat and easily become airborne. Organisms are found in soil, moldy and decaying plant materials, and composts. By their 16S rRNA gene sequences and G+C contents, members of this genus are more closely related to the genus *Bacillus* than they are to the other genera considered aerobic actinomycetes, but they are considered with this group because of their similar morphologic features. Optimum growth temperatures vary by species, but most species grow between 35 and 58°C (31).

Tsukamurella

The genus *Tsukamurella* was created to accommodate organisms with a specific cell wall chemistry that separated them from other aerobic actinomycetes (32). The type species of the genus, *Tsukamurella paurometabola*, was previously known as *Corynebacterium paurometabolum*. There are approximately 12 named species in the genus. The taxonomy of the genus remains confusing; see below for additional details (Fig. 2J and K and 3J).

Williamsia

The genus *Williamsia* was recently established to include environmental organisms that resembled those in genera belonging to the family *Nocardiaceae* but that had unusual cell morphology. When examined by electron microscopy, cells of *Williamsia* show hairy structures distributed over the whole surface of the cell. These structures are not visible in negatively staining preparations, indicating that the structures are composed of fibrillar capsular material and not true fimbriae (33). The genus contains approximately eight species (Fig. 2N and O and 3O).

EPIDEMIOLOGY AND TRANSMISSION

While the aerobic actinomycetes are widely distributed in the environment, the extent to which particular species are geographically restricted is not well known. Their primary ecological niche is probably the decomposition of plant material (30).

The majority of infections caused by aerobic actinomycetes stem from environmental sources, and even most nosocomial infections appear attributable to an environmental source, such as dust from construction work (34). There has not yet been a documented case of direct patient-to-patient spread of an aerobic actinomycete infection without the intermediation of another human agent or of environmental contamination.

Two outbreaks of *Gordonia bronchialis* sternal wound infections were traced to nurses from whose hands and other body sites the organism was isolated. In one case, the organism was also isolated from two of the nurse's dogs (35, 36).

An outbreak of pseudoinfection attributed to *T. paurometabola*, involving specimens from 10 different patients and attributed to a common source somewhere in the laboratory, has also been reported (37). See the 9th edition of the *Manual of Clinical Microbiology* for a discussion of additional outbreaks and pseudo-outbreaks (38).

CLINICAL SIGNIFICANCE

While the number of recognized species of aerobic actinomycetes is rapidly increasing, assessment of the clinical significance of many species is becoming increasingly difficult. In some cases, a new species is described on the basis of a single isolate, and while careful attention is paid to its molecular features, little or no information may be provided to document that the isolate was actually a cause of disease in the patient from whom it was isolated. On the other hand, reports continue to be published regarding organisms that, while unquestionably the cause of disease, have been misidentified because of failure to use molecular methods; in other cases, even when molecular methods have been used, the accuracy of the reported identifications may be in doubt because of the provision of inadequate detail regarding the molecular technique used and the methods of interpretation used. Furthermore, many identifications reported in the older literature would be considered incorrect by currently accepted species criteria; perhaps the best example is that of *Nocardia asteroides*, which has only rarely been documented to be a human pathogen using currently accepted taxonomy.

Unfortunately, all these factors hinder the accurate association of clinically useful information with a given species. There is perhaps a growing tendency to assemble species into "groups" on the basis of certain features, such as antimicrobial susceptibility, as was originally done by Wallace et al. for what were considered antibiogram types of *N. aster-*

FIGURE 2 Colonial morphology of type strains of various aerobic actinomycetes grown on Sabouraud dextrose agar (Emmons modification), unless otherwise noted. While appearances are typical for the species illustrated, the extent of possible colonial variation within a given species is not known. (A) *Actinomadura latina* at 21 days (smooth umbilicate colonies with no aerial hyphae); (B) *Actinomadura pelletieri* at 10 days (ropy but smooth-surfaced colony with no aerial hyphae); (C) *Streptomyces griseus* at 10 days (rugose colonies with some aerial hyphae); (D) *Nocardia brasiliensis* at 10 days (umbilicate colonies; sparse aerial hyphae are present, but visible only on microscopic inspection of colonies); (E) *Nocardia carnea* at 25 days (with aerial hyphae present); (F) *Amycolatopsis orientalis* at 21 days (with aerial hyphae present); (G) *Nocardia otitidiscaviarum* at 7 days (rugose colony with sparse aerial hyphae present, visible only on careful microscopic inspection); (H) *Rhodococcus equi* at 10 days (smooth colonies with no aerial hyphae); (I) *Gordonia bronchialis* at 10 days (irregular but smooth surface with no aerial hyphae); (J) *Tsukamurella pulmonis* at 10 days (smooth colonies with no aerial hyphae); (K) *Tsukamurella strandjordii* at 10 days (rough colonies with no aerial hyphae); (L) *Nocardia cyriacigeorgica* at 10 days (with dense powdery aerial hyphae covering most or all the surface of the colonies); (M) *Segniliparus rugosus* at 15 days on Middlebrook agar (rough colonies with no aerial hyphae); (N) *Williamsia deligens* at 15 days (smooth colonies with no aerial hyphae); (O) *Williamsia muralis* at 15 days (mucoid colonies).
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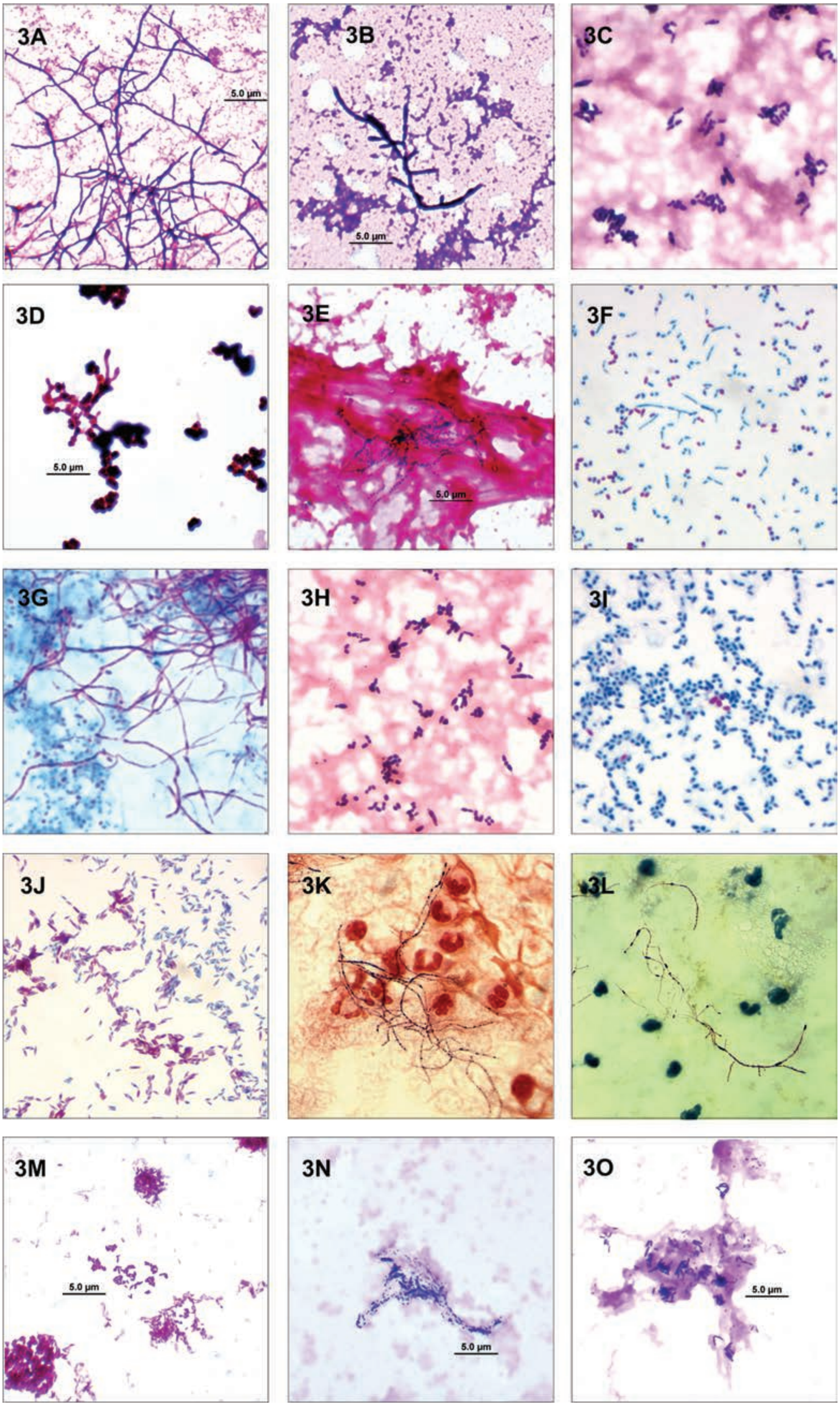
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TABLE 3 Species of aerobic actinomycetes other than *Nocardia* species more frequently reported as human pathogens and GenBank accession numbers of type strain sequences[a]

Genus and species	GenBank accession no. of type strain sequence
Actinomadura madurae	NR_026343
Actinomadura pelletieri	AJ293710
Dermatophilus congolensis	AJ243918
Dietzia species	See the text for species identification-related issues
Gordonia bronchialis	NR_027594
Gordonia terrae	X79286[b]
Nocardiopsis dassonvillei	NR_029314
Rhodococcus equi	FJ468344
Streptomyces somaliensis	AJ007403
Tsukamurella paurometabola	AF283281
Tsukamurella tyrosinosolvens	AY238514

[a] GenBank sequences are available at http://www.ncbi.nlm.nih.gov/.
[b] The best sequence available for the *G. terrae* type strain.

TABLE 4 Species of *Nocardia* more frequently reported as human pathogens and GenBank accession numbers of type strain sequences

Nocardia species	GenBank accession no. of the type strain sequence
N. abscessus	AF430018
N. brasiliensis	AF430038
N. cyriacigeorgica	GQ376180
N. farcinica	AF430033
N. nova	AF430028
N. otitidiscaviarum	AF430067
N. pseudobrasiliensis	AF430042
N. veterana	AY171039
N. wallacei	GQ853074

oides but which are now divided into different species (39). While currently "species" are delineated and identified primarily, if not entirely, on the basis of molecular features, possibly a separate but more relevant clinically based taxonomy may emerge, based not only on molecular features but also on such aspects as pathogenicity, antimicrobial susceptibility, and type of disease produced, all of which presumably have a basis in the fundamental molecular features of the organism.

Tables 3 and 4 list species of aerobic actinomycetes other than those in the genus *Nocardia* and those in the genus *Nocardia*, respectively, that are considered relatively frequent human pathogens or, in a few cases, are clearly established pathogens but probably have previously been unrecognized as separate species. Tables 5 and 6 list species of aerobic actinomycetes other than those in the genus *Nocardia* and species in the genus *Nocardia*, respectively, that have only rarely (often only once) been reported as human isolates; their actual causative role in human disease does not seem well established, and there are no recent reports for many of them. Tables 5 and 6 should by no means be considered exhaustive. Whenever a laboratory isolates an aerobic actinomycete whose sequence indicates that the organism belongs to a species that has rarely been isolated, the circumstances surrounding the isolation of the organism should be carefully evaluated to assess its potential clinical significance. A search of the current literature may be conducted to determine what is already established regarding that species, including its known pathogenicity and antibiotic susceptibilities. For such searches, the LPSN and PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) may be good starting points; current nomenclature and taxonomic standing should be followed as stated in the LPSN.

Actinomadura

A useful clue to the identification of organisms in the genus *Actinomadura* is the nature of the lesion from which an isolate originates. Most commonly, this organism causes a mycetoma, a chronic, invasive, slowly progressive infection usually occurring in the foot (Madura foot) and nearby anatomic structures and nearly always resulting from traumatic implantation of the organism. Draining sinuses are typically present in a mycetoma; macroscopically visible grains (organism aggregates or microcolonies) may be visible in the discharge from the lesions. The infection occurs most frequently in tropical regions, where people are more likely to walk barefoot. The word "mycetoma" is used only to describe the clinical nature of the infection, not the etiologic agent. Actinomycotic mycetomas are caused by aerobic acti-

FIGURE 3 Microscopic morphology of various aerobic actinomycetes. All photomicrographs were taken at a ×1,000 magnification. (A) *Actinomadura latina* (Gram stain from Tween-albumin broth [TAB] at 4 days, with long, branching, relatively solidly staining rods; (B) *Amycolata autotrophica* (Gram stain from TAB at 4 days, with long beaded rods with some branching); (C) *Gordonia bronchialis* (Gram stain from TAB at 4 days, showing coryneform rods with no obvious branching); (D) *Dermatophilus congolensis* (Gram stain from Sabouraud dextrose agar at 14 days in CO_2, with dense aggregates of cells of various sizes [note the chains of longitudinally and transversely dividing cells near the top]); (E) *Nocardia abscessus* (Gram stain from TAB at 10 days, with long, beaded, branching rods [note that the beads generally do not abut one another]); (F) *Nocardia farcinica* (modified acid-fast stain from horse blood agar at 2 to 3 days showing many coccal forms [it is mostly these that are modified acid-fast stain positive]); (G) *N. veterana* (modified acid-fast stain from TAB at 25 days showing some long, branching forms that are modified acid-fast stain positive); (H) *Rhodococcus equi* (Gram stain from TAB at 4 days showing coccobacilli and short coryneform rods without obvious branching); (I) *Rhodococcus equi* (modified acid-fast stain from charcoal yeast extract [CYE] agar at 10 days, on which only a small percentage of the cells stain positive); (J) *Tsukamurella pulmonis* (modified acid-fast stain from Lowenstein-Jensen medium at 4 days showing thin rods, many of which stain positive); (K) direct Gram stain of sputum that grew a *Nocardia* species (note the lacy network of long, thin, branching, beaded rods [courtesy of Daniel P. Fedorko]); (L) direct modified acid-fast stain of the same specimen as in panel K (the beads are purplish, but the intervening areas of the organism stain positive [courtesy of Daniel P. Fedorko]); (M) *Segniliparus rotundus* (Kinyoun stain from Middlebrook at 12 days); (N) *Segniliparus rugosus* (Gram stain from TAB at 7 days); (O) *Williamsia deligens* (Gram stain from TAB at 5 days). doi:1128/9781555817381.ch29.f3

nomycetes; eumycotic mycetomas are caused by true fungi. Organisms in the genus *Actinomadura* have very rarely been implicated in other types of infection. *Actinomadura madurae* and *Actinomadura pelletieri* have been the two species most frequently reported as pathogens. In many reports mentioning the species causing mycetomas, identification has been made solely on the basis of the histological appearance of the grains, on the basis of a small number of phenotypic tests, or in ways not specified in any detail (40–43). As with virtually all the aerobic actinomycetes, molecular methods are the only procedures that allow definitive species-level identification of organisms in this genus.

TABLE 5 Species of aerobic actinomycetes other than *Nocardia* species infrequently reported or poorly documented as human pathogens and GenBank accession numbers of type strain sequences

Aerobic actinomycete species	GenBank accession no. of the type strain[a] sequence
Actinomadura chibensis	AB264086
Actinomadura cremea	AF134067
Actinomadura latina	AY035998
Actinomadura sputi	FM957483
Actinomadura nitritigenes	AY035999
Actinomadura vinacea	AF134070
Amycolatopsis benzoatilytica	AY957506
Amycolatopsis orientalis	AJ400711
Amycolatopsis palatopharyngis	AF479268
Desmospora activa	AM940019
Dietzia species[b]	See the text for species identification-related issues
Gordonia araii	AB162800
Gordonia effusa	AB162799
Gordonia otitidis	AB122026
Gordonia polyisoprenivorans	NR_026500
Gordonia rubripertincta	X80632
Gordonia sputi	X80634
Kroppenstedtia eburnea	FN665656
Rhodococcus corynebacterioides[c]	AF430066
Rhodococcus erythropolis	X79289[d]
Rhodococcus fascians[e]	X79186
Rhodococcus globerulus	NR_026184
Rhodococcus gordoniae	AY233201
Rhodococcus rhodochrous	FJ468342
Segniliparus rotundus	AY608918
Segniliparus rugosus	AY608920
Streptomyces albus	AJ621602
Streptomyces bikiniensis	X79851
Streptomyces cinereoruber	AY999771
Streptomyces griseus	AY207604[f]
Streptomyces sudanensis[g]	EF515876
Streptomyces thermovulgaris	Z68094
Tsukamurella inchonensis	AF283281
Tsukamurella pulmonis	X92981
Tsukamurella strandjordii	AF283283
Tsukamurella spumae	Z37150
Tsukamurella tyrosinosolvens	AY238514
Williamsia deligens	AJ920290
Williamsia muralis	Y17384[h]

[a] From the LPSN (http://www.bacterio.net/); sequences submitted prior to 2000 are replaced when possible with similar, more-recent sequences.

[b] Some species of *Dietzia* are more frequently isolated. See Table 3.

[c] Previously *Nocardia corynebacterioides*.

[d] GenBank record X79289 lists the name for this species as *Rhodococcus erythreus* strain DSM 43066, the type strain of *R. erythropolis*.

[e] The later synonym for *Rhodococcus luteus*. This is the best sequence found for the type strain of *R. fascians*.

[f] The GenBank record is for strain KACC 20084.

[g] See reference 244.

[h] The best sequence found for the type strain of *W. muralis*.

TABLE 6 Species of *Nocardia* infrequently reported or poorly documented as human pathogens and GenBank accession numbers of type strain sequences

Nocardia species	GenBank accession no. of the type strain sequence
N. africana	AF430054
N. amikacinitolerans	GU985442
N. anaemiae	GQ376192
N. aobensis	GQ376159
N. araoensis	GQ376160
N. arthritidis	GQ217494
N. asiatica	GQ217495
N. asteroides	DQ659898
N. beijingensis	GQ217493
N. blacklockiae	GQ376162
N. brevicatena	AF430040
N. carnea	AF430035
N. concava[a]	EF177464
N. corynebacterioides[b]	AF430066
N. elegans	GQ376166
N. exalbida	GQ376167
N. harenae	DQ282122
N. higoensis	GQ376169
N. ignorata[a]	DQ659907
N. inohanensis	DQ659908
N. kruczakiae	DQ659909
N. mexicana	GQ376178
N. mikamii	EU484388
N. neocaledoniensis	GQ853080
N. niigatensis	GQ853079
N. ninae	GQ853078
N. niwae	FJ765056
N. paucivorans	GQ376188
N. pneumoniae	GQ853075
N. puris	GQ217500
N. shimofusensis	NR_028650
N. sienata	AB516654
N. takedensis	GQ376185
N. terpenica	GQ376183
N. testacea	AB192415
N. thailandica	AB126874
N. transvalensis	AF430047
N. vermiculata	GQ853068
N. vinacea	DQ659919
N. yamanashiensis[a]	DQ659920

[a] Type strains of these species are known to contain multiple differing copies of the 16S rRNA gene (see the text).

[b] The later synonym of *Rhodococcus corynebacterioides*.

Amycolata and *Amycolatopsis*

There are no recent reports documenting human infection caused by species in either the genus *Amycolata* or the genus *Amycolatopsis*. Three species in the genus *Amycolatopsis* and several other aerobic actinomycete species may be causative agents of equine placental infection and abortion (44).

Dermatophilus

Dermatophilus congolensis causes dermatitis in a wide variety of animals worldwide, including cattle, horses, goats, and sheep, but has only rarely been noted as a cause of human infection (45). In humans, the organism has been reported to cause a variety of cutaneous manifestations, including scaling and exudative lesions, pustules, pitted keratolysis (46), and hairy leukoplakia of the tongue (47). Filamentous and coccoid forms of the organism may be visualized directly in tissue specimens. Optimal therapy has not been defined, but infections may be self-limiting. This organism should be considered in travelers returning from tropical areas, particularly if lesions develop subsequent to animal contact at a site of previous skin trauma (48).

Dietzia

Until recently, the few reported cases of infection attributed to *Dietzia* species have been attributed to *Dietzia maris*; these include isolates from blood and an intravascular catheter (49), from an infection associated with a hip prosthesis (50), and a case of aortic dissection (51). However, recent reports highlight the problems of distinguishing between *Dietzia* species and *Rhodococcus equi* using traditional biochemicals or with the use of commercially available Gram-positive rod identification panels (52, 53). One of these reports also notes the impossibility of distinguishing between certain pairs of *Dietzia* species (e.g., *D. maris* and *D. schimae*), even with the use of gene sequencing of two different molecular targets (52). Until the distinctions among the various species are resolved by molecular methods, it may be most useful to refer to all isolates as *Dietzia* species.

Gordonia

There have been relatively few reports of infections attributed to species in the genus *Gordonia*. However, an unknown number of *Gordonia* infections may be missed, either because the isolate is considered an insignificant coryneform Gram-positive rod or the isolate is misidentified as belonging to another genus, such as *Nocardia* or *Rhodococcus* (54). In five patients with catheter-related *Gordonia* species infection, the organism was correctly identified to the genus level in only one case until 16S rRNA gene sequencing was employed (54). In three of these five patients, *Gordonia terrae* was the infecting organism, one was *Gordonia bronchialis*, and one was *Gordonia otitidis*. There has been a review published on medical-device-associated *Gordonia* infections in connection with a report of infection of an orthopedic device caused by *Gordonia araii*; most of the infections reviewed were attributed to *G. terrae* (55). Two of the very few clusters of infection attributable to any aerobic actinomycete involved patients who developed sternal-wound infections following coronary artery bypass surgery (35, 36). In one of these outbreaks, the organism involved, *Gordonia bronchialis*, was identified by biochemical testing and cell wall mycolic acid analysis only. Immunocompromise and/or the presence of foreign bodies appears to have been a contributing factor in many of the infections caused by *Gordonia* species.

Kroppenstedtia

Fourteen clinical isolates of *K. eburnea* have been reported, mostly from blood (11) or other sterile fluids, and one from skin. It is not clear that any of the isolates were of clinical significance. However, as most isolates were recovered from sterile fluids, this genus is included here, pending future determination of its true clinical significance.

The isolates were identified by partial 16S sequencing and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. Of the 11 isolates available for antimicrobial susceptibility testing, all were susceptible to most agents tested but resistant to clarithromycin. The authors of that report noted that the organisms were not acid fast and did not stain with auramine O. No mention was made in their report of the presence of an aerial mycelium (56).

Nocardia

Nocardia infections generally result either from trauma-related introduction of the organism or, particularly in immunocompromised patients, from inhalation and the resulting establishment of a pulmonary focus. *Nocardia* species have also been implicated in extrapulmonary disease in a variety of body sites which usually results from hematogenous spread from a pulmonary site (57). The brain is one of the most common secondary sites of infection (57). Various *Nocardia* species have also been implicated as the causal agents of keratitis and other ocular infections, but the accuracy of the species-level identification is not always clear (58, 59). In addition, a few cases of catheter-related *Nocardia* infections have been reported, but the precise species involved has not always been clearly identified (60). A well-documented case of catheter-related infection caused by *N. farcinica* was recently reported (61).

In 1988, a breakthrough in the clinically useful categorization of pathogenic nocardial isolates was provided by Wallace and his coworkers (39). They divided organisms phenotypically resembling *N. asteroides* into six different drug pattern types and one additional miscellaneous group. With more-recent molecular characterizations, numerous different species have been described within this set of organisms, which came to be known as the *Nocardia asteroides* complex. These include *Nocardia abscessus* (drug pattern I), *Nocardia cyriacigeorgica* (drug pattern VI), *N. farcinica* (drug pattern V), *Nocardia nova* (drug pattern III), *Nocardia wallacei* (drug pattern IV), and isolates of drug pattern II. Other new *Nocardia* species are continually being described, and undoubtedly, by current species definition criteria, many more will be described in the future.

Given the current state of the literature, it is possible to make only the most tentative statements regarding geographic distribution and disease correlates of different species. While accurate species assignment allows one to make some predictions regarding likely antimicrobial susceptibility patterns, such a species assignment today requires the use of molecular methods (57). Nonetheless, susceptibility testing of all clinically significant isolates is recommended, whether or not molecular techniques have been used for their identification. The current Clinical and Laboratory Standards Institute (CLSI) document lists typical susceptibility patterns of the most commonly isolated *Nocardia* species (62).

Of the 84 species of *Nocardia* currently recognized, 49 have been reported as human isolates, and several others have been reported as pathogens of animals. Importantly, isolates in many reports of infection, even in the recent literature, are incorrectly identified because of failure to use optimal identification methods.

Nocardia abscessus

Nocardia abscessus was formally named in 2000 by Yassin et al. (63). ATCC strain 23824, the reference strain of *N. asteroides* drug pattern type I (64), was one of the isolates found to be *N. abscessus* on the basis of 16S rRNA sequencing and DNA-DNA hybridization. In their description in 1988 of the antimicrobial susceptibility patterns of 78 clinical isolates of *Nocardia* from various sources, Wallace et al.

noted that 20% of the isolates had this drug pattern type (39). Several of the strains in the report naming the species were from abscesses; a subsequent report from Japan reported several isolates from pulmonary sources and one from a brain abscess (65). Two isolates have been reported from Germany, one from pericardial fluid (66) and the other from a posttraumatic wound (67). More-recent cases have included a report of disseminated infection in an AIDS patient (68).

According to the CLSI, the expected antimicrobial susceptibility pattern of *N. abscessus* is as follows: susceptibility to amikacin, amoxicillin-clavulanic acid, ceftriaxone, linezolid, and sulfonamides and resistance to ciprofloxacin, clarithromycin, and imipenem (62).

Nocardia asteroides and the "*N. asteroides* Complex"

There are innumerable reports of human infection attributed to *N. asteroides* (38); it has probably been considered the most commonly isolated human-pathogenic *Nocardia* species. However, molecular analyses of the pathogenic isolates attributed to this species that have been conducted thus far have indicated that all belong to some other named or as-yet-unnamed species. It is currently believed that *N. asteroides sensu stricto* is rarely pathogenic.

The term "*N. asteroides* complex" has been used for organisms phenotypically resembling the *N. asteroides* type strain. However, several distinct species have now been described within that complex, and precisely what the complex is intended to designate is usually unclear. Given the current ability to identify *Nocardia* isolates molecularly, the phrase "*N. asteroides* complex" should be avoided. The term "complex" is best restricted to groups of species that are related on a molecular basis and have similar phenotypic features. Such groups would include the *N. nova* complex and the *Nocardia transvalensis* complex. Whenever the term "complex" is used, it is best to state initially precisely which species are intended to be included in the complex.

Nocardia brasiliensis

N. brasiliensis appears to be the most common cause of actinomycotic mycetoma (see "*Actinomadura*" above) in the Western Hemisphere, especially in Mexico (40, 69). The organism probably occurs worldwide; there are reports of infection from Australia (70), West Bengal (43), and Europe (71) and many reports from North America (72). A variety of cutaneous manifestations in addition to mycetoma, including cellulitis, abscesses, and lymphocutaneous infection, have been reported. Nearly all cases are a result of trauma, including that caused by thorns (71), cat scratch (73), and insect bite (74). Most of the trauma-related infections have occurred in immunocompetent individuals. Disseminated infection, usually originating from a pulmonary focus, has also been reported (72); such infections are more likely to occur in immunocompromised patients (75). Some cases, such as one of a brain abscess resulting from dissemination from a pulmonary focus (76), occur in patients who appear to be immunocompetent. However, most of the cases of invasive disease, as well as some of the cases of cutaneous infection, attributed to *N. brasiliensis* (prior to 1995) almost certainly have been caused by *Nocardia pseudobrasiliensis* (see below).

According to the CLSI, the expected antimicrobial susceptibility pattern of *N. brasiliensis* is as follows: susceptibility to amikacin, amoxicillin-clavulanic acid, linezolid, minocycline, sulfonamides, and tobramycin and resistance to ciprofloxacin, clarithromycin, and imipenem (62).

Nocardia brevicatena/Nocardia paucivorans

Brown et al. suggested in 1997 that *Nocardia* isolates sharing both an unusual drug susceptibility pattern and one or another of three different restriction fragment length polymorphism (RFLP) patterns of an amplified portion of the 65-kDa heat shock protein (HSP) gene should be considered to form the *Nocardia brevicatena* complex (B. A. Brown, R. W. Wilson, V. A. Steingrube, Z. Blacklock, and R. J. Wallace, Jr., *Abstracts of the 97th General Meeting of the American Society for Microbiology, 1997*, abstr. C-65, p. 131, 1997). These organisms included 19 clinical isolates from the United States, 10 clinical isolates from Australia, and three ATCC reference strains of *N. brevicatena*. There have been no subsequent reports of clinical isolates or taxonomic studies of these organisms.

The species *N. paucivorans* was described by Yassin et al. in 2000 on the basis of a respiratory isolate from a patient with chronic lung disease (77). By 16S rRNA gene sequencing, this organism showed 99.6% sequence similarity to *N. brevicatena*; results of DNA-DNA hybridization indicated that they were distinct species. *N. paucivorans* and *N. brevicatena*, along with isolates belonging to the unnamed group *N. asteroides* drug pattern II (39), are sometimes considered to belong to the *N. paucivorans/N. brevicatena* complex based on similar phenotypic characteristics and antibiograms (57).

N. paucivorans has been recovered from cerebrospinal fluid in a case of cerebral nocardiosis in an immunocompromised individual (78), an intracerebral abscess in an immunocompetent patient (79), and a mitral valve (80). Two of 86 (2.3%) clinical *Nocardia* isolates from Belgium were identified as belonging to this species (81).

Nocardia cyriacigeorgica

N. cyriacigeorgica was described on the basis of an isolate obtained from the sputum of a patient with chronic bronchitis (82). Subsequent isolates were obtained from brain abscesses in an immunocompromised patient (83) and from a patient with pneumonia following a near-drowning incident, from whom *N. farcinica* and several other pathogens were isolated (84). Isolates of this species were reported to constitute 13 of 96 (14%) clinical *Nocardia* isolates from Thailand (85) and 13 of 86 (15%) such isolates from Belgium (81). The 16S rRNA sequence of the type strain of this species (1,400 bp) was found to be identical to that of the reference strain of *N. asteroides* drug pattern type VI (ATCC 14759) (64, 86). DNA-DNA hybridization studies subsequently established that this drug pattern type VI reference strain and *N. cyriacigeorgica* belong to the same species (87). In the original work describing the different *N. asteroides* drug pattern types, type VI strains were the most commonly isolated strains (35%) (39). *N. cyriacigeorgica* is probably the most frequent human nocardial pathogen, at least in areas where actinomycotic mycetomas are relatively rare. While the species *N. cyriacigeorgica* has been designated an emerging pathogen (88), it is actually a relatively common and long-recognized pathogen that has recently acquired a valid name. In fact, many isolates previously reported as *N. asteroides* almost certainly belong to this species.

According to the CLSI, the expected antimicrobial susceptibility pattern of *N. cyriacigeorgica* is as follows: susceptibility to amikacin, ceftriaxone, imipenem, linezolid, and sulfonamides and resistance to amoxicillin-clavulanic acid, ciprofloxacin, and clarithromycin (62).

Nocardia farcinica

The organisms initially described by Wallace et al. as belonging to the group *N. asteroides* drug pattern type V (39) were subsequently found to belong to *N. farcinica* (89). *N. farcinica* isolates that showed *in vitro* resistance to trimethoprim-sulfamethoxazole have been reported to respond to meropenem alone (90) and to the combination of linezolid and minocycline (91). This species may have a particular propensity for causing disseminated disease; Wallace et al. reported that among 30 patients for which the disease extent was known, 57% had disseminated disease and one-third had central nervous system (CNS) involvement (89). Most patients infected by this species, especially those with disseminated disease, have some type of immunocompromise (89), but cutaneous and other infections have been reported to occur in the apparently immunocompetent as well (92). The lung is a common site of involvement, affecting 59.7% of patients according to one review (92). As verified by molecular analysis, *N. farcinica* has been isolated from brain abscesses (93), blood (93), cases of keratitis (94), and an infected cochlear implant (95). *N. farcinica* has also been isolated from the bronchoalveolar wash fluid of a cystic fibrosis patient (96). Isolates of this species were reported to make up 34 of 96 (35%) clinical *Nocardia* isolates from Thailand (85) and 38 of 86 (44%) such isolates from Belgium (81).

According to the CLSI, the expected antimicrobial susceptibility pattern of *N. farcinica* is as follows: susceptibility to amikacin, amoxicillin-clavulanic acid, ciprofloxacin, linezolid, and sulfonamides and resistance to ceftriaxone, clarithromycin, and tobramycin (62).

Nocardia nova

N. nova was described by Tsukamura in 1982, distinguished from *N. asteroides* by several phenotypic tests (97), and confirmed to be a separate and distinct species by DNA-DNA hybridization (98). Wallace et al. found that 18% of 78 clinical isolates fell into their type III drug susceptibility pattern, characterized by susceptibility to ampicillin and erythromycin and resistance to carbenicillin (39). In a subsequent study of 223 clinical isolates, by both biochemical and susceptibility testing procedures, 17% of the isolates, as well as the type strain of *N. nova*, had similar characteristics, including the type III drug pattern; these isolates were all then considered *N. nova* strains (99). Of the patients for whom clinical information was available, 35% were thought to have disseminated disease; organisms were obtained from many sites, including blood, lung, CNS, skin and soft tissue, joints, and cornea. An upper extremity sporotrichoid form of nocardiosis attributed to *N. nova* in a human immunodeficiency virus (HIV)-positive patient who sustained a thumb injury while working in a field has been reported (100). Hamdad et al. reported the isolation of *N. nova*, identified by 16S rRNA gene sequencing, from a case of spondylodiscitis and psoas abscess (101).

Recent investigations have revealed that the organisms identified as *N. nova* by phenotypic testing, including antibiogramming, as well as by the RFLP patterns obtainable from the *hsp*65 gene, actually may belong to other species in addition to *N. nova*, including *Nocardia africana*, *Nocardia kruczakiae*, and *Nocardia veterana* (102). These species can be distinguished from one another and from *N. nova sensu stricto* only by gene sequencing. Phylogenetic analysis using sequence data of the HSP and *secA1* genes consistently place these species in the same clade as *N. nova sensu stricto*, indicating the close relationship among these species (P. S. Conville and F. G. Witebsky, unpublished data). Phylogenetic analysis of the 16S rRNA, HSP, and *secA1* genes also places *Nocardia aobensis* and *Nocardia elegans* in the *N. nova* complex clade; no phenotypic characteristics of these two species have been examined to determine their similarity to other species in the complex. All species included in the *N. nova* complex (including *N. aobensis* and *N. elegans*) have been isolated from humans or implicated in human disease. Isolates identified only by phenotypic testing as belonging to one or another of these species are probably best reported as members of the *N. nova* complex.

According to the CLSI, the expected antimicrobial susceptibility pattern of members of the *N. nova* complex is as follows: susceptibility to amikacin, ceftriaxone, clarithromycin, imipenem, linezolid, and sulfonamides and resistance to amoxicillin-clavulanic acid and ciprofloxacin (62).

Nocardia otitidiscaviarum

The initial isolate of the species *N. otitidiscaviarum*, described by Snijders in 1924, was obtained from the infected middle ear of a guinea pig; for a time this species was known as *N. caviae* (103). Clark et al. reviewed 28 cases of cutaneous infection, including several mycetoma cases, attributed to this species; many of those for which information was available were considered trauma related (104). There are a few reports of infection at other sites, including a brain abscess (105), a pyothorax infection (106), a catheter-related infection (107), disseminated infection (108), and cavitary pneumonia (109). This species is relatively reliably identified on the basis of its decomposition reactions; it decomposes xanthine and hypoxanthine but not casein or tyrosine. In a molecular study of numerous clinical isolates, several different sequences were obtained from nine different strains that had been phenotypically identified as *N. otitidiscaviarum* (110). These results were interpreted as suggesting the presence of several different species within this group, but some of the results also suggested that individual isolates might contain two or more differing copies of the 16S rRNA gene.

According to the CLSI, the expected antimicrobial susceptibility pattern of *N. otitidiscaviarum* is as follows: susceptibility to amikacin, ciprofloxacin, linezolid, and sulfonamides and resistance to amoxicillin-clavulanic acid, ceftriaxone, and imipenem (62).

Nocardia pseudobrasiliensis

In 1995, Wallace et al. (111) reported on a subset of organisms that had been identified as *N. brasiliensis* that were sometimes isolated from cutaneous infections but were predominantly associated with noncutaneous invasive disease. This subset of isolates appeared to belong to another taxon, formally named *N. pseudobrasiliensis* the following year (112). Most infections have occurred in immunocompromised patients; cases have been reported from North and South America, Japan, and Australia (111, 113, 114).

Unlike true *N. brasiliensis* isolates, most *N. pseudobrasiliensis* isolates hydrolyze adenine, are susceptible to ciprofloxacin and clarithromycin, and are resistant to minocycline. The two species also differ in their mycolic acid patterns and 16S rRNA, HSP, and *secA1* gene sequences. Most cases of infectious disease of a nonmycetomatous nature previously attributed to *N. brasiliensis* have probably been caused by this species (see "*Nocardia brasiliensis*" above).

According to the CLSI, the expected antimicrobial susceptibility pattern of *N. pseudobrasiliensis* is as follows: susceptibility to amikacin, ciprofloxacin, clarithromycin, linezolid, sulfonamides, and tobramycin and resistance to amoxicillin-clavulanic acid, imipenem, and minocycline (62).

Nocardia transvalensis and the N. transvalensis Complex

N. transvalensis was originally described in 1927 on the basis of an isolate from an African patient with a mycetoma (115). In 1997, Wilson et al. published a study of 58 clinical isolates from the United States and Australia that showed resistance to amikacin (116). Using biochemical and molecular methods, these authors were able to separate the isolates into four distinct groups. One group comprised 53% of the isolates studied, were all recovered from patients in the United States, and were similar to isolates classified as *N. asteroides* drug pattern type IV as defined by Wallace et al. (39). Isolates belonging to this group were later officially designated *Nocardia wallacei* (117) (see below). Another group (17% of isolates) included organisms similar to the type strain of *N. transvalensis*. Two additional groups, new taxon 1 (14% of isolates) and new taxon 2 (16% of isolates), were also defined. Australian isolates belonged to the *N. transvalensis sensu stricto* group and to new taxon 1. Isolates belonging to new taxon 1 have been given the species designation *Nocardia blacklockiae* (117). Because of their similar phenotypic and molecular characteristics, Wilson proposed that these three species and the unnamed new taxon 2 be considered to belong to the *N. transvalensis* complex.

There have been numerous reports in the literature describing the isolation of *N. transvalensis* from clinical specimens; many of these reports base the identification of the isolate on a small number of phenotypic tests. It is unclear if these reports represent accurate species assignments, given the similarity of related species as described above. McNeil et al. reported on 16 patients from whom isolates attributed to this species had been obtained (118). The isolates from 10 of the patients were considered clinically significant; the other isolates were considered colonizers or of uncertain significance. A variety of sites of infection were involved, some of the infections were disseminated, and several patients were known to be immunocompromised. There are a few other case reports of molecularly characterized disseminated infection attributed to the *N. transvalensis* complex, including a brain abscess in a cancer patient (119), brain and cutaneous infection in a heart transplant patient (120), and pulmonary infection and subcutaneous abscess in a patient with histiocytosis X (121). Three clinical isolates assigned to this species complex have been reported from Japan (122). A well-documented case of *Nocardia transvalensis* has also been reported in a case of pneumonia in a patient with cystic fibrosis (123).

Those using 16S rRNA gene sequencing for identification of members of the *N. transvalensis* complex should be aware that numerous sequences for *N. transvalensis* have been submitted to GenBank. Many of these sequences are more closely related to the type strain of *N. wallacei* than to the type strain of *N. transvalensis*.

According to the CLSI, the expected antimicrobial susceptibility pattern of members of the *N. transvalensis* complex is as follows: susceptibility to ceftriaxone, ciprofloxacin, linezolid, and sulfonamides and resistance to amikacin, clarithromycin, and tobramycin (62).

Nocardia veterana

N. veterana was first described on the basis of an isolate obtained from the bronchial lavage fluid of a patient with pulmonary lesions, but that isolate was thought not to be clinically significant (124). Subsequently, *N. veterana* has been implicated as the causative agent in cases of mycetomas (125), three patients with pulmonary disease (126), ascitic fluid infection in an HIV patient (127), brain abscess in a diabetic patient (128), and bloodstream infection in a cancer patient (129). In a report of three additional clinical isolates, two of which were shown to have been causative agents of pulmonary disease, it was noted that the isolates had an antimicrobial susceptibility pattern essentially identical to the patterns of *N. africana* and *N. nova* (130), demonstrating that species identification could not be definitively established by susceptibility testing alone. It was also noted that the isolates of this species that were studied showed 16S rRNA gene similarities to the type strains of *N. africana* and *N. nova* of 99.0 and 97.7%, respectively. *N. veterana* is one of the species included in the *N. nova* complex (see "*Nocardia nova*" above). HSP, *secA1*, and 16S rRNA gene sequencing place *N. veterana* in a clade with other members of the complex. The expected antimicrobial susceptibility pattern of *N. veterana* is consistent with the susceptibility pattern of members of the *N. nova* complex (see above).

Nocardia wallacei

Officially described in 2008, *N. wallacei* is the most commonly isolated member of the *N. transvalensis* complex (117). 16S rRNA, HSP, and *secA1* gene sequencing showed ≥99.8% sequence similarity among five clinical isolates obtained from sputum samples; one of these was also recovered from pleural fluid. This species was initially designated *N. asteroides* drug pattern IV by Wallace et al. and comprised 5% of 78 isolates identified as *N. asteroides* by phenotypic methods but unique in their resistance to amikacin (39). The expected antimicrobial susceptibility pattern of *N. wallacei* is consistent with the susceptibility pattern of members of the *N. transvalensis* complex (see above).

Nocardiopsis

Nearly all of the very few infections attributed to organisms in the genus *Nocardiopsis* have been attributed to *Nocardiopsis dassonvillei*. In a letter to the editor regarding a case of actinomycetoma attributed to this species, it was stated that *N. dassonvillei* was "regularly encountered" at the Centers for Disease Control and Prevention, with 21 isolates having been identified from 1981 through 1986; no clinical details were provided (131). In the case of a blood isolate, a variety of methods, including 16S rRNA gene sequencing, were used to identify the isolate; the same report provided references to a number of other cases of infection, including mycetomas and cutaneous infections, attributed to this species (132).

Rhodococcus

As described previously, reassignment of *Rhodococcus equi* to the genus *Prescottella* has been proposed (24, 25). However, since this change had not been validated at the time of writing, the name *Rhodococcus equi* is used in the text here. The most commonly isolated pathogen in the genus *Rhodococcus* is *R. equi*. While other species have occasionally been reported to cause disease, in most but not all of these reports, gene sequencing was not used, and it is impossible to obtain reliable species-level identifications without this technique (22, 133). There is some evidence that *R. equi* itself may be a complex of several different species (134). Infections caused by *R. equi* have been the subject of several reviews (135–137). The organism has long been known as a significant pulmonary pathogen in horses (hence the species name) (138). The initial report of human disease caused by the organism (reported as *Corynebacterium equi*) involved a patient on high-dose steroids with a pulmonary abscess; he had worked briefly in stockyards contaminated with animal feces shortly before the onset of his illness (139). Herbivore manure provides an ideal growth medium for the organism, and inhalation of the organism is presumed to be the major mode of infection in horses (138) and is probably also the principal mechanism for human infections. Direct inoculation and oral ingestion are other possible routes of infection (140). Most patients who develop *R. equi* infection are immunocompromised, and at least until recently, approximately two-thirds of infected patients were also HIV infected (140). Essentially any body site can be involved, but the lung is a site of involvement in approximately 80% of immunocompromised patients and at least 40% of immunocompetent patients. Bacteremia has been reported in >80% of immunocompromised patients and approximately 30% of immunocompetent patients (140). Pulmonary cavitation is a frequent finding. Malacoplakia, an aggregation of histiocytes containing concentrically layered basophilic structures known as Michaelis-Gutmann bodies, has been noted as a histopathologic finding in several studies (137, 141). Two virulence-associated antigens (VapA and VapB) encoded by plasmids have been identified. Isolates producing the VapA antigen are the predominant, possibly the sole, cause of disease in horses, but isolates producing the VapA antigen, the VapB antigen, or neither antigen have been isolated from human cases of infection (142). A combination of antimicrobials is generally used for the treatment of infections. Agents used include aminoglycosides, erythromycin, imipenem, quinolones, rifampin, and vancomycin. The current CLSI document *Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes, Approved Standard, Second Edition*, recommends that a regular Gram-positive panel be inoculated (if such panels include vancomycin and rifampin, critical antimicrobials for this species) (62). Linezolid may also be efficacious (140); in an *in vitro* study of 102 *R. equi* isolates obtained from humans and animals, the linezolid MIC ranged from 0.5 to 2.0 μg/ml (143). Some rifampin-resistant strains with *rpoB* gene mutations have been reported (144). Without careful assessment, *R. equi* isolates can easily be mistaken for coryneform bacteria and dismissed as insignificant (Fig. 2H). One report describes two patients who died of overwhelming *R. equi* infection; for both patients, isolates from multiple positive blood cultures were initially misidentified as a *Corynebacterium* species (145).

Segniliparus

Although the two species that make up this genus (*Segniliparus rotundus* and *Segniliparus rugosus*) were originally recovered from clinical material, no information was provided concerning the significance of these isolates except that they were from "nonsterile human sources" (28). Subsequent reports of the recovery of *S. rugosus* seem to indicate the predilection of this organism for patients with cystic fibrosis. A report of the recovery of *S. rugosus* from the bronchoalveolar lavage fluid or induced sputum of three cystic fibrosis patients emphasized the microscopic and colonial similarities of this species with those of rapidly growing mycobacteria (Fig. 2M and 3M and N). In a subsequent report from Australia, *S. rugosus* was isolated from the mycobacterial culture of sputum from a cystic fibrosis patient (146). A case of *S. rotundus* pneumonia in a non-cystic fibrosis patient with bronchiectasis has recently been reported (147).

Streptomyces

The most common type of infection attributed to species in the genus *Streptomyces* is mycetoma (see "*Actinomadura*" above), and the most commonly mentioned etiologic agent is *Streptomyces somaliensis*, although in many reports the identification procedures employed could not have ensured that other species were not involved. There are a few reports implicating other species in the genus as occasional pathogens. In case reports of bacteremias attributed to *Streptomyces bikiniensis* (148) and *Streptomyces thermovulgaris* (149), molecular methods were used in identifying the isolates, and such methods were described in detail in a case report of a mycetoma attributed to *Streptomyces albus* (150). While the majority of isolates from nonmycetomatous lesions probably represent either contamination or colonization, these organisms are capable of occasionally causing disease other than mycetoma. Six cases of invasive *Streptomyces* infections have been reported, and there is a literature review of such infections (151). Because of the huge number of validly described species of *Streptomyces* and the lack of information about the clinical significance of many of these species, identification to the genus level is probably sufficient in most cases. In a study of the susceptibility of 92 *Streptomyces* species from clinical specimens, 100% of those tested were found to be susceptible to amikacin and linezolid, 77% to minocycline, 67% to imipenem, and 51% to clarithromycin and amoxicillin-clavulanate (152).

Tsukamurella

Tsukamurella infections have been most commonly reported in connection with a foreign body, such as an intravenous catheter (153), but have also been reported even in apparently immunologically normal patients in the absence of any foreign body (154). The literature on catheter-related infections caused by *Tsukamurella* species has recently been reviewed in connection with two additional reports of such infection (153); several other of the relatively few infections reportedly caused by *Tsukamurella* species are also briefly mentioned in that review. *Tsukamurella tyrosinosolvens* has been implicated in cases of keratitis, bacteremia, and catheter-related infections (155). *Rhodococcus aurantiacus* ATCC 25938 (the initial type strain of the species), which has had a convoluted taxonomic history, has been placed in the species *Tsukamurella paurometabola* (32). In the older literature, there are a few reports of infection attributed to *R. aurantiacus*, such as pulmonary infection, meningitis, peritonitis, and subcutaneous abscesses (156). Organisms isolated from clinical material have generally been found to resemble strain ATCC 25938. Pseudo-outbreaks attributed to *T. paurometabola* (37) and *T. tyrosinosolvens* (due to improperly wrapped specimen processing equipment) have been reported (157).

Williamsia

The first clinical isolate of the genus *Williamsia* was recovered from a protected bronchial brush sample of a patient with bilateral alveolar infiltrates following aortic valve replacement. A Gram stain of the sample showed numerous Gram-positive rods, and culture of the fluid grew >1,000 CFU/ml of an organism identified by 16S rRNA gene sequencing as *Williamsia muralis* (99.9% sequence similarity). Using breakpoints established for *Nocardia* species, susceptibility testing of the *W. muralis* isolate indicated that the isolate was susceptible to amoxicillin-clavulanate, cefotaxime, imipenem, ciprofloxacin, tobramycin, gentamicin, and trimethoprim-sulfamethoxazole (158). The recovery of *W.*

muralis was also reported from a case of endophthalmitis. The isolate showed only 98.4% 16S rRNA gene similarity, but 100% DNA-DNA hybridization, with the type strain of *W. muralis* (159). Two isolates of *Williamsia deligens* have been reported from human blood, but no clinical information was provided (160).

Other Genera

Some other genera of aerobic actinomycetes have roles in human diseases but are unlikely to be encountered in the clinical laboratory. Organisms in three genera of thermophilic aerobic actinomycetes, *Saccharomonospora*, *Saccharopolyspora*, and *Thermoactinomyces*, have been implicated in the etiology of hypersensitivity pneumonitis. Spores of these organisms may be encountered when performing air sampling; molecular methods are helpful for species-level identification when needed (161, 162). A recently described sputum isolate has been placed in a new genus and species, *Desmospora activa*, in the family *Thermoactinomycetaceae*, but its pathogenic significance is unclear from the report (8).

COLLECTION, TRANSPORT, AND STORAGE

In temperate climates, the respiratory tract is the most frequent portal of entry for the aerobic actinomycetes and therefore the primary site of nocardial infections in immunocompromised hosts. Sputum is the most easily obtained pulmonary specimen, and examination of several fresh early-morning samples collected on separate days (163) may maximize the chances of organism recovery; isolation of an aerobic actinomycete from multiple samples may help to establish the clinical significance of the isolated organism (11). *Nocardia* species and other aerobic actinomycetes may, however, be difficult to recover from sputum even in documented cases of pulmonary infection (15), either because of low numbers of organisms present in the sample or because they may be overgrown by more rapidly growing bacteria in the specimen. More-invasive procedures, such as bronchoalveolar lavage or fine needle or open lung biopsy, may be required to obtain a definitive diagnosis (15). These more-invasive procedures may be necessary for diagnosis of as many as 44% of primary pulmonary infections (70); macrophage-rich samples may be necessary to maximize recovery of organisms such as rhodococci, which tend to localize within these cells (19).

Exudates from abscesses or mycetomas should be delivered to the laboratory in a sterile container for macroscopic and microscopic examination for characteristic granules and for smear tests and culture. In the case of disseminated cutaneous lesions or small lesions secondary to trauma, a skin biopsy can be useful. The use of swabs is not recommended, as fibers can make smear interpretation difficult (163).

In immunocompromised patients, the aerobic actinomycetes, especially *Nocardia*, can disseminate to almost any organ. A biopsy sample or aspirate, when obtainable, may be the best specimen to evaluate for the presence of such organisms. Normally sterile body fluids should be collected in a sterile container and sent immediately to the laboratory. *Nocardia* isolates are infrequently recovered from cerebrospinal fluid, even if numerous brain lesions are present, because the organisms may be confined to the brain abscess itself. Blood should be inoculated directly into blood culture media; many commercially available blood culture systems have been shown to support the growth of *Nocardia* species. *Nocardia* and *Tsukamurella* spp. have been implicated as causes of catheter-related bacteremia; it is likely that other aerobic actinomycetes are also involved in catheter-related infections. Potentially infected catheter tips should be transported to the laboratory in a sterile container and cultured by appropriate methods.

In all cases where infection with an aerobic actinomycete is suspected, it is of utmost importance that the laboratory be notified of the suspected diagnosis. This will ensure that samples will receive appropriate handling, that the correct direct smears will be prepared, and that the sample will be inoculated onto the appropriate media and incubated for an extended period of time (a minimum of 2 weeks, preferably for up to 3 weeks) at the appropriate temperature.

All samples should be transported promptly to the laboratory following the specimen collection and handling procedures outlined in chapter 18.

DIRECT EXAMINATION

Microscopy

Careful microscopic examination of clinical specimens suspected of containing aerobic actinomycetes is extremely important. The observation of organisms characteristic of *Nocardia* species and other aerobic actinomycetes should alert laboratory personnel to inoculate appropriate media and to extend incubation at the appropriate temperature. In addition, the detection of organisms directly in the specimen may assist in the interpretation of culture results; smears of such specimens may show more-characteristic diagnostic morphologies of organisms than smears from colonial growth.

Smears can be made directly from sputum, drainages, and aspirates; however, liquid specimens, such as bronchoalveolar lavage fluid or normally sterile body fluids that are not excessively cellular, should be concentrated before smear preparation and medium inoculation. For example, a 5-ml aliquot of the sample can be centrifuged for 10 min at 2,800 × *g* and the pellet can be used for smears and medium inoculation. Alternatively, smears can be prepared using a cytocentrifuge. For tissue samples, smears should be made from ground material and from touch preps. Two important stains that should be used in clinical laboratories for direct samples are the Gram stain and the modified acid-fast stain. Histopathologic examination of fixed tissue by use of special stains (including Fite stain and Grocott-Gomori methenamine silver stain) may also reveal the presence of organisms belonging to these genera (164).

Gram stain morphologies of the aerobic actinomycetes vary by genus; organisms appear as Gram-positive rods ranging in shape from coccoid to bacillary. Filamentous and branching forms may be present, depending on the species involved and the stage of growth in infected tissues (Fig. 3). See Table 1 for a summary of the microscopic morphologies of the aerobic actinomycetes.

The modified acid-fast stain used on direct specimens may more accurately reflect the true partially acid-fast nature of the organisms than do modified acid-fast stains prepared from colonial growth. The modified acid-fast stain uses a weaker decolorizer (1% H_2SO_4) than does the mycobacterial stain (3% HCl). The decolorization step of the staining process should be carefully monitored to reduce the risk of over- or underdecolorization. See chapter 19 for details on reagent preparation and staining methodology. Because of the difficulty of standardizing the modified acid-fast staining technique, it is imperative that positive and negative controls be run simultaneously with patient smears. The control slides can be made from growing suspensions of *Streptomyces* species (negative control) and *Nocardia* species (positive control). Smears should be evaluated by experienced laboratory personnel, and the quality of the stain itself should be evaluated before results are reported. *Gordonia*, *Nocardia*, *Rhodococcus*, and *Tsukamurella* (and possibly *Dietzia*) are known to be partially acid fast with this stain; *Segniliparus* is strongly acid fast.

When smears are read, careful attention should be paid to the cellular material present in the sample (Fig. 3K and L). *Nocardia* species are frequently seen in association with polymorphonuclear leukocytes (57). Phagocytized Gram-positive or modified-acid-fast-stain-positive organisms can sometimes be seen within macrophages and mononuclear cells; in the modified acid-fast smear, these may appear as "beaded" cells with strongly acid-fast granules within non-acid-fast or weakly acid-fast rods (14, 19).

In cases of suspected actinomycetoma, aspirated material should be examined grossly for the presence of granules by spreading the sample in a sterile petri dish. Granules in infections caused by *Nocardia* species are small (80 to 130 μm), may be kidney-shaped with club-like structures in the periphery, and are usually yellow to orange in color (165, 166). In infections caused by *A. madurae*, granules can be yellowish-white and large enough to be seen with the naked eye. Microscopically, a zone of pseudoclubs can be seen in the periphery (165). Granules in infections caused by *A. pelletieri* are red to pink in color (165). Granules should be washed and crushed, smears prepared from the crushed material, and appropriate media inoculated. Granules are most often seen in infections caused by *N. brasiliensis* or *Actinomyces* species but can also be seen in infections caused by other species of *Nocardia* (57). Adequate data are not available to allow using the presence of granules or granule color for precise species or even genus-level discrimination of the etiologic agent.

Nucleic Acid Detection

Several authors have reported the use of molecular methodologies for the detection of *Nocardia* directly from clinical specimens. Targets include the 65-kDa HSP gene (167), the 16S rRNA gene (168, 169), and the *secA1* gene (G. Fahle, personal communication). These techniques have the potential to provide rapid diagnosis of nocardiosis from samples exhibiting bacterial morphologies suggestive of this genus. Further assessment of these techniques will be needed to assess their sensitivities and overall clinical utility.

Isotalo et al. describe a method using *in situ* hybridization for detection of *Nocardia* species in formalin-fixed, paraffin-embedded tissue specimens. Their procedure showed 77% sensitivity compared to that of standard histologic stains (170).

ISOLATION PROCEDURES

Blood agar, chocolate agar, brain heart infusion agar, Sabouraud dextrose agar, and Lowenstein-Jensen medium support the growth of most aerobic actinomycetes; *Dermatophilus congolensis* may not grow on Sabouraud dextrose agar or Lowenstein-Jensen medium (7). Buffered charcoal yeast extract agar (BCYE) is particularly useful for the recovery of *Nocardia* species. Specimens from sterile sites or concentrated sterile body fluids can be inoculated directly onto these media. Specimens from respiratory sites, skin, and other potentially contaminated sites, such as mycetomas, should additionally be inoculated onto selective media, such as modified Thayer-Martin agar (171) and selective BCYE (containing polymyxin B, anisomycin, and either vancomycin or cefamandole) (172). Sabouraud agar with added chloramphenicol may not be useful, as it may also suppress the growth of some *Nocardia* species (173). A specialized medium for the recovery of *R. equi* using a Mueller-Hinton agar-based medium with added ceftazidime and novobiocin has been described (174). Members of the aerobic actinomycetes may also grow in liquid media (such as broth media for mycobacterial cultures), in which they may appear as solid granules. These particles may be captured with a pipette and plated to solid media. Such granules may also be crushed between two slides, dried, and stained (Y. Shea, personal communication).

Cultures for aerobic actinomycetes should be handled as fungal cultures, thus ensuring that the cultures will be incubated and regularly examined over an extended period. Two BCYE plates (for samples from sterile sites) or selective BCYE plates (for respiratory or other potentially contaminated sites) should be inoculated. One BCYE or selective BCYE plate should be incubated at 30°C and the other at 35°C, both in ambient air. It should be noted, however, that *Streptomyces* species may show best growth at 25°C and that *Dermatophilus congolensis* grows better and shows enhanced production of aerial hyphae in increased CO_2 (7). For all cultures, plates should be held for a minimum of 2 weeks, preferably for up to 3 weeks, and should be sealed to prevent dehydration.

A low-pH decontamination procedure has been successfully used for pretreatment of heavily contaminated specimens suspected of harboring *Nocardia* species. The sample is diluted 1:10 in 0.2 M HCl–0.2 M KCl at pH 2.2, mixed, and allowed to stand for 3 to 5 min, after which it is inoculated onto selective and nonselective media (172, 175). Murray et al. reported a drop in the viability of *Nocardia* species after 30-min exposures to *N*-acetyl-L-cysteine (NALC), NaOH-NALC, or benzalkonium chloride (Zephiran)-trisodium phosphate (176). The key to improved recovery of *Nocardia* spp. from NaOH-NALC-treated specimens may be a shorter exposure to the decontaminating reagents (15 min), as is in fact also recommended for the recovery of mycobacteria (177).

Aerobic actinomycetes have been recovered from blood using a variety of commercially available blood culture systems, including conventional 2-bottle systems, biphasic bottles, automated blood culturing systems, and lysis-centrifugation systems. Using various blood culture systems, aerobic actinomycetes have been recovered after 3 to 19 days of incubation, which in some cases included the incubation times of terminal subcultures (148, 178). Studies employing newer blood culture systems have resulted in recommendations that extended incubation is no longer necessary (179); however, such studies have not established that such shorter incubation would generally be adequate for the isolation of aerobic actinomycetes. If the possibility of bacteremia with a member of one of these genera is suspected, it would be advisable to perform fungal blood cultures (for the expanded incubation period, at least 3 weeks) or to perform terminal subcultures if the incubation period of routine blood cultures cannot be extended.

Plated cultures from all sources should be examined daily for the first week of incubation and then weekly thereafter, preferably using a dissecting microscope, which will allow detection of tiny colonies. Such microscopic examination is particularly important for specimens that contain contaminating microbiota, as an aerobic actinomycete can be quickly overgrown by more rapidly growing organisms. Care should also be exercised when Lowenstein-Jensen or Middlebrook media are examined for mycobacteria or when BCYE plates are examined for *Legionella* species, as *Nocardia* and other aerobic actinomycetes can grow on these media.

IDENTIFICATION

Microscopic Morphology

Evaluation of the Gram stain morphology of a suspected aerobic actinomycete should be the initial step in organism identification, as microscopic morphologies can vary among the genera (Table 1 and Fig. 3). A sufficient number of fields should be reviewed to allow determination of the most prevalent morphology and to detect the sometimes-rare branching forms. Care should be taken not to confuse perpendicular aligning of the organisms with true branching. Smears made from colonial growth of filamentous isolates may fragment and appear as bacillary or coccoid forms (57).

A properly prepared and carefully interpreted modified acid-fast smear can assist in the preliminary identification of the organism (Fig. 3F, G, I, J, and L). Quality control slides should be stained along with stains of colonies from cultures. With the modified acid-fast stain, the background should be blue; slides that have a pink background may be inadequately decolorized and should be repeated. The smear should be scanned for areas where individual bacterial cells can be seen or areas where single layers of cells allow clear differentiation of cell borders. The acid-fast reaction of tightly packed clumps of organisms may not represent the true partially acid-fast nature of the cells; be wary of large clumps of cells which all appear to be acid-fast stain positive. Acid-fast cells will clearly be red; cells that stain purple or light pink may or may not be truly acid fast. A stain that shows an unambiguous positive acid-fast reaction may frequently show only a few clearly red cells, with a majority of blue cells. Frequently, only the beads appear acid-fast positive. If modified-acid-fast-stain results are ambiguous, transfer of the organism to a lipid-rich medium, such as Lowenstein-Jensen medium or Middlebrook 7H11 agar (to increase the organism's lipid content), and repeat staining may give a more clear-cut stain result. Acid fastness may become more evident as colonies age; the acid-fast reaction has been reported to be most reliable when the test is performed with colonies after 1 to 4 weeks of growth (180). Occasionally, coccoid forms of *Streptomyces* may appear partially acid fast; hyphae, however, are acid-fast negative. Because of the difficulties of interpretation of the modified acid-fast smear, results of this stain should be considered preliminary and must be used only in conjunction with results from other tests. Very rarely, an isolate of a species that is considered to be modified-acid-fast-stain positive may not give a positive stain reaction. If the microscopic and colonial morphology is consistent with an aerobic actinomycete, and if the isolate might be clinically significant, an attempt should be made to determine the identification of the isolate.

Slide Cultures

Slide cultures are probably the best way to evaluate the morphology of some genera, such as *Amycolata*, *Amycolatopsis*, and *Nocardiopsis*. Differences among the genera of aerobic actinomycetes in the growth characteristics seen on slide culture may be subtle; experience in recognizing these morphologic differences is required for correct interpretation of these tests. Further information on characteristic slide culture morphologies can be found in references 181 and 182.

Colonial Morphology

The colonial appearance of members of the aerobic actinomycetes is extremely variable among genera and even between isolates of the same species (Fig. 2). Environmental factors, such as growth medium, incubation temperature, air circulation, presence of CO_2, and age of culture, can affect the size and consistency of the colonies and the production of aerial hyphae (183). Some species produce a diffusible pigment that can vary from strain to strain. See Table 1 for information on specific colonial morphologies.

Aerial Hyphae

Aerial hyphae project away from the surface of the colony into the air but may not be apparent until 7 to 14 days for some species that form such hyphae. The use of a dissecting microscope for observation of aerial hyphae is recommended. *Nocardia* species and *Streptomyces* species usually produce abundant aerial hyphae that give the colonies their characteristic powdery or velvety appearance (Fig. 2C, E, and L); rare strains of *Nocardia* produce sparse or no aerial hyphae (Fig. 2D and G). Of the genera that are partially acid fast, only *Nocardia* species regularly produce aerial hyphae. For some genera of aerobic actinomycetes, the presence of spores and their relative numbers and arrangement on the aerial hyphae can also give some clue to genus identification (182).

Genus Assignment

To determine the genus of an unknown isolate, observation of microscopic and colonial morphology is especially important (Table 1 and Fig. 2 and 3). In assessing the characteristics of colonial growth, the presence or absence of aerial hyphae should be determined. *Nocardia* species and *Streptomyces* species generally produce aerial hyphae, with other less commonly isolated genera also showing this morphologic trait (Table 1). Positive results of a carefully prepared and interpreted modified acid-fast smear combined with the presence of aerial hyphae help to distinguish *Nocardia* from the other genera.

Some recent reports have documented the inadequacy of traditional and commercial biochemical methods in identifying isolates correctly to the genus level. For example, isolates initially misidentified as *Rhodococcus equi* using commercial test strips have on further study been found to belong to the genus *Dietzia* (52); isolates initially identified as *Rhodococcus* species have been found to belong to the genus *Tsukamurella* (155).

Laurent et al. describe a PCR-based method that allows differentiation of members of the genus *Nocardia* from other genera of aerobic actinomycetes (184). It is not known if this method is applicable for the discrimination of newly described *Nocardia* species from other genera.

Species Assignment

Limitations

Given the increasing number of species of aerobic actinomycetes, the use of phenotypic or biochemical tests to obtain exact species or even genus-level identification has become impossible. In addition, phenotypic attributes may be unstable based on environmental and procedural variation. Among the *Nocardia*, most species are nonreactive in most commercially available biochemicals, precluding the definitive identification of these isolates by these methods. For laboratories without molecular capabilities, assignment to the genus level should be attempted. When a precise identification is required for a significant patient isolate, molecular testing is generally required.

Some new species have been described based on a single isolate; such species present problems for laboratories attempting to make identification decisions phenotypically, as characteristics described may not reflect the typical reactions of the species that would be determined if more isolates were analyzed (185).

Biochemicals

Because of the increasing number of described species and the low discrimination power and small number of commercially available phenotypic tests, biochemical testing is not recommended for definitive identification of the aerobic actinomycetes. For laboratories without molecular capabilities, the use of antibiotic susceptibility patterns and basic biochemical results may provide preliminary identifications of frequently isolated *Nocardia* species or complexes obtained from clinical specimens. See the 9th and 10th editions of the *Manual of Clinical Microbiology* for information on species discrimination based on biochemical testing (38, 186) and CLSI document M24-A2 (62) for a list of expected susceptibility results for various *Nocardia* species. When biochemical tests are performed, it is extremely important to include appropriate positive and negative controls to ensure that tests are inoculated, incubated, and interpreted correctly. See Evaluation, Interpretation, and Reporting of Results below for recommendations on reporting preliminary identifications obtained using these methods.

Susceptibility Testing

For *Nocardia* species, basic biochemical testing may be paired with susceptibility testing to achieve preliminary identifications. Some species or species complexes have predictable susceptibility patterns that may assist in isolate characterization (57, 62). These patterns should not be used exclusively as identification techniques, as many newly described species have not been tested for their antibiotic susceptibilities, and some intraspecies variability has been noted.

Cell Wall and Cell Membrane Analysis

Analysis of the cell wall chemistry of the aerobic actinomycetes can aid in the identification of isolates to the genus level (Table 2). While these organisms have distinct cell walls that are characteristic for each genus, methodologies for the investigation of these chemical structures are generally available only in research or reference laboratories. Cell wall analysis is not useful for species-level identification.

Analysis of whole-cell hydrolysates has been used to identify various forms of diaminopimelic acid (DAP) and sugars present in the peptidoglycan layer of the cell walls. The form of DAP combined with the type of sugars present in the cell wall allows classification of the various genera into cell wall types (187, 188) (Table 2).

Members of the aerobic actinomycetes and related genera contain elevated levels of cell wall lipids; analysis of these lipids might be useful for genus-level assignment (189, 190).

Mycolic acids, high-molecular-weight fatty acids with an α-branched, β-hydroxy structure, are present in the cell walls of some genera of aerobic actinomycetes. Mycolic acids are thought to contribute to the staining characteristics and pathogenicity of these organisms (191). The aerobic actinomycetes containing mycolic acids can be differentiated at the genus level based on the number of carbon atoms of their constituent mycolic acids (Table 2). It should be noted that the number of carbon atoms making up the mycolic acids of some genera varies from one report to another. See Table 2.

Menaquinones, the type of isoprenoid quinone present in the cell membranes of the aerobic actinomycetes, vary among genera in the numbers of their isoprene units and in the degrees of unsaturation of the C_3 isoprenyl side chain (192, 193) (Table 2). Menaquinone analysis has been particularly useful for distinguishing *Nocardia* species from other mycolic acid-containing aerobic actinomycetes (Table 2).

Molecular Identification

Molecular techniques, particularly gene sequencing, are currently the only methods that can provide definitive identification of most isolates of aerobic actinomycetes. These methods have the added benefit of providing identifications in a fraction of the time needed for biochemical tests.

PCR with Amplicon Detection

The use of PCR and amplicon detection by gel electrophoresis, with genus- or species-specific primers, enables the identification of aerobic actinomycetes that have unique gene regions. Regions of the 16S rRNA gene have been targeted for the genus-level identification of *Nocardia* (184), *Pseudonocardia* and *Saccharopolyspora* (194), *Gordonia* (195), and *Nocardiopsis* (196). PCR and amplicon detection of the 16S rRNA and *choE* genes have also been used for the species-level identification of *Rhodococcus* species (197, 198).

Wehrhahn et al. describe the use of capillary gel electrophoresis to evaluate amplified regions of the 16S-23S intergenic spacer region for identification of *Nocardia* species. The resulting electropherogram is examined for the presence and pattern of peaks indicating amplified fragments of various sizes. The type strains studied showed unique peak patterns; however, there was significant intraspecies heterogeneity observed (199). The usefulness of this methodology with unknown clinical isolates remains to be determined.

PCR with REA

PCR paired with restriction endonuclease analysis (REA) has been used for the identification of commonly isolated *Nocardia* species. The procedure takes advantage of the presence of restriction endonuclease recognition sites within the variable regions of an organism's gene sequence (38). However, because of the increasing number of recognized *Nocardia* species, and because these REA methodologies are able to detect alterations only within the relatively short restriction endonuclease recognition sequence, the usefulness of these techniques is limited to the identification of only a few species. If a laboratory decides to use REA of any gene as an identification tool, the RFLP patterns for the specific gene and endonuclease combinations for each species newly described must be determined (experimentally or by *in silico* analysis) to prevent misidentifications. The assessment of each newly described species is necessary to ensure that the pattern obtained from a newly described species is different from that obtained from all other species. For information on which species were presumptively identifiable by the use of REA of the HSP gene, see the report by Rodríguez-Nava et al. (200). Presumptive identification of *N. abscessus*, *N. brasiliensis*, *N. cyriacigeorgica*, and *N. otitidiscaviarum* was achievable using REA of the 16S rRNA gene for isolates described prior to 2009; see edition 9 of the *Manual of Clinical Microbiology* (38) for information on this technique.

In addition to being used for the identification of *Nocardia* species, REA has been used for differentiation among numerous genera of aerobic actinomycetes and identification of some species of thermophilic actinomycetes (161, 201). It is likely that the usefulness of these procedures, too, will be limited as the number of recognized species within these genera increases.

Gene Sequencing

Gene sequencing has become a powerful and, currently, a necessary tool for the identification of isolates within this complex group of organisms. Sequence analysis of the 16S rRNA gene has been used for the species-level identification of members of nearly all genera of the aerobic actinomycetes (12, 33, 86, 132, 150, 162, 202–205).

Several genes have been shown to discriminate adequately among *Nocardia* species. By far the most sequence information is available for the 16S rRNA gene sequences of members of this genus. See chapter 6 for a discussion of the phylogenetic attributes of this gene. The presence of a variable sequence region, located near the 5′ terminus of the gene (206), has made partial 16S rRNA sequencing of a 500-bp region of the gene a useful method for the identification of many *Nocardia* species. Cloud et al. (207) compared identifications obtained with a commercial 16S rRNA gene sequencing kit and an expanded database with results obtained using phenotypic identifications and with sequencing results from a 999-bp region of the same gene. The 500-bp-based identification of 94 clinical isolates representing 10 species showed 72% agreement with identifications obtained by phenotypic methods and 90% agreement with identifications obtained by sequencing a larger portion of the gene. At present, this commercial system is capable of distinguishing among the more commonly isolated *Nocardia* species and was also shown to be useful for the genus-level identification of *Gordonia*, *Rhodococcus*, *Streptomyces*, and *Tsukamurella* (110).

Sequencing a region of the 16S rRNA gene larger than 500 bp is necessary for the separation of some species that have identical or nearly identical sequences within the 500-bp region (for example, *N. abscessus*, *Nocardia asiatica*, and *Nocardia arthritidis*; *N. elegans* and *N. veterana;* and *Nocardia higoensis* and *Nocardia shimofusensis*). These species are clearly distinguished with sequence analysis of 1,300 bases.

There is no consensus on how similar 16S rRNA gene sequences must be to consider isolates of *Nocardia* to belong to the same species. It is well known that the interspecies heterogeneity of closely related *Nocardia* species can be quite low, making unambiguous identification of some isolates difficult, if not impossible. The species pairs *N. brevicatena*/*N. paucivorans* and *N. kruczakiae*/*N. veterana* show 99.5% (1,352 bp) and 99.8% (1,379 bp) 16S rRNA gene sequence similarity, respectively (77, 102). *Nocardia testacea* and *Nocardia sienata* differ in their 16S rRNA gene sequences by only 2 bases. These species pairs were all shown to be distinct by DNA-DNA hybridization studies. The CLSI document MM18-A indicates that for aerobic actinomycetes, greater than 99.6% similarity is required for identification to the species level (208). In addition, the document recommends a sequence similarity of 99.0 to 99.5% for identification to the genus level. We suggest that for accurate identification of some members of the *N. nova* complex (*N. kruczakiae* and *N. veterana*), sequence similarity of greater than 99.8% be employed.

The presence of multiple copies of the 16S rRNA gene in some isolates of *Nocardia* has been reported (209, 210). In these studies, the presence of up to five gene copies per organism was noted, and in some isolates, the various copies showed different base sequences. Sequence chromatograms from such isolates show multiple overlapping peaks at a particular position, especially within variable regions of the gene. These multiple peaks remain unresolved with repeat testing; only cloning and sequence analysis of the resulting clones allow clear definition of the gene sequences in these regions. If the various copies of a particular gene are different, it is impossible to determine the identification of an unknown isolate using the sequence of this gene. Unfortunately, the type strains of several species contain multiple copies of the 16S rRNA gene (Table 6) (209). The sequences initially deposited in GenBank at the time the species were described do not indicate the presence of ambiguous bases in the 16S rRNA gene sequence and, therefore, do not reflect the presence of multiple gene copies. It is unclear how the ambiguous base sequences were resolved prior to the deposition of the sequence in GenBank. If the sequences of unknown isolates are compared to these type strain sequences, an acceptable match could not occur. There have been several reports of isolation of species for which the type strain contains multiple copies; in these reports, the authors have sometimes reported 100% agreement of their 16S rRNA gene sequences with that the type strain, but given the presence of multiple differing copies, the resulting identification is questionable.

Other gene targets have been examined for their ability to discriminate among closely related *Nocardia* species. Rodríguez-Nava et al. evaluated a 441-bp region of the HSP gene for its discriminatory ability for the identification of 44 *Nocardia* species (200). They found this gene to have more variable sequence regions than found in the 16S rRNA gene, resulting in more sequence dissimilarity among species. This level of sequence heterogeneity will allow discrimination of some species that are very similar by 16S rRNA gene sequencing.

The *secA1* gene is a housekeeping gene that codes for the SecA1 protein. This protein is essential for the export of proteins across the bacterial cytoplasmic membrane (211). Sequence analysis of a 468-bp region of the *secA1* gene has been shown to give good discrimination among *Nocardia* species (212). In a study of 30 type or reference strains of clinical significance, clear differentiation of all species was obtained. In addition, alignment of the deduced amino acid sequence (156 amino acid residues) showed good separation of all type and reference strains and eliminated some within-species microheterogeneities seen in the gene sequences. The deduced amino acid sequences of 38 of 40 clinical isolates tested were identical to that of the type strain of the species to which that isolate was assigned. Kang et al. reported that the analysis of the *gyrB* gene and the *secA1* gene sequences of *Gordonia* species allow greater discrimination among species than does the 16S rRNA gene (213).

In a study of 120 *Nocardia* isolates (10 ATCC strains and 110 clinical isolates), Kong et al. noted some discordant identifications with the *secA1* genes from clinical isolates compared to the 5′ ends of 606-bp 16S rRNA gene sequences, especially with isolates identified as *N. nova* on the basis of 16S rRNA sequences (214). With the *secA1* gene target, the authors noted a high level of intraspecies diversity, especially among *N. nova*, *N. cyriacigeorgica*, *N. farcinica*, and *N. veterana*. The authors suggest that the diversity of this gene may make it a useful target for epidemiologic evaluations.

The *gyrB* gene may also be a useful target for the identification of *Nocardia* species, although it has been noted to be present in multiple copies in some strains (215). This gene encodes the β-subunit of DNA gyrase, a type II topoisomerase. In an evaluation of 56 *Nocardia* type strains, Takeda et al. (216) noted that with the *gyrB* gene, the interspecies variation ranged from 82.4% to 99.9% (270 to 2 base differences) in sequences of 1,200 bp. The 16S rRNA gene sequences of the same species (as determined from GenBank entries) showed 94.4 to 100% interspecies variation, indicating that sequencing of the *gyrB* gene may enable the discrimination of some closely related species. Phylogenetic trees created using this gene were similar to trees created using the 16S rRNA gene. This gene also clearly discriminated *Nocardia* species from other related genera.

The *rpoB* gene codes for the β-subunit of RNA polymerase and has also been used for the identification of *Nocardia* species. While there are *rpoB* gene sequences for *Nocardia* species in the GenBank database, we are not aware of any publication documenting the usefulness of this gene for identification. Tamura et al. (215) noted that some *Nocardia* strains may harbor multiple copies of the *rpoB* gene.

For some genera, such as *Nocardia*, sequence analysis of more than one gene may be useful for unambiguous identification of a clinical isolate and for the recognition of an unusual species. Rodríguez-Nava et al. (200) noted that a combined analysis using both the 16S rRNA and the HSP gene resolved identification inconsistencies of some *Nocardia* isolates.

Multilocus sequence analysis (MLSA) may also help clarify taxonomic relationships of other members of the aerobic actinomycetes. In 2010, McTaggart et al. reported the usefulness of MLSA for identification and phylogenetic studies of *Nocardia* species and noted that MLSA may soon surpass the use of DNA-DNA hybridization as the gold standard for bacterial taxonomy (217). The authors created sequence-based phylogenetic clusters using concatenated partial sequences of five genes, *gyrB*, 16S rRNA gene, *secA1*, *hsp65*, and *rpoB*, in a study of 36 type and reference strains and 190 clinical isolates. The results showed that 71.3% of the clinical isolates clustered with type strains and were thus identified to the species level. The authors noted that when target sequences of individual genes were evaluated, the resulting identification differed from that obtained using MLSA. This was especially evident for sequences targeting *hsp65* and *rpoB*. The authors noted that the sequences from multiple genes appeared to "buffer" the effect of discrepant alleles in the sequences of the individual genes. The authors indicate that results using a 4-locus (*gyrB*, 16S rRNA gene, *secA1*, *hsp65*) or a 3-locus (*gyrB*-16S rRNA, *secA1*) scheme were nearly as reliable results using a 5-locus scheme.

An important component of identification of isolates by gene sequencing is the quality of the database used for sequence comparison. Users of any sequence database should be aware of the limitations inherent in that database. The public database GenBank (NCBI, National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov/) includes the most extensive collection of sequences from many gene targets. The drawback to its use is that entries in the general database are not curated, and comparison of an unknown sequence with some poor-quality entries may result in an erroneous identification. GenBank contains a reference sequence (RefSeq) collection that contains nonredundant, annotated sequences that are curated by NCBI staff and collaborators (http://www.ncbi.nlm.nih.gov/refseq). See Tables 3 through 6 for GenBank type strain sequences deemed to be reliable for the identification of *Nocardia* and other aerobic actinomycetes. For organisms not listed in those tables, laboratories may want to obtain specific type strains and determine the sequence of that isolate themselves to use as a comparison for unknown patient isolates. Proprietary 16S rRNA gene databases for the identification of *Nocardia* species may be limited in the number of species represented and in the number of sequences of each species included. Cloud et al. expanded a commercial database with additional entries to obtain more-reliable matches for query organisms (207). Other useful databases include the Ribosomal Database Project (http://rdp.cme.msu.edu/) for 16S rRNA gene sequences, BiBi (https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi), which includes sequences from a variety of gene targets, and a commercially available database which includes curated 16S rRNA and *secA1* gene sequences.

Other Molecular Methods

Pyrosequencing provides sequence information for short regions of a DNA target and takes advantage of variable regions of otherwise conserved genes. The usefulness of pyrosequencing is limited due to the short region sequenced, the difficulty of interpretation of results from G+C-rich organisms, and the inability of this technique to easily distinguish among some closely related species. There is some evidence that the use of pyrosequencing may be useful for the identification of *Nocardia* to the genus level (218).

A PCR and reverse line blot hybridization assay was evaluated by Xiao et al. using a total of 135 reference and clinical *Nocardia* isolates encompassing 14 species. Amplification of a region of the 16S rRNA gene and a region of the 16S-23S gene spacer region was followed by hybridization with a panel of species- or group-specific probes. The assay correctly identified 91.8% of the clinical isolates as determined by 16S rRNA gene sequencing (219).

Yassin and Muller recently reported a real-time PCR assay that enables the identification of members of the genus *Tsukamurella* from colonial growth. This method is able to discriminate *Tsukamurella* from other aerobic actinomycetes (220).

Proteomics

MALDI-TOF mass spectrometry is a novel methodology that is being used to identify species of numerous genera of bacteria, especially isolates from clinical specimens. Identifications are based on proteomic profiling by mass spectral analysis, and results obtained from individual isolates are compared to spectra included in databases. There have been only a few published reports describing the use of this technology with the aerobic actinomycetes. Verroken et al. describe the use of MALDI-TOF for the identification of 43 clinical isolates of *Nocardia* species initially identified using a combination of phenotypic and enzymatic tests and 16S rRNA gene sequencing (221). The nocardial proteins were first extracted using a process involving boiling, centrifugation, and the addition of ethanol, formic acid, and acetonitrile prior to spotting. Using the conventional database, the authors were able to identify only 28/43 isolates (65%) to the species or genus level; 15 isolates (35%) were misidentified. With the addition of the spectra of 100 additional isolates to the database, the authors were able to increase the percentage of isolates identified to the species or genus level to 95% (41/43 isolates correctly identified), with only 2 misidentifications. Analysis of resulting dendrograms indicated that some species contained isolates with very diverse spectra but still clustered together. Farfour et al. reported the accurate identification of 44 isolates of *Nocardia* (42 to the species level and 2 to the genus level) and 2 isolates of *Rhodococcus equi* (222). This study utilized an alternate database that was built using direct-colony testing, with no extraction process involved.

As indicated above, one of the limitations of the use of MALDI-TOF for the identification of aerobic actinomycetes is the lack of spectra for these organisms in the instrument databases. In cases of uncommonly isolated organisms, users must create their own databases with reference strains and well-characterized clinical isolates related to the organisms that they wish to identify. It is important to note that the spectra of some *Nocardia* type strains are different from the spectra of clinical isolates of the same species and that a species may contain isolates with many different, but related, spectra. Comparison to all spectra from the same species may improve the accuracy of the resulting identifications. It is also important to note that it may be difficult to accurately identify closely related species (such as members of the *N. nova* complex) using MALDI-TOF (S. Antonara, personal communication).

TYPING SYSTEMS

Among the aerobic actinomycetes, reports on various strain typing methodologies are limited to studies on *Rhodococcus* species, *Tsukamurella* species, and *Nocardia* species. For *Nocardia*, there is no consensus on the best method for performing strain typing. Lack of reproducibility and/or insufficient differences among strains are common difficulties seen with most methods. In addition, interpretive criteria for the various methods are undefined.

Among *Nocardia* species, *N. farcinica* is most frequently implicated in infection outbreaks. There are several reports of strain typing of *N. asteroides* using various methods, but it is unclear if results reflect differences in strains or genetic differences inherent in members of the "*N. asteroides* complex." See Table 7 for a summary of methods and organism targets.

SEROLOGIC METHODS

There have been no recent publications (since 1988) describing serologic methods for the diagnosis of infections caused by aerobic actinomycetes.

ANTIMICROBIAL SUSCEPTIBILITIES

Susceptibility testing should be performed on all isolates of *Nocardia* and other aerobic actinomycetes thought to be of possible clinical significance. CLSI has recently published a revised approved standard for susceptibility testing of both mycobacteria and aerobic actinomycetes (62). The recommended procedure for susceptibility testing of *Nocardia* and the other aerobic actinomycetes is broth microdilution; panels containing the appropriate dilutions of antimicrobials specifically active against these genera are commercially available, but at the time of this writing, none are cleared for use in the United States. The CLSI document outlines methods for inoculum preparation, panel inoculation, incubation, and result interpretation. For *Nocardia* species, panels are read after 3 to 5 days of incubation, with the incubation duration dependent on the particular species being tested. Antimicrobial agents recommended for primary susceptibility testing of all aerobic actinomycete genera include amikacin, amoxicillin-clavulanic acid, ceftriaxone, ciprofloxacin, clarithromycin, imipenem, linezolid, minocycline, trimethoprim-sulfamethoxazole, and tobramycin. Agents to be considered for secondary testing include cefepime, cefotaxime, and doxycycline. The CLSI document lists both the breakpoints and the interpretive criteria for all drugs to be tested for *Nocardia* isolates; both an MIC and an interpretation of the MIC result should be provided in the report to the physician. The document also indicates that the breakpoints listed for *Nocardia* species can be used for other aerobic actinomycetes but should be reported as tentative pending accumulation of further information on these drug/organism combinations (62).

In 2012, the results of a multisite reproducibility study of the broth microdilution method for *Nocardia* species were published (223). Six U.S. testing sites with various levels of experience in performing susceptibility testing reported results for five frequently recovered *Nocardia* species using a commercially prepared panel. Results indicated that interlaboratory reproducibility was related to the antimicrobial being tested; ceftriaxone, imipenem, sulfamethoxazole, trimethoprim-sulfamethoxazole, and tigecycline results were found to be the least reproducible. Results for ceftriaxone with *N. cyriacigeorgica* and *N. wallacei* were most problematic and should probably not be reported for these species.

Sulfonamides remain the mainstay of treatment of nocardial infections, often in combination with other antimicrobial agents, because of the historical *in vitro* susceptibility of *Nocardia* isolates to these drugs and because of a long history of the clinical effectiveness of these drugs if administered in adequate doses (223, 224). There have been several recent reports indicating an increase in resistance to the sulfonamides among *Nocardia* species. Ameen et al. reported that 8 of 19 patients with actinomycetomas caused by *Nocardia* failed sulfonamide treatment in Mexico; the authors proposed that sulfonamides should be used only for limited actinomycotic disease of comparatively short duration and only for those patients with actinomycetomas who are at low risk for visceral dissemination (225). In 2010, Uhde et al. reported that 61% of *Nocardia* isolates submitted for susceptibility testing to the Centers for Disease Control and Prevention (CDC) from 1995 to 2004 were resistant to sulfamethoxazole and 42% were resistant to trimethoprim-sulfamethoxazole (226). The authors note that their results may not represent the resistance profile of all U.S. isolates, as only organisms recovered from patients with severe infections or from patients with treatment failures may have been submitted for testing. The authors also indicate that they did not receive information on any possible antibiotic prophylaxis or on clinical outcome. Larruskain et al. reported in 2011 that the majority of *N. farcinica* isolates from northern Spain were resistant to trimethoprim-sulfamethoxazole (227). In contrast, results from Taiwan indicate that

TABLE 7 Typing methods used for aerobic actinomycetes

Method[a]	Organism(s)	Reference(s)
Ribotyping	*R. equi* and *R. rhodochrous*	245, 246
RAPD	*N. farcinica*	247, 248
PFGE	*N. farcinica*, *Tsukamurella* spp.	34, 157, 248
Repetitive extragenic palindromic-PCR (REP-PCR)	*Nocardia* species	249
Amplified fragment length polymorphism (AFLP)	*N. farcinica*	248
PCR-capillary gel electrophoresis	*Nocardia* species	199

[a] RAPD, randomly amplified polymorphic DNA; PFGE, pulsed-field gel electrophoresis.

only 2% of 138 *Nocardia* isolates tested over an 11-year period were determined to be sulfonamide resistant (228). In response to the report from the CDC on sulfonamide resistance in the United States, Brown-Elliott et al. reported a retrospective analysis of 552 *Nocardia* isolates recovered and tested at 6 U.S. laboratories, among which only 2% were resistant to sulfonamides (229). The authors propose that reports of increased resistance may be related to the difficulties in interpretation of sulfonamide broth dilution tests, the lack of a recommended *Nocardia* species quality control organism, and the lack of proficiency testing programs to ensure accurate interpretation. There are also limited data to indicate the *in vivo* action of sulfonamides in cases of *in vitro* resistance. In a recent report, a patient with an arterial-stent-related *N. farcinica* infection was treated with multiple antibiotics, including trimethoprim-sulfamethoxazole, despite *in vitro* resistance to this drug. It appeared that the trimethoprim-sulfamethoxazole was useful in controlling the patient's infection (230).

The multisite broth microdilution study published in 2012 noted a significant lack of reproducibility among testing sites for the sulfonamides (223). Of a total of 1,681 replicate sulfonamide MIC results for 5 species (one isolate each of *N. brasiliensis*, *N. cyriacigeorgica*, *N. farcinica*, *N. nova*, and *N. wallacei* determined by previous testing to be susceptible to sulfonamides), 14.6% of results indicated resistance to sulfamethoxazole or trimethoprim-sulfamethoxazole. Since criteria for inoculum preparation and assay performance were similar at all testing sites, the authors noted that the most likely explanation for this lack of reproducibility with these drugs is the difficulty of determination of the endpoint, which is defined as the dilution that results in 80% inhibition compared to growth in the control well. Interestingly, only 1.7% of 296 concurrently run disk diffusion tests using the same isolates indicated resistance to sulfamethoxazole. The authors propose that when performing broth microdilution tests for sulfonamides, a disk diffusion test be performed for validation of the MIC result. The inoculated disk diffusion plate may also be useful for checking the inoculum concentration (223). The CLSI document states that isolates showing a discrepancy between the broth microdilution and disk diffusion tests for sulfonamides should be retested or referred to a reference laboratory for testing (62).

The 2012 multicenter study also highlighted additional difficulties that may be encountered in the setup and interpretation of broth microdilution tests for *Nocardia* (223). Preparation of an adequate inoculum is an important concern; the clumping nature of *Nocardia* isolates often prevents the preparation of an adequately homogeneous inoculum suspension. The current CLSI document suggests the use of a "pellet pestle" to assist in creating a dense suspension with a minimum of organism clumps (62). In addition, the use of a nephelometer to standardize the inoculum suspension is recommended to provide an appropriately dense inoculum (62). However, even with careful inoculum preparation, resulting colony counts prepared using traditional spread plate techniques may not accurately represent the actual organism concentration in the inoculum, nor do such colony count results correspond to accurate or inaccurate MIC values (223). Because endpoint determination for *Nocardia* species is more technically challenging than for other organisms, the multisite study manuscript recommends that technical staff become familiar with endpoint determinations as illustrated in the CLSI guidelines, especially for the sulfonamides. Additional recommendations to improve susceptibility testing results include thorough training of technical staff in inoculum and panel preparation, comparison of results with expected results for a particular species (as noted in the current CLSI document), the testing of an appropriate quality control strain (*N. nova* ATCC BAA-2227 was recommended [223]), and participation in proficiency testing with another laboratory. The multisite study manuscript also recommends referral of any isolate to a reference laboratory if the susceptibility pattern obtained is different from the expected result for that organism (223).

In a study of 51 clinical isolates of *Nocardia* species and 12 other species of aerobic actinomycetes, Lowman and Aithma compared results from the Etest to results obtained by broth microdilution. Results with the Etest showed only 46.2 to 81.6% agreement within ±1 dilution and 67.5 to 100% categorical agreement with broth microdilution. There were very major errors reported with the Etest for the more rarely isolated species. The authors concluded that the Etest is not an acceptable alternative to broth microdilution for the aerobic actinomycetes and note specific problems with inoculum preparation and reading/interpretation of the test (231).

There is limited information in the literature regarding susceptibility testing of members of the aerobic actinomycetes other than *Nocardia*. Erol et al. report MIC_{90} results of testing isolates of *Amycolatopsis* species from cases of nocardioform placentitis in horses using a commercially available panel (232).

For *R. equi*, the current CLSI document recommends that a regular Gram-positive panel be inoculated (if such panels include vancomycin and rifampin, critical antimicrobials for this species). Results can be read after 24 h of incubation, and results should be reported as "tentative." Breakpoints for *Staphylococcus aureus* should be used (62).

Liu et al. recently reported susceptibility results for *Tsukamurella tyrosinosolvens*, *Tsukamurella spumae*, and *Tsukamurella pulmonis* using a short incubation antimicrobial susceptibility testing system and noted that these three species were susceptible to amoxicillin-clavulanic acid, ciprofloxacin, moxifloxacin, and linezolid. *T. tyrosinosolvens* and *T. spumae* were reported as susceptible to sulfamethoxazole, while *T. pulmonis* was reported as resistant (155). The current CLSI document indicates that susceptibility testing for *Tsukamurella* species may be read after 48 h of incubation for some species (62).

Moser et al. reported that 13 clinical isolates of *Gordonia polyisoprenivorans* were susceptible to amikacin, amoxicillin-clavulanic acid, ampicillin, ceftriaxone, ciprofloxacin, imipenem, linezolid, and vancomycin using the CLSI-recommended procedure. Eight of the 13 isolates were reported to be resistant to sulfamethoxazole (233).

Drug susceptibility testing of isolates of *Segniliparus rugosus* may require the use of Middlebrook 7H9 broth, as the use of cation-adjusted Mueller-Hinton broth may provide insufficient growth (234). Using breakpoints recommended for *Nocardia* species and for rapidly growing mycobacteria (38), Butler et al. (234) indicated that the *S. rugosus* isolates were susceptible to imipenem, rifabutin, sulfamethoxazole, and trimethoprim-sulfamethoxazole and resistant or intermediate to amikacin, amoxicillin-clavulanate, ceftriaxone, ciprofloxacin, clarithromycin, linezolid, minocycline, and tobramycin.

Isolates in the genera *Dietzia* tend to be susceptible to a broad range of antimicrobials, and at present, there is no clear evidence that susceptibility can be predicted by species identification (52).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The aerobic actinomycetes are widespread in the environment. Therefore, particularly when an aerobic actinomycete is isolated in culture in small amounts without having been visualized in the direct patient specimen, it may be impossible for the laboratory to determine if the isolate is the result of specimen or laboratory contamination, patient colonization, or actual infection. Some indication of the quantity of the organism present should be given for any isolate of an aerobic actinomycete. Discussion between the clinical and laboratory staff is extremely useful for selection of additional patient specimens and laboratory procedures for determination of the clinical significance of an isolate from a given patient. (See the Clinical Significance section of this chapter for information on species known to cause human disease. Additionally, see Tables 3 through 6 for information on the frequency of isolation of species known to be clinically significant.) Identification of an organism to the genus level may help determine its significance. However, even for accurate genus assignment, molecular methods may need to be employed. In some cases, genus-level identification (if possible) suffices.

When an isolate is reported to the species level, the laboratory should be certain that clinicians are aware of the reliability of the identification method used. Reports should indicate how the isolate was identified. It may be possible to preliminarily identify the more commonly encountered aerobic actinomycetes, particularly *Nocardia* species, to the species level using phenotypic methods (81, 186), but some are inevitably misidentified by such procedures. Precise identification can be achieved only by gene sequencing; if identification is obtained by other methods, the report should indicate that the identification is presumptive. Susceptibility results should be reported for isolates considered to be clinically significant. For accurate determination of both identification and susceptibility results, referral to a laboratory with expertise in working with these organisms may be necessary.

Whenever a laboratory isolates an aerobic actinomycete for which sequencing indicates that the organism has been rarely isolated, the circumstances surrounding the isolation of the organism should be carefully evaluated to assess its potential clinical significance, and a search of recent literature might be conducted to determine what is already established regarding pathogenicity and antibiotic susceptibility for that species. For such searches, the LPSN and PubMed may be good starting points; care should be taken to consider current nomenclature and taxonomy as stated by the LPSN. The report of a rare or unusual species should be accompanied by a brief summary of whatever is known regarding its clinical significance.

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Mycobacterium: General Characteristics, Laboratory Detection, and Staining Procedures*

GABY E. PFYFFER

30

Many species within the genus *Mycobacterium* are prominent pathogens, above all, the members of *Mycobacterium tuberculosis* complex, *Mycobacterium leprae*, and *Mycobacterium ulcerans*. In addition, there are more than 150 species of environmental mycobacteria, called nontuberculous mycobacteria (NTM), which exert various degrees of pathogenicity and virulence.

Tuberculosis is second only to HIV/AIDS as the greatest global killer due to a single infectious agent. Based on the most recent survey data of the World Health Organization (WHO), about one-third of the world's population has latent tuberculosis, corresponding to approximately 2.4 billion people (1). It was estimated that 8.7 million new cases of tuberculosis occurred and that 1.4 million people died of tuberculosis in 2011. Of particular concern is the increasing extent of estimated multidrug-resistant tuberculosis (MDR-TB) (>300,000 cases; 3.7% of new cases and 20% of previously treated cases) and extensively drug-resistant tuberculosis (XDR-TB) cases, identified to date in 81 countries, as well as the problem of people coinfected with HIV. According to the WHO, the infections of 1.1 million (13%) of the 8.7 million people who have developed tuberculosis worldwide were HIV related, among which 79% were in the African region (1). Reducing the burden of tuberculosis depends largely on how rapidly DOTS (directly observed therapy, short-course) programs can be implemented. Among the prime obstacles for a successful elimination of the disease are lack of political commitment, shortages of trained staff, and poor laboratory services, together with inadequate patient management. Although the total number of global tuberculosis cases is still increasing in absolute terms as a result of population growth, it has, nevertheless, been observed that the number of incident cases per capita has been falling globally for several years at a rate of 2.2% between 2010 and 2011. Likewise, the tuberculosis mortality rate has decreased 41% since 1990, reflecting the effectiveness of prevention strategies and control measures implemented by health authorities, including the use of more-rapid and efficient laboratory algorithms to detect M. *tuberculosis* and susceptibility testing against antituberculosis drugs. If these common efforts are not jeopardized in the future, it appears that the world is on track to achieve the global target of a 50% reduction of tuberculosis cases by 2015 (1). In this context, the clinical mycobacteriology laboratory plays a pivotal role.

Apart from the members of the M. *tuberculosis* complex, NTM species are increasing in number, and some of them are sources of important diseases in humans (2–6). Thus, rapid and reliable identification of NTM is essential. Overall, the level of service and the choice of methods applied in clinical mycobacteriology laboratories should be determined by the patient population served and by the resources available.

TAXONOMY AND DESCRIPTION OF THE GENUS

The genus *Mycobacterium* is the only genus in the family *Mycobacteriaceae* (7) and is related to other mycolic acid-containing genera. The high G+C contents of the DNA of *Mycobacterium* species (61 to 71 mol%, except in M. *leprae* [55%]) are within the range of those of the other mycolic acid-containing genera, *Gordonia* (63 to 69 mol%), *Tsukamurella* (68 to 74 mol%), *Nocardia* (64 to 72 mol%), *Rhodococcus* (63 to 73 mol%), and *Segniliparus* (68 to 72 mol%) (8, 9).

Mycobacteria are mainly aerobic, non-spore-forming (except M. *marinum* [10]), nonmotile, slightly curved or straight rods (0.2 to 0.6 µm by 1.0 to 10 µm) that may branch. Colony morphology varies among the species, ranging from smooth to rough and from nonpigmented (nonphotochromogens) to pigmented. Pigmented colonies are regularly or variably yellow, orange, or, rarely, pink, usually due to carotenoid pigments. Some species require light to form pigment (photochromogens), while other species form pigment in either the light or the dark (scotochromogens). Aerial filaments are very rarely formed and never visible without magnification. Filamentous or mycelium-like growth may sometimes occur, but upon slight disturbance, the organism easily fragments into rods or coccoid elements.

The cell wall peptidoglycolipid contains *meso*-diaminopimelic acid, alanine, glutamic acid, glucosamine, muramic acid, arabinose, and galactose. Mycolic acids (whose numbers of carbon atoms range from 70 to 90), together with free lipids (e.g., trehalose-6,6′-dimycolate), provide for a

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*This chapter contains information (safety and transport issues) presented in chapter 36 by Barbara A. Brown-Elliott and Richard J. Wallace, Jr., and in chapter 37 by Véronique Vincent, Barbara A. Brown-Elliott, Kenneth C. Jost, Jr., and Richard J. Wallace, Jr., in the 8th edition of this *Manual*.

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hydrophobic permeability barrier (11). Other important fatty acids are waxes, phospholipids, and mycoserosic and phthienoic acids. Various patterns of cellular fatty acids (whose numbers of carbon atoms range from 10 to 20) are found as well, among which is tuberculostearic (10-*R*-methyloctadecanoic) acid, a unique cell component for a number of aerobic actinomycetes (11).

The high content of complex lipids of the cell wall prevents the access of common aniline dyes. Not readily stained by Gram's method, mycobacteria are stained with special procedures (e.g., Ziehl-Neelsen staining). They are not easily decolorized, even with acid-alcohol, and they are, therefore, acid fast. However, acid fastness can be partly or completely lost at some stage of growth by a proportion of the cells of some species, particularly the rapidly growing ones.

Compared to the growth of other bacteria, the growth of most mycobacterial species is slow, with generation times of up to approximately 20 h (*M. ulcerans*'s is up to 36 h) on commonly used media. A natural division exists between slowly and rapidly growing species of mycobacteria. Slow growers require more than 7 days to produce colonies on solid media from a dilute inoculum under ideal culture conditions. Rapid growers, by definition, require less than 7 days when subcultured on Löwenstein-Jensen (L-J) medium but may also take several weeks to appear in primary cultures from clinical specimens.

Nutritional Requirements and Growth

Most species adapt readily to growth on relatively simple substrates, using ammonia or amino acids as nitrogen sources and glycerol as a carbon source in the presence of mineral salts. A few species (e.g., *Mycobacterium haemophilum* and *Mycobacterium genavense*) are fastidious and require supplements such as mycobactin, hemin, or other compounds. To date, *M. leprae* has not been cultured outside living cells. Growth of mycobacteria is stimulated by carbon dioxide and by fatty acids, which may be provided in the form of egg yolk or oleic acid, even though the latter is toxic in high concentrations (≥1%) and has to be neutralized by albumin. Optimum temperatures for growth vary widely among species (from <30 to 45°C).

With the genomes of several mycobacterial species deciphered, functional genomics have provided new insights into their physiological and metabolic regulation and their relation to virulence (12).

Susceptibility to Physical and Chemical Agents

Mycobacteria are able to survive for weeks to months on inanimate objects if protected from sunlight. *M. tuberculosis* complex organisms, for instance, survive for several months on surfaces or in soil or cow dung, from which other animals may be infected (13). Mycobacteria are not easily killed by freezing or desiccation but can be killed by heat (for *Mycobacterium avium*, for instance, the time necessary to kill 90% of the cells at 60°C is 4 min, but for *Mycobacterium xenopi*, it is 33 min) and by ultraviolet (sun)light (NTM, for instance, are 2- to 10-fold more resistant to UV irradiation than *Escherichia coli* [4]). Mycobacteria are more resistant to acids, alkali, and some chemical disinfectants than most other non-spore-forming bacteria. Quaternary ammonium compounds, hexachlorophene, and chlorhexidine are bacteriostatic at best. Proprietary disinfectants recommended as suitable for use in tuberculosis laboratories are those containing phenols, chlorine, or alcohol (14–16). Other commonly used agents, such as ethylene oxide and formaldehyde vapor, as well as disinfectants, such as 2% alkaline glutaraldehyde, 2% peracetic acid, and stabilized hydrogen peroxide, are effective in killing *M. tuberculosis* as well. With iodophors, the bactericidal effect depends on the content of available iodine as well as on the presence of organic matter. In their guidelines, the Centers for Disease Control and Prevention (CDC), National Institutes of Health (NIH), suggest intermediate-level disinfectants (reference 15; see also chapter 13).

EPIDEMIOLOGY AND TRANSMISSION

The genus *Mycobacterium* includes obligate pathogens, opportunistic pathogens, and commensals. Because *M. leprae* and *M. tuberculosis* complex organisms are incapable of replication in an inanimate environment, the major ecological niche for them is tissues of humans and warm-blooded animals. *M. tuberculosis* is carried in airborne particles (droplet nuclei) generated when patients with pulmonary tuberculosis cough. These particles, 1 to 5 μm in size, are kept suspended by normal air currents. Infection occurs when a susceptible person inhales the droplet nuclei. Once in the alveoli, the organisms are engulfed by alveolar macrophages. Usually, the host cell-mediated immune response limits the multiplication and spread of *M. tuberculosis*. However, some bacilli can remain viable but dormant for many years after initial infection. Patients latently infected with *M. tuberculosis* are asymptomatic and not infectious but usually have a positive tuberculin skin test (TST) or a positive result with one of the interferon gamma (IFN-γ) release assays (IGRAs). In general, persons with a latent infection have a 10% risk during their lifetime of developing active tuberculosis, whereas patients with HIV infection have a 10 to 15% risk per year of progressing to disease manifestation (17).

In contrast, NTM are free-living mycobacteria usually found in association with watery habitats, such as lakes, rivers, and soil (4). While for some human-pathogenic NTM species, e.g., *Mycobacterium haemophilum* and *Mycobacterium szulgai*, the reservoir has not yet been defined (2), for others, such as *M. avium* complex (MAC) organisms, *Mycobacterium chimaera*, *Mycobacterium genavense*, *Mycobacterium gordonae*, *Mycobacterium kansasii*, *Mycobacterium lentiflavum*, *Mycobacterium simiae*, *Mycobacterium xenopi*, and some rapidly growing mycobacteria, tap water and/or showerheads have served as reservoirs (18–21). Some NTM species are associated with nosocomial disease and/or pseudo-outbreaks (2). Other well-known sources of positive mycobacterial cultures are bronchoscopes and related devices. Organisms isolated from these pseudoinfections include *M. tuberculosis* and *M. xenopi* as well as other NTM. Although not components of the microbiota of humans or animals, NTM may simply be isolated as "bystanders" from the skin, upper respiratory tracts, and intestinal and genital tracts in asymptomatic individuals (3). Due to their ubiquitous nature, the question of their clinical significance is, therefore, important but often difficult to answer (2). The major route of infection of NTM leading to pulmonary disease is via aerosols. Other possible routes of NTM infection from their reservoirs may occur via ingestion of soil or water, inhalation of dusts, and aspiration due to gastric reflux of NTM that entered the stomach via ingestion (4).

CLINICAL SIGNIFICANCE AND DESCRIPTION OF SPECIES

With the advent of molecular techniques for appropriate identification, close to 200 mycobacterial species have now been described, and their number is increasing steadily. This chapter will focus on the slowly growing mycobacteria only; rapidly growing mycobacteria are described in chapter 32.

Mycobacterium tuberculosis Complex

The *M. tuberculosis* complex taxon includes *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *Mycobacterium africanum*, *Mycobacterium caprae*, *Mycobacterium microti*, *Mycobacterium canettii*, *Mycobacterium pinnipedii*, and the recently described *Mycobacterium mungi* and *Mycobacterium orygis*. Although the members of the *M. tuberculosis* complex are characterized by different phenotypes and mammalian host ranges, they display a very extreme genetic homogeneity, with only approximately 0.01 to 0.03% synonymous nucleotide variation and no significant trace of genetic exchange among them (22–24). Irrespective of this, identification to the species level is not merely an academic exercise but justified for epidemiologic, public health, and therapeutic reasons.

Mycobacterium tuberculosis

In the industrialized world, a higher prevalence of tuberculosis occurs among the medically underserved ethnic minorities, the urban poor, homeless persons, prison inmates, alcoholics, intravenous drug users, the elderly in general, foreign-born persons from areas of high prevalence, and contacts of persons with active tuberculosis than among other populations. The greatest known risk factor for progression of latent infection to active tuberculosis is HIV infection. Combined HIV and tuberculosis infections, especially in combination with drug resistance, have caused outbreaks in the past with extremely high mortality rates. In addition, the emergence of XDR-TB has had a dramatic impact on the changing patterns of global tuberculosis (25). Groups with a higher likelihood of progression also include individuals with underlying medical conditions, persons who have been infected within the past 2 years, children ≤4 years old, and persons with fibrotic and/or cancerous lesions on chest radiographs.

Tuberculosis in adults is a slowly progressive process characterized by chronic inflammation and caseation and formation of cavities. These foci may rupture into the bronchi, allowing very large numbers of organisms to spread to other areas of the lungs and to be aerosolized by coughing, hence infecting other persons. Important clinical features of pulmonary tuberculosis are cough, weight loss, night sweat, low-grade fever, dyspnea, and chest pain. Extrapulmonary manifestations of *M. tuberculosis* infection include cervical lymphadenitis, pleuritis, pericarditis, synovitis, meningitis, and infections of the skin, joints, bones, and internal organs (26). Pulmonary disease in AIDS patients often differs in radiologic findings from the ordinary clinical picture of tuberculosis and usually progresses rapidly. In these patients, extrapulmonary manifestation and disseminated disease, sometimes even without the formation of granulomas, are seen more frequently (26).

In culture, colonies of *M. tuberculosis* are off-white and rough on solid medium (Fig. 1). The genome of *M. tuberculosis* (4,411,529 bp) was deciphered more than 15 years ago (27). Studies have shown that modern strains of *M. tuberculosis* have an extremely low level of genetic variation, suggesting that the entire population of *M. tuberculosis* strains resulted from clonal expansion after an evolutionary bottleneck some 35,000 years ago (24, 28). Today's strains constitute just the visible tip of a much broader progenitor species. Based on very recent molecular data, the *M. tuberculosis* genome appears to be a composite assembly resulting from horizontal gene transfer events predating clonal expansion. The amount of synonymous nucleotide variation in housekeeping genes suggests that tuberculosis bacilli were contemporaneous with early hominids in East Africa and coevolved with their human host much longer than previously thought (24, 29).

Mycobacterium bovis

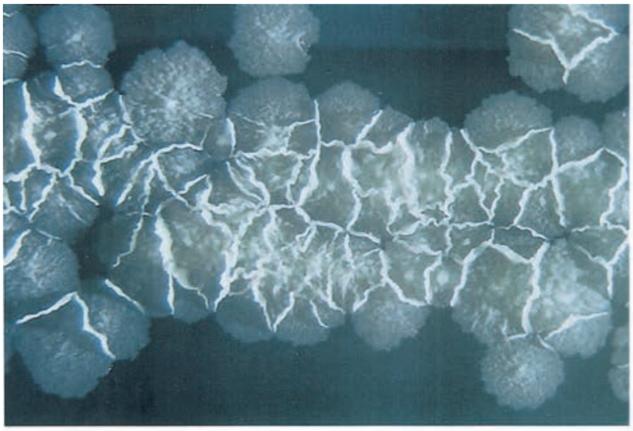
M. bovis causes tuberculosis in warm-blooded animals, such as cattle, dogs, cats, pigs, parrots, badgers, deers, camelids, some birds of prey, and also primates, including humans. *M. bovis* caused as many as 25% of cases of human tuberculosis in developed countries in the late 19th century; the number of tuberculosis cases caused by *M. bovis* has dropped to 1 to 2% today (30), but this organism still remains an important problem in nonindustrialized nations. Human disease is very similar to that caused by *M. tuberculosis* and is treated accordingly, except that pyrazinamide is ineffective due to the inherent resistance of *M. bovis*. Colonies on egg-based media are small and rounded, with irregular edges and a granular surface; on agar media, colonies are small and flat (7). The genome of *M. bovis* has a size of 4,345,492 bp and a G+C content of 65.6%. The sequence is >99.9% identical to that of *M. tuberculosis*. There are some deletions in the genome which led to a reduced genome size (31).

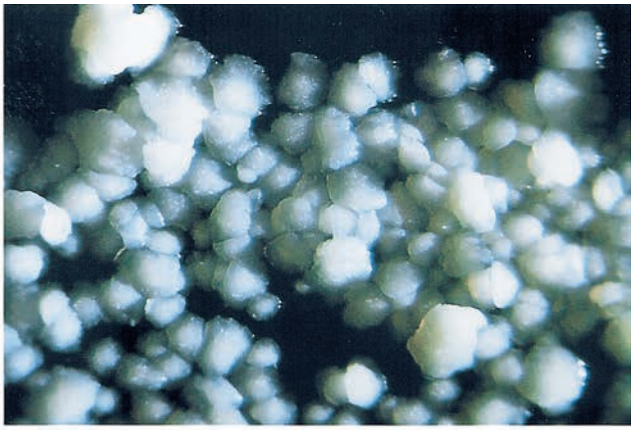
Mycobacterium bovis BCG

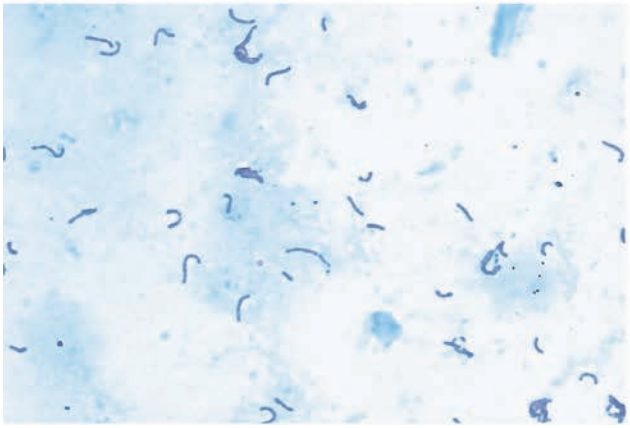
In many parts of the world, *Bacillus* Calmette-Guérin (BCG) is still used for vaccine purposes. Distributed by Calmette in 1924 to laboratories around the world, the strain has been maintained *in vitro* by serial passaging. Today, there exists a genetically heterogeneous conglomerate of BCG strains (Fig. 2) (32, 33; www.bcgatlas.org) which predominantly conform to the properties described for *M. bovis*, except that they are attenuated in virulence. In rare instances, BCG may, however, disseminate as a complication of intravesical BCG immunostimulation against bladder cancer (34, 35). The 4,374,522-bp genome contains nearly 4,000 protein-coding genes, 58 of which are present in two copies as a result of two independent tandem duplications, DU1 and DU2. Also, mutations in genes encoding σ factors and pleiotrophic transcription regulators, like PhoRn and Crp, were uncovered in various BCG strains. Together with gene amplification, these mutations affect gene expression levels, immunogenicity, and possibly protection against tuberculosis, suggesting that early BCG vaccines may even be superior to the later ones, which are more widely used (36). Single nucleotide polymorphism analysis is able to differentiate the *M. bovis* BCG lineage from the virulent *M. bovis* lineage (37).

Mycobacterium africanum

M. africanum, with a genome size of 4,389,314 bp (G+C content of 65.6%), causes up to half of the cases of human tuberculosis in West Africa. Infections with *M. africanum* display differences from infections with *M. tuberculosis* in patients' characteristics and immunoepidemiological features (38). The organism has also been reported from other continents, such as the United States, mainly in patients who have lived in Africa (39). Resembling the colonies of *M. tuberculosis*, *M. africanum* has physiological and biochemical properties that position the organism between *M. tuberculosis* and *M. bovis*. Prior to molecular genetics, the definition of *M. africanum* was difficult and its validity questioned by some authors. *M. africanum* strains were classified into two major subgroups on the basis of geographical origin







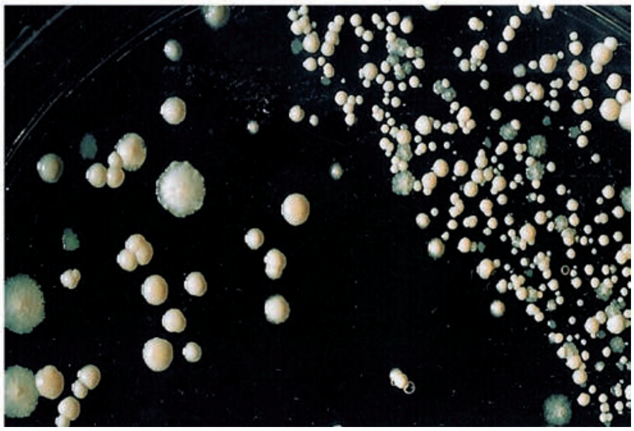


FIGURE 1 (Top left) *Mycobacterium tuberculosis* on Middlebrook 7H10 agar. Note the dry and rough colonies, which sometimes have a nodular or wrinkled surface. doi:1128/9781555817381.ch30.f1

FIGURE 2 (Top right) *Mycobacterium bovis* BCG on Middlebrook 7H10 agar. Colonies may be flat as well as round with irregular edges. doi:1128/9781555817381.ch30.f2

FIGURE 3 (Bottom left) Acid-fast staining (Ziehl-Neelsen) of *Mycobacterium microti*. Note the characteristic curved ("croissant-like") microscopic morphology. doi:1128/9781555817381.ch30.f3

FIGURE 4 (Bottom right) *Mycobacterium canettii* on Middlebrook 7H10 agar. Note the heterogeneous colony morphology consisting of some flat and smooth but predominantly domed and glossy colonies (photograph kindly provided by D. van Soolingen). doi:1128/9781555817381.ch30.f4

and biochemical properties, i.e., M. *africanum* subtype I from West Africa and M. *africanum* subtype II from East Africa. Based on genotypic analyses, subtype II has recently been described as M. *tuberculosis*, thus leaving the West African type as the only M. *africanum* strain (with the two subtypes MAF1 and MAF2 [38, 40]). The species is characterized by the lack of a region of difference (RD9), the presence of RD12, and a specific *gyrB* gene polymorphism (22). These findings are corroborated by most recent genome analyses of M. *africanum*, which reveal a lineage-specific locus and gene erosion common to the M. *tuberculosis* complex (41).

Mycobacterium caprae

M. *caprae*, with the former names M. *tuberculosis* subsp. *caprae* (42) and M. *bovis* subsp. *caprae* (43), was originally described as preferring goats to cattle as hosts. M. *caprae* is also seen in sheep, pigs, wild boars, red deers, and foxes (44) and accounted for 31% of human tuberculosis cases, mostly as pulmonary manifestation, in Germany between 1999 and 2001 (43). Easily recognized by its susceptibility to pyrazinamide, M. *caprae* can be added to the list of agents of human tuberculosis contracted from animals. On the basis of mycobacterial interspersed repetitive unit (MIRU) genotyping, it was later demonstrated that M. *caprae* is closely related to the branches of classical M. *bovis*, M. *pinnipedii*, M. *microti*, and ancestral M. *tuberculosis* but separate from modern M. *tuberculosis* (45). With a heterogeneous RD4, M. *caprae* can be differentiated by PCR from M. *bovis*, and the three variants of alpine M. *caprae* isolates can be identified as well (46).

Mycobacterium microti

Originally isolated from rodents, such as voles and shrews, M. *microti* also causes naturally acquired tuberculosis in guinea pigs, rabbits, llamas (47, 48), cats (49, 50), meerkats (51), and other warm-blooded animals as well as both immunocompetent and immunosuppressed humans (52, 53, 54). Usually revealing a characteristic "croissant"-like morphology in stained smears (Fig. 3), the organism normally fails to grow in culture. At least the vole type of M. *microti* can easily be recognized upon spacer oligotyping (see chapter 31) since it contains an exceptionally short genomic direct repeat region resulting in identical two-spacer sequence reactions (52). Numerous deletions relative to M. *tuberculosis* have been discovered (55).

Mycobacterium canettii

M. *canettii* was collected by Georges Canetti in 1969. van Soolingen et al. (56) and Pfyffer et al. (57) were the first to report M. *canettii*, the "smooth TB bacillus," causing lymphadenitis in a child and generalized tuberculosis in an HIV-positive patient, respectively. The facts that both patients were exposed in Africa and that more cases of cervical lymphadenitis have subsequently been reported from Djibouti (58) support the hypothesis that M. *canettii* might be more abundant on the African continent than elsewhere. In 2002, pulmonary manifestation of M. *canettii* was reported in Africa (59), and in 2009, the first case of M. *canettii* meningitis was recognized in a Sudanese refugee living in the United States (60).

With its smooth, round, and glossy colonies (Fig. 4), M. *canettii* differs considerably from all other members of the M. *tuberculosis* complex and can even be mistaken for an NTM. However, M. *canettii* may, in rare instances, also have a stable rough morphotype, mimicking that of M. *tuberculosis*. As a consequence, adequate molecular techniques have to be applied to identify the organism to the species level (e.g., by the line probe assay [Hain Lifescience, Nehren, Germany] and 65-kDa *hsp* PCR restriction enzyme analysis [PRA]). Studies by Brosch et al. (22) and Marmiesse et al. (61) of 20 regions where insertion/deletion events took place in the genome of M. *tuberculosis* suggested that M. *canettii* diverged first from the rest of the M. *tuberculosis* complex. On the basis of results of variable-number tandem repeat (VNTR) genotyping and analysis of *hsp65* gene polymorphisms in 44 strains of M. *canettii*, Fabre et al. (58) confirmed that M. *canettii* is the most probable source species of M. *tuberculosis* complex, rather than just another branch of the taxon. In fact, it is assumed that M. *canettii* appeared some 2.8 million years ago and may, therefore, be the ancestor of all members of the present-day M. *tuberculosis* complex (24). Based on sequencing and whole-genome analyses, strains of M. *canettii* are highly recombinogenic and exhibit early branching, with larger genome sizes, higher rates of genetic variation, and fewer molecular scars than M. *tuberculosis* and with distinct CRISPR (clustered regularly interspaced short palindromic repeats)-Cas system-associated sequences (62).

Mycobacterium pinnipedii sp. nov.

On the basis of host preference as well as phenotypic and genotypic characteristics, M. *pinnipedii* sp. nov., another member of the M. *tuberculosis* complex, was defined by Cousins et al. in 2003 (63). Pinnipeds appear to be the natural host, but the organism is also pathogenic for guinea pigs, rabbits, and possibly cattle. The "seal bacillus" also affects animals in zoological gardens, e.g., camels (*Camelus bactrianus*) and tapirs (*Tapirus indicus*) (64). Transmission of M. *pinnipedii* infection from a tapir to other tapirs (64) and from sea lions to humans was recently demonstrated by the TST and IGRAs (65). Infections with M. *pinnipedii* manifest with granulomatous lesions in lymph nodes, lungs, pleura, and spleen and are able to disseminate.

Novel Proposed Species within the *M. tuberculosis* Complex

Based on molecular characteristics, two novel taxa of the M. *tuberculosis* complex have been described. The proposed new species, *Mycobacterium mungi*, is the causative agent of tuberculosis in the banded mongoose (*Mungo mungo*) (66), and M. *orygis* affects larger mammals, such as oryxes, gazelles, antelopes, and waterbucks on the African continent (67–69).

Mycobacterium leprae

In past centuries, leprosy (Hansen's disease) occurred on a large scale in Europe, in particular in Norway. Today, rigorous control programs in many areas of endemicity, such as southern and Southeast Asia, Africa, and Latin America, have led to a considerable reduction in leprosy burden. Nevertheless, new cases continue to occur in almost all countries where leprosy is endemic, and according to the WHO, high-burden pockets can exist against a low-burden background (70). For 2011, the registered prevalence of leprosy as reported by 105 countries was 219,075, compared to >763,000 in 2001 (70). Currently, there are 18 countries that reported >1,000 new cases. These countries contributed 94% of the new cases detected globally in 2011. With India contributing 58%, Brazil 16%, and Indonesia 9% to all new cases detected, these three countries contributed 83% of the new cases detected globally in 2011. Access to diagnosis and treatment with multidrug therapy (dapsone, rifampin, and clofazimine; available by the WHO free of cost) remain key elements in the strategy to eliminate the disease. With the implementation of the enhanced global strategy from 2011 to 2015, which emphasizes reducing grade 2 disabilities among new cases, it is crucial that leprosy programs focus on underserved populations and remote areas to improve access and coverage, while not neglecting urban centers.

Leprosy is a chronic, granulomatous, and debilitating disease. Its principal manifestations include anesthetic skin lesions and peripheral neuropathy with nerve thickening. Leprosy illustrates a continuous spectrum of disease, from one with very few demonstrable bacilli (tuberculoid leprosy) to a progressive, widespread, and very severe form with massive numbers of organisms due to the absence of cell-mediated immunity (lepromatous leprosy). The majority of leprosy patients show manifestations between these two polar forms and are clinically unstable. Medical complications arise from nerve damage and immune reactions.

Shedding from the nose, rather than from skin lesions, is important for transmission, which results most likely from prolonged and intimate contact with a person with multibacillary disease. The natural reservoir for M. *leprae* is not well established, but naturally occurring infections in the nine-banded armadillo (*Dasypus novemcinctus*) have been documented in the southern United States, with a prevalence of 0 to 10% in those animals (71).

Together with *Mycobacterium tilburgii*, M. *leprae* differs from all other mycobacteria in that it cannot be cultured *in vitro*. By tradition, the diagnosis of leprosy is essentially a clinical one, based on finding one or more signs of disease which are supported by the presence of acid-fast bacilli (AFB) on slit-skin smears or in skin biopsy specimens. Since leprosy bacilli are much less acid and alcohol fast than M. *tuberculosis*, 10% sulfuric acid is preferentially used as a decolorizer in place of an acid-alcohol solution (Fite-Faraco stain [72]). In the case of lepromatous disease, nodules and plaques are the preferred sites for biopsies, which reveal numerous AFB. Conversely, in patients with tuberculoid leprosy, the rims of lesions should be biopsied, where usually only a few or no AFB are found. A number of PCR assays have been established to conclusively detect the organism and to characterize M. *leprae* genotypes (73). A recently developed line probe assay (GenoType LepraeDR; Hain Lifescience, Nehren, Germany) directly detects the organism and its antibiotic resistance in clinical specimens via detection of mutations in the *rpoB* gene (conferring resistance to rifampin), the *gyrA* gene (conferring resistance to ofloxacin), and the *folP1* gene (conferring resistance to dapsone [74]). The complete genome of *M. leprae* is 3,268,203 bp in length, with a G+C content of 57.8%. As in *M. bovis*, the deletions observed in the genome of *M. leprae* have lost >1.1 Mb and accumulated >1,100 pseudogenes during reductive evolution (75).

Naturally acquired murine leprosy has been observed in rats, mice, and cats and is caused by *Mycobacterium lepraemurium*, a species which may be cultured on egg yolk-based media (76). Finally, unique clinicopathologic features of diffuse lepromatous leprosy in humans plus phylogenetic analyses of the 16S rRNA, *rpoB*, *hsp65*, *rrs*, and *sigA* genes led to the discovery of a new mycobacterial species for which *M. lepromatosis* has been proposed (77–79).

Nontuberculous Slowly Growing Mycobacteria Frequently Involved in Human Disease

With the increased awareness of NTM disease in humans and the molecular achievements in the diagnosis of these organisms, a large number of prevalence studies have become available (80). The American Thoracic Society (ATS) and the Infectious Diseases Society of America (IDSA) have revised their comprehensive guidelines for the diagnosis, treatment, and prevention of NTM disease in HIV-positive and HIV-negative individuals (2). Although applied widely, the guidelines have recently been challenged by a retrospective analysis of Scandinavian patients from whose respiratory tracts NTM were isolated (81).

Mycobacterium avium Complex

MAC organisms have been isolated from water, soil, plants, animals, indoor water systems, hot tubs, and pools. They are important pathogens of poultry and swine but were not recognized as a cause of human disease until the 1940s. Generally, MAC organisms are of low pathogenicity. Single positive specimens with low numbers of AFB are not infrequently observed in individuals without apparent disease. This complicates the interpretation of culture results, particularly from specimens of the respiratory tract (2, 3).

Before the advent of AIDS, the most common presentation of MAC infection was pulmonary disease, which manifested several different clinical patterns, i.e., tuberculosis-like infiltrates, nodular bronchiectasis, and solitary nodules, as well as diffuse infiltrates in immunocompromised patients (82). Tuberculosis-like upper lobe fibrocavitary disease due to MAC organisms typically occurs in white men 45 to 60 years of age who are heavy smokers, many of whom abuse alcohol and some of whom have preexisting lung disease. The clinical presentation is similar to that of tuberculosis. In women, nodular bronchiectasis usually occurs in elderly nonsmoking individuals with no predisposing disorders of the lungs or immune system other than associated bronchiectasis ("Lady Windermere syndrome"). These patients usually present with a persistent cough only; the disease tends to have a much slower progression than cavitary disease. Less frequent are thoracic infections in otherwise healthy children (83). In this patient group, MAC organisms are also the leading cause of localized mycobacterial lymphadenitis, which is usually unilateral and involves lymph nodes in the submandibular, submaxillary, or periauricular areas (84). Generalized MAC infections in non-AIDS patients remain extremely rare.

In patients ($n = 385$) with cystic fibrosis, the overall prevalence of NTM was 8%, with the most prominent species being *Mycobacterium abscessus* (39%), MAC organisms (21%), and *M. gordonae* (18%) (85). MAC species affect predominantly adult patients with a mild form of cystic fibrosis, whereas *M. abscessus* affects younger patients with more severe disease and more frequent intravenous treatment (86).

In conjunction with HIV infection, MAC species have become the most common environmental NTM causing disease in humans. Patients with AIDS may present with disseminated or focal infections, mostly when the CD4 count is below 100 cells/mm^3. Bacteremia occurs in nearly all those patients, its magnitude ranging from <1 to 10^2 CFU/ml. The organisms are found predominantly in circulating monocytes. Almost any organ may be involved, with levels of mycobacteria as high as 10^{10} CFU/g of tissue. Focal infections commonly involve the lungs or the gastrointestinal tract and occasionally also the peripheral lymph nodes (87).

MAC organisms are well known for their heterogeneous colony morphology. Glossy, whitish colonies may often occur together with smaller translucent colonies. A third, less frequent, morphology resembles the dry and flat colonies of *M. tuberculosis*. With age, some MAC strains may develop a yellowish pigment.

MAC organisms are a very heterogeneous group of AFB comprised of, by classical definition, the two taxa *M. avium* and *Mycobacterium intracellulare*. Clinically, the former seems to be the more important pathogen in disseminated disease, while the latter is more often seen in respiratory disease. As more-sophisticated molecular tools have become available in laboratories, the taxonomy of the MAC has become increasingly complex. New species within the complex have been proposed and new subspecies discovered. At present, the species *M. avium* consists of four subspecies, namely, subsp. *avium*, subsp. *silvaticum*, subsp. *paratuberculosis*, and subsp. *hominissuis* (88). *M. avium* subsp. *paratuberculosis* is responsible for paratuberculosis (Johne's disease), a chronic inflammation of the gastrointestinal tract that affects mainly lifestock and wild ruminants, while in humans, the association of the organism with Crohn's disease seems to be specific. However, its role in the etiology of the disease remains to be defined (89). Since *M. avium* subsp. *paratuberculosis* is one of the slowest-growing mycobacterial species, primary isolation can take several months, and the medium needs special supplements (88). Direct detection of the organism from veterinary and clinical specimens is possible via PCR methods (90).

There are several important differences at the genetic level which distinguish *M. avium* subsp. *hominissuis* from *M. avium* subsp. *avium* (88). By IS*1245*-based restriction fragment length polymorphism (RFLP) analysis, *M. avium* isolates from birds have been identified as *M. avium* subsp. *avium*. Since highly variable RFLP patterns were found among all *M. avium* isolates which belonged to the *M. avium* subsp. *hominissuis*, a relation to pet birds in the etiology of lymphadenitis could not be established; i.e., the source of infection may be environmental (91, 92).

There are other taxa very closely related to MAC organisms that could not be assigned to one or the other classical MAC species. For instance, Tortoli et al. (93) proposed to elevate a genetic MAC variant (MAC-A) to the species rank as *M. chimaera* sp. nov. (see also reference 94). Likewise, the former MAC-X sequevar gave rise to the species *Mycobacterium colombiense* (95, 96), and the scotochromogenic sequevar MAC-Q is now considered *Mycobacterium vulneris* (97). Finally, Ben Salah et al. (98) found clinical MAC isolates which appear to represent three other new species, *Mycobacterium marseillense*, *Mycobacterium timonense*, and *Mycobacterium bouchedurhonense*. These new species, together with *Mycobacterium arosiense* (99), add to the heterogeneity of MAC members. To date, MAC consists of nine known species, four subspecies of *M. avium* plus certain isolates that are designated "MAC-other" (100).

Mycobacterium genavense

M. genavense is a slow-growing, fastidious organism (101) that was isolated in 1991 from the blood of an AIDS patient in Geneva, Switzerland, and subsequently in the United States and in several European countries (2, 3, 102). It has been associated with enteritis, genital infections, soft tissue infections, and lymphadenitis in HIV-positive and in HIV-negative immunocompromised individuals. *M. genavense* causes up to 12.8% of all NTM infections in AIDS patients. These infections are similar to those caused by MAC organisms, except that stool specimens are more often smear positive in *M. genavense* infections (103). *M. genavense* is also the most common cause of mycobacterial disease in a variety of pet birds, including parrots and parakeets (104).

Mycobacterium haemophilum

M. haemophilum, another fastidious mycobacterial organism, was first isolated in 1978 from a subcutaneous lesion in a patient with Hodgkin's disease (105). Approximately 50% of infections have been in patients with AIDS, with a relatively large number reported from New York City. The other cases have been in other immunosuppressed individuals (105) but also in immunocompetent pediatric patients with localized cervical lymphadenopathy or with a pulmonary nodule. The classical clinical presentation is that of multiple skin nodules in clusters or without a definite pattern, commonly involving the extremities and occasionally associated with abscesses, draining fistulas, cellulitis, endophthalmitis, and osteomyelitis (3, 105).

Mycobacterium kansasii

In the United States and many other countries, *M. kansasii* is second to members of the MAC as a cause of NTM lung disease (2, 3). The organism has been cultured from its major reservoir, tap water, in municipalities around the world where clinical disease occurs. It is common in mine workers in both the United Kingdom and South Africa (106), and its infections differ from those due to MAC organisms in that the response to chemotherapy is much better (2).

Chronic pulmonary disease resembling classical tuberculosis is the most common manifestation of *M. kansasii*. Extrapulmonary infections include cervical lymphadenitis in children, cutaneous and soft tissue infections, tenosynovitis, uveitis, musculoskeletal disease, and, most recently, pericarditis (107). *M. kansasii* rarely disseminates, except in patients with severely impaired cellular immunity (for instance due to organ transplants and AIDS [2]). Connick and Levi (108) reported a unique case of cutaneous *M. kansasii* abscess in an AIDS patient that worsened following initiation of antiretroviral therapy (ART).

M. kansasii is a photochromogenic species. Studies of the base sequences of the 16S rRNA gene suggest that, phylogenetically, it is closely related to the slowly growing, nonpigmented species *M. gastri*. Molecular studies have defined up to seven genotypes of *M. kansasii*, with subtypes I and II being the predominant subspecies responsible for human infection (2, 109).

Mycobacterium malmoense

The species name *M. malmoense* is derived from the city of Malmö in Sweden, where the first strains were isolated from patients in 1977. Disease due to this organism was later found in other European countries, with an increasing incidence in Scandinavia (2). It remains rare in the United States, Canada, and other areas of the world. However, in these countries, *M. malmoense* infection may be more common than suspected, because it may require 8 to 12 weeks to isolate some strains, which is longer than most laboratories hold mycobacterial cultures.

M. malmoense isolates are clinically significant in 70 to 80% of patients. Patients with *M. malmoense* infection are usually adults with chronic difficult-to-treat pulmonary disease (mostly middle-aged men with previously documented pneumoconiosis) or young children with cervical lymphadenitis (2, 3). Septic arthritis of the shoulder (110) and disseminated infections have rarely been reported (111).

Mycobacterium marinum

M. marinum causes cutaneous infections as a result of trauma to the skin and subsequent exposure to contaminated freshwater fish tanks (fish tank granuloma) or salt water. The disease occurs worldwide. In the United States, it is most common in the southern coastal states. The typical presentation is a single papulonodular lesion confined to one extremity, usually involving the elbow, knee, foot, toe, or finger. The lesion appears 2 to 3 weeks after inoculation and, with time, may become verrucous or ulcerated (2, 3). A second type resembles cutaneous sporotrichosis, in which the primary inoculation is followed by spread along the lymphatics. More-severe complications include tenosynovitis, arthritis, bursitis, and osteomyelitis. Disseminated infections, including infections in patients with AIDS or persons under systemic steroid therapy, have been rare (2). The *M. marinum* population appears to be genetically structurized, i.e., according to host, ecosystem, and tissue tropism in humans. Only certain genotypes appear to be present in humans, and not all of them can produce deep infections, which suggests that the immune system may play a filtering role (112).

M. marinum is photochromogenic and requires 28 to 30°C for primary isolation. Ghosh et al. (10) observed spores in old cultures of *M. marinum*, which, upon exposure to fresh medium, germinated into vegetative cells and reappeared again in the stationary phase with endospore formation. With its genome comprising approximately 6,636,827 bp (G+C content, 65.8%), *M. marinum* is genetically very closely related to *M. ulcerans* but also to *M. tuberculosis*, the latter having undergone genome downsizing and external lateral gene transfer to become a specialized pathogen of humans and other mammals (113). Israeli *M. marinum* isolates from humans and fish were compared by direct sequencing of the 16S rRNA and *hsp65* genes, restriction mapping, and amplified fragment length polymorphism analysis. Surprisingly, significant molecular differences separated all clinical isolates from the piscine isolates (114).

Mycobacterium simiae

First isolated in 1965 from rhesus macaques, more than 400 cases have been reported from a few geographic areas, including the southwestern United States and the Caribbean (2, 115). The environmental niche is assumed to be aquatic. The majority of cases relate to HIV-positive patients, involving primarily the lungs and the reticuloendothelial system. In non-HIV patients, pulmonary manifestations are common, but lymphadenopathy, skin lesions, genitourinary tract infections, and uveitis also occur (2). In Israeli cystic fibrosis patients, *M. simiae* was the organism seen most often (40.5%), followed by *M. abscessus* (31%), and MAC organisms (14.3%) (116). Unless tested for pigment production under the influence of light, it may be misidentified morphologically as *M. tuberculosis* by an inexperienced observer.

Mycobacterium szulgai

M. szulgai was first described as a distinct species in 1972 and is rarely recovered from the environment. Therefore, isolation of this organism is almost always considered clinically significant. Patients are mainly middle-aged men presenting with chronic pulmonary disease indistinguishable from tuberculosis (2). The remaining presentations include rare cases of bursitis, cervical adenitis, tenosynovitis, cutaneous infections, and osteomyelitis (2, 3). Cases of *M. szulgai* infection in AIDS patients and disseminated disease in an immunocompetent patient have been reported as well. Although *M. szulgai* is closely related to *M. malmoense* based on 16S rRNA gene sequences, phenotypic distinction between the two species is easy. *M. szulgai* is scotochromogenic at 37°C and photochromogenic at 25°C (117).

Mycobacterium ulcerans

The frequency of *M. ulcerans* infection has long been underestimated due to difficulties in isolating the pathogen. Today, it is the third-most-frequent mycobacterial disease in humans after tuberculosis and leprosy. In Africa, the disease is known as Buruli ulcer and in Australia as Bairnsdale ulcer (2, 3, 118, 119). Cases of Buruli ulcer have also become evident in Peru and Japan. Closely associated with tropical wetlands, *M. ulcerans* most likely proliferates in mud beneath stagnant waters.

All ages and both sexes are affected, among them many children under 15 years. Clinical manifestation typically begins as a painless lump under the skin at the site of previous trauma on the lower extremities. After a few weeks, a shallow ulcer develops at the site of the lump. *M. ulcerans* produces a cytotoxin (mycolactone) with immunomodulating properties that causes necrosis. The type of disease ranges from a localized nodule or ulcer to widespread ulcerative or nonulcerative disease, including osteomyelitis. If untreated, severe limb deformities with contractures and scarring are common. There is growing evidence that *M. ulcerans* also produces disease in wild animals, such as lizards, possums, koala bears, armadillos, rats, mice, and cattle (120).

Failure to cultivate this organism in the past is due to its fastidious, heat-sensitive nature (optimum temperature, 30°C) as well as to an excessively long generation time (up to 36 h). Isolation from clinical specimens is a slow and difficult process due to many factors (118). Molecular techniques which may provide a more rapid result have been developed (121). Comparative genomic analysis has revealed that *M. ulcerans* arose from *M. marinum* by horizontal gene transfer of a virulence plasmid that carries a cluster of genes for mycolactone production, followed by reductive evolution (122, 123).

Mycobacterium xenopi

M. xenopi was first isolated in 1957 from skin lesions on an African toad (*Xenopus laevis*), but it was not recognized as a human pathogen until 1965. In some areas, such as Canada and southeast England, it is second only to MAC organisms as an NTM clinical isolate (2). Together with *M. simiae*, this organism is the most prevalent species of NTM isolated from respiratory specimens in northern Israel (115). Increased isolation of *M. xenopi* from clinical specimens may also be due to improved laboratory techniques. Nosocomial infection and pseudoinfection via water storage tanks in hospitals have also been described.

Most *M. xenopi* infections occur in the lungs, usually occurring in male adult patients with underlying lung disease, such as chronic obstructive pulmonary disease or bronchiectasis. Extrapulmonary infections, such as septic arthritis, spondylitis, and disseminated disease, have also been described in immunocompromised individuals (2). Skin and soft tissue manifestations as well as a recent case report of *M. xenopi* spondylodiscitis in an AIDS patient not only highlight its potential pathogenic role but also point to the uncertainties in therapeutic management. With an optimum growth temperature of 45°C, it seems to frequently occur in hot water systems.

Nontuberculous Slowly Growing Mycobacteria Rarely Recovered or Rarely Causing Human Disease

Several species of slowly growing mycobacteria are frequently recovered but are rarely associated with human disease. Some of the case reports of infections attributable to these mycobacteria, especially from the era before the introduction of molecular laboratory techniques, lack sufficient documentation of identification or disease association. Other species are so rarely recovered that most laboratories will never see them.

Mycobacterium asiaticum

M. asiaticum was not recognized as a distinct species until 1971. This photochromogenic organism has since very infrequently been isolated from patients with respiratory disease in Australia, the United States, and elsewhere (124). Cases of bursitis and tenosynovitis have been described as well.

Mycobacterium celatum

First described in 1993, *M. celatum* has been isolated from diverse geographic areas (throughout the United States, Finland, and Somalia), mostly from respiratory tract specimens but also from stool and blood. In one series, 32% of the patients from whom *M. celatum* was isolated were infected with HIV. *M. celatum* has also been isolated from immunocompetent patients (a child with lymphadenitis and an elderly patient with a fatal pulmonary infection [5]).

M. celatum shares phenotypical characteristics with MAC organisms, *M. malmoense*, and *M. shimoidei* and can thus be identified only with molecular tests. Within the bacterial chromosome, *M. celatum* has two copies of the 16S rRNA gene. Several subtypes (1 to 3) have been identified using 16S rRNA gene sequencing or RFLP analysis of the gene encoding the 65-kDa heat shock protein (*hsp65*).

Mycobacterium gordonae

M. gordonae is the most commonly encountered "nonpathogenic" species in clinical mycobacteriology laboratories. This scotochromogenic species is widely distributed in soil and water. A pseudo-outbreak associated with drinking water in a French hospital underlined the necessity for proper maintenance of water supply equipment (125). Convincing evidence that *M. gordonae* plays a role in disease is difficult to find (2). There are a few reports of peritonitis in patients undergoing continuous ambulatory peritoneal dialysis and in renal transplant patients (126) and a report of disseminated disease in a child with cystic fibrosis (127).

Eckburg et al. (128) have reviewed clinical and chest radiographic findings among persons who are sputum culture positive for M. *gordonae* and concluded that it is a nonpathogenic colonizing organism, even among persons with local or general immune suppression and abnormal chest radiograph findings.

Mycobacterium scrofulaceum

The name of this species was derived from scrofula, a historical term used to describe mycobacterial infections of the cervical lymph glands. Until the 1980s, M. *scrofulaceum* was the most common cause of mycobacterial cervical lymphadenitis in children. Since then, it has been replaced primarily by "MAC" (84). Other types of clinical disease are rare. They include pulmonary disease, conjunctivitis, osteomyelitis, meningitis, granulomatous hepatitis, and disseminated disease (2, 3). M. *scrofulaceum* accounted for 14% of the isolates tested in respiratory specimens collected from South African miners (106) and for approximately 2% of the mycobacterial infections in AIDS patients (2).

Mycobacterium shimoidei

M. *shimoidei* was first described in 1988 in a Japanese patient with chronic cavitary lung disease. Only a few clinical cases have been reported since, mainly in Japan and Finland. It is a thermophilic organism growing well at 45°C.

Mycobacterium terrae Complex

The M. *terrae* complex consisted originally of the three species M. *terrae*, *Mycobacterium nonchromogenicum*, and *Mycobacterium triviale*. Clinical disease due to M. *terrae* is generally limited to tenosynovitis of the hand following local trauma and pulmonary disease (2). M. *nonchromogenicum*, ubiquitous in aquatic environments, has been the cause of bacteremia in an AIDS patient (2). Molecular analyses of several genes (16S RNA gene, *hsp65*, *rpoB*) have unveiled many previously unreported sequevars within these species but also new species belonging to the complex, such as *Mycobacterium arupense*, *Mycobacterium senuense*, and *Mycobacterium kumamotonense* (129). The same study confirmed the inappropriate inclusion of M. *triviale* within the M. *terrae* complex and allowed the recognition of novel species, such as *Mycobacterium engbaekii*, with its pink photochromogenic pigmentation (phylogenetically related to the likewise pink *Mycobacterium hiberniae*), *Mycobacterium heraklionense*, and *Mycobacterium longobardum* (129).

Newer Species of Nontuberculous Mycobacteria

As a consequence of more-sophisticated molecular technologies applied in the clinical mycobacteriology laboratory, the number of newer species is rapidly increasing (Table 1). However, much less is known about these species, and their clinical relevance remains to be elucidated.

SAFETY, TRANSPORT, AND COLLECTION OF SPECIMENS

Laboratory Safety Procedures

Nosocomial transmission of M. *tuberculosis* from patients or specimens is of major concern to health care workers and laboratory personnel. Because of the low infective dose of M. *tuberculosis* for humans (50% infective dose [ID_{50}], <10 AFB [acid-fast bacilli]), specimens from suspected or known cases of tuberculosis must be considered potentially infectious and handled with appropriate precautions (15, 16). Risk assessment and the use of appropriate biosafety practices are of prime importance (14, 15). Control of aerosol production and other forms of mycobacterial contamination requires the use of properly functioning biosafety cabinets (BSCs), centrifuges with safety carriers, and meticulous processing techniques (references 16 and 130; see also chapter 12).

In light of the three-times-higher incidence of tuberculosis in laboratory personnel working with M. *tuberculosis* complex than in those not working with these agents, the low infective dose of tubercle bacilli, and the rapid spread by infecting aerosols, the CDC states in their guidelines on biosafety in microbiological and biomedical laboratories (15) that for laboratory personnel working with M. *tuberculosis* complex, biosafety level 2 (BSL 2) practices are not ideal. BSL 2 practices and procedures, containment equipment, and facilities are suggested only for non-aerosol-producing manipulations of clinical specimens, such as preparation of AFB smears. All aerosol-generating activities must be conducted in a class II BSC. Efforts should be made to apply rigorous BSL 3 practices and containment equipment and to provide BSL 3 facilities for laboratories associated with higher risk, namely, those involved in the manipulation of cultures (identification and susceptibility testing) of M. *tuberculosis* complex organisms. Such practices require that laboratory access be restricted, that directional airflow be used to maintain the laboratory under negative pressure, and that workers wear special laboratory clothing and gloves (15, 130). The stringent biosafety measures may, in the future, invite more laboratories to abandon mycobacterial culture if BSL 3 facilities cannot be afforded.

All respiratory protective devices (respirators) used in the workplace should be certified by the National Institute for Occupational Safety and Health (NIOSH) (131). Respirators that contain a NIOSH-certified N-series (not resistant to solids and liquids that contain oil) filter with a 95% efficiency rating (N-95) are appropriate for use. They meet the recommendations from the CDC for selection of respirators for protection against M. *tuberculosis*, as follows: (i) the unloaded filter must filter particles 0.3 μm in size with an efficiency of 95% at flow rates up to 50 liters/min; (ii) the respirator must be qualitatively or quantitatively fit tested to obtain a face-seal leakage rate of no more than 10%; (iii) the respirator must fit different facial sizes and characteristics, which is attained by making the respirators available in at least three sizes; and (iv) it must be checked for face piece fit by the person wearing the respirator each time it is worn, in accordance with the Occupational Safety and Health Administration (OSHA) standards. Surgical masks are not NIOSH-certified respirators and must not be worn to provide respiratory protection.

The decision when and if to use respiratory protective devices in the laboratory should be based on risk assessment. A respirator program should be implemented by the laboratory and include a written protocol describing when respirator use is necessary and the procedures addressing (i) selection of the appropriate respirator, (ii) how to conduct fit testing, and (iii) training of personnel in the use, fit-checking, and storage of the respirator.

All work involving specimens or cultures, such as making smears, inoculating media, adding reagents, opening centrifuge cups, sonication, and setting up molecular tests, must be performed in a BSC. The handling of all specimens suspected of containing mycobacteria (including specimens processed for other microorganisms), with the exception of centrifugation for concentration purposes, must be done within the BSC. Specimens that are to be taken out of the

TABLE 1 Newer slowly growing NTM species associated with human disease[a]

Mycobacterium species	Clinical manifestation(s) and/or origin of specimen	Remarks	Yr detected (reference[s])
M. arosiense	Osteomyelitis	Scotochromogenic, member of the MAC	2008 (99)
M. arupense	Tenosynovitis	Formerly MCRO 6, genetically related to *M. nonchromogenicum*, belongs to *M. terrae* complex	2006 (226, 227)
M. bohemicum	Lymphadenitis, respiratory tract, skin	Scotochromogenic, HPLC and GLC profiles overlap those of the MAC and *M. scrofulaceum*	1998 (5, 228)
M. bouchedurhonense	Respiratory tract	Member of the MAC	2009 (98)
M. branderi	Ulcerative tenosynovitis, respiratory tract	Photochromogenic, genetically related to *M. celatum*	1995 (5)
M. chimaera	Respiratory tract	Genetic variant MAC-A	2004 (93)
M. colombiense	Respiratory tract	Genetic variant MAC-X, positive with MAC probes, negative with *M. avium* and *M. intracellulare* species-specific probes	2006 (95, 100, 229)
M. conspicuum	Respiratory tract	Pale yellow, cocco-bacillary temperature optimum is 22–31°C, grows at 37°C in liquid media only, genetically related to *M. asiaticum* and *M. gordonae*; a few clinical cases only	1995 (5, 230)
M. doricum	Meningitis, osteomyelitis, soft tissue infections	Scotochromogenic, pathogenic potential unclear	2001 (231, 232)
M. engbaekii	Urinary and respiratory tracts	Pink pigment, phylogenetically related to *M. hiberniae*, member of the *M. terrae* complex	2013 (129)
M. europaeum	Respiratory tract, lymph adenopathy	Scotochromogenic, member of the *M. simiae* complex	2011 (233)
M. florentinum	Lymphadenitis, respiratory tract	Genetically related to *M. triplex* and *M. lentiflavum*	2005 (234)
M. fragae	Respiratory tract	Genetically related to *M. celatum* and *M. kyorinense*	2012 (235)
M. heckeshornense	Cavitary lung disease, lymphadenitis, tenosynovitis	Scotochromogenic, grows poorly on solid media, colonies resemble *M. xenopi*	2000 (5)
M. heidelbergense	Lymphadenitis, pulmonary disease	Nonpigmented, grows poorly on egg-based medium, biochemically indistinguishable from *M. malmoense*, genetically related to *M. simiae*	1997 (5)
M. heraklionense	Respiratory tract	Member of the *M. terrae* complex	2013 (129)
M. interjectum	Lymphadenitis, chronic lung disease, polyangitis	Scotochromogenic as well as nonpigmented, cocco-bacillary, genetically related to *M. simiae*	1993 (5)
M. intermedium	Chronic bronchitis, dermatitis	Photochromogenic and scotochromogenic, cocco-bacillary	1993 (5)
M. kubicae	Respiratory tract	Scotochromogenic, domed colonies, cells are rod shaped and frequently bent, genetically related to *M. simiae*, pathogenic potential questionable	2000 (5)
M. kumamotonense	Respiratory tract	Nonchromogenic	2006 (226)
M. kyorinense	Lymphadenitis, respiratory tract	Genetically related to *M. celatum*	2009 (236, 237)
M. lacus	Bursitis with caseating granulomas after trauma	Large bacilli with prominent beading, nonpigmented, genetically related to *M. malmoense* and *M. marinum*	2002 (5)
M. lentiflavum	Lymphadenitis, cavitary pulmonary disease, chronic pulmonary disease, spondylodiscitis, disseminated disease, skin, pleural effusion	Scotochromogenic, cocco-bacillary, colonies are pale yellow and tiny, genetically related to *M. simiae*	1996 (5)
M. longobardum	Osteomyelitis	Member of the *M. terrae* complex	2013 (129, 238)
M. mantenii	Lymphadenitis, pulmonary disease	Scotochromogenic, genetically related to *M. scrofulaceum*	2009 (239)
M. marseillense	Respiratory tract	Member of the MAC	2009 (98)
M. nebraskense	Pulmonary disease	Photochromogenic, genetically related to the MAC, *M. scrofulaceum*, *M. malmoense*, and *M. kansasii*	2004 (240)
M. palustre	Lymphadenitis	Scotochromogenic, genetically related to the *M. simiae* group and *M. kubicae*, a single case reported	2002 (5)
M. parascrofulaceum	Cervix, respiratory tract	Scotochromogenic, formerly MCRO 33, genetically related to *M. simiae*	2004 (241)
M. paraseoulense	Respiratory tract	Scotochromogenic, 100% identity with 16S rRNA gene of *M. seoulense* (552 bp), but unique sequences in the *hsp65* and *rpoB* genes as well as the ITS region	2010 (242)
M. parmense	Lymphadenitis	Scotochromogenic	2004 (243)
M. riyadhense	Maxillary sinusitis, respiratory tract	Nonchromogenic	2009 (244, 245)

(Continued on next page)

TABLE 1 Newer NTM species associated with human disease[a] *(Continued)*

Mycobacterium species	Clinical manifestation(s) and/or origin of specimen	Remarks	Yr detected (reference[s])
M. saskatchewanense	Sputum, pleural fluid	Scotochromogenic, formerly MCRO 8, genetically related to *M. interjectum*	2004 (246)
M. sherrisii	"Type strains" from various clinical sources, including patients with disseminated disease	Previously considered to be *M. simiae* serotype 2	2004 (247, 248, 249)
M. senuense	Respiratory tract	Nonchromogenic, member of *M. terrae* subclade	2008 (250)
M. seoulense	Respiratory tract	Scotochromogenic, genetically related to *M. nebraskense* and *M. scrofulaceum*	2007 (251)
M. shigaense	Skin nodules	Scotochromogenic, unique *hsp65* and *rpoB* genes as well as unique ITS region, misidentified as *M. simiae* on account of 16S rRNA gene sequence analysis	2012 (252)
M. tilburgii	Dysuria, hematuria, intestinal lesions, pulmonary nodules, disseminated disease	Not cultivable	2009 (253, 254)
M. timonense	Respiratory tract	Member of the MAC	2009 (98)
M. triplex	Lymphadenitis, pulmonary disease, disseminated disease	Nonphotochromogenic, genetically related to *M. genavense* and *M. simiae*	1996 (5)
M. tusciae	Lymphadenitis, respiratory tract (cystic fibrosis)	Scotochromogenic, rough colonies, role as a pathogen unclear	1999 (5)
M. vulneris	Lymphadenitis, wound infection	Scotochromogenic, similar to *M. colombiense* and the MAC-Q 16S–23S ITS sequence	2009 (97)
M. yongonense	Pulmonary disease	Nonchromogenic, *rpoB* gene related to that of *M. intracellulare*	2013 (255, 256)

[a] MAC, *M. avium* complex; MCRO, relates to groups of unidentified mycobacterial organisms whose GenBank accession numbers have been deposited and which were thereafter described as new species; CSF, cerebrospinal fluid; HPLC, high-performance liquid chromatography; GLC, gas-liquid chromatography; 16S–23S ITS, 16S to 23S internal transcribed spacer sequence.

BSC should be covered before transport. All work surfaces, including benchtops and inside the BSC, should be cleaned with an appropriate disinfectant before and after work. Effective disinfectants include Amphyl (Reckitt Benckiser North America, Wayne, NJ) or other phenol-soap mixtures and 0.05% to 0.5% sodium hypochlorite (concentrations vary according to the nature of the contaminated surface). Five percent phenol is no longer recommended as a surface disinfectant due to the documented toxicity of this compound to personnel. Ultraviolet light is a useful adjunct for surface decontamination and may be used to irradiate the work area when it is not in use. Centrifuges should be used with aerosol-free safety carriers to contain debris in the event that tubes break. Use of electric incinerators rather than open flames is recommended. The excess inoculum from inoculating loops, wire, or spades may be removed by dipping the tool into a container of 95% ethanol in washed sand prior to insertion in an incinerator. Disposable inoculating loops are recommended, as are syringes with permanently attached needles if needles are required. An autoclave should be available in an easily accessible area and used to decontaminate infectious waste before its removal to disposal areas.

Personnel should be regularly monitored with either the TST or one of the IGRAs annually and more often if a conversion in the laboratory/institution has been documented. Those with a positive TST or positive IGRAs should be evaluated for active tuberculosis with a chest radiograph and clinical evaluation. Physical examinations should be obtained when necessary. New converters should be referred to the employee health and the infection control departments for epidemiological evaluation. Laboratories should have written protocols describing procedures for handling laboratory accidents. In case of a laboratory accident with possible formation of aerosols, personnel should hold their breath as much as possible, make sure that biological safety cabinets are on and centrifuges are turned off, and then leave the area to get protection as soon as possible, with the door closed for at least 30 min (the amount of time depends on the type of accident and the amount of risk). Using appropriate respiratory protection devices, personnel can return to the accident area to clean the spill. After such an incident, TST/IGRA-negative personnel should be monitored and retested, preferably using the same method longitudinally. Persons who are pregnant or immunocompromised should be discouraged from working in a mycobacteriology laboratory. (See also "Biosafety and Regulatory Issues" in chapter 12.)

Transportation and Transfer of Biological Agents

Mycobacteria are on the list of infectious agents being regulated for shipping and transfer. Stringent regulations on the transportation of biological agents have been enacted in the United States (for instance, *M. tuberculosis* cultures are no longer accepted by the U.S. Postal Service) as well as in other countries to ensure that the public and workers in the transportation chain are protected from exposure to any infectious agents. Protection is achieved through (i) requirements for rigorous packaging that will withstand rough handling and contain all liquid material within the package without leakage to the outside, (ii) appropriate labeling of the package with the biohazard symbol and other labels to alert the workers in the transportation chain to the hazardous contents of the package, (iii) documentation of the hazardous contents of the package should such information be necessary in an emergency situation, and (iv) training of workers in the transportation chain to familiarize them with the hazardous contents in order to be able to respond to emergency situations. Details regarding the transport of specimens within and outside a facility have been well described (130).

The reader is referred to the regulatory documents of his/her respective country. For the United States, further information is available in the following documents:

42 CFR, Part 72, Interstate Transportation of Etiologic Agents (257) (this regulation is in revision to harmonize it with other U.S. and international regulations);

49 CFR, Hazardous Materials Regulations, Parts 171 to 180 (258) (applies to the shipment of infectious substances in commercial transportation within the United States);

39 CFR, Part 20, International Postal Service (259) (regarding international mail);

39 CFR, Part 111, General Information on Postal Service (260) (regarding regulations on transporting infectious substances through the U.S. Postal Service [USPS], which are codified in section 346 of publication 52 [261] and section 135 of the *International Mail Manual* [262]; a copy of the *Domestic Mail Manual* may be obtained from the U.S. Government Printing Office by phone at 1-202-512-1800 or 1-866-512-1800 or from the USPS website [http://bookstore.gpo.gov/ or http://pe.usps.gov);

29 CFR, Part 1910.1030, Bloodborne Pathogens (263) (these regulations provide minimal packaging and labeling for blood and body fluids when transported within a laboratory or outside it; information may be obtained from your local OSHA office or at the OSHA website http://www.osha.gov);

Technical Instructions for the Safe Transport of Dangerous Goods by Air published by the International Civil Aviation Organization (ICAO) (264) (these instructions apply to the shipment of infectious substances by air and are recognized in the United States and by most countries worldwide; a copy of these regulations may be obtained from the ICAO Document Sales Unit by phone at 1-514-954-8022, by e-mail [sales_unit@icao.int], or from the ICAO website http://www.icao.int);

Dangerous Goods Regulations (DGR), published by the International Air Transport Association (IATA) (265) (these regulations are issued by an airline association, are based on the ICAO Technical Instructions, and are followed by most airline carriers; a copy of these regulations is available by phone at 1-800-716-6326 for the United States and Canada, at 41-22-770-2751 for Europe, Africa, and the Middle East, or from http://www.iata.org/index.htm or http://www.who.int/en/; permits must be obtained for importation and exportation of biological and infectious agents by contacting the Centers for Disease Control and Prevention, Atlanta, GA www.cdc.gov).

Collection and Storage of Specimens

General Rules

Many different types of clinical specimens may be collected for mycobacteriological analyses (117, 132). The majority originate from the respiratory tract (sputum, tracheal and bronchial aspirates, bronchoalveolar lavage specimens), but urine, gastric aspirates, tissues, biopsy specimens, and normally sterile body fluids, such as cerebrospinal fluid, pleural aspirates, and pericardial aspirates, are other commonly submitted specimens. Blood and fecal specimens are usually submitted from immunocompromised patients only.

Specimens should always be collected and submitted in sterile, leakproof, disposable, appropriately labeled, laboratory-approved containers without any fixatives. Generally, transport media or preservatives are not necessary owing to the robust nature of mycobacteria. Minute biopsy material (e.g., fine needle aspirates) may be immersed in a small amount of sterile physiological saline. Collection should bypass areas of possible contamination as much as possible, i.e., tap water, since the presence of environmental mycobacteria may result in false-positive smear and/or culture results (133). In general, swabs are not optimal for the recovery of AFB since they provide limited material and the hydrophobicity of the mycobacterial cell envelope often compromises a transfer from swabs onto solid media or into broth media. If transport to the laboratory is delayed more than 1 h, specimens (except blood) should be refrigerated at 4°C. Likewise, upon arrival in the laboratory, specimens should be refrigerated until processed (see chapters 11 and 18 for additional information on specimen collection).

Sputum

Sputum, expectorated or induced, is the principal specimen obtained for the diagnosis of pulmonary tuberculosis. Recent recommendations from the ATS/CDC/IDSA are to collect three sputa at least 8 h apart, one of which should be an early morning specimen, rather than to require collection of sputa over 3 days (130, 134). The number of specimens has, however, been questioned for quite some time. Recent meta-analyses confirmed the low yield of the third specimen in the classical spot-morning-spot strategy (135, 136); the average incremental yield and/or the increase in sensitivity of examining a third specimen ranged between 2% and 5% only. Similarly, Monkongdee et al. (137) and Noeske et al. (138) concluded that a third sputum smear added little to the diagnosis of tuberculosis, even in populations in which the incidence of HIV is high. Pooled sputum specimens are unacceptable for mycobacterial processing because of increased contamination (117). Follow-up cultures should be considered because it is culture (and not a smear) which yields a definite answer as to whether chemotherapy has been effective. Sputum induction increases tuberculosis case detection, and yield was, as demonstrated by a systematic review of the literature (139), generally higher than that with nasopharyngeal aspiration and gastric lavage and compared equally well to that for bronchoalveolar lavage. Children may have difficulties producing sputum. In the age group up to 12 years, a gastric aspirate is usually the specimen of choice for the diagnosis of pulmonary tuberculosis.

Bronchial Aspirates, Bronchoalveolar Lavage Specimens, Fine Needle Aspirates, and Lung Biopsy Specimens

In some patients unable to produce sputum, invasive collection techniques, such as bronchoscopy, may be necessary to diagnose pulmonary tuberculosis or mycobacteriosis. Special care is imperative for cleansing the bronchoscope to avoid cross-contamination with AFB from a preceding patient who underwent bronchoscopy. Also, the bronchoscope should not be in contact with tap water, which may contain environmental mycobacteria. Specimens collected by other invasive techniques, such as fine needle aspiration and open lung biopsy, may be submitted in difficult-to-diagnose cases.

Gastric Lavage Fluids

Aspiration of swallowed sputum from the stomach by gastric lavage may be necessary for infants, young children, and the obtunded. Fasting, early-morning specimens are recommended in order to obtain sputum swallowed during sleep. Samples of 5 to 10 ml, adjusted to neutral pH, should be collected on three consecutive days. If they cannot be processed within 4 h, the laboratory should provide sterile disposable containers with 100 mg of sodium carbonate for collection. Nonneutralized specimens are not acceptable because long-term exposure to acid is detrimental to mycobacteria.

Urine

The first morning specimen should be collected on three consecutive days by the clean catch method (midstream) into a sterile container. The first morning specimen provides the best results because organisms accumulate in the bladder overnight. A minimum of 40 ml of urine is usually required for culture. Twenty-four-hour pooled specimens and small-volume specimens (unless a larger volume is not obtainable) are unacceptable. Catheterization should be used only if a midstream sample cannot be obtained.

Body Fluids

As much body fluid as possible (e.g., cerebrospinal, pleural, peritoneal, pericardial, or synovial [joint] fluid) is aseptically collected by aspiration or during surgical procedures. Bloody specimens may be anticoagulated with sodium polyanethol sulfonate (SPS). Certain body fluids (such as cerebrospinal fluid [CSF] and peritoneal dialysis effluent) may contain very small numbers of mycobacteria. It is advisable to submit larger specimen volumes (e.g., >5 ml for CSF) to increase culture yields and the chance of detecting mycobacterial organisms. Never submit a swab dipped in fluid.

Tissues (Lymph Node, Skin, and Other Biopsy Material), Abscess Contents, Aspirated Pus, and Wounds

Specimens submitted in formalin are unacceptable for smear and culture. As much material as possible should be aspirated aseptically into a sterile container. Tissues must not be immersed in saline or other liquid or wrapped in gauze. For cutaneous ulcers, biopsy material should be collected from the periphery of the lesion. Minute biopsy material may be moistened with a small amount of sterile saline. It is advisable to incubate a second set of cultures at 30°C since one of the organisms with a lower optimum growth temperature (*M. haemophilum*, *M. marinum*, *M. ulcerans*) may be the infectious agent. Swabs are strongly discouraged, unless it is the only specimen available.

Blood

The majority of disseminated mycobacterial infections are due to MAC organisms. Therefore, if such an organism is isolated from blood, it is always associated with clinical disease. If blood has to be transported before inoculation of the medium, SPS, heparin, or citrate may be used as anticoagulants. Blood collected in EDTA or in conventional blood culture bottles and coagulated blood are not acceptable. Direct inoculation of blood onto a solid medium is not recommended either.

For many years, the Isolator system (Wampole Laboratories, Cranburry, NJ) and the radiometric Bactec 13A blood culture bottle (Becton Dickinson Microbiology Systems, Sparks, MD) were the only reliable and recommended systems for mycobacterial blood cultures (140). Since the Bactec 13A medium is no longer available, cultures from blood and bone marrow specimens have to be obtained with alternative media, e.g., the Myco/F Lytic bottles (Becton Dickinson) (140, 141) or the BacT/Alert MB Blood medium (bioMérieux, Marcy-L'Etoile, France) (142).

Stool Specimens

Generally, cultures from feces (>1 g) for mycobacteria are not encouraged, except for patients with AIDS to detect MAC organisms. Past recommendations have been that stool be cultured for mycobacteria only if the direct smear of unprocessed stool is positive for AFB. The sensitivity of the stool smear, however, is only 32 to 34% (143), suggesting that its results should not determine whether a culture for mycobacteria be performed. Screening with smears is, therefore, not an effective way to identify patients at risk for developing disseminated MAC infection.

Inadequate Specimens

Processing of inappropriate clinical specimens for mycobacteria is a waste of both financial and personnel resources. There are quite a few reasons why a specimen should not be accepted (and the clinician should be notified), e.g., (i) too small an amount was submitted; (ii) the "sputum" specimens consist of saliva; (iii) the swabs are dried (a biopsy specimen is preferable); (iv) the sputum or urine is pooled; (v) sample containers are broken; and (vi) the interval between specimen collection and processing is too long (>7 days) (130). Clinical staff must be properly trained to prevent submission of unacceptable specimens.

For additional information regarding the collection and storage of specimens, the reader is referred to a CLSI document (130).

ISOLATION AND STAINING PROCEDURES

Because mycobacteria usually grow slowly and require long incubation times, a variety of microorganisms other than mycobacteria can grow over cultures of specimens obtained from nonsterile sites. Appropriate pretreatment and processing procedures (homogenization, decontamination, concentration), culture media, and conditions of incubation must be selected to facilitate the optimum recovery of mycobacteria (117, 130, 132). In particular, pretreatment of specimens has to be done carefully to eliminate contaminants as much as possible while not seriously affecting the viability of mycobacteria.

Processing of Specimens

Decontamination of a specimen should be attempted only if it is thought to be contaminated. Tissues or body fluids collected aseptically usually do not require pretreatment. If the need to decontaminate a specimen is not clear, the specimen may be refrigerated until routine bacteriologic cultures are checked the next day. It may, however, be easier to initially inoculate a chocolate agar plate to check for sterility overnight before a sample is processed for mycobacteria.

Normally Sterile Specimens

Normally sterile tissue samples may be ground in sterile 0.85% saline or 0.2% bovine albumin and then inoculated directly onto the media. Because body fluids commonly contain only small numbers of mycobacteria, they should be concentrated to maximize the yield of mycobacteria, i.e., centrifuged at ≥3,000 × *g* for 15 min prior to inoculation of the sediment onto media. If the volume of fluid submitted for culture is small and cannot be obtained again, it may be added directly to liquid media.

Contaminated Specimens

The majority of specimens submitted for mycobacterial culture consist of a complex organic matrix contaminated with a variety of organisms. Mucin may trap mycobacterial cells and protect contaminating bacteria from the actions of decontaminating agents. Thus, mycobacteria are recovered optimally from clinical specimens through the use of procedures that reduce or eliminate contaminating bacteria while releasing mycobacteria trapped in mucin and cells. Liquefaction of certain specimens, particularly sputum, is often necessary. Mycobacteria are then concentrated to enhance detection in stained smears and by culture.

Digestion and Decontamination Methods

Sodium hydroxide (NaOH), the most commonly used decontaminant, also serves as a mucolytic agent but must be used cautiously because it is only slightly less harmful to tubercle bacilli than to the contaminating organisms. The stronger the alkali, the higher its temperature during the time that it acts on the specimen, and the longer it is allowed to act, the greater will be the killing action on both contaminants and mycobacteria. Harsh decontamination can kill 20 to 90% of the mycobacteria in a clinical specimen (117, 130, 132). Homogenization should occur by centrifugal swirling, and this swirling should not be vigorous enough to allow material to rise to the cap. After agitation, there should be at least a 15-min delay before opening the tube to allow any fine aerosol droplets formed during the mixing to settle. All such procedures should be carried out in a class II BSC.

Most commonly, a combination liquefaction-decontamination mixture is used. *N*-Acetyl-L-cysteine (NALC), dithiothreitol, and several enzymes effectively liquefy sputum. These agents have no direct inhibitory effect on bacterial cells; however, their use permits treatment with lower concentrations of NaOH, thereby indirectly improving the recovery of mycobacteria. The appropriate concentration of NaOH is dependent on the observed contamination rate for the individual laboratory. If specimens are heavily contaminated, NaOH may be increased (but cannot exceed 5 to 6%). The most widely used digestion-decontamination method is the NALC–2% NaOH method (117, 130, 132; see also the Appendix). Pretreatment of clinical specimens with sodium dodecyl (lauryl) sulfate (SDS)-NaOH is, in contrast, not suitable for the *Mycobacterium* Growth Indicator Tube (MGIT) cultivation method (144), since it results in poor recovery of mycobacteria and a delayed mean time to detection of AFB. When C_{18}-carboxypropylbetaine was used, culture and smear sensitivity significantly improved compared to those of the NALC-NaOH procedure. However, the contamination rate was extremely high (20.8%) (145). Pretreatment of sputum from cystic fibrosis patients with chlorhexidine has yielded twice as many NTM-positive cultures as specimens treated with NALC-NaOH. This option is interesting if one takes into account that recovery of NTM is hampered by the presence of *Pseudomonas aeruginosa* in the respiratory tracts of 80% of these patients (146).

Addition of cetylpyridinium chloride (CPC) (see the Appendix) to specimens mailed from remote collection stations to a central processing station has yielded a good recovery of *M. tuberculosis* without overgrowth by contaminating bacteria.

In addition to the use of CPC, the use of sodium carbonate and sodium borate has been recommended for rural areas, allowing *M. tuberculosis* to remain viable for 5 to 18 days (147). Under field conditions, liquefaction and concentration of sputum for acid-fast staining may also be conducted by treating the specimens with an equal volume of 5% sodium hypochlorite solution (undiluted household bleach) and waiting 15 min before centrifugation (117). Such treated specimens cannot be cultured, however, because the chemical seriously affects the viability of AFB. The major limitation is, therefore, that a second specimen must be collected for culture. The method is very useful, however, for rapid smear preparation and interpretation in laboratories that do not process specimens for culture or that do not have a BSC. Finally, a universal sample processing method utilizing guanidium isothiocyanate did not provide any significant advantage over the standard NALC method in a field study performed in Uganda (148).

Many conventional decontamination protocols used in mycobacteriology interfere with the viability of *M. ulcerans*. Oxalic acid, NaOH, and, to a lesser extent, mild HCl have a detrimental impact on the viability of *M. ulcerans* (149). However, treatment with *mild* hydrochloric acid (final concentration, 0.03 N [149]) or oxalic acid (150) as a decontamination agent provides the best results. A mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) may control secondary contamination.

No one method of digestion and decontamination is ideal for all clinical specimens, all laboratories, and all circumstances. The laboratorian must be aware of the inherent limitations of the various methods used. Even under the best of conditions, all currently available procedures are toxic for mycobacteria to some extent. Thus, the best yield of mycobacteria may be expected to result from the use of the mildest decontamination procedure that sufficiently controls contaminants. Strict adherence to specimen processing is mandatory to ensure the survival of the maximal number of mycobacteria.

Commonly used digestion-decontamination methods are described with step-by-step instructions elsewhere (117, 130, 132) and in the Appendix of this chapter. In general, the specimen is diluted with an equal volume of digestant and allowed to incubate for some time. A neutralizing buffer is added, and the specimen is centrifuged in order to sediment any AFB present. Centrifugation should be carried out at ≥3,000 × *g* for 15 min to get maximum recovery. The sediment is then inoculated onto the appropriate liquid and solid media.

Whatever method is used, care must be taken to prevent laboratory cross-contamination of patient specimens during processing due to aerosols (130, 151). A single false-positive culture for *M. tuberculosis* could easily be the basis of a diagnosis of tuberculosis, with profound consequences for the patient, clinical management, epidemiologic investigations, and public health control measures.

Optimizing Decontamination Procedures

While no contamination or very low rates of contamination indicate that the pretreatment conditions were too harsh and eliminated not only bacteria and fungi but also mycobacteria, a contamination rate exceeding 5% of all digested and decontaminated specimens cultured is generally defined as excessive contamination. A high contamination rate suggests either too weak decontamination or incomplete digestion. One or a combination of several of the following measures may be used to help decrease the contamination rate.

1. Cautiously and slightly increase the strength of the alkali treatment. Be aware that >4% NaOH will affect the viability of tubercle bacilli.
2. Use a selective medium (one containing antibiotics) in addition to a nonselective primary culture medium to inhibit the growth of bacterial and fungal contaminants. Selective 7H11 agar (Mitchison medium), Mycobactosel agar (BBL Microbiology Systems, Cockeysville, MD), or the Gruft modification of L-J medium should be considered. The most useful media for recovering MAC organisms from stool specimens have been Mitchison's selective 7H11 agar and Mycobactosel L-J medium (152).
3. Make sure that specimens are completely digested; partially digested specimens may not be completely decontaminated. Increase the NALC concentration to digest thick, mucoid specimens.
4. Use an alternative digestion-decontamination procedure for problem specimen types. Respiratory secretions from patients with cystic fibrosis, often overgrown with pseudomonads, can successfully be decontaminated with NALC-NaOH followed by the addition of 5% oxalic acid to the concentrated sediment.

To determine the decontaminating capabilities of each new batch of reagents, the laboratory may wish to inoculate blood agar plates with four to six decontaminated sputum specimens in addition to inoculating mycobacterial media. Numbers of contaminants that grow after 48 h of incubation at 35°C should be minimal to none (117, 130, 132).

Acid-Fast Stain Procedures

Smear microscopy, the most rapid and inexpensive way to diagnose tuberculosis, is preferentially done from pretreated and concentrated specimens. In parallel, it is a rapid means of identifying the most contagious patients (153). The predictive value of the AFB smear for M. *tuberculosis* was 92% for expectorated sputum specimens, 71% for induced sputum specimens, and 71% for bronchoalveolar lavage specimens (154). Current recommendations in the United States, Canada, and Europe support a three-smear approach when screening patients suspected of having an M. *tuberculosis* infection for isolation purposes. In a large retrospective study involving 3 smears/patient, 89% of patients were identified as being AFB smear positive by the first specimen, 7.8% were identified by the second smear, and 3.2% by further smears. This suggests that a two-smear approach for discontinuation of respiratory isolation precautions may be safe and reduce hospital expenditures (155).

The common Gram stain is not suitable for mycobacteria. The organisms may be Gram invisible, may appear as clear zones or "ghosts," or may appear as beaded Gram-positive rods, particularly with rapidly growing mycobacteria (156). Special acid-fast staining procedures are necessary to promote the uptake of dyes. Although the exact nature of the acid-fast staining reaction is not completely understood, phenol allows penetration of the stain, which is facilitated by high temperatures, such as those applied, for instance, with Ziehl-Neelsen staining. Mycobacteria are able to form stable complexes with certain arylmethane dyes, such as fuchsin. The cell wall mycolic acid residues retain the primary stain even after exposure to acid-alcohol or strong mineral acids. This resistance to decolorization is required for an organism to be termed acid fast. Certain staining protocols include a counterstain to highlight the stained organisms for easier microscopic recognition. A modified Ziehl-Neelsen stain involving cytospin slides with Triton processing has improved the detection rate of extracellular M. *tuberculosis* in CSF and also identified intracellular M. *tuberculosis* in neutrophils, monocytes, and lymphocytes (157).

Alternatively, mycobacteria can be stained by fluorescent dyes (auramine O alone or in combination with rhodamine B). Unlike with carbol fuchsin, auramine O fluorescence is enhanced upon binding to both DNA and RNA. The advantages of fluorescence microscopy over conventional light microscopy outweigh the higher cost for the microscope for the following reasons: (i) there is a much shorter observation time than with Ziehl-Neelsen staining, (ii) there is no requirement to heat the smear, and (iii) the fluorochrome protocol is no more complex than that for carbol fuchsin staining.

Because acid-fast artifacts may be present in a smear, it is necessary to view the cell morphology carefully. AFB are approximately 1 to 10 µm long and typically slender rods, 0.2 to 0.6 µm wide, that may appear curved or bent. Individual bacilli may display heavily stained areas and areas of alternating stain, producing a beaded appearance. Assessing AFB morphology for presumptive identification of mycobacterial species has to be done with caution and requires the laboratory personnel to have ample training and experience. In liquid medium, M. *tuberculosis* often exhibits serpentine cording, but cords are also seen with some NTM species, such as MAC organisms, M. *gordonae*, M. *chelonae*, M. *abscessus*, and M. *marinum* (158, 159). NTM may appear pleomorphic, i.e., as long filaments or coccoid forms, with uniform staining properties. M. *kansasii* organisms can often be suspected in stained sputum smears by their large size and cross-banding appearance (160). Less than 10% of cells of rapidly growing mycobacteria may be acid fast and may not stain with the fluorochrome stain (161). If the presence of a rapid grower is suspected and the results of acid-fast stains, in particular fluorochrome stains, are negative, it may be worthwhile to stain the smear with carbol fuchsin and to use a weaker decolorizing process. Organisms that are truly acid fast are difficult to overdecolorize. The laboratory must be aware that there are nonmycobacterial organisms with various degrees of acid fastness, such as *Rhodococcus* species, *Nocardia* species, and *Legionella micdadei*, as well as the cysts of *Cryptosporidium*, *Isospora*, *Cyclospora*, and *Microsporidium* spores. Kinyoun's cold carbol fuchsin method appears inferior to both the Ziehl-Neelsen and fluorochrome methods (162).

Each slide made from a clinical specimen should be thoroughly examined for the presence of AFB. When a carbol fuchsin-stained smear is read, a minimum of 300 fields should be examined (magnification, ×1,000) before the smear is reported as negative (117, 130, 132). The fluorochrome stain is read at a lower power (×250) than the carbol fuchsin stain; therefore, more material can be examined in a given period. At the lower magnification, a minimum of 30 fields of view should be examined. This requires as little as 90 s. This ease of detection of AFB with the fluorochrome stain makes it the preferred staining method for clinical specimens, although an inexperienced observer may misinterpret fluorescent debris as bacilli. Information about specific staining procedures is given in chapter 19 as well as references 117, 130, and 132.

Low-cost, robust light-emitting diode (LED) microscopes have facilitated the expansion of fluorescence microsopy into low-resource settings (163). This prompted the WHO to recommend in 2010 that LED fluorescence microscopy should replace Ziehl-Neelsen stain microscopy for tuberculosis diagnosis. Fluorescent LED requires a shorter smear reading time (1.5 min per slide) than light microscopy (4 min for Ziehl-Neelsen-stained slides) ($P < 0.001$) (164). In a multicountry cross-sectional evaluation, LED fluorescence microscopy and Ziehl-Neelsen smears were compared (three slides for LED fluorescence microscopy versus three Ziehl-Neelsen slides per patient). Sensitivity was 77% for LED fluorescence microscopy and 70% for Ziehl-Neelsen-stained smears, whereas specificity attained 88.1% and 96.5% for the two methods ($P < 0.001$) (165). As documented by a multicenter study analyzing 11,276 slides, the smear positivity rate for LED fluorescence microscopy was 11.2% compared to 8.6% for Ziehl-Neelsen staining (166). Independently of the fluorescence microscope used, it should be noted that auramine-rhodamine-stained slides fade very quickly, and this occurs irrespective of the environment for storage (167).

All smears in which no AFB are seen should be reported as negative. Conversely, when AFB are detected on a smear, the smear should be reported as AFB positive and the staining method should be specified. It is best to confirm positive smears by having them reviewed by another experienced reader. Ideally, all positive fluorochrome-stained smears should be confirmed by a carbol fuchsin-based staining method, e.g., Ziehl-Neelsen staining, and slides stored for future reference (117, 130, 132). The widely accepted practice of confirming positive fluorochrome stains may be challenged in the future. Murray et al. (168) have demonstrated that stain applied to a liquefied (dithiothreitol), concentrated sample and examined before the decontamination process (NaOH) was the most effective method for the detection of AFB.

Information about the quantity of AFB observed on the smear should be provided. The recommended interpretations and reporting of smear results are given in Table 2 (see also reference 130). If only one or two organisms are seen on an entire smear, this should be noted but not reported. Confirmation of this finding should be attempted by preparation of additional smears from the same specimen or, if possible, smears prepared from a new specimen. Observations made with the fluorochrome smears should be converted to a format that equates these observations with those made with a 100× oil immersion objective.

The reliability of smear microscopy is highly dependent not only on the experience of the laboratory personnel but also on the number of AFB present in the specimen. While 10^6 AFB/ml of specimen usually result in a positive smear, only 60% of the smears are positive if 10^4 AFB/ml are present (169). The overall sensitivity of the smear has been reported to range from 22 to 80% (153). An important factor influencing sensitivity is the minimum amount of sputum submitted to the laboratory. In a long-term study, the sensitivity of a concentrated smear from >5 ml of sputum was significantly greater than the sensitivity of a smear processed regardless of volume (170). Other factors influencing smear sensitivity include the type of specimens examined, staining techniques, the experience of the reader, the patient population being evaluated, and whether the smear has been done with or without pretreatment (indirect versus direct smear). Respiratory specimens yield the highest smear positivity rate (153). In practice, the fluorochrome stain is more sensitive than the carbol fuchsin stain, even when read at lower magnification, probably because the fluorochrome-stained smears are easier to read.

The specificity of the smear for the detection of mycobacteria is very high. Prolonged or very harsh specimen decontamination and short incubation of cultures may account for smear-positive but culture-negative results. Patients with pulmonary tuberculosis may have positive smears with negative cultures (for 2 to 10 weeks on average) during a course of appropriate treatment.

Cytocentrifugation of sputum has resulted in controversial results concerning the sensitivity of smear microscopy (171, 172). The concentration of sputum by centrifugation after liquefaction with 5% sodium hypochlorite is a possible means of increasing smear sensitivity, in particular in developing countries.

The diagnostic yield of acid-fast stains of body fluids is less than for respiratory specimens because the number of mycobacteria is usually lower. A variety of techniques have been used to concentrate mycobacteria from CSF and other body fluids, but comparative data are lacking. Centrifugation is not an effective way to concentrate mycobacteria in body fluids since mycobacteria have a buoyant density of approximately 1; therefore, many organisms remain in the supernatant.

With each new batch of staining reagents, good laboratory practice includes the preparation of a positive and a negative smear for internal quality assessment (130). Smears containing *M. tuberculosis* or an NTM (positive control) and a Gram-positive organism, preferentially a *Nocardia* sp. strain which is not totally acid fast (negative control), may be prepared in advance. Cross-contamination of slides during the staining process and the use of water contaminated with NTM during staining procedures are potential sources of false-positive results (133). Staining jars or dishes should not be used. Transfer of AFB in the oil used for microscopy may also occur. Troubleshooting protocols to prevent false-positive and false-negative smear results are available (130).

Culture

Culture is more effective than smearing since it can detect as few as 10^1 to 10^2 viable organisms/ml specimen. Media

TABLE 2 Acid-fast smear evaluation and reporting[a]

Report	No. of AFB seen by the following staining method and at the indicated magnification, no. of microscope fields (no. of sweeps)[b]:		
	Fuchsin stain at ×1,000	Fluorochrome stain at:	
		×250	×450
No AFB seen	0	0	0
Indeterminate; test was repeated	1–2, 300 (3)	1–2, 30 (1)	1–2, 70 (1.5)
+	1–9, 100 (1)	1–9, 10	2–18, 50 (1)
++	1–9, 10	1–9	4–36, 10
+++	1–9	10–90	4–36
++++	>9	>90	>36

[a] Adapted from reference 117.

[b] In all cases, one full sweep refers to scanning the full length (2 cm) of a smear 1 cm wide by 2 cm long.

available for the recovery of mycobacteria include nonselective and selective ones (117, 130, 132), the latter containing one or more antibiotics to prevent overgrowth by contaminating bacteria or fungi. Broth media are preferred for a rapid initial isolation of mycobacteria.

Solid Media

Egg-Based Media

Egg-based media contain whole eggs or egg yolk, potato flour, salts, and glycerol and are solidified by inspissation. These media have a good buffer capacity, a long shelf life (several months, when refrigerated), and support good growth of most mycobacteria. Also, materials in the inoculum or medium toxic to mycobacteria are neutralized. Disadvantages of these media include variations from batch to batch depending on the quality of the eggs used, difficulties in discerning colonies from debris, and the inability to achieve accurate and consistent drug concentrations for susceptibility testing. When egg-based media become contaminated, they may liquefy.

Of the egg-based media, L-J medium is most commonly used in clinical laboratories. The concentration of malachite green in standard acid-fast media (e.g., L-J) was selected to maximize the growth of mycobacteria while inhibiting other microorganisms. In general, it recovers M. *tuberculosis* well but is not as reliable for the recovery of other species. M. *bovis*, for instance, grows less well on L-J medium, but growth is stimulated if glycerol is replaced by pyruvate. In contrast to most members of the M. *tuberculosis* complex, M. *bovis* is able to grow in a reduced-O_2 atmosphere. M. *genavense* fails to grow on L-J medium. Good recovery of M. *ulcerans* is obtained on L-J medium with glycerol (150, 173). Petragnani medium contains about twice as much malachite green as does L-J medium and is most commonly used for recovery of mycobacteria from heavily contaminated specimens. American Trudeau Society medium contains a lower concentration of malachite green than L-J medium and is, therefore, more easily overgrown by contaminants, but the growth of mycobacteria is less inhibited, resulting in earlier growth of larger colonies.

Agar-Based Media

Compared to egg-containing media, agar-based media are chemically better defined. They do not readily support the growth of contaminants; however, the plates are expensive to prepare and their shelf life is relatively short (1 month in the refrigerator). Care should be exercised in the preparation, incubation, and storage of the media, because excessive heat or light exposure may result in deterioration and in the release of formaldehyde, which is toxic to mycobacteria. Agar-based media are transparent and provide a ready means of detecting the early growth of microscopic colonies easily distinguished from inoculum debris. Colonies may be observed in 10 to 12 days, in contrast to 18 to 24 days with egg-based media. Microscopic examination can be performed by simply turning over the plate and examining it by focusing on the agar surface through the bottom of the plate at a ×10 to ×100 magnification. This may provide both earlier detection of growth than unaided visual examination and presumptive identification of the species of mycobacteria present. The use of thinly poured 7H11 agar plates (10 by 90 mm; Remel, Lenexa, KS) facilitates this process, as microcolonies are visible after 11 days (174). Agar-based media can also be used for susceptibility testing (175). In the quest for more-rapid and affordable growth techniques, microcolony-based culture methods have been developed. However, as shown by a recent systematic review, there is sufficient evidence neither for the feasibility and costs of implementation of these tests for growth detection and drug susceptibility testing nor for the impact on patient outcomes (176).

Middlebrook medium contains 2% glycerol, which enhances the growth of MAC organisms. Nonantibiotic supplements may be helpful for the recovery of other mycobacteria and in special situations. Addition of 0.2% pyruvic acid is recommended if M. *bovis* is suspected, and 0.25% L-asparagine or 0.1% potassium aspartate added to 7H10 agar maximizes the production of niacin. Addition of 0.1% enzymatic hydrolysate of casein to the Middlebrook 7H11 formulation (the only difference from 7H10) improves the recovery of isoniazid-resistant strains of M. *tuberculosis*. M. *genavense* fails to grow on 7H11 agar as well. However, Middlebrook 7H11 agar supplemented with mycobactin J (Allied Monitor, Fayette, MO) supports the growth of M. *genavense*, as do microaerophilic conditions, the radiometric Bactec 7H12 pyrazinamide test medium (101), or addition of blood and charcoal to acidified Middlebrook agar (177).

Selective Media

The addition of antimicrobial agents may be helpful in eliminating the growth of contaminating organisms. If a selective medium is used for a particular specimen, it should not be used alone but in conjunction with a nonselective agar- or egg-based medium. Egg-based selective media include L-J Gruft medium with penicillin and nalidixic acid and Mycobactosel L-J medium with cycloheximide, lincomycin, and nalidixic acid. Mitchison selective 7H11 medium and its modifications contain carbenicillin (especially useful for inhibiting pseudomonads), polymyxin B, trimethoprim lactate, and amphotericin B.

Heme-Containing Medium for the Growth of M. *haemophilum*

M. *haemophilum* will grow on egg- or agar-based media only if they are supplemented with hemin, hemoglobin, or ferric ammonium citrate (105). Thus, clinical specimens should be inoculated either on chocolate agar or on media with supplements (e.g., Middlebrook 7H10 agar with hemolyzed sheep erythrocytes, hemin, or a factor X disk, or on L-J medium containing 1% ferric ammonium citrate) to enhance the recovery of this organism. Broth media should be similarly supplemented. M. *haemophilum* can be isolated from the MGIT (178), whereas the MB/BacT Alert 3D system is less optimal for this organism (179). As a whole, M. *haemophilum* infections may be underrecognized because of its predilection for a low incubation temperature (30°C) and its unique nutritional requirements.

Liquid Media

Broth media may be used for both primary isolation and subculturing of mycobacteria. Cultures based on liquid media yield significantly more rapid results than solid-medium-based cultures. Also, isolation rates for mycobacteria are higher. Middlebrook 7H9 and Dubos Tween-albumin broths are commonly used for subculturing stock strains of mycobacteria and preparing the inoculum for drug susceptibility tests and other *in vitro* tests. 7H9 broth is used as the basal medium for several biochemical tests. Tween 80 can be added to liquid media and acts as a surfactant that allows the dispersal of clumps of mycobacteria, resulting in a more homogeneous growth.

At present, the commercially available culture systems marketed for the isolation of mycobacteria range from simple tubes, such as the MGIT (Becton, Dickinson Microbiology Systems), to the fully automated systems (e.g., Bactec MGIT 960 [Becton, Dickinson], ESP culture system II and VersaTREK culture system II [Trek Diagnostic Systems, Cleveland, OH], and MB/BacT Alert 3D system [bioMérieux]). These nonradiometric techniques have been tested in various studies against solid media and the radiometric Bactec 460 technique, which has long been considered the gold standard but has been discontinued by the manufacturer.

Mycobacterium Growth Indicator Tube (MGIT)

The MGIT (Becton Dickinson Microbiology Systems) contains a modified Middlebrook 7H9 broth in conjunction with a fluorescence quenching-based oxygen sensor (silicon rubber impregnated with a ruthenium pentahydrate) to detect growth of mycobacteria. The large amount of oxygen initially present in the medium quenches the fluorescence of the sensor. Growth of mycobacteria or other microorganisms in the broth depletes the oxygen, and the indicator fluoresces brightly when the tubes are illuminated with UV light at 365 nm. For the manual version, a Wood's lamp or a transilluminator can be used as the UV light source, while in the automated Bactec MGIT 960 system (see below), tubes are continuously monitored by the instrument. Prior to use, the 7H9 broth is supplemented with oleic acid-albumin-dextrose to promote the growth of mycobacteria and with polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) to suppress the growth of contaminants.

Overall, the sensitivity and time to growth detection of the MGIT system are similar to those of the Bactec 460TB system and have been superior to solid media in clinical evaluations (180). However, contamination rates for the MGIT system are slightly higher than for the Bactec 460TB system, probably owing to the enrichments added to the MGIT broth that enhance the growth of both mycobacteria and nonmycobacterial organisms.

The principal advantages of the manual MGIT system over the Bactec 460TB system include reduced opportunity for cross-contamination of cultures, no need for needle inoculation, no radioisotopes, and no need for special instrumentation other than a UV light source. Its limitations include higher contamination rates, masking of fluorescence by blood or grossly bloody specimens, and lack of compatibility with some methods of digestion and decontamination of specimens (144).

Automated, Continuously Monitoring Systems

Several automated, continuously monitoring systems have been developed for the growth and detection of mycobacteria, e.g., the Bactec MGIT 960 (Becton Dickinson), the VersaTREK culture system (VersaTREK Diagnostic Systems), and the MB/BacT Alert 3D (bioMérieux). All have in common that they are no longer based on the use of radioisotopes. The MGIT system uses a fluorescence quenching-based oxygen sensor (ruthenium pentahydrate) to detect growth. The technology used in the VersaTREK culture system is based on the detection in a sealed bottle of pressure changes in the headspace above the broth medium that result from gas production or consumption due to the growth of microorganisms. The MB/BacT Alert 3D system employs a colorimetric carbon dioxide sensor in each bottle to detect the growth of mycobacteria. Each of the systems includes a broth similar to 7H9 broth supplemented with a variety of growth factors and antimicrobial agents. All three systems are FDA cleared for the isolation of mycobacteria; the VersaTREK culture system and the Bactec MGIT 960 system are also cleared for drug susceptibility testing of *M. tuberculosis* complex organisms to various extents, though.

These systems have similar levels of performance and operational characteristics. In clinical evaluations, recovery rates were similar to those of the Bactec 460TB system and superior to those of conventional solid media (Bactec MGIT 960 [181]; VersaTREK culture system [formerly the ESP culture system II] [182]; MB/BacT Alert 3D [179, 181]). In a meta-analysis of 10 published studies encompassing 1,381 strains from 14,745 clinical specimens, the Bactec MGIT 960 and Bactec 460TB systems revealed sensitivities/specificities in detecting mycobacteria of 81.5%/99.6% and 85.8%/99.9%, respectively. Combined with solid media, the sensitivities of the two systems increased to 87.7 and 89.7%, respectively (183). For some systems, time to detection of mycobacteria is similar to those of the old radiometric Bactec 460TB technique. Parrish et al. (184) demonstrated for the Bactec MGIT 960 system a shorter time to detection (13.5 versus 25.2 days) and a greater sensitivity (100% versus 66.6%) for the recovery of members of the *M. tuberculosis* complex than the MB BacT/Alert system. For blood specimens, the BacT/Alert MB system, the manual Myco/F Lytic medium, and the Isolator 10 lysis-centrifugation system detected *M. tuberculosis* in 16.4 days, 20.0 days, and 23.8 days, respectively (185).

Throughout the literature, contamination rates are reported to have been higher with these new systems than with the Bactec 460TB system. However, all of them share advantages over the radiometric broth system in having no potential for cross-contamination by the instrument, being less labor-intensive, having continuous monitoring, using no radioisotopes, addressing safety more appropriately, and offering electronic data management. Since these systems monitor continuously, bottles are incubated in the instruments for their entire life in the laboratory. As a consequence, these systems are both instrument and space intensive. Some automated systems also lack the versatility of the Bactec 460TB system in that inoculation of blood is not possible, and therefore, additional instruments, e.g., the Bactec 9050 apparatus with Bactec Myco/F Lytic medium, have to be used for this purpose; alternatively, the bottles can be put into the standard Bactec 9240 or FX instrument. The same holds for the incubation of cultures harboring mycobacteria with a lower optimum temperature, such as *M. chelonae*, *M. haemophilum*, *M. marinum*, or *M. ulcerans*. Susceptibility testing applications for the primary and second-line antituberculosis drugs are available for the Bactec MGIT 960 system and VersaTREK system (formerly ESP culture system II [175]; see chapter 76).

Whenever one works with automated continuously monitoring systems, mycobacterial growth can go undetected. As demonstrated by Pena et al. (186), 1,323 (13%) of 10,263 tested specimens were culture positive for mycobacteria, but approximtely 1% of instrument-negative MGIT cultures contained mycobacterial growth when tubes had been inspected visually for clumps before the tubes were discarded after 8 weeks.

Medium Selection

Medium selection for the isolation of mycobacteria and culture reading schedules are usually based on personal preferences and/or laboratory tradition. Both should be optimized for the most rapid detection of positive cultures and identification of mycobacterial isolates. The variety of media and methods available today is sufficient to permit laboratories to develop an algorithm that is optimal for their patient population and administrative needs. Workload, financial resources, and, in particular, the limited amounts of processed sediments are, however, restraining factors in working with too many different types of media. Thus, cultivation of mycobacteria always involves a compromise.

Today, it is generally accepted that the use of a liquid medium in combination with at least one solid medium is essential for good laboratory practice in the isolation of mycobacteria (130). Addition of a solid medium is advantageous for those strains which occasionally do not grow in liquid medium, aids in the detection of mixed mycobacterial infections, and can serve as a backup for broth, which has a higher contamination rate. All positive cultures, even if identified directly from the broth, must be subcultured to solid media to detect mixed cultures and to correlate direct identification results with colony morphology. The nonradiometric growth systems cannot serve as stand-alone culture systems for mycobacteria for the reasons stated above.

Detection of colonies on solid medium certainly offers several advantages over detection of growth in broth, because colonial morphology can provide clues to identification and facilitate the selection of confirmatory tests. However, smears from broth-based systems can sometimes provide microscopic clues, such as cord formation (see above), although the reliability for presumptive identification of M. *tuberculosis* should be applied with caution since the phenomenon is also observed with some NTM species (158, 159, 160).

Incubation

Temperature

The optimum incubation temperature for most cultures is 35 to 37°C. Exceptions to this include cultures obtained from skin and soft tissue suspected of containing M. *marinum*, M. *ulcerans*, M. *chelonae*, or M. *haemophilum*, which have lower optimum temperatures. For such specimens, a second set of media has to be inoculated and incubated at 25 to 33°C. Lower temperatures increase detection time. The newer automated liquid-medium-based culture systems do not offer the possibility of incubating at temperatures lower than 36 ± 1°C.

Atmosphere

Five percent to 10% CO_2 in air stimulates the growth of mycobacteria in primary isolation cultures using conventional media. Middlebrook agar requires a CO_2 atmosphere to ensure growth, while it is necessary to incubate egg media under CO_2 for only the first 7 to 10 days after inoculation, i.e., during the log phase of growth. Subsequently, L-J cultures can be removed to ambient-air incubators if space is limited. In the absence of CO_2 incubators, plates may be incubated in commercially available bags with CO_2-generating tablets. Candle extinction jars are unacceptable for use in mycobacteriology laboratories because the oxygen tension is less than that required for the growth of mycobacteria. Broth systems usually do not require incubation at increased CO_2 concentrations.

Time

Mycobacterial cultures on solid and in liquid media are generally held for 6 to 8 weeks before being discarded as negative. Specimens with positive smears that are culture negative should be held for an additional 4 weeks. The same should be done for culture-negative specimens that were positive for mycobacteria by one of the nucleic acid-based amplification assays or for cases for which there is a persisting suspicion of tuberculosis. Plates should be incubated with the medium side down until the entire inoculum has been absorbed. Once this has happened, media should be incubated inverted in CO_2-permeable polyethylene bags or sealed with CO_2-permeable shrink-seal bands to prevent them from drying up during the incubation period. Tubed media should be incubated in a slanted position with the screw caps loose for at least a week until the inoculum has been absorbed; they can then be incubated upright if space is at a premium. Caps on the tubes should be tightened at 2 to 3 weeks to prevent desiccation of the media. Specimens from skin lesions should be incubated for 8 to 12 weeks if M. *ulcerans* is suspected.

Reading Schedule

Since many mycobacteria are slowly growing organisms, cultures can be examined less frequently than routine bacteriologic cultures. All solid media should be examined within 3 to 5 days after inoculation to permit early detection of rapidly growing mycobacteria and to enable prompt removal of contaminated cultures. Young cultures (up to 4 weeks of age) should be examined twice a week, whereas older cultures could be examined at weekly intervals. Use of a hand lens for opaque media and a microscope for agar media will facilitate early detection of microcolonies.

The manual MGIT may be inspected for growth daily for the first 1 to 2 weeks. Afterwards, it should be inspected twice weekly or weekly for growth.

When using one of the nonradiometric continuously monitoring systems, laboratory personnel are automatically alerted by the instrument if a specimen turns positive. Irrespective of the system used, the acid fastness of the organism has to be confirmed by smear staining. Also, it is highly advisable to subculture the broth on a sheep blood or chocolate agar plate to rule out contaminants. Once growth of AFB is detected, susceptibility testing can be performed, always according to the instructions specified by the manufacturers.

Reporting

Traditionally, solid culture media are being kept for up to 8 weeks. Even though liquid media detect mycobacteria much earlier, most incubation protocols still require a minimum of 6 weeks (130). A large multicenter evaluation involving >1,500 positive cultures (among them, 466 M. *tuberculosis* isolates) stressed that laboratories using MGIT may issue reports of no growth of M. *tuberculosis* as early as at 4 weeks (187). This was confirmed by Pfyffer and Wittwer (188); 58.3% of all mycobacteria were detected within 14 days, 37.5% were detected within 21 days, and 4.2% were detected within 28 days if a combination of solid media (L-J/Middlebrook) and liquid medium (Bactec MGIT 960) were utilized. In this context, 50% of species in the M. *tuberculosis* complex were detected within 14 days, and 50% were detected within 21 days. As much as 70% of NTM appeared within 14 days, 20% were detected within 21 days, and 10% were detected within 28 days.

Storage of Positive Cultures

Positive cultures may be kept at room temperature for several weeks. If subcultured, they may be saved at room temperature for several months. Solid cultures have to be sealed to avoid dehydration of the medium. The CLSI (130) recommends that cultures that may be needed for possible follow-up in the future be frozen at −70°C (a minimum of 1 year is recommended) or cryopreserved as suggested by Shu et al. (189).

IMMUNODIAGNOSTIC TESTS FOR TUBERCULOSIS

Historically, the first immunodiagnostic test was the TST. The shortcomings of this test are well known and include the inability to distinguish active tuberculosis disease from past sensitization by BCG, unknown predictive values, and cross-reaction with NTM.

Over time, much effort has been devoted to the development of serological tests for the diagnosis of tuberculosis, but no test has found widespread clinical use. The sensitivity and specificity of serological tests with crude antigen preparations are too low for clinical application.

In contrast, the two commercially available whole-blood IGRAs have become a more promising, though not perfect, tool to detect tuberculosis infection, in particular, if TST remains equivocal. Both tests are FDA approved but not for testing individuals under the age of 17, individuals with immunedepression due to AIDS, persons taking immunosuppressive drugs (e.g., anti-tumor necrosis factor or corticosteroids), or transplant recipients. Two test systems, the T-Spot.TB (Oxford Immunotec, Oxford/United Kingdom) and the QuantiFERON gold in-tube (QFNG-IT; Cellestis, Victoria/ Australia) tests, are not affected by BCG vaccination, do not cross-react with the majority of NTM, and are less prone to variability and subjectivity associated with placing and reading of the TST. Also, individuals to be tested have to see a doctor or health care personnel only once. As shown by a recent systematic review of studies performed in high-, middle-, and low-income countries, the higher per-test cost of IGRAs may be compensated for by low postscreening costs (medical attention, chest X rays, chemoprevention), given the higher specificity of the IGRAs than that of conventional TST (190). In particular, there was strong evidence of the cost-effectiveness of the IGRAs in screening risk groups, such as immigrants from high-incidence countries, close contacts, and health care workers. Disagreement between QFNG-IT and TST results as well as the high reversion rate with QFNG-IT, however, raised concerns about the effectiveness of QFNG-IT as a sole screening test for health care workers (191).

Although both tests measure T cell IFN-γ responses to two or three *M. tuberculosis*-specific antigens (ESAT-6, CFP-10, TB 7.7) over a 16- to 24-h incubation period, they are based on different technologies. The T-SPOT.TB assay is based on the enzyme-linked immunosorbent spot (ELISPOT) methodology and requires the isolation and incubation of peripheral blood mononuclear cells (PBMC) and the standardization of 250,000 PBMC in each of its test wells. The assay requires, overall, two working days and may be more laborious than the QFNG-IT. Nevertheless, the use of a standardized number of washed PBMC may represent another advantage. In contrast, the QFNG-IT assay has technical advantages over the T-SPOT.TB assay, since the stimulation of a T cell IFN-γ response in whole blood is performed in tubes precoated with the *M. tuberculosis* antigens. Also, the enzyme-linked immunosorbent assay (ELISA) is simple to perform and requires one working day. Since background noise may occur, a "nil" control is required to adjust for this background, as well as for heterophile antibody effects and nonspecific IFN-γ in blood samples. The reproducibility of QFNG-IT assay results in duplicate tests is excellent (192).

It is important to stress that neither of these new tests distinguishes between latent and active infection. To date, there are several guidelines available, among them the one for the United States (193). A large number of publications focus on the performance characteristics of each test compared to TST. As a whole, IGRAs are better correlated with the intensity of tuberculosis exposure than the TST (194).

There are several systematic reviews and meta-analyses which document the performance characteristics of the two types of IGRAs in both immunocompetent and immunocompromized patients. In a head-to-head comparison of the QFNG-IT and the T-SPOT.TB assays, Pai et al. (195) concluded that the QFNG-IT test has a specificity of 99% (T-SPOT.TB test, 96%) among non-BCG-vaccinated participants and a specificity of 96% (T-SPOT.TB test, 93%) among BCG-vaccinated participants, while Higuchi et al. (196) reported equal specificities for the two assays. For latent tuberculosis, specificity varied between 98 and 100%. In immunocompetent adults, the negative predictive values for progression to tuberculosis within 2 years were 97.8% for the T-SPOT.TB assay and 99.8% for the QFNG-IT assay (197). In contrast, it is well understood that IGRAs have limited accuracy in diagnosing active tuberculosis, as evidenced by a meta-analysis of 884 studies (198). This also holds true for HIV-positive individuals (199). Also, QFNG-IT results do not offer much value for treatment monitoring of tuberculosis disease (200).

Diel et al. (201) compared both IGRAs in TST-positive persons recently exposed to pulmonary tuberculosis cases. In that study, factors independently influencing the risk of *M. tuberculosis* infection and their interactions with each other were evaluated by multivariate analysis. There were five variables which significantly predicted a positive IGRA result, i.e., age, AFB positivity of the source case, cough, cumulative exposure time, and foreign origin of the patient. There was excellent agreement between the two assays (93.9%, kappa = 0.85), with QFNG-IT finding 30.2% of contacts positive and T-SPOT.TB finding 28.7% of them. Again, the IGRAs were more accurate indicators of the presence of latent tuberculosis than the TST (201).

In HIV-positive asymptomatic individuals (n = 286), both the QFNG-IT and T-SPOT.TB assays were more sensitive than the TST (20.0% and 25.2%, respectively, compared with 12.8% for the TST) but seemed, as a whole, to be less sensitive than in immunocompetent patients (202). The value of TST and serial QFNG-IT tests in patients with rheumatic diseases during long-term treatment with tumor necrosis factor blockers has not been defined to date (203).

The performance of IGRAs in children is less understood. Without having the inconveniences and complications associated with the TST, IGRAs are acceptable substitutes for it. The sensitivity and specificity of IGRAs are, however, not significantly higher than the values observed for the TST (204). In children with latent tuberculosis, the agreement between the QFNG-IT and T-SPOT.TB assays was very good (92%), with moderate agreement between the TST and the QFNG-IT assay (77%) and the TST and the T-SPOT.TB assay (75%) (205). Available data suggest that the TST and IGRAs have similar levels of accuracy for the detection of tuberculosis infection or the diagnosis of disease in children (206).

Since the experience with IGRAs is still limited, longitudinal studies are needed to define their predictive values, especially in children and high-risk populations (207). A large meta-analysis has pointed out very clearly present-day limitations of IGRAs, such as with their performance in children, in immunocompromized persons, and in the elderly (208). Other problems concern altered performance characteristics of the assays in conjunction with ethnicity

(209), specifically with the phenomena of conversions, reversions, and nonspecific variations in serial testing (210–212). Assessment of within-subject IGRA variability is important in establishing thresholds for conversions and reversions. With the QFNG-IT assay, Detjen et al. (192) observed considerable intraindividual variability in serial analyses within 3 days. van Zyl-Smit et al. (213) concluded from their study that a 3-spot or 80% IFN-γ response variation on either side of the baseline values explains 95% of the short-term variability and may be useful for interpreting conversions, reversions, and values close to the cutoff point. Therefore, these authors have proposed a borderline or uncertainty zone for both tests (4 to 8 spots [inclusive] for the T-SPOT.TB assay and 0.2 to 0.7 IU/ml for the QFNG-IT assay) instead of strictly adhering to the manufacturer-defined cutoff value (T-SPOT.TB assay, > 6 spots; QFNG-IT test, >0.35 IU/ml).

Basically, the problem of indeterminate results occurs with both IFN-γ release assays. In HIV-infected individuals, the T-SPOT.TB assay provided more indeterminate results than the QFNG-IT test (8 versus 1/256, $P < 0.01$) (202), similar to what has been confirmed by others (14% versus 1.8%) (214). If preanalytical errors can be excluded, indeterminate results appear to be dependent on the number of CD4 cells, inasmuch as patients with a CD4 count of ≤200 cells/ml were significantly more likely to have an indeterminate result (202, 214). In children <4 years of age, indeterminate results were more often seen using the QFNG-IT than the T-SPOT.TB assay (215). After the T-SPOT.TB assay was performed, when indeterminate results were obtained with the QFNG-IT test, 65% of the 40 patients yielded a valid result (216). In addition, age, race/ethnicity, and sex were also associated with indeterminate QFNG-IT results (217). There is a growing number of studies pointing to the need for standardizing IGRA preanalytical practices. Herrera et al. (218), for instance, have demonstrated that a delay in incubation of up to 12 h (which is well within the manufacturer's maximum delay of 16 h) increased the number of indeterminate QFNG-IT results. Six- and 12-h delays resulted in rates of positive-to-negative reversion (from immediate-incubation results) of 19% and 22%, respectively (219).

The optimal strategy for the diagnosis of latent tuberculosis infection is controversial. Adoption of a two-step strategy (TST followed by an IGRA) may be limited by TST-mediated boosting of subsequent IGRA responses. van Zyl-Smit et al. (213) have demonstrated that it appears safe to perform a QFNG-IT or T-SPOT.TB assay within 3 days of performing the TST.

From the present state of knowledge, it is obvious that the applications of the IGRAs for tuberculosis infection in different high-risk groups have to be tailored. Also, caution has to be exerted in their current use in immunosuppressed patients.

CROSS-CONTAMINATION

With the advent of molecular techniques designed for molecular epidemiology, cross-contamination linked either to laboratory procedures or, more rarely, to contaminated bronchoscopes can easily be proven. False-positive results may be generated at any step between specimen collection and reading of cultures (130, 220). Laboratory personnel should be alerted for a possible laboratory error if (i) the culture result is not compatible with the clinical picture, (ii) there is a late-appearing cluster of cultures which have scanty growth (<10 colonies on solid medium) or a significant delay in recovering mycobacteria from a liquid system, (iii) there is a large number of isolates of a particular species that is usually rare in the laboratory or of an organism that is normally considered an environmental contaminant, or (iv) there is only one positive culture from multiple specimens submitted from a single patient. Practices which can lead to false-positive culture results are numerous and include inadequate sterilization of instruments or equipment (such as bronchoscopes), use of contaminated water for specimen collection or for laboratory procedures, transfer of organisms from one specimen to another through direct contact or via common reagents or equipment, mix-up of testing samples or lids of specimen containers, failure to take precautions which minimize the production of aerosols, etc. Laboratory aspects of cross-contamination are addressed in more detail in the following section.

QUALITY ASSURANCE

General Aspects

Quality control (QC) is vital for monitoring a laboratory's effectiveness in detecting and isolating mycobacteria. This includes standard components of laboratory quality assurance, such as personnel competency, procedure manuals, external proficiency testing, and QC of media, tests, and reagents (see chapters 18 and 19). Laboratories performing mycobacterial testing should follow QC recommendations in the scientific literature and in *ad hoc* publications (130, 132,175). The Clinical Laboratory Improvement Amendments (CLIA) have reported that applications of a principal sanction against laboratories for a proficiency test violation were rare during a 14-year period of study (221).

The Public Health Service introduced the "levels of service concept" for mycobacteriology laboratories in 1967. In this scheme, laboratories define the level of service which best fits the needs of the patient population that they serve, the experience of their personnel, their laboratory facilities, and the number of specimens that they receive, always keeping the biosafety risk in mind. Levels of service were promulgated by the CDC, the ATS, and the College of American Pathologists (CAP) and are based on workload, personal experience, and cost-effectiveness (222). Personnel working in a clinical mycobacteriology laboratory must have proper training and certification in the specific functions that they perform. In the United States, all laboratories performing mycobacteriology testing must be enrolled in an external proficiency test program approved by federal and state regulatory agencies. Level I laboratories (acid-fast smear) must prepare at least 15 specimens per week, and level II laboratories (smear, culture, identification, and susceptibility testing of *M. tuberculosis* complex organisms) must process at least 15 specimens per week. Level III laboratories perform all the activities of a level II lab and, in addition, identify all NTM (performing susceptibility testing, where applicable).

Multiple test parameters are monitored by adherence to the quality assurance guidelines described in the recent CLSI standard documents (130, 175). Acceptable results derived from testing quality control reference strains do not guarantee accurate results with all clinical isolates. If inconsistent results are seen with clinical isolates (220), the test should be repeated in an attempt to ensure accuracy. Each laboratory should put its own policies into effect regarding the verification of atypical test results.

Laboratories must maintain a collection of well-characterized mycobacterial strains that are used for QC of test systems. These controls may be obtained from the American Type Culture Collection (ATCC) and proficiency testing programs. Frequently used stock cultures can be maintained on L-J slants or in 7H9 broth at 37°C or room temperature if subcultured monthly. Cultures on L-J slants may be held for up to 1 year if stored at 4°C. Such maintenance is not recommended for strains with drug resistance. Freezing of organisms suspended in water, saline, or 7H9 broth and storage at –70°C ± 10°C is the best option for long-term maintenance of stock cultures (130).

QC of Smear, Culture, and Molecular Tests

Apart from submitting to external QC tests, laboratories should make every possible effort to perform internal QC tests, e.g., with each new lot of medium commercially obtained or prepared in-house. Detailed procedures for monitoring the many different working steps in a clinical mycobacteriology laboratory are outlined in references 130 and 132.

Ideally, positive-control slides should be prepared from a concentrated sputum sample obtained from a patient with active tuberculosis. In practice, many laboratories use suspensions of stock cultures or seeded negative sputa as positive controls for acid-fast staining procedures. Control slides are also commercially available (BBL AFB QC slides; Becton, Dickinson Diagnostic Systems, Sparks, MD). An increase in the percentage of smear-positive but culture-negative specimens of >2% that cannot be attributed to a response to mycobacterial therapy or the presence of AFB in the negative controls suggests that water or reagents used in the pretreatment or staining procedures were contaminated with NTM. M. *gordonae* or M. *terrae* complex are most often involved. AFB may also be carried over from one slide to another if slides are not set properly apart from each other during the staining process. AFB may also be found in the oil used with the immersion lens after a positive slide is examined. The sensitivity of the AFB smear is directly related to the relative centrifugal force (RCF) (*g* force) attained during centrifugation. Thus, laboratories should calculate the RCF of their centrifuge and periodically monitor and document that they are reaching sufficient RCF by checking the revolutions per minute with a tachometer (117).

Laboratories should also monitor contamination rates (percentage of specimens producing contaminating growth on culture media) for decontaminated specimens. Contamination rates of 3 to 5% are generally considered acceptable. Rates below 3% usually indicate that the decontamination procedure is too harsh and that the procedure needs to be modified to minimize the lethal effect on mycobacteria. Contamination rates above 5% often indicate a too-weak decontamination, which could compromise mycobacterial cultures due to overgrowth of contaminants. It should be emphasized that the widespread use of liquid media increases the generation of aerosols; as a consequence, the risk of contamination between samples also increases. Laboratories that handle large numbers of isolates of MAC, M. *abscessus*, or specimens from patients with cystic fibrosis will probably have much higher contamination rates due to the high incidence of colonization of the sputum with Gram-negative bacteria, especially *Pseudomonas aeruginosa*.

Culturing mycobacteria is naturally prone to errors because of the multiple steps involved in processing cultures, the viability of mycobacteria for long periods in a laboratory environment, and the large number of mycobacteria present in some specimens (151). False-positive cultures may result from mislabeling, specimen switching during handling, specimen carryover (including proficiency testing specimens), contaminated reagents, or cross-contamination between culture tubes or vials (130, 151). Inclusion of a positive control (e.g., a suspension of M. *tuberculosis*) in the processing of patient specimens is discouraged due to the risk of cross-contamination. Standardized laboratory procedures that minimize the potential for errors leading to false-positive cultures should be followed, and mechanisms should be in place to rapidly recognize their occurrence. Transfers or inoculation of cultures must be accomplished by using individual transfer pipettes, single-delivery diluent tubes, or disposable labware. The order in which specimens are processed and media are inoculated should be recorded. A negative-control specimen following processing of patient specimens with the same digestion or decontamination solution can be used for detecting possible specimen contamination of the solutions. Alternatively, processing solutions may be cultured directly. Laboratories should prospectively track positivity rates. The significance of an isolate may be determined by reviewing the order in which specimens were handled for all manipulations (e.g., initial processing, liquid-medium readings, subculturing), the direct-smear results, the time to positivity, and the clinical history. Since the introduction of molecular fingerprinting of M. *tuberculosis* strains, false-positive cultures have been demonstrated to occur more frequently than previously assumed. False-positive cultures were identified in a number of studies that evaluated ≥100 patients, with a median false-positivity rate of 3.1%. Remarkably, of the 236 patients with false-positive cultures, 67% were treated and underwent unnecessary hospitalization (151). The deleterious impact of these undesirable events may be minimized if the evidence of false positivity is established in a timely manner and a rapid molecular method of fingerprinting is available. Single positive cultures of M. *tuberculosis* strains grown from AFB smear-negative specimens in the absence of clinical signs of tuberculosis should be analyzed by a PCR-derived typing technique to rule out laboratory contamination (130). Strains with identical fingerprints isolated within a 1-week period from different patients should be considered probably false positive.

The CDC and others (130) have recommended that AFB smear results be available and that positive results be reported within 24 h of specimen receipt. The time required for identification and susceptibility testing of M. *tuberculosis* should average 14 to 21 days and 15 to 30 days from the time of specimen receipt, respectively (223).

Nucleic acid amplification (NAA)-based assays require several levels of controls (e.g., to detect amplification inhibition as well as contamination between specimens), in addition to positive or negative controls (224). When used as approved by the FDA, NAA tests for M. *tuberculosis* diagnosis do not replace any previously recommended tests (225). Laboratories that test patient specimens by using laboratory-developed methods or commercially available NAA assays for nonapproved or off-label indications and that report their results must validate the assays and establish their performance characteristics prior to diagnostic use. Approved guidelines for molecular diagnostic methods in clinical microbiology are available from the CLSI; in these documents, the development, validation/verification, quality assurance, and routine use of NAA assays are addressed in detail (224). However, basing the identification of M. *tuberculosis* on a sole positive laboratory-developed test result is not recommended because the results of such assays vary considerably.

Potential probes and/or primers must be selected for sensitivity by using multiple clinical and reference strains of the target organism. Additionally, specificity must be evaluated by testing for cross-hybridization with other organisms which may be present in patient samples (224).

Testing to assess amplification should include positive and negative controls and controls for detection of the presence of inhibitors, such as endogenous nucleic acid. Other QC measures include those referring to assays of restriction enzymes, reagents, inspection of equipment, and laboratory design (i.e., separate areas for processing, amplification, and detection steps) (224). Excellent proficiency testing schemes to assess laboratory performance of nucleic acid amplification tests are currently available in both the United States and Europe.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Adequate funding and focused training are critical in maintaining state-of-the-art mycobacteriology laboratories (130, 175, 224). Laboratories play a pivotal role in the diagnosis and control of tuberculosis, and every effort should be made to implement sensitive and rapid methods for the detection, identification, and susceptibility testing of MAC organisms as well as other mycobacterial species. Specifically, these include the use of (i) fluorochrome stain for mycobacteria in smears, (ii) a broth-based or microcolony method for culture, and (iii) rapid identification methods (e.g., molecular assays).

The 24-h turnaround time for AFB smear results presents a challenge for most laboratories. The daily processing of specimens required to meet this goal adds considerable expense to the laboratory budget.

NAA assays offer the promise of same-day detection and identification of *M. tuberculosis*. Implementation of these new technologies presents several new challenges. Although the performance characteristics of many of these assays are quite good for smear-positive respiratory specimens, limited information exists on the use of these tests for the diagnosis of paucibacillary pulmonary or extrapulmonary disease. The new technologies have been shown to supplement rather than replace culture. Culture will still be required to obtain organisms for susceptibility testing and detect mycobacteria other than *M. tuberculosis*.

The significance of the isolation of NTM may be difficult to assess since many species are opportunistic pathogens, and the reader is referred to the criteria suggested by the ATS for their evaluation (2). In addition, accurate identification of NTM will prevent rarely encountered pathogens from being mistaken for nonpathogenic species.

Thus, accurate and timely reporting of the results of AFB microscopy, molecular tests, culture, identification, and drug susceptibility tests is essential to the effective management of individual patients and to the appropriate implementation of public health and infection control measures (130).

APPENDIX

Commonly Used Digestion-Decontamination Methods (117, 130, 132)

NALC-NaOH Method

Reagents

Digestant. For each 100 ml, combine 50 ml of sterile 0.1 M (2.94%) trisodium citrate with 50 ml of 4% NaOH. The NaOH and citrate mixtures can be mixed, sterilized, and stored for future use. To this solution, add 0.5 g of powdered NALC just before use. Use within 24 h of addition of the NALC because the mucolytic action of NALC is inactivated upon exposure to air.

Phosphate buffer. The buffer is 0.067 M and pH 6.8. Mix 50 ml of solution A (0.067 M Na_2HPO_4, 9.47 g of anhydrous Na_2HPO_4 in 1 liter of distilled water) and 50 ml of solution B (0.067 M KH_2PO_4, 9.07 g of KH_2PO_4 in 1 liter of distilled water). If the final buffer requires pH adjustment, add solution A to raise the pH or solution B to lower it.

BSA (*optional*). Use sterile 0.2% bovine serum albumin (BSA) fraction V (pH 6.8).

Procedure

1. Transfer up to 10 ml of specimen to a sterile, graduated, 50-ml plastic centrifuge tube labeled with appropriate identification. The tube should have a leakproof, aerosol-free screw cap. Add an equal volume of the NALC-NaOH solution. The final concentration of NaOH in the tube is 1%.
2. Tighten the cap completely. Invert the tube so that the NALC-NaOH solution contacts all the inside surfaces of the tube and cap, and then mix the contents for approximately 20 s on a vortex mixer. If liquefaction is not complete during this time, agitate the solution at intervals during the following decontamination period.
3. Allow the mixture to stand for 15 min at room temperature, with occasional gentle shaking by hand. Avoid movement that causes aeration of the specimen. A small pinch of crystalline NALC may be added to viscous specimens for better liquefaction. Specimens should remain in contact with the decontaminating agent for only 15 min, since overprocessing results in reduced recovery of mycobacteria. If more-active decontamination is needed, slightly increase the concentration of NaOH.
4. Add phosphate buffer (pH 6.8) up to the 50-ml mark on the tube.
5. Centrifuge the solution for at least 15 min at ≥3,000 × *g*.
6. Decant the supernatant fluid into a splashproof discard container containing a suitable disinfectant. Do not touch the lip of the tube to the discard container. Wipe the lip of each tube with disinfectant-soaked gauze (separate piece for each tube) to absorb drips, and recap.
7. Using a separate sterile pipette for each tube, add to the sediment 1 to 2 ml of sterile, 0.2% BSA fraction V (pH 6.8) or 1 to 2 ml of phosphate buffer (pH 6.8), and resuspend the sediment with the pipette or by shaking the tube gently by hand. BSA may have a buffering and detoxifying effect on the sediment and increases the adhesion of the specimen to solid media. However, BSA may lengthen detection times (as, for instance, in the Bactec 460TB system).
8. Inoculate the specimens onto appropriate solid culture media and into broth media. Use a separate disposable capillary pipette for each specimen to deliver 3 drops to solid medium.
9. Prepare a smear for acid-fast staining. Use a sterile disposable pipette to place 1 drop of the sediment onto a clean, properly labeled microscope slide covering an area approximately 1 by 2 cm. Place the smears on an electric slide warmer at 65 to 75°C for 2 h to dry and fix them. Alternatively, air dry the smears and fix them by passing the slide three or four times through the blue cone of a flame (heat fixing does not always kill mycobacteria, and the slides are potentially infectious).
10. Refrigerate (4°C) the remaining sediment for later use if needed (e.g., for direct susceptibility testing or further treatment if the specimen is contaminated, etc.).

The NALC-NaOH method can be used to process gastric lavage specimens, tissues, stool, urine, and other body fluids. For neutralized gastric lavage specimens and other body fluids (≥10 ml), centrifuge at ≥3,000 × *g* for 30 min in sterile screw-cap 50-ml centrifuge tubes, decant the supernatants, resuspend the sediments in 2 to 5 ml of sterile distilled water, and proceed as for sputum. If a gastric lavage specimen is mucopurulent, add 50 mg of NALC powder per 50 ml of lavage fluid and vortex before centrifugation. Tissue that is not collected aseptically can be ground, placed in a tube, homogenized by vortexing, and processed as for sputum. For stool specimens, place approximately 1 g of a formed specimen or 1 to 5 ml of a liquid specimen in a total volume of 10 ml of 7H9 broth, sterile water, or sterile saline; vortex vigorously for 30 s, and then allow large particles to settle to the bottom of the tube for 15 min. Remove 7 to 8 ml of supernatant, place it into a 50-ml centrifuge tube, and process as for sputum.

Sodium Hydroxide Method

Reagents

Digestant. NaOH solution (2 to 4%). Sterilize by autoclaving.

2 N HCl. Dilute 33 ml of concentrated HCl to 200 ml with water. Sterilize by autoclaving.

Phenol red indicator. Combine 20 ml of phenol red solution (0.4% in 4% NaOH) and 85 ml of concentrated HCl with distilled water to make 1,000 ml.

Phosphate buffer. The buffer is 0.067 M and pH 6.8. See the NALC-NaOH procedure for buffer preparation.

Procedure

Follow the steps described for the NALC-NaOH method, substituting 2% NaOH for the NALC-alkali digestant.

1. Transfer a maximum volume of 10 ml of specimen to a sterile 50-ml screw-cap plastic centrifuge tube. Add an equal volume of NaOH.
2. With the cap tightened, invert the tube and then agitate the mixture vigorously for 15 min on a mechanical mixer or vortex vigorously and let stand for exactly 15 min. If it is necessary to reduce excessive contamination, the NaOH concentration can be increased to 3 or 4%.
3. Add phosphate buffer (pH 6.8) up to the 50-ml mark on the tube. Recap the tube, and swirl by hand to mix well.
4. Centrifuge the specimen at ≥3,000 × *g* for 15 min, decant the supernatant, and add a few drops of phenol red indicator to the sediment. Neutralize the sediment with HCl. Thoroughly mix the contents of the tube thoroughly. Stop acid addition when the solution is persistently yellow.
5. Resuspend the sediment in 1 to 2 ml of phosphate buffer or sterile 0.1% BSA fraction V.
6. Inoculate the resuspended sediment to appropriate culture media, and prepare a smear.

Zephiran-Trisodium Phosphate Method

Principle

This system can be used when the laboratory cannot monitor the exposure time to the decontaminating agent, since the timing of this digestion-decontamination process is not critical. Benzalkonium chloride (Zephiran), a quaternary ammonium compound, together with trisodium phosphate selectively destroys many contaminants with little activity on tubercle bacilli. Zephiran is bacteriostatic to mycobacteria, and so the digested, centrifuged sediment must be neutralized with buffer before being inoculated onto agar medium. The phospholipids of egg medium neutralize this compound. This procedure is incompatible with the Bactec 460TB system.

Reagents

Zephiran-trisodium phosphate digestant. Dissolve 1 kg of trisodium phosphate (Na_3PO_4 $12H_2O$) in 4 liters of hot distilled water. Add 7.5 ml of Zephiran concentrate (17% benzalkonium chloride [Winthrop Laboratories, New York, NY]), and mix. Store at room temperature.

Neutralizing buffer. Neutralizing buffer has a pH of 6.6. Add 37.5 ml of 0.067 M disodium phosphate to 62.5 ml of 0.067 M monopotassium phosphate (for preparation of buffer solutions, see the NALC-NaOH procedure).

Procedure

1. Transfer a maximum volume of 10 ml of specimen to a sterile, 50-ml screw-cap plastic centrifuge tube. Add an equal volume of the Zephiran-trisodium phosphate digestant.
2. Tighten the cap, invert the tube, and then agitate the mixture vigorously for 30 min on a mechanical shaker. Permit the material to stand, without shaking, for an additional 20 to 30 min at room temperature.
3. Centrifuge the specimen at ≥3,000 × *g* for 15 min, decant the supernatant, and add 20 ml of neutralizing buffer. Vortex for 30 s to thoroughly suspend the sediment in the buffer (the neutralizing buffer serves to inactivate traces of Zephiran in the sediment, which is critical if inoculation of an agar-based medium is intended).
4. Centrifuge the specimen again for 15 min.
5. Decant the supernatant, retaining some fluid to resuspend the sediment.
6. Inoculate egg-based medium, and make a smear. The phospholipids of egg medium provide neutralization for this quaternary compound.

Oxalic Acid Method

Principle

The oxalic acid method is superior to alkali methods for processing specimens consistently contaminated with *Pseudomonas* species and certain other contaminants. Specimens processed by this method may be used with the Bactec 460TB system. It can also be used to decontaminate a previously processed sediment when cultures are contaminated with *Pseudomonas* and other non-glucose-fermenting Gram-negative bacilli.

Reagents

5% oxalic acid

Physiological saline (0.85%)

4% NaOH

Phenol red indicator or pH paper

Procedure

1. Add an equal volume of 5% oxalic acid to 10 ml, or less, of specimen in a 50-ml centrifuge tube (1/1, vol/vol).
2. Vortex the solution, and then allow it to stand at room temperature for 30 min with occasional shaking.
3. Add sterile saline to the 50-ml mark on the centrifuge tube. Recap the tube, and invert it several times to mix the contents.
4. Centrifuge for 15 min at ≥3,000 × *g*, decant the supernatant fluid, and add a few drops of phenol red indicator to the sediment. Alternatively, use pH paper.
5. Neutralize with 4% NaOH.
6. Resuspend the sediment, inoculate it to media, and make a smear.

CPC Method

Principle

Cetylpyridinium chloride (CPC), a quaternary ammonium compound, is used to decontaminate specimens, while sodium chloride effects liquefaction. CPC is bacteriostatic for mycobacteria inoculated onto agar-based media. This effect is not neutralized in the digestion process, and thus sediments from specimens treated with CPC should be inoculated only on egg-based media. This method is incompatible with the Bactec 460TB system.

This method is a means of digesting and decontaminating specimens in transit (>24 h). Mycobacteria remain viable for 8 days in the solution.

Reagents

CPC digestant-decontaminant. Dissolve 10 g of CPC and 20 g of NaCl in 1,000 ml of distilled water. The solution is self-sterilizing and remains stable if protected from light, extreme heat, and evaporation. Dissolve with gentle heat any crystals that might form in the working solution. Other reagents used in processing include sterile water and sterile saline or 0.2% sterile BSA fraction V.

Procedure

1. Collect 10 ml or less of sputum in a 50-ml screw-cap centrifuge tube.
2. Inside a BSC, add an equal volume of CPC-NaCl, cap securely, and shake by hand until the specimen liquefies.
3. Package the specimen appropriately as specified by current postal regulations, and send it to a processing laboratory.
4. Upon receipt in the processing laboratory (allow at least 24 h for digestion/decontamination to be completed), dilute the digested/decontaminated specimen to the 50-ml mark with sterile distilled water and recap securely. Invert the tube several times to mix the contents.
5. Centrifuge at ≥3,000 × *g* for 15 min, decant the supernatant fluid, and suspend the sediment in 1 to 2 ml of sterile water, saline, or 0.2% BSA fraction V.

6. Inoculate the resuspended sediment onto egg medium, and make a smear.

Sulfuric Acid Method

Principle

The sulfuric acid method may be useful for urine and other body fluids that yield contaminated cultures when processed by one of the alkaline digestants.

Reagents

4% sulfuric acid
4% sodium hydroxide
Sterile distilled water
Phenol red indicator

Procedure

1. Centrifuge the entire specimen for 30 min at ≥3,000 × *g*. This may require several tubes.
2. Decant the supernatant fluids; pool the sediments if several tubes were used for a single specimen.
3. Add an equal volume of 4% sulfuric acid to the sediment.
4. Vortex and let stand for 15 min at room temperature.
5. Fill the tube to the 50-ml mark with sterile water.
6. Centrifuge at ≥3,000 × *g* for 15 min and decant the supernatant.
7. Add 1 drop of the phenol red indicator and neutralize with 4% NaOH until a persistent pale pink color forms.
8. Inoculate the media and make a smear.

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Mycobacterium: Laboratory Characteristics of Slowly Growing Mycobacteria*

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This chapter represents a transition from previous chapters that detailed the general characteristics and phenotypic methods of identification of slowly growing mycobacteria. Although phenotypic characterization remains important, molecular techniques are increasingly employed as the gold standard for definitive identification to the species level.

As Tortoli noted in 2003, mycobacterial taxonomy can be divided into two major periods defined by methods used for identification to the species level (1). The first period, characterized by utilization of phenotypic studies, lasted from the late 1880s to the end of the 1980s. The second major era, characterized by a shift to genotypic studies, began during the last decade of the 20th century and has continued to the present time.

The *Mycobacterium tuberculosis* complex (MTBC) remains the most important group within the genus *Mycobacterium* from a global and clinical perspective. The MTBC currently includes not only the most significant human mycobacterial pathogens, *M. tuberculosis*, *M. bovis*, and *M. bovis* bacillus Calmette-Guérin (BCG), but also the less frequently encountered pathogens *M. caprae*, *M. microti*, *M. africanum*, *M. canettii* (the smooth variant of *M. tuberculosis*), and *M. pinnipedii*. Further discussion regarding the MTBC can be found in chapter 30 in this *Manual*.

Of the more than 150 currently validated species of nontuberculous mycobacteria (NTM), approximately 80 are slowly growing species (Table 1). The most clinically significant and/or most frequently encountered slowly growing NTM species include *Mycobacterium avium*, *M. intracellulare*, *M. kansasii*, *M. marinum*, *M. xenopi*, *M. malmoense*, and *M. ulcerans*. *M. gordonae*, although rarely a pathogen, occurs frequently in human samples, usually as a consequence of contamination from tap water (Table 2). Like the rapidly growing mycobacterial (RGM) species (see chapter 32 in this *Manual*), the majority of species of the slowly growing NTM have been described since the early 1990s with the advent of molecular technology. From 2007 to mid-2013, approximately 25 new species were described, including *M. europaeum*, *M. koreense*, *M. longobardum*, *M. minnesotense*, *M. parakoreense*, *M. sherrisii*, *M. shinjukuense*, and *M. yongonense* (2–23).

EPIDEMIOLOGY AND TRANSMISSION

Unlike *M. tuberculosis*, for which humans are the definitive host, most species of NTM are widely distributed in the environment, and the occurrence of NTM disease is attributed to a combination of host factors, such as age, body weight, the presence of chronic lung diseases (such as cystic fibrosis, bronchiectasis, or chronic obstructive pulmonary disease), alterations of chest structure, and other conditions, along with exposure. Organisms can be found in samples of soil and water, including both natural and treated water sources. For example, *M. kansasii*, *M. xenopi*, and *M. simiae* are regularly recovered from municipal water and only rarely from other environmental sources.

Furthermore, unlike MTBC, there has been no evidence of animal-to-human (except *M. marinum* from fish/fish tanks) or human-to-human transmission with slowly growing NTM. Human disease due to NTM is assumed to be acquired from environmental sources either directly by inhaling organisms in aerosols or traumatic implantation or indirectly by ingesting contaminated food or water. The source of infection may not always be detected for NTM (24).

Incidence rates of NTM disease are only estimates since, unlike tuberculosis (TB), NTM disease is noncommunicable from human to human, and therefore numbers of infections are not required to be reported to public health agencies. One publication from 2007 reported that the isolation prevalence of all NTM species (excluding *M. gordonae*) in pulmonary disease in Ontario, Canada, increased from 9.1/100,000 in 1997 to 14.1/100,000 by 2003, with a mean annual increase of 8.4%. Similar increases were noted for individual species. These findings indicate a significant rise in pulmonary disease caused by NTM in Canada (25). Increasing numbers of NTM isolates were also reported for several European countries (26). Although the data are scarce, NTM disease prevalence is believed to be increasing in the United States as well (24). Nationwide studies by the Centers for Disease Control and Prevention (CDC) have revealed that NTMs

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TABLE 1 Characteristics of currently recognized slowly growing species of NTM[h]

Species	Pigmentation	Established pathogenicity	Unique *hsp65* gene	Unique 16S rRNA gene	Date of description
M. algericum	N	Y[a]	Y	Y	2011
M. arosiense	Y	Y	Y	Y	2008
M. arupense	N[b]	Y	Y	Y	2006
M. asiaticum	Y	Y	Y	Y	1971
M. avium subsp. *avium*	N[b]	Y[c]	Y[d]	N	1901
"*M. avium* subsp. *hominissuis*"	N[b]	Y[e]	Y[d]	N	2002
M. avium subsp. *paratuberculosis*	N[b]	Y	Y[d]	N	1900
M. bohemicum	Y	Y	U	Y	1998
M. botniense	Y	N	U	Y	2000
M. bouchedurhonense	N	Y	Y	Y	2009
M. branderi	N	Y	U	Y	1995
M. celatum	N	Y	Y	Y	1993
M. chimaera	N	Y	N	Y	2004
M. colombiense	Y	Y	N	Y	2006
M. conspicuum	Y	Y	U	Y	1996
M. cookii	Y	N	U	Y	1990
M. doricum	Y	Y	U	Y	2001
M. engbackii	Y[g]	N	Y	Y	2013
M. europaeum	Y	Y	Y	Y	2011
M. farcinogenes	Y	Q	Y	Y	1973
M. florentinum	N	Y	U	Y	2005
M. fragae	N	U	Y	Y	2013
M. gastri	N	N	Y	N	1966
M. genavense	N	Y	U	Y	1993
M. gordonae	Y	N	U	V	1962
M. haemophilum	N	Y	Y	Y	1978
M. heckeshornense	Y	Y	Y	Y	2001
M. heidelbergense	N	Y	Y	Y	1998
M. heraklionense	N	Y	Y	Y	2013
M. hiberniae	Y	N	Y	Y	1993
M. interjectum	Y	Y	Y	Y	1993
M. intermedium	Y	Q	Y	Y	1993
M. intracellulare	Y/N	Y	Y	Y	1965
M. kansasii	Y	Y	Y	N	1955
M. kubicae	Y	N	U	Y	2000
M. koreense	N	Y	Y	Y	2012
M. kumamotonense	N	Q	Y	Y	2006
M. kyorinense	N	Q	Y	Y	2009
M. lacus	N	Q	Y	Y	2002
M. lentiflavum	Y	Y	Y	Y	1996
M. leprae	—[h]	Y	Y	Y	1880
M. lepraemurium	—	Y	Y	Y	1912
M. longobardum	N	Y	Y	Y	2013
M. malmoense	N	Y	Y	Y	1977
M. mantenii	Y	Y	Y	Y	2009
M. marinum	Y	Y	Y	N	1926
M. marseillense	N	Y	Y	Y	2009
M. minnesotense	Y	N	Y	Y	2013
M. monteofiorense	N	Y[i]	Y	Y	2003
M. nebraskense	Y	Q	Y	Y	2004
M. nonchromogenicum	N	Q	N	Q	1965
M. noviomagense	N	N	Y	Y	2009
M. palustre	Y	Q	U	Y	2002
M. paraffinicum	Y	U	Y	Y	2009
M. paragordonae	Y	N	Y	Y	2014
M. parakoreense	N	Y	Y	Y	2012
M. parascrofulaceum	Y	Y	Y	Y	2004
M. paraseoulense	Y	U	Y	N	2010
M. paraterrae	Y	Y	Y	Y	2010
M. parmense	Y	Y	Y	Y	2004
M. pseudoshottsii	Y	Y[j]	N	N	2005
M. pulveris	N	N	U	Y	1983

(*Continued on next page*)

TABLE 1 Characteristics of currently recognized slowly growing species of NTM[h] *(Continued)*

Species	Pigmentation	Established pathogenicity	Unique *hsp65* gene	Unique 16S rRNA gene	Date of description
M. *riyadhense*	N	Y	Y	Y	2009
M. *saskatchewanense*	Y	Y	Y	Y	2004
M. *scrofulaceum*	Y	Y	Y	Y	1956
M. *senuense*	N	Q	U	Y	2008
M. *seoulense*	Y	Q	Y	Y	2007
M. *sherrisii*	N	Y	Y	Y	2011
M. *shigaense*	Y	Y	Y	N	2011
M. *shimoidei*	N	Y	Y	Y	1982
M. *shinjukuense*	N	Y	Y	Y	2011
M. *shottsii*	N	Y[j]	U	Y	2003
M. *simiae*	Y	Y	Y	Y	1965
M. *stomatepiae*	N	Y[j]	Y	Y	2008
M. *szulgai*	Y	Y	Y	Y	1972
M. *terrae*	N	Q	N	Y	1966
M. *timonense*	N	Y	Y	Y	2009
M. *triplex*	N	Y	Y	Y	1997
M. *triviale*	N	N	U	U	1970
M. *tusciae*	Y	Y	U	Y	1999
M. *ulcerans*	N	Y	U	N	1950
M. *vulneris*	Y	Q	Y	Y	2009
M. *xenopi*	N	Y	Y	Y	1959
M. *yongonense*	N	Y	Y	N	2013

[a] Fish and goats.
[b] A few strains may show light pigmentation.
[c] Birds.
[d] Sequence outside the 441-bp Telenti fragment.
[e] Humans and swine.
[f] Late yellow pigment
[g] Some strains show pink pigment.
[h] Y, yes; N, no; U, undetermined; Q, questionable; —, not grown on artificial media.
[i] Eels.
[j] Fish.

made up one-third of mycobacterial isolates reported in the 1980s (prevalence of 1.8/100,000) and that by the early 1990s they accounted for up to two-thirds of mycobacterial isolates (27–29). A more recent study correlating clinical and microbiologic data of Oregon residents who had at least one NTM isolate between 2005 and 2006, using the American Thoracic Society (ATS) and Infectious Diseases Society of America (IDSA) criteria for defining NTM infection, revealed a prevalence of 8.6 per 100,000 in the general population and 20.4 per 100,000 for individuals ≥50 years of age (30).

The most common infections with NTM are pulmonary diseases, but skin and soft tissue, lymphatic, and disseminated infections also occur. Disseminated infections most often occur in the setting of advanced HIV disease, but non-HIV-infected patients can also be affected. Although regional variations in species isolation have been shown, *Mycobacterium avium* complex (MAC) strains are the most commonly isolated, pathogenic, slowly growing NTM, but numerous other NTM species also cause disease. In the United States, M. *kansasii* is the second most frequently recovered pathogenic species (24).

CLINICAL SIGNIFICANCE

Because of the presence of multiple species of NTM in the environment and their opportunistic pathogenic nature, the determination of the clinical significance of the isolation of these species is based upon multiple factors, including the clinical setting, host-specific factors, the species, the pathogenic potential of the organism, the number of positive cultures, the source of the culture isolate, and quantitation of the organisms detected by smear and culture (24). For example, although the incidence of a specific NTM such as M. *gordonae* in cultures is high, the pathogenicity of this species is very low, in contrast to that of species such as MAC and M. *kansasii* (31).

In contrast to NTM, the isolation of MTBC is always an important finding in a clinical laboratory. The finding of this complex has vital epidemiologic and public health consequences. Further details on clinical significance of the MTBC may be found in chapter 30 in this *Manual*.

DIRECT EXAMINATION

Microscopy

One of the first, easiest, and least expensive means of detecting the presence of mycobacteria in clinical samples has been microscopic examination. Special acid-fast stains, along with the use of bright-field and fluorescence microscopy, are needed for staining of the organisms since the routine Gram stain is not optimal for staining mycobacteria. Further discussion of the specific techniques can be found in chapter 30 of this *Manual*.

Antigen Detection

Antigen detection is generally not performed for direct detection of mycobacteria in diagnostic laboratories. However, more recently antigen detection assays that detect lipoarabi-

TABLE 2 Important properties of selected slowly growing NTM species[h]

Species	Optimal growth temp or range (°C)	Colony morphology	Niacin	Nitrate reduction	Features of 16S rRNA gene (GenBank accession no. and/or reference)	Clinical relevance,[a] specimens of first isolation, or important laboratory feature(s)
M. *avium* subsp. *avium*, M. *avium* subsp. *paratuberculosis*, M. *avium* subsp. *silvaticum*	30–37	Smooth (rough)	Neg	Neg	3 subspecies established (188), another subspecies proposed ("M. *avium* subsp. *hominissuis*") (74); identical 16S rRNA gene sequences for all subspecies (GQ153272)	Lymphadenitis in children; pulmonary disease in adults[a]; often disseminated infection in HIV patients (all caused by M. *avium* subsp. *hominissuis*)
M. *intracellulare*	30–37	Smooth (rough)	Neg	Neg	Several sequence variants known, some elevated to species level (see following rows) (GQ153276)	Pulmonary disease in adults[a]; often disseminated infection in HIV patients
Other MAC members						
M. *arosiense*	42	Smooth	ND	Pos	6-bp difference from M. *intracellulare* (EF054881)	Osteomyelitis in a child (189)[d]
M. *chimaera*	25–37	Smooth	Neg	Neg	1-bp difference (position 403) from M. *intracellulare* (AJ548480)	Probably similar to M. *intracellulare* (190)[b]
M. *colombiense*	20–37	Rough	Neg	Neg (85%)	7-bp difference from M. *intracellulare* (AM062764)	HIV patients, blood, sputum (191)[c]
M. *vulneris*	37	Smooth	Neg	Neg	3-bp difference from M. *colombiense* (EU834055)	Lymphadenitis in a child and infection after a dog bite (192)[d]
Other slowly growing NTMs						
M. *bohemicum*	37–40	Smooth	Neg	Neg	Unique 16S rRNA gene sequence (98) (U84502)	Lymphadenitis in children (193)
M. *celatum*	33–42	Smooth	Neg	Neg	3 sequence types are published; type 2 is very different from type 1, type 3 is similar to type 1 (194, 195); unclear taxonomic situation; possesses 2 rRNA operons (196) (L08169, type 1; L08170, type 2; Z46664, type 3)	Pulmonary disease in adults[a]
M. *genavense*	31–42	Smooth	Neg	Neg	Unique 16S rRNA gene sequence (197) (X60070)	First detection in HIV patients; single cases also from nonimmunocompromised patients; almost no growth on solid media, scarce growth in liquid media, growth enhanced by Mycobactin J
M. *gordonae*	30–37	Smooth/ rough	Neg	Neg	Several sequence variants of the 16S rRNA gene (75) (AJ581472) Sequence variants (FJ418069,[e] EU486080, GUI42930)	Present in running water systems; usually without clinical relevance
M. *haemophilum*	28–32	Rough	Neg	Var[f]	Unique 16S rRNA gene sequence (X88923)	Lymphadenitis in children; skin lesion with immunosuppression; growth usually dependent on the addition of hemin to the medium
M. *heidelbergense*	30–37	Smooth	Neg	Neg	Unique 16S rRNA gene sequence (X70960)	Lymphadenitis in a child (198)
M. *hiberniae*	37	Rough	ND	Pos	Unique 16S rRNA gene sequence (1-bp difference from M. *engbaeckii*) (AY438069[e])	From environmental specimens; usually without clinical relevance
M. *interjectum*	31–37	Smooth	Neg	Neg	Unique 16S rRNA gene sequence (X70961)	Lymphadenitis in a child (199)

(Continued on next page)

TABLE 2 Important properties of selected slowly growing NTM species[h] *(Continued)*

Species	Optimal growth temp or range (°C)	Colony morphology	Niacin	Nitrate reduction	Features of 16S rRNA gene (GenBank accession no. and/or reference)	Clinical relevance,[a] specimens of first isolation, or important laboratory feature(s)
M. *intermedium*	31–37	Smooth	Neg	Neg	Unique 16S rRNA gene sequence (X67847)	Isolated from sputum specimens (200)
M. *kansasii*	35–37	Rough	Neg	Pos	6 subspecies differing in several genes (76, 168); subspecies 1 is prevalent worldwide (171, 201, 202) (AF480601)	Pulmonary disease in adults[a]; rarely associated with lymphadenitis in children
M. *lentiflavum*	22–37	Smooth	Neg	Neg	3 sequence variants (X80769, X80770, X93995)	Most isolates obtained from pulmonary specimens (77)[a]
M. *malmoense*	30	Smooth	Neg	Neg	Unique 16S rRNA gene sequence yet very similar to that of M. *szulgai* (GQ153278)	Lymphadenitis in children; pulmonary disease in adults[a]; rare in USA but common in North European countries
M. *marinum*	30	Rough (smooth)	Var[g]	Neg	16S rRNA gene sequence has only a 2- to 5-bp difference (none in first 500 bp) from that of M. *ulcerans* (203) (AJ536032)	Swimming pool granuloma; growth rate differentials: M. *ulcerans*, extremely slow growing; M. *marinum*, <7 days
M. *scrofulaceum*	37	Smooth	Neg	Neg	Unique 16S rRNA sequence (GQ153271)	Pulmonary disease in adults[a]; rarely associated with lymphadenitis in children
M. *shimoidei*	37	Rough	Neg	Neg	Unique 16S rRNA gene sequence (no correct sequence available)	
M. *simiae*	37	Smooth	Var	Pos	Unique 16S rRNA gene sequence (GQ153280)	Pulmonary disease in adults[a]; also in water supplies depending on geographic regions
M. *szulgai*	37	Rough/ smooth	Neg	Pos	Unique 16S rRNA gene sequence, yet very similar to that of M. *malmoense* (AF547969[e])	Pulmonary disease in adults[a]
M. *terrae* complex (other clinically significant species include M. *arupense*, M. *heraklionense*, M. *kumamotonense*)	25–37	Rough/ smooth	Neg	Pos/Neg	Group of species with similar phenotypic characteristics and clinical significance; require 16S rRNA gene sequencing for species ID	Tenosynovitis/osteomyelitis usually of the hand/ fingers; isolates from other sources are usually without clinical relevance. Most common species is M. *arupense*. M. *nonchromogenicum* rarely seen when ID based on 16S sequencing (M. *arupense*, DQ157760; M. *heraklionense*, GU084182; M. *kumamotonense*, AB239925; M. *terrae*, DQ058407).
M. *ulcerans*	30	Rough	Neg	Neg	16S rRNA gene sequence has only a 2-bp difference from M. *marinum* (nucleotides 1248 and 1289) (203) (no correct sequence available)	Extensive skin ulceration in tropical environments; requires lengthy incubation for growth on artificial media; M. *marinum* grows <7 days at 30°C.
M. *xenopi*	40–45	Smooth	Neg	Neg	Unique 16S rRNA gene sequence (AJ536033 [at positions 182 and 408, C or T may be present])	Present in hot water systems; often without clinical relevance; rare cause of chronic pulmonary disease

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[a] Clinical relevance in pulmonary specimens must be proven according to the ATS guidelines.

[b] AccuProbe test is positive for MAC- and for M. *intracellulare*-specific probes but negative for M. *avium*-specific probe.

[c] AccuProbe test is positive for MAC-specific probe but negative for M. *avium*- and for M. *intracellulare*-specific probes.

[d] No data available for AccuProbe tests.

[e] Sequence data exist for only a fragment of the gene.

[f] Different reports with inconsistent data.

[g] See reference 204. The test method differed from the current strip method.

[h] Abbreviations: Neg, negative; Pos, positive; ND, not determined; Var, varies.

nomannan (LAM), a major lipopolysaccharide component of the cell wall of M. *tuberculosis*, have shown promise and have been commercially developed into an enzyme-linked immunosorbent assay (ELISA) (Clearview TB ELISA; Alere Inc., Waltham, MA) and point-of-care lateral flow assays (Determine TB; Alere) (32). A recent meta-analysis of commercially available urinary LAM assays in patients with microbiologically confirmed pulmonary tuberculosis revealed that the sensitivity ranged from 13 to 93% while specificity ranged from 87 to 99% (33). Although the sensitivity of the assay is not optimal for screening all populations, the highest sensitivities were found among HIV-positive patients and increased with advanced immunosuppression ($CD4^+$ T-cell counts, <100 cells/μl) (33).

Nucleic Acid Detection

Early detection of disease caused by members of the MTBC is essential for initiating the appropriate therapy and controlling the transmission of tuberculosis. Direct nucleic acid amplification (NAA) techniques for the detection of MTBC nucleic acids from patient specimens are increasingly being used (34–42). These tests can provide results in as little as 2 hours. In addition to laboratory-developed PCR assays, several commercially available tests are available (Table 3). Two tests have been FDA approved or FDA cleared for the detection of MTBC from smear-positive and smear-negative respiratory specimens, the Amplified *Mycobacterium tuberculosis* direct (AMTD) test (Hologic GenProbe, San Diego, CA) and the Xpert MTB/RIF test (Cepheid, Sunnyvale, CA) (43). In addition, the Xpert MTB/RIF test is also FDA cleared for the detection of mutations associated with rifampin resistance. For nonrespiratory specimens, no FDA-cleared test is available.

As with all NAA tests, quality control for MTBC nucleic acid amplification tests should include the use of inhibition controls in each specimen to decrease the potential for falsely negative results, unless the laboratory has collected sufficient data to demonstrate that inhibition is negligible in the specimen type being analyzed. In order to prevent cross-contamination with amplicon, a strict unidirectional workflow must be followed, equipment must be dedicated to its respective area, and an effective cleaning procedure must be performed. The use of closed amplification systems has the advantage that tubes containing amplified products do not need to be reopened, and thus the risk of cross-contamination with amplicon is reduced. When NAA tests are being implemented, performance characteristics, such as sensitivity and specificity, need to be established for the laboratory by use of well-known samples. Laboratory proficiency should be examined regularly by comparing NAA test results with microbiological and clinical data and through participation in proficiency testing events sponsored by various regulatory agencies (e.g., the CDC and the College of American Pathologists [CAP]).

In response to the increasing demand for MTBC NAA testing and in recognition of the importance of prompt laboratory results in tuberculosis diagnosis and control, updated guidelines for the use of nucleic acid amplification tests in the diagnosis of tuberculosis were released by the Centers for Disease Control and Prevention in 2013 (44). The recommendations state that NAA testing should be performed on at least one respiratory specimen from each patient with signs and symptoms of pulmonary tuberculosis for whom a diagnosis of tuberculosis is being considered but has not yet been established and for whom the test result would alter case management or infection control activities. However, culture remains the reference standard for laboratory confirmation of tuberculosis and is required for drug susceptibility testing and genotyping.

Several reviews and meta-analyses have evaluated the literature on the diagnostic accuracy of NAA for the direct detection and identification of MTBC from respiratory specimens (35–40, 42, 45–47). A high degree of variability was noted across various studies. Sensitivity values ranged from 36 to 100% (38) or 27 to 100% (42), while specificity ranged from 54 to 100% (38) or 91 to 100% (42). The highest sensitivity and specificity were achieved when smear-positive specimens were analyzed, with smear-negative specimens generally providing lower sensitivity for detection of MTBC. Thus, a positive NAA test result combined with a high clinical probability provides a rapid diagnosis of tuberculosis. In contrast, a patient can be presumed to be infected with an NTM if a negative NAA result (with inhibitors excluded) was obtained from

TABLE 3 Commercially available NAA tests for detection of M. *tuberculosis* complex

NAA test	Manufacturer	Method
AMTD[a]	Hologic Gen-Probe Inc., San Diego, CA	Transcription-mediated amplification of rRNA
GenoType Mycobacteria Direct assay	Hain Lifescience, Nehren, Germany	Nucleic acid sequence-based amplification of 23S rRNA of MTBC, M. *avium*, M. *malmoense*, M. *kansasii*, and M. *intracellulare*; line probe assay
artus M. *tuberculosis* PCR kit	Qiagen GmbH, Hilden/Hamburg, Germany	Real-time PCR, amplification of 16S rRNA gene
Loop-mediated isothermal amplification	Eiken Chemical Co. Ltd., Tokyo, Japan	Isothermal amplification, visual observation using UV light
RealArt M. *tuberculosis* TM PCR reagents	Abbott Laboratories, Abbott Park, IL	Real-time PCR using the ABI Prism 7000 system
Xpert MTB/RIF[a]	Cepheid, Sunnyvale, CA	Real-time PCR, detection of MTBC and resistance to rifampin; amplification of *rpoB*

[a] Amplified Mycobacterium tuberculosis Direct Test (AMTD) received FDA approval and Xpert MTB/RIF test received FDA clearance for smear-positive respiratory specimens and smear-negative respiratory specimens from patients suspected of having tuberculosis.

a smear-positive specimen. A negative NAA test result, especially from a smear-negative specimen, does not rule out MTBC infection.

For the AMTD test, sensitivity was reported to be 90 to 100% for smear-positive specimens and 63.6 to 100% for smear-negative specimens (42). Specificity is excellent. The most significant drawback of the AMTD test is the use of an open amplification system, which increases the potential for cross-contamination between specimens. In addition, the test is labor intensive and technically complex to perform.

The Xpert MTB/RIF assay is an automated, cartridge-based technique that identifies M. *tuberculosis* complex and rifampin resistance simultaneously in primary patient respiratory samples using a molecular beacon-based real-time PCR assay. The assay is simple and robust, allowing it to be used outside the conventional microbiology laboratory, and it yields results within 2 hours (48). The Xpert MTB/RIF assay has a sensitivity of >90% in smear-positive respiratory samples and >70% in smear-negative respiratory samples (49, 50). The World Health Organization (WHO) recommends employing the Xpert MTB/RIF as the initial diagnostic test in individuals suspected of having multiple-drug-resistant (MDR) or HIV-associated tuberculosis, particularly in areas of high prevalence (51). In low-incidence countries, samples with high suspicion of MDR (e.g., origin of the patient from Eastern Europe or sub-Saharan Africa; HIV infection; history of tuberculosis) should be considered for referral to a reference laboratory for performance of the Xpert MTB/RIF. Some false rifampin resistance has been reported for the Xpert MTB/RIF assay particularly when used in low-prevalence settings, so confirmation of the NAA result for RIF with conventional drug susceptibility testing is required (52, 53). The Xpert MTB/RIF assay complements, but does not replace, culture or antimicrobial susceptibility testing.

In general, commercially available tests are preferred over laboratory-developed assays because of the standardized protocols and improved quality control. A meta-analysis for the use of in-house NAA tests reported a highly heterogeneous estimate of diagnostic accuracy (range of sensitivity, 9.4 to 100%; range of specificity, 5.6 to 100%) (37). Laboratory-developed tests (LDTs) must be rigorously verified against culture and/or the FDA-approved/cleared tests prior to use. LDTs do offer advantages such as the ability to verify and test a wider variety of specimen sources than the approved/cleared tests, and they are often less costly.

IMMUNODIAGNOSTIC TESTS

Several immunodiagnostic tests are available for the detection of latent infection with MTBC. The tuberculin skin test (TST) has been available for nearly a century and continues to be widely used today. Since 2001, two gamma interferon release assays (IGRAs; QuantiFERON Gold In-Tube from Cellestis/Qiagen, Gaithersburg, MD, and T-SPOT.TB from Oxford Immunotec, Oxford, United Kingdom) have been introduced as an alternative to the TST. The IGRAs provide a number of advantages over the TST, the most notable of which is the lack of a positive response in persons vaccinated with the M. *bovis* BCG vaccine. Additional information on the two commercially available IGRAs can be found in chapter 30 of this *Manual*.

IDENTIFICATION

The rapid detection and identification of M. *tuberculosis* complex have been the most important tasks of a clinical mycobacteriology laboratory. However, within the last 30 years, the number of currently validated NTM species has increased from approximately 30 to more than 150 species. This development has been paralleled by an increasing incidence of infections due to NTM. Thus, clinical mycobacteriology laboratories today also encounter the challenge of providing accurate identification of NTM species. The use of molecular techniques has dramatically accelerated the diagnostic process. MTBC bacilli can be detected rapidly in clinical specimens by NAA tests. DNA probes for the confirmation of MTBC and a few NTM species (M. *avium* complex, M. *gordonae*, and M. *kansasii*) grown on culture media have been available for many years. Historically, species identification has relied, with few exceptions, on the analysis of a series of phenotypic tests for which performance was restricted mostly to specialized laboratories. These tests require a sufficient amount of bacterial cells and several weeks of incubation. It is now recognized that most mycobacterial species cannot be reliably identified by biochemical and other phenotypic tests. Some species may exhibit convergent characteristics, and strains of one species may show variability in certain features. In addition, many new species have not been studied in detail. The most reliable methods for identification of all mycobacterial species today involve molecular analyses of selected genes. These techniques have the additional advantage that they can be performed from liquid culture media, which in general enable more-rapid growth and a more sensitive detection of mycobacteria than solid culture media. Mixed cultures with NTM are not rare, and thus there is a danger of working with cultures in broth only. Thus, all results obtained by molecular methods should be confirmed, even after the reporting of the results, by some important phenotypic characteristics, such as growth rate, colony morphology, and pigmentation (Table 2). Nevertheless, it needs to be stressed that phenotypic tests (e.g., biochemical analyses) are not sufficient for the definitive identification of NTM at the species level.

The Clinical and Laboratory Standards Institute (CLSI) recognizes that not all laboratories have the necessary funds or instrumentation to provide molecular testing in mycobacteriology. Laboratories that have access only to probe technology and that have the appropriate safety equipment (e.g., a class II biological safety [BSL-2] cabinet) can utilize the probes on isolates to rule out MTBC, M. *avium* complex, M. *gordonae*, and M. *kansasii*. If molecular identification techniques are not available, laboratories should refer the other NTM isolates to a reference laboratory with DNA sequencing capabilities for accurate identification (54).

Phenotypic Methods

Current guidelines recommend at least two different media for the culture of mycobacteria. While liquid media (usually Middlebrook 7H9 or 7H12) allow for rapid detection of mycobacterial growth, phenotypic evaluation requires that mycobacteria be grown on solid egg- or agar-based culture media. The rapid growth and subsequent molecular identification of mycobacteria in liquid broth are generally sufficient for definitive species identification, although as discussed above, care must be taken to ensure that the broth culture contains a single organism and is not a mixed culture. Phenotypic analysis alone, including growth characteristics and biochemical properties, are not sufficiently specific to substitute for molecular identification. Therefore, the major application of phenotype-based analyses is to provide orientation for the subsequent diagnostic steps and complement or confirm molecular identification, which may not always be unambiguous.

The key methods for the phenotypic classification of mycobacteria are the growth rate, optimal growth temperature, morphology, and pigmentation. Biochemical tests have been largely discontinued, and their application in the routine mycobacteriology laboratory has become obsolete.

Growth Rate

Growth rate is an obvious property that can be observed, and mycobacterial growth rate is dependent on temperature, the initial bacterial inoculum, and the species. The estimation of growth rate is most reliably performed on solid medium, even though protocols using standardized growth curves have been developed for growth in liquid culture medium (the Bactec MGIT system from Becton, Dickinson and Company, Franklin Lakes, NJ; or the VersaTREK system from Thermo Scientific/Trek Diagnostic Systems, Oakwood Village, OH). To perform a standardized growth test in the routine laboratory, defined suspensions of mycobacteria (e.g., 0.5 McFarland standard) are inoculated on solid media and incubated at 30 ± 1°C and 36 ± 1°C. Cultures are observed for growth at 5 to 7 days and weekly thereafter. Mycobacteria can thus be classified into the slowly and rapidly growing species. RGM are able to form mature colonies within 7 days of incubation, whereas slowly growing species require a longer incubation time for colony formation.

Temperature

Mycobacterium species differ in the ability to grow at certain temperatures. For determination of the preferred growth temperature, solid culture media are inoculated with defined suspensions of mycobacteria (e.g., 0.5 McFarland standard) and incubated at various temperatures. For slowly growing species, the minimum set of temperatures for incubation comprises 30 ± 1°C and 36 ± 1°C. Most slowly growing mycobacterial species grow well at 35 to 37°C, but others, including M. *marinum*, grow better at a lower temperature (30°C), especially on primary isolation. Therefore, samples from skin or lymph nodes and other tissue specimens from the body periphery should routinely be incubated both at 30 ± 1°C and at 36 ± 1°C. Additional media incubated at 22 to 25°C and 42°C may be necessary for optimal growth of some species, such as M. *haemophilum* (28 to 32°C), M. *xenopi* (42°C), M. *conspicuum* (22 to 31°C), and M. *stomatepiae* (22°C). Cultures to determine temperature requirements of known RGM can be read within 1 week, while slowly growing mycobacteria require a longer period of incubation.

Colony Morphology

Colony morphology is a phenotypic property of mycobacteria that can easily be determined. Morphologic characteristics such as size and colony description (flat, raised, etc.) should be noted, but the most discriminative and relevant property is the smooth or rough growth form of the colonies. M. *tuberculosis* usually grows in rough, nonpigmented colonies on Löwenstein-Jensen slants (Fig. 1) as well as on agar-based media. In contrast, M. *bovis* grows in flat, smooth, nonpigmented colonies. The colony morphology may vary when different formulations of solid media are used. Slowly growing NTM species may also exhibit either rough or smooth variants or both types, but colony morphology is not a reliable parameter for the discrimination of NTM. Recent studies have suggested that smooth/rough colony morphology among the RGM may be related to pathogenicity, with rough colony morphology representing the invasive phenotype.

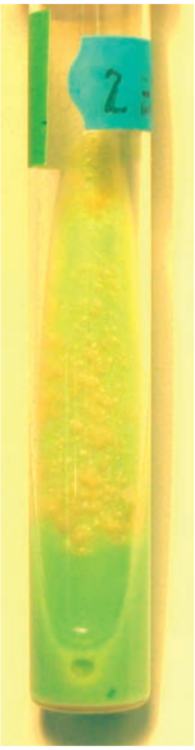

FIGURE 1 M. *tuberculosis* grown on a Löwenstein-Jensen slant for 14 days. Colonies are dry, wrinkled, off-white, and eugenic. doi:10.1128/9781555817381.ch31.f1

Pigmentation and Photoreactivity

Pigmentation of mycobacteria has been used for primary classification into nonchromogenic and chromogenic species (Runyon classification system, named for Ernest Runyon, a pioneer taxonomist in mycobacteriology). Species of the MTBC do not produce pigment, while NTM species can be chromogenic (scoto- or photochromogenic) or nonchromogenic. Chromogenic NTM species produce pigment that can range from light yellow and yellow to orange or even rose-colored. Within the pigmented mycobacteria, the photochromogens are nonpigmented in the dark and produce pigmentation only after exposure to light (Fig. 2). Since pigment production is an oxygen-dependent reaction, primary cultures of these species may remain nonpigmented even when exposed to light if the caps are tightly closed. Loosening of the caps, done in a biological safety cabinet, helps promote the color change. The scotochromogens produce pigment when grown in the dark or when exposed to light (Fig. 3). The color may intensify with the increasing age of the culture. The nonchromogenic species do not produce pigmentation when grown either in the dark or after exposure to light. The color of nonchromogenic colonies may remain off-white, buff, or pale yellow and does not intensify when exposed to light (Fig. 4). This separation, however, is no longer performed in most laboratories.

Niacin Accumulation

Some *Mycobacterium* species cannot use nicotinic acid, although they produce it in their biosynthetic pathways. Consequently, nicotinic acid is excreted into the medium and can be detected. Paper strip format tests (BD Diagnostics,

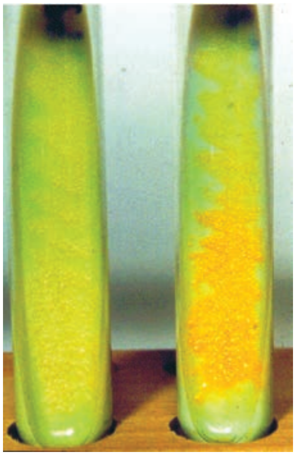

FIGURE 2 M. *marinum* on Löwenstein-Jensen slants after 2 weeks of growth before exposure to light (left) and after exposure to light (photochromogenic) (right). doi:10.1128/9781555817381.ch31.f2

Sparks, MD) to detect niacin or isoniazid are commercially available, are easy to perform, and avoid the use of hazardous reagents. Better results can be obtained with the isoniazid test strips than with the niacin test strips. The detection of niacin is one of the key phenotypic tests for the identification of M. *tuberculosis*, even though molecular techniques have replaced this test in most laboratories. Within the NTM, M. *simiae* can also be positive in the niacin test. M. *tuberculosis* strain H37 and M. *avium* can be used as positive and negative controls, respectively.

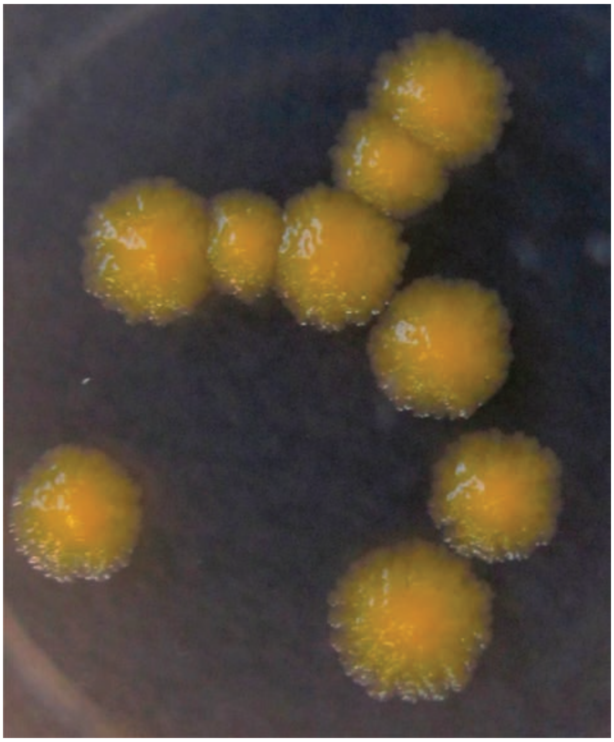

FIGURE 3 M. *gordonae* grown on 7H10 Middlebrook agar for 10 days. Colonies are yellow and smooth (scotochromogenic). doi:10.1128/9781555817381.ch31.f3

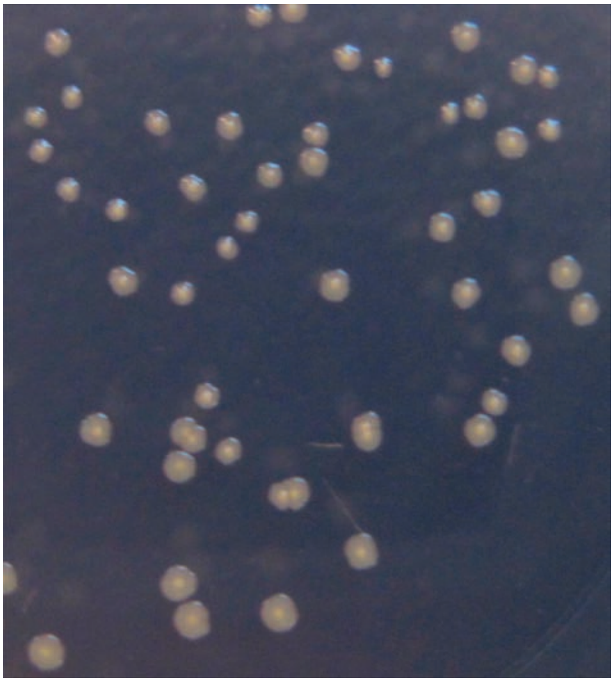

FIGURE 4 M. *celatum* grown on 7H10 Middlebrook agar for 10 days. Colonies are small, colorless, and smooth (nonchromogenic). doi:10.1128/9781555817381.ch31.f4

Nitrate Reduction

Several mycobacterial species produce nitrate reductase, which reduces nitrate to nitrite. This ability is assessed by the formation of a diazonium salt, which becomes visible through the production of a red water-soluble dye (55). As for the measurement of niacin production, a paper strip assay has proved to be reliable and efficient for the identification of nitrate reduction (BD Diagnostics). The confirmation of the presence of a nitrate reductase is another key phenotypic property for the identification of M. *tuberculosis*. M. *tuberculosis* strain H37 and M. *avium* can be used as positive and negative controls, respectively.

Identification of Species of MTBC Using Phenotypic Markers

If a strain is identified as a member of the MTBC using the AccuProbe test (Hologic GenProbe, San Diego, CA) or another molecular method, further differentiation of the species between M. *tuberculosis*, M. *bovis*, and M. *bovis* BCG can be performed reliably using simultaneous evaluation of several phenotypic markers (Table 4). Species identification is considered acceptable if all test results are unambiguous and all controls give correct results.

However, molecular techniques should always be given priority over conventional phenotypic methods to achieve unambiguous and definitive results for species identification. Furthermore, other members of the MTBC, including M. *africanum*, M. *microti*, M. *pinnipedii*, and M. *canettii* can be identified only by molecular tests. Sufficient information on phenotypic properties is not available for these rarely isolated members of the MTBC.

Identification of NTM Species Using Phenotypic Markers

As previously emphasized, species identification of NTM is not feasible using phenotypic tests alone. The number of

TABLE 4 Phenotypic identification of M. *tuberculosis*, M. *bovis*, and M. *bovis* BCG

Species	Niacin	Nitrate reduction	Colony morphology[a]	Susceptibility to:	
				TCH[b]	Pyrazinamide
M. *tuberculosis*	Positive	Positive	Rough	Resistant	Susceptible[e]
M. *bovis*	Negative	Negative	Smooth	Susceptible[d]	Resistant
M. *caprae*	Negative	Negative	Smooth	Susceptible[d]	Susceptible
BCG	Negative[c]	Negative	Rough	Susceptible	Resistant

[a] On Löwenstein-Jensen medium.
[b] TCH, thiophen-2-carboxylic acid hydrazide (tested on solid Löwenstein-Jensen medium).
[c] Some strains are weakly positive.
[d] Isoniazid-resistant M. *bovis* strains are also resistant to TCH.
[e] Can be resistant if the strain is already resistant to other first-line drugs.

species continues to grow, which highlights the lack of distinctive phenotypic properties. In general, only molecular tests provide a reliable identification. However, as previously stated, all results obtained by molecular methods should be consistent with established key phenotypic characteristics (Table 2). As an example, M. *chelonae* is an RGM with a preferred temperature of 30 ± 2°C. Incubating media inoculated with M. *chelonae* at higher temperatures may result in delayed growth, which might erroneously suggest a slowly growing NTM rather than a rapidly growing NTM.

Mycolic Acid Analysis

The various compositions of the cell wall mycolic acids of different species of mycobacteria were used for diagnostic purposes for many years (56–58). Use of high-performance liquid chromatography (HPLC) analysis of the mycolic acid pattern for identification of *Mycobacterium* species has largely been replaced by other methods (e.g., sequencing, mass spectrometry) that provide improved species discrimination.

GENOTYPIC IDENTIFICATION OF NONTUBERCULOUS MYCOBACTERIAL SPECIES

Complete Genome Sequences

Complete genomic sequences have been established for >40 mycobacterial species to date. Detailed information on all genome projects either completed or still in progress can be obtained at http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genomeprj&Cmd=Search&TermToSearch=txid176.

Single Gene or 16S-23S rRNA Spacer Region Analyses

Partial sequence analysis of selected genes or sequencing of the 16S-23S rRNA internal transcribed spacer region (ITS) is the most practical method for identification of mycobacterial species. Sequence variability among species, but homogeneity within species, is the basic prerequisite for this application. For several genes (16S rRNA, *hsp65*, and *rpoB*), specific regions within the gene that work well for species identification have been identified (34, 41, 59–66). The most important target for identification of slowly growing mycobacteria is the 16S rRNA gene.

16S rRNA Gene

For routine identification of mycobacteria, sequence analysis of the complete 16S rRNA gene (approximately 1,500 bp) is not practical and also usually not necessary. The information content of a sequence stretch of approximately 500 bp located at the 5′ end of the gene is sufficient for identification of most species and is the only sequencing kit currently commercially available. Specific primers for amplification and sequencing of the mycobacterial 16S rRNA gene (Table 5) have been described and validated (61, 67–74).

Primers 264 and 247 target genus-specific sequence regions and can be used for the detection of mycobacteria even in the presence of contaminating bacteria. Furthermore, the 285 and B9 primers can be used for the sequencing reaction. Either of these can be combined with any of the reverse

TABLE 5 Primers for amplification and sequencing of several mycobacterial target genes/sequences

Target	Primer—orientation[a]	Sequence	Product size (bp)	Reference
16S rRNA	285—F	5′-GAGAGTTTGATCCTGGCTCAG	~1,000	61
16S rRNA	264—R	5′-TGCACACAGGCCACAAGGGA		61
16S rRNA	B9—F	5′-CGTGCTTAACACATGCAAGTC	~550	205
16S rRNA	247—R	5′-TTTCACGAACAACGCGACAA		
ITS 1	Sp1—F	5′-ACC TCC TTT CTA AGG AGC ACC	~300	81
ITS 1	Sp2—R	5′-GAT GCT CGC AAC CAC TAT CCA		81
ITS 1	ITS 1—F	5′-GAT TGG GAC GAA GTC GTA AC	~400	76
ITS 1	ITS 2—R	5′-AGC CTC CCA CGT CCT TCA TC		76
hsp65	Tbll—F	5′-ACCAACGATGGTGTGTCCAT	441	86
hsp65	Tb12—R	5′-CTTGTCGAACCGCATACCCT		
gyrB	MTUB—F	5′-TCG GAC GCG TAT GCG ATA TC	1,020	94
gyrB	MTUB—R	5′-ACA TAC AGT TCG GAC TTG CG		
rpoB	Myco—F	5′-GGCAAGGTCACCCCGAAGGG-3′	723	34
rpoB	Myco—R	5′-AGCGGCTGCTGGGTGATCATC-3′		

[a] F, forward; R, reverse.

primers. Both forward primers are located upstream of hypervariable regions A and B, which enable the species-specific identification of most mycobacterial species (61). A few slowly growing species have identical hypervariable regions A and B or an identical complete 16S rRNA gene sequence. These include (i) M. *marinum* and M. *ulcerans* and (ii) M. *kansasii* (sequence variants I and IV) and M. *gastri*. For accurate identification of these species, sequence analysis of other genes or regions (e.g., ITS, *hsp65*, and *rpoB*) is required. All members of the MTBC have identical 16S rRNA gene sequences and thus cannot be discriminated by this technique. In addition, some species have minor intraspecies 16S rRNA gene sequence variants that differ in a few base pairs (e.g., M. *gordonae*, M. *kansasii*, and M. *lentiflavum* [75–77]).

23S rRNA Gene

The 23S rRNA gene is also known to contain conserved and variable sequence regions that enable the specific amplification and species identification of mycobacteria (78, 79). Variable regions can also be found in the 5′ region. The disadvantage of this target is the length of the gene (3,100 bp), which is not readily analyzed for most of the *Mycobacterium* species since amplification and sequencing must be performed with multiple sets of primers (79).

ITS 1 Region

Another target is the internal transcribed spacer sequence, which separates the 16S and 23S rRNA genes in the operon and is denominated ITS 1. The sequence of this fragment comprises only 200 to 330 bp and thus can easily be analyzed. Several sets of primers that enable the amplification and sequencing of the complete fragment have been published (Table 5) (76, 80, 81).

Primers Sp1 and Sp2 allow the genus-specific amplification of the region. Since they target the start site of the ITS, they are not optimal for analysis of the whole ITS 1 sequence. Primers ITS 1 and ITS 2 are located in the 16S and 23S rRNA gene regions, respectively. Using these primers, the entire ITS can be amplified and sequenced. However, they are not genus specific and cannot be used when cultures of mycobacteria are contaminated with other bacteria.

For the ITS 1 sequence, a high variability that could be used for species identification has been shown (80, 82–84). However, for some species, mainly for rapidly growing species but also for some slowly growing species (M. *simiae* and M. *xenopi*), two or more sequence variants have been observed. As with the 16S rRNA gene sequence, M. *marinum* and M. *ulcerans* have identical ITS 1 sequences and thus cannot be differentiated by this analysis.

hsp65 Gene

One of the first genetic targets used for the differentiation of mycobacteria is an approximately 440-bp fragment of *hsp65*, which codes for the 65-kDa heat shock protein and is also known as the *groEL2* gene (64). The amplification of this fragment, followed by a restriction enzyme digestion using the restriction enzymes BstEII and HaeIII and by analysis of the obtained digestion products using agarose gel electrophoresis, provides restriction fragment length polymorphism (RFLP) patterns that are specific for most clinically significant species (Table 5) (85, 86). An algorithm for the differentiation of the most clinically important species showing the apparent molecular sizes of the fragments has been devised. This PCR-restriction enzyme analysis technique has been used widely, since this is a simple and rapid method with no need for more-sophisticated sequencing techniques. However, technical difficulties, such as small size differences between the fragments or the incidence of similar or identical restriction patterns for some species of closely related mycobacteria and, in particular, new species of mycobacteria, are problems encountered with this method. With the greater availability and decreasing cost of sequencing, sequence analysis of this *hsp65* gene fragment has been increasingly used instead of the PCR-restriction enzyme analysis (63, 87).

Advantages of sequencing *hsp65* rather than the 16S rRNA gene are especially evident in the identification of closely related species, as *hsp65* is much less conserved than the 16S rRNA gene. *hsp65* sequencing allows for differentiation of M. *marinum* from M. *ulcerans*, M. *gastri* from M. *kansasii*, and, with some restrictions, M. *avium* subsp. *avium* from M. *avium* subsp. *hominissuis*.

rpoB Gene

The *rpoB* gene encodes the β subunit of the bacterial RNA polymerase. For M. *tuberculosis*, mutations in an 81-bp region of *rpoB* (rifampin resistance-determining region) are known to confer resistance to rifampin. For NTM species, sequence variability within *rpoB* can be used for species identification (34, 60, 88). Several different sequence fragments of the approximately 3,600-bp gene have been used for amplification and sequence determination. Kim et al. (60) amplified a fragment comprising 306 bp at positions 1362 to 1668 (referred to as region 2/3), whereas Lee et al. (88) targeted a 360-bp sequence at positions 902 to 1261 (referred to as region 1/2) (34). In contrast, Adékambi et al. (34) chose a fragment more distant (region 5) and analyzed an approximately 760-bp fragment at positions 2573 to 3337 (Table 5). In the studies of Kim et al. (60) and Lee et al. (88), type strains of many slowly growing mycobacteria were included, whereas detailed analyses using region 5 (34) have been performed mainly for rapidly growing mycobacteria. Extensive investigations using clinical isolates of slowly growing mycobacteria are not available for any part of the *rpoB* gene to date.

Use of Sequence Databases

For identification of mycobacteria by sequencing analysis, public databases are frequently used, which can present challenges. Public databases contain sequences from strains that are not validly published as species, sequences from strains identified as known species but having divergent sequences, and many sequences from uncultured bacteria that are not further characterized. Furthermore, old sequences that are fragmentary or faulty are still included in the public databases (89).

Thus, the evaluation of the search result has to be done carefully. For example, in the case of an uncommon species, cross-checking of the valid description as a species can easily be performed by using the *List of Prokaryotic Names with Standing in Nomenclature* (http://www.bacterio.net). When obtaining several correct results deriving from different species, it may help to check the database entries if the sequences have been obtained from type strains.

The International Nucleotide Sequence Database Collaboration database (INSDC; http://www.insdc.org/) can be used for the analysis of the sequences derived from the different genes as detailed above. There are some curated databases and analysis tools that have been constructed for the analysis of the 16S rRNA gene sequences, such as the commercially available tools SmartGene IDNS (SmartGene, Lausanne, Switzerland), RipSeq (Isentio, Sunnyvale, CA), and the MicroSeq microbial identification system (Applied Biosystems, Foster City, CA) or two tools with free access, the Ribosomal

Database Project (RDP; http://rdp.cme.msu.edu/) and the RIDOM (Ribosomal Differentiation of Medical Microorganisms) database (http://www3.ridom.de/rdna/). The advantage of these databases is the quality control of the entries, ensuring that most of the difficulties associated with public databases will not be encountered. For the commercial Web-based databases, the user also has the option to update the system as needed. However, these curated databases can lag behind the updates of the INSDC database and may not include sequence variants of species.

Pyrosequencing

Pyrosequencing differs from Sanger sequencing in that it relies on the detection of pyrophosphate release on nucleotide incorporation rather than chain termination with dideoxynucleotides. Identification of mycobacteria by pyrosequencing relies on a 20- to 30-bp region within the hypervariable region A of the 16S rRNA gene (90–92). A study evaluating pyrosequencing for the analysis of AFB isolates was performed by Bao et al. in comparison to phenotypic analysis and Sanger sequencing. Pyrosequencing was capable of identifying 114 (98%) of the 117 AFB isolates and correctly identified most of the slowly growing mycobacteria to the species level (92). Of the mycobacteria tested, it was not able to identify *M. scrofulaceum* and *M. simiae* to the species level. Similar to Sanger sequencing of the 16S rRNA gene, pyrosequencing has the same limitation for identification of some of the closely related organisms, as discussed in "Single Gene or 16S-23S rRNA Spacer Region Analyses" above.

Pyrosequencing has the advantage of being a rapid, simple, and inexpensive molecular method for identification of *Mycobacterium* species. Additional sequencing may be required to differentiate some species due to the short sequence length generated by this technique (91, 93).

GENOTYPIC IDENTIFICATION OF SPECIES OF THE MTBC

Within the MTBC, the most prevalent species is *M. tuberculosis* (~93 to 97%), followed in frequency by *M. bovis* and *M. bovis* BCG. Identification of *M. bovis* is of clinical relevance because of the inherent resistance of this species to pyrazinamide, one of the first-line agents for treatment of TB. Strains that are monoresistant to pyrazinamide should be analyzed by molecular techniques. These strains can be *M. bovis*, *M. bovis* BCG, or, although rare, monoresistant *M. tuberculosis* strains. Moreover, *M. bovis* BCG is used for treatment of bladder cancer, and thus correct identification of *M. bovis* BCG isolated from patients treated for bladder cancer is necessary to enable a correct decision for treatment of a patient. The identification of the other species is mainly of epidemiological importance and may be an indication of the source of the infection.

gyrB Gene Sequencing

The *gyrB* gene encodes the B subunit of DNA gyrase (topoisomerase II), an enzyme essential for bacterial replication. In 2000, Kasai et al. (94) showed single nucleotide polymorphisms (SNPs) in an approximately 1.2-kbp fragment of the *gyrB* gene which were specific for some species of the MTBC (Table 5). More-detailed analyses confirmed these results and determined that they could be extended to most members of the MTBC (95).

RD/Spoligotyping

Although all members of the MTBC are characterized by a high genetic similarity, several sequence polymorphisms that can be used for species identification have been discovered. Comparative genomic analysis has shown that members of the MTBC have evolved from a common ancestor through sequential DNA deletions with precise genomic deletions (96). These areas in the genome are exploited for the identification of the members of the MTBC and are referred to as regions of difference (RD). The presence or absence of these deletions can be analyzed by PCR assays (97). Spoligotyping, a technique applied primarily for strain typing, identifies most species within the MTBC. However, with this technique, *M. bovis* BCG cannot be discriminated from *M. bovis*.

Line Probes Specific for MTBC

A commercially available assay (GenoType MTBC; Hain Lifescience, Nehren, Germany) based on line probe technology enables the identification of species within the MTBC (98–101). The test can be performed from solid or liquid media, with a total test time of 4 to 6 hours. The assay is based on an MTBC-specific 23S rRNA gene fragment, *gyrB* DNA sequence polymorphisms, and the RD1 deletion of *M. bovis* BCG. Specific oligonucleotides targeting these polymorphisms are immobilized on membrane strips. Amplicons derived from a multiplex PCR react with these probes during hybridization. Species can be identified according to the interpretation table provided with the kit. The inclusion of an MTBC-specific 23S rRNA gene fragment confirms the presence of MTBC in order to rule out possible cross-reactivity. Specific patterns can be obtained for *M. tuberculosis*/*M. canettii*, *M. africanum*/*M. pinnipedii*, *M. microti*, *M. caprae*, *M. bovis*, and *M. bovis* BCG. *M. tuberculosis* and *M. canettii* as well as *M. africanum* and *M. pinnipedii* share identical *gyrB* sequences, and thus they can be identified by specific patterns, but they cannot be differentiated from each other.

The specificity of this test was 100% when strains from culture collections or clinical isolates were compared (99–101). With strains from culture collections, all species, with the exception of an *M. canettii* strain (identified as *M. tuberculosis*), were correctly identified (101). Additionally, 100% of the clinical strains studied were correctly identified (99–101). No false-positive results were obtained with several NTM strains (101).

Real-Time PCR Assays

Laboratory-developed, multiplex real-time PCR assays have been described for the rapid identification of MTBC members to the species level from both culture and clinical specimens. The presence or absence of regions of difference (RD) allows identification to the species level of the MTBC using real-time PCR analysis (96, 102).

Other Molecular Tests for NTM and MTBC

Sequence analysis of target genes for the identification of mycobacteria may not be practical for routine clinical laboratories. Commercially available assays that are based on liquid- or solid-phase hybridization have been shown to be easily implemented into a routine workflow. They are intended for the detection of some of the most important *Mycobacterium* species and can be performed from both solid and liquid media.

AccuProbe Culture Identification Tests

The AccuProbe test was the first commercial molecular assay for the identification of selected mycobacterial species from positive culture media. Probes are available for the identification of MTBC, *M. avium*, *M. intracellulare*, MAC, *M. gordonae*, and *M. kansasii*. All probes are FDA cleared and commercially available. Species identification is based on the hybridization of specific DNA probes to rRNA of the bacteria. Briefly, by heat treatment and sonication, nucleic acids, including the target 16S rRNA, are released from the mycobacteria. A specific DNA probe hybridizes with the target rRNA. Finally, the DNA-rRNA hybrid molecule can be detected by chemiluminescence. The results are obtained within 2 hours. The utility of these tests has been proven in many studies and by usage in many laboratories worldwide (76, 103–107). Specificities of the tests are usually reported to be 100%, or sometimes lower (96%), for MAC. Sensitivity values vary with the test used: 100% for *M. gordonae*, 95.2% for MAC, and 97.4 to 100% for *M. kansasii* (76, 103, 104, 107). It has also been shown that MTBC organisms can reliably be identified in the presence of *M. avium* (105). However, there are also some disadvantages of these tests. The probes are limited to a few, although important, mycobacterial species, necessitating the performance of additional tests for identification of species for which there are no probes. The need to perform individual tests for each target species renders the tests expensive and, if not performed in parallel, also time-consuming. False-positive results for MAC probes have been reported, but they can be prevented if a higher cutoff value (80,000 relative light units instead of the 30,000 relative light units recommended in the technical insert) is used (108). Studies have also shown cross-reactions with the *M. intracellulare* probe and several slowly growing mycobacterial species (*M. arosiense*, *M. chimaera*, *M. nebraskense*, and *M. saskatchewanense*) (109). Similarly, cross-hybridizations have been documented with the MAC probe and *M. nebraskense*, *M. palustre*, *M. saskatchewanense*, and *M. paraffinicum*. Cross-reactivity has been seen with the MTBC probe and *M. celatum* (110) and *M. holsaticum* (109), although this cross-reactivity can be avoided when the selection step of the procedure is performed for 10 min, according to the manufacturer's instructions (111).

Line Probe Assays

Alternatively, techniques based on the application of PCR plus reverse-hybridization-designed DNA strip assays (line probe assays), have been developed. Briefly, the target sequences are amplified by PCR using biotinylated primers. The amplified PCR products are allowed to hybridize to immobilized, membrane-bound probes covering the species-specific sequence fragment, followed by an enzyme-mediated color reaction. The banding patterns can be analyzed by eye by comparing the patterns on an interpretation chart. The identification of the species relies on specific banding patterns (Fig. 5).

Three tests for the identification of several mycobacterial species are commercially available but are not yet FDA cleared. The INNO-LiPA Mycobacteria v2 assay (Innogenetics, Ghent, Belgium) is based on the nucleotide differences in the 16S-23S rRNA gene spacer region and can detect the following slowly growing *Mycobacterium* species: MTBC, *M. kansasii*, *M. xenopi*, *M. gordonae*, *M. genavense*, *M. simiae*, *M. marinum*, *M. ulcerans*, *M. celatum*, *M. avium*/*M. intracellulare*/*M. scrofulaceum* complex, *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. malmoense*, and *M. haemophilum*.

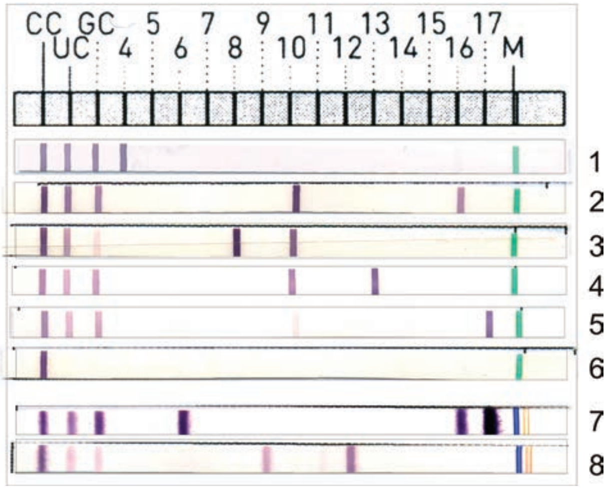

FIGURE 5 Line probe assay (GenoType Mycobacterium CM/AS) for the identification of *Mycobacterium* species. CC, conjugate control; UC, universal control; GC, genus-specific control; 4 to 17, specific bands for identification of the species; M, marker line (flags the upper side of the strip). Lines 1 to 6, CM strips; lines 7 and 8, AS strips. First lane, *M. avium*; second lane, MTBC; third lane, *M. gordonae*; fourth lane, *M. malmoense*; fifth lane, *M. xenopi*; sixth lane, negative control; seventh lane, *M. genavense*; eighth lane, *M. haemophilum*. doi:10.1128/9781555817381.ch31.f5

The GenoType Mycobacterium CM/AS test (Hain Lifescience, Nehren, Germany), is based on the detection of species-specific sequences in the 23S rRNA gene. It is composed of two different strips (strip CM is for common mycobacteria, and strip AS is for additional species) and can identify the following slowly growing mycobacteria: with strip CM, *M. avium*, *M. gordonae*, *M. intracellulare*/*M. chimaera*, *M. scrofulaceum*/*M. paraffinicum*/*M. parascrofulaceum*, *M. interjectum*, *M. kansasii*, *M. malmoense*/*M. haemophilum*/*M. palustre*/*M. nebraskense*, *M. marinum*/*M. ulcerans*, MTBC, and *M. xenopi*; and with strip AS, *M. simiae*, *M. celatum* (types 1 and 3), *M. genavense*/*M. triplex*, *M. lentiflavum*, *M. heckeshornense*, *M. szulgai*/*M. intermedium*, *M. haemophilum*/*M. nebraskense*, *M. kansasii*, *M. ulcerans*, *M. gastri*, *M. asiaticum*, and *M. shimoidei* (a slash separating two or more species means that they share the same pattern).

Both the Innogenetics and Hain Lifesciences tests can be conducted on isolates from liquid or solid culture media, and the results are available in approximately 4 to 6 hours. Several studies have evaluated the usefulness of the tests. Concordant results of 89.9 to 98.6% for the GenoType CM and AS assays with standard methods (e.g., sequencing) have been reported (112–117). Most (up to 96%) of the regularly encountered species already can be identified using the GenoType CM assay alone (116). A limitation of this test is the resolution of patterns that are shared by more than one species, which necessitates additional techniques for further discrimination (e.g., sequencing of additional gene targets or biochemical tests) leading to a delay in obtaining a final result. For the INNO-LiPA assay, 92.2% to 98.4% concordant results with standard methods have been reported (115, 118). The assay can further differentiate *M. kansasii* and *M. chelonae* strains into subtypes. The clinical significance of this information so far is unclear, and thus this knowledge is mainly of epidemiological value.

Using line probe assays, more than 90% of the commonly encountered mycobacteria can rapidly be identified. In a few cases, false identification has been reported (109, 116, 118). For the GenoType AS assay, results for *M. celatum* should be reported only if they are obtained from solid cultures with appropriate growth rates and typical colony morphology. Recent studies have shown that the GenoType

M. *intracellulare* probe cross-hybridizes with several slowly growing mycobacterial species, including M. *arosiense*, M. *chimaera*, M. *colombiense*, M. *mantenii*, and M. *saskatchewanense*. Two infrequently encountered NTM species, M. *riyadhense* and the nonvalidated species "M. *simulans*," were incorrectly assigned to the MTBC by the GenoType assay (109). Similarly, the INNO LiPA MAIS assay cross-reacts with M. *arosiense*, M. *heidelbergense*, M. *mantenii*, M. *nebraskense*, M. *parascrofulaceum*, and M. *paraffinicum* (109). Importantly, neither of the line probe systems has been reported to misidentify members of the MTBC as an NTM (109). All results obtained with line probe tests should be confirmed by key established phenotypic characteristics, such as growth rate, colony morphology, and pigmentation.

A third line probe assay was recently described, the Speed-oligo Mycobacteria (Vircel, Granada, Spain), and is based on targeting both 16S rRNA and 16S-23S rRNA regions. The assay can identify 19 mycobacterial species, including MTBC (M. *tuberculosis*, M. *bovis*, M. *microti*, M. *africanum*, M. *caprae*) and 10 additional NTMs (M. *avium*, M. *gordonae*, M. *interjectum*, M. *intracellulare*, M. *kansasii*, M. *malmoense*, M. *marinum*/M. *ulcerans*, M. *scrofulaceum*, and M. *xenopi*). The Speed-oligo Mycobacteria system has a limited spectrum of species identification compared to the other assays and identifies many of the mycobacterial species to the complex or group. It can be performed on isolates from solid or liquid media within 3 hours. The only clinically relevant misidentification reported in the limited studies published to date was the misidentification of M. *marinum* as M. *kansasii* (119, 120). The Speed-oligo Mycobacteria system has also been evaluated for use directly from respiratory specimens and demonstrated an overall sensitivity of 76% (93% from smear-positive and 56% from smear-negative specimens) and specificity of 99%. The study concluded that the assay is an easy and fast alternative for detecting mycobacteria and differentiating between MTBC and NTM, especially from smear-positive specimens (121).

Multiplex Real-Time PCR Assays

There are a handful of reports in the literature on the use of multiplex real-time PCR with probe-based detection for the rapid identification of MTBC and NTMs from culture isolates (122) or for the differentiation of members of the MTBC (123, 124). More recently, multiplex real-time PCR coupled with high-resolution melting curve analysis has been reported for the detection of mutations in MTBC isolates associated with resistance to first- and second-line antituberculous agents (125, 126). A commercial multiplex real-time PCR assay (Seegene, Seoul, South Korea) uses a proprietary technology that enables simultaneous screening and detection of multiple targets using a single fluorescent channel. This technology has been used for discrimination between NTM and MTBC directly from clinical specimens using the AnyplexTM MTB/NTM assay. In addition, the Seegene Anyplex II MTB/MDR/XDR detects MTB, MDR-TB, and extensively drug-resistant (XDR) TB in a single real-time PCR from clinical specimens. Although the Seegene assays are not U.S. FDA cleared, they have received CE certification. Studies demonstrating the clinical utility of the Seegene multiple PCR assays for detection of *Mycobacterium* species are yet to be performed.

M. leprae PCR

As M. *leprae* is a nonculturable organism, PCR techniques have been developed and applied for its detection from a variety of specimen sources, such as slit-skin smears, blood, nasal cavity, biopsy specimens, and urine (127–132). Molecular targets for detection of M. *leprae* PCR have included protein antigens of 18 kDa, 36 kDa (known as the proline-rich antigen [pra]), and 65 and 85 kDa (the 16S rRNA gene and repetitive sequences) (127, 128, 130, 132–136). Many studies have shown that PCR methods are specific and more sensitive than conventional methods (split skin smears, histopathology, and serologies) for the diagnosis of M. *leprae* (127, 128, 130, 132–137).

Mass Spectrometry

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is revolutionizing the identification of microorganisms and has been recently applied to the identification of mycobacteria. MALDI-TOF MS analysis of mycobacteria involves several steps including inactivation, extraction, and analysis. Several different methods have been described for the inactivation and extraction of mycobacterial cells. Since there are additional safety concerns when dealing with acid-fast bacillus (AFB)-positive cultures, these methods differ from the extraction methods described for other bacteria and fungi. All inactivation and extraction steps should be performed within a BSL-3 facility using the appropriate personal protective equipment (PPE). MALDI-TOF analyses of both whole-cell (138) and purified protein extraction (139–141) methods have been described. Inactivation of mycobacterial cells is usually achieved either by ethanol or by heat treatment and dissociation/lysis of cells by mechanical lysis using either a micropestle or bead beating techniques. Extraction is performed by adding formic acid and acetonitrile to the inactivated mycobacterial cells (139, 141). Following inactivation and extraction, an aliquot is spotted onto a solid target support plate and overlaid with a chemical matrix (for example, alpha-cyano-4-hydroxy-cinnamic acid). The sample plate is loaded into the instrument, and mycobacterial proteins are ionized using a laser. The ionized proteins then move through the time of flight chamber and are separated based on the mass-to-charge ratio of the ions, such that the lighter proteins hit the detector first, followed by the heavier proteins. A mass spectrum is produced as the ions collide with the ion detector and serves as a "fingerprint" for identification of the mycobacterial species. Currently, there are two commercial MALDI-TOF platforms available for microbial identification, the Vitek MS (bioMerieux, Durham, NC) and the Biotyper system (Bruker Daltonics, Billerica, MA) (138–141). Databases supplied with both instruments contain entries for MTBC and many NTMs, including those most commonly encountered in the clinical laboratory. MALDI-TOF MS databases continue to improve in terms of species coverage and the depth of strain diversity, but more esoteric species may be underrepresented and may need to be added by the end user or may await future database updates by the manufacturers.

Recent studies have demonstrated the ability of MALDI-TOF MS to serve as a rapid and accurate means for the identification of mycobacteria from both solid and liquid media (138–141). Saleeb et al. (142) reported some of the earliest use of MALDI-TOF MS with the identification of 104 mycobacterial isolates comprising 17 species. Since this early report, others have also demonstrated that MALDI-TOF MS can unambiguously identify the most commonly encountered species of mycobacteria (143). However, species of the MTBC cannot be differentiated and the closely related M. *chimaera* and M. *intracellulare* are not separated, according to recent studies (138, 144).

A potential pitfall of the identification by MALDI-TOF MS in comparison to molecular sequencing methods is the requirement for a moderate amount of growth present on solid media rather than the scant growth required for sequencing. Not surprisingly, this problem is most evident when dealing with the slowly growing mycobacteria (141). In such circumstances, sequencing may provide a more rapid turnaround time (TAT; i.e., 12 to 24 h) for identification than MALDI-TOF analysis.

Although the costs of MALDI-TOF MS instrumentation are not insignificant, studies have shown that this technique has minimal associated reagent costs, requires minimal technologist time, can be utilized for a wide variety of microorganisms, and provides a reduction in TAT for organism identification, leading to an overall reduction in costs (145).

A second MS method is a novel technology coupling PCR amplification and electrospray ionization mass spectrometry (ESI-MS) to identify M. *tuberculosis* complex, MTBC resistance determinants, and NTM from culture within ~6 hours. This system measures the mass/charge ratio of PCR amplicons generated from several loci, focusing on conserved, species-specific regions and resistance determinants, to identify base compositions comparative to a database of mycobacteria and resistance determinants. Using the base composition as unique molecular signatures ("fingerprints"), the PLEX-ID system (Abbott Molecular, Des Plaines, IL) is able to identify the organism as an MTBC or NTM and provide resistance markers if an MTBC is identified (146, 147). A recent study demonstrated the ability of the MDR-TB assay on the PLEX-ID to appropriately identify all MTBC and all slowly growing NTMs tested from both broth (MGIT tubes) and solid media using database version NFDU.415.455.349. In comparison to the agar proportion method, the sensitivity and specificity for the detection of MTBC drug resistance using the MDR-TB assay were 100% and 92.3% for rifampin, 100% and 93.8% for isoniazid, 91.0% and 94.4% for ethambutol, and 100% and 100% for fluoroquinolones, respectively (148). Further studies need to be performed to determine if the MDR-TB assay is capable of detecting AFB directly from clinical specimens.

High instrument costs of the PCR ESI-MS system may hamper the implementation of this technology into small clinical microbiology laboratories, and therefore this technology may be limited to use in reference laboratories or state public health laboratories.

TYPING SYSTEMS

Historically, the first strain typing method for M. *avium* was serotyping based on seroagglutination procedures. Combined use of serotyping and species-specific DNA probes has shown that the organisms previously named serovars 1 through 6 and 8 through 11 are M. *avium* and that serovars 7, 12 through 17, 19, 20, and 25 are M. *intracellulare*. Finally, multilocus enzyme electrophoresis has been shown to provide a wider range of polymorphisms than serotyping (149). Serotyping, for both MAC and other mycobacteria, has been replaced by molecular strain typing.

Genotyping of *M. tuberculosis* Strains

The genotyping of M. *tuberculosis* isolates contributes to the knowledge and control of TB by indication of epidemiological links between patients, discrimination between exogenous reinfections and endogenous reactivations, outbreak detection, and the recognition of laboratory cross-contaminations. The CDC has initiated a laboratory program to provide genotyping services for TB control programs to public health laboratories. Further information may be obtained from their website at http://www.cdc.gov/tb/programs/default.htm.

The introduction of the IS*6110*-RFLP technique marked a milestone in determining the molecular epidemiology of M. *tuberculosis* and has been the reference standard for many years. More recently, several alternative PCR-based techniques, which are less time-consuming and in part more discriminative than the classical IS*6110*-based technique, have been developed.

IS*6110* RFLP Typing

IS*6110* is an insertion sequence that is present in variable numbers (0 to >20 [150]) and has been inserted at various positions in the genomes of MTBC isolates. IS*6110*-based RFLP analysis is performed using an internationally standardized protocol that facilitates comparison of patterns generated by different laboratories (151). High-quality DNA is isolated from mature culture isolates and subjected to restriction enzyme digestion. The DNA fragments are electrophoretically separated, transferred to a membrane, and hybridized with an IS*6110* probe. The resulting patterns are compared to an internal size standard. For large-scale comparisons, the patterns should be digitalized by scanning in order for them to be stored in a database. Using computer analysis, the results from separate runs can be compared to detect identical patterns.

The IS*6110* fingerprint patterns are highly discriminatory, but only for patterns with seven or more bands. The complete analysis is laborious and time-consuming if the time to obtain sufficient growth on solid media and the multiple technical steps are considered. Furthermore, the analysis of the complex banding patterns requires sophisticated pattern-matching computer software.

Spoligotyping

Spoligotyping (an abbreviation for *sp*acer *oligo*nucleotide *typing*) is a PCR-based technique that targets the variability in the direct repeat (DR) locus of M. *tuberculosis*. The DR region that is present in all MTBC strains consists of multiple DRs of a conserved 36-bp sequence separated by nonrepetitive unique spacer sequences. Of these, 43 spacer sequences identified in the genomes of M. *tuberculosis* and M. *bovis* BCG are used for the spoligotyping assay (152). The spacer segments are amplified by PCR using primers that target the DR sequence. Amplified DNA fragments are hybridized to a membrane with 43 covalently bound oligonucleotides complementary to the 43 spacers.

There are several advantages of spoligotyping compared to IS*6110* RFLP analysis. Since the technique is PCR based, a small amount of DNA is sufficient for analysis. Thus, spoligotyping can be performed from scantily grown solid as well as liquid cultures, enabling a more rapid turnaround time. The technique is less laborious and more rapid than the classical IS*6110* RFLP method. Additionally, the format of the result can easily be transferred into a binary code that can be handled in common computer programs, rendering large-scale comparisons less difficult than the classical method. Unfortunately, spoligotyping is less discriminatory than IS*6110* RFLP typing and not sufficient for population-based studies. It can be used for M. *tuberculosis* strains with few IS*6110* copies or for M. *bovis* isolates that also have few IS*6110* copies. This technique is useful for the analysis of assumed laboratory cross-contaminations, as the results can be obtained rapidly.

MIRU-VNTR Typing

Genotyping may be performed based on the variable numbers of tandem repeats (VNTR) of different classes of genetic elements named mycobacterial interspersed repetitive units

(MIRU) (153, 154). This technique is based on the amplification of multiple repeat loci and the subsequent determination of the amplicon size, which depends directly on the number of VNTR copies. Initially, 12 different loci were shown to be suitable for analysis and were considered to possess discriminatory power comparable to that of IS*6110* RFLP analysis (155). This method was adopted in the previously discussed CDC TB genotyping program. Subsequent studies, however, have found the 12-locus assay, even when combined with spoligotyping, to be less discriminatory than the classic RFLP method. The use of a new set of 24 loci for epidemiological studies has been proposed (153, 156, 157). A subset of 15 highly discriminatory loci among the 24 loci provides sufficient discriminatory information for routine epidemiological discrimination.

The amplification of the various loci is usually performed in a multiplex format. The PCR products can be analyzed using a DNA sequencing instrument. For small-scale comparisons, analysis of PCR products using classical agarose gel electrophoresis is also possible. The result format is a series of 12, 15, or 24 digits (depending on the MIRU format employed). The number designates the number of repeats at each locus. This format is portable between laboratories and thus simplifies the comparison of large databases. The MIRU-VNTR technique is rapid, with minimal growth required, and is increasingly replacing the IS*6110* RFLP reference standard method.

Multilocus Sequence Typing

Multilocus sequence typing (MLST) directly measures the DNA sequence variations in a set of housekeeping genes in M. *tuberculosis* and characterizes strains by their unique allelic profiles. MLST involves PCR amplification of 7 housekeeping genes (*gyrA*, *gyrB*, *katG*, *purA*, *recA*, *rpoB*, and *sodA*) followed by DNA sequencing (158). A recent study comparing MLST to MIRU-VNTR and spoligotyping was performed to evaluate the discriminatory power between the three methods to assess the genetic diversity of M. *tuberculosis*. Of the three methods, MIRU-VNTR showed the highest discriminatory power followed by spoligotyping followed by MLST. The authors of the study conclude that the combination of MIRU-VNTR and spoligotyping provides the greatest level of discriminatory power and therefore is the most useful genotyping tool to be applied to M. *tuberculosis* isolates (158).

Genotyping of Slowly Growing NTM

Different approaches have been used for strain comparisons of NTM. Whole-genome analyses have included pulsed-field gel electrophoresis (PFGE) (159–163), amplified fragment length polymorphism analysis (AFLPA) (164), or RFLP typing using specific molecular markers (162, 165). For PFGE and AFLPA, no genetic information is necessary and the procedures can be performed without further knowledge of the species genome. For RFLP typing, some genetic information, such as the presence of specific IS elements, is a prerequisite.

Pulsed-Field Gel Electrophoresis

PFGE has been a useful technique for strain typing of the slowly growing NTM. This technique requires an actively growing culture and 3 to 4 weeks for completion. Problems with standardization may result from cell clumping of rough strains. This clumping may also result in different amounts of DNA even from different batches of the same strain, causing problematic uneven (light and overloaded) lanes. Previous studies have detailed the method of PFGE related to clinical and epidemiologic studies of M. *kansasii*, M. *szulgai*, and MAC (166–171). Recently, PFGE has been applied to study macrolide-susceptible and -resistant isolates and multiple cultures of patients with nodular MAC disease and bronchiectasis (166).

Multilocus Sequence Typing

Another approach for NTM typing is analysis of multiple genes with a high sequence variation, like *hsp65* or ITS. However, available sequences have not provided the level of variability needed for epidemiological analyses. With the progress of whole-genome analysis and the possibility of large-sequence determination, however, multilocus sequence typing is likely to prove increasingly useful for typing of NTM.

Repetitive-Unit Sequence-Based PCR

Repetitive-unit sequence-based PCR (rep-PCR) has been used for outbreak control of several slowly growing mycobacterial species, including M. *simiae*, M. *gordonae*, M. *terrae*, M. *tuberculosis*, and M. *avium* complex (172–174). The method is commercially available through the DiversiLab System (bioMérieux, Durham, NC). Although the technology is proprietary, the company offers a Web-based library of rep-PCR sequences that can be searched for matches. Few studies have compared rep-PCR results to those obtained with PFGE.

Variable-Number Tandem Repeat

MIRU-VNTRs were initially applied to typing of M. *avium* subsp. *paratuberculosis* (MAP) strains but now have been expanded to other members of the MAC (175–177). Although the VNTR loci are usually species specific, the low cost, reproducibility, smaller volume of DNA requirement, and speed of the VNTR technique suggest that it could be a substitute method for PFGE strain comparison with MAC (178).

IS*1245*/IS*900*

The most extensive investigations for typing of NTM strains have been undertaken for M. *avium*. RFLP analysis based on the insertion sequence IS*1245*, which is restricted to subspecies of M. *avium*, has been used for genotyping. Standardization of this technique was proposed in 1998 (179), but this technology never became widespread. However, Mijs et al (180) used this method as one tool for distinguishing M. *avium* subsp. *avium* strains obtained from bird specimens from swine- and human-derived strains, for which they proposed the name "M. *avium* subsp. *hominissuis*." RFLP analysis based on the insertion sequence IS*900* can be used for genotyping of MAP.

The availability of the complete genome sequences of the "M. *avium* subsp. *hominissuis*" strain 104 and the MAP strain K-10 allowed the search for divergent regions that can be used for standardization of other techniques for genotyping (165). Based on the genomic sequence data, large-sequence polymorphisms, which were analyzed for their variability among different M. *avium* strains, could be identified. By the use of multilocus sequence typing, many genes that contain multiple sequence variations can now be analyzed in parallel (181).

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial susceptibility testing (AST) of clinically relevant mycobacteria is crucial for the control of infections caused by many species of mycobacteria and should be performed for all species, for which CLSI guidelines are available (182). Procedures are technically demanding and require rigid safety precautions, especially when liquid cultures are involved. When handling positive cultures with a high concentration of bacilli, cross-contamination is another critical issue. Therefore, antimicrobial susceptibility testing should be performed only by experienced personnel.

Nontuberculous Slowly Growing Mycobacteria

AST of the NTM requires skill and knowledge of the individual species characteristics. Therefore, the CLSI has advised laboratories that infrequently encounter NTM to refer those isolates to a qualified reference laboratory. Laboratories that elect to perform in-house NTM AST should carefully validate their results and monitor their proficiency regularly, as required by specific accrediting agencies, such as the College of American Pathologists (182).

Generally, broth micro- or macrodilution techniques in serial 2-fold concentrations are recommended. Testing is most efficiently performed in 96-well microtiter panels. Breakpoints for some species of slowly growing mycobacteria have been proposed for the following antimicrobials: rifampin, rifabutin, amikacin, ethambutol, ciprofloxacin, moxifloxacin, minocycline, doxycycline, clarithromycin, trimethoprim-sulfamethoxazole, and linezolid (182).

The CLSI recommendation specifies testing for slowly growing species, including *M. kansasii*, MAC, and *M. marinum*. The CLSI and the statement of the American Thoracic Society and the Infectious Diseases Society of America have recommended that only rifampin and clarithromycin results be reported for *M. kansasii* unless the isolate is rifampin resistant; if so, all of the previously listed antimicrobials should be tested (24, 182).

For isolates of *M. marinum*, experts in mycobacterial susceptibility testing concur that AST is unnecessary in most cases due to the narrow range of MICs for this species. In cases of intolerance to agents typically effective for the treatment of infections due to *M. marinum* (i.e., clarithromycin, rifampin, and ethambutol), antimicrobial testing of the previously listed agents should be performed (182).

For MAC, because previous studies have shown that no correlation exists between MICs and the clinical response of the patient to agents other than clarithromycin, testing of only clarithromycin is recommended. However, a recent study of patients with MAC has shown a correlation of amikacin MICs to clinical response and amikacin breakpoints have been proposed to the CLSI (183). Interpretive criteria are also provided for moxifloxacin and linezolid, but no clinical correlation studies have been performed to date (182).

Other species of slowly growing NTM have insufficient data to make specific recommendations for testing, and therefore the CLSI recommends that testing of these NTM should follow the guidelines for testing rifampin-resistant *M. kansasii*.

Fastidious species that require iron- or hemin-containing media for growth, such as *M. haemophilum*, may be tested using an alternative agar disk elution method with commercial antimicrobial disks in molten agar. The CLSI has recommended testing of antimicrobials, including rifampin, clarithromycin, amikacin, ciprofloxacin, trimethoprim-sulfamethoxazole, linezolid, and doxycycline or minocycline (182).

Specific details and discussion of the AST procedures can be found in chapter 76 of this *Manual*.

M. tuberculosis Complex

Currently, methods for testing MTBC are based on the method of proportion, which relies on a clinical definition of drug resistance. Susceptibility testing of MTBC may be performed in agar-based (agar proportion method) or broth-based (commercial) systems. Agar proportion methods may be performed using either commercially prepared or in-house media.

Both the agar proportion and newer liquid detection systems define resistance as growth of >1% of an inoculum of bacterial cells in the presence of a "critical" concentration of the drug. By convention, the critical concentration corresponds to the lowest concentration of drug that inhibits 95% of "wild-type strains" of MTBC that have never been exposed to the antimicrobials without simultaneously inhibiting strains of MTBC from patients who do not respond to treatment and that are considered resistant. The critical concentration is thus the standard concentration by which susceptibility and resistance are established.

A newer method for MTBC susceptibility testing uses a 96-well microtiter panel containing 12 lyophilized first- and second-line antimycobacterial agents. In contrast to the more common agar proportion methods, the MycoTB panel (Thermo Scientific/TREK Diagnostics, Oakwood Village, OH) tests a range of concentration of each antimycobacterial providing a MIC that is consistent with AST formats for other bacterial organisms. Two studies comparing the Sensititre MycoTB panel have demonstrated 94 to 100% categorical agreement with the reference agar proportion method. The Sensititre MycoTB panel appears to be a more rapid (10 to 14 days versus 21 days), quantitative, and efficient method than the gold standard agar proportion method (184, 185). An additional advantage to the Sensititre MycoTB panel is the availability of second-line susceptibility results in the case of a resistant strain. The disadvantage is the absence of pyrazinamide in the panel, which would require testing by another method. Another disadvantage is potential safety concerns associated with working in microtiter plates with possible drug-resistant strains.

Generally, the first isolate of MTBC cultures from each patient should be tested, and susceptibility testing should be repeated if the patient fails to respond to therapy or if the cultures remain positive at 2 to 3 months. Primary susceptibility testing includes a battery of antimicrobials, including isoniazid at two concentrations (critical and higher concentrations), rifampin, ethambutol, and pyrazinamide. When an isolate is resistant to rifampin or any two of the other primary drugs, a secondary panel including a higher concentration of streptomycin and additionally capreomycin, ethionamide, amikacin, *p*-aminosalicylic acid, rifabutin, cycloserine, linezolid, moxifloxacin, and levofloxacin should be tested (182).

Direct susceptibility testing of smear-positive samples from patients known to have or suspected of having *M. tuberculosis* involves inoculation of drug-containing medium with a directly processed sample (i.e., concentrated after decontamination and digestion). Indirect susceptibility testing may be performed using cultures already growing either in liquid or in solid medium. This method is usually used on smear-negative samples or if the direct test results are invalid due to contamination, insufficient numbers of colonies in the drug-free quadrants, or insufficient growth after 3 weeks of incubation. Details on both methods of susceptibility testing can be found in chapter 76. Standardized agar proportion methods are not considered rapid methods. Therefore, the addition of a commercial liquid susceptibility system with shorter incubation is strongly recommended for patient testing. It is imperative, however, that any commercial system be validated to produce results that correlate with the standard susceptibility agar proportion methods. As with any other laboratory test, adequate and consistent quality control and proficiency testing should be performed to ensure accurate and consistent results (182).

Molecular detection of drug resistance markers in MTBC generally makes use of laboratory-developed PCR or sequencing methods that target well-characterized genes (*katG*, *rpoB*, *pncA*, *gyrB*, etc.) that have mutations associated with resistance to first- and second-line agents (186). These LDTs have been used for detection of resistance markers from culture isolates and, in some instances, directly from respiratory specimens. In addition, the commercially available Xpert TB/RIF assay (Cepheid), discussed earlier in this chapter, provides information about rifampin resistance directly from respiratory specimens. The CDC offers a service for the rapid molecular detection of drug resistance in MTBC using sequencing methods from culture isolates or NAA test-positive sediments. Targets associated with resistance to both first- and second-line agents are interrogated with reported sensitivities of 97.1% for *rpoB*, 85.4% for *katG*, 84.6% for *pncA*, 78.6% for *embB*, 81.6% for *gyrA*, and 90.8% for MDR TB (187).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Implementation of sensitive and rapid methods for the detection, identification, and susceptibility testing of MTBC and NTM is of paramount importance to the diagnosis and control of TB and NTM disease. The use of the fluorochrome stain for mycobacterial smears, a broth-based culture system, and the use of DNA probes or sequence analysis for identification along with direct susceptibility testing of smear-positive specimens are optimal for supporting the critical decisions required for optimal patient care. As emphasized in this chapter, phenotypic testing of the slowly growing mycobacterial species has limited use and for other mycobacteria (i.e., rapidly growing mycobacteria) should be applied only in conjunction with molecular assays. Laboratory work should be organized to prioritize timely and accurate identification of MTBC and susceptibility testing of clinically significant isolates.

In order to optimize rapid detection, identification, and AST for the shortest turnaround times, the U.S. Association of Public Health Laboratories has recommended that the Fast Track Referral Model System be implemented. This network system helps to ensure that quality state-of-the-art technology is used, results are reported in a timely manner, and the tracking and fingerprinting of isolates of MTBC occur efficiently for optimal exchange of information between medical centers and the public health sector (54).

CLSI guidelines recommend reporting results within 24 hours for acid-fast bacterium smears and interim reports if growth is seen on inoculated solid medium or when a positive signal is detected on liquid medium. A confirmatory report should be issued once growth is evident, and an identification of either NTM or MTBC should be reported. Moreover, reporting should be done as soon as species level identification is available for NTM and, finally, when susceptibility testing is completed (usually within 7 to 14 days for NTM with broth systems). Additionally, for MTBC, identification from culture results should be reported within an average of 14 days, and a complete culture report with susceptibility results should be reported within an average of 4 to 6 weeks. The use of liquid medium rapid detection systems along with solid medium is critical in order to meet these guidelines (24, 54, 182).

In an effort to develop a more consistent and practical method of reporting identifications performed by molecular techniques, the CLSI (54) has published interpretive criteria for the identification of mycobacterial species by DNA target sequencing, including consensus cutoff values for percent identity. The document states that mycobacteria with 100% 16S rRNA partial (500-bp) gene sequence identity for genus and species may be definitively reported with both genus and species. However, for those isolates whose identity is 99.0% to 99.9%, the recommendation is to report as "*Mycobacterium* most closely related to [the species given]." However, if the identity to a recognized species is ≥95 to 98.9%, the results should state that the isolate cannot be definitely identified by 16S rRNA gene sequencing; identification should be given as "most closely related to *Mycobacterium* sp."

Microheterogeneity within an NTM species (i.e., 1 to 5 bases in the 16S rRNA gene are different from the sequence of a known reference strain) has been described for several slowly growing species, including *M. gordonae*, *M. bohemicum*, *M. kansasii*, *M. celatum*, and *M. lentiflavum*. In general, members of the genus *Mycobacterium* are closely related to each other in their 16S rRNA gene sequences and may differ by only a few bases or not at all. In the absence of strong phenotypic differences, definitive NTM identification may be difficult, if not impossible. Turenne et al. advocate that the individual strain ambiguities should be examined carefully using optimum quality sequence databases for comparison, and even then, results may remain inconclusive without resorting to sequencing of alternate targets (89).

As has been discussed, 16S rRNA partial gene sequencing is not useful for separation of some species, including the species within the MTBC. Thus, with MTBC, reporting results of only the 16S rRNA gene sequence should state "*Mycobacterium tuberculosis* complex." When species level identification is necessary, alternate targets, such as *gyrB*, may provide species differentiation, except for MTB and *M. canettii*.

Among the slowly growing NTM, several species are not identifiable with 16S rRNA gene sequencing alone. For example, *M. kansasii* and *M. gastri* share sequence identity in their complete 16S rRNA genes, and thus alternate gene targets or determination of photochromogenicity and/or growth rates are required to provide species resolution. Alternate gene targets that may help in cases where sequencing of the 16S rRNA gene is not adequate include the ITS 1 region, *hsp65*, *gyrB*, *rpoB*, *recA*, and *dnaA* genes. Specific numerical cutoff values such as are recommended for the 16S rRNA gene are not yet recommended for these genes.

The more accurate characterization of new species by molecular technology and the enhancement of clinical data with standardized AST results continue to advance our knowledge of the MTBC, NTM, and disease caused by these species. Proper interpretation and reporting of species identification, even by molecular methods, should be checked against fundamentally established phenotypic characteristics, such as growth rate, colony morphology, and pigmentation. Determination of temperature requirements also may be useful for select isolates.

Finally, appropriate AST of clinically significant species is vital to recognize and effectively treat multiple-drug-resistant mycobacterial species, including multidrug-resistant and extensively drug-resistant MTBC strains, as well as NTM such as *M. simiae*, MAC, rifampin-resistant *M. kansasii*, and other clinically significant drug-resistant species.

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Mycobacterium: Clinical and Laboratory Characteristics of Rapidly Growing Mycobacteria

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TAXONOMY AND DESCRIPTION OF THE AGENTS

The rapidly growing mycobacteria (RGM) are generally defined as nontuberculous species that grow on laboratory media within 7 days (1). RGM contain long-chain fatty acids, known as mycolic acids, that can be quantitated using chromatographic techniques, such as high-performance liquid chromatography (HPLC). Prior to the molecular era, HPLC was used for identification of species of the RGM in most major reference laboratories. However, this method has been replaced in most laboratories by more definitive molecular identification methods for more accurate species identification (2).

Currently, there are more than 140 species of nontuberculous mycobacteria (NTM), among which approximately 70 are species of RGM. More than half of the RGM species have been described since the early 1990s. Since 2010, only three new validated species, *Mycobacterium litorale*, *M. bacteremicum*, and *M. iranicum*, have been added to the list (3–5). Although the species *M. algericum* was described as an RGM, only microcolonies were evident at 1 week. Moreover, this organism is most closely related to the slowly growing mycobacteria of the *M. terrae* complex and thus, by definition, should not be considered an RGM (6). Additionally, most recently, the species *M. abscessus* was subdivided into two subspecies. The former species *M. abscessus* is now designated *M. abscessus* subsp. *abscessus*, and two previously described species, *M. massiliense* and *M. bolletii*, have been combined and proposed to form another subspecies of *M. abscessus*, *M. abscessus* subsp. *bolletii* (7, 8). The latter designation is controversial since the recent discovery that no functional *erm* gene is present in isolates previously named *M. massiliense*, in contrast to the case for isolates previously called *M. bolletii*, with a functional *erm* gene conferring inducible macrolide resistance. Moreover, the 2012 descriptions of the genomic sequences of *M. abscessus* subsp. *bolletii* (previously *M. bolletii*) and *M. abscessus* subsp. *massiliense* (previously *M. massiliense*) may help to resolve some of the questions surrounding the species and provide information to better discriminate between the subspecies (9, 10). Interestingly, Choi et al. noted two rRNA gene operons in the species, which is a distinctly different finding from previous reports of a single rRNA gene operon in *M. abscessus* (11, 12). Moreover, recent studies showed that *M. abscessus* subsp. *massiliense* is composed of several different genotypes, which are also related to colony morphology (13). Thus, for ease of reading in this chapter, we use the terminology "*M. abscessus* subsp. *massiliense*" to refer to the former species *M. massiliense*.

Two newly described species, *M. bacteremicum* and "*M. franklinii*," were described in 2011, although the latter species has not yet been validated (4, 14). The second recently validated species, *M. litorale*, was described from soil samples in China in 2012 but has not been recovered from clinical samples (3). A third newly described species, *M. iranicum*, has been reported as a pathogen in the United States, Europe, and Asia and has been isolated from respiratory, wound, and cerebrospinal fluid cultures (5).

There are currently six major groups or complexes of RGM, based on pigmentation and genetic relatedness (Table 1). The first group of nonpigmented pathogenic species includes 10 species, some of which once belonged to the *M. fortuitum* group (Table 1) (1, 15–19).

The second group of nonpigmented RGM is the *M. chelonae*/*M. abscessus* group listed in Table 1 (1, 7, 20–23). A previously described species, *M. salmoniphilum*, has been revived and also considered to be related to this group. Although this species has been recovered from disseminated disease of salmon and trout, it has not been recovered from humans (24). "*M. franklinii*," the currently nonvalidated species, has been described for multiple patients with sinopulmonary disease, primarily from the northeastern United States (14). Six isolates from extrapulmonary sources have also been described. The organism is closely related to the *M. chelonae*/*M. abscessus* group.

A third nonpigmented group, the *M. mucogenicum* group, currently includes three species, as noted in Table 1 (1, 20, 25).

The fourth group, the *M. smegmatis* group, is currently composed of the two late-pigmented species, i.e., *M. smegmatis* (formerly known as *M. smegmatis sensu stricto*) and *M. goodii* (1, 16, 26).

The fifth group of RGM includes the early-pigmented species, which traditionally have been difficult to identify by conventional (phenotypic) laboratory methods. The only proven pathogens in this group are *M. neoaurum* and *M. bacteremicum* (Table 1), which have been associated with mycobacteremia in patients with catheter infections, and *M. iranicum*, which has been recovered from five respiratory

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TABLE 1 Six major groups of RGM

Group or taxon	Species within group or taxon
Mycobacterium fortuitum group	*M. fortuitum*, *M. peregrinum*, *M. senegalense*, *M. setense*, *M. septicum*,[a] *M. porcinum*,[a] *M. houstonense*,[a] *M. boenickei*,[a] *M. brisbanense*,[a] *M. neworleansense*[a]
M. chelonae/*M. abscessus* group	*M. chelonae*, *M. immunogenum*, *M. abscessus* subsp. *abscessus* (formerly *M. abscessus*), *M. abscessus* subsp. *bolletii* (formerly *M. massiliense* and *M. bolletii*), *M. salmoniphilum*, "*M. franklinii*"[b]
M. mucogenicum group	*M. mucogenicum*, *M. aubagnense*, *M. phocaicum*
M. smegmatis group	*M. smegmatis* (formerly *M. smegmatis sensu stricto*), *M. goodii*
Early pigmented RGM	*M. neoaurum*, *M. canariasense*, *M. cosmeticum*, *M. monacense*, *M. bacteremicum*
M. mageritense/*M. wolinskyi* group	*M. mageritense*, *M. wolinskyi*

[a] Includes the former third-biovariant complex.
[b] "*M. franklinii*" is a nonvalidated species.

samples, a hand wound, and two cerebrospinal fluid samples (4, 5, 27). Several newly described early-pigmented species, including *M. canariasense*, *M. cosmeticum*, and *M. monacense*, are also clinically significant (28–30). There are a number of previously listed environmental (nonpathogenic) species as well (1, 17, 28, 31–33).

Current studies based on DNA sequence analysis suggest the presence of a sixth, nonpigmented pathogenic group, composed of *M. mageritense* and *M. wolinskyi* (2, 21, 22).

The introduction of the new species noted above within the RGM emphasizes the importance of molecular identification to the species level and questions the meaningfulness of the current "group" classifications, especially for the *M. fortuitum* group. However, because previous data (prior to the reliance on genetic testing) and publications use this "group" nomenclature, these designations are retained in this chapter for ease of discussion (1). As more information is obtained using advanced molecular technology and more laboratories adopt this newer methodology, the group designations may become even more obsolete.

CLINICAL SIGNIFICANCE

The RGM are opportunistic pathogens that produce disease in a variety of clinical settings. The three major clinically important species of RGM, responsible for approximately 80% of mycobacterial disease in humans, are the *M. fortuitum* group (including *M. porcinum*), *M. chelonae*, and *M. abscessus* (19, 34, 35). Other potentially pathogenic and clinically significant RGM species are included in Table 2 (1, 2, 15–17, 20–24, 26, 28, 30, 32, 33, 36–39). RGM are presumed to be common in the environment and have been identified most often in tap water when associated with outbreaks of catheter sepsis in bone marrow transplants, wound infections, and associated pseudo-outbreaks of disease (1, 35). The specific reservoir for *M. abscessus* chronic lung infections has yet to be identified.

Community-Acquired Skin and Soft Tissue Infections

The most common infection seen with the RGM is a post-traumatic-wound infection. The patients are generally healthy, and drug-induced immune suppression shows a minimal increase in risk for this type of infection. The *M. fortuitum* group accounts for approximately 60% of cases of localized cutaneous infections, but any of the more than 30 pathogenic RGM species listed in Table 2 can cause disease (1, 15, 33, 40, 41).

Traumatic wound infections, especially open fractures, often involve species within the former *Mycobacterium fortuitum* third-biovariant complex (i.e., *M. porcinum*, and *M. houstonense*) (Table 1) (1, 40). More than 75% of the infections reported in a series of 85 isolates of the *M. fortuitum* third biovariant from the United States and the Queensland, Australia, state laboratory were associated with skin, soft tissue, or bone infections (1). The majority of infections occurred 4 to 6 weeks following puncture wounds or open fractures. Metal puncture wounds (48%) and injuries from motor vehicle accidents (26%) were the most common antecedent injuries, and approximately 40% of the injury sites involved the foot or leg. Stepping on a nail was the most frequently related scenario. None of the isolates in this series were studied by molecular techniques that would identify them as one of the species within the former *M. fortuitum* third-biovariant complex. A 2011 report of an outbreak from 2003 until 2010 in a large university medical center in Texas, involving *M. porcinum*, included not only multiple respiratory isolates but also environmental water and ice isolates, along with clinical isolates from localized abscesses (spinal and paraspinal sites, finger, neck, and breast), peritoneal fluid, blood, and/or Port-a-Cath sites (35).

Sporadic cases of localized wound infections following medical or surgical procedures, including needle injections, can occur with *M. chelonae* but are less common than those with *M. fortuitum*.

In late 2011 and early 2012, an outbreak of 19 cases of *M. chelonae* associated with tattoo ink were reported in New York (42). This was the largest documented outbreak of this type involving *M. chelonae*. The clinical picture of post-traumatic-wound infection ranges from localized cellulitis or abscesses to osteomyelitis (1). A 2006 report from a major U.S. clinical referral center for patients from Minnesota, Wisconsin, Iowa, and South Dakota characterized 63 HIV-negative patients with RGM infections involving *M. abscessus* or *M. chelonae* (71%) or involving the *M. fortuitum* group (29%). Moreover, patients with *M. chelonae* or *M. abscessus* usually had multiple (disseminated) cutaneous

TABLE 2 Currently recognized species of RGM[l]

Species	Established clinical significance	Pigmentation	Unique phenotype	Unique *hsp65* PRA pattern	Unique (complete) 16S rRNA gene sequence
Common human pathogens					
M. *abscessus* subsp. *abscessus*[a]	Yes	No	No	Yes	Yes
M. *chelonae*	Yes	No	Yes	Yes	Yes
M. *fortuitum*	Yes	No	Yes	Yes	Yes
Less common human pathogens (>10 clinical isolates or cases)					
M. *abscessus* subsp. *bolletii*[b]	Yes	No	No	No	No
M. *abscessus* subsp. *massiliense*[b]	Yes	No	No	No	No
M. *bacteremicum*	Yes	Yes	No	Yes	Yes
M. *boenickei*	Yes	No	No	Yes	Yes
M. *canariasense*	Yes	No	No	Yes	Yes
M. *cosmeticum*	Yes	Yes	Yes	Yes	Yes
"M. *franklinii*"[c]	Yes	No	No	Yes	No
M. *goodii*	Yes	Yes	No	Yes	Yes
M. *houstonense*	Yes	No	No	No	No[d]
M. *immunogenum*	Yes	No	No	Yes	Yes
M. *mageritense*	Yes	No	Yes	Yes	Yes
M. *mucogenicum*	Yes	No	Yes	Yes	Yes
M. *neoaurum*	Yes	Yes	No	Yes	Yes
M. *peregrinum*	Yes	No	No	Yes	No[e]
M. *porcinum*	Yes	No	No	Yes	Yes
M. *senegalense*	Yes	No	No	Yes	Yes
M. *smegmatis*	Yes	Yes	No	Yes	Yes
Rare human pathogens (<10 cases or isolates)					
M. *aubagnense*	Yes	No	No	No	Yes
M. *brisbanense*	Yes	No	No	Yes	Yes
M. *brumae*	Yes	No	No	ND	Yes
M. *elephantis*	Yes	Yes	No	Yes	Yes
M. *iranicum*	Yes	Yes	No	Yes	Yes
"M. *lacticola*"[e]	Yes	Yes	Unknown	Yes	Yes
M. *monacense*	Yes	Yes	No	Yes	Yes
M. *moriokaense*	Yes	No	No	Yes	Yes
M. *neworleansense*	Yes	No	No	No	Yes
M. *novocastrense*	Yes	Yes	No	ND	No
M. *phocaicum*	Yes	No	No	Yes	No
M. *septicum*	Yes	No	No	Yes	No[f]
M. *setense*	Yes	No	Yes	Yes	Yes
M. *wolinskyi*	Yes	No	No	Yes	Yes
Unproven human pathogens					
M. *agri*	No	No	No	ND	Yes
M. *aichiense*	No	No	No	ND	Yes
M. *alvei*	No	No	No	ND	No
M. *aromaticivorans*	No	Yes	No	ND	No
M. *aurum*	No	Yes	No	ND	Yes
M. *austroafricanum*	No	Yes	No	ND	Yes
M. *chitae*	No	No	No	ND	Yes
M. *chlorophenolicum*	No	Yes	No	ND	Yes
M. *chubuense*	No	Yes	No	ND	Yes
M. *confluentis*	No	No	No	ND	Yes
M. *crocinum*	No	Yes	No	ND	No
M. *diernhoferi*	No	Yes	No	ND	ND
M. *duvalii*	No	Yes	No	ND	Yes
M. *fallax*[g]	No	No	No	ND	Yes
M. *flavescens*	No	Yes	No	ND	Yes
M. *fluoranthenivorans*	No	No	No	ND	No[h]
M. *frederiksbergense*	No	Yes	No	ND	No
M. *gadium*	No	Yes	No	ND	Yes

(Continued on next page)

TABLE 2 Currently recognized species of RGM[l] *(Continued)*

Species	Established clinical significance	Pigmentation	Unique phenotype	Unique *hsp65* PRA pattern	Unique (complete) 16S rRNA gene sequence
M. *gilvum*	No	Yes	No	ND	Yes
M. *hassiacum*[i]	No	Yes	No[i]	ND	Yes
M. *hodleri*[j]	No	Yes	No	ND	No
M. *holsaticum*	No	Yes	No	Yes	Yes
M. *insubricum*	No	No	No	Yes	Yes
M. *komossense*	No	Yes	No	ND	Yes
M. *litorale*	No	Yes	Yes	ND	Yes
M. *llatzerense*	No	No	Yes	ND	Yes
M. *madagascariense*	No	Yes	No	ND	Yes
M. *murale*	No	Yes	No	ND	Yes
M. *obuense*	No	Yes	No	ND	Yes
M. *pallens*	No	Yes	No	ND	No
M. *parafortuitum*	No	Yes	No	ND	Yes
M. *phlei*	No	Yes	No	ND	Yes
M. *poriferae*	No	Yes	No	ND	ND
M. *psychrotolerans*	No	Yes	No	ND	Yes
M. *pyrenivorans*	No	Yes	No	ND	Yes
M. *rhodesiae*	No	Yes	No	ND	Yes
M. *rufum*	No	Yes	No	ND	No
M. *rutilum*	No	Yes	No	ND	No
M. *salmoniphilum*[k]	Yes	No	No	Yes	Yes
M. *sphagni*	No	Yes	No	ND	Yes
M. *thermoresistibile*	No	Yes	No	ND	Yes
M. *tokaiense*	No	Yes	No	ND	Yes
M. *vaccae*	No	Yes	No	ND	Yes
M. *vanbaalenii*	No	Yes	No	ND	Yes

[a] Formerly M. *abscessus*.
[b] Formerly M. *massiliense* and M. *bolletii* (the taxonomic change is currently controversial).
[c] "M. *franklinii*" is currently a nonvalidated species.
[d] M. *houstonense* is 100% identical to M. *farcinogenes*.
[e] "M. *lacticola*" is currently a nonvalidated species.
[f] M. *peregrinum* (type I) is 100% identical to M. *septicum*.
[g] RGM at 30°C and slowly growing mycobacterium at 35°C.
[h] The 16S rRNA gene sequence is identical to that of the unvalidated species "M. *hackensackense*."
[i] Grows at 65°C.
[j] Grows on or degrades a variety of organic substrates.
[k] Pathogenic for fish.
[l] ND, no data available.

lesions, in contrast to those with single (localized) lesions due to M. *fortuitum*. Most patients with M. *fortuitum* had undergone a prior surgical procedure or had experienced a penetrating trauma at the infected site. Patients with M. *chelonae* or M. *abscessus* were older and more likely to be on some type of immunosuppressive agent (43). Localized or disseminated infections with M. *chelonae* most frequently occur in patients receiving long-term corticosteroids and/or chemotherapy, organ transplant recipients, and patients with rheumatoid arthritis or other autoimmune disorders or suppressive therapy (43). Immune suppression in diseases such as AIDS has not been a significant risk factor for development of localized or disseminated M. *chelonae* infections (1, 43).

Starting in 2000, an outbreak of furunculosis on the lower extremities, caused by M. *fortuitum*, was described for 32 otherwise healthy patients who were patrons of a nail salon in California ("foot spa disease") (44). The organism was also cultured from contaminated footbaths and from inlet suction screens containing hair and other debris, and shaving the legs prior to the footbath and pedicure was an identified risk factor (44, 45). Other species, including M. *fortuitum*, M. *abscessus*, and M. *mageritense*, were subsequently recovered from cases in California and Georgia (1, 46–48). Strains from the footbath and patients were identical by DNA strain typing.

Occasionally, members of group 6 (M. *mageritense*, and M. *wolinskyi*), and members of group 4 (M. *smegmatis* group, including M. *smegmatis* [*sensu stricto*], and M. *goodii*) (49) have been reported from infections following traumatic injury and surgical or medical procedures, such as cardiac surgery, breast reduction surgery, and facelift plastic surgery. Cellulitis and localized abscess are the most common manifestations (1, 26, 34).

Disseminated Cutaneous Disease

For unknown reasons, disseminated cutaneous disease is rare for all taxonomic groups other than the M. *abscessus*/M. *chelonae* group, even in immunocompromised patients, including those with AIDS (1, 34, 50).

In contrast, disseminated cutaneous disease due to both M. *abscessus* and M. *chelonae* is much more common. It typically presents as multiple chronic painful red nodules, usually involving the lower extremities (1, 34). These lesions then drain spontaneously, with the drainage usually being acid-fast bacillus smear positive. With disease due to M. *chelonae*, almost all patients are immunosuppressed, usually from low-dose corticosteroid therapy. Although the disease is presumably a consequence of hematogenous spread, bacteremia is rarely identified. A portal of entry for the infection is rarely evident (1, 34). In a series of 100 clinical isolates from skin and soft tissue, we reported that 53% were from patients with disseminated cutaneous infections (1).

Disseminated cutaneous disease due to M. *abscessus* occurs less frequently but is serious (1). Like disseminated M. *chelonae* disease, most cases occur in chronically immunosuppressed patients receiving corticosteroids, and the disease has no apparent portal of entry. Also like the case with M. *chelonae*, patients with disseminated cutaneous infection due to M. *abscessus* rarely have detectable bacteremia and/or endocarditis but usually present with multiple draining cutaneous nodules, usually in the lower extremities (1, 34).

A rare type of disseminated infection due to RGM in immunocompetent hosts presenting with lymphadenopathy was recently described for patients from Thailand (51).

Bone and Joint Infections

The RGM may also cause bone and joint infections. Like bacterial disease, osteomyelitis may follow open bone fractures, puncture wounds, and hematogenous spread from another source. The most common scenario is an open fracture of the femur, often followed by orthopedic surgical procedures. The most frequent pathogen recovered in this setting is the M. *fortuitum* group, including the newly described species M. *houstonense*, M. *boenickei*, and M. *setense* (1, 15, 17, 52, 53). The two newly described species in the M. *smegmatis* group, M. *goodii* and M. *wolinskyi*, have also been associated with osteomyelitis (1, 26). Bone involvement secondary to a puncture wound is likely the second major cause of osteomyelitis. Infections most commonly involve the M. *fortuitum* group (1). M. *fortuitum* infections in prosthetic knees and joints have also been reported (52). Vertebral osteomyelitis has also been described (54, 55).

Pulmonary Infections

Chronic Lung Infection and Bronchiectasis

As with disseminated cutaneous disease, chronic lung disease is rare among RGM groups, except for the M. *chelonae*/M. *abscessus* group (55). Chronic lung infections with RGM, most often in nonsmoking older women with associated bronchiectasis, are sometimes associated with the M. *avium* complex (MAC) as well. M. *abscessus* is the causative agent in >80% of cases of pulmonary disease due to RGM (34).

Similarities exist between patients with MAC and those with M. *abscessus*, such that a common pathogenicity or host susceptibility factor may be involved (34). Multiple cultures of M. *abscessus* from respiratory samples are usually associated with significant pulmonary disease.

Cystic Fibrosis

M. *abscessus* subsp. *abscessus* and M. *abscessus* subsp. *massiliense* have been isolated with increasing frequency from the respiratory tracts of patients with cystic fibrosis (CF) (1, 57–62). A 2012 study concluded that CF patients with M. *abscessus* were younger and had more severe disease and more frequent intravenous antimicrobial treatment (62). M. *abscessus* is the second most common species of nontuberculous mycobacteria recovered from CF patients (after the MAC), and it may be the most common species associated with clinical disease in this setting (59). Patients with CF also have bronchiectasis, in addition to chronic, recurrent airway and parenchymal infections that may be the primary risk factors for susceptibility to NTM disease (58, 59). Bronchiectasis, an almost universal factor in M. *abscessus* infection, appears to be less predominant in patients with M. *abscessus* subsp. *massiliense* infection (63). Recent studies have indicated that because of a nonfunctional *erm* gene in this species, patients with the former M. *abscessus* subsp. *massiliense* are successfully treated with macrolides more often than patients with M. *abscessus* subsp. *abscessus* (80% success rate, in contrast to 25% success for patients with M. *abscessus* subsp. *abscessus*) (63, 64).

At present, there is no explanation for the apparently varying geographic distribution of M. *abscessus* subsp. *massiliense* strains in the general population with respiratory disease. Recent data showed a much higher prevalence (55%) among 150 patients studied in South Korea than in other populations, such as only 28% of 40 patients at the National Institutes of Health in the United States, 21% of 39 isolates in the Netherlands, 22% of 50 patients with CF in France, and 26% of 102 patients in Japan (63).

Chronic Aspiration Syndrome

M. *fortuitum* has been reported as a pathogen in half of the cases of chronic aspiration disease secondary to underlying gastroesophageal disorders, such as achalasia (1, 34). Pulmonary disease with M. *fortuitum* in the absence of these disorders is rare. Pulmonary disease with M. *chelonae* and M. *smegmatis* has been described in only a few cases, including lipoid pneumonia.

Miscellaneous Lung Disease

A rare case of pulmonary disease with M. *mageritense* was recently described for a patient with a compromised immune system (1, 34, 56). However, the previously discussed recent outbreak of M. *porcinum* described the first reported cases of clinically significant pulmonary disease associated with this species, a member of the M. *fortuitum* group (35).

Hypersensitivity Pneumonitis

Hypersensitivity pneumonitis among metal grinders working with contaminated metalworking fluids in industrial plants has been associated with a newly described species of RGM, M. *immunogenum* (1, 34, 65). Multiple pseudo-outbreaks associated with this species have been reported, resulting from contaminated automated bronchoscope cleaning machines and metalworking fluids. This species is able to grow and remain viable in degraded metalworking fluid and is resistant to the routine biocides used for disinfection of metalworking fluids (1, 65). However, as yet, this species has not been reported from open lung biopsy specimens from these patients.

Central Nervous System Disease

Central nervous system disease involving the RGM is rare, but morbidity and mortality are high. Most of the reported cases have been associated with M. *fortuitum* (1, 66, 67).

Corneal Infections (Keratitis)

The number of RGM recovered from ocular infections has been increasing over the last 20 years. A retrospective review of cases of NTM keratitis from 1982 to 1997 at an eye institute in Florida showed that 19 of 24 cases were due to RGM (68). A recent study which identified 113 ophthalmic isolates by molecular methods showed that the most common RGM were M. *chelonae* (45%), M. *abscessus* (42%), and the M. *fortuitum* group (8%) (69).

Since the early 1990s, other descriptions of epidemic and sporadic ocular infections associated with the RGM have been published, including postkeratoplasty infections and infections following laser *in situ* keratomileusis (LASIK) surgery (1, 69).

Otitis Media

The most common NTM ear disease is chronic otitis media due to M. *abscessus*. In a 1988 outbreak of 17 cases of otitis media in two ear-nose-throat clinics, patients presented with chronic ear drainage with a perforated tympanic membrane and a prior tympanostomy tube (1). In another series, 20 of 21 cases of sporadic chronic otitis media (some with associated mastoiditis) were due to M. *abscessus* infection following ear tube placement. Approximately one-half of the isolates from these cases were aminoglycoside resistant, resulting from the long-term use of aminoglycoside ear drops (1). Sporadic cases of this disease continue to be seen. The key to the disease appears to be the presence of the foreign body (tympanostomy tube).

Recently, otitis media and otomastoiditis caused by M. *abscessus* subsp. *massiliense* were described for middle-aged adults with underlying otologic disease. These patients were treated successfully with clarithromycin-containing regimens for up to 9 months (70).

Health Care-Associated Infections

Health care-associated disease with RGM has been reported most commonly with M. *fortuitum*, M. *chelonae*, M. *abscessus*, and M. *mucogenicum*, although any species may be involved. Most infections occur following contamination with tap water (1, 40, 71, 72). Types of infections include postsurgical wound infections, catheter sepsis, infections following hemodialysis, postinjection abscesses, vaccine-related outbreaks, and otitis media following tympanostomy tube replacement (1, 73, 74). These have been seen as both sporadic cases and localized outbreaks. Recent outbreaks have involved cosmetic procedures, such as liposuction, liposculpture, acupuncture, and mesotherapy, a procedure comprising multiple subcutaneous injections of pharmaceutical or homeopathic medications for cosmetic purposes (41, 73–80).

In addition to true outbreaks of infection, numerous health care-associated pseudo-outbreaks have been described. Contaminated or malfunctioning bronchoscopes, automated endoscope cleaning machines, and contaminated laboratory reagents and ice have been implicated (1, 73, 81–83).

Catheter-Associated Infections

Central catheter-associated infections are the most common health care-associated infections due to the RGM (1, 39, 85). They are the most common cause of RGM bacteremia, but the disease may also present as local wound drainage as part of an exit site or tunnel infection (1, 85). Other types of catheters can also become infected, including peritoneal catheters, ventriculo-peritoneal shunts, and shunts for hemodialysis (1). The most common species are M. *mucogenicum*, M. *fortuitum*, M. *abscessus*, and the newly described species M. *bacteremicum*. An outbreak of M. *phocaicum* and M. *mucogenicum* was described for five patients with central venous catheters in an oncology unit in a Texas hospital (86). This outbreak represents the first report of clinical isolates of M. *phocaicum* in a hospital in the United States.

Surgical Wound Infections

Surgical wound infections due to RGM are a well-recognized clinical entity. In the 1970s and 1980s, these were most commonly associated with augmentation mammaplasty and coronary artery bypass surgery, and multiple disease outbreaks occurred (1, 87). Infections following these types of surgery are now less common, although a cluster of 12 cases of post-augmentation mammaplasty surgical site infections due to M. *fortuitum* and M. *porcinum* was reported between 2002 and 2004 in Brazil (87). More often, however, these types of infections have been replaced by infections following other types of cosmetic surgeries, such as liposuction, and other types of prosthetic surgeries, such as knee replacements.

In a 1989 report (15), approximately 80% of RGM wound isolates related to cardiac surgery were from seven southern coastal states, including Texas, Louisiana, Georgia, Maryland, Alabama, Florida, and South Carolina. A second report, published in the same year, showed that 92% of 37 identified cases of surgical wound infection following augmentation mammaplasty were also from patients in southern coastal states, with the majority located in Texas, Florida, and North Carolina, suggesting that the disease risk was highest in the southeastern United States (1).

Recovery of both subspecies of M. *abscessus* has been reported from outbreaks of infections associated with laparoscopic surgeries and cosmetic surgeries in Brazil, the Dominican Republic, and Korea (76, 78). These and other recent reports suggest that although few studies have identified these newer subspecies in invasive infections, they have been misclassified in previous studies (88).

Prosthetic Device Infections

Infections following insertion of prosthetic devices, including prosthetic heart valves, artificial knees and hips, lens implants, and metal rods inserted into the vertebrae to stabilize bones following fractures, have also been described (1, 52). Again, M. *fortuitum* is the most common pathogen, but any of the pathogenic RGM, including the M. *smegmatis* group, can be associated with this type of infection (1).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Details of standard methods are included in chapter 30. Transport of species is accomplished by using leakproof containers and proper safety protocols. Specimens for detection of RGM should be delivered to the laboratory in a timely manner following appropriate shipping and handling regulations for shipping biological or infectious materials (89).

Disinfectants

The RGM are generally resistant to the activity of biocides, such as organomercurials, chlorine, 2% formaldehyde, and alkaline glutaraldehyde, all of which are commonly used disinfectants (1). A report of the contamination of benzalkonium chloride, a widely used antiseptic compound, with M. *abscessus* and of a resulting outbreak of this species in several patients following steroid injections after skin disinfection emphasizes the limitations of disinfectants against the RGM (90).

DIRECT EXAMINATION

Microscopy

The use of mycobacterial smears is a rapid and reasonably sensitive step in the diagnosis of RGM disease. Gram stains of colonies showing faintly staining, "ghost-like" beaded

Gram-positive bacilli are often helpful in establishing a diagnosis of mycobacteriosis. Ziehl-Neelsen or Kinyoun staining may also be useful. However, the use of smears alone is not sufficient for species identification. A large study found that NTM, including RGM, are likely to be detected by fluorochrome staining of specimens, especially those from patients at low risk for AIDS in areas where lung disease is endemic (89, 91). Further details of the staining procedures are found in chapter 30.

Nucleic Acid Detection

Currently, there are no commercial nucleic acid detection systems available for direct detection of NTM. An indirect method could be identifying an acid-fast bacillus smear-positive sample that is negative by a nucleic acid amplification test specific for *Mycobacterium tuberculosis*, but this does not prove the presence of RGM compared to the slowly growing NTM.

ISOLATION PROCEDURES

Primary isolation of RGM optimally requires culture at 28 to 30°C rather than 35°C, especially for recovery of *M. chelonae* and *M. immunogenum* (1). Direct examination and isolation procedures are detailed in chapter 30.

Recovery of RGM from routine blood culture systems does not appear to be problematic. However, no large comparative studies of commercial systems have been performed to date.

IDENTIFICATION

Biochemical Testing

As previously stated, the RGM are defined as NTM that grow within 7 days (most species grow within 3 to 4 days) (1). Until the advent of more modern molecular techniques, traditional laboratory identification of the RGM was based primarily upon growth rate, pigmentation, colonial morphology, and a select battery of biochemical tests (1). These standard tests included tests of arylsulfatase production, tolerance to 5% NaCl, nitrate reductase activity, and iron uptake. All members of the *M. fortuitum* group and *M. chelonae*/*M. abscessus* group exhibit a strongly positive arylsulfatase reaction at 3 days. The *M. smegmatis* group (*M. smegmatis* and *M. goodii*) and *M. wolinskyi* have similar growth rates but do not exhibit arylsulfatase activity at 3 days (1, 26). Approximately 95% of the isolates of *M. smegmatis (sensu stricto)* and 80% of *M. goodii* isolates develop a late (7 to 10 days) yellow-orange pigmentation (1, 26).

The current proposal for the clinical laboratory is that biochemical testing of the RGM should be replaced by molecular methods. Moreover, biochemical testing should be performed only when describing a new species as part of a polyphasic identification algorithm.

Supplemental Biochemical Testing: Carbohydrate Utilization

The supplementation of standard biochemical tests with carbohydrate utilization has allowed more complete and accurate laboratory identification of established species and discrimination of some (but not all) new species (1). Identification to the species level and susceptibility testing (see chapter 76) should be performed on isolates of RGM considered to be clinically significant.

However, as previously stated, molecular testing is the only definitive means of identifying the RGM species, and laboratories should proceed cautiously in identifying these species by biochemical testing alone (92, 93).

Antimicrobial Susceptibility Tests for Taxonomic Identification

As discussed above, other adjunctive nonmolecular tests, including antimicrobial susceptibility tests, have also been utilized for identification of the RGM (1, 93). They are less commonly used currently, with the advent of molecular techniques, but can provide species confirmation in conjunction with molecular results. As a screening tool, isolates of the *M. fortuitum* group and the *M. chelonae*/*M. abscessus* group can be differentiated by the use of a polymyxin B (300 IU) disk diffusion method. Generally, isolates of the *M. fortuitum* group exhibit a partial or clear zone of inhibition (≥10 mm) around the polymyxin disk, whereas isolates of the *M. chelonae*/*M. abscessus* group show no zone of inhibition (1).

Moreover, *M. chelonae* and *M. abscessus* also have different antimicrobial susceptibility patterns. One major difference between the two species is resistance to cefoxitin. By agar disk diffusion, *M. chelonae* shows complete resistance to cefoxitin, with no partial or complete zones of inhibition, in contrast to the partial or complete zones seen with *M. abscessus*. Isolates of *M. chelonae* generally have cefoxitin MICs of ≥256 µg/ml, whereas the modal MIC for isolates of *M. abscessus* is 32 to 64 µg/ml (1). Furthermore, recent studies have shown that isolates of *M. abscessus* subsp. *abscessus*, but not *M. chelonae* or *M. abscessus* subsp. *massiliense*, have an inducible *erm* gene similar to the gene in *M. fortuitum*, which conveys macrolide resistance (94, 95).

Isolates of *M. abscessus* also have lower amikacin MICs and are resistant to tobramycin, whereas tobramycin is more active than amikacin with *M. chelonae*. Additionally, isolates of *M. chelonae* are more susceptible *in vitro* to some of the newer antibiotics, including linezolid and moxifloxacin, than are isolates of *M. abscessus* (92).

HPLC Identification

HPLC analysis of the mycobacterial cell wall mycolic acid content is routinely used in large reference or state health department laboratories to identify slowly growing isolates of NTM, but it has been problematic with RGM (1, 96). HPLC can be helpful for placing RGM isolates into groups or complexes, but it is not specific enough to identify most species with a high degree of accuracy.

Molecular Identification Methods

Nucleic Acid Probes

The INNO-LiPA multiplex probe assay (Innogenetics, Ghent, Belgium) is based on the principle of reverse hybridization (97). Although the assay has not received U.S. Food and Drug Administration clearance, it can identify both rapidly and slowly growing mycobacterial species. Biotinylated DNA obtained by PCR amplification of the 16S-23S internal transcribed spacer (ITS) is hybridized with specific oligonucleotide probes immobilized as parallel lines on membrane strips. The main advantage of this system is that a large variety of species may be identified by a single assay, without the need to select an appropriate probe. One limitation of the assay is the cross-reactivity that may be detected with species of the *M. fortuitum* group and several species, such as *M. thermoresistibile*, *M. agri*, and *M. alvei*, that are rarely found in clinical samples (1, 97). Additionally, it failed to differentiate isolates of closely related species, such as M. *chelonae* and M. *abscessus* (98). Since the original studies with the INNO-LiPA assay, however, the system has been improved to include additional probes for the M. *fortuitum-peregrinum* complex and M. *smegmatis*.

A similar commercial PCR method which targets the 23S rRNA gene, the GenoType Mycobacterium assay (Hain Lifescience, GmbH, Nehren, Germany), provides probes for simultaneous identification of M. *chelonae* and specific probes for M. *peregrinum*, M. *fortuitum*, and M. *phlei*. These two systems are widely used in Europe for NTM identification (99, 100).

Sequence Analysis

Nucleic acid sequence analysis has been performed for the identification of mycobacteria for several years. This identification tool has been useful for the discrimination of most of the newly described species of RGM (38, 101–103).

Partial 16S rRNA Gene Sequence Analysis

Generally, the identification of mycobacteria, including RGM, focuses on two main hypervariable domains, known as region A and region B, located at the 5′ end of the 16S rRNA gene. These regions correspond to *Escherichia coli* positions 129 to 267 and 430 to 500, respectively. Hypervariable region A, especially, contains most of the species-specific sequence variations (so-called "signature sequences") in mycobacterial species, and sequencing of this region allows taxonomic identification of most mycobacteria, including many species of RGM (2, 38).

A commercial gene sequencing system, the MicroSeq 500 16S rDNA bacterial sequencing kit (Life Technologies, Foster City, CA), analyzes the first 500-bp sequence and compares the sequence with those in a commercially prepared database. The use of this commercial system alone cannot differentiate some major species of RGM, such as M. *chelonae* and M. *abscessus*, which require sequencing of other regions or other genes for identification (101, 103). For this reason, clinical laboratories have supplemented the commercial database with additional sequences from their own or other libraries, such as RIDOM (Würzburg, Germany) or GenBank.

The currently commercially available partial 16S rRNA gene sequencing system (MicroSeq 'first 500 bp') has a number of large gaps for identification of RGM. For example, isolates of two major RGM pathogens, M. *chelonae* and M. *abscessus*, require sequencing of sites outside the first 500 bp, as they are identical in this region but differ at other 16S rRNA gene sites (in the 3′ region) (38, 101, 102). To ensure accurate identification, at least 300 bp of quality sequence should be compared between the reference and query sequences and should cover at least one region of the gene where variations are to be expected. Therefore, most clinical laboratories currently sequence between 450 and 480 bp in order to provide an adequate sequence (104).

In general, members of the genus *Mycobacterium* are closely related to each other, and closely related species may differ by only a few base pairs, or none at all. For example, M. *goodii*, which is difficult to distinguish from M. *smegmatis* phenotypically, except by susceptibility pattern, has only a four-base difference in the entire 16S rRNA gene (26).

A quality-controlled database is indispensable for the evaluation and accurate identification of unknown strains (103). The laboratorian should also recognize that sequence analysis is an important component in a polyphasic approach to the identification of unknown strains. While in some instances molecular analysis-based identifications without conventional testing may be adequate, more often there are cases in which the broader picture must be reviewed. Some investigators suggest that key phenotypic tests, including colonial morphology, pigmentation, and growth rate, are necessary, especially for the differentiation of closely related species (103).

The lack of consensus on standard reporting criteria or a cutoff value has been a major obstacle in interpretation of sequence data (103, 104). Reporting criteria, such as (i) distinct species, (ii) "related" to a species, and (iii) "most closely related to" a species, depending upon the amount of sequence difference between the unknown isolate and the 16S rRNA gene database entries (40), have been recommended but not validated (38, 103).

The 2008 CLSI MM18-A (104) document recommends guidelines for 16S rRNA gene sequencing in order to identify *Mycobacterium* spp. in a consistent, practical manner. For sequences with 100% sequence probability, the definite genus and species may be assigned. However, for sequence probabilities of 99.0 to 99.9%, the document recommends reporting "genus, most closely related to species," and for isolates with sequence probabilities of ≥95% to 98.9%, laboratories should consider reporting "unable to definitively identify by 16S rRNA gene sequencing, most closely related to *Mycobacterium* sp." (104). We agree that although 100% identity is mandatory for signature sequences, one or a few mismatches at other positions may be acceptable for identification to the species level (104).

Sequencing remains a complex and often cost-prohibitive expense for a routine clinical laboratory, which also may not have an adequate volume of isolates to warrant sequencing. Therefore, the consensus opinion is that not all laboratories should attempt to incorporate sequencing into their laboratory routine. Moreover, requests for sequencing should instead be sent to a qualified reference laboratory with skill and experience in the method (103, 104).

Sequencing of the *hsp65* Gene

Although the 65-kDa heat shock protein gene (*hsp65*) is highly conserved among species of mycobacteria, it exhibits more interspecies and intraspecies polymorphisms than those in the 16S rRNA gene sequence (105–107). This variability can be advantageous for the development of other strategies for the identification of genetically related species of RGM (106, 107). Most sequencing or restriction fragment length polymorphism analyses have utilized a 441-bp sequence identified by Telenti et al. (107), often referred to as the Telenti fragment.

Studies based on DNA sequencing have demonstrated interspecies allelic diversity within the RGM. Detailed studies of several RGM species, including M. *peregrinum*, M. *porcinum*, M. *senegalense*, M. *chelonae*, and M. *abscessus*, have shown 4 to 6 sequence variants (sequevars) per species that differ by 4 to 6 nucleotides within the 441-bp Telenti fragment (107, 108).

Additionally, unlike 16S rRNA gene sequencing, the *hsp65* sequencing method is able to differentiate isolates of M. *abscessus* from those of M. *chelonae* (they differ by almost 30 bp in the 441-bp *hsp65* sequence, compared to only 4 bp in the entire 1,500-bp 16S rRNA gene sequence) (108). In contrast to the case with 16S rRNA gene sequences, with sequencing of the *hsp65* gene, even RGM species with a high degree of 16S rRNA gene similarity, such as M. *fortuitum*, M. *septicum*, M. *peregrinum*, M. *houstonense*, and M. *senegalense*, can be discriminated as distinct species.

Like the case for other sequencing methods, one limitation of sequencing of the *hsp65* gene is that few or no sequences of newer RGM species are available in databases, and detailed sequencing of older species (i.e., multiple strains) has not been done, such that only one sequence per species is generally available. Thus, development of a comprehensive database and in-house validation are essential (2, 103, 108).

rpoB Sequence Analysis

Initial studies using *rpoB* (the gene encoding the β-subunit of RNA polymerase) sequencing for description of species were based upon partial *rpoB* gene sequence analysis, comprising only about 20% of the entire gene length. Other investigators have suggested that species identification of a variety of RGM species is possible using a 340- to 360-bp region, but extensive variation may require development of more species-specific probes (20–22, 109–111).

The utility of *rpoB* was recently emphasized in a study comparing the phylogenetic relationships among 19 RGM species, including the major pathogens in this group, by comparing *rpoB* to several different sequence targets, including the 16S rRNA gene, *hsp65*, *sodA*, and *recA*. All 19 species showed good discrimination with *rpoB* (22, 112).

Not only has the *rpoB* sequence been useful for the identification of established species, but it has also helped to enable the discrimination of species that cannot be differentiated by the 16S rRNA gene or the *hsp65* sequence alone. Newly described species that are usually differentiated by *rpoB* sequencing include M. *abscessus* subsp. *massiliense*, M. *phocaicum*, and M. *aubagnense* (20, 21, 23). However, recent studies have shown that multilocus sequencing is necessary to identify M. *abscessus* subsp. *massiliense* (7, 8, 115).

Sequence Analysis of Other Gene Targets

Other molecular targets for taxonomic identification, including the 32-kDa protein gene, the superoxide dismutase gene (*sodA*), the 16S-23S rRNA ITS, *dnaJ*, *secA1*, and *recA*, have been suggested for mycobacterial identification utilizing either PCR-restriction fragment length analysis (PRA) or direct sequencing (22, 51, 113–115). However, preliminary data suggest that these gene sequences are more variable than *hsp65*, and, to date, they have been utilized less commonly for the laboratory identification of the species of RGM (22, 113, 114).

Moreover, a major limitation of all sequence-based testing is the lack of sufficient databases (21, 103). Additionally, a multigenic approach for taxonomic evaluation of species has been suggested widely by investigators and was recently proposed by an *ad hoc* committee for the reevaluation of the species definition in bacteriology (116).

Recent studies by Kim et al. used *erm*(41) gene sequences to differentiate M. *abscessus* subsp. *massiliense* from M. *abscessus* subsp. *abscessus* and M. *abscessus* subsp. *bolletii* (117). The results of species identification using *erm*(41) were concordant with those of multilocus sequence analysis including *rpoB*, *hsp65*, *sodA*, and the 16S-23S ITS (117). However, Blauwendraat et al. reported that sequencing of *sodA* provides little value in species differentiation (98).

Multilocus Sequence Analysis

Recent studies have shown the inaccuracy of single-target sequencing for differentiation of species within the M. *abscessus* complex (i.e., M. *abscessus* subsp. *abscessus*, the former species M. *massiliense*, and the former species M. *bolletii*) (118). The previous strategy, using *rpoB* sequencing for identification of species within the M. *abscessus* complex, appears to be unacceptable due to the composite genetic structure, which suggests that genetic exchanges among members of the group led to legitimate recombination events among homologous housekeeping genes in a large number of isolates of the M. *abscessus* complex studied thus far (114, 118, 119).

These studies also emphasize the need for multiple-gene sequencing in the definition of species (118). A 2011 study by Macheras et al. suggested the use of eight housekeeping gene sequences (4,071 bp), including *argH*, *cya*, *glpK*, *gnd*, *murC*, *pgm*, *pta*, and *purH* (120). Their work also brings into question the previous criteria for differentiation of species based on >3% *rpoB* sequence divergence between two RGM species (36, 120). Furthermore, their work substantiates a later study by Leao and colleagues asserting that M. *massiliense* and M. *bolletii* are not different species (7, 8).

PCR-Restriction Enzyme Analysis

PRA of *hsp65* has become a valuable tool for the identification of RGM. With the nonsequencing PRA method used on *hsp65*, minor differences (sequevars) within the species rarely involve a restriction site, so most species have only one PRA pattern. Currently, the 441-bp Telenti fragment of *hsp65* remains the most useful sequence for PRA identification of RGM, although it has not been evaluated extensively with the pigmented RGM and with the newer species and subspecies of RGM, such as M. *phocaicum*, M. *aubagnense*, M. *abscessus* subsp. *massiliense*, and others (1, 107, 108). However, use of another restriction enzyme (SmlI) in addition to the BstEII and HaeIII enzymes may enable differentiation of M. *abscessus* subsp. *massiliense* from the former species M. *bolletii* and M. *abscessus* (R. J. Wallace, unpublished data). Additionally, the correlation of rough/smooth colony morphology of M. *abscessus* subsp. *massiliense* has been demonstrated by *hsp65* PRA with Hinf1 (13).

The advantages of PRA are that the method of identification does not rely upon growth rate and nutritional requirements, the equipment is relatively inexpensive, and the results for a large number of mycobacterial species can be generated rapidly. The disadvantages are that it requires knowledge of PCR and is a relatively complex procedure that requires extensive in-house validation, since there are no available commercial systems. Furthermore, the method is not approved by the U.S. Food and Drug Administration. However, as with all sequence-based methods of identification, its utility is limited by the availability of an updated public database.

Algorithms for identification of mycobacterial species, including RGM, by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene have been proposed (89, 106, 107). Figure 1 shows a PRA gel of the most commonly encountered RGM in the clinical laboratory.

Variable-Number-Tandem-Repeat (VNTR) Analysis

Recently, Choi and colleagues demonstrated identification to the species level of 85 isolates of the M. *abscessus* complex (M. *abscessus* subsp. *abscessus*, M. *abscessus* subsp. *massiliense*, and M. *abscessus* subsp. *bolletii*), using VNTR11 and VNTR23 as targets (121). The assay was also able to differentiate the M. *abscessus* complex from other mycobacterial species, including M. *fortuitum* and M. *chelonae*. Complete agreement of identification was noted among the 85 strains. VNTR11 showed a polymorphism of two to four copies (179 to 278 bp) in M. *abscessus* but only one copy in

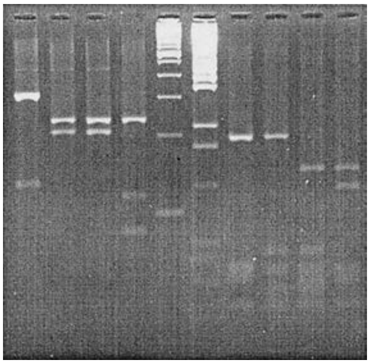

FIGURE 1 PRA patterns of commonly encountered species of RGM (106, 107). Lane 1, M. *chelonae*, BstEII; lane 2, M. *abscessus* subsp. *massiliense*, BstEII; lane 3, M. *abscessus* subsp. *abscessus*, BstEII; lane 4, M. *fortuitum*, BstEII; lane 5, 100-bp ladder; lane 6, pGem ladder; lane 7, M. *chelonae*, HaeIII; lane 8, M. *abscessus* subsp. *massiliense*, HaeIII; lane 9, M. *abscessus* subsp. *abscessus*, HaeIII; lane 10, M. *fortuitum*, HaeIII. doi:10.1128/9781555817381.ch32.f1

M. *abscessus* subsp. *massiliense* and M. *abscessus* subsp. *bolletii*. VNTR23 was present as one to three copies (196 to 238 bp) in M. *abscessus*, in contrast to one pattern with two copies in M. *abscessus* subsp. *bolletii*. None of the M. *abscessus* subsp. *massiliense* isolates were positive for VNTR23 (121).

Pyrosequencing

Pyrosequencing technology (Biotage, Uppsala, Sweden) employs nucleic acid sequencing of a 20- to 30-bp segment of hypervariable region A of the 16S rRNA gene. The method is based on the detection of pyrophosphate during DNA synthesis. During sequencing, visible light is produced that is proportional to the number of incorporated nucleotides.

The method is not as discriminating as traditional Sanger sequencing but is an attractive and less expensive alternative. Another advantage of the pyrosequencing technology is its commercial availability. Like the case for other sequencing methods, the major limitation is the quality of the databases for interpretation and comparison of sequences (122). In a recent study of 50 RGM isolates (M. *chelonae*/M. *abscessus* and M. *mucogenicum*), consensus sequences were obtained for 40 isolates compared to traditional sequencing results. Of 10 isolates of M. *fortuitum*, 3 had identical sequences which matched those of M. *fortuitum*/M. *peregrinum*, and the remaining 7 isolates matched M. *fortuitum* (123). To date, this method has provided reliable and rapid identification of a variety of RGM species (123).

A real-time PCR with melting curve analysis has been developed that consistently detects and differentiates M. *tuberculosis* from NTM. In a recent study, 20 isolates previously identified as M. *fortuitum* were confirmed to be M. *fortuitum*. However, 7 of 24 isolates previously identified as M. *chelonae* or M. *abscessus* were found by pyrosequencing analysis to have been misidentified by traditional methods (124). The application of these methods is currently used only in research or large reference laboratories, until more extensive evaluation can be done.

Recently, investigators proposed the use of a segment of the *rpoB* gene in conjunction with pyrosequencing to determine the sequence of a 60-bp portion of the *rpoB* gene. The sequence, located in variable region II according to the numbering of Adékambi et al. (20), at positions 1105 to 1219 (including primer sequences), successfully identified 99/100 isolates of M. *abscessus* and M. *chelonae* (125).

Matrix-Assisted Laser Desorption Ionization—Time of Flight Mass Spectrometry

Matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF MS) technology was recently demonstrated to be a reliable and rapid (approximately 90 min to identification of most RGM species) technological advance for the identification of NTM, including the RGM. The current method produces unique spectral fingerprints based on extracted proteins. Moreover, MALDI-TOF MS provides a less expensive and less labor-intensive procedural alternative to gene sequencing for identification of the RGM (126, 127). In a recent study by Lotz and colleagues, the investigators were able to differentiate several clinically relevant species of RGM, including M. *immunogenum*/M. *chelonae*/M. *abscessus* subsp. *abscessus*, but not M. *abscessus* subsp. *abscessus*, from M. *abscessus* subsp. *bolletii* or M. *abscessus* subsp. *massiliense* (128). Additionally, 6/8 isolates of M. *fortuitum*, 8/8 isolates of M. *mucogenicum*, and 6/7 isolates of M. *peregrinum* were also correctly identified in liquid media, compared to 25/26 isolates of M. *chelonae*, 25/26 isolates of M. *fortuitum*, 13/16 isolates of M. *peregrinum*, and 19/20 isolates of M. *mucogenicum* correctly identified from solid media (128). Overall identification for both slowly and rapidly growing mycobacterial species was 97% (128).

The original studies using MALDI-TOF MS began in 2004, using more complex strategies, with whole cells for mycobacterial identification, than the protein extract methods available at present. In early studies, Hettick et al. tested eight strains of RGM (M. *fortuitum*) and were able to illustrate a high degree of reproducibility between culture strains (129, 130). Additionally, Wallace and colleagues tested 22 isolates of RGM and showed that the clustering of strains by MALDI-TOF MS was comparable to that by PRA and genetic sequencing (18). In 2006, Pignone et al. used root mean square values for the comparison of different mycobacterial profiles of 14 strains of RGM (5 M. *fortuitum* group, 3 M. *mucogenicum*, 3 M. *abscessus*, and 3 M. *chelonae* strains) (131). These investigators showed reproducible identification, with unique mass spectral profiles of all 14 strains of the RGM, extending the work of the previous studies by Hettick et al. (129, 131). Although Lefmann et al. identified 24 clinical and 12 type strains of mycobacteria (including 9 strains of the M. *tuberculosis* complex) by using MALDI-TOF MS, they only tested two clinical and two type strains of RGM, and their strategy entailed initial PCR and transcription-RNase cleavage steps, unlike the systems currently being evaluated (132).

Today's MALDI-TOF MS systems are more easily incorporated into the routine workflow of the laboratory and provide rapid and accurate identification of many strains of mycobacteria, as shown by Saleeb and colleagues (126). Currently, the two predominant commercial MALDI-TOF MS systems are the Bruker Biotyper (Bruker Daltonics, Billerica, MA) and bioMerieux Vitek MS (bioMerieux, Durham, NC) systems. The current systems also contain a spectral database and identification algorithms for the detection of conserved and microbe-specific peak patterns in whole-cell mass spectra (127). The most time-consuming portion of the current method for identification of mycobacteria is the inactivation and extraction procedure (126). Although MALDI-TOF MS is able to identify multiple species of

RGM, the method is still inadequate to distinguish closely related RGM species, including *M. abscessus* subsp. *abscessus* versus *M. abscessus* subsp. *massiliense*, and *M. mucogenicum* versus *M. phocaicum* (126).

Just as for genetic sequencing methods, the importance of an extensive database for MALDI-TOF MS cannot be overemphasized. Most users are currently supplementing the available commercial databases with their own in-house-developed databases to allow species-level differentiation of a greater number of species of NTM (126).

Future applications suggested for MALDI-TOF MS include refinement of the databases, possible typing of organisms, detection of the absence or presence of specific proteins associated with virulence and antimicrobial resistance, and development of methods for direct detection and identification from clinical samples (127).

TYPING SYSTEMS

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) is the most widely used method for molecular strain typing of the RGM. Although PFGE has never been standardized for RGM, most investigators concur that small (2 or 3 bands) differences between isolates indicate that the isolates are closely related; differences of 4 to 6 bands indicate that the strains are possibly related, and differences of ≥7 bands indicate that the isolates are genetically different (16, 82, 84, 133). Because unrelated strains of most RGM contain highly diverse PFGE patterns, this technique has been useful in epidemiological investigations. With the addition of thiourea as a modification of the original method, it is now possible to obtain reliable results by PFGE for all species of RGM, including isolates previously affected by DNA degradation (134, 135). Recent studies have shown that DNA smear patterns of previous isolates are correlated with the presence of *dnd*, a DNA degradation gene (136).

Random Amplified Polymorphic DNA PCR

In the random amplified polymorphic DNA PCR (RAPD-PCR) method, using one arbitrary primer and low-stringency conditions, the primer hybridizes to both strands of template DNA where it is matched or partially matched, resulting in strain-specific heterogeneous DNA products. Zhang and colleagues applied the RAPD-PCR or arbitrarily primed PCR analysis method to compare strains of *M. abscessus* (134). They were able to confirm several previous observations about prior nosocomial RGM outbreaks, including a 1988 epidemic of otitis media due to aminoglycoside-resistant *M. abscessus* in children with prior tympanostomy tubes, as well as a cardiac surgery outbreak (1, 134).

VNTR Analysis

The development of strain typing methods using VNTR would be valuable for helping to explain differences between isolates from chronic new infections and relapse infections. Blauwendraat and colleagues also indicated that the method would show whether or not cross-infection occurs and, moreover, enable prospective studies of strains of the *M. abscessus* complex correlated with clinical outcomes (98). Recently, Wong et al. (137) described a VNTR typing assay (MavA) using 18 tandem repeats in the *M. abscessus* genomic sequence, which was found to be superior to multilocus sequencing, with 100% typeability, 100% locus stability, and 100% reproducibility.

Repetitive-Sequence-Based PCR

A commercial system, DiversiLab system (bioMerieux, Durham, NC), is available for strain typing of organisms, including mycobacteria, by using repetitive elements interspersed throughout the genome. The system electrophoretically separates repetitive-sequence-based PCR (rep-PCR) amplicons on microfluidic chips to provide computer-generated readouts. The discriminative power has been reported to equal or exceed that of standard restriction fragment length polymorphism analysis for some species of mycobacteria, with a smaller sample size than that for standard PFGE, and in a much shorter time frame (114, 138). Limitations of the system include the lack of an extensive established database and the cost of the system.

Enterobacterial Repetitive Intergenic Consensus PCR (ERIC PCR)

Enterobacterial repetitive consensus sequences are repetitive elements distributed along the bacterial chromosome, at intergenic regions of polycistronic operons or flanking open reading frames. The method was recently evaluated with isolates of the *M. abscessus*/*M. chelonae* complex and with isolates of *M. fortuitum* (87, 139). Typing of isolates by ERIC PCR works in mycobacteria as a RAPD PCR because the presence of ERIC repeats has never been demonstrated in available *Mycobacterium* genomes, and amplification with appropriate ERIC primers can occur in the absence of genuine ERIC sequences (139). In a study of outbreak strains of *M. abscessus* in Brazil, ERIC PCR showed higher discriminatory power than PFGE for typing of strains which had shown smear patterns with PFGE using thiourea (139), although this method was not as discriminatory when testing isolates of *M. fortuitum* (87, 139).

SEROLOGIC TESTS

Serologic classification of mycobacteria has been attempted starting in 1925 with *M. avium* complex. However, serotyping has not been suitable for routine species identification of mycobacteria including the RGM and early studies served to emphasize the complexity of the antigenic composition of mycobacteria with many antigens shared by more than one species (140).

ANTIMICROBIAL SUSCEPTIBILITIES

Several different methods have been used for susceptibility testing of RGM for clinical purposes. These methods include agar disk diffusion, broth microdilution, agar disk elution and E-test. Each method has proved useful but none of the methods were well standardized until 2003 with the publication of the Clinical and Laboratory Standards Institute (CLSI) guidelines (141). In the M24A document, the CLSI recommended broth microdilution as the reference method for susceptibility testing of the RGM (142). Nine antimicrobials including amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, linezolid, imipenem, sulfamethoxazole and tobramycin have been recommended for testing and breakpoints have been established for these agents. A recent tentative CLSI guideline revision to include additional agents such as moxifloxacin, meropenem, minocycline, and trimethoprim-sulfamethoxazole has also been published (142).

Additionally, a change of the imipenem intermediate breakpoint from 8 μg/ml to 8–16 μg/ml has enabled reporting of this drug with isolates of *M. abscessus* and *M. chelonae* which was not previously recommended (142).

Briefly for the broth microdilution method, drug dilutions are prepared using serial 2-fold dilutions of cation-adjusted Mueller-Hinton broth. Suspensions of organisms are prepared to match a 0.5 McFarland turbidity standard. The suspensions are then diluted to a concentration of approximately 10^6 CFU/ml. From that suspension, 100 µl is delivered into the wells of a 16-well microtiter plate with a final concentration of approximately 10^4 CFU/well (142). MICs are optimally read after incubation at 30°C for 3 days.

Several specific recommendations about test results have also been made. Tobramycin should only be reported for isolates of M. *chelonae*. Any RGM isolate with an amikacin MIC of ≥64 µg/ml should be retested and/or sent to a reference laboratory in order to confirm resistance (although mutational resistance involving the 16S rRNA gene does occur). Also, if any isolate of the M. *fortuitum* group has an imipenem MIC >8 µg/ml, this should also be repeated with careful attention paid to inoculum density and a maximum incubation time of 3 days because of the instability of imipenem over time.

Another caveat is that the MICs of sulfamethoxazole and trimethoprim sulfamethoxazole are read using 80% inhibition of growth as the susceptibility endpoint not 100% inhibition as is used for the other antimicrobials. Overinoculation of the MIC panels is often most obvious with sulfonamides. An inexperienced laboratorian may interpret the sulfonamide MIC as resistant when in reality the inoculum was too heavy. Rarely isolates of the M. *fortuitum* group are resistant to sulfonamides. If an isolate in this group is found resistant, a repeat test with lower inoculum is warranted (142).

The recent finding of the presence of a functional *erm* gene that induces macrolide resistance in many isolates of the M. *fortuitum* group, and M. *abscessus* subsp. *abscessus* but not M. *chelonae* or M. *abscessus* subsp. *massiliense* also has made changes to the manner in which clarithromycin reporting should be done (94, 95). A major revision to the CLSI document has been added to recommend an initial 3-day reading of MICs followed by a final reading at 14 days (unless the isolate becomes resistant before that time) to detect a functional *erm* gene in RGM isolates (142). The significance of the finding of these genes has not yet been assessed in clinical trials. However, a recent landmark study in Korea among 64 patients with M. *abscessus* subsp. *abscessus* and 81 patients with M. *abscessus* subsp. *massiliense* lung disease showed that approximately 80% of the patients with M. *abscessus* subsp. *massiliense* could be successfully treated with a macrolide containing multidrug regimen in contrast to only about 20% of the patients with M. *abscessus* subsp. *abscessus* (64). The investigators suggested that inducible resistance to clarithromycin could explain the decreased efficacy of clarithromycin containing antibiotic regimens against M. *abscessus* lung disease (64).

A 2012 study by Choi and colleagues has also shown other susceptibility differences between M. *abscessus* subsp. *massiliense* (143). These investigators observed evidence of *in vitro*, *ex vivo*, and *in vivo* activity of moxifloxacin and two macrolides, clarithromycin and azithromycin against M. *abscessus* and M. *abscessus* subsp. *massiliense* (143). When moxifloxacin was combined with a macrolide against M. *abscessus*, antagonism (defined as fractional inhibitory concentration (FIC) ≥2) was observed in 65.4% (7/26) strains with clarithromycin and 46.2% (12/26) with azithromycin *in vitro*. Similar results were seen in macrophage cultures and a murine model. In contrast, however, either indifferent (FIC >0.5 and <2) or synergistic (FIC ≤0.5) effects of the same combinations were seen in strains of M. *abscessus* subsp. *massiliense*. These findings may help to further explain the differences in efficacy of macrolide-moxifloxacin treatment regimens against M. *abscessus* and M. *abscessus* subsp. *massiliense* (144). Further details of the antimicrobial susceptibility method and guidance for patient therapy may be found in chapter 76.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Dramatic taxonomic changes largely attributed to the advent of molecular testing, have occurred over the past 10 to 20 years. Multiple new species have been introduced and some former subspecies have attained species status. Of the more than 70 valid species of RGM currently described, almost half have been described within the past 10 years.

The recommended species identification methods are evolving, with declining interest and efficiency of phenotypic testing including HPLC and increasing availability and accuracy of molecular methods. Phenotypic tests have limited utility (e.g., citrate utilization to separate M. *chelonae* from M. *abscessus*), and are best applied in conjunction with molecular methods. Currently, molecular methods are preferred and generally are the only way to identify many newer species or subspecies such as M. *goodii*, M. *abscessus* subsp. *massiliense*, M. *wolinskyi*, and others. Laboratories in which molecular testing is unavailable should consider referring RGM isolates to a reference laboratory with molecular capabilities (93).

The major species of RGM (see Table 2) each have different virulence in different clinical settings, and different drug susceptibilities. The diagnostic priorities in terms of disease include bloodstream infection (usually catheter sepsis), disseminated or post-traumatic wound infection, and pulmonary disease (especially in the setting of bronchiectasis). For example, M. *mucogenicum* is a recognized cause of catheter sepsis, but because of its common presence in tap water, the species is usually considered a contaminant in sputum. The most common RGM isolated from pulmonary specimens and associated with disease is M. *abscessus*. However, other RGM such as M. *fortuitum*, which is rarely considered a pathogen except in the setting of achalasia or lipoid pneumonia, may also be recovered from respiratory samples. The pathogenic potential of the RGM is generally related to clinical findings (unexplained fever, dimorphic inflammatory lesions, etc.), immune status of the patient, number of positive cultures, quantity of organisms in the sample recovery from smear positive samples, and sources of the recovered species. Some established and newly described species have been identified from environmental samples but as yet not identified as human or animal pathogens (89). These factors emphasize the necessity to perform species level identification and susceptibility testing on clinically significant isolates of RGM. Furthermore, isolates recovered from a single sample are less likely to be significant than those from multiple samples.

When species identification of a clinically significant isolate is not available (i.e., susceptibility may be finalized prior to identification of species), MICs should be reported for antimicrobials as recommended by the CLSI. An identification such as "Rapidly growing *Mycobacterium* sp." may be acceptable until identification has been performed. However, the report should include a caveat that tobramycin is reported only for M. *chelonae* (142).

When MIC results are reported, an interpretation of MIC values (i.e., "susceptible," "intermediate," or "resistant") should be given for each antimicrobial for which there are recommended breakpoints (142). For agents such as tigecycline where no breakpoints have been recommended, an MIC value with a notation that "no CLSI breakpoints have been established for this species" should accompany the report. Moreover, because of the documented lack of efficacy for the use of first-line tuberculosis drugs (i.e., ethambutol, rifampin, isoniazid), laboratories should not report MICs for these agents against RGM (92).

Furthermore, for cultures that remain positive for the same species after 6 months of appropriate antimicrobial therapy, confirmation of species identification by molecular methods and repeat antimicrobial susceptibility testing is warranted (142).

Although recent advances in antimicrobial therapies including the new macrolides, fluoroquinolones, oxazolidinones and tigecycline have enhanced the therapeutic options and the prognosis for RGM disease, there is still a compelling need for the development of more efficient, effective, and safe oral antimicrobials for treatment. For example, *M. abscessus* subsp. *abscessus* lung disease is still generally considered incurable with the currently available antimicrobials. Susceptibility testing of the RGM is necessary in order to select optimal antimicrobial therapy and to monitor the development of mutational drug resistance which may occur with the prolonged therapy required for RGM disease.

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Approaches to the Identification of Aerobic Gram-Negative Bacteria*

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33

This chapter describes the approaches used to identify Gram-negative rods, with emphasis on the greater difficulty in identifying non-glucose-fermenting organisms. We present a scheme to identify these organisms centered around three enzymatic activities, i.e., oxidase, trypsin (benzyl-arginine arylamidase or benzyl-arginine aminopeptidase), and pyrrolidonyl aminopeptidase. These enzymatic reactions are fast and easy to interpret, and they are stable markers in almost all taxa discussed; i.e., there are few species for which these tests yield variable intraspecies results.

Before presenting the identification scheme, several issues of terminology and methodology need to be addressed. First, the use of only the substrate name to designate a biochemical test is imprecise and can lead to confusion. We suggest referring to the specific activity of the biochemical test. For example, we use "gelatin hydrolysis" or "gelatinase" instead of "gelatin" to indicate that the test demonstrates the degradation of and not the production of gelatin.

The term "production" is used when a specific metabolic product is sought, as in the case of indole, which is produced as a degradation product of tryptophan. The term "hydrolysis" is used when it is the disappearance of the substrate (and not the appearance of a degradation product) which is being demonstrated. For instance, tyrosine is present as a granulous deposit in agar, and a clearance zone is observed around the colonies or streak when tyrosine is hydrolyzed.

Although all biochemical tests rely on the presence of enzymes, the term "enzymatic activity" is used for tests in which a bacterial suspension is added to a substrate and the enzymatic activity is read without the need for bacterial growth. Enzymatic activity is detected by (i) a chromogenic shift of the substrate, e.g., by the degradation of colorless *o*-nitrophenyl-β-D-galactopyranoside (ONPG) by β-galactosidase, which results in liberation of *o*-nitrophenol, a yellow compound; (ii) by the color change of a pH indicator added to the suspension, e.g., phenol red for assessing tributyrate esterase activity; or (iii) by another indicator of enzymatic activity that causes a color change by interacting with the enzymatic product (e.g., Griess reagents turn red after interacting with nitrite [nitrate reductase activity], Kovác's reagents turn red after interacting with indole [indole production], or cinnamaldehyde turns orange to pink after interacting with free β-naphthylamine [aminopeptidase activity]).

This chapter also uses precise terminology for the terms "utilization," "oxidation," and "glycolysis." "Utilization" is not a precise term to address either assimilation or the usage of any substrate being metabolized, and therefore we avoid using it. "Oxidizer" is often used to describe saccharolytic nonfermenters as opposed to asaccharolytic nonfermenters. However, in strict biochemical terminology, it refers to oxidative phosphorylation as opposed to fermentation as an alternative route after glycolysis. In this chapter, we do not use the term "oxidation" to mean "saccharolysis," but we specifically use the term "acidification of carbohydrates" or "saccharolysis." In addition, we avoid the use of "glycolysis" for "saccharolysis," because "glycolysis" may be interpreted as referring to the acidification of glucose only or can be understood as the biochemical metabolic cycle preceding fermentation or respiration.

TEST METHODS WITH EMPHASIS ON GRAM-NEGATIVE NONFERMENTERS

Careful attention to testing methods is critical when describing their application to nonfermenters. Slight variations of reagents or incubation conditions can cause numerous misidentifications and have caused erroneous descriptions of new species. Also, although several media and biochemical tests are used for the identification of fermenting *Enterobacteriaceae*, these are not necessarily optimal when testing nonfermenters. Nonfermenters require good aeration because of their strict respiratory metabolism, most often with atmospheric oxygen as the terminal electron acceptor. The growth of most nonfermenters in liquid media is moderate or weak due to the intrinsically poor aeration of liquid media. Therefore, a small inoculum such as that used for *Enterobacteriaceae* is not appropriate. Instead, liquid media should be inoculated with a turbid suspension of nonfermenters. Whenever bacterial growth is not required, as with testing enzymatic activity, it is preferable to use a heavy suspension of bacterial cells in an aqueous solution of the substrate rather than to inoculate a liquid medium. Further detailed remarks can be found below where the different biochemicals used for identification are described.

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*This chapter contains information presented in chapter 31 by Georges Wauters and Mario Vaneechoutte in the 10th edition of this *Manual.*

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Below we briefly describe some specific tests or modifications used in the identification algorithms of this chapter and of chapter 44 and highlight protocols that should be followed when these tests are performed and their results are interpreted, with an emphasis on their application to nonfermenters. The descriptions of the other tests discussed in this chapter can be found in chapter 19.

Assimilation or Utilization of Organic Compounds as the Sole Carbon Source for Growth

Different approaches exist to determine the capacities of strains to assimilate, i.e., utilize organic compounds as the sole carbon source for growth. Microbial ID/Characterization (Biolog, Hayward, CA) and API NE and API 32GN (BioMérieux, Marnes-la-Coquette, France) are commercial systems that score growth by assessing the optical density of the minimal broths to which the organic compound has been added. Another method to assess the assimilation of an organic compound as the sole carbon source is to observe the alkalinization of a minimal medium to which the sole carbon source and a pH indicator have been added. Therefore, the term "alkalinization" is frequently used as synonymous to the term "assimilation," although "assimilation" may be preferred as a more general term (see below). Simmons' citrate agar base medium is frequently used, with bromothymol blue as the pH indicator (yellow at pH 6.0, green at pH 6.9, and blue at pH 7.6). Citrate as the sole carbon source can be replaced by any compound to be tested (1). For most substrates, the medium is also alkalinized as a result of the production of hydroxyl radicals and CO_2 by the metabolism of the sole carbon source added. Because CO_2 can escape, the remaining radicals alkalinize the medium. Therefore, it is essential that the tubes be incubated with a loose lid to allow the carbon dioxide to escape. Alkalinization facilitates the interpretation, but as stated above, growth without alkalinization can occur with some substrates and should be interpreted as assimilation positive, as well.

In this chapter, we limit the description of assimilation assays for the identification of nonfermenters to assays for acetamide and acetate, used solely as additional characteristics to differentiate between some species. However, as described in chapter 44, *Acinetobacter* identification has been based largely on assimilation tests by supplementing the basal mineral medium of Cruze et al. (2) with a 0.1% (wt/vol) concentration of the carbon source. We refer the reader to that chapter for basal medium composition, inoculation, and interpretation.

Acid Production from Carbohydrates or Sugars: Saccharolysis

The term "acid production" may refer to acidification of a carbohydrate by fermenters as well as by nonfermenters. For diagnostic purposes, while there is no need to differentiate between these modes of acid production (fermentation or oxidation), it is important to distinguish acidification from assimilation. Assimilation is tested on minimal media lacking all carbon sources except the one tested for utilization, whereas more-complex media (peptone-rich media) aimed at supporting the optimal growth of the organism tested are used to assess the acidification of the single carbohydrate substrate added.

Except with the strong acid producers *Pseudomonas aeruginosa* and *Acinetobacter* spp. and some rare species, like those of the genus *Asaia*, acid production by nonfermenters is usually much weaker than acid production by fermenters. Nonfermenters acidify only as a result of their general metabolic activity; this usually leads to weaker acidification than fermentation, which produces strong acids like lactic acid. Nonfermenters acidify only under well-aerated incubation conditions. Therefore, the conditions to assess this weaker acidification by nonfermenters should be optimized to avoid false-negative results. API kits, like API 20 E (*Enterobacteriaceae*), API 20 NE (non-*Enterobacteriaceae*), and API ID 32 GN (Gram negatives), are not suited to detect the acidification of carbohydrates by nonfermenters. API 20 NE and API ID 32 GN only score assimilation, which, as already stated above, is sometimes confused with acidification. Two examples are presented to emphasize this point. The results of carbohydrate acidification for *Chryseobacterium caeni* N4^T, obtained by Kämpfer et al. (3), were substantially different from the original description of this species (4). Quan et al. (4) described *C. caeni* as an asaccharolytic species, whereas Kämpfer et al. (3) found the type strain of this species to be one of the most saccharolytic strains among the members of the *Chryseobacterium* genus, rapidly acidifying glucose, maltose, sucrose, trehalose, and L-arabinose. This contradiction may be explained by the fact that Quan et al. (4) used the API 20 E, API 20 NE, and API 32 GN galleries to assess the acidification of carbohydrates. Another example comes from the description of *Ralstonia insidiosa* (5), which mentions that no acid is produced from glucose, sucrose, or lactose when the organism is tested with RapID NF Plus (Remel) and API 20 NE (BioMérieux), whereas this species clearly acidifies glucose, L-arabinose, D-xylose, and maltose (6).

Because acid production from carbohydrates by nonfermenting Gram-negative rods results in a weaker pH change than that caused by fermentation, the medium and incubation conditions should be optimized to detect the weak acid production. This can be achieved by (i) heavy inoculation; (ii) good aeration; (iii) using media with only small amounts of peptones, because nonfermenters' aerobic metabolism promotes alkalinization and neutralizes the (weak) acidification; and (iv) using media with an appropriate pH indicator.

The oxidation/fermentation medium, with its low peptone content of 0.1%, as originally described by Hugh and Leifson (7), is widely used for this purpose, but acidification of carbohydrates by nonfermenters is better demonstrated on slanted solid media. Ammonium salt agar (ASA) has been recommended (8), but better results can be obtained with low-peptone phenol red (LPPR) agar, as originally described for testing the acidification of ethylene glycol (9). The slant is heavily inoculated, and the medium is incubated for up to 7 days. LPPR agar is more sensitive to acidification of a substrate than ASA, because it is not buffered and because phenol red shifts color to yellow already at pH 6.8, whereas ASA is buffered and the indicator used, i.e., bromocresol purple, shifts only when the pH has dropped to as low as 5.2.

Acid production from the sugar alcohol ethylene glycol, best tested on LPPR agar with 2% ethylene glycol (9), is interesting for the identification of nonfermenters, because it is independent of acid production from sugars. This method is helpful in identifying species that acidify ethylene glycol but not sugars, such as *Alcaligenes faecalis*, *Oligella* spp., and *Kerstersia gyiorum*. Conversely, species like *Burkholderia* spp. and *Ralstonia pickettii* acidify sugars but not ethylene glycol.

Arginine Dihydrolase

Arginine dihydrolase activity by nonfermenters can best be tested by dense inoculation (McFarland standard, 4 to 5) in Moeller broth with 1% arginine. After inoculation, the broth is overlaid with paraffin oil. Alkalinization, caused by the production of ammonia, is detected by the pH indicators. A positive result is indicated by a purple color, which can be read after 4 h to up to 2 days. These enzymatic reactions can proceed in the absence of oxygen and therefore are not hampered by the presence of paraffin, which is needed to prevent false-positive alkalinization of the broth due to aerobic metabolic pathways, like peptone metabolism. This is a problem especially when prolonged incubation is needed, as is the case with some *Haematobacter* strains.

Esculin Hydrolysis

Esculin hydrolysis by nonfermenters is preferably tested on esculin agar without bile, because bile can inhibit some nonfermenters, such as *Moraxella* spp. Broad streak inoculation should be applied. Strains positive for esculin hydrolysis cause a blackening of the agar after overnight incubation.

Flagellum Stains

The detection of flagella is very useful for the identification of some groups of nonfermenters that are otherwise biochemically very similar. Since flagellum staining and microscopy require additional efforts, we limit their use to those species that cannot be differentiated by other methods. There are several types of flagellation. The genera *Achromobacter*, *Alcaligenes*, *Bordetella*, and *Cupriavidus*, including *Cupriavidus gilardii* (originally described as polar [10]), have peritrichous flagella (9). *Ochrobactrum* and *Rhizobium* spp. also exhibit peritrichous flagella, but they frequently have only one or two lateral flagella. *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Pseudomonas alcaligenes*, *Pseudomonas pseudoalcaligenes*, *Pseudomonas oryzihabitans*, the genera *Brevundimonas* and *Shewanella*, *Ralstonia pickettii*, and *Sphingomonas paucimobilis* have polar monotrichous flagellation. In the last two species, the flagella are rarely detected. *Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas luteola*, the *Burkholderia cepacia* group, and *Stenotrophomonas maltophilia* have polar multitrichous flagella, and the genera *Comamonas*, *Delftia*, and *Herbaspirillum* have bipolar multitrichous tufts.

We have found that flagella are best stained as follows (adapted from the work of Kodaka et al. [11]). Ten volumes of solution A [10 ml of 5% phenol, 2 g of tannic acid, and 10 ml of $KAl(SO_4)_2 \cdot 12H_2O$ (saturated solution)] are mixed with 1 volume of solution B (12 g of crystal violet in 100 ml of 96% ethanol). The mixture is allowed to first stand for 2 to 3 days, without filtration, centrifugation, or shaking. This stain can be used for up to 1 year when stored at room temperature. Staining is carried out by making a suspension with a density of <0.5 McFarland standard from fresh colonies on tryptic soy agar (TSA) in a mixture of 30% ethanol and 70% tap water, briefly heating a coverslip (24 by 60 mm) (not a microscopic slide) by holding it in a flame, and adding a small drop (5 μl) of the suspension. This drop spreads spontaneously and is allowed to dry. The staining solution is added for 1 to 4 min (longer for longer-stored staining solution), and the coverslip is rinsed with tap water and allowed to dry in an incubator. Pritt glue is applied to the coverslip for better adherence, and the coverslip is then placed onto a slide, with the colored face upward. It is observed under a ×100 magnification.

Flexirubin Pigment Production

Flexirubin pigment production can be assessed for yellow-pigmented colonies of the *Flavobacteriaceae*. A small mass of bacterial cells is collected with a loop and deposited in a drop of a 20% KOH solution on a slide; a red color indicates the presence of flexirubin pigments (8). *Sphingomonas paucimobilis* produces yellow colonies, but not flexirubin, whereas most *Myroides* strains produce weakly yellow colonies and are always flexirubin positive.

Gelatin Hydrolysis

Gelatin hydrolysis is best assessed by means of the plate method. Inoculate the plate (nutrient agar plus 0.4% gelatin) with a spot or a streak and incubate until luxuriant growth is obtained. When the isolate is gelatin hydrolysis positive, visual clearing of the agar is usually obvious. If it is not, add an $HgCl_2$ solution (12 g of $HgCl_2$, 16 ml of HCl [35%], and 80 ml of distilled water [dH_2O]) and observe the clearing. The use of $HgCl_2$ solution is generally avoided because of its high toxicity.

Growth at 41°C

Growth at 41°C should be tested by adding 1 drop of a small inoculum (e.g., as for swabbing susceptibility testing plates) in broth tubes incubated in a warm water bath, with a precision of ±0.1°C. Care should be taken that the broth is still completely clear, i.e., not turbid at all, after inoculation. Use of 1.5 to 2 ml of tryptic soy broth (TSB) with a maximal air surface (e.g., in tubes of 16 mm) instead of 5 ml is preferred for better aeration and optimal growth of nonfermenters. The test is read as positive when growth, observed as the presence of any turbidity, occurs. The use of a positive-control tube at 37°C is recommended to exclude the possibility that negativity for growth at 41°C is caused by a general loss of viability.

H_2S Production or Sulfite Reductase Activity

H_2S production or sulfite reductase activity is best tested on Kligler's iron agar (KIA), containing thiosulfate and Fe salt. The H_2S produced from thiosulfate by sulfite reductase will react with the Fe salt to produce FeS, a strong black precipitate which for clinically relevant nonfermenters is observed only for *Shewanella* species. Lead acetate paper, held over the medium to detect evaporating H_2S fumes, is not very useful. This method detects the weak production of H_2S from the amino acids methionine and cysteine. This reaction is widely observed among *Enterobacteriaceae* and is therefore not very discriminative. (Note that when this reaction is present with nonfermenters, the observed result is usually weak and can yield variable or false-negative results.) Amino acid-derived H_2S production is too weak to be detected on KIA.

Indole Production

Indole production can be detected by different methods. The broth growth method includes inoculation of a broth culture, e.g., heart infusion broth, incubation for 48 h, extraction with xylene, and addition of Ehrlich's reagent (see chapter 19). However, as is the case for the spot indole test (see chapter 19), the broth growth method is not advisable for nonfermenters, because it may lack sensitivity and may be difficult to interpret, yielding a false-negative result for indole production. For example, *Flavobacterium mizutaii*, which is clearly positive for indole production, was described as negative (12), because indole production was assessed using the broth growth method. Similarly, *Elizabethkingia meningoseptica* is mentioned as only 50% indole production positive (13), but using the heavy-suspension method, we have never encountered an *E. meningoseptica* isolate that was negative for indole production. In general, most members of the genera *Chryseobacterium*, *Elizabethkingia*, and *Empedobacter* are indole positive, but this reaction is sometimes weak and difficult to demonstrate by conventional broth methods. Therefore, we recommend a simple, rapid, growth-independent method for demonstration of indole production. A heavy suspension (4 to 5 McFarland standard) is prepared in a 0.3% tryptophan solution in dH_2O. After 3 to 4 h of incubation, indole is detected by adding Kovác's reagent (for its composition, see chapter 19). This method allows detection of all indole-producing nonfermenting Gram-negative rods within a short time.

Motility

Motility can easily be determined by microscopic observation of a wet-mount preparation of a young colony from an agar plate. Motility can be studied by inoculation of sulfide-indole motility (SIM) medium, described in chapter 19. Another culture method, circumventing the need for microscopy, uses inoculation of a large inoculum of a fresh culture in soft agar (0.3% agar), preferably in a small petri dish (5-cm diameter); this provides better aeration than tubes, reducing the potential for false-negative results. For some species, such as *Sphingomonas*, motility can best be demonstrated after incubation of cultures at room temperature, instead of the usual 30 to 35°C for other nonfermenters.

NaCl Requirement

Most commercially available basic media (like TSB) already contain NaCl and should not be used to assess the requirement for NaCl. It is best to use well-aerated tubes with peptone water, i.e., an aqueous solution of 1% tryptone or Casitone, without NaCl rather than peptone water to which 1% NaCl has been added. Because one is testing the ability of an organism to grow under these conditions, a small inoculum should be used to allow facile detection of turbidity. Also, care should be taken that the inoculum is prepared from a suspension in dH_2O or 1% peptone water without NaCl. The halotolerance of isolates can be studied using peptone water containing increasing concentrations of NaCl, up to 12%.

Nitrite and Nitrate Reductase Activities

Nitrite and nitrate reductase activities can be determined together in nitrate broth (see chapter 19), but for nonfermenters, they are best determined by heavy inoculation of separate nitrate and nitrite broths, in which (after incubation) the presence of nitrite is detected by the addition of Griess reagents and, eventually, of zinc dust.

Nitrate can be reduced to nitrite by nitrate reductase, and nitrite can be reduced by nitrite reductase to either nitrogen gas (N_2) or ammonium for assimilation into amino compounds. Some species reduce only nitrate, others reduce both nitrate and nitrite, and a few may reduce nitrite without reducing nitrate.

Nitrate reduction to nitrite by nitrate reductase is best detected in 0.5 or 1 ml of 0.1% KNO_3 broth (for prolonged incubation) or aqueous solution (4 h), using a turbid inoculum (McFarland standard of 4 to 5). The organism is positive for nitrate reductase activity when the addition of Griess reagents (solution A first and then solution B) results in a pink color within 2 min, indicating the presence of nitrite, i.e., the reduction of nitrate to nitrite.

Nitrite reductase activity can be tested by adding a very heavy inoculum (McFarland standard of 8 to 10) to a small volume, e.g., 0.5 or 1 ml, of nitrite broth (0.001% $NaNO_2$ in 1% peptone). The organism is positive for nitrite reductase activity when, after 4 to 24 h of incubation, the addition of Griess' reagents results in a colorless solution, indicating the absence of nitrite, i.e., the reduction of nitrite to N_2, or ammonium.

In summary, certainly for nonfermenters, it may be advisable to use separate nitrate and nitrite broths, to eventually add a Durham tube to the nitrite broth (instead of to the nitrate broth, as is usually done), and to add zinc dust to the nitrate broth (the last only for strains for which both nitrate and nitrite broths remain colorless after the addition of the Griess reagents). Interpretation can be done according to Table 1 in chapter 19 of the previous edition of this *Manual* (17).

Pyrrolidonyl Aminopeptidase (Pyrrolidonyl Arylamidase) Activity

Pyrrolidonyl aminopeptidase (pyrrolidonyl arylamidase) activity has been shown to be very helpful in the identification of nonfermenters (14, 15) and is a central characteristic, together with oxidase and trypsin, in the identification scheme proposed here. The test is described in chapter 19.

Starch Hydrolysis

Starch hydrolysis is tested by inoculation onto Mueller-Hinton agar (MHA), which contains starch. Flooding with iodine solution (Lugol) yields a purple-blue color in the presence of starch. Starch hydrolysis is read as a non-purple-stained zone around the streak.

Susceptibility to Colistin

Susceptibility to colistin can be tested on common agars, using 10-µg colistin disks. For diagnostic purposes, any zone of inhibition is interpreted as susceptible.

Susceptibility to Desferrioxamine

Susceptibility to desferrioxamine, an Fe chelator, can be tested on MHA (or on TSA for strains growing poorly on MHA) by using paper disks loaded with 250 µg of desferrioxamine or by using commercially available Rosco tablets (15). Media should not contain blood, because this provides large amounts of bioavailable Fe, which, in turn, causes false-positive resistance results. Any inhibition zone is interpreted as susceptible.

Susceptibility to Vancomycin

Susceptibility to vancomycin is tested by disk diffusion, using 30-µg vancomycin disks. Some Gram-negative organisms are vancomycin susceptible, and this can be used as a rapid and distinctive identification tool.

Trypsin or Benzyl-Arginine Arylamidase Activity

Trypsin or benzyl-arginine arylamidase activity is very discriminative for nonfermenters, since the strains of almost all taxa exhibit an all-or-nothing positive result, providing a test with high discriminatory value. Therefore, this test can act as a first step in identification. Testing for trypsin activity is described in chapter 19, but the procedure is also easily performed by adding a trypsin tablet (Rosco) to a dense bacterial suspension in 0.5 ml of dH_2O, the results of which can be read within 4 h of incubation by the addition of cinnamaldehyde [see "Pyrrolidonyl Aminopeptidase (Pyrrolidonyl Arylamidase) Activity" above] (15).

IDENTIFICATION SCHEME AND IDENTIFICATION TABLES

Below, we outline a proposal for rapid and simplified biochemical testing, with emphasis on the identification of

TABLE 1 Dichotomous identification algorithm for Gram-negative bacteria with poor or no growth on SBA[a]

1a. Growth on SBA p^{w} → 2
1b. Growth on SBA n → 14
2a. Cells are tiny coccobacilli → 3
2b. Cells are diplococci or coccobacilli → *Neisseria*, except *Neisseria gonorrhoeae* (chapter 34), *Moraxella* (chapter 44)
2c. Cells are fusiform rods → *Capnocytophaga* (chapter 35)
2d. Cells are rods → 6
3a. Urease p → *Brucella* spp. (chapter 47)
3b. Urease n → 4
4a. Oxidase n → *Francisella* (chapter 46)
4b. Oxidase p → 5
5a. Hydrogen sulfide production on KIA p → *Francisella philomiragia* (chapter 46)
5b. Hydrogen sulfide production on KIA n → *Bordetella* spp. (chapter 45)
6a. Colony color pink → *Asaia*, *Azospirillum*, *Methylobacterium*, *Roseomonas* (chapter 44)
6b. Colony color other → 7
7a. Cauliflower-like colonies → *Bartonella* (chapter 48)
7b. Other colonies → 8
8a. Ornithine decarboxylase p, acidification of glucose n → *Eikenella* (chapter 35)
8b. Ornithine decarboxylase n, acidification of glucose p → 9
9a. Acidification of lactose p → 10
9b. Acidification of lactose n → 11
10a. Acidification of xylose p, sucrose n → *Dysgonomonas* (chapter 35)
10b. Acidification of xylose n, sucrose p → *Aggregatibacter aphrophilus* (chapter 35)
11a. Acidification of sucrose p → 12
11b. Acidification of sucrose n → 13
12a. Alkaline phosphatase p → *Suttonella* (chapter 35)
12b. Alkaline phosphatase n → *Cardiobacterium* (chapter 35)
13a. Catalase p → *Aggregatibacter actinomycetemcomitans* (chapter 35)
13b. Catalase n → *Simonsiella* (chapter 35)
14a. Regan-Lowe agar or Bordet-Gengou agar required for growth → *Bordetella pertussis* (chapter 45)
14b. Chocolate agar required for growth → 15
14c. Buffered charcoal yeast extract agar required for growth → 18
14d. Brain heart infusion agar with serum required for growth → 19
15. Chocolate agar required for growth
15a. Cells are diplococci or coccobacilli → *Neisseria gonorrhoeae* (chapter 35)
15b. Cells are bacilli → 16
16a. Cauliflower-like colonies → *Bartonella*, *Afipia* (chapter 48)
16b. Other colonies → 17
17a. Requirement for hemin (X) and/or adenine dinucleotide (V) p → *Haemophilus* (chapter 36)
17b. Requirement for hemin (X) and/or adenine dinucleotide (V) n → *Francisella* (chapter 46)
18. Buffered charcoal yeast extract agar required for growth
18a. Cells are long rods → *Legionella* (chapter 49)
18b. Cells are regular rods → *Francisella* (chapter 46)
19. Brain heart infusion agar with serum required for growth
19a. Cells are pleomorphic, bent filamentous rods → *Streptobacillus* (chapter 35)
19b. Cells are small rods → *Bartonella*, *Afipia* (chapter 48)

[a] p, positive; p^{w}, weakly positive; n, negative; → [number], go to number indicated further on in the table; SBA, 5% sheep blood agar; KIA, Kligler's iron agar. Chapter numbers in parentheses indicate the chapters in which the organism(s) is described further in this *Manual*.

Gram-negative nonfermenters. Most nonfermenting Gram-negative bacteria of clinical relevance have an optimal growth temperature between 30 and 37°C and grow on simple media, such as TSA, MHA, and sheep blood agar (SBA). A few species, however, may grow better below 30°C (e.g., *Methylobacterium*), and some *Moraxella* species are more fastidious and require SBA at 37°C for optimal growth.

The scheme below makes it possible in most cases to record the following characteristics within 3 days, starting with KIA: growth on TSA, MHA, and/or SBA; oxidase; hydrogen sulfide production; fermentation of lactose and glucose; colony pigmentation; motility; susceptibility to colistin, desferrioxamine, and vancomycin; alkaline phosphatase; benzyl-arginine aminopeptidase (trypsin); pyrrolidonyl aminopeptidase; urease; β-galactosidase; indole production; nitrate reductase; nitrite reductase; lysine decarboxylase; arginine dihydrolase; and acidification of glucose, mannitol, xylose, and ethylene glycol. The reagents used can be prepared once each year and stored at 4°C.

For the sake of manageability, *Advenella incenata* (see chapter 45) and the species of the genus *Pandoraea* (see chapter 43) have not been included in this simplified scheme for aerobically growing Gram-negative bacteria. The extreme variability of the phenotypic characteristics within these species and the biochemical inactivity of some *Pandoraea* species would have rendered the scheme proposed here impractical. However, these species are listed below (see Table 5), and information on their characteristics has been filled out as completely as possible.

Gram-negative bacteria can be identified using the scheme below. It should be mentioned that *Acinetobacter*

TABLE 2 Dichotomous identification algorithm for aerobically growing Gram-negative bacteria which form purple or pink colonies on SBA[a]

1a. Colony is purple on SBA → *Chromobacterium* (chapter 35)
1b. Colony is pink on SBA → 2
2a. Fermentation p, butt of KIA tube is yellow → some *Serratia* species (chapter 38)
2b. Fermentation n, butt of KIA tube is unchanged → 3
3a. β-Galactosidase p → *Azospirillum* spp. (chapter 44)
3b. β-Galactosidase n → 4
4a. Desferrioxamine S, nitrate reductase p → *Roseomonas* genomospecies 4 (chapter 44)
4b. Desferrioxamine R, nitrate reductase n → 5
5a. Trypsin p → 6
5b. Trypsin n → 7
6a. Urease p → *Methylobacterium* spp. (chapter 44)
6b. Urease n → *Asaia* spp. (chapter 44)
7a. Pyrrolidonyl aminopeptidase p → 8
7b. Pyrrolidonyl aminopeptidase n → 9
8a. Oxidase p^{w} → *Roseomonas gilardii* (chapter 44)
8b. Oxidase n → *Roseomonas mucosa* (chapter 44)
9a. Acidification of ethylene glycol p → *Roseomonas cervicalis* (chapter 44)
9b. Acidification of ethylene glycol n → *Roseomonas* genomospecies 5 (chapter 44)

[a] p, positive; p^{w}, weakly positive; n, negative; → [number], go to number indicated further on in the table; S, susceptible; R, resistant; SBA, 5% sheep blood agar; KIA, Kligler's iron agar. Chapter numbers in parentheses indicate the chapters in which the organism(s) is described further in this *Manual.*

cells can stain Gram variable; sometimes one of two cells in a pair of diplococci is Gram negative and the other is Gram positive. Also, some phylogenetically Gram-positive bacteria may stain Gram negative, e.g., *Lactobacillus iners* and *Gardnerella vaginalis* (16). These have not been included in the scheme below. The scheme begins with observation of growth on SBA and takes advantage of observed pigment production.

Table 1 lists the organisms that are very fastidious and grow poorly or not at all on SBA. Species like *Acinetobacter parvus* or most *Moraxella* spp. also grow poorly or slowly, but clearly visible colonies are formed after 48 h at 37°C; these are described below (see Table 4). The organisms that grow on SBA and that exhibit pink or purple colonies are included in Table 2. For those species that grow well on SBA and do not form pink or purple colonies, proceed according to the steps outlined below.

Day 1. Stab inoculate a KIA tube.
Incubate overnight at 30 to 37°C.

Day 2. Interpret the KIA results (differentiate between fermenters and nonfermenters) as follows.

If both the butt of the tube and the slant are yellow, the culture is positive for the fermentation of both lactose and glucose. Refer to Table 3 (glucose-fermenting Gram-negative organisms). If only the butt is yellow, the organism is negative for lactose and positive for glucose fermentation. Refer to Table 3. If there is no yellow color, the organism is negative for both lactose and glucose fermentation; i.e., the organism is a nonfermenter. Proceed with the testing outlined for day 3 below.

(Text continues on page 623)

TABLE 3 Dichotomous identification algorithm for aerobically growing Gram-negative bacteria fermenting glucose (facultative anaerobes)[a]

1a. Oxidase p → 2
1b. Oxidase n → 6
2a. Growth with 6% NaCl p, halotolerance p → *Vibrio* spp. (chapter 41)
2b. Growth with 6% NaCl n, halotolerance n → 3
3a. Motility p → 4
3b. Motility n → 5
4a. Lysine decarboxylase p → *Plesiomonas* spp. (chapter 38), *Aeromonas hydrophila*, *Aeromonas veronii* bv. sobria (chapter 40)
4b. Lysine decarboxylase n → other *Aeromonas* spp. (chapter 40)
5a. Fermentation of sucrose p → *Pasteurella* spp., *Actinobacillus* spp. (chapter 35)
5b. Fermentation of sucrose n → *Pasteurella bettyae*, *partim* oxidase p (chapter 35)
6a. Growth with 6% NaCl p, halotolerance p → *Vibrio metschnikovii* (chapter 41)
6b. Growth with 6% NaCl n, halotolerance n → 7
7a. Growth on MacConkey agar n → *Pasteurella bettyae*, *partim* oxidase n, *partim* growth on MacConkey agar n (chapter 35)
7b. Growth on MacConkey agar p → 8
8a. Fermentation of lactose, trehalose, or xylose p → *Enterobacteriaceae* (chapters 37–39)
8b. Fermentation of lactose, trehalose, and xylose n → 9
9a. Phenylalanine deaminase p → *Providencia* spp., *Morganella* spp. (chapter 38)
9b. Phenylalanine deaminase n → 10
10a. Hydrogen sulfide production in KIA p → *Edwardsiella* spp. (chapter 38)
10b. Hydrogen sulfide production in KIA n → *Pasteurella bettyae*, *partim* oxidase n, *partim* growth on MacConkey agar p (chapter 35)

[a] p, positive; n, negative; → [number], go to number indicated further on in the table; KIA, Kligler's iron agar; *partim*, Latin term used to indicate "the part that is." Chapter numbers in parentheses indicate the chapters in which the organism(s) is described further in this *Manual.*

TABLE 4 Dichotomous identification algorithm for aerobically growing Gram-negative nonfermenters (strict aerobes)[a]

Gram-negative nonfermenters
1a. Hydrogen sulfide production in KIA p → 2
1b. Hydrogen sulfide production in KIA n → 3

Hydrogen sulfide production-positive, Gram-negative nonfermenters
2a. Requirement for NaCl n, acidification of sucrose p → *Shewanella putrefaciens* (chapter 44)
2b. Requirement for NaCl p, acidification of sucrose n → *Shewanella algae* (chapter 44)

Hydrogen sulfide production-negative, Gram-negative nonfermenters
3a. Pyoverdin production p (green pigment on MHA) → 4
3b. Pyoverdin production n (no green pigment on MHA) → 10
4a. Growth at 41°C p, assimilation of acetamide p → *P. aeruginosa*, *partim* pyoverdin production p (65%) (chapter 42)
4b. Growth at 41°C n, assimilation of acetamide n → 5
5a. Acidification of xylose n → 6
5b. Acidification of xylose p → 7
6a. Growth with 6% NaCl p, halotolerance p → *Pseudomonas mosselii* (chapter 42)
6b. Growth with 6% NaCl n, halotolerance n → *Pseudomonas monteilii* (chapter 42)
7a. Nitrate reductase p → 8
7b. Nitrate reductase n → 9
8a. Nitrite reductase p → *Pseudomonas veronii* or *Pseudomonas fluorescens*, *partim* pyoverdin production p (95%), *partim* nitrate reductase p (20%), *partim* nitrite reductase p (5%) (chapter 42)
8b. Nitrite reductase n → *Pseudomonas fluorescens*, *partim* pyoverdin production p (95%), *partim* nitrate reductase p (20%), *partim* nitrite reductase n (95%) (chapter 42)
9a. Gelatinase p → *Pseudomonas fluorescens*, *partim* pyoverdin production p (95%), *partim* nitrate reductase n (80%) (chapter 42)
9b. Gelatinase n → *Pseudomonas putida*, *partim* pyoverdin production p (95%) (chapter 42)
10a. Indole p → 11
10b. Indole n → 23

Hydrogen sulfide production-negative, indole production-positive, Gram-negative nonfermenters
11a. Trypsin n → *Balneatrix alpica* (chapter 44)
11b. Trypsin p → 12
12a. Pyrrolidonyl aminopeptidase n → *Bergeyella zoohelcum* (chapter 44)
12b. Pyrrolidonyl aminopeptidase p → 13
13a. Acidification of glucose n → *Weeksella virosa* (chapter 44)
13b. Acidification of glucose p → 14
14a. Acidification of mannitol p → 15
14b. Acidification of mannitol n → 16
15a. Urease p → *Elizabethkingia miricola* (chapter 44)
15b. Urease n → *Elizabethkingia meningoseptica* (chapter 44)
16a. Urease p → 17
16b. Urease n → 18
17a. Growth at 41°C p → *Chryseobacterium gleum* (chapter 44)
17b. Growth at 41°C n → *Wautersiella falsenii* (chapter 44)
18a. Acidification of ethylene glycol p → *Chryseobacterium hominis* (chapter 44)
18b. Acidification of ethylene glycol n → 19
19a. Acidification of xylose p → 20
19b. Acidification of xylose n → 21
20a. Gelatinase p → *Chryseobacterium anthropi* (chapter 44)
20b. Gelatinase n → *Sphingobacterium mizutaii* (chapter 44)
21a. Gelatinase n → *Chryseobacterium treverense* (chapter 44)
21b. Gelatinase p/pp → 22
22a. Esculin hydrolysis p → *Chryseobacterium indologenes* (chapter 44)
22b. Esculin hydrolysis n → *Empedobacter brevis* (chapter 44)

Hydrogen sulfide production-negative, indole production-negative, Gram-negative nonfermenters
23a. Oxidase n → 24
23b. Oxidase p → 34

Hydrogen sulfide production-negative, indole production-negative, oxidase-negative, Gram-negative nonfermenters
24a. Brown pigment on TSA, i.e., tyrosine hydrolase p → 25
24b. Other → 26
25a. Urease p → *Bordetella parapertussis* (chapter 45)
25b. Urease n → *Bordetella holmesii* (chapter 45)

(Continued on next page)

TABLE 4 Dichotomous identification algorithm for aerobically growing Gram-negative nonfermenters (strict aerobes) *(Continued)*

26a. Trypsin p → 27
26b. Trypsin n → 29
27a. Pyrrolidonyl aminopeptidase n → *Stenotrophomonas maltophilia* (chapter 43)
27b. Pyrrolidonyl aminopeptidase p → 28
28a. Arginine dihydrolase p, esculin hydrolysis p → *Pseudomonas luteola* (chapter 42)
28b. Arginine dihydrolase n, esculin hydrolysis n → *Pseudomonas oryzihabitans* (chapter 42)
29a. Motility n → *Acinetobacter* (chapter 44)
29b. Motility p → 30
30a. Acidification of glucose, of mannitol, of xylose p → *Burkholderia gladioli* (chapter 43)
30b. Acidification of glucose, of mannitol, of xylose n → 31
31a. Gelatinase p → "*Bordetella ansorpii*" (chapter 45)
31b. Gelatinase n → 32
32a. Desferrioxamine S, growth at 41°C p → *Kerstersia gyiorum* (chapter 45)
32b. Desferrioxamine R, growth at 41°C n → *Bordetella trematum* (chapter 45)

Hydrogen sulfide production-negative, indole production-negative, oxidase-positive, Gram-negative nonfermenters
33a. Trypsin p → 34
33b. Trypsin n → 65
34a. Pyrrolidonyl aminopeptidase p → 35
34b. Pyrrolidonyl aminopeptidase n → 51

Hydrogen sulfide production-negative, indole production-negative, oxidase-positive, Gram-negative nonfermenters; trypsin positive, pyrrolidonyl aminopeptidase positive
35a. Urease n → 36
35b. Urease p → 42
36a. Desferrioxamine S → 37
36b. Desferrioxamine R → 38
37a. Alkaline phosphatase p/pp → *Brevundimonas diminuta*, *partim* pyrrolidonyl aminopeptidase p (20%) (chapter 43)
37b. Alkaline phosphatase n → *Pseudochrobactrum asaccharolyticum* (chapter 44)
38a. Alkaline phosphatase p → *Sphingomonas* spp., *partim* pyrrolidonyl aminopeptidase p (25%) (chapter 44)
38b. Alkaline phosphatase n → 39
39a. Colistin S → 40
39b. Colistin R → 41
40a. Growth at 41°C p → *Pseudomonas aeruginosa*, *partim* pyoverdine production n (35%), *partim* pyrrolidonyl aminopeptidase p (95%) (chapter 42)
40b. Growth at 41°C n → *Pseudomonas fluorescens*, *partim* pyoverdine production n (5%), *partim* pyrrolidonyl aminopeptidase p (60%) (chapter 42)
41a. Acidification of ethylene glycol p → *Ochrobactrum intermedium*, *partim* urease n (56%) (chapter 44)
41b. Acidification of ethylene glycol n → *Inquilinus limosus*, *partim* urease n (65%) (chapter 44)
42a. Nitrate reductase n → 43
42b. Nitrate reductase p → 47
43a. Desferrioxamine S → *Myroides odoratus* (chapter 44)
43b. Desferrioxamine R → 44
44a. Alkaline phosphatase n, mucoid colonies → *Inquilinus limosus*, *partim* urease p (35%) (chapter 44)
44b. Alkaline phosphatase p → 45
45a. Acidification of mannitol p, of ethylene glycol p → *Sphingobacterium spiritivorum* (chapter 44)
45b. Acidification of mannitol n, of ethylene glycol n → 46
46a. Gelatinase p, esculin hydrolysis n → *Myroides odoratimimus* (chapter 44)
46b. Gelatinase n, esculin hydrolysis p → *Sphingobacterium multivorum* (chapter 44)
47a. Alkaline phosphatase p, motility n, acidification of ethylene glycol n → *Sphingobacterium thalpophilum* (chapter 44)
47b. Alkaline phosphatase n, motility p, acidification of ethylene glycol p → 48
48a. β-Galactosidase (ONPG) p → 49
48b. β-Galactosidase (ONPG) n → 50
49a. Tributyrate esterase pp, phenylalanine deaminase n → *Pannonibacter phragmitetus* (chapter 44)
49b. Tributyrate esterase n/(p), phenylalanine deaminase p → *Rhizobium radiobacter* (chapter 44)
50a. Colistin S, growth at 41°C n → *Ochrobactrum anthropi* (chapter 44)
50b. Colistin R, growth at 41°C p → *Ochrobactrum intermedium*, *partim* urease p (44%) (chapter 44)

Hydrogen sulfide production-negative, indole production-negative, oxidase-positive, Gram-negative nonfermenters; trypsin positive, pyrrolidonyl aminopeptidase negative
51a. Urease p → *Alishewanella fetalis* (chapter 44)
51b. Urease n → 52

(Continued on next page)

TABLE 4 *(Continued)*

52a. Alkaline phosphatase p → 53
52b. Alkaline phosphatase n → 55
53a. Esculin hydrolysis n → *Brevundimonas diminuta*, *partim* pyrrolidonyl aminopeptidase n (80%) (chapter 43)
53b. Esculin hydrolysis p → 54
54a. Desferrioxamine R, vancomycin S → *Sphingomonas* spp., *partim* pyrrolidonyl aminopeptidase n (75%) (chapter 44)
54b. Desferrioxamine S, vancomycin R → *Brevundimonas vesicularis* (chapter 43)
55a. Nitrate reductase n → 56
55b. Nitrate reductase p → 58
56a. Desferrioxamine S, nitrite reductase p, arginine dihydrolase n, acidification of glucose n, of xylose n → *Alcaligenes faecalis*, *partim* trypsin p (30%) (chapter 45)
56b. Desferrioxamine R, nitrite reductase n, arginine dihydrolase p, acidification of glucose p, of xylose p → 57
57a. Gelatinase p → *Pseudomonas fluorescens*, *partim* pyoverdin production n (5%), *partim* pyrrolidonyl aminopeptidase n (40%), *partim* nitrate reductase n (80%) (chapter 42)
57b. Gelatinase n → *Pseudomonas putida*, *partim* pyoverdin production n (5%) (chapter 42)
58a. Starch hydrolysis p, cauliflower-like and often yellowish colonies → *Pseudomonas stutzeri* (chapter 42)
58b. Starch hydrolysis n → 59
59a. Nitrite reductase p → 60
59b. Nitrite reductase n → 62
60a. Growth at 41°C n → *Pseudomonas fluorescens*, *partim* pyoverdin production n (5%), *partim* pyrrolidonyl aminopeptidase n (40%), *partim* nitrate reductase p (20%), *partim* nitrite reductase p (5%) (chapter 42)
60b. Growth at 41°C p → 61
61a. Gelatinase n, acidification of mannitol n, often yellowish → *P. mendocina* (chapter 42)
61b. Gelatinase p (80%), acidification of mannitol p (70%) → *P. aeruginosa*, *partim* pyoverdin production n (35%), *partim* pyrrolidonyl aminopeptidase n (5%), *partim* nitrate reductase p (5%), *partim* nitrite reductase p (95%) (chapter 42)
62a. Acidification of glucose p → 63
62b. Acidification of glucose n → 64
63a. Growth at 41°C p → *P. aeruginosa*, *partim* pyoverdin production n (35%), *partim* pyrrolidonyl aminopeptidase n (5%), *partim* nitrite reductase n (5%), or *P. pseudoalcaligenes*, *partim* acidification of glucose p (10%) (chapter 42)
63b. Growth at 41°C n → *Pseudomonas fluorescens*, *partim* pyoverdin production n (5%), *partim* pyrrolidonyl aminopeptidase n (40%), *partim* nitrate reductase p (20%), *partim* nitrite reductase n (95%) (chapter 42)
64a. Acidification of fructose p^{w} → *P. pseudoalcaligenes*, *partim* acidification of glucose n (90%) (chapter 42)
64b. Acidification of fructose n → *P. alcaligenes* (chapter 42)
65a. Pyrrolidonyl aminopeptidase p → 66
65b. Pyrrolidonyl aminopeptidase n → 87

Hydrogen sulfide production-negative, indole production-negative, oxidase-positive, Gram-negative nonfermenters; trypsin negative, pyrrolidonyl aminopeptidase positive

66a. Fastidious, small colonies on SBA, plump coccobacilli → *Moraxella atlantae* (chapter 44)
66b. Good growth → 68
67a. Desferrioxamine S → 68
67b. Desferrioxamine R → 69
68a. Urease p/(p), nitrite reductase p, acidification of glucose p, of xylose p, colistin R → *Ralstonia pickettii* (chapter 43)
68b. Urease n, nitrite reductase n, acidification of carbohydrates n, colistin S → *Comamonas terrigena* (chapter 43)
69a. Lysine decarboxylase p → *Burkholderia cenocepacia, partim* pyrrolidonyl aminopeptidase p (50%) (chapter 43)
69b. Lysine decarboxylase n → 70
70a. β-Galactosidase p → *Herbaspirillum* spp. (chapter 55)
70b. β-Galactosidase n → 71
71a. Motility n → *Bordetella petrii* (chapter 45)
71b. Motility p → 72
72a. Nitrate reductase p → 73
72b. Nitrate reductase n → 81
73. Urease p/(p), arginine dihydrolase p/(p) → 74
73b. Urease n, arginine dihydrolase n → 75
74a. Gelatinase p → *Acidovorax facilis* (chapter 43)
74b. Gelatinase n → *Acidovorax delafieldii* (chapter 43)
75a. Colistin R → 76
75b. Colistin S → 77
76a. Acidification of mannitol p, of xylose n → *Delftia acidovorans* (chapter 43) (in addition, assimilation of acetamide p ↔ *Comamonas testosteroni* [chapter 43])
76b. Acidification of mannitol n, of xylose p → *Achromobacter xylosoxidans* (chapter 45)
77a. Nitrite reductase p → 78
77b. Nitrite reductase n → 79

(Continued on next page)

TABLE 4 Dichotomous identification algorithm for aerobically growing Gram-negative nonfermenters (strict aerobes) *(Continued)*

78a. Acidification of glucose (p) → *Acidovorax temperans* (chapter 43)
78b. Acidification of glucose n → *Achromobacter denitrificans* (chapter 45)
79a. Alkaline phosphatase p^{w} → *Cupriavidus gilardii*, *partim* pyrrolidonyl aminopeptidase p (15%), *partim* nitrate reductase p (50%) (chapter 43)
79b. Alkaline phosphatase n → 80
80a. Flagellation polar or bipolar → *Comamonas testosteroni* (chapter 43) (in addition, assimilation [alkalinization] of acetamide n ↔ *Delftia acidovorans*)
80b. Flagellation peritrichous → *Achromobacter piechaudii* (chapter 45)
81a. Acidification of glucose p, of xylose p → 82
81b. Acidification of glucose n, of xylose n → 84
82a. Acidification of mannitol n → *Ralstonia insidiosa* (chapter 43)
82b. Acidification of mannitol p → 83
83a. Acidification of ethylene glycol n, growth at 41°C p → *Acidovorax wautersii* (chapter 43)
83b. Acidification of ethylene glycol p, growth at 41°C n → *Ralstonia mannitolilytica* (chapter 43)
84a. Urease p → *Cupriavidus pauculus* (chapter 43)
84b. Urease n → 85
85a. Alkaline phosphatase p^{w} → *Cupriavidus gilardii*, *partim* pyrrolidonyl aminopeptidase p (15%), *partim* nitrate reductase n (50%) (chapter 43)
85b. Alkaline phosphatase n → 86
86a. Acidification of ethylene glycol p, growth at 41°C p → *Bordetella hinzii* (chapter 45)
86b. Acidification of ethylene glycol n, growth at 41°C n → *Cupriavidus respiraculi* (chapter 43)

Hydrogen sulfide production-negative, indole production-negative, oxidase-positive, Gram-negative nonfermenters; trypsin negative, pyrrolidonyl aminopeptidase negative
87a. Acidification of glucose p → 88
87b. Acidification of glucose n → 94
88a. Colistin R → 89
88b. Colistin S → 91
89a. Acidification of ethylene glycol p → *Burkholderia pseudomallei* (chapter 43)
89b. Acidification of ethylene glycol n → 90
90a. Acidification of sucrose n, desferrioxamine S → *Burkholderia multivorans*, *partim* desferrioxamine S (85%) (chapter 43)
90b. Acidification of sucrose p (90%), desferrioxamine R → *B. cenocepacia*, *partim* PAP n (50%) (chapter 41) or *Burkholderia multivorans*, *partim* desferrioxamine R (15%) (chapter 43)
91a. Urease p → 92
91b. Urease n → 93
92a. Arginine dihydrolase p → *Haematobacter missouriensis* (chapter 44)
92b. Arginine dihydrolase n → *Paracoccus yeei*, mucoid, yellowish colonies, O-shaped coccoid cells (chapter 44)
93a. Nitrate reductase p, nitrite reductase p → *Psychrobacter faecalis* (chapter 44)
93b. Nitrate reductase p, nitrite reductase n → *Wohlfartiimonas chitiniclastica* (chapter 44)
94a. Desferrioxamine S → 95
94b. Desferrioxamine R → 101
95a. Urease p → *Oligella ureolytica* (chapter 44)
95b. Urease n → 96
96a. Motility p → 97
96b. Motility n → 99
97a. Nitrite reductase p → *Alcaligenes faecalis*, *partim* trypsin n (70%) (chapter 45)
97b. Nitrite reductase n → 98
98a. Growth at 41°C p → *Comamonas kerstersii* (chapter 43)
98b. Growth at 41°C n → *Comamonas aquatica* (chapter 43)
99a. Nitrite reductase p → *Oligella urethralis* (chapter 44)
99b. Nitrite reductase n → 100
100a. Acidification of ethylene glycol p, tributyrate esterase p → *Moraxella osloensis* (chapter 44)
100b. Acidification of ethylene glycol n, tributyrate esterase n → *Moraxella lincolnii* (chapter 44)
101a. Urease p → 102
101b. Urease n → 105
102a. Motility p → *Bordetella bronchiseptica* (chapter 45)
102b. Motility n → 103
103a. Arginine dihydrolase p → *Haematobacter massiliensis* (chapter 44)
103b. Arginine dihydrolase n → 104
104a. Growth on 12% NaCl p, growth at 41°C p → *Psychrobacter phenylpyruvicus* (chapter 44)
104b. Growth on 12% NaCl n, growth at 41°C n (7% positive) → *Psychrobacter sanguinis* (chapter 44)
105a. Motility p → *Cupriavidus gilardii*, *partim* PAP n (85%) (chapter 43)
105b. Motility n → 106

(Continued on next page)

TABLE 4 *(Continued)*

106a. Gelatinase p → *Moraxella lacunata* (chapter 44)
106b. Gelatinase n → 107
107a. Acidification of ethylene glycol p → 108
107b. Acidification of ethylene glycol n → 109
108a. Tween 80 esterase p → *Psychrobacter pulmonis* (chapter 44)
108b. Tween 80 esterase n → *Moraxella canis* (chapter 44)
109a. Nitrite reductase p → *Moraxella catarrhalis* (chapter 44)
109b. Nitrite reductase n → *Moraxella nonliquefaciens* (chapter 44)

[a] Nonfermenters are usually referred to as strict aerobes, although some nonfermenters, such as all denitrifiers (producing nitrogen gas from nitrite), can grow anaerobically when nitrate and/or nitrite is available as an alternative electron acceptor in oxidative respiration, instead of gaseous oxygen. p, positive; pp, strongly (promptly) positive; (p), positive after a delay; p^{w}, weakly positive; p/(p), positive or positive after a delay; n, negative; S, susceptible; R, resistant; → [number], go to number indicated further on in the table; MHA, Mueller-Hinton agar; PAP, pyrrolidonyl aminopeptidase; *partim*, Latin term used to indicate "the part that is." Chapter numbers in parentheses indicate the chapters in which the organism(s) is described further in this *Manual*.

[b] *P. monteilii*, *P. mosselii*, and *P. veronii* are 100%, *P. fluorescens* and *P. putida* are 95%, and *P. aeruginosa* is 65% positive for pyoverdin production.

Day 3. Begin the identification of nonfermenters (non-glucose-fermenting organisms) (Table 4).

Inoculate two TSA plates and incubate them overnight at 30 to 35°C. Two SBA plates can be used (more appropriate for more-fastidious organisms), but pigmentation is sometimes more clear on TSA. Inoculation of TSA (or SBA) is used in order to record pigment production, to obtain dense growth on day 4, and to prepare a dense inoculum to carry out rapid enzymatic tests and to inoculate other media, as described below. Two plates are inoculated to keep one for prolonged testing, if necessary. Proceed to day 4.

Day 4. Record the results of oxidase; perform and record the results of 10 enzymatic tests.

A. Perform and record the oxidase test by picking colonies from TSA with a swab to which a drop of oxidase reagent has been added. The test is positive when colonies turn red to purple-blue within 2 min.
B. To perform the following 10 enzymatic tests, use one of the two TSA plates. As previously mentioned, all of these reactions require a heavy inoculum, that is, a McFarland standard of at least 4 to 5 (critical to the accuracy of test results).
   1. Alkaline phosphatase. Prepare the inoculum in 0.5 ml of dH_2O containing one alkaline phosphatase tablet (Rosco). Incubate for 4 to 5 h. A yellow color indicates a positive result.
   2. Trypsin, i.e., benzyl-arginine aminopeptidase. Prepare a suspension in 0.5 ml of dH_2O containing one trypsin tablet (Rosco). Incubate for 4 to 5 h. Add 1 drop of cinnamaldehyde reagent. An orange-to-pink color within 5 min indicates a positive result.
   3. Pyrrolidonyl aminopeptidase. Prepare a suspension in 0.5 ml of dH_2O containing one pyrrolidonyl aminopeptidase tablet (Rosco). Incubate for 4 to 5 h. Add 1 drop of cinnamaldehyde reagent. An orange-to-pink color, usually within 5 min, indicates a positive result.
   4. β-Galactosidase. Prepare a suspension in 0.5 ml of dH_2O containing one ONPG tablet (Rosco). Incubate for 4 to 5 h. A yellow color indicates a positive result.
   5. Indole production. Prepare a suspension in 0.5 ml of 0.3% tryptophan solution. Incubate for 4 to 5 h. Add 8 to 12 drops of Kovác's reagent. Indole production is positive when the supernatant turns red.
   6. Urease. Prepare a suspension in 1 ml of liquid Christensen urea. Incubate for 4 to 5 h. A pink color indicates a positive result. Monitor the suspension for up to 1 to 2 days if results are still negative after 4 to 5 h and when no final identification has yet been reached.
   7. Nitrate reductase. Prepare a suspension to a McFarland standard of 4 to 5 in 1 ml of 0.1% KNO_3 broth. Incubate for 4 to 5 h. Take 0.5 ml of the solution and add a drop of Griess reagent A and subsequently 1 drop of Griess reagent B. The test is positive when the suspension immediately turns red, indicating the presence of nitrite. The test may also be positive when the suspension is colorless in cases of strong nitrite reductase activity. When the result is not clear, repeat the test after 24 h with the remaining 0.5 ml of solution.
   8. Nitrite reductase. Prepare a suspension to a McFarland standard of 6 to 8 in 1 ml of 0.001% $NaNO_2$ broth. Incubate it for 4 to 5 h. Take 0.5 ml of the solution and add a drop of Griess A reagent and subsequently add a drop of Griess B reagent. The test is positive when the suspension is colorless, indicating the absence of nitrite. When the result is not clear, repeat the test after 24 h with the remaining 0.5 ml of solution.
   9. Lysine decarboxylase. Prepare a suspension in 1 ml of Moeller broth plus 1% lysine. Cover the suspension with paraffin oil. Incubate for 4 to 5 h. A purple color of the medium indicates a positive result. Monitor the suspension for up to 1 or 2 days.
   10. Arginine dihydrolase. Prepare a suspension in 1 ml of Moeller broth plus 1% arginine. Cover the suspension with paraffin oil. Incubate it for 4 to 5 h. A purple color of the medium indicates a positive result. Monitor the suspension for up to 1 or 2 days, e.g., for some strains of *Pseudomonas pseudoalcaligenes* or of *Haematobacter* spp. that are positive after a delay.

Further simplification is possible by preparing a suspension at a McFarland standard of 4 to 5 in 1.5 ml of dH_2O and dividing it into three aliquots of 0.5 ml, to which disks for tests 1 to 3 can be added. Also, tests 4 and 5 can be carried out in the same tube by adding the ONPG disk to the tryptophan solution. In that case, after 4 to 5 h of incubation, read the color for β-galactosidase before adding Kovác's reagent to read the color for indole production.

C. Testing for susceptibility to colistin, desferrioxamine, and vancomycin. Starting from TSA, prepare a suspension at a McFarland standard of 0.5, inoculate one MHA

(*Text continues on page 634*)

TABLE 5 Overview of biochemical characteristics of the Gram-negative nonfermenters[a]

Category and item no.	Species	Chapter	Cell morphology	Colony color/pigment/ morphology	Hydrogen sulfide production	Pyoverdin production	Indole production	Oxidase	Trypsin	PAP	Susceptibility to desferrioxamine
A. H_2S production positive											
2a	*Shewanella putrefaciens*	44	Rods	o	***p***	n	n	p	p	p	R
2b	*Shewanella algae*	44	Rods	o	***p***	n	n	p	p	p	R
B. H_2S production negative, pyoverdin production positive											
4a	*Pseudomonas aeruginosa*, *partim* pyoverdin production p (65%)	42	Rods	Diffusible green pigment on MHA	**n**	***p***	n	p	p	95	R
6a	*Pseudomonas mosselii* (*n* = 12)	42	Rods	Diffusible green pigment on MHA	**n**	***p***	n	p	o	o	o
6b	*Pseudomonas monteilii* (*n* = 10)	42	Rods	Diffusible green pigment on MHA	**n**	***p***	n	p	o	o	o
8a	*Pseudomonas veronii* (*n* = 8)	42	Rods	Diffusible green pigment on MHA	**n**	***p***	n	p	o	o	o
8a	*Pseudomonas fluorescens*, *partim* pyoverdin production p (95%), *partim* nitrate reductase p (20%), *partim* nitrite reductase p (5%)	42	Rods	Diffusible green pigment on MHA	**n**	***p****	n	p	p	n*	R
8b	*Pseudomonas fluorescens*, *partim* pyoverdin production p (95%), *partim* nitrate reductase p (20%), *partim* nitrite reductase n (95%)	42	Rods	Diffusible green pigment on MHA	**n**	***p****	n	p	p	n*	R
9a	*Pseudomonas fluorescens*, *partim* pyoverdin production p (95%), *partim* nitrate reductase n (80%)	42	Rods	Diffusible green pigment on MHA	**n**	***p****	n	p	p	n*	R
9b	*Pseudomonas putida*, *partim* pyoverdin production p (95%)	42	Rods	Diffusible green pigment on MHA	**n**	***p****	n	p	p	n*	R
C. H_2S production negative, pyoverdin production negative, indole production positive											
11a	*Balneatrix alpica*	44	Rods	Yellowish	**n**	**n**	***p***	p	***n***	n	S
12a	*Bergeyella zoohelcum*	44	Rods	Mucoid	**n**	**n**	***p***	p	**p**	***n***	S
13a	*Weeksella virosa*	44	Rods	Mucoid	**n**	**n**	***p***	p	**p**	**p**	S
15a	*Elizabethkingia miricola*	44	Rods	PY or no pigment, flexirubin n	**n**	**n**	***p***	p	**p**	**p**	R
15b	*Elizabethkingia meningoseptica*	44	Rods	PY or no pigment, flexirubin n	**n**	**n**	***p***	p	**p**	**p**	R
17a	*Chryseobacterium gleum*	44	Rods	Yellow, flexirubin p	**n**	**n**	***p***	p	**p**	**p**	R
17b	*Wautersiella falsenii*	44	Rods	PY or no pigment, flexirubin n	**n**	**n**	***p***	p	**p**	**p**	12S
18a	*Chryseobacterium hominis*	44	Rods	PY or no pigment, flexirubin n	**n**	**n**	***p***	p	**p**	**p**	R
20a	*Chryseobacterium anthropi*	44	Rods	Flexirubin n, often sticky	**n**	**n**	***p***	p	**p**	**p**	78S
20b	*Sphingobacterium mizutaii*	44	Rods	PY, flexirubin n	**n**	**n**	***p***	p	**p**	**p**	R
21a	*Chryseobacterium treverense*	44	Rods	Yellow, flexirubin n	**n**	**n**	***p***	p	**p**	**p**	S
22a	*Chryseobacterium indologenes*	44	Rods	Beta-hemolysis, yellow, flexirubin p (75 %)	**n**	**n**	***p***	p	**p**	**p**	R
22b	*Empedobacter brevis*	44	Rods	PY, flexirubin n	**n**	**n**	***p***	p	**p**	**p**	R
D. H_2S production negative, pyoverdin production negative, indole production negative, oxidase negative											
25a	*Bordetella parapertussis*	45	Small cells	***Brown on TSA***	**n**	**n**	**n**	***n***	n	n	R

Urease	Nitrate reductase	Nitrite reductase	Motility	Lysine decarboxylase	Arginine dihydrolase	Alkaline phosphatase	Growth at 41°C	Gelatinase	Acidification of glucose	Acidification of mannitol	Acidification of xylose	Acidification of ethylene glycol	Susceptibility to colistin	Esculin hydrolysis	Starch hydrolysis	β-Galactosidase (ONPG)	Tween 80 esterase	Tributyrin esterase	Phenylalanine deaminase	Additional characteristic(s)
n	p	p	p	n	n	p	v	p	***p***^{w}	n	n	p	S	n	o	o	o	o	o	***Requirement for NaCl p***
n	p	p	p	n	n	p	v	p	**n**	n	n	p	S	n	o	o	o	o	o	**Requirement for NaCl n**
n	p	97	p	n	p	n	***p***	80	p	70	90	p	S	n	n	o	o	o	o	
o	n	o	o	n	p	o	**n**	n	p	75	***n***	o	o	n	8	o	o	o	o	***Growth with 6% NaCl p***
50	n	p	o	n	p	o	**n**	15	p	n	***n***	o	o	n	n	o	o	o	o	**Growth with 6% NaCl n**
25	***p***	***p***	o	o	p	o	**n**	15	p	o	**p**	o	o	o	o	o	o	o	o	
n	***p****	***p****	p	n	p	n	**n**	p	p	55	**p**	38	S	n	n	n	?	?	n	
n	***p****	**n***	p	n	p	n	**n**	p	p	55	**p**	38	S	n	n	n	?	?	n	
n	**n***	v	p	n	p	n	**n**	***p***	p	55	**p**	38	S	n	n	n	?	?	n	
n	**n***	n*	p	n	p	n	**n**	**n**	p	25	**p**	p	S	n	n	n	o	o	o	
n	p	n	p	n	n	p	n	n	p	p	n	n	S	n	p	n	o	o	o	
p	n	n	n	n	n	p	n	p	n	n	n	n	R	n	n	n	o	o	o	
n	n	n	n	n	n	p	p	p	***n***	n	n	n	S	n	n	n	o	o	o	
p	n	n	n	n	n	n	n	p	**p**	***p***	n	p	R	p	n	p	o	o	o	
n	n	85	n	n	n	p	50	p	**p**	***p***	n	p	R	p	n	p	o	o	o	
p	75	50	n	n	n	p	***p***	p	**p**	**n**	25	p	R	p	p	n	o	o	o	
p	n	15	n	n	n	p	**n**	p/(p)	**p**	**n**	n	45	R	55		30	o	o	o	
n	75	75	n	n	n	p	n	p	**p**	**n**	n	***p***	15	p	p	n	o	o	o	
n	n	n	n	n	n	p	n	***p***	**p**	**n**	***p***	**n**	R	n	o	n	o	o	o	MacConkey n
n	n	75	n	n	n	p	n	**n**	**p**	**n**	***p***	**n**	R	p	n	n	o	o	o	
n	60	p	n	n	n	p	n	***n***	**p**	**n**	**n**	**n**	R	20	n	n				
n	30	15	n	n	n	p	n	**p**	**p**	**n**	**n**	**n**	R	***p***	p	n	o	o	o	
n	n	n	n	n	n	p	n	**pp**	**p**	**n**	**n**	**n**	R	**n**	60	n	o	o	o	MacConkey p
p	n	n	n	n	n	n	18	n	n	n	n	n	S	n	o	n	o	o	o	Tyrosine hydrolase p, cf. brown on TSA

(Continued on next page)

TABLE 5 Overview of biochemical characteristics of the Gram-negative nonfermenters *(Continued)*

Category and item no.	Species	Chapter	Cell morphology	Colony color/pigment/ morphology	Hydrogen sulfide production	Pyoverdin production	Indole production	Oxidase	Trypsin	PAP	Susceptibility to desferrioxamine
25b	*Bordetella holmesii* (*n* = 13)	45	Small cells	***Brown on TSA***	**n**	**n**	**n**	***n***	o	o	o
27a	*Stenotrophomonas maltophilia*	43	Rods	**Not brown on TSA**	**n**	**n**	**n**	***n***	***p***	***n***	R
28a	*Pseudomonas luteola*	42	Rods	**Not brown on TSA, yellow**	**n**	**n**	**n**	***n***	***p***	**p**	R
28b	*Pseudomonas oryzihabitans*	42	Rods	**Not brown on TSA, yellow**	**n**	**n**	**n**	***n***	***p***	**p**	R
29a	*Acinetobacter*	44	Gram stain variable CB	**Not brown on TSA**	**n**	**n**	**n**	***n***	**n**	n	R/(S?)
30a	*Burkholderia gladioli*	43	Rods	**Not brown on TSA**	**n**	**n**	**n**	***n***	**n**	80	R
	B. cepacia complex, *partim* oxidase n (1–4%, up to 14% for *B. pyrrocinia*)	43	Rods	**Not brown on TSA**	**n**		**n**	***n***[*]	**n**	v	v
31a	"*Bordetella ansorpii*" (*n* = 1)	45	Rods	**Not brown on TSA**	**n**	**n**	**n**	***n***	**n**	o	o
32a	*Kerstersia gyiorum*	45	Rods	**Not brown on TSA, dry, spreading**	**n**	**n**	**n**	***n***	**n**	n	S
32b	*Bordetella trematum*	45	Rods	**Not brown on TSA**	**n**	**n**	**n**	***n***	**n**	n	R
E. H_2S production negative, pyoverdin production negative, indole production negative, oxidase positive											
E (i). Trypsin production positive, PAP positive											
37a	*Brevundimonas diminuta*, *partim* PAP p (20%)	43	Rods	o	**n**	**n**	**n**	**p**	***p***	***p***[*]	***S***
38a	*Pseudochrobactrum asaccharolyticum*	44	Rods	o	**n**	**n**	**n**	**p**	***p***	***p***	***S***
36a	*Sphingomonas* spp., *partim* PAP p (25%)	44	Rods	Yellow, flexirubin n	**n**	**n**	**n**	**p/p**[w]	***p***	***p***[*]	**R**
40a	*Pseudomonas aeruginosa*, *partim* pyoverdin production n (35%), *partim* PAP p (95%)	42	Rods	Not green on MHA	**n**	**n**[*]	**n**	**p**	***p***	***p***[*]	**R**
40b	*Pseudomonas fluorescens*, *partim* pyoverdin production n (5%), *partim* PAP p (60%)	42	Rods	Not green on MHA	**n**	**n**[*]	**n**	**p**	***p***	***p***[*]	**R**
41a	*Ochrobactrum intermedium*, *partim* urease n (56%)	44	Rods	Creamy or mucoid	**n**	**n**	**n**	**p**	***p***	***p***	**R**
41b	*Inquilinus limosus*, *partim* urease n (65%)	44	Rods	Mucoid	**n**	**n**	**n**	**p**	***p***	***p***	**R**
43a	*Myroides odoratus*	44	Rods	Yellow, flexirubin p, Spreading	**n**	**n**	**n**	**p**	***p***	***p***	***S***
44a	*Inquilinus limosus*, *partim* urease (p) (35%)	44	Rods	Mucoid	**n**	**n**	**n**	**p**	***p***	***p***	**R**
45a	*Sphingobacterium spiritivorum*	44	Rods	Yellowish, flexirubin n	**n**	**n**	**n**	**p**	***p***	***p***	**R**
46a	*Myroides odoratimimus*	44	Rods	Yellow, flexirubin p, spreading	**n**	**n**	**n**	**p**	***p***	***p***	**R**
46b	*Sphingobacterium multivorum*	44	Rods	Yellowish, flexirubin n	**n**	**n**	**n**	**p**	***p***	***p***	**R**
47a	*Sphingobacterium thalpophilum*	44	Rods	Yellowish, flexirubin n	**n**	**n**	**n**	**p**	***p***	***p***	R
49a	*Pannonibacter phragmitetus*	44	Rods	Creamy	**n**	**n**	**n**	**p**	***p***	***p***	S
49b	*Rhizobium radiobacter*	44	Rods	Creamy	**n**	**n**	**n**	**p**	***p***	***p***	30S
50a	*Ochrobactrum anthropi*	44	Rods	Creamy or mucoid	**n**	**n**	**n**	**p**	***p***	***p***	R
50b	*Ochrobactrum intermedium*, *partim* urease p (44%)	44	Rods	Creamy or mucoid	**n**	**n**	**n**	**p**	***p***	***p***	R

Urease	Nitrate reductase	Nitrite reductase	Motility	Lysine decarboxylase	Arginine dihydrolase	Alkaline phosphatase	Growth at 41°C	Gelatinase	Acidification of glucose	Acidification of mannitol	Acidification of xylose	Acidification of ethylene glycol	Susceptibility to colistin	Esculin hydrolysis	Starch hydrolysis	β-Galactosidase (ONPG)	Tween 80 esterase	Tributyrin esterase	Phenylalanine deaminase	Additional characteristic(s)
n	N	o	n	n	n	o	n	n	n	n	n	o	o	n	o	o	o	o	o	
n	40	n	p	p	n	pp	50	p	(p)	n	35	n	v	40	n	v	o	o	o	
n	95	n	p	n	***p***	30	n	n	p	94	p	p	S	***p***	n	p	o	o	o	
n	n	n	p	n	**n**	n	n	n	p	p	p	p	S	**n**	n	n	o	o	o	
v	v	v	***n***	v	v	o	v	v	v	N	v	o	o	n	o	o	o	o	o	
n	35	n	**p**	n	n	***n***	n	70	***p***	***p***	***p***	***p***	R	10	o	o	o	o	o	
v	v	n	**p**	v	n	**pp**	v	v	***p***	v	v	**n**	R	v	o	v				
N	N	o	**p**	o	N	o	o	***p***	**n**	**n**	**n**	o	o	n	o	o	o	o	o	
n	n	n	**p**	n	n	n	***p***	**n**	**n**	**n**	**n**	p	S	n	n	n	o	o	o	Looks like *A. faecalis*, but organism is nitrate and nitrite n
n	n	n	**p**	n	n	n	**n**	**n**	**n**	**n**	**n**	p	S	n	n	n	o	o	o	
n	n	n	p	n	n	***p/pp***	40	70	n	n	n	50	R	n	n	n	o	o	o	Vancomycin R
n	n	n	n	n	n	**n**	n	n	p^{w}	n	p^{w}	p	R	n	o	n				Vancomycin R
n	n	n	65	n	n	***p***	n	n	p	n	p	95	5	p	n	p	o	o	o	Vancomycin S
n	p	95	p	n	p	**n**	***p***	80	p	70	90	p	***S***	n	n	o	o	o	o	Vancomycin R
n	20	5	p	n	p	**n**	**n**	p	p	55	p	40	***S***	n	n	n	?	?	n	Vancomycin R
n*	p	p	p	n	70	**n**	65	n	p	v	p	***p***	**R**	35	o	n	o	n	o	Vancomycin R
n*	n	n	65	n	n	n	35	n	p	p	p	**n**	**R**	p	o	p	o	o	o	Vancomycin R
p	***n***	n	n	n	n	p	30	p	n	n	n	n	R	n	n	n	o	o	o	
(p)*	***n***	n	65	n	n	***n***	35	n	p	p	p	n	R	p	o	p	o	o	o	
p	***n***	n	n	n	n	**p**	n	n	p	***p***	p	***p***	R	p	o	75	o	o	o	
p	***n***	n	n	n	n	**p**	30	***p***	n	**n**	n	**n**	R	***n***	n	n	o	o	o	
p	***n***	n	n	n	n	**p**	n	**n**	p	**n**	p	**n**	R	**p**	n	p	o	o	o	
p/(p)	**p**	n	n	n	n	***p***	p	n	p	n	p	***n***	R	p	n	p	o	o	o	
p	**p**	p	p	n	30	**n**	p	n	p	60	p	**p**	S	35	n	***p***	o	***pp***	***n***	
p	**p**	40	p	n	n	**n**	30	n	p	p	p	**p**	80	p	o	***p***	o	**n/(p)**	**p**	
p	**p**	p	p	n	72	**n**	***n***	n	p	v	p	**p**	***S***	35	o	**n**	o	n	o	
p*	**p**	p	p	n	11	**n**	**p**	n	p	v	p	**p**	**R**	n	o	**n**				

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TABLE 5 Overview of biochemical characteristics of the Gram-negative nonfermenters (*Continued*)

Category and item no.	Species	Chapter	Cell morphology	Colony color/pigment/ morphology	Hydrogen sulfide production	Pyoverdin production	Indole production	Oxidase	Trypsin	PAP	Susceptibility to desferrioxamine
E (ii). Trypsin production positive, PAP negative											
51	*Alishewanella fetalis*	44	Rods	o	**n**	**n**	**n**	**p**	***p***	**n**	S
53a	*Brevundimonas diminuta*, *partim* PAP n (80%)	43	Rods	o	**n**	**n**	**n**	**p**	***p***	**n***	S
54a	*Sphingomonas* spp., *partim* PAP n (75%)	44	Rods	Yellow, flexirubin n	**n**	**n**	**n**	**p/p**^{w}	***p***	**n***	***R***
54b	*Brevundimonas vesicularis*	43	Rods	Mostly orange	**n**	**n**	**n**	**p**	***p***	**n**	**S**
56a	*Alcaligenes faecalis*, *partim* trypsin p (30%)	45	Rods	Usually dry, often have a fruity smell	**n**	**n**	**n**	**p**	***p****	**n**	***S***
57a	*Pseudomonas fluorescens*, *partim* pyoverdin production n (5%), *partim* PAP n (40%), *partim* nitrate reductase n (80%)	42	Rods	Not green on MHA	**n**	**n***	**n**	**p**	***p***	**n***	**R**
57b	*Pseudomonas putida*, *partim* pyoverdin production n (5%)	42	Rods	Not green on MHA	**n**	**n***	**n**	**p**	***p***	**n**	**R**
58a	*Pseudomonas stutzeri*	42	Rods	Cauliflower (rough), often yellowish	**n**	**n**	**n**	**p**	***p***	**n**	R
60b	*Pseudomonas fluorescens*, *partim* pyoverdin production n (5%), *partim* PAP n (40%), *partim* nitrate reductase p (20%), *partim* nitrite reductase p (5%)	42	Rods	Not green on MHA	**n**	**n***	**n**	**p**	***p***	**n***	R
61a	*Pseudomonas mendocina*	42	Rods	Often yellowish	**n**	**n**	**n**	**p**	***p***	**n**	R
61b	*Pseudomonas aeruginosa*, *partim* pyoverdin production n (35%), *partim* PAP n (5%), *partim* nitrite reductase p (97%)	42	Rods	Not green on MHA	**n**	**n***	**n**	**p**	***p***	**n***	R
63a	*Pseudomonas aeruginosa*, *partim* pyoverdin production n (35%), *partim* PAP n (5%), *partim* nitrite reductase n (3%)	42	Rods	Not green on MHA	**n**	**n***	**n**	**p**	***p***	**n***	R
63a	*Pseudomonas pseudoalcaligenes*, *partim* acidification of glucose p (10%)	42	Rods	Not green on MHA	**n**	**n**	**n**	**p**	***p***	**n**	70
63b	*Pseudomonas fluorescens*, *partim* pyoverdin production n (5%), *partim* PAP n (40%), *partim* nitrate reductase p (20%), *partim* nitrite reductase n (95%)	42	Rods	Not green on MHA	**n**	**n***	**n**	**p**	***p***	**n***	R
64a	*Pseudomonas pseudoalcaligenes*, *partim* acidification of glucose n (90%)	42	Rods	Not green on MHA	**n**	**n**	**n**	**p**	***p***	**n**	70
64b	*Pseudomonas alcaligenes*	42	Rods	Not green on MHA	**n**	**n**	**n**	**p**	***p***	**n**	25

Urease	Nitrate reductase	Nitrite reductase	Motility	Lysine decarboxylase	Arginine dihydrolase	Alkaline phosphatase	Growth at 41°C	Gelatinase	Acidification of glucose	Acidification of mannitol	Acidification of xylose	Acidification of ethylene glycol	Susceptibility to colistin	Esculin hydrolysis	Starch hydrolysis	β-Galactosidase (ONPG)	Tween 80 esterase	Tributyrin esterase	Phenylalanine deaminase	Additional characteristic(s)
p	p	n	n	n	n	p	p	p	p	n	n	n	S	n	o	n	o	o	o	
n	n	n	p	n	n	***p***	40	70	n	n	n	50	R	***n***	n	n	o	o	o	
n	n	n	65	n	n	***p***	n	n	p	n	p	95	5	**p**	n	p	o	o	o	Vancomycin S
n	n	n	p	n	n	***p***	20	25	90	n	25	p	R	**p**	o	n	o	o	o	Vancomycin R
n	***n***	p	p	n	n	**n**	20	20	n	n	n	80	S	n	n	n	o	o	o	
n	***n****	v	p	n	p	**n**	n	***p***	p	25	p	p	S	n	n	n	?	?	n	
n	***n***	n	p	n	p	**n**	n	**n**	p	25	p	p	S	n	n	n	o	o	o	
n	**p**	p	p	n	n	**n**	70	n	p	p	p	p	S	n	***p***	n	o	o	o	
n	**p***	***p****	p	n	p	**n**	***n***	p	p	25	p	p	S	n	**n**	n	?	?	n	
n	**p**	***p***	p	n	p	**n**	**p**	***n***	p	***n***	p	p	S	n	**n**	n	o	o	o	
n	**p**	***p****	p	n	p	**n**	**p**	**80**	p	**70**	90	p	S	n	**n**	n	o	o	o	
n	**p**	**n***	p	n	p	**n**	p	80	***p***	70	90	p	S	n	**n**	n	o	o	o	
n	**p**	**n**	p	n	78	**n**	p	n	***p****	n	70	p	S	n	**n**	n	o	o	o	Fructose p^{w}
n	**p***	**n***	p	n	p	**n**	n	p	***p***	25	p	p	S	n	**n**	n	?	?	n	
n	**p**	**n**	p	n	78	**n**	p	n	**n***	n	70	p	S	n	**n**	n	o	o	o	***Fructose p^{w}***
n	**p**	**n**	p	n	12	**n**	v	n	**n**	n	n	p	S	n	**n**	n	o	o	o	**Fructose n, but type strain is fructose p^{w}**

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TABLE 5 Overview of biochemical characteristics of the Gram-negative nonfermenters *(Continued)*

Category and item no.	Species	Chapter	Cell morphology	Colony color/pigment/ morphology	Hydrogen sulfide production	Pyoverdin production	Indole production	Oxidase	Trypsin	PAP	Susceptibility to desferrioxamine
E (iii). Trypsin production negative, PAP positive											
66a	*Moraxella atlantae*	44	Rods	***Fastidious, small colonies on SBA, plump CB***	**n**	**n**	**n**	**p**	n	p	60S
68a	*Ralstonia pickettii*	43	Rods	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**S**
68b	*Comamonas terrigena*	43	Rods	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**S**
69a	*Burkholderia cenocepacia*, *partim* PAP p (50)	43	Rods	**Normal growth**	**n**	**n**	**n**	**p/p^{w}**	**n**	***p****	**R**
70a	*Herbaspirillum* spp.	55	Curved rods	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**R**
71a	*Bordetella petri*	45	Small cells	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**R**
74a	*Acidovorax facilis*	43	Rods	**Normal growth,** pigment n, hemolysis n	**n**	**n**	**n**	**p**	**n**	***p***	**R**
74b	*Acidovorax delafieldii*	43	Rods	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**R**
76a	*Delftia acidovorans*	43	Rods	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**R**
76b	*Achromobacter xylosoxidans*	45	Rods	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**R**
78a	*Acidovorax temperans*	43	Rods	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**R**
78b	*Achromobacter denitrificans*	45	Rods	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**R**
80a	*Comamonas testosteroni*	43	***Polar or bipolar***	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**R**
80b	*Achromobacter piechaudii*	45	**Peritrichs**	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**R**
79a	*Cupriavidus gilardii*, *partim* PAP p (15%), *partim* nitrate reductase p (50%)	43	Rods	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p****	**R**
82a	*Ralstonia insidiosa*	43	Rods	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**R**
83b	*Ralstonia mannitolilytica*	43	Rods	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**R**
83a	*Acidovorax wautersii*	43	Rods	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**R**
84a	*Cupriavidus pauculus*	43	Rods	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**R**
85a	*Cupriavidus gilardii*, *partim* PAP p (15%), *partim* nitrate reductase n (50%)	43	Rods	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p****	**R**
86a	*Bordetella hinzii*	45	Small CB	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***	**R**
86b	*Cupriavidus respiraculi*	43	Rods	**Normal growth**	**n**	**n**	**n**	**p**	**n**	***p***^{w}	**R**
E (iv). Trypsin production negative, PAP negative											
89a	*Burkholderia pseudomallei*	43	Rods		**n**	**n**	**n**	**p**	**n**	**n**	v
90a	*Burkholderia multivorans*, *partim* desferrioxamine S (85%)	43	Rods	Normal growth	**n**	**n**	**n**	**p/p^{w}**	**n**	**n**	***S****
90b	*Burkholderia multivorans*, *partim* desferrioxamine R (15%)	43	Rods	Normal growth	**n**	**n**	**n**	**p/p^{w}**	**n**	**n**	**R***

Urease	Nitrate reductase	Nitrite reductase	Motility	Lysine decarboxylase	Arginine dihydrolase	Alkaline phosphatase	Growth at 41°C	Gelatinase	Acidification of glucose	Acidification of mannitol	Acidification of xylose	Acidification of ethylene glycol	Susceptibility to colistin	Esculin hydrolysis	Starch hydrolysis	β-Galactosidase (ONPG)	Tween 80 esterase	Tributyrin esterase	Phenylalanine deaminase	Additional characteristic(s)
n	n	n	n	n	n	p	45	n	n	n	n	n	S	n	n	n	n	n	o	
p/(*p*)	p	p	p	n	n	n	v	n	*p*	n	*p*	n	*R*	n	o	n	o	o	o	
n	p	n	p	n	n	n	n	n	**n**	n	**n**	n	**S**	n	n	n	o	o	o	
n/p	v	n	p	*p*	n	p/(p)	p/n	v	p	p	p	n	R	v	o	v	o	o	o	
p	n	n	p	**n**	n	25	P	n	(p)	p	p	n	25S	n	o	*p*	o	o	o	Flagellar, polar, multitrichous (often bipolar)
n	p	p	*n*	**n**	n	n	o	n	n	n	n	n	S	n	o	**n**	o	o	o	
p	*p*	o	**p**	**n**	*p*	n	n	*p*	p	p	p	n	S	n	o	**n**	o	o	o	Gelatinase p, differentiation from A. *delafieldii* and A. *temperans*
(p)	*p*	n	**p**	**n**	*(p)*	n	50	**n**	p	p	(p)	n	50S	n	o	**n**	o	o	o	
n	*p*	n	**p**	**n**	**n**	n	30	n	n	*p*	n	40	*R*	n	n	**n**	o	o	o	Acetamide assimilation p
n	*p*	60	**p**	**n**	**n**	n	85	n	80	**n**	p	p	*R*	n	o	**n**	o	o	o	
n	*p*	*p*	**p**	**n**	**n**	n	p	n	*(p)*	p^{w}	n	p(p)	**S**	n	o	**n**	o	o	o	
n	*p*	*p*	**p**	**n**	**n**	n	25	n	**n**	n	n	p	**S**	n	n	**n**	o	o	o	
n	*p*	**n**	**p**	**n**	**n**	*n*	n	n	n	n	n	20	**S**	n	n	**n**	o	o	o	Acetamide assimilation n, differentiation from *Delftia*
n	*p*	**n**	**p**	**n**	**n**	*n*	60	n	n	n	n	p	**S**	n	n	**n**	o	o	o	
n	p*	**n**	**p**	**n**	**n**	p^{w}	p	n	n	n	n	60	**S**	n	n	**n**	o	o	o	
(p)	**n**	n	**p**	**n**	n	n	p	n	*p*	*n*	*p*	n	R	n	o	**n**	o	o	o	
p	**n**	n	**p**	**n**	n	n	*p*	n	*p*	**p**	*p*	*n*	R	n	o	**n**	o	o	o	Flagellar, polar, monotrichous
p	**n**	n	**p**	**n**	n	n	n	n	*p*	**p**	*p*	**p**	S	n	o	**n**				
p	**n**	n	**p**	**n**	n	n	85	n	**n**	n	**n**	n	S	n	n	**n**	o	o	o	
n	n*	n	**p**	**n**	n	*p*^{w}	p	n	**n**	n	**n**	60	S	n	n	**n**	o	o	o	
n	**n**	n	**p**	**n**	n	**n**	*p*	n	**n**	n	**n**	*p*	S	n	n	**n**	o	o	o	
n	**n**	n	**p**	**n**	n	**n**	n	n	**n**	n	**n**	**n**	S	n	n	**n**	o	o	o	
n	p	p	p	n	p	p	p	v	*p*	p	p	*p*	*R*	v	o	n				
40	95	n	p	50	n	p/ (p)	p	<5	*p*	p	p	**n**	*R*	<5	o	>95	o	o	o	Acidification of 90% sucrose
40	95	n	p	50	n	p/ (p)	p	<5	*p*	p	p	**n**	*R*	<5	o	>95	o	o	o	Acidification of 90% sucrose

(Continued on next page)

TABLE 5 Overview of biochemical characteristics of the Gram-negative nonfermenters *(Continued)*

Category and item no.	Species	Chapter	Cell morphology	Colony color/pigment/ morphology	Hydrogen sulfide production	Pyoverdin production	Indole production	Oxidase	Trypsin	PAP	Susceptibility to desferrioxamine
90b	*Burkholderia cenocepacia*, *partim* PAP n (50%)	43	Rods	Normal growth	**n**	**n**	**n**	**p/p**^{w}	**n**	**n***	**R**
92a	*Haematobacter missouriensis*	44	Pleomorphic rods or CB	Variable colony size, mucoid or sticky	**n**	**n**	**n**	**p**	**n**	**n**	R
92b	*Paracoccus yeei*	44	DC or CB, O shaped	Mucoid, yellowish	**n**	**n**	**n**	**p**	**n**	**n**	R
93a	*Psychrobacter faecalis*	44	Large CB	Large, creamy	**n**	**n**	**n**	**p**	**n**	**n**	R
93b	*Wohlfahrtiimonas chitiniclastica*	44	Short rods	Brown, spreading colonies	**n**	**n**	**n**	**p**	**n**	**n**	S
95a	*Oligella ureolytica*	44	Small CB	Normal growth	**n**	**n**	**n**	**p**	**n**	**n**	***S***
97a	*Alcaligenes faecalis*, *partim* trypsin n (70%)	45	Flagellation p, peritrichous	Usually dry; often have a fruity smell	**n**	**n**	**n**	**p**	**n***	**n**	***S***
98a	*Comamonas kerstersii*	43	Rods	Normal growth	**n**	**n**	**n**	**p**	**n**	**n**	***S***
98b	*Comamonas aquatica*	43	Rods	Normal growth	**n**	**n**	**n**	**p**	**n**	**n**	***S***
99a	*Oligella urethralis*	44	Flagellation n, small CB	Normal growth	**n**	**n**	**n**	**p**	**n**	**n**	***S***
100a	*Moraxella osloensis*	44	Plump rods or CB	Small	**n**	**n**	**n**	**p**	**n**	**n**	***S***
100b	*Moraxella lincolnii*	44	Plump rods or CB	Fastidious, small	**n**	**n**	**n**	**p**	**n**	**n**	***S***
102a	*Bordetella bronchiseptica*	45	Small CB	Normal growth	**n**	**n**	**n**	**p**	**n**	**n**	**R**
103a	*Haematobacter massiliensis*	44	Pleomorphic rods or CB	Variable colony size, mucoid or sticky	**n**	**n**	**n**	**p**	**n**	**n**	**R**
104a	*Psychrobacter phenylpyruvicus*	44	Plump rods or CB	Small, promoted by Tween 80	**n**	**n**	**n**	**p**	**n**	**n**	**R**
104b	*Psychrobacter sanguinis*	44	Rods	Promoted by Tween 80	**n**	**n**	**n**	**p**	**n**	**n**	**R**
105a	*Cupriavidus gilardii*, *partim* PAP n (85%)	43	Rods	Normal growth	**n**	**n**	**n**	**p**	**n**	**n***	**R**
106a	*Moraxella lacunata*	44	Plump rods or CB	Small	**n**	**n**	**n**	**p**	**n**	**n**	**R**
108a	*Psychrobacter pulmonis*	44	Large CB	Large, creamy, not promoted by Tween 80	**n**	**n**	**n**	**p**	**n**	**n**	**R**
108b	*Moraxella canis*	44	DC	Normal growth	**n**	**n**	**n**	**p**	**n**	**n**	**R**
109a	*Moraxella catarrhalis*	44	DC	Normal growth	**n**	**n**	**n**	**p**	**n**	**n**	**R**
109b	*Moraxella nonliquefaciens*	44	Plump rods or CB	Small	**n**	**n**	**n**	**p**	**n**	**n**	**R**
Species not included in dichotomous table											
	Advenella incenata/Tetrathiobacter kashmirensis (*n* = 6)	45	Plump rods	Creamy	n	n	n	***p***	n	n	R/S
	Pandoraea pulmonicola	43	Rods	Pigment n	n	n	n	***p***^{w}	n	n	R
	Pandoraea apista	43	Rods	Pigment n	n	n	n	**n**	n	***p***	R
	Pandoraea norimborgensis/ Pandoraea pnomenusa	43	Rods	Pigment n	n	n	n	**n**	n	**n**	R
	Pandoraea sp.	43	Rods	Pigment n	n	n	n	**n**	n	**n**	R
	Pandoraea sputorum	43	Rods	Pigment n	n	n	n	**n**	n	**n**	R

Urease	Nitrate reductase	Nitrite reductase	Motility	Lysine decarboxylase	Arginine dihydrolase	Alkaline phosphatase	Growth at 41°C	Gelatinase	Acidification of glucose	Acidification of mannitol	Acidification of xylose	Acidification of ethylene glycol	Susceptibility to colistin	Esculin hydrolysis	Starch hydrolysis	β-Galactosidase (ONPG)	Tween 80 esterase	Tributyrin esterase	Phenylalanine deaminase	Additional characteristic(s)
n/p	v	n	p	p	n	p/(p)	p/n	v	***p***	p	p	**n**	***R***	v	o	v	o	o	o	Acidification of 90% sucrose
p	n	n	n	n	***p***	n	n	n	***p***	(p)	(p)	p	**S**	n	o	n	n	n	p	
p	p	n	n	n	**n**	n	35	n	***p***	45	p	p	**S**	n	o	o	n	p	n	
n	***p***	***p***	n	n	n	n	n	n	***p***	n	p	p	**S**	n	o	n	p	p	10	
n	**n**	**n**	n	n	n	n	p	n	***p***^{w}	n	p^{w}	p	**S**	n	n	n			pp	
p	p	p	70	n	n	n	20	n	**n**	n	n	p	S	n	n	n	n	n	n	
n	n	***p***	***p***	n	n	n	20	v	**n**	n	n	80	S	n	n	n	o	o	n	
n	p	**n**	***p***	n	n	n	*p*	n	**n**	n	n	n	S	n	n	n	o	o	o	
n	p	**n**	***p***	n	n	n	n	n	**n**	n	n	n	S	n	n	n	o	o	o	
n	n	***p***	**n**	n	n	n	60	n	**n**	n	n	p	S	n	n	n	n	n	p^{w}	
n	25	**n**	**n**	n	n	75	50	n	**n**	n	n	***p***	S	n	n	n	95	***p***	n	
n	n	**n**	**n**	n	n	n	n	n	**n**	n	n	**n**	S	n	n	n	n	**n**	n	
p	p	n	***p***	n	n	n	80	n	**n**	n	20	n	S	n	n	n	o	o	o	
p	n	n	**n**	n	***p***	n	n	n	**n**	n	n	p	S	n	o	n	n	n	p	
p	20	n	**n**	n	**n**	n	***p***	n	**n**	n	n	p	S	n	n	n	p	p	p	***Growth with 12% NaCl p***
p	33	n	**n**	n	**n**	n	**7**	n	**n**	n	n	87	S	n	n	n	p	p	33	**Growth with 12% NaCl n**
n	v	n	***p***	n	n	p^{w}	p	n	**n**	n	n	60	S	n	n	n	o	o	o	
n	p	n	**n**	n	n	75	n	***p***	**n**	n	n	25	S	n	n	o	p	p	n	
n	p	p	**n**	25	n	n	p	**n**	**n**	n	n	***p***	S	n	n	n	***p***	p	n	
n	80	20	**n**	n	n	n	p	**n**	**n**	n	n	***p***	S	n	n	o	**n**	p	n	
n	p	***p***	**n**	n	n	n	25	**n**	**n**	n	n	**n**	S	n	n	n	n	p	n	
n	p	**n**	**n**	n	n	n	15	**n**	**n**	n	n	**n**	S	n	n	n	n	p	n	
n/p	n/p	n	p/n	n	n	n/p^{w}	v	n	p/n	n	p/n	n/p	***S***	n	o	n	o	o	o	
n	n	n	p	n	n	p	o	n	n	n	n	n	**R**	n	o	o	o	o	o	
(p)	n	p	p	n	n	n	o	n	(p)^{w}	n	n	n	S	n	o	o	o	o	o	
(p)	***p***	n	p	n	n	n	o	n	n	n	n	n	R	n	o	o	o	o	o	
p	**n**	***p***	p	n	n	p^{w}	o	n	(p)^{w}	n	n	n	R	***p***	o	o	o	o	o	
p	**n**	**n**	p	n	n	n	o	n	n	n	n	n	R	**n**	o	o	o	o	o	

[a] Characteristics useful for identification according to the dichotomous Table 4 are listed in boldface type; the distinction between groups in each dichotomy is indicated by roman rather than italic type. Alphanumeric data are percentages of positive isolates. PAP, pyrrolidonyl aminopeptidase; MCA, MacConkey agar; SBA, sheep blood agar; TSA, tryptic soy agar; CB, coccobacilli; DC, diplococci; PY, partly yellow; n, negative; n*, negative, but some isolates of this species are positive for this characteristic (dealt with elsewhere in the table); n/p, most strains are negative; o, data not listed; p, positive; pp, strongly (promptly) positive; p*, positive, but some isolates of this species are negative for this characteristic (dealt with elsewhere in the table); p^{w}, weakly positive; (p), positive after a delay; (p)^{w}, weakly positive after a delay; p/(p), most strains are positive, but some are positive after a delay; p/n, most strains are positive; p/p^{w}, most strains are positive, but some are weakly positive; R, resistant; R/S, most strains are resistant; S, susceptible; S/R, most strains are susceptible; S [number], percentage of susceptible strains; v, strain dependent.

plate to obtain confluent growth, and add colistin (10 μg), desferrioxamine (250 μg), and vancomycin (30 μg) disks. Incubate overnight at 30 to 37°C. Proceed to the instructions for day 5 (A).

D. MHA is preferred for susceptibility testing, because green pigmentation due to pyoverdin (fluorescein production) can be scored more easily on this medium. Proceed to the instructions for day 5 (B).

E. Testing for motility. Starting with TSA, stab inoculate underneath the surface of the soft agar (0.5% TSA), preferably in a small (e.g., 5-ml) petri dish. Incubate overnight at 30°C. Proceed to the instructions for day 5 (C).

F. Testing for acidification of carbohydrates. Starting with TSA, inoculate four LPPR agar tubes, one each with 1% glucose, 1% mannitol, 1% xylose, and 2% ethylene glycol, and incubate them for 24 h at 30°C. Proceed to the instructions for day 5 (D).

Day 5. Record susceptibility, green colony pigmentation (pyoverdin production), motility, and acidification of carbohydrates.

A. Record susceptibilities to different antibacterial agents. Any clear zone of inhibition is considered to indicate susceptibility for diagnostic purposes. A complete lack of a zone of inhibition is interpreted as resistance.

B. Record pyoverdin production as the presence of diffusible green pigment on MHA.

C. Record motility. Motility is positive when the culture spreads from the inoculation zone.

D. Record the acidification of glucose, mannitol, xylose, and ethylene glycol. The test is positive when the red color of the medium turns orange to yellow. Extend incubation up to 7 days when necessary.

Prolongation of reading is done when a test is still negative or doubtful and when no final identification (on the basis of the other test results) has yet been reached. When final identification is not reached on the basis of these rapid tests, additional tests should be used (see above, chapter 19, and individual chapters on organism groups). These include flagellum stain, gelatin hydrolysis, esculin hydrolysis, starch hydrolysis, growth on MacConkey agar, acidification of sucrose or of raffinose, tributyrate esterase and Tween 80 esterase, acetamide assimilation (alkalinization), requirement for NaCl, halotolerance, and growth at 41°C. Selection of these tests depends on the group of species that has been suggested already on the basis of the rapid tests and can be deduced from the dichotomous identification algorithm in Table 4 and/or from Table 5.

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Neisseria

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TAXONOMY

According to the second edition of *Bergey's Manual of Systematic Bacteriology*, the genus *Neisseria* belongs to the family *Neisseriaceae* of the order *Neisseriales* (1), which is placed in the class *Betaproteobacteria*. Since the 1980s, several alterations have been made within the taxonomy and classification of the family *Neisseriaceae*, due to knowledge gained from molecular analyses. The exclusion and subsequent reassignment of the genera *Moraxella*, *Acinetobacter*, and *Psychrobacter* to the *Gammaproteobacteria* were first proposed by the use of DNA-rRNA and DNA-DNA hybridization techniques (2) and later confirmed by 16S rRNA gene sequencing (3). Today, the family *Neisseriaceae* is the only family within the order *Neisseriales*, and, in addition to the genus *Neisseria*, contains *Eikenella*, *Kingella*, and 32 other genera.

DESCRIPTION OF THE GENUS *NEISSERIA*

Most members of the genus *Neisseria* are cocci with a diameter of up to 2 µm, presenting as single bacteria or in pairs. The species *Neisseria elongata*, *N. weaveri*, *N. bacilliformis*, and *N. shayeganii* sp. nov. (4) are notable exceptions that consist of short rods, frequently arranged as diplobacilli or in chains. While *Neisseria* species are Gram negative, they occasionally show a tendency to withstand decolorization. Capsules (*N. meningitidis*) and pili (*N. meningitidis* and *N. gonorrhoeae*) may be present, but flagella are not formed. *N. meningitidis* is the only species expressing a polysaccharide capsule, of which 12 different serogroups are distinguishable (5). Strains of several species, such as *N. flavescens*, *N. sicca*, and *N. subflava*, may produce a yellowish pigment. *Neisseria* spp. grow optimally under aerobic conditions and at temperatures of 35 to 37°C. Nevertheless, isolation of *N. gonorrhoeae* from body sites with reduced oxygen tensions suggests a capability of anaerobic growth, which was suggested to be due to nitrite respiration (7). Microaerobic growth by denitrification of nitrite via NO has also been shown for *N. meningitidis* (8). While many species are not nutritionally demanding, the human-pathogenic species *N. gonorrhoeae* and *N. meningitidis* are fastidious, showing particular susceptibility to unfavorable environmental factors, such as extreme temperatures, desiccation, and alkaline or acidic conditions. All species are oxidase positive and, with the exception of *N. elongata* subsp. *nitroreducens* (9) and some *N. bacilliformis* strains (10), catalase positive. *Neisseria* species produce acid from carbohydrates by oxidation, not fermentation. Some species, such as *N. elongata* and *N. cinerea*, are asaccharolytic. Most members of the genus are able to reduce nitrite. The natural habitats of the members of this genus are the mucous membranes of animals and humans. The species *N. gonorrhoeae* and *N. meningitidis* are exclusively human pathogens. Exotoxins are typically not produced (1). All species classified in the genus *Neisseria* are naturally competent for DNA uptake and display a high frequency of horizontal gene transfer (11). As a consequence, phylogenetic analyses of this genus based on different genes may yield incongruent results (12). Note that this distortion also applies to 16S rRNA gene sequencing, which has been used to define inter- and intrageneric relationships within the *Neisseraceae* and *Moraxellaceae* (3). Multiple-locus instead of single-locus approaches might therefore be more suitable for the resolution of species of *Neisseria* (13). According to Euzeby's "List of Prokaryotic Names with Standing in Nomenclature" (http://www.bacterio.net/n/neisseria.html), the genus *Neisseria* presently consists of 28 species.

EPIDEMIOLOGY AND TRANSMISSION

N. gonorrhoeae causes gonorrhea, which is the second most commonly reported notifiable disease in the United States (http://www.cdc.gov/std/stats11/gonorrhea.htm). *N. gonorrhoeae* is always considered pathogenic, and humans are the only hosts of this bacterium. It is transmitted mainly through sexual practices and infects the mucosal surfaces of the urethra, cervix, rectum, pharynx, and eyes. The risk of infection is greatly influenced by sexual behavior yet can be reduced by the use of condoms (14). Furthermore, the eye can be infected as an intrapartum event during passage of the fetus through the birth canal. The rate of gonorrhea in the United States decreased by 74% from 1975 to 1997, following implementation of the gonorrhea control program in the mid-1970s (http://www.cdc.gov/std/stats11/gonorrhea.htm); it further decreased to the lowest recorded figure, 98.1/100,000, in 2009, and was slightly increased (to 104.2/100,000) in 2011. Rates vary considerably between states, with values below 8/100,000 in Vermont and over 400/100,000 in the District of Columbia. Women have acquired more infections than men since 2002; in 2011, rates in women and men were 108.9/100,000 and 98.7/100,000, respectively. The group with the highest burden of disease is young adults between 20 and 24 years of age. In 2011, the gonorrhea rate remained highest among African Americans

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(427.3/100,000), with African American women between 20 and 24 years of age being particularly affected (2,050/100,000). The overall rate in the United Kingdom (37.2/100,000) was lower than that in the United States in 2011 (http://www.gov.uk/phe). While the age distribution is very similar to that in the United States, men have consistently higher rates than women in the United Kingdom. In 2011, both sexes had the highest burden of disease in the age group of 20- to 24-year-olds, with rates of 112.9/100,000 and 192.4/100,000 in women and men, respectively.

N. meningitidis also occurs exclusively in humans and plays dual roles as a commensal and a potentially invasive pathogen. On average, the mucosal surfaces of the oro- and nasopharynges of 10% of the population are colonized by this bacterium (15). Carriage is strongly age dependent, with adolescents and young adults attaining rates of over 30%, in contrast to infants, with carriage rates of a few percent (15). As a consequence of repeated episodes of carriage, the percentage of sera with bactericidal activity against pathogenic strains increases with age. Transmission occurs through large-droplet secretions from the oropharynx and is favored by repeated or close contact. Nevertheless, outbreaks and clusters are rare in developed countries (16). Disease occurs in only a minute proportion of individuals acquiring *N. meningitidis* and follows a typical age distribution, with infants and adolescents having the highest incidences in developed countries. Apart from genetic host polymorphisms (17), individuals with underlying conditions, such as properdin deficiencies (18), late complement deficiencies (18), and splenic impairment including asplenia (19), are at increased risk for invasive meningococcal disease (IMD). Also, behavioral risk factors, including exposure to smokers (20) and kissing (21), have been described to contribute to acquisition of disease.

IMD is rare in developed countries. In the United States, the incidence for the year 2011 was estimated to be 0.20/100,000 (22), with serogroups B, C, and Y constituting 91% of all cases. Incidences in European countries are variable, with England and Wales reaching a rate of 1.65/100,000, with an 82% dominance of serogroup B, in 2011 (http://www.gov.uk/phe) and Germany reporting a rate of 0.43/100,000, with a 67% proportion of serogroup B, in 2012 (23). In contrast, African countries in the so-called meningitis belt regularly report epidemic waves, mainly caused by serogroup A, with rates soaring to values over 300/100,000 (24).

A number of vaccines have been developed for the prevention of IMD. In 2007, the Advisory Committee on Immunization Practices recommended vaccination of all adolescents (preferably at 11 to 12 years of age) with a quadrivalent polysaccharide-protein conjugate vaccine covering serogroups A, C, W, and Y (25), emphasizing in 2010 the addition of a booster dose at 16 years of age (26). Due to high rates of serogroup C disease in the 1990s and early 2000s, several European countries implemented vaccination campaigns with a conjugate vaccine against serogroup C, which led to a dramatic reduction of disease with this capsule type (27). Poor immunogenicity of the serogroup B capsular polysaccharide, consisting of (2→8)-α-Neu5Ac (a polysialic acid composed of *N*-acetylneuraminic acid subunits), and concerns about induction of autoimmunity due to the presence of (2→8)-α-Neu5Ac on normal tissues have thus far precluded the development of glycoconjugate vaccines against serogroup B. Nevertheless, outer membrane vesicle vaccines have been used to combat local epidemics caused by serogroup B, e.g., in New Zealand (28). Also, a recombinant protein-based vaccine with an outer membrane vesicle component targeting serogroup B (29) was recently approved by the European Medicines Agency and the Australian Therapeutic Goods Administration.

CLINICAL SIGNIFICANCE

Members of the genus *Neisseria* have a high affinity for mucosal membranes. A wide variety of species can be isolated from humans, including *N. gonorrhoeae*, *N. cinerea*, *N. elongata*, *N. flavescens*, *N. lactamica*, *N. meningitidis*, *N. mucosa*, *N. polysaccharea*, *N. sicca*, and *N. subflava*. Several species are predominantly recovered from animals, such as *N. animalis*, *N. animaloris*, *N. zoodegmatis* (from throats of cats and dogs) (30), *N. denitrificans* (from throats of guinea pigs), *N. dentiae* (from dental plaques of domestic cows), *N. macacae* (from oropharynges of rhesus monkeys), and *N. weaveri* (from the oral flora of dogs). Similar to *N. elongata*, the new species *N. bacilliformis* likely colonizes the oral cavity and respiratory tract of humans (10). Most human *Neisseria* species are considered normal inhabitants of the upper respiratory tract which cause disease in an opportunistic fashion. Rarely, species of animal origin can cause wound infections in humans after bites (31). *N. meningitidis* mostly appears as a mere commensal of the human oropharynx yet can cause life-threatening, acute disease in previously healthy individuals. *N. gonorrhoeae*, however, is always considered a pathogen, even if obvious signs of disease are absent.

Uncomplicated infection by *N. gonorrhoeae* (gonorrhea) manifests most commonly as acute urethritis in men. The major symptoms are urethral discharge, sometimes associated with dysuria, typically without frequency or urgency. Coinfection of the preputial (Tyson's), urethral (Littré's), and bulbo-urethral (Cowper's) glands is possible. Also, completely asymptomatic infections occur in up to 10% of cases. Most cases of untreated urethritis resolve spontaneously after several weeks. Further localized complications after gonococcal urethritis include acute epididymitis, penile edema, and abscesses of the above-mentioned glands. In women, the endocervix is the primary site of genital infection. Additionally, *N. gonorrhoeae* may infect the urethra, the rectum, the peri-urethral (Skene's) glands, and the ducts of the greater vestibular (Bartholin's) glands. The squamous epithelium of the vagina is typically not infected in sexually mature women. In contrast to infection in men, asymptomatic infection in women is common (32). Also, if symptoms appear, they often cannot clearly be attributed to infection by *N. gonorrhoeae*, as concurrent infection by *Chlamydia trachomatis* and *Mycoplasma genitalium* is common. The main complaints include increased vaginal discharge, dysuria, and intermenstrual bleeding. Ascension of the infection may result in pelvic inflammatory disease, which manifests as various combinations of endometritis, salpingitis, tubo-ovarian abscess, and peritonitis. Acute perihepatitis (Fitz-Hugh-Curtis syndrome) can develop following direct extension of *N. gonorrhoeae* from the fallopian tube to the liver capsule and the surrounding peritoneum. While over 80% of rectal infections remain asymptomatic, some patients complain of acute proctitis. Pharyngeal infection is acquired by oral sexual exposure and is mostly asymptomatic yet can also cause overt pharyngitis or tonsillitis (33). While probably less transmissible than rectal or urethral gonorrhea, its silent nature and considerable prevalence among men who have sex with men (MSM) render pharyngeal infection a common reservoir for gonorrhea in sexually active MSM. Gonococcal conjunctivitis in adults usually results from autoinoculation or oculogenital or orogenital exposure. If the infection is not treated promptly, corneal ulceration may rapidly develop. Conjunctivitis of the newborn (ophthalmia neonatorum) is transmitted during birth and is favored by premature rupture of the membranes and preterm delivery. Historically a common cause of blindness, it can be prevented by administering a 1% aqueous solution of silver nitrate or an antibiotic ointment (usually containing erythromycin) into the conjunctivae after delivery. Disseminated gonococcal infection reflects bacteremic dissemination, possibly generation of immune complexes, and indirect immunological mechanisms. Disseminated gonococcal infection complicates fewer than 1% of mucosal infections (34). It usually manifests as septic arthritis and a characteristic syndrome of polyarthritis and dermatitis and should be suspected in patients presenting with tenosynovitis, arthritis, and vasculitic skin lesions (35).

IMD commonly presents as meningitis, acute sepsis, or a combination of both. In addition, unusual presentations include transient mild bacteremia, chronic meningococcal sepsis, pneumonia (mainly by serogroup Y), septic arthritis, and endocarditis (36). Symptoms of meningitis vary widely and can include a stiff neck, headache, confusion, and photophobia. Lethality of meningococcal meningitis without sepsis can be as low as 3% (37). Sequelae, such as sensorineural hearing loss, developmental delay, and speech defects, afflict a substantial portion of survivors, yet in a smaller proportion than in other forms of acute bacterial meningitis (38). Petechial lesions are telltale signs of meningococcal sepsis, and they can coalesce and become ecchymotic. Nevertheless, nonpurpuric maculopapular rashes, readily confused with viral exanthems, have also been associated with meningococcemia. Meningococcal septic shock can take a fulminant course with a lethality of 30% (39), and plasma concentrations can reach up to 10^8 meningococci/ml (40), which in turn leads to massive activation of cytokines and vasoactive anaphylatoxins. Meningococcal shock syndrome is characterized by myocardial depression, vasoplegia, capillary leakage, and disseminated intravascular coagulation (39). Complications of IMD include arthritis, pericarditis, cranial nerve dysfunction, meningococcal pericarditis, and, rarely, cerebral or spinal infarction. In addition, adolescent survivors of IMD have been described as suffering from a series of long-term consequences, including poorer physical and mental health, quality of life, and educational achievement (41). Bacteremia can also manifest without signs of sepsis, in the form of chronic meningococcemia, a condition associated with low-grade relapsing fever, arthritis, and rash (42). Meningococcal pneumonia has been recognized as an infrequent clinical syndrome for more than 100 years (43). Most meningococcal pneumonias are caused by serogroup Y and affect adults disproportionately (44). A preceding viral illness, notably pandemic influenza (45), has been reported to promote its development. *N. meningitidis* is an uncommon cause of acute bacterial conjunctivitis and can also be the etiologic agent of urethritis in men.

The clinical significance of *Neisseria* species other than *N. gonorrhoeae* and *N. meningitidis* is covered in "Evaluation, Interpretation, and Reporting of Results" at the end of the chapter.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Neisseria gonorrhoeae

The selection of specimens for culture-based diagnosis of gonorrhea depends on sex, the level of sexual maturity, and the exposed anatomical sites. The anterior portion of the male urethra is sampled by introducing a swab up to 2 cm in a rotatory fashion. Samples from the endocervical canal are obtained by introducing a swab after removal of mucus plugging the cervical orifice. For MSM and women who practice anal intercourse, rectal samples should be taken. Swabs heavily contaminated with feces must be discarded. For symptomatic patients, direct swabbing of lesions under rectoscopic guidance improves culture yields. A pharyngeal swab should be obtained from individuals in high-risk groups, including MSM. Vaginal swabs are inadequate for culture-based diagnosis for sexually mature women yet can be used for prepubescent females. If the hymen is intact, however, the specimen is collected from the vaginal orifice.

Dacron (polyethylene terephthalate)- or rayon (viscose)-tipped swabs, e.g., Transwab (Medical Wire, Corsham, United Kingdom), Bactiswab (Remel, Lenexa, KS), or Minitip Amies (Copan Innovation, Brescia, Italy), are preferable for culture-based diagnosis of gonorrhea. Calcium alginate swabs should be avoided due to reported toxicity (46). Also, cotton buds and oil-based lubricants can contain unsaturated fatty acids, which inhibit *N. gonorrhoeae*. Although direct plating maximizes the culture yields of gonococci, this approach is not always practical or possible. In such cases, Amies-based semisolid transport media can be used to transport swabs to the processing laboratory. There are, however, considerable performance differences in commercial Amies-based transport systems after 24 and 48 h that are not uniformly rectifiable by the addition of charcoal (47). Therefore, it is advisable to process swabs transported in these media within 6 h after collection. During the time of transport, media should be kept at room temperature and not refrigerated.

Survival and transport of gonococci for over 24 h can be achieved by culture medium transport systems which allow direct plating of specimens in a clinical environment. These usually consist of a solid medium, onto which swabs are inoculated directly after collection, and a CO_2-generating system within a resealable container. A CO_2-rich atmosphere is generated by tablets containing citric acid and sodium bicarbonate that are activated after addition of water. Commercially available systems include Biocult-GC (Orion Diagnostica, Espoo, Finland) and JEMBEC GC-Lect agar (Becton, Dickinson and Company, Franklin Lakes, NJ).

Similar to culture samples, specimens for molecular detection of *N. gonorrhoeae* are best sampled by use of rayon- or Dacron-tipped swabs, since calcium alginate was reported to inhibit PCR (48). The inhibitory influence of aluminum shafts is contentious, and preliminary testing in conjunction with the employed molecular kit is advisable. Transport and collection systems specifically designed for molecular detection include the Digene female swab specimen collection kit (Qiagen Inc., Valencia, CA) and the STD swab specimen collection and transport kit (Roche Diagnostics, Basel, Switzerland). Some molecular kits can also be used for urine and vaginal swabs (see "Nucleic Acid Amplification Tests").

Neisseria meningitidis

The types of specimens that can be used for the detection of *N. meningitidis* include blood, cerebrospinal fluid (CSF), nasopharyngeal and oropharyngeal swabs, bronchoalveolar lavage fluid, joint aspirates, urethral and endocervical swabs, petechial aspirates, and biopsies. Pharyngeal swabs used for determination of meningococcal carriage are best taken from the posterior pharyngeal wall through the mouth and plated directly after sampling (49). Alternatively, swabs may be put into Amies-based transport media and plated, preferably within 5 h after collection. Growth of *N. meningitidis* and *N. gonorrhoeae* in commercial blood culture media is adversely affected by the anticoagulant sodium polyanetholesulfonate (50), for which no suitable substitute is currently available. The inhibitory action is reduced by the addition of gelatin at a concentration of 1 g/liter to most commercially available blood culture media.

Laboratory Safety Issues for Handling of Meningococcal Cultures

Rare cases of fatal meningococcal disease have been described for laboratory staff (51). Risk factors for laboratory-acquired infection are exposure to droplets or aerosols containing *N. meningitidis* (51). Laboratories working with live *N. meningitidis* isolates should comply with biological safety containment level 2 standards, including the use of class II biological safety cabinets whenever infectious splashes or aerosols may be created, e.g., during mobilization of organisms from culture plates, handling of liquid cultures, and performance of carbohydrate utilization tests, oxidase testing, and slide agglutination tests.

DIRECT EXAMINATION

Microscopy

In cases of urethritis, the direct smear for Gram staining should be prepared with a swab that is different from that used for the collection of a culture specimen. A presumptive diagnosis of gonococcal urethritis in men is made by visualization of Gram-negative diplococci associated with or within polymorphonuclear leukocytes. The sensitivity of microscopy depends on the anatomical site investigated and is highest for urethral slides of men, where it reaches 89% (52). For endocervical smears and rectal smears of MSM, however, it drops to 51% and 54%, respectively (52). The specificity of microscopic diagnosis for these sites has been reported to be over 90%. Microscopy is not useful for the diagnosis of pharyngeal gonorrhea. Nevertheless, microscopic diagnosis is mandatory for normally sterile material.

A Gram stain of CSF is required for all cases of suspected bacterial meningitis sent to the laboratory. Visualization of Gram-negative diplococci is sufficient for the presumptive diagnosis of meningococcal meningitis (Fig. 1). If more than 1 ml of CSF is available, the specimen should be centrifuged at 1,000 × *g* for 10 min and the pellet used for microscopic examination and culture. Cytocentrifugation also increases the sensitivity of microscopic investigation. On Gram-stained smears from CSF, meningococci appear as Gram-negative diplococci both inside and outside polymorphonuclear leukocytes, which are typically abundant in samples from cases of bacterial meningitis. Organisms may tend to resist decolorization.

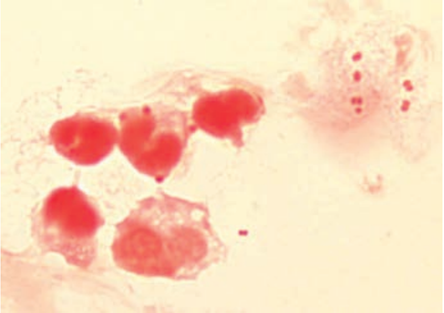

FIGURE 1 Gram stain of CSF from a patient with meningococcal meningitis. doi:10.1128/9781555817381.ch34.f1

Antigen Detection

Diagnosis of meningococcal meningitis can be made through the use of commercially available antigen detection kits. These methods are useful if no or only limited access to microscopes is available. They are of questionable clinical usefulness compared with Gram staining (53) and should therefore not be used as a substitute for microscopy. Commercially available latex agglutination tests, which consist of latex particles coated with monoclonal antibodies targeting the capsular polysaccharides of common serogroups, include the Pastorex meningitis test (Alere Inc., Waltham, MA) and the Wellcogen N. meningitidis A, C, Y, and W135 test (Oxoid Ltd., Basingstoke, United Kingdom). These assays have a reasonable sensitivity and specificity (54) yet are useless for the detection of uncommon serogroups (55). In laboratories handling only a small number of cases, the cost of purchasing and storing antigen detection kits outweighs any potential benefits for patient management.

Currently, no commercially available antigen detection kits with adequate sensitivity and specificity exist for the diagnosis of gonococcal infection.

Nucleic Acid Detection

Neisseria gonorrhoeae

Nucleic acid tests permit the rapid and sensitive detection of *N. gonorrhoeae* from clinical samples without the requirement of bacterial viability. They have been in use since the early 1990s and can be categorized as hybridization assays and nucleic acid amplification tests (NAATs).

Hybridization Assays

The two commercially available hybridization assays include the Digene CT/GC dual ID HC2 (Qiagen Inc., Valencia, CA) and Gen-Probe Pace 2 (P2 assay; Gen-Probe Inc., San Diego, CA) tests, which use RNA probes targeting genomic DNA and DNA probes targeting rRNA, respectively. The detection method for the RNA-DNA hybrids in the HC2 assay involves antibody-mediated recognition of the hybrids and subsequent binding of alkaline phosphatase-conjugated antibodies, which act on a chemiluminescent substrate. Signal amplification results from multiple alkaline phosphatase molecules being attached to a conjugated antibody, several of which bind to a single captured hybrid. In the P2 assay, the DNA probes are labeled with a chemiluminescent substrate, which is quantified after separation of the stable DNA-RNA hybrids from the nonhybridized probe. The sensitivities of hybridization tests are probably higher than that of culture (56, 57). Today, however, hybridization tests have largely been replaced by NAATs (see below).

Nucleic Acid Amplification Tests

Most commercial NAAT assays developed to date are multiplex NAAT assays targeting both *N. gonorrhoeae* and *C. trachomatis*. The Roche Amplicor CT/NG assay (Roche Diagnostics, Basel, Switzerland) is a first-generation assay that uses PCR for amplification of the DNA cytosine methyltransferase gene. It has shown cross-reactivity with strains of several commensal *Neisseria* species, contributing to low positive predictive values (PPV) with urogenital specimens in several studies (58). Additional testing has been carried out with real-time PCR assays targeting the *porA* pseudogene (59), *gyrA* (60), and the 16S rRNA gene (61). Nevertheless, confirmatory tests themselves can introduce shortcomings, as shown for the poorly specific target *cppB* (encoding cryptic plasmid protein B) (61, 62) and the insufficiently sensitive *porA* pseudogene (61). The Becton Dickinson ProbeTec SDA assay (Becton, Dickinson and Company, Franklin Lakes, NJ) is a second-generation test that uses strand displacement amplification for the multiplication of DNA. It targets a region within the multicopy pilin gene-inverting protein homologue (63). Due to its limited specificity and cross-reactivity with commensal *Neisseria* species (64), confirmatory tests using the *porA* pseudogene (65), *cppB* (66), and *opa* (67) have been employed. Additional testing by other NAATs, the Aptima Combo 2 (AC2) and Aptima GC (AG) tests (see below), showed high concordances for cervical and urethral swabs (68) and for male urethral swabs and first-catch urine (69). The AC2 test (Gen-Probe, San Diego, CA) is also a second-generation test, and it uses transcription-mediated amplification for the replication of gonococcal 16S rRNA and a chemiluminescent single-stranded DNA probe for product detection. The AG test represents a confirmatory assay based on the same technology and even uses the same capture probe as the AC2 test, yet it targets a slightly different region of the rRNA subunit. Evaluations regarding the performance of this assay have largely been favorable. Specificity and sensitivity were shown to be higher than those for the Amplicor assay in Australia (70). While a study using the AC2 test with the AG test as a confirmatory assay demonstrated a PPV of 97% among 60,000 female urine and cervical swabs, despite a low prevalence (71), PPV varied between 75% and 100% for urogenital specimens in a multicenter study (69). Specificities of the AC2 test compared with the SDA test were very similar and always over 94% in several studies investigating first-void urine (72), vaginal (73), rectal (74, 75), and pharyngeal (75, 76) specimens. The Abbott RealTime CT/NG assay (Abbott Molecular, Maidenhead, Berkshire, United Kingdom) and the Cobas 4800 CT/NG test (Roche Molecular Systems, Pleasanton, CA) constitute further additions to the arsenal of NAATs, targeting a region in the *N. gonorrhoeae* opacity (Opa) gene (77) and the direct repeat region DR9 (78), respectively. Studies including several thousand clinical specimens have shown high sensitivities (>96%) and specificities (>99%) for both assays (79, 80). Finally, the Cepheid CT/NG Xpert Rapid PCR test (Cepheid, Sunnyvale, CA), which targets several undisclosed genomic targets, showed a favorable PPV of over 91% in a large population with an overall prevalence of 2.3% (81).

In summary, NAATs provide several advantages over culture-based diagnosis, yet they also have a series of important limitations. The main advantages are their superior sensitivity over culture, as evidenced in numerous clinical studies (74–76, 82), and their less stringent collection and transport conditions. The current list of NAATs with FDA approval includes the Amplicor, Abbott RealTime, AC2, ProbeTec, Cobas 4800 CT/NG, and Xpert tests. Most NAATs are licensed for male urine specimens; further approved specimens differ by test and are listed in Table 1. Importantly, no NAAT is currently cleared for oropharyngeal, rectal, ocular, or pediatric specimens. Major limitations of NAATs include a high cost, carryover contamination, high-quality control requirements, an absence of antibiotic resistance data (58), and varying specificity (83). When challenged on a panel of *Neisseria* and *Moraxella* isolates, most tests show specificities between 85.9% and 99.1% (83); this implies that PPV of most presently used NAATs may not reach the recommended value of 90% (84, 85) in settings with low prevalences of around 1% (86). Assays may also be susceptible to inhibition by substances present in patient samples, e.g., in urine (87), and competing amplification in the case of coinfection with *C. trachomatis* (58). The assay complexity, involving steps such as nucleic acid extraction, amplification, and detection, requires stringent quality control and staff training. Nevertheless, the latest commercial assays, such as the AC2, Abott RT, and Cobas 4800 CT/NG tests, can be integrated into fully automated molecular testing systems, such as the Panther (Hologic, Bedford, MA), m2000 (Abbott Molecular, Maidenhead, Berkshire, United Kingdom), and Cobas 4800 (Roche Diagnostics, Rotkreuz, Switzerland) systems, respectively. Furthermore, the Xpert test is a cartridge-based point-of-care test with minimal hands-on and turnaround times (81).

TABLE 1 Commercially available NAATs for detection of *N. gonorrhoeae*

Test name	Producer	Method[a]	Genetic target(s)	Specimen types	Laboratory platform(s)	Reference(s)
Amplicor CT/NG	Roche Diagnostics	PCR	DNA cytosine methyltransferase gene	Urine, endocervical swab, male urethral swab		58–61, 83
ProbeTec SDA	Becton, Dickinson and Company	SDA	Pilin gene-inverting protein homologue	Urine, endocervical swab, male urethral swab, vaginal swab	BD Viper	63, 64, 81, 83, 161
Aptima Combo 2 and GC	Gen-Probe	TMA	16S rRNA	Urine, endocervical swab, male urethral swab, vaginal swab	Tigris, Panther (Hologic)	69–76, 81, 83
Abbott RealTime CT/NG	Abbott Molecular	PCR	*opa* gene	Urine, endocervical swab, male urethral swab, vaginal swab	m2000	77, 79, 80, 83
Cobas 4800 CT/NG	Roche Molecular Systems	PCR	Direct repeat region DR9	Male urine, vaginal swab	Cobas 4800	78–80, 83
Cepheid CT/NG Xpert	Cepheid	PCR	Multiple undisclosed targets	Urine, endocervical swab, vaginal swab	GeneXpert	81

[a] SDA, strand displacement amplification; TMA, transcription-mediated amplification.

Neisseria meningitidis

Several in-house methods have been developed to enable culture-independent diagnosis of IMD, and these are especially useful when previous antibiotic treatment or unfavorable transport conditions lead to a negative culture. The DNA targets used for molecular diagnosis include *ctrA* (88), IS*1106* (89), *siaD* (90, 91), *porA* (92), *porB* (93), *fetA* (94), and housekeeping genes used for multilocus sequence typing (MLST). Specifically, *ctrA* was evaluated as a target for real-time detection of meningococcal DNA (95). Apart from facilitating laboratory confirmation of meningococcal disease, the polysialyltransferase gene (*siaD*) can be used for serogrouping (serogroups B, C, W, and Y), while the *porA*, *porB*, *fetA*, and housekeeping genes allow culture-independent typing (16). False-positive results have been reported for IS*1106*, which should therefore not be used as a single target for routine screening (89). Moreover, *ctrA* testing is negative in rare cases of IMD caused by *N. meningitidis* harboring the capsule-null locus (96).

ISOLATION PROCEDURES

Cultivation of *N. gonorrhoeae* requires the use of chocolate agar, which supports the growth of many other commensal bacteria. To isolate *N. gonorrhoeae* from mucosal and other nonsterile body sites, several selective media containing a mixture of inhibitory agents have been developed. All of them contain the antibiotics vancomycin and colistin for the suppression of Gram-positive and Gram-negative bacteria, respectively. The prototype medium, developed by Thayer and Martin (97), consists of a chocolate agar base which, in addition to the above antibiotics, contains nystatin for the inhibition of yeasts. The addition of trimethoprim to the modified Thayer-Martin medium and following formulations prevents swarming of *Proteus* species. Martin-Lewis medium contains anisomycin, which has increased activity against *Candida albicans*, instead of nystatin. Further modifications include GC-Lect agar (Becton, Dickinson and Company), which provides additional control against *Capnocytophaga* species and against vancomycin-resistant Gram-positive contaminants by the addition of lincomycin. Moreover, the reduced vancomycin concentration in GC-Lect agar enhances the recovery of uncommon vancomycin-susceptible *N. gonorrhoeae* strains. In contrast to the media described above, New York City medium is a clear peptone-corn starch agar containing yeast dialysate, citrated horse plasma, and lysed horse erythrocytes. It contains the antibiotics vancomycin, colistin, amphotericin B, and trimethoprim.

Specimens should be inoculated on warmed or room temperature media. Plates should be incubated at 35 to 37°C with 3 to 7% CO_2 in a moist atmosphere after inoculation. This is accomplished in a commercially available CO_2 incubator equipped with a humidifier. A moist, CO_2-rich atmosphere can also be generated with a candle extinction jar, using white, nonscented candles. Cultures should be examined daily for growth and held for a minimum of 72 h.

For culture-based detection of *N. meningitidis* from primarily sterile materials, such as CSF or joint fluid, specimens should be inoculated onto sheep blood agar and chocolate agar. Specimens from mucosal surfaces (e.g., respiratory material) must be inoculated additionally on selective media (see above) which exclude growth of most commensal *Neisseria* spp. Incubation conditions are identical to those for *N. gonorrhoeae*. Nevertheless, in contrast to *N. gonorrhoeae*, *N. meningitidis* tends to grow more readily on solid media and almost invariably grows on blood agar plates. Media must be examined for suspicious growth at 24, 48, and 72 h. After 72 h of no growth, a negative culture result can be issued.

IDENTIFICATION

Presumptive Identification

Colonial Morphology

After 48 h of growth on chocolate agar, colonies of *N. gonorrhoeae* are up to 1 mm in diameter, opaque, grayish white, glistening, and convex. Morphology can vary subject to the presence of pili and opacity proteins. Colonies of *N. gonorrhoeae* expressing pili and opacity proteins are wrinkled and well defined, with a clear edge, while nonpiliated colonies have more diffuse edges and are more glistening. Due to rapid pilus phase variation, colonial morphology can appear heterogeneous after primary inoculation.

Colonies of *N. meningitidis* have smooth entire edges and are about 1 mm in diameter after 18 h of growth on blood agar. They are gray, convex, glistening, and occasionally mucoid. Blood agar beneath the colonies may display a gray-green discoloration.

Microscopic Morphology

A Gram stain must be performed on suspected *N. gonorrhoeae* and *N. meningitidis* colonies to confirm the presence of uniform Gram-negative diplococci. Consistent results are obtained with <24-h-old colonies, before autolytic processes appear. Microscopic examination of suspicious colonies growing on selective plates is essential to rule out Gram-negative rods belonging to the genera *Moraxella* (e.g., *Moraxella osloensis*), *Acinetobacter*, and *Kingella* occasionally growing on them. While *Acinetobacter* spp. can be indistinguishable from *N. meningitidis* and *N. gonorrhoeae* by Gram staining, they can be discerned by their negative oxidase reaction (see below).

Oxidase Test

Performance of the oxidase test is mandatory for colonies suspected to belong to *Neisseria*. Both *N. gonorrhoeae* and *N. meningitidis* give a positive reaction. In the filter paper method, an oxidase reagent (1% dimethyl-*p*-phenylene-diaminedihydrochloride or tetramethyl-*p*-phenylene-diamine-dihydrochloride) is placed on filter paper, onto which a colony is rubbed with a wooden stick (Nichrome loops may give a false-positive reaction). A fresh isolate should produce a deep purple color within 10 s. Commercial strips (e.g., Microbact oxidase strips [Oxoid, United Kingdom]) are a useful alternative.

Definitive Identification

Carbohydrate Utilization Assays

Neisseria species produce acid from carbohydrates by oxidation, not fermentation. The only carbohydrate used by *N. gonorrhoeae* is glucose, while *N. meningitidis* additionally catabolizes maltose (Table 2). Rarely, however, *N. gonorrhoeae* (98) and *N. meningitidis* (99) fail to acidify carbohydrate-containing media. Also, several asaccharolytic species, including *N. cinerea*, *N. flavescens*, and *N. elongata*, do not produce acid at all from sugars. The traditional cystine tryptic agar sugar method has been replaced by rapid carbohydrate utilization tests in most routine laboratories. These tests give results within 4 h and are integrated into commercial kits, including the ApiNH (bioMérieux,

TABLE 2 Characteristics of medically relevant *Neisseria* species[a]

Species	Morphology	Growth on selective media	Acid production from:					Nitrate reduction	Polysaccharide from SUC	Reference
			GLU	MAL	LAC	SUC	FRU			
N. animaloris	CR	ND	+	0	0	0	ND	+	ND	146
N. bacilliformis	R	0	0	0	0	0	ND	V	ND	10
N. cinerea	C	V	0	0	0	0	0	0	0	
N. elongata	C									1
subsp. *elongata*		0	0	0	0	0	0	0	0	
subsp. *glycolytica*		0	(+)	0	0	0	0	0	0	
subsp. *nitroreducens*		0	0	0	0	0	0	+	0	
N. flavescens	C	0	0	0	0	0	0	0	+	
N. gonorrhoeae	C	+	+	0	0	0	0	0	0	
N. lactamica	C	+	+	+	+	0	0	0	0	
N. meningitidis	C	+	+	+	0	0	0	0	0	
N. mucosa	C	0	+	+	0	+	+	+	+	
N. polysaccharea	C	V	+	+	0	V	0	0	+	
N. sicca	C	0	+	+	0	+	+	0	+	
N. subflava	C									1
bv. flava		V	+	+	0	0	+	0	0	
bv. perflava		V	+	+	0	+	+	0	+	
bv. subflava		V	+	+	0	0	0	0	0	
N. weaveri	R	ND	0	0	0	0	0	0	ND	159
N. zoodegmatis	CR	ND	V	0	0	0	ND	V	ND	146

[a] Symbols and abbreviations: 0, negative; +, positive; (+), weakly positive; R, rods; C, cocci; CR, coccoid rods; ND, not done; GLU, glucose; MAL, maltose; LAC, lactose; SUC, sucrose; FRU, fructose; V, variable.

Marcy-l'Etoile, France) and RapID NH (Remel, Lenexa, KS) kits.

Chromogenic Enzyme Substrate Tests

Identification of *N. gonorrhoeae* can be confirmed by direct detection of enzyme activities by use of chromogenic substrates. The tested enzymes usually include β-galactosidase, γ-glutamyl-aminopeptidase, and proline-iminopeptidase (Pip), which are specific for *N. lactamica*, *N. meningitidis*, and *N. gonorrhoeae*, respectively. The substrates used for the above enzymes, i.e., bromo-chloro-indolyl-β-galactoside (or -galactopyranoside), γ-glutamyl-nitroanilide, and proline-methoxynaphthylamide, change their colors after positive reaction to blue, yellow, and red, respectively. The three enzymes can be assayed in a one-tube format in the two commercially available kits, GonoCheck II (EY Laboratories Inc., San Mateo, CA) and Neisseria PET (BioConnections, Wetherby, United Kingdom). In both tests, β-galactosidase and γ-glutamyl-aminopeptidase are assayed first. This requires an incubation step at 37°C for 30 min. In a second step, which takes up to 2 min, Pip is tested by the addition of the appropriate reagent (Neisseria PET test) or replacement of the screw cap and subsequent inversion of the tube (GonoCheck II test).

A study reported poor sensitivity of these tests for the confirmation of *N. gonorrhoeae* (100). False-negative results were obtained with Pip-negative *N. gonorrhoeae* isolates, which were shown to constitute 4% of isolates in a recent survey in England and Wales (101). Moreover, *N. meningitidis* may occasionally be γ-glutamyl-aminopeptidase negative (98).

Immunologic Methods for Culture Confirmation

All commercially available tests for the culture confirmation of *N. gonorrhoeae* rely on the recognition of gonococcal protein I (with its IA and IB variants) by a pool of monoclonal antibodies. The Phadebact Monoclonal GC test (Bactus AB, Huddinge, Sweden) is a coagglutination assay employing inactivated *Staphylococcus aureus* cells coated with antibodies bound via their Fc portions to staphylococcal protein A. Cross-reactions with *Moraxella catarrhalis*, *N. cinerea*, and *N. lactamica* have been reported. Nevertheless, more recent studies found the test to be highly sensitive and specific for culture confirmation of *N. gonorrhoeae* (100, 102). The BD GonoGen II test (Becton, Dickinson and Company) is a colorimetric test employing antibodies adsorbed to metal sol particles, which give the reagent its raspberry red color. False-positive reactions with *N. lactamica* and *N. meningitidis* have been observed (103). Furthermore, the solubilizing buffer of the GonoGen II kit was described to only insufficiently extract protein I (100), resulting in false-negative reactions for some isolates. However, repeat testing with an extended extraction method led to a high specificity and sensitivity of the test (100). The MicroTrak culture confirmation test (Trinity Biotech, County Wicklow, Ireland) uses fluorescein isothiocyanate-labeled antibodies for confirmation of *N. gonorrhoeae*. Positive specimens are identified by the presence of apple-green fluorescent diplococci under a fluorescence microscope. Among the immunologic methods, the MicroTrak test was appraised as being the most labor-intensive (100). While earlier evaluations pointed to a high specificity yet limited sensitivity (103), false-negative reactions were not observed in a later study (100).

Multitest Identification Systems

Several kits combine carbohydrate utilization tests and direct enzyme detection assays for rapid confirmation of isolates belonging to *Neisseria*. The ApiNH system can be used for the identification of *Neisseria*, *Haemophilus*, and *M. catarrhalis* and uses 13 miniaturized tests. In total, the test comprises four sugar utilization tests (assessing glucose, fructose, maltose, and sucrose), eight enzyme substrate tests, and an acidimetric penicillinase test. In contrast, the RapID

NH (Remel, Lenexa, KS) system contains only 2 carbohydrate utilization tests (for glucose and sucrose), 10 enzyme substrate tests, and a resazurin reduction test. The ApiNH and RapID NH kits are inoculated with dense bacterial suspensions adjusted to McFarland standards of 4 and 3, respectively. Results are obtained after incubation at 37°C for 2 and 4 h, respectively. The denser bacterial inoculum used in the ApiNH system might explain its slightly higher sensitivity than that of the RapID NH test (100). The automated bacterial identification platform Vitek II (bioMérieux) can also be used for identification of *Neisseria* species. Its NHI card contains 30 biochemical tests. Valenza and colleagues reported misidentification of *N. gonorrhoeae* as *N. cinerea* for one isolate, owing to the lack of glucose utilization (99). In another study, all *N. gonorrhoeae* isolates were identified correctly, yet 6% of them received a low discrimination result (104).

Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry has generated a lot of interest as an emerging technique in the identification of bacterial pathogens. A pioneering study analyzing 29, 13, and 15 strains of *N. gonorrhoeae*, *N. meningitidis*, and other *Neisseria* species, respectively, reported that direct bacterial profiles were sufficiently different to allow species identification of pathogenic *Neisseria* isolates (105). Further studies assessing the performance of two major mass spectrometer systems confirmed its usefulness for identification of *Neisseria* species yet included only very few isolates (106, 107). An analysis focusing on *Neisseria meningitidis* verified the ability of MALDI-TOF mass spectrometry to correctly identify all 100 study strains and additionally categorize them into groups of clonal complexes (108). While these reports are promising, more studies are needed to quantify the method's ability to accurately identify species within the genus *Neisseria*.

Hybridization Test

The Accuprobe culture identification test (Gen-Probe Inc., San Diego, CA) is a DNA probe assay for *N. gonorrhoeae* strains isolated from culture. Similar to the AC2 and AG tests, the Accuprobe test targets gonococcal rRNA. After lysis of bacteria, released rRNA is bound by single-stranded DNA probes labeled with a chemiluminescent substrate. Labeled DNA-RNA hybrids are detected in a luminometer. While the test has not been evaluated lately, an older study confirmed its high sensitivity and specificity (109).

DNA Sequencing

Interpretive criteria for identification of bacteria and fungi by use of DNA target sequences have been published by the Clinical and Laboratory Standards Institute (CLSI) (110). Harmsen et al. established a reference database for 16S rRNA gene sequence fragments, including a representative set of *Neisseria* spp. obtained from reference strain collections (http://rdna.ridom.de/), which in most cases allows the identification of an organism belonging to the genus *Neisseria* to the species level (3). Nevertheless, sequence diversity between different species of the genus can be as low as 1% by 16S rRNA gene sequencing. A 413-bp fragment of the gene encoding 50S ribosomal protein L6 (*rplF*) has been shown to provide a higher resolution (111). Also, multilocus approaches, including 7-locus (112) and 52-locus (ribosomal) (13) MLST, are robust methods for species assignment within the genus *Neisseria*.

TYPING SYSTEMS

Neisseria gonorrhoeae

Methods used for typing of *N. gonorrhoeae* include *opa* typing, pulsed-field gel electrophoresis, multiantigen sequence typing, and MLST. While both *opa* typing and pulsed-field gel electrophoresis are highly discriminatory, they are cumbersome and poorly portable. Multiantigen sequence typing, on the other hand, represents a portable, sequence-based typing method for *N. gonorrhoeae*, based on sequencing of coding regions of the highly polymorphic antigens Por and TbpB (β subunit of transferrin-binding protein) (113). Moreover, MLST (114) based on the sequence typing of seven housekeeping gene fragments was also shown to be highly discriminatory for *N. gonorrhoeae* typing (112).

Neisseria meningitidis

N. meningitidis is a highly variable organism, and a vast array of techniques has been developed to describe variants. The simplest method of typing is based on the nature of the polysaccharide capsule. In total, 12 different serogroups can be distinguished, which include A, B, C, E, H, I, K, L, W, X, Y, and Z (5). The nomenclature was recently updated, with serogroups W135 and 29E being renamed W and E, respectively (5). Serogrouping of common serogroups is usually performed by slide agglutination with a set of commercially available sera (supplied by Remel, Lenexa, KS, or Becton, Dickinson and Company, Franklin Lakes, NJ). A further level of differentiation can be achieved by serotyping and serosubtyping, which designate the serological characterization of the outer membrane proteins PorB and PorA, respectively. Today, however, DNA sequence-based typing schemes for hypervariable outer membrane proteins have replaced sero(sub)typing. Protocols are available at http://neisseria.org. Typing targets have been harmonized throughout Europe (115) (Table 3). Protocols for multiple-locus variable-number tandem-repeat analysis have also been developed for meningococci (116). Finally, use of whole-genome sequencing, as employed for the analysis of historical outbreaks (117, 118), is likely to be extended in the coming years.

SEROLOGIC TESTS

Serologic tests are used for the determination of protection against IMD after vaccination or for seroepidemiologic studies. They do not contribute to the ascertainment of invasive disease, since asymptomatic carriage can also elicit protective (i.e., high) titers. The serum bactericidal assay is a functional assay using an external complement source, such as baby rabbit or human complement, that determines a bactericidal titer. It is currently regarded as the best surrogate test for vaccine protection across all serogroups (119). In addition to the serum bactericidal assay, serum IgG concentrations against serogroups A, C, W, and Y can be determined by enzyme-linked immunosorbent assay (ELISA) or bead assays (120).

ANTIMICROBIAL SUSCEPTIBILITIES

Neisseria gonorrhoeae

The CLSI recommends the use of GC agar containing 1% growth supplement for disk diffusion testing of *N. gonorrhoeae* (121). Colony suspensions of isolates need to be adjusted to a 0.5 McFarland standard before inoculation onto media. The CLSI further recommends agar dilution for the

TABLE 3 Molecular typing methods for *N. meningitidis*

Target[a]	Method used		Value or use
	Culture isolates	Native samples	
Serogroup	Slide agglutination	PCR (caveat: sequencing required for serogroups W and Y [142])	Vaccine preventability
PorA	PCR, DNA sequencing	PCR, DNA sequencing	Routine fine typing (in combination with FetA and serogroup typing)
FetA	PCR, DNA sequencing	PCR, DNA sequencing	Routine fine typing (in combination with PorA and serogroup typing)
PorB	PCR, DNA sequencing		Additional typing method
Housekeeping genes: *abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC*, *pgm* (MLST)	PCR, DNA sequencing	PCR, DNA sequencing	Global epidemiology; species status
penA	PCR, DNA sequencing		Confirmation of reduced penicillin susceptibility
Factor H binding protein	PCR, DNA sequencing		Analysis of possible coverage by new-generation meningococcal vaccines

[a] PorA, porin A; FetA, ferric enterobactin transport protein A; PorB, porin B; *abcZ*, putative ABC transporter gene; *adk*, adenylate kinase gene; *aroE*, shikimate dehydrogenase gene; *fumC*, fumarate hydratase gene; *gdh*, glucose-6-phosphate dehydrogenase gene; *pdhC*, pyruvate dehydrogenase subunit gene; *pgm*, phosphoglucomutase gene; *penA*, gene encoding penicillin binding protein 2.

measurement of MICs, yet due to their ease of use, gradient test systems (e.g., Etest) represent an acceptable and frequently used surrogate.

Treatment and control of gonorrhea are aggravated by the ability of *N. gonorrhoeae* to mount resistance against a wide range of antibiotics. While penicillin was the treatment of choice up to the 1970s, the emergence and increase of penicillinase (122) and chromosomally mediated penicillin resistance led to the abandonment of penicillin as a treatment option. Similarly, plasmid- and chromosomally mediated resistance against tetracycline resulted in the replacement of this drug by broad-spectrum cephalosporins in the 1980s, and later by the fluoroquinolones. Nevertheless, resistance against fluoroquinolones emerged in Southeast Asia in the 1990s (123) and spread widely to many countries, including the United States (124). Since April 2007, quinolones are no longer recommended to treat gonococcal infections in the United States (125). According to the Centers for Disease Control and Prevention, 30.4% of strains in 2011 were resistant to penicillin, tetracycline, ciprofloxacin, or a combination of these antimicrobials (126). Ciprofloxacin resistance was detectable in 13.3% of strains in 2011. In contrast, the rate of ciprofloxacin resistance in Europe was 53% in 2010, and it was close to 100% in many Asian settings, including China and Vietnam, in 2009 (127). The proportion of strains with decreased susceptibility to azithromycin, defined by the Gonococcal Isolate Surveillance Project (GISP) as having a MIC of ≥2 mg/liter, was 0.3% in 2011 (126). The higher overall azithromycin resistance of 7.2% in Europe in 2010 may have been due partly to the different cutoff (≥1 mg/liter) used by the European Gonococcal Antimicrobial Surveillance Programme (Euro-GASP) (128). Nevertheless, high-level azithromycin resistance, defined as having a MIC of ≥256 mg/liter (129), was not documented in recent reports from the United States and Europe (126, 128). In contrast, the global rise in cefixime resistance continues to give rise to concern, as this antibiotic, until recently, was recommended as an oral single-dose treatment. While the proportion of isolates with decreased susceptibility in the United States was low, with rates of 0.20% and 0.05% for 2010 and 2011 (126), respectively, the share of strains with decreased susceptibility against cefixime rose by 4%, to 8.7% in 2010, in Europe (128). Again, this difference may have been enhanced by distinct cutoffs for cefixime resistance of ≥0.5 mg/liter and ≥0.25 mg/liter for GISP and Euro-GASP, respectively; nevertheless, the percentage of isolates with a MIC of 0.25 mg/liter was comparably low, i.e., 1.3% of isolates within GISP in 2011 (126), suggesting a true difference between surveillance networks. A further concern is the emergence of resistance against ceftriaxone, due to alterations within the *penA* gene (130). As with cefixime, cutoff MIC values for decreased susceptibility against ceftriaxone differ, with cutoffs of ≥0.5 mg/liter and ≥0.25 mg/liter for GISP and Euro-GASP, respectively. While neither network recently reported strains meeting its own definition of decreased susceptibility, 0.05% and 0.02% of strains in GISP had MICs of 0.25 mg/liter in 2010 and 2011, respectively.

Due to the above-described developments, several guidelines now list a combination of intramuscular ceftriaxone and oral azithromycin as a first-line treatment regimen (85, 131). Ceftriaxone may be replaced by cefixime if ceftriaxone is not available. As described before, fluoroquinolones may be used for treatment only after antimicrobial susceptibility is documented by culture.

Neisseria meningitidis

According to the CLSI, testing should be performed by disk diffusion on Mueller-Hinton agar with 5% defibrinated sheep blood or by broth microdilution using cation-adjusted Mueller-Hinton broth with 2.5 to 5.0% lysed horse blood (121). Alternatively, gradient test systems (e.g., Etest) are frequently used. In contrast to *N. gonorrhoeae*, *N. meningitidis* is usually penicillin susceptible, and β-lactamase production is rare (132). In many countries, penicillin is still regarded as a treatment of choice for IMD, although reduced susceptibility, resulting from modification of penicillin binding protein 2, has increasingly been recorded in several countries (133, 134). Its molecular basis lies in a combination of five amino acid polymorphisms, at positions 504, 510, 515, 541, and 566 of the PenA protein's transpeptidase region (135). In analogy to cefixime resistance in *N. gonorrhoeae* (123), *penA* genes of meningococcal strains with intermediate resistance were found to have a mosaic structure, suggesting multiple events of interspecies horizontal DNA transfer originating from commensal *Neisseria* species (135). Moreover, MICs of cefotaxime were reported to be higher in penicillin-intermediate strains (136). The CLSI defines cefotaxime nonsusceptibility as having a MIC of >0.12 mg/liter, yet it does not provide a threshold for resistance (121). While cefotaxime nonsusceptibility is rare globally, disquietingly high MICs of up to 8 mg/liter were reported from a sample of 8 nonsusceptible strains in India (137). Nevertheless, spread to other countries has not taken place, and the mechanism of nonsusceptibility has not been verified. Rifampin and ciprofloxacin are used for chemoprophylaxis in close contacts of patients. Rifampin-resistant strains have MICs of >2 mg/liter and result from point mutations in the RNA polymerase β subunit (*rpoB*) gene (138). Despite this one-step mechanism, the rate of resistance is very low (139). Resistance against ciprofloxacin emerged in the United States in 2007 (140) and is associated with a point mutation at position 91 of the gene encoding subunit A of DNA gyrase (*gyrA*). While the rate of resistant isolates is low in the United States (140) and Europe, an alarmingly large proportion (65% of isolates) was reported in an outbreak of IMD in India (141). The CLSI defines ciprofloxacin resistance as having a MIC of ≥0.12 mg/liter or a zone diameter of ≤32 mm, using the 5-μg ciprofloxacin disk diffusion method (121). Azithromycin is used for mucosal eradication in patient contacts in areas with high rates of ciprofloxacin resistance (140). Due to the lack of resistant strains (143), "susceptibility-only" criteria were issued by the CLSI, according to which strains with azithromycin MICs of ≤2 mg/liter are considered susceptible (121).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Neisseria gonorrhoeae

Due to the imperfect specificity of many diagnostic methods used for identification of *N. gonorrhoeae*, results have to be interpreted in the context of local disease prevalence, which considerably influences the test's PPV. This particularly applies to NAATs, which, due to varying specificities, cannot be recommended uniformly for use in low-prevalence settings. If medicolegal ramifications are likely to result from a positive test, as is the case, e.g., in victims of sexual assault, special scrutiny has to be applied to all laboratory procedures involved in the issuing of a positive result.

In low-prevalence settings, special protocols should be in place to ensure confirmation of results. This is also important for specimens from children and adolescents and for the documentation of sexual abuse. In such cases, suspected *N. gonorrhoeae* should be confirmed by at least two different methods, including (i) multitest identification systems, (ii) immunologic methods, (iii) DNA probe culture confirmation, (iv) sequencing of the 16S rRNA gene, or (v) MALDI-TOF mass spectrometry. Additionally, strains and DNA need to be conserved.

In settings of high prevalence, such as in laboratories serving genitourinary medicine clinics, tests giving a "yes-no answer" may be preferable over systems identifying the exact species. In these settings, two levels of confidence may be attached to the laboratory report. A presumptive diagnosis of gonorrhea may be issued if either of the following two criteria is met: (i) microscopic visualization of typical Gram-negative diplococci on examination of a smear of male urethral exudate or endocervical secretions; and (ii) growth of oxidase-positive bacteria from the male urethra or endocervix on selective media, with a colonial morphology and microscopic appearance (Gram-negative diplococci) suggestive of *N. gonorrhoeae*. A definitive diagnosis requires (i) isolation of oxidase-positive Gram-negative diplococci from sites of exposure (e.g., urethra, endocervix, throat, or rectum) by culture on selective media and (ii) confirmation by biochemical or molecular methods.

The choice of approach is often determined by the workload and prevalence of gonorrhea in the service area of the laboratory. Laboratories that rarely encounter *N. gonorrhoeae* should prefer kits which give a full identification to the species level.

Neisseria meningitidis

N. meningitidis is always considered a pathogen when isolated from usually sterile body fluids, such as blood or CSF. Also, if it is isolated from the urethra, cervix, or conjunctiva, a pathogenic role is likely. In such cases, *N. meningitidis* should always be reported and the strain be forwarded to a reference laboratory. Many national guidelines consider meningococcal conjunctivitis an indication for chemoprophylaxis of the patient and close contacts, due to a high immediate risk of invasive disease (144, 145). Detection of *N. meningitidis* from bronchoalveolar lavage fluid or sputum has to be interpreted in liaison with the clinician, as it does not automatically imply a pathogenic role. Growth from oropharyngeal or nasopharyngeal specimens usually reflects asymptomatic carriage and may be omitted from laboratory reports, as it readily leads to confusion regarding the pathogenic significance. Eradication of asymptomatic carriers should not be recommended. Similarly, typing of meningococcal carriage isolates should not routinely be performed. Furthermore, obtaining nasopharyngeal swabs to detect meningococci from close contacts of a patient with invasive disease should not be done on a routine basis, as this may reveal the presence of unrelated strains and delay the decision regarding whom should be offered chemoprophylaxis.

Other *Neisseria* Species

Neisseria animaloris

N. animaloris strains are Gram-negative coccoid rods that may constitute currently underrecognized agents of dog bite infections (31). *N. animaloris* is a commensal of the oral cavity of cats and dogs and was formerly named CDC group EF-4a. *N. animaloris* produces yellowish white, convex, opaque, shiny, smooth, and hemolytic colonies on blood agar (146). In contrast to most other *Neisseria* species, these strains grow on MacConkey agar. As a distinctive characteristic, *N. animaloris* is arginine dihydrolase positive. Isolates have been described as being ampicillin susceptible (31). Further characteristics are detailed in Table 2.

Neisseria bacilliformis

Like *N. elongata*, *N. weaveri*, and *N. shayeganii* sp. nov., *N. bacilliformis* organisms are rods, not cocci. On blood agar, colonies are smooth and glistening and have sizes of up to 1 mm after 24 h (10). The colors of colonies range from light gray to buff (Fig. 2). Catalase reaction and reduction of nitrate are variable, and strains are asaccharolytic (10). Strains are associated with the human respiratory tract and

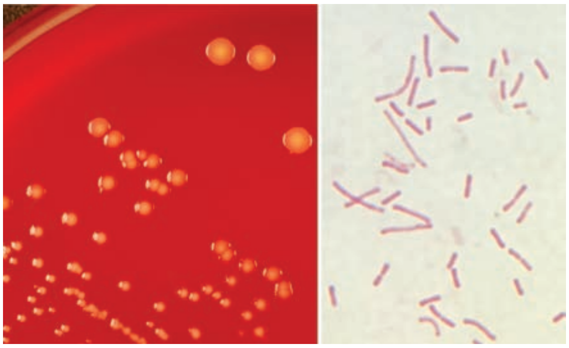

FIGURE 2 Colony morphology (left) and Gram stain (right) of *N. bacilliformis* (10). The strain was isolated from a human periodontal pocket. It was characterized by nearly complete sequencing of the 16S rRNA gene. The species is noteworthy due to its rod-like morphology.
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have occasionally been recovered as causative agents of endocarditis (10, 147).

Neisseria cinerea

As the species name suggests, colonies of *N. cinerea* have an ash-gray color, and they are up to 1.5 mm in diameter. Isolates are asaccharolytic; i.e., they do not acidify carbohydrate-containing media. Due to their carbohydrate utilization profile, *N. cinerea* strains can be confused with glucose-negative *N. gonorrhoeae* (99). Furthermore, growth on selective media is occasionally possible despite colistin susceptibility (148). *N. cinerea* colonizes the oropharynges of over 24% of adults (149). Furthermore, it has been attributed a role in ocular infections in infants (150).

Neisseria elongata

N. elongata forms grayish white, semiopaque colonies, which have a diameter of up to 3 mm after 48 h of incubation. In contrast to the majority of species within *Neisseria*, *N. elongata* organisms are short rods of ca. 0.5 μm in diameter. The species consists of three subspecies: *N. elongata* subsp. *elongata*, *N. elongata* subsp. *glycolytica*, and *N. elongata* subsp. *nitroreducens* (Table 2). As an exception within the genus *Neisseria*, *N. elongata* subsp. *nitroreducens* is catalase negative. Like other *Neisseria* species, *N. elongata* mainly appears as a colonizer of the human oropharynx. Nevertheless, several cases of endocarditis caused by *N. elongata* have been published (151).

Neisseria flavescens

N. flavescens produces smooth and opaque yellow colonies. *N. flavescens* does not generate acid from sugars yet produces polysaccharide from sucrose, which can be detected by pouring an iodine-containing solution (e.g., Lugol's solution) over colonies growing on brain heart infusion agar with sucrose. The iodine test is positive if colonies develop a deep blue color, indicating the presence of a starch-like polysaccharide. *N. flavescens* colonizes the pharynx of humans and only rarely causes disease, such as endocarditis (152). Recent genomic analyses suggest that this species should be relabeled a variant of *N. subflava* (13).

Neisseria lactamica

N. lactamica is readily confused with *N. meningitidis*, since it is morphologically similar and may grow on selective media. Nevertheless, it acidifies lactose in addition to glucose and maltose and is γ-glutamyl-aminopeptidase negative. *N. lactamica* is a commensal of the upper respiratory tract in infants and children. In contrast to the case for *N. meningitidis*, colonization of the oropharynx with *N. lactamica* begins as soon as 2 weeks after birth (153). It is rarely pathogenic, although exceptional cases of meningitis and septicemia have been described. *N. lactamica* usually displays reduced susceptibility to penicillin.

Neisseria mucosa

N. mucosa typically grows in large, adherent, mucoid colonies, which are mostly nonpigmented. In carbohydrate utilization tests, strains of this species are glucose, maltose, fructose, and sucrose positive. *N. mucosa* is usually an apathogenic commensal in the nasopharynx of humans. However, it has been associated with infective endocarditis (154) and with bacteremia in a neutropenic patient (155). Due to variable susceptibility to penicillin, the choice of antibiotic treatment must be supported by susceptibility testing.

Neisseria polysaccharea

Strains of *N. polysaccharea* present as small, grayish yellow, translucent colonies. Similar to *N. meningitidis*, they acidify glucose and maltose but not fructose or lactose. In addition, they may grow on selective media. In contrast to *N. meningitidis*, however, the iodine test is positive, indicating the production of polysaccharide from sucrose (similar to the case for *N. flavescens*). This species colonizes the nasopharynx of children and has so far not been associated with disease.

Neisseria sicca

The colonies formed by *N. sicca* are large (≤3 mm), dry, wrinkled, and grayish white, although some strains may produce a yellowish pigment. Its carbohydrate utilization profile is indistinguishable from that of *N. mucosa*, but it does not reduce nitrate. This bacterium is a common oropharyngeal commensal in humans. Nevertheless, it can appear as an opportunistic pathogen. *N. sicca* has been implicated, e.g., as the causative agent of endocarditis (156). Recent genomic analyses suggest that *N. sicca* and *N. macacae* should be categorized as variants of *N. mucosa* (13).

Neisseria subflava

N. subflava appears as smooth, variably transparent colonies with a yellowish pigment. This species contains the previous species *N. subflava*, *N. perflava*, and *N. flava* (1). Strains of *N. subflava* acidify glucose and maltose. In addition, *N. subflava* bv. subflava and *N. subflava* bv. flava produce acid from fructose, while *N. subflava* bv. perflava acidifies sucrose and produces polysaccharide from sucrose. *N. subflava* is a common commensal of the human oropharynx but has occasionally been associated with invasive diseases, such as meningitis, endocarditis, and bacteremia (157). Similar to the case for *N. lactamica*, reduced susceptibilities to penicillin, and also cefixime and ciprofloxacin, have been reported (158).

Neisseria weaveri

Colonies of *N. weaveri* are of variable size (1 to 2 mm), smooth, flat, and slightly glistening (1). They have entire edges and are grayish. Like most species of the genus *Neisseria*, *N. weaveri* is strongly catalase and oxidase positive. Like *N. bacilliformis* and *N. elongata*, *N. weaveri* organisms are rods, not cocci. *N. weaveri* does not use carbohydrates and does not reduce nitrate (159). Strains are infrequently recovered from human dog bite wounds and oral cavities of dogs (159). While septicemia in an immunosuppressed individual has been described (160), invasive disease is very rare.

Neisseria zoodegmatis

Similar to *N. animaloris*, *N. zoodegmatis* is a Gram-negative coccoid rod (30) and was formerly named CDC group EF-4b. Its habitat (30), colony morphology, growth on MacConkey agar (146), and clinical significance (31) are identical to those of *N. animaloris*. Nevertheless, in contrast to *N. animaloris*, *N. zoodegmatis* is arginine dihydrolase negative (146).

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Aggregatibacter, Capnocytophaga, Eikenella, Kingella, Pasteurella, and Other Fastidious or Rarely Encountered Gram-Negative Rods*

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35

The bacterial genera covered in this chapter are taxonomically diverse and belong to the families *Cardiobacteriaceae*, *Flavobacteriaceae*, *Leptotrichiaceae*, *Neisseriaceae*, *Pasteurellaceae*, and *Porphyromonadaceae*, but common traits justify their discussion as a group. They are isolated infrequently and constitute part of those Gram-negative genera that stand apart from the *Aeromonadaceae*, *Enterobacteriaceae*, *Vibrionaceae*, and *Rickettsiales*. With the exception of *Chromobacterium* and some *Pasteurella* species, for aerobic growth they require supplemented media, on which they often grow slowly (48 h at 35 to 37°C), and fail to grow on enteric media. A 5 to 10% CO_2 atmosphere may be necessary for growth initiation and improves growth on subculture. With the exception of *Chromobacterium*, they do not possess flagella but may show gliding or twitching motility, resulting in limited spreading of colonies and pitting of the agar surface.

The identification of these genera is difficult by conventional phenotypic methods. Commercially available systems (1–5) allow identification of the frequently encountered human isolates. If accurate identification of uncommon isolates is of concern, then molecular methods, e.g., 16S rRNA gene analysis, are necessary (6). The application of matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) shows promising results (7, 8). Molecular methods and MALDI-TOF MS are both discussed in this chapter. The patterns of the cellular fatty acids are no longer integrated in the tables; these data can be found in chapter 33 of the 10th edition of this *Manual*. This chapter discusses only species that can be isolated from humans.

TAXONOMY AND DESCRIPTION OF THE AGENTS

The taxonomy of the family *Pasteurellaceae* has undergone important changes (9; http://www.icsp.org/subcommittee/pasteurellaceae/taxa.html); the family consists of >80 named species distributed in 17 genera, of which 4 are known to contain human pathogens: *Actinobacillus*, *Aggregatibacter*, *Haemophilus*, and *Pasteurella*. Minimal standards for the description of genera, species, and subspecies of the *Pasteurellaceae* have been proposed and might create further changes (10). *Aggregatibacter* spp., formerly named *Haemophilus* (11), are included in this chapter. The genus *Haemophilus*, as presently constituted, is described in chapter 36. *Neisseria* spp. with rod forms, i.e., *Neisseria elongata* subsp. *glycolytica* and subsp. *nitroreducens*, *Neisseria weaveri*, and CDC group EF-4a, renamed *Neisseria animaloris* (12), are described in chapter 34 but are included in a differentiation table in this chapter (see Table 4).

Actinobacillus

The genus *Actinobacillus* in the family *Pasteurellaceae* consists of facultatively anaerobic, nonmotile, Gram-negative rods and comprises animal (*A. equuli*, *A. lignieresii*, and *A. suis*) and exclusively human (*A. hominis* and *A. ureae*) species but no longer includes *Actinobacillus actinomycetemcomitans* (11). The G+C content of the DNA of *Actinobacillus* spp. is between 40 and 43 mol% (13).

Aggregatibacter

Multilocus sequence analysis of *A. actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus*, and *Haemophilus segnis* (11) had suggested that these bacteria represented a monophyletic group. In addition, DNA-DNA relatedness between *H. aphrophilus* and *H. paraphrophilus* was 77%, and DNA from *H. aphrophilus* was able to transform *H. paraphrophilus* to have an NAD-independent phenotype (11). It was, therefore, proposed to transfer *A. actinomycetemcomitans*, *H. aphrophilus*, *H. paraphrophilus*, and *H. segnis* into a new genus, *Aggregatibacter* in the family *Pasteurellaceae*, with the species *Aggregatibacter aphrophilus* comprising the former *H. aphrophilus* and *H. paraphrophilus*, i.e., including V factor-dependent and -independent isolates (11). The G+C content of the DNA of *Aggregatibacter* spp. is 42 to 44 mol% (13).

Capnocytophaga

The *Capnocytophaga* genus in the family *Flavobacteriaceae* at present consists of nine species (*C. canimorsus*, *C. cynodegmi*, *C. ochracea*, *C. gingivalis*, *C. sputigena*, *C. haemolytica*, *C. granulosa*, *C. leadbetteri*, and genospecies AHN8471) of

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facultatively anaerobic, nonmotile, Gram-negative rods, but with gliding motility (14). The G+C content of the DNA of this genus is 34 to 44 mol% (15).

Cardiobacterium

The *Cardiobacterium* genus in the family *Cardiobacteriaceae* consists of facultatively anaerobic, nonmotile, Gram-negative rods, with the species *C. hominis* and *C. valvarum* (16). The G+C content of the DNA of these species is 59 to 60 mol% (17).

Chromobacterium

The *Chromobacterium* genus in the family *Neisseriaceae* (18) contains several facultatively anaerobic, motile species, of which *C. violaceum* is at present the only agent of human disease. Its DNA content is between 65 and 68 mol% (18). *C. haemolyticum*, isolated from a sputum culture, is so far represented by one strain only (19) and is not covered here.

Dysgonomonas

The genus *Dysgonomonas* in the family *Porphyromonadaceae* consists of facultatively anaerobic, nonmotile, Gram-negative rods (20). Four species have been described: *D. capnocytophagoides* and *D. gadei* (21), *D. mossii* (22), and *D. hofstadii* (23). The G+C content of the DNA is ~38 mol% (20).

Eikenella

The genus *Eikenella* in the family *Neisseriaceae* (18) consists of facultatively anaerobic, nonmotile, Gram-negative rods. Thus far, only one species, *E. corrodens*, has been recognized. The G+C content of the DNA of *Eikenella* is between 56 and 58 mol% (18).

Kingella

The genus *Kingella* in the family *Neisseriaceae* consists of the facultatively anaerobic, nonmotile species *K. kingae*, *K. denitrificans*, *K. oralis*, and *K. potus* (18, 24). The G+C content of the DNA of these species is between 47 and 58 mol% (18).

Pasteurella

The taxonomy of the genus *Pasteurella* in the family *Pasteurellaceae* has been in flux for some time (10, 13; http://www.bacterio.net). *P. multocida* can be separated into the subspecies *multocida*, *septica*, and *gallicida*; *P. canis*, *P. dagmatis*, and *P. stomatis* are other species isolated from humans. In spite of the genotypical homogeneity of *P. multocida* isolates, phenotypically diverse lineages have been observed, e.g., sucrose-negative variants from infections from bite wounds made by large cats (25). The G+C content of the DNA of *Pasteurella* species is between 38 and 46 mol% (13). New genera or reclassifications may be necessary for species discussed below that are preceded by "*(P.)*" or "*(Pasteurella)*" (26).

Simonsiella

The genus *Simonsiella* in the family *Neisseriaceae* consists of several obligately aerobic species that may show gliding motility (18). The only species isolated from humans is *S. muelleri*. This genus has a G+C content in its DNA of 40 to 50 mol% (18).

Streptobacillus

The *Streptobacillus* genus in the family *Leptotrichiaceae* (27) consists of one facultatively anaerobic, nonmotile species, *S. moniliformis*, with a G+C content in its DNA of 24 to 26% mol% (27). See chapter 54 for the genus *Leptotrichia* of the family *Leptotrichiaceae*.

Suttonella

The *Suttonella* genus in the family *Cardiobacteriaceae* (17) consists of facultatively anaerobic, nonmotile, Gram-negative rods and so far contains only one species, *S. indologenes* (formerly *Kingella indologenes*) (http://www.bacterio.net). The G+C content of its DNA is 49 mol% (17).

EPIDEMIOLOGY AND TRANSMISSION

Most bacteria in this group are part of the microbiota of the nasopharynx and/or the oral cavity of animals and/or humans and are parasitic, with the only environmental genus being *Chromobacterium*. Transmission from animals occurs by contact (e.g., bites and licking of wounds), from humans to humans by droplets (e.g., directly with *Kingella* spp. or by paraphernalia or human bites with *E. corrodens*). They may cause infections anywhere in the human body. Risk factors exist for certain types of septicemia (e.g., liver cirrhosis for *P. multocida*, neutropenia for oxidase-negative *Capnocytophaga* spp., and chronic granulomatous disease for *C. violaceum*). Endogenous infections occur as well, e.g., HACEK (*Haemophilus* spp., *Aggregatibacter* spp., *Cardiobacterium* spp., *E. corrodens*, and *Kingella* spp.) endocarditis (28).

The habitat of the HACEK group, e.g., *Haemophilus* spp. and *Aggregatibacter* spp., is the human oral cavity, including dental plaque; infections are endogenous (11, 29). The normal habitat of *Cardiobacterium* spp. is the human oral cavity and nasopharynx but possibly also the gastrointestinal and urogenital tracts (17, 28). Infections are endogenous. The natural habitat of *E. corrodens* is the oral cavities and possibly the gastrointestinal tracts of humans and some mammals, from which it can be transmitted via saliva (bites, syringes) to other individuals (30–32). Endogenous infections prevail, however. The natural habitat of *Kingella* spp. is the upper respiratory tract and oral mucosa of humans and possibly other primates. *K. kingae* colonizes the throat but not the nasopharynx of many children aged 6 months to 4 years (33). Ribotyping and pulsed-field gel electrophoresis have shown that *K. kingae* can be transmitted via respiratory droplets (34), although most infections are endogenous. *K. oralis* has been isolated from the human mouth (18). *S. indologenes* is not an official constituent of HACEK but is well known as an agent of endocarditis (35). However, its natural habitat is not known (17).

A. lignieresii (primary habitat in the oral cavities of sheep and cattle), *A. equuli* (in the oral cavities of horses and pigs), and *A. suis* (in the oral cavities of pigs) can be transmitted to humans by animal contact (29). Exclusively human are *A. hominis* and *A. ureae*, whose normal habitat is unknown (29, 36).

The oxidase- and catalase-negative species *C. ochracea*, *C. gingivalis*, *C. sputigena*, *C. haemolytica*, and *C. granulosa*, as well as the recently described *C. leadbetteri* and genospecies AHN8471, are normal but not prominent members of the human oral microbiota (14). The first three have been isolated from adults with periodontal disease but also from periodontitis-free adults; the other four have been isolated from supragingival and subgingival plaque in children and adults (14). Infections are endogenous. The oxidase- and catalase-positive species *C. canimorsus* and *C. cynodegmi* reside in the oral cavities of healthy dogs (25% of dogs have *C. canimorsus* as determined by culture and 85 to 100% of dogs have it as determined by PCR) and cats (15%, as determined by culture) (37).

Chromobacterium inhabits soil and water in tropical and subtropical climates between latitudes of 35°N and 35°S (South Africa, Southeast Asia, Australia, southeastern United States, and, rarely, South America) (38, 39). The portal of entry is usually the skin, but oral intake has also been reported (40).

Most *D. capnocytophagoides* strains have been isolated from stools of immunocompromised patients, and a few strains have been isolated from other sources (41). The natural habitats of this and the other *Dysgonomonas* spp. are unknown (22, 23).

Pasteurella spp. are widespread in healthy and diseased wild and domestic animals, including rodents, dogs, and cats, inhabiting the nasopharynx and gingiva (13). Human isolates are transmitted predominantly from animals by contact (bites or licking or scratching of wounds). Of the "related" species, *(Pasteurella) aerogenes* occurs primarily in pigs (42), *(Pasteurella) caballi* in pigs and equines (42, 43), and *(Pasteurella) pneumotropica* in rodents and dogs (44); the natural habitat of *(Pasteurella) bettyae* is uncertain.

The natural habitat of *S. muelleri* is the oral cavity of humans. In healthy human populations, the incidence of *Simonsiella* spp. is in the range of 30 to 40%. Children possibly have a higher incidence than adults. In dogs and cats, *Simonsiella* spp. are common and abundant (18).

S. moniliformis occurs naturally in the upper respiratory tract of up to 100% of wild and laboratory rats and other rodents (mice, gerbils, squirrels, ferrets, and weasels) and occasionally of dogs and cats preying on rodents. Transmission to humans occurs either from bites of those animals (rat bite fever) or from consumption of contaminated food or water (Haverhill fever) (45).

CLINICAL SIGNIFICANCE

Actinobacillus spp.

A. lignieresii causes actinobacillosis, a granulomatous disease in cattle and sheep in which, similar to actinomycosis, sulfur granules form in tissues (46). A few human soft tissue infections after a cow or sheep bite or other contacts have been reported (42). *A. equuli* and *A. suis* have caused a variety of diseases in horses and pigs; human infections are generally due to horse or pig bites or contact (42). Both species have also been isolated, albeit rarely, from the human upper respiratory tract (13, 47). *A. ureae* is most often a commensal in the human respiratory tract, particularly in patients with lower respiratory tract disease (13), but has also been found as an agent of meningitis following trauma or surgery (48) and of other infections in immunocompromised patients (49). *A. hominis* has also been isolated from such patients but has occurred as a commensal as well, albeit rarely (36). Virulence factors belong to the pore-forming protein toxins of the RTX family; RTX toxins have repeats in the structural toxin peptide and exhibit a cytotoxic and often also a hemolytic activity. They are particularly widespread in species of the family *Pasteurellaceae* (50).

Aggregatibacter spp.

A. actinomycetemcomitans is one of the major agents of juvenile and adult periodontitis (51) and may occur together with *Actinomyces* spp. in actinomycotic sulfur granules (11). Furthermore, it may cause HACEK endocarditis (28), soft tissue infections, and other infections (52). In a large, multinational cohort study, HACEK organisms were the causes of 1.4% of all cases of endocarditis (53). HACEK endocarditis is characterized by a relatively long interval between first symptoms and diagnosis (range, 2 weeks to 6 months), large vegetations on native or artificial valves of the left side, and frequent embolizations. Prognosis is good with appropriate antibiotic treatment (28, 53). Virulence factors are an RTX leukotoxin (50), a cytotoxic distending toxin (54), and the adhesin EmaA (55), as well as fimbriae (56).

A. aphrophilus may cause systemic disease, particularly bone and joint infections, spondylodiscitis, and endocarditis (52, 57, 58).

A. segnis, whose frequency may be underestimated due to apparent misdiagnoses, may cause endocarditis (59). It may be an important cause of bacteremia and sometimes of other infections, e.g., pyelonephritis (60).

Capnocytophaga spp.

C. ochracea, *C. gingivalis*, *C. sputigena*, *C. haemolytica*, and *C. granulosa* have been reported as agents of septicemia and other endogenous infections (endocarditis, endometritis, osteomyelitis, soft tissue infections, peritonitis, ophthalmic lesions, and noma) (30, 47, 61–63) in immunocompetent and immunosuppressed (mainly neutropenic) patients. They are able to suppress neutrophilic chemotaxis and lymphocyte proliferation (64). The association with periodontitis remains unclear (14).

Infections with *C. canimorsus* and *C. cynodegmi* are associated mainly with dog or cat bites or contact. Patients infected with *C. canimorsus* most often present with septicemia and have previously been splenectomized or are alcoholics. In fulminant cases with a poor prognosis, disseminated intravascular coagulation, acute renal failure, respiratory distress syndrome, and shock may develop (65). Hemolytic-uremic syndrome and thrombotic thrombocytopenic purpura are other possible sequelae (66). Meningitis (67, 68), keratitis (65), and endocarditis (69) have been reported as well. *C. cynodegmi* has been isolated more rarely, mainly from localized or systemic infections (70). *C. canimorsus* resists phagocytosis by macrophages and killing by complement and leukocytes; macrophages incubated with the bacterium fail to produce several proinflammatory cytokines (71).

Cardiobacterium spp.

Disease caused by both *Cardiobacterium* species is mainly HACEK endocarditis (16, 53); *C. hominis* prosthetic valve endocarditis has been described after upper endoscopy and colonoscopy (72), and on rare occasions, *C. hominis* has been isolated from other body sites (30, 73). Most reported cases of *C. valvarum* endocarditis are related to periodontal diseases (74). In blood culture-negative cases, the diagnosis has been made by broad-range PCR applied to valve tissue (75).

Chromobacterium violaceum

Localized infections usually arise from contaminated wounds, and septicemia with multiple organ abscesses may follow. They are significantly associated with neutrophil dysfunction (glucose-6-phosphate dehydrogenase deficiency, chronic granulomatous disease). Children without these conditions and those with bacteremia show a high fatality rate (38–40). A number of virulence factors other than endotoxin, i.e., adhesins, invasins, and cytolytic proteins, have been described (76).

Dysgonomonas spp.

Diarrhea was reported to have occurred in 10 of 20 patients with fecal isolates of *C. capnocytophagoides*, whereas routine stool cultures yielded the organism in 1.1 to 2.3% of cultures (77). Bacteremia occurs as well (41); one blood isolate was found to be identical by ribotyping to one in the stool of the same patient (78). One strain of *D. mossii* was isolated from intestinal juice of a patient with pancreatic carcinoma (79). *D. gadei* has been isolated from a human gallbladder (21) and from blood (80), and *D. hofstadii* has been isolated from a wound (23).

Eikenella corrodens

E. corrodens is associated with juvenile and adult periodontitis (51) but is also an agent of infections of the upper respiratory tract, pleura and lungs, abdomen, joints, bones, wounds (e.g., from a human bite), and, rarely, other infections, like noma (30–32, 81). These organisms are often indolent and found mixed with other members of the oropharyngeal microbiota, particularly staphylococci and streptococci. Risk factors are dental manipulations and intravenous drug abuse. Endocarditis is of the HACEK type if monomicrobial, but polymicrobial non-HACEK cases are known (28). *E. corrodens* can trigger a cascade of events that induce inflammation in periodontal tissue (82).

Kingella spp.

Infections with *K. kingae* show a predilection for bones and joints of previously healthy children <4 years of age (33). The use of culture and broad-range and *K. kingae*-specific real-time PCR shows it to be the most common cause of osteoarthritic infection in this age group (83). Septic arthritis, discitis, and osteomyelitis of the lower extremities as well as occult bacteremia are conspicuous. Stomatitis and/or upper respiratory infections may precede systemic disease, suggesting entry through a damaged mucosa (33). In adults, systemic infections occur in immunocompromised individuals (84) or may present as HACEK endocarditis (28, 53). Virulence factors are an RTX toxin (85) and type IV pili (86).

K. denitrificans has been reported as an agent of endocarditis (28, 53). *K. oralis* has been isolated from patients with periodontitis, but its relationship to the disease is unclear. *K. potus* has caused a wound infection following a kinkajou bite (24).

Pasteurella spp.

Human isolates of *P. multocida* are mostly found in wound or soft tissue infections. Less frequent are colonization or infection of the respiratory tract and (by hematogenous or contiguous route) systemic disease, such as meningitis, dialysis-associated peritonitis, endocarditis, osteomyelitis, urinary tract infection, and septicemia, with cirrhosis of the liver being a particular risk factor (87–90). The subspecies most frequently encountered is subsp. *multocida*, which is also more frequent in respiratory infections and bacteremias than subsp. *septica*, which is most often associated with wound infections and central nervous system infections (42, 88). Infected cat bite wounds contain pasteurellae significantly more often than infected dog bite wounds, reflecting a higher oropharyngeal colonization rate in cats than in dogs (88). In the respiratory tract, colonization may eventually lead to sinusitis or bronchitis as well as pneumonia and empyema, the latter two mostly in patients with prior respiratory disease (87). Virulence factors are capsules (five serotypes, of which A and D account for most human isolates), lipopolysaccharide (88), sialidases, hyaluronidase, surface adhesins, iron acquisition proteins (91), and the *P. multocida* toxin (PMT) (92). The PMT has many properties that mark it as a potential carcinogen; the PMT is a highly potent mitogen and has been demonstrated to block apoptosis (92).

Cases of human infection with *P. canis* (from dogs), *P. dagmatis*, and *P. stomatis* (from dogs or cats) are infrequent (88, 93, 94); however, *P. canis* is the most common species isolated from infected dog bite wounds (42). *P. dagmatis* has been reported in systemic infections such as pneumonias, peritonitis, septicemia, and endocarditis; furthermore, *P. dagmatis* can cause exacerbation of bronchiectasis symptoms in chronic obstructive pulmonary disease patients (93). A prosthetic valve endocarditis caused by a *P. dagmatis*-like isolate originating from a patient's cat was recently described (95). In some cases of pasteurellosis, animal contact could not be established (96). Double infections with two *Pasteurella* spp. have also been observed (93).

Of the "related" species, *(P.) aerogenes* has caused wound infections from pig and hamster bites (42, 97), *(P.) bettyae* has been found in infections of newborns and in infections of the male and female genital tracts (98), and *(P.) caballi* has been isolated from horse bites (42); *(P.) pneumotropica* is a rare agent of systemic infection in humans (44). Reported cases of human infection with *Avibacterium gallinarum*, *Bibersteinia trehalosi*, *Gallibacterium anatis*, and *Mannheimia haemolytica*, all formerly in the genus *Pasteurella*, remain doubtful when stricter identification criteria are employed (99) or require confirmation (100).

Simonsiella spp.

S. muelleri has not been found associated with disease. *Simonsiella* can be encountered in exfoliative cytology specimens of the upper gastrointestinal tract, erosions of the oral mucosa, and the respiratory tract and should not be confused with a truly pathogenic organism (101).

Streptobacillus moniliformis

Rat bite fever is a systemic illness beginning with fever and chills, followed by migratory, sometimes even suppurative, polyarthritis and a maculopapular rash on the extremities. Rare complications include endocarditis, myo- or pericarditis, pneumonia, septicemia, and abscess formation (45, 102, 103).

Suttonella indologenes

Human isolates of *S. indologenes* have been very rare; they have been isolated from ocular sources (47) and a blood culture in a case of endocarditis (35).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Collection and transport of specimens should follow the guidelines described in chapter 18; the low viability of many species makes the use of transport media, e.g., eSwab (Copan Diagnostics Inc., Murrieta, CA), whenever indicated, mandatory. Cultures of most bacteria described in this chapter can be stored at room temperature for 1 to 2 weeks. However, for some other very fastidious bacteria, e.g., *C. canimorsus*, subcultures must be performed frequently. For keeping strains in a culture collection, the isolates should be frozen in a cryoprotective solution, e.g., skim milk, at –70°C.

DIRECT EXAMINATION

Direct microscopy of specimens or of positive blood cultures supported by clinical information may identify the corresponding bacteria; e.g., the typical morphology of spindle-shaped cells in the Gram stain and the anamnestic history of a dog bite allow the presumptive identification of *C. canimorsus*. In a few instances of blood culture-negative cases, microscopy of the resected valve and/or broad-range 16S rRNA gene sequence PCR of the valve or emboli have allowed the diagnosis of endocarditis (75). When the question arises as to whether an isolate is a Gram-negative coccus or coccobacillus, a penicillin disk can be added to the agar plate after streaking with the isolate. After incubation, the Gram stain from growth around the disk shows coccoid forms in the case of typical *Neisseria* spp.; otherwise it shows long rods (104).

Actinobacillus spp. are medium-sized, Gram-negative, rod-shaped or coccoid bacteria with a tendency to bipolar staining. Their arrangement is single, in pairs, and, rarely, in short chains.

Aggregatibacter spp. are coccoid to rod-shaped, Gram-negative bacteria, occasionally exhibiting filamentous forms. A commercial PCR-based hybridization method for the detection of *A. actinomycetemcomitans* and other periodontal pathogenic bacteria, e.g., *E. corrodens* and *Capnocytophaga* spp., has been described (105).

Capnocytophaga spp. are mainly fusiform, medium-to-long cells with tapered ends (Fig. 1a). *L. buccalis* is a long, fusiform, Gram-negative rod but with one tapered and one square end (Fig. 1b). Differentiation is supported by biochemical reactions; furthermore, lactic acid is the major fermentation end product of *L. buccalis*, and succinic acid is the major fermentation end product of *Capnocytophaga* spp. (47). *C. canimorsus* can also be detected by broad-range PCR (68).

Cardiobacterium spp. are pleomorphic on blood agar. *C. hominis* stains irregularly and appears as straight, Gram-negative rods in short chains, pairs, or rosettes, sometimes with bulbous ends; occasionally, filaments are formed. On chocolate agar, this morphology is less distinct than on blood agar. In a few instances, broad-range PCR of valve or emboli have led to the diagnosis (75).

C. violaceum is a medium-sized, Gram-negative rod or coccus that is motile by one polar flagellum and one to four lateral flagella (18). A PCR technique for its detection has been developed (106).

Dysgonomonas spp. are small, Gram-negative rods or cocci similar to *Moraxella* spp. (Fig. 1c) (20). In contrast to coccoid *Neisseria* spp., these two genera show long rods in the Gram stain from growth around the penicillin disk (104).

E. corrodens is a slender, straight, small, Gram-negative rod with rounded ends (Fig. 1d). A specific PCR is also commercially available (105).

Kingella spp. are short, Gram-negative rods with square ends that lie together in pairs or clusters (Fig. 1e). They tend to decolorize unevenly on Gram stains. A broad-range PCR has detected *K. kingae*-specific sequences in culture-negative osteoarticular specimens (83).

Pasteurella spp. are small, Gram-negative rods or cocci that occur singly, in pairs, or in short chains. Bipolar staining is frequent. "Related" species are more rod-like. A PCR technique for their detection is mainly applied in the veterinary field (107).

Simonsiella spp. are Gram-negative, crescent-shaped rods (0.5 to 1.0 μm long) arranged in multicellular filaments (10 to 50 μm by 2 to 8 μm) and segmented into groups of mostly eight cells, resulting in a caterpillar-like appearance. The long axis of each cell is perpendicular to the long axis of the filament. Incomplete decolorization on Gram stains is common.

S. moniliformis is a Gram-negative rod with a variable morphology. Depending on age and culture conditions, cells may appear as straight, small to medium-size rods or as 100- to 150-μm-long tangled chains and filaments with bulbar swellings (Fig. 1f). In culture-negative cases, broad-range PCR of fluids has been employed (108). A PCR assay that uses primers designed on the basis of 16S rRNA gene sequence data has been described; the PCR product treated with the restriction enzyme BfaI generates three fragments specific for *S. moniliformis* (109).

S. indologenes is a plump, irregularly staining, Gram-negative rod; occasionally, pairs, chains, or rosettes are formed.

ISOLATION PROCEDURES

For culture of members of this group, the use of blood or chocolate agar and, wherever normal microbiota and specific bacteria are suspected, of selective media is mandatory. Most of them are facultatively anaerobic and do not grow on MacConkey agar. HACEK members do not need more than 5 days of incubation in modern blood culture systems (110).

Actinobacillus spp. and *Aggregatibacter* spp. require enriched media, and they grow better in a 5 to 10% CO_2 atmosphere. Some *A. aphrophilus* (formerly *H. paraphrophilus*) strains and *A. segnis* require V factor; none requires hemin. Selective media for *A. actinomycetemcomitans* have employed bacitracin and vancomycin (111).

Primary isolation of *Capnocytophaga* spp. requires 5 to 10% CO_2 and enriched media; the composition of the blood agar base influences the ability to grow (112). For detection in mixed cultures, selective media containing bacitracin, polymyxin B, vancomycin, and trimethoprim have been used (113), as have Thayer-Martin and Martin-Lewis agars (15). *C. canimorsus* has most often been isolated from blood cultures. However, it may not be detected by commonly used automated blood culture systems; therefore, clinicians should inform the laboratory about risk factors for *C. canimorsus* infection so that subcultures on enriched media can be performed in a blind manner (112).

Cardiobacterium spp. mostly require 5 to 10% CO_2 and increased humidity for initial growth on blood agar. *Cardiobacterium* spp. have most often been isolated from blood.

C. violaceum grows on routine media, even on most enteric ones, at 30 to 35°C, its optimal temperature (18).

Dysgonomonas spp. grow slowly on blood agar in CO_2-enriched atmosphere (20). A selective medium containing cefoperazone, vancomycin, and amphotericin B has been used for stool cultures (78).

With a few exceptions, *E. corrodens* strains require hemin for growth unless 5 to 10% CO_2 is present (18). *E. corrodens* grows well on commercial Columbia agar (112).

Recovery of *K. kingae* from body fluids and pus can be difficult because these specimens seem to be inhibitory. For isolation from mixed cultures, media containing clindamycin or vancomycin as well as Thayer-Martin agar have been recommended (18). The use of various blood culture media has significantly improved the detection rate (33).

In contrast to some *Haemophilus* spp., *Pasteurella* spp. are hemin and CO_2 independent and will, therefore, grow on media without blood. "Related" species may even grow on enteric media (13).

S. muelleri grows aerobically on blood agar. There are no enrichment procedures for *Simonsiella*, but an isolation

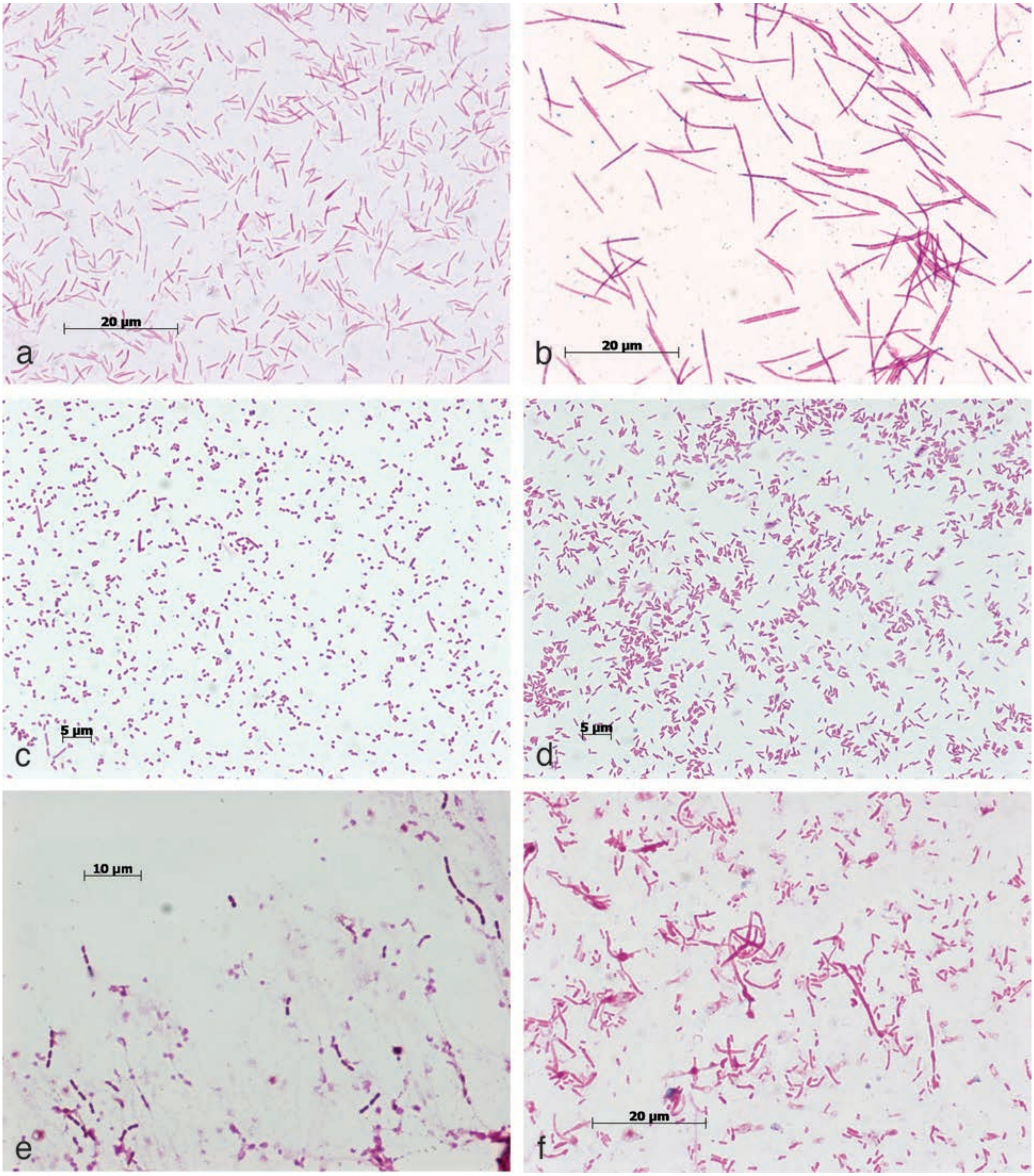

FIGURE 1 (a) *Capnocytophaga ochracea*. Gram stain of a 48-h culture grown on sheep blood agar (Institute of Medical Microbiology, University of Zurich, Zurich, Switzerland).(b) *Leptotrichia buccalis*. Gram stain of a 48-h culture grown on sheep blood agar (Institute of Medical Microbiology, University of Zurich). (c) *Dysgonomonas capnocytophagoides*. Gram stain of a 48-h culture grown on sheep blood agar (Institute of Medical Microbiology, University of Zurich). (d) *Eikenella corrodens*. Gram stain of a 48-h culture grown on sheep blood agar (Institute of Medical Microbiology, University of Zurich). (e) *Kingella kingae*. Gram stain of a 48-h culture grown in Trypticase soy broth (Institute of Medical Microbiology, University of Zurich). (f) *Streptobacillus moniliformis*. Gram stain of a 48-h culture grown on sheep blood agar (Institute of Medical Microbiology, University of Zurich). doi:10.1128/9781555817381.ch35.f1

method with tryptic soy broth without dextrose agar has been described (18).

S. moniliformis is best isolated from blood, joint fluid, or abscess material. For culture, media enriched with sheep, horse, or rabbit blood (15% seems to be optimal), serum, or ascitic fluid and a 5 to 10% CO_2 atmosphere at 37°C are required. Sodium polyanethol sulfonate in blood culture media is inhibitory (45).

S. indologenes grows slowly on blood agar. Aerobic growth is enhanced by high humidity and CO_2, which makes the organism appear to be facultatively anaerobic (17).

IDENTIFICATION

Phenotypic identification of fastidious Gram-negative rods presents several challenges. Triple sugar iron or Kligler's agar may not support the growth of fastidious genera (e.g., *Eikenella*). Media should be rich in peptones (e.g., cystine Trypticase agar); serum (except rabbit serum) should not be used, because it may split maltose. The inoculum should be large (cell paste or agar blocks). Unsupplemented media used to check acid formation from carbohydrates may yield false-negative reactions. Gas formation from carbohydrates is scant or absent in most species. Indole may have to be extracted with xylene. Furthermore, phenotypic characteristics used for identification are subject to variation and dependent on individual interpretation and expertise. Correct identification to the species level often requires multiple substrates that may not be available to routine laboratories (60, 99) and may not even be provided by automated systems (4). However, the VITEK 2 *Neisseria-Haemophilus* identification card (VITEK 2 ID-NH card; BioMérieux, Marcy l'Etoile, France) allows for the identification of most bacteria of the HACEK group (2, 4). *Pasteurella* spp. are not in the database of the VITEK 2 ID-NH card, but they are in the database of the identification card for Gram-negative bacilli (VITEK 2 ID-GNB card; BioMérieux) (1). See chapter 33 for the dichotomous identification algorithms for Gram-negative bacteria. Determination of key reactions may be used for a rapid presumptive identification, e.g., with Api NH (BioMérieux) (114). A positive ornithine decarboxylase reaction and missing sugar acidification in the cystine Trypticase agar are typical for *E. corrodens* (104); a blood culture isolate with a positive indole reaction and a negative catalase reaction is diagnostic for *C. hominis*; *P. multocida* has a typical pattern of acidification of sugars and a positive indole reaction, and together with a history of cat bite the diagnosis is feasible.

The application of newer identification methods like MALDI-TOF MS shows promising results regarding the identification of HACEK group members (7, 8). The inclusion of the species in the databases of the two MALDI-TOF MS systems, i.e., VITEK MS (BioMérieux) and Bruker MALDI Biotyper (Bruker Daltonik GmbH, Bremen, Germany), is mentioned below. Accurate identification of fastidious Gram-negative rods isolated from normally sterile body sites is important for initiation of appropriate patient management. In view of the phenotypic closeness of the species, molecular methods (e.g., 16S rRNA gene or *rpoB* gene sequencing) seem optimal for the identification to the species level of *Actinobacillus*, *Aggregatibacter*, *Capnocytophaga*, and *Pasteurella* spp. (6, 9, 44, 60, 100). Furthermore, molecular methods can identify new species in association with clinical entities.

Colonies of *Actinobacillus* spp. are ~2 mm in diameter after 24 h of growth at 37°C, smooth or rough, viscous, and often adherent to the agar. Smooth colonies are dome shaped and have a bluish hue when viewed by transmitted light. Biochemical reactions are listed in Table 1. Some *Actinobacillus* spp. are included in both MALDI-TOF MS databases. *A. ureae* is included in the VITEK 2 ID-NH card database.

A. actinomycetemcomitans colonies initially show a central dot and a slightly irregular edge and, on further incubation, develop a star-like configuration resembling "crossed cigars" and pit the agar. After several subcultures, this rough morphology may give way to smooth and opaque, nonpitting colonies, reflecting loss of fimbriae. In liquid media, the bacterium forms granules that adhere to the sides and to the bottom of the tube. Colonies of other *Aggregatibacter* spp. are granular or smooth, grayish white to yellowish, and opaque; without CO_2, there is pleomorphism, with small and large colonies. For species identification, a battery of tests (requirements for V and X factors, biochemical tests, colonial morphology [Table 1]) is necessary to avoid confusion with *Haemophilus* spp. *Aggregatibacter* spp. have negative reactions for production of indole, ornithine decarboxylase, and urease and are not dependent on X factor; *Haemophilus* spp. are at least positive for one of those three reactions or cannot synthesize heme components from δ-aminolevulinic acid. Automated systems may present difficulties in identifying these bacteria to the species level (4, 59, 60, 99). *A. segnis* is identified by PCR (59, 60). *Aggregatibacter* spp. mentioned in Table 1 are included in both MALDI-TOF MS databases as well as in the VITEK 2 ID-NH card database.

Colonies of *Capnocytophaga* spp. on blood agar are very small after 24 h at 37°C and reach 2 to 4 mm in diameter after 2 to 4 days; they are convex or flat and often slightly yellow when scraped off agar, show regular or spreading edges with gliding motility, and adhere to the agar surface (15). Phenotypic differentiation of species in the oxidase-negative group may be inconclusive due to the similarity of many biochemical reactions (Table 2) and the lack of suitable substrates even in automated systems (4). This has frequently given rise to identification as "*Capnocytophaga* sp." 16S rRNA gene sequencing is at present the most adequate diagnostic tool (6, 14, 115). All *Capnocytophaga* spp. mentioned in Table 2 are included in the database of the Bruker MALDI Biotyper, and some of these species are in the database of the VITEK MS system. The VITEK 2 ID-NH card database includes *Capnocytophaga* spp. at the genus level.

Colonies of *Cardiobacterium* spp. attain a diameter of ~1 mm after 48 h at 37°C on blood agar; they are circular, smooth, and opaque, and they may pit the agar. Biochemical test reactions are recorded in Table 3. Differences between the two species are minimal and concern indole production in some strains, mannitol fermentation, and quantitative composition of cellular fatty acids (16). Some automated systems may misidentify *C. valvarum* (114). Both species are included in the database of the Bruker MALDI Biotyper. The VITEK 2 ID-NH card database includes *C. hominis*.

Colonies of *C. violaceum* measure 1 to 2 mm in diameter after 24 h of growth, are round and smooth, have an almond-like smell, and may be beta-hemolytic. Most strains produce a violet pigment called violacein, which is soluble in ethanol but not in water. Identification is easy if this pigment is produced, although the positive oxidase reaction will be detected only by a modified technique (116). Biochemical reactions are listed in Table 3. This species may even grow on most enteric media. Nonpigmented strains may be confused with *Aeromonas* spp. but are lysine, maltose, and mannitol negative. *C. violaceum* is included in both MALDI-

TABLE 1 Biochemical reactions of *Actinobacillus* and *Aggregatibacter* spp.[a]

Reaction	*Actinobacillus lignieresii*	*Actinobacillus equuli*	*Actinobacillus suis*	*Actinobacillus ureae*	*Actinobacillus hominis*	*Aggregatibacter actinomycetemcomitans*	*Aggregatibacter aphrophilus*	*Aggregatibacter segnis*
Requirement for V factor	–	–	–	–	–	–	v	+
Beta-hemolysis	–	v	+	–	–	–	–	–
Catalase	v	v	+/+^{w}	+	+	+	–	v
Oxidase	+	+	+	+	+	v	v	–
Esculin hydrolysis	–	–	+	–	v	–	–	–
Urease	+	+	+	+	+	–	–	–
ONPG	+	+	v	–	+	–	+	–
Growth on MacConkey agar	v	+	v	–	–	–	v^{w}	–
Gas from glucose	–	–	–	–	–	v	+	–
Acid from:								
Lactose	v	+	+	–	+	–	+^{D}	–
Sucrose	+	+	+	+	+	–	+	+^{w}
D-Xylose	+	+	+	–	+	v	–	–
Maltose	+	+	+	+	+	v	+	+^{w}
D-Mannitol	+	+	–	+	+	v	–	–
Trehalose	–	+	+	–	+	–	+^{D}	–
D-Melibiose	–	+	+	–	+	–	v	–

[a]Data are from references 29, 36, 47, and 104. +, ≥90% of strains positive; –, ≥90% of strains negative; D, delayed reaction; ONPG, *ortho*-nitrophenyl-β-D-galactopyranoside; v, variable; w, weak; /, or. All species are indole negative, reduce nitrate to nitrite, and are positive for alkaline phosphatase.

TOF MS databases and in the VITEK 2 ID-GNB card database, e.g., for the identification of Gram-negative rods including members of the family *Enterobacteriaceae* and non-enteric bacilli (1).

Colonies of *Dysgonomonas* spp. are entire, measure 1 to 2 mm in diameter after 24 h of growth, have a strawberry-like odor, and do not spread or adhere. The species show few biochemical differences (Table 2). Aerobically growing isolates of *L. buccalis* may be confused with *Dysgonomonas*; microscopic examination of morphology, different cellular fatty acid profiles, and determination of the production of lactic acid from glucose in *Leptotrichia* (*Dysgonomonas*

TABLE 2 Biochemical reactions of *Capnocytophaga* spp., *Dysgonomonas* and related species, and *Streptobacillus*[a]

Reaction	*C. ochracea*	*C. sputigena*	*C. gingivalis*	*C. granulosa*	*C. haemolytica*	*C. canimorsus*	*C. cynodegmi*	*D. capnocytophagoides*/ *D. gadei*[b]	*D. mossii*[b]	*D. hofstadii*[b]	*S. moniliformis*
Catalase	–	–	–	–	–	+	+	–	–	–	–
Oxidase	–	–	–	–	–	+	+	–	–	–	–
Indole	–	–	–	–	–	–	–	–	+	+	–
Arginine dihydrolase	–	–	–	ND	ND	+	+	–	–	–	+
Nitrate to nitrite	v	v	–	–	+	–	v	–	–	–	–
Esculin hydrolysis	v	+	–	–	+	v	+	v	+	+	v
Gelatinase	–	v	–	–	–	–	–	–	–	–	–
ONPG	+	+	–	+	+	+	+	+	+	+	–
Acid from:											
Lactose	+	v	v	+	+	+	+	+	+	ND	–
Sucrose	+	+	+	+	+	–	+	+	+	ND	–
Xylose	–	–	–	–	–	–	–	+	+	ND	–

[a]Data are from references 14, 21, 23, 47, and 104. +, ≥90% of strains positive; –, ≥90% of strains negative; ND, no data; ONPG, *ortho*-nitrophenyl-β-D-galactopyranoside; v, variable. All species are negative for urease and ornithine decarboxylase and form acid from glucose (sometimes only with addition of serum).

[b]The isolates of *D. gadei* were catalase and indole positive (21, 80). *D. mossii* and *D. hofstadii* (one strain) differ in a few reactions in the Api Rapid ID32A system (23).

TABLE 3 Biochemical reactions of some rod-shaped species of the *Neisseriaceae* and of the *Cardiobacteriaceae*[a]

Reaction	*Chromobacterium violaceum*	*Eikenella corrodens*	*Kingella kingae*	*Kingella denitrificans*	*Kingella oralis*	*Kingella potus*	*Simonsiella muelleri*	*Cardiobacterium hominis*	*Cardiobacterium valvarum*	*Suttonella indologenes*
Catalase	+	–	–	–	–	–	–	–	–	v
Oxidase	v	+	+	+	+	+	+	+	+	+
Indole	v	–	–	–	–	–	–	+w	v	+
Arginine dihydrolase	+	–	–	–	–	–	–	–	ND	–
Nitrate to nitrite	+	+	–	+/G	–	–	v	–	–	–
Esculin hydrolysis	–	–	–	–	–	–	–	–	–	–
Ornithine decarboxylase	–	+	–	–	–	–	–	–	ND	–
Growth on MacConkey agar	+	–	–	–	–	–	–	–	–	–
Alkaline phosphatase	+	–	+	–	+	–	–	–	ND	+
Acid from:										
Glucose	+[b]	–[c]	+	+	+w	–	+	+	v	+
Sucrose	v	–	–	–	–	–	–	+	v	+
Maltose	–	–	+	–	–	–	+	+	v	+
D-Mannitol	–	–	–	–	–	–	–	+	v	–
Special feature(s)	Violacein v	LD v	Beta-hemolysis	Growth on TMA		DNase +, yellow pigment	Microscopic morphology			

[a] Data are from references 16, 24, 47, 74, and 104. +, ≥90% of strains positive; –, ≥90% of strains negative; G, gas; LD, lysine decarboxylase; ND, no data; TMA, Thayer-Martin agar; v, variable; w, weak. All species are negative for urease and acid production from lactose and D-xylose.

[b] Some strains form small amounts of gas.

[c] Weakly positive reactions may be observed in oxidation-fermentation media.

produces propionic and succinic acid) (47) are of help if 16S rRNA gene sequencing is not available (21, 23, 47). *Dysgonomonas* spp. are not yet in the MALDI-TOF MS databases nor in the VITEK 2 card databases.

Colonies of *E. corrodens* are 1 to 2 mm in diameter after 48 h of growth, show clear centers that are often surrounded by spreading growth, may pit the agar, and assume a slightly yellow hue after several days. In liquid media, granules are produced. Typical isolates fail to form acid from carbohydrates in nonsupplemented media and are ornithine decarboxylase and nitrate reduction test positive; lysine decarboxylase activity is variable (Table 3). *E. corrodens* grows poorly or not at all on triple sugar iron or Kligler's agar. *E. corrodens* is well identified by the VITEK 2 ID-NH card and by MALDI-TOF MS (4, 7).

Kingella colonies on blood agar in 5 to 10% CO_2 (which enhances growth) are 1 to 2 mm in diameter after 48 h of growth. One type is smooth with a central papilla, and the other spreads and pits the medium. Only *K. kingae* shows a small but distinct zone of hemolysis on blood agar. Colonies have a short viability and have to be subcultured frequently. Biochemical test results are listed in Table 3. In addition to microscopic and colonial morphology, the tests serve to separate kingellae from rod-shaped members of the genus *Neisseria* (Table 4), with which they may be confused in automated systems (4). Since *K. denitrificans* may grow on Thayer-Martin or Martin-Lewis agar (18), it may be misidentified as *Neisseria gonorrhoeae* unless the catalase reaction, negative for *Kingella*, is performed. *Kingella* spp. are in both MALDI-TOF MS databases as well as in the VITEK 2 ID-NH card database.

Colonies of *Pasteurella* spp. are 1 to 2 mm in diameter after 24 h of growth at 37°C and are opaque and grayish. Encapsulated strains tend to be mucoid. A slight greening underneath the colonies may be noted. Indole-positive isolates exhibit a mouse-like odor. Biochemical reactions are listed in Table 5. The oxidase test has to be performed on blood agar; a weak reaction after 30 s is characteristic for many *Pasteurella* spp. (117). Nutritionally fastidious, nonmotile strains of *Enterobacteriaceae*, e.g., *Escherichia coli*, may be misidentified as *Pasteurella* unless an oxidase test is performed. In view of the phenotypic closeness of some taxa (25; http://www.bacterio.net), species identification in automated systems may be unsatisfactory (1, 93, 94). 16S rRNA gene sequencing and *sodA* gene sequencing have provided reliable species identification, the latter with even more discriminatory power (100). All *Pasteurella* spp. mentioned in Table 5 are included in the database of the Bruker MALDI Biotyper, and some *Pasteurella* spp. are included in the database of the VITEK MS system. The VITEK 2 ID-GNB card database includes *Pasteurella* spp.

Colonies of *Simonsiella* spp. are 1 to 2 mm in diameter after 24 h, may show gliding motility, and produce a pale yellow pigment. *S. muelleri* is beta-hemolytic. Biochemical tests are listed in Table 3. *Simonsiella* spp. are not yet in the MALDI-TOF MS databases nor in the databases of the VITEK 2 cards.

S. moniliformis may show wild-type and L-phase colonies in the same culture. The former are 1 to 3 mm in diameter after 48 to 72 h of growth on blood agar and are round and smooth. L-phase colonies grow better on clear media, yielding the "fried egg" appearance, with irregular outlines and coarse lipid globules. In liquid media, growth occurs mainly in the form of "puff balls" at the bottom of the tube. The organism dies quickly unless subcultured (45). It is

TABLE 4 Differentiation between *Kingella* and rod-shaped *Neisseria* species[a]

Reaction	*Kingella kingae*	*Kingella denitrificans*	*Kingella oralis*	*Kingella potus*	*Neisseria elongata*		*Neisseria weaveri*	*Neisseria animaloris* (EF-4a)
					subsp. *glycolytica*	subsp. *nitroreducens*		
Catalase	–	–	–	–	+	–	+	+
Nitrate to nitrite	–	+	–	–	–	+	–	+
Nitrate to gas	–	+	–	–	–	–	–	v[b]
Alkaline phosphatase	+	–	+	–	–	–	ND	–
Arginine dihydrolase	–	–	–	–	–	–	–	v[b]
Beta-hemolysis	+	–	–	–	–	–	–	–
Acid from:								
Glucose	+	+	$+^{w}$	–	$+^{w}$	v	–	+[c]
Maltose	+	–	–	–	–	–	–	–

[a] Data are from references 18, 24, and 47. +, ≥90% of strains positive; –, ≥90% of strains negative; ND, no data available; v, variable; w, weak.

[b] *Neisseria zoodegmatis* (EF-4b) is negative.

[c] *N. zoodegmatis* is positive only in oxidation-fermentation media (oxidative).

biochemically inert; glucose is acidified weakly and in a delayed fashion (Table 2). Fatty acid analysis can confirm the diagnosis, as can 16S rRNA gene sequencing (47, 102, 108). *S. moniliformis* is included in the database of the Bruker MALDI Biotyper.

Colonies of *S. indologenes* may spread or pit the agar surface of the blood agar. Biochemical tests are listed in Table 3. *S. indologenes* is included in both MALDI-TOF MS databases.

TYPING SYSTEMS AND SEROLOGIC TESTS

On the basis of surface polysaccharides, six serotypes of *A. actinomycetemcomitans* can be distinguished, of which a, b, and c are most common. Serotype b is associated with periodontitis, endocarditis, and penicillin resistance; serotype c is associated with periodontal health and extraoral infections (118, 119).

Typing of *Capnocytophaga* spp. has been done by multilocus enzyme electrophoresis or by restriction fragment length polymorphism analysis (14). Typing of *C. violaceum* has employed *recA* PCR-restriction fragment length polymorphism analysis (120). For *E. corrodens*, typing has been done by arbitrarily primed PCR demonstrating unstable clonality of *E. corrodens* in the oral cavity (121). For typing of *Pasteurella* spp., PCR profiling, restriction endonuclease analysis, ribotyping, multilocus sequence typing, and pulsed-field gel electrophoresis have been employed (25, 122).

Detection of antibodies directed against any of the bacteria discussed in this chapter has been tried on a small scale only and does not seem to offer much value.

ANTIMICROBIAL SUSCEPTIBILITIES

Approved guidelines for broth microdilution susceptibility testing of the HACEK group and for antimicrobial dilution and disk susceptibility testing of *Pasteurella* spp. have been published by the Clinical and Laboratory Standards Institute (CLSI) (123) (see chapter 74). β-Lactamase production among members of the HACEK group is well documented, and β-lactamase-producing isolates are ampicillin resistant; some isolates may be resistant to ampicillin due to mechanisms other than β-lactamase production; routine performance of a β-lactamase test is recommended for all HACEK isolates (123). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has recently published breakpoints for antimicrobial dilution and disk susceptibility testing of *P. multocida*; for all other species, EUCAST has defined non-species-related pharmacokinetic/pharmacodynamic breakpoints (124).

TABLE 5 Biochemical reactions of human *Pasteurella* and "related" species[a]

Reaction	*P. multocida*[b]	*P. canis*	*P. dagmatis*	*P. stomatis*	*(P.)*[c] *aerogenes*	*(P.) bettyae*	*(P.) caballi*	*(P.) pneumotropica*
Catalase	+	+	+	+	+	–	–	+
Oxidase	+	+	+	+	+	v	v	+
Indole	+	+	+	+	–	$+^{w}$	–	+
Urease	–	–	+	–	+	–	–	+
Ornithine decarboxylase	+	+	–	–	v	–	+	+
Growth on MacConkey agar	–	–	–	–	+	v	–	v
Gas from glucose	–	–	v	–	+	$+^{w}$	+	–
Acid from:								
Lactose	–	–	–	–	v	–	+	–
Sucrose	+	+	+	+	+	–	+	+
D–Xylose	v	–	–	–	v	–	+	+
Maltose	–	–	+	–	+	–	+	+
D-Mannitol	+	–	–	–	–	–	+	–

[a] Data are from references 13, 29, 43, and 47. +, ≥90% of strains positive; –, ≥90% of strains negative; v, variable; w, weak. All species reduce nitrate to nitrite and are negative for arginine dihydrolase and esculin hydrolysis.

[b] The three subspecies, *multocida*, *septica*, and *gallicida*, can be separated on the basis of sorbitol and dulcitol fermentation (+/– in subsp. *multocida*, –/– in subsp. *septica*, and –/+ in the mostly avian subsp. *gallicida*); weakly sorbitol-positive strains of subsp. *multocida* can be recognized by a specific PCR profile (130).

[c] New genera or reclassifications may be necessary for species preceded by "*(P.)*" (26).

Susceptibility studies of *Actinobacillus* spp. are extant for a few isolates of the human-pathogenic species that are susceptible to many antimicrobials, including penicillin (36, 49).

Aggregatibacter spp. are susceptible to cephalosporins, tetracyclines, and aminoglycosides (52, 53, 57, 60, 125). Resistance to ampicillin is not uncommon, but amoxicillin combined with a β-lactamase inhibitor has been effective (125).

Capnocytophaga spp. are usually susceptible to broad-spectrum cephalosporins, carbapenems, clindamycin, macrolides, tetracyclines, and fluoroquinolones but are resistant to aminoglycosides (63). Multidrug-resistant isolates have occasionally been encountered (62).

C. hominis and *C. valvarum* are susceptible to many antimicrobials, including penicillin (16, 72, 125). β-Lactamase production is rare, and its effect can be neutralized by clavulanic acid (123, 126).

C. violaceum is resistant to many antimicrobials (β-lactams and colistin) but is mostly susceptible to imipenem, fluoroquinolones, gentamicin, tetracyclines, and co-trimoxazole (39).

D. capnocytophagoides strains are susceptible to tetracyclines, clindamycin, macrolides, and co-trimoxazole, whereas they are resistant to cephalosporins, aminoglycosides, and fluoroquinolones and variably susceptible to other β-lactam antibiotics and to imipenem (77, 78).

E. corrodens is generally susceptible to penicillin, cephalosporins, carbapenems, doxycycline, azithromycin, and fluoroquinolones but is often resistant to narrow-spectrum cephalosporins, macrolides, and clindamycin (32, 125, 127). β-Lactamase-positive strains have been reported, but the enzyme was inhibited by β-lactamase inhibitors (123, 127).

Kingella spp. are generally susceptible to β-lactam antibiotics, macrolides, tetracyclines, co-trimoxazole, and quinolones (33, 125). β-Lactamase-positive isolates have been reported to be susceptible to combinations with β-lactam inhibitors (33).

Pasteurella spp. are generally susceptible to penicillin, broad-spectrum cephalosporins, tetracyclines, quinolones, co-trimoxazole, and azithromycin; resistance to erythromycin can occur (128). Rare penicillin-resistant isolates of *Pasteurella* spp. have been encountered, but their effect can be neutralized by clavulanic acid (123, 129). For isolates of *Pasteurella* spp. from bite wounds, routine testing is usually not necessary; multiple organisms are often present in these specimens. Empiric therapy directed toward these organisms is generally effective for *P. multocida* as well (123).

S. muelleri is not yet an established pathogenic bacterium, and susceptibility data are scarce.

S. moniliformis is susceptible to penicillin and tetracyclines, the mainstays of treatment, and to cephalosporins, carbapenems, aztreonam, clindamycin, erythromycin, and tetracycline; it shows intermediate susceptibility to aminoglycosides and fluoroquinolones and is resistant to colistin and co-trimoxazole (45, 103). Patients with *S. moniliformis* endocarditis require dual therapy with high-dose penicillin G in combination with an aminoglycoside; the use of an aminoglycoside appears to enhance activity against the cell wall-deficient L forms of *S. moniliformis* (45, 102, 103).

The susceptibility of *S. indologenes* resembles that of HACEK organisms (35).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Some bacteria of the group are colonizers of the human or animal oral cavity; therefore, the evaluation of their isolation may be difficult. All should be identified to the species level if isolated as pure cultures from normally sterile body sites. Interpretation as infectious agents and results of susceptibility testing should be clearly reported to the physician.

With specimens normally colonized with aerobic and anaerobic bacteria, as well as with specimens from wounds, e.g., bite wounds, the significance of the bacteria discussed in this chapter depends on their predominance and the absence of other potentially pathogenic bacteria. If these conditions are met, identification to the species level is needed for adequate interpretation and reporting as infectious agents and for susceptibility testing. If none of these conditions is present, a repeat culture and close cooperation between the microbiology laboratory and the physician are necessary for interpretation, for identification to the species or genus level, and for susceptibility testing.

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Haemophilus

NATHAN A. LEDEBOER AND GARY V. DOERN

36

TAXONOMY AND DESCRIPTION OF THE GENUS

Haemophilus spp. are members of the family *Pasteurellaceae* (1). While the number of *Haemophilus* species described greatly exceeds the number of human pathogens, eight species affecting humans currently included in this genus are *H. influenzae*, *H. aegyptius*, *H. ducreyi*, *H. pittmaniae*, *H. parainfluenzae*, *H. haemolyticus*, *H. parahaemolyticus*, and *H. paraphrohaemolyticus*. Recently a new taxon, *H. sputorum*, has been proposed by Norskov-Lauritsen et al. (2) based on a cluster analysis of 300 commensal *Haemophilus* isolates. Isolates of the proposed taxon *H. sputorum* did not exhibit β-galactosidase activity and formed a unique cluster closely related to *H. paraphrohaemolyticus*. *H. sputorum* has been isolated from blood, sputum from cystic fibrosis patients, and sockets of extracted teeth (2). *Aggregatibacter aphrophilus*, *Aggregatibacter paraphrophilus*, and *Aggregatibacter segnis* were formerly included in the genus *Haemophilus* but have been reclassified into the genus *Aggregatibacter* (formerly *Actinobacillus*) based on molecular taxonomy (3). Additionally, *A. aphrophilus* and *A. paraphrophilus* have been combined into a single species, *A. aphrophilus* (3). A description of the characteristics and epidemiology of the newly reclassified *A. aphrophilus* species group can be found in chapter 35 in this *Manual.* A comprehensive review of the taxonomy of *Haemophilus* species has been provided by Kilian (4, 5).

Even given the recent reclassification of species, significant genetic diversity still exists in the *Haemophilus* genus. The genomes of these species range in size from 1.8 Mb for *H. influenzae* to 2.8 Mb for *H. ducreyi* (5). DNA-DNA hybridization studies demonstrate significant heterogeneity between species; studies conducted by Burbach et al. as discussed in reference 5 demonstrate binding ratios between *H. influenzae* and other species to range from 10% (*H. paracuniculus*) to 70% (*H. aegyptius*). *H. influenzae* is most closely related to *H. aegyptius*, with 90% homology, but is most distant from *H. ducreyi*, with only 18% homology. Intraspecies heterogeneity is also significant, ranging from 50 to 100% in *H. influenzae* and *H. parainfluenzae* strains (5).

Members of the *Haemophilus* genus are small, nonmotile, non-spore-forming, non-acid-fast, pleomorphic, Gram-negative bacilli with fastidious growth requirements. Cells in this genus are coccobacilli or short rods. The cell wall resembles those of other Gram-negative bacilli but contains fewer fatty acids than occur in other members of the family *Pasteurellaceae* (1, 5, 6); the lipopolysaccharide of *Haemophilus* is structurally different from those of members of the *Enterobacteriaceae* (1, 5, 6). The fatty acid composition of the cell wall includes *n*-tetradecanoate (14:0), 3-hydroxy-tetradecanoate (3-OH-14:0), *n*-hexadecanoate (16:0), and hexadecanoate (16:1) (7). Fimbriae have been observed on the cell walls of certain species of *H. influenzae* and *H. aegyptius* (5). The genome of *Haemophilus* spp. is characterized by a G+C content of 37 to 45% (1, 5, 8).

Haemophilus spp. are facultatively anaerobic, with requirements for X and/or V factors for growth. X factor is protoporphyrin IX, a metabolic intermediate in the hemin biosynthetic pathway (5). V factor is composed of nicotinamide complexed as NAD or NADP. Both factors are present in erythrocytes ("haemophilus" means "blood loving" in Greek). Requirements for these compounds vary based on the species, with *H. influenzae*, *H. aegyptius*, and *H. haemolyticus* requiring both X and V factors for growth, whereas others require only a single factor (Table 1). Optimal growth occurs at 35 to 37°C in the presence of 5 to 7% CO_2. All species are CAMP reaction negative and produce alkaline phosphatase (9).

Organisms within the *Haemophilus* genus typically grow on chocolate agar, producing colonies that are usually smooth, with a flat or convex shape. They are nonpigmented (i.e., buff or light tan) or slightly yellow and are 0.5 to 2.0 mm in diameter. Certain *Haemophilus* spp. produce beta-hemolysis when grown on sheep blood agar plates (Table 1). Growth in broth can vary between homogeneous and granular.

Species of *Haemophilus*, other than *H. ducreyi*, typically ferment a wide range of different biochemical substrates. In particular, fermentation of glucose, sucrose, lactose, mannose, and xylose are useful characteristics in the species identification of organisms in this genus. Production of indole, ornithine decarboxylase, urease, catalase, and β-galactosidase plus the ability to produce beta-hemolysis when grown on blood-containing media are other variable properties of *Haemophilus* spp. that aid in the species identification of organisms in this genus (Table 1).

Strains of *H. influenzae* may produce one of six distinct capsular polysaccharides or may be nonencapsulated. Nonencapsulated *H. influenzae* strains are referred to as nontypeable (NTHi) (10) (Fig. 1). The presence of polysaccharide capsular antigen provides the basis for serotype designations, "a" to "f." Capsular serotyping is based on the polysaccharide composition of the capsular structure. Depending on the

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TABLE 1 Differential characteristics of *Haemophilus* species[a]

Haemophilus species	Requirement for:		Catalase	Fermentation of:					Production of:				Hemolysis
	X factor	V factor		Glucose	Sucrose	Lactose	Xylose	Mannose	Indole	Urease	Ornithine decarboxylase	β-Galactosidase	
H. influenzae	+	+	+	+	–	–	+	–	v[b]	v[b]	v[b]	–	–
H. aegyptius	+	+	+	+[c]	–	–	–	–	–	+	–	–	–
H. haemolyticus	+	+	+	+	–	–	v	–	v	+	–	–	+
H. parainfluenzae	–	+	v	+	+	–	–	+	v[b]	v[b]	v[b]	v	–
H. ducreyi	+	–	–	v	–	–	–	–	–	–	–	–	–[d]
H. parahaemolyticus	–	+	v	+	+	–	–	–	–	+	–	v	+
H. pittmaniae	–	+	w	+	+	–	–	+	uk	uk	uk	+	+
H. paraphrohaemolyticus[e]	–	+	+	+	+	–	–	–	–	+	–	v	+
H. sputorum	–	+	uk	+	uk	–	–	–	+	+	–	+	+[f]

[a] +, positive; –, negative; uk, unknown; v, variable reaction; w, weak reaction.
[b] Indole, urease, and ornithine decarboxylase production are the basis for biotyping schemes with *H. influenzae* and *H. parainfluenzae* as depicted in Table 2.
[c] A delayed positive reaction occurs in >90% of strains.
[d] Delayed development of hemolysis occurs in 11 to 89% of strains.
[e] Elevated concentrations of CO_2 of ≥10% enhance growth.
[f] Beta-hemolysis is produced on horse and sheep blood agar; occasional strains fail to produce beta-hemolysis.

strain type, the capsule is composed of ribose and fructose in the furanose ring and Glc, Gal, GlcNAc, or ManANAc in the pyranose ring. The structures of the capsules belonging to each serotype can be found in reference 5. Indole, ornithine decarboxylase, and urease production are the basis for a biotyping scheme with both *H. influenzae* and *H. parainfluenzae* (11) (Table 2).

EPIDEMIOLOGY AND TRANSMISSION

Haemophilus species can be found as part of the commensal biota of mucous membranes in humans. Colonization of the oral cavity superior to the palatal arches by *H. parainfluenzae* and *H. pittmaniae* is normal (5, 12–15). *H. parahaemolyticus* and *H. haemolyticus* colonization in healthy individuals remains rare, although *H. haemolyticus* has been isolated from subgingival dental plaques (5). Colonization of the cervix with *H. ducreyi* has been documented following sexual intercourse (16, 17).

H. influenzae may also be found as part of the commensal bacterial biota of the mucosal surfaces of the upper respiratory tract (URT) of many healthy individuals (18); however, asymptomatic colonization of the URT with encapsulated strains of *H. influenzae* type b (Hib) is rare. Carriage of Hib was reported in only 2 to 5% of healthy children in the prevaccine era and has further decreased (~0.06%) since the introduction of the pediatric Hib conjugate antigen vaccine, HIB, in the 1980s (19). In contrast, NTHi, together with strains of *H. parainfluenzae*, represents a major portion of the cultured bacterial microbiota of the pharynx and nasopharynx of >90% of healthy individuals (13, 20). Clones of NTHi present in the URT differ when asymptomatic carriers are compared to those with infection (20, 21). In asymptomatically colonized individuals, the clones vary continuously, with a mean duration of carriage of 1 to 2 months (21). However, during infection, a single clonal group predominates. As a result of widespread use of HIB, the distribution of serotypes causing invasive infection has shifted. A recent report by the U.S. Centers for Disease Control and Prevention (CDC) classified 69.5% of invasive isolates as nontypeable, 2.2% as type a, 3.6% as type b, 0.3% as type c, 0.3% as type d, 5.7% as type e, and 18.3% as type f from 1999 to 2008 (22). Despite the reduction in Hib carriage and the efficacy of the vaccine in preventing invasive infections, the prevalence of invasive *H. influenzae* and Hib remains high among Alaskan Natives and Native Americans (22). Recently, an increase in the incidence of invasive *H. influenzae* infections has been observed in patients aged >65 years (22, 23). MacNeil et al. (22) reported that from 1999 to 2008 the incidence of invasive *H. influenzae* was 4.09 per 100,000 population, with 19.5% of invasive infections occurring in nursing home residents (22).

The incubation period for *H. influenzae* is poorly understood. The presence of a concomitant or preceding viral infection can predispose previously healthy carriers to infection. In these instances, the colonizing bacteria invade the inflamed or damaged mucosa and enter the bloodstream. The antiphagocytic nature of the Hib capsule and the absence of the anticapsular antibody lead to increasing bacterial proliferation (18). When the bacterial concentration exceeds a critical level, it can disseminate to various sites, including the meninges, subcutaneous tissue, joints, pleura, pericardia, and lungs. The presence of antibody, complement, and phagocytic cells determines the clearance of the bacteremia and can influence dissemination (18).

Host defenses include activation of the alternative and classical complement pathways and production of antibodies

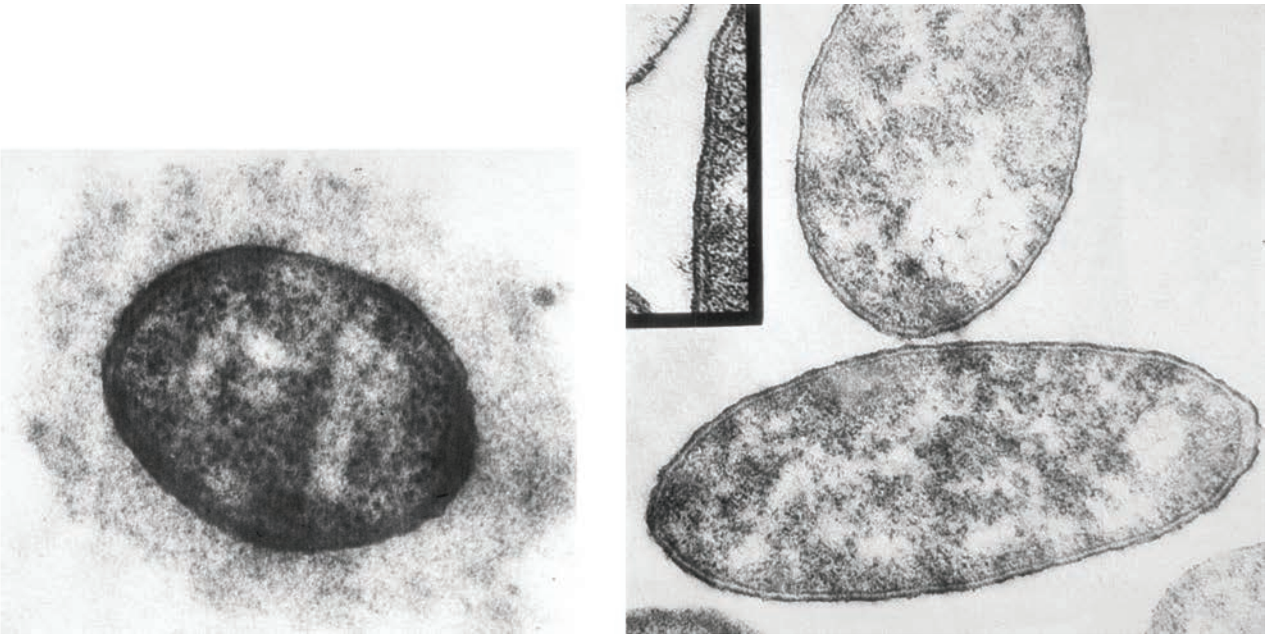

FIGURE 1 Electron micrographs depicting an encapsulated type b strain (left) and a nonencapsulated, nontypeable strain (right) of *Haemophilus influenzae*.
doi:10.1128/9781555817381.ch36.f1

directed against the polyribosylribitol phosphate (PRP) capsule of Hib. Antibody reactive with the Hib capsule plays a primary role in conferring immunity. Newborns have a low risk of infection because of the presence of maternal antibodies acquired through colostrum. Beginning at about 2 months of age, when maternal antibodies to the PRP capsule begin to wane, infants are at high risk for developing invasive *H. influenzae* disease. In the absence of maternal antibodies, and even following natural infection, the anti-PRP immune response of infants is diminished (18). Therefore, infants are at risk for repeat infections because prior episodes of infection do not confer immunity. By the age of 5 years, most children have naturally acquired antibodies.

TABLE 2 Biotypes of *Haemophilus influenzae* and *Haemophilus parainfluenzae*

Species	Biotype	Production of:		
		Indole	Urease	Ornithine decarboxylase
H. influenzae	I	+	+	+
	II	+	+	−
	III	−	+	−
	IV	−	+	+
	V	+	−	+
	VI	−	−	+
	VII	+	−	−
	VIII	−	−	−
H. parainfluenzae	I	−	−	+
	II	−	+	+
	III	−	+	−
	IV	+	+	+
	V	−	−	−
	VI	+	−	+
	VII	+	+	−
	VIII	+	−	−

CLINICAL SIGNIFICANCE

H. influenzae

Invasive infections caused by *H. influenzae*, such as meningitis, epiglottitis, orbital cellulitis, and bacteremia, are usually caused by capsular type b strains and generally fall within biotypes I and II of this species (24). Life-threatening *Haemophilus* infections, however, have fortunately become exceedingly uncommon in developed countries since the development and introduction of the pediatric HIB vaccine (25–27). When infections caused by Hib occur today, it is usually in the setting of an unvaccinated child, although they may also arise in both children and adults as a result of head trauma or cerebrospinal fluid (CSF) leak or following a neurosurgical procedure. Biotype IV strains, at least in the pre-HIB vaccine era, were often found to cause systemic infections in neonates as well as aggressive infections of the genital tract in postpartum women (28, 29).

The vast majority of *H. influenzae* infections today are caused by NTHi (10, 20). This organism is an important cause of acute conjunctivitis, acute otitis media, acute maxillary sinusitis, acute bacterial exacerbation of chronic bronchitis, and pneumonia (18). The organism gains access to the site of infection by direct contiguous spread from its reservoir in the URT. Spread via respiratory secretions, usually on the hands of patients, NTHi can also lead to conjunctival infection. Antecedent viral infections with resultant inflammation of the eustachian tubes and sinus ostea predispose patients to infection of the middle ear cavity and maxillary sinuses, respectively, by compromising egress from and ingress to these closed spaces (30). Establishment of infection in the lungs is facilitated by any condition that diminishes mucociliary clearance of organisms from the respiratory tree (18, 31, 32). Examples include smoking, chronic obstructive pulmonary disease, viral infection, recurrent bacterial infection, and physiological alterations, such as those that occur in individuals with cystic fibrosis

(18, 31, 32). Persons at risk for systemic NTHi infection, particularly the elderly, also include those with functional or anatomic asplenism, sickle cell disease, complement deficiencies, Hodgkin's lymphoma, congenital or acquired hypogammaglobulinemia, and T-cell immunodeficiency states (e.g., HIV infection). Rarely is NTHi documented to be a cause of bacteremia; in a recent study, Laupland et al. (33) found the incidence of NTHi bacteremia to be 304 cases per 33,601,000 person-years. This may be due to the relative avirulence of the organism, the inadequacy of conventional blood culture techniques in propagating this fastidious bacterium, or the lack of contemporary studies investigating the epidemiology of invasive *H. influenzae* infections.

H. ducreyi

Chancroid is a sexually transmitted disease caused by *H. ducreyi* that is usually characterized by the development of a single painful genital ulcer, with associated inguinal lymphadenopathy occurring 2 to 7 days following exposure (34–36). Keratinocytes are likely the first cell type encountered by *H. ducreyi* upon infection of human skin; thus, the interaction between *H. ducreyi* and keratinocytes is likely important in establishing infection (37). Chancroid occurs most often in developing countries, including much of Asia, Africa, and Latin America. Epidemics of disease are associated with low socioeconomic status, poor hygiene, prostitution, and drug abuse. Commercial sex workers are also believed to serve as reservoirs for *H. ducreyi*. After 1987, reported cases of chancroid in the United States declined steadily until 2001. Since then, the number of cases reported has fluctuated from 17 to 55 cases annually. In 2010, only 24 cases were reported to the CDC, with 12 of these cases from Texas (38). Because of difficulties in establishing an etiologic diagnosis of *H. ducreyi* infection and limited resources in many countries of endemicity, the true incidence of chancroid is unknown. The incidence of *H. ducreyi* in the United States and other industrialized countries also remains unknown due to poor diagnostic techniques and underreporting, likely making chancroid a more important cause of genital ulcers than is currently reported.

Other *Haemophilus* spp.

H. parainfluenzae remains the predominant species colonizing the URT, accounting for fully 75% of the *Haemophilus* biota in the oral cavity and in the pharynx. Interestingly, *H. parainfluenzae* does not routinely colonize the nasal cavity. *H. parainfluenzae* is thought to account for at least some cases of acute otitis media, acute sinusitis, and acute bacterial exacerbation of chronic bronchitis, although its role in these diseases is often inconclusive. Infrequently, it has also been identified as a cause of subacute bacterial endocarditis. As is the case with systemic infections due to NTHi, blood cultures can be falsely negative in patients with *H. parainfluenzae* endocarditis due to the fastidious nature of the pathogen and the potential lysis of the organism in the high concentrations of sodium polyanethol sulfonate present in blood culture bottles (39–41).

H. aegyptius, a distinct species of *Haemophilus* that closely resembles biotype III strains of *H. influenzae* and that has been referred to as the Koch-Weeks bacillus, is an important cause of acute purulent conjunctivitis (42). This disease, often called pinkeye, occurs most often in younger children, especially those having extensive contact with other children in closed settings, such as day care centers and grammar school classrooms. It is characterized by the rapid onset of conjunctival inflammation, visual disturbance, ocular pain and pruritus. It often involves both eyes and is highly transmissible.

Brazilian purpuric fever, a condition that occurs most often in South America, is characterized by rapid onset of high fevers, hypotension, diffuse cutaneous hemorrhaging, and abrupt vascular compromise (43). The causative agent is often mistaken to be *H. aegyptius* but is instead an organism that is classified in biogroup III of *H. influenzae* (43, 44). These strains are characterized by the inability to ferment D-xylose, by a particular pattern of their housekeeping genes, by a distinct rRNA restriction pattern, and by resistance to serum bactericidal activity, making them unique among known *H. influenzae* biogroups (5).

H. haemolyticus has recently been reported as a cause of invasive disease in the United States, mischaracterized as *H. influenzae* (45). The report by Anderson et al. (45) retrospectively evaluated 161 NTHi isolates collected from 2009 to 2010 and 213 isolates collected from 1991 to 2000 and identified 7 strains associated with bacteremia, septic arthritis, or peritonitis that were mischaracterized as *H. influenzae*. In each instance, strains were poorly hemolytic or nonhemolytic and characterized by phenotypic microbiological identification techniques as *H. influenzae*. The report emphasized the importance of molecular identification techniques in characterizing NTHi strains.

Other *Haemophilus* species have only rarely been implicated as causes of infection in humans, although lower respiratory tract infection, sinusitis, conjunctivitis, bacteremia, meningitis, wound infections, peritonitis, arthritis, osteomyelitis, and brain abscess have been documented in individual case reports or small case series (7, 46).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

The collection of specimens for the diagnosis of *Haemophilus* infections is predicated on the nature of the infection being evaluated. Details of specimen collection and transport can be found in chapter 18. In patients suspected of having meningitis, blood and CSF cultures should be performed. Middle ear fluid obtained by tympanocentesis is the specimen of choice for patients with otitis media; however, in patients with perforated tympanic membranes and otorrhea, an aseptically collected aspirate of middle ear fluid from the external auditory canal is also satisfactory. In cases of maxillary sinusitis, direct sinus aspirates or middle meatal swab specimens collected under endoscopic guidance should be obtained. Conjunctival swab specimens are required in the evaluation of patients thought to have *Haemophilus* conjunctivitis. In patients suspected of having bronchopulmonary infections due to *Haemophilus* spp., specimens representative of lower respiratory tract secretions should be obtained in such a way as to avoid contamination with oropharyngeal commensal biota. This means that collection of optimal specimens, such as by bronchoalveolar lavage or bronchial washing (less preferable), should be performed to provide optimum specificity when evaluating patients suspected of having *Haemophilus* bronchopulmonary infections. While collection of sputum and tracheal aspirates is less invasive, distinguishing between pathogens and oral biota can be nearly impossible by this means. When bacterial pneumonia is suspected, blood cultures should also be obtained. Importantly, with one exception, nasal, nasopharyngeal, and nasal swab specimens are of no value whatsoever in evaluating patients suspected of having *Haemophilus* infections at any of these respiratory tract sites. The one possible exception is in cystic fibrosis patients experiencing an exacerbation. In this setting, an induced deep-cough specimen collected on a swab inserted into the posterior pharynx may be rewarding (47). In patients suspected of having *Haemophilus* infections in normally sterile sites, such as the pleural space, synovium, pericardium, or peritoneum, fluid aspirated aseptically from the site of involvement represents the specimen of choice. Concomitant blood cultures should also be performed.

Finally, specimens for culture of *H. ducreyi* should be collected from the margins of genital lesions with a saline- or broth-moistened swab. The swab should be immediately transported to the laboratory and plated without delay to avoid loss of organism viability. It is imperative that health care providers inform the laboratory of the clinical suspicion of chancroid so that appropriate media for culture of *H. ducreyi* can be employed. If extended transport is required, swab specimens should be plated directly at the time of collection in the patient care area or the specimen swab should be placed in transport medium containing hemin (48). When refrigerated (4°C), the use of Amies transport medium has been demonstrated to maintain the viability of *H. ducreyi* for up to 3 days. Alternatively, specially formulated thioglycolate-hemin-based media containing albumin and glutamine can also be used to preserve organism viability for transport taking >3 days (35, 49). Studies evaluating the viability of *H. ducreyi* using flocked swabs and liquid Amies transport media are limited, and additional studies are needed. While optimal cultivation of *H. ducreyi* is based on collection of ulcer materials, lymph node aspirates, pus, and aspirates from buboes can also be submitted for culture, albeit with less sensitivity than ulcer material. When cultivating *H. ducreyi* from these specimens, laboratories should consider allowing clinicians to directly plate specimens to maintain optimal recovery. Specimens for *H. ducreyi* nucleic acid amplification techniques should be collected using standard collection techniques for nucleic acid amplification from genital specimens, although large-scale studies evaluating specific transport media have not been conducted.

Long-term storage of *Haemophilus* spp. is usually accomplished by lyophilization or freezing of isolates at −60 to −80°C in tryptic soy broth with >10% glycerol or on porous beads (Pro-Lab Diagnostics, Round Rock, TX).

DIRECT EXAMINATION

Microscopy

On Gram stain, *Haemophilus* spp. appear as small, pleomorphic, Gram-negative coccobacilli with coccoid, coccobacillary, rod-shaped, or filamentous forms (Fig. 2). Because of the pleomorphism of *Haemophilus* spp., careful interpretation of the Gram stain smears must be undertaken to avoid confusion with other Gram-negative bacteria, such as *Neisseria meningitidis*. Underdecolorization of Gram stains may erroneously suggest the presence of *Streptococcus pneumoniae*, *Listeria monocytogenes*, or *Streptococcus agalactiae*.

Gram stain smears of CSF should be prepared and examined and the results reported within 1 h of receipt of the specimen in the laboratory directly to the health care provider who requested the test. With nonturbid specimens, following centrifugation in a cytocentrifuge at 10,000 × *g* for 10 min, a concentrated smear is prepared. With visibly turbid specimens, a direct smear should be prepared in addition to the cytospin smear. A Gram stain is performed immediately and examined for the presence of polymorphonuclear leukocytes and bacteria morphologically compatible with *Haemophilus* spp. In rare cases, the CSF Gram stain may reveal many polymorphonuclear leukocytes but no bacteria. When this occurs, prepare another cytospin smear and stain with acridine orange (BD, Sparks, MD; or Remel, Lenexa, KS).

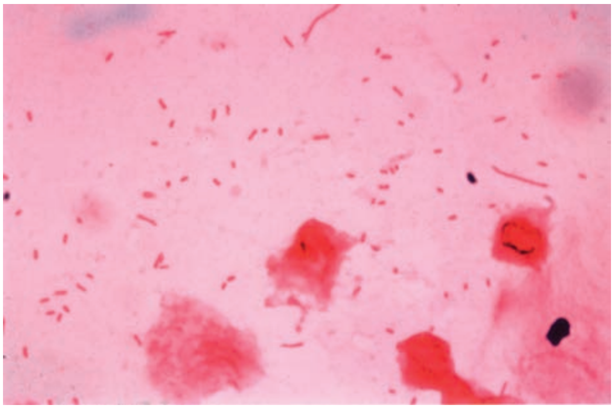

FIGURE 2 Gram stain of *Haemophilus influenzae* present in CSF. doi:10.1128/9781555817381.ch36.f2

The same approach as that applied to Gram staining CSF should be applied to pleural, peritoneal, synovial, and pericardial fluid specimens. Middle ear fluid specimens and sinus aspirate Gram stains should be prepared directly from the specimen without cytocentrifugation.

Gram stains of lower respiratory tract secretions may be prepared directly from the specimen (e.g., expectorated sputa, endotracheal suction specimens, transbronchial biopsy specimens, bronchial brush biopsy specimens, and thoracotomy specimens) or following cytocentrifugation (e.g., bronchial washes and bronchoalveolar lavage fluid).

Strands of small, Gram-negative bacilli arranged in a railroad track-like manner on a direct Gram stain are highly suggestive of *H. ducreyi*. However, Gram staining of genital specimens for *H. ducreyi* is controversial because most genital ulcers contain a mixed bacterial biota, making Gram stain interpretation difficult. Furthermore, the positive yield of a Gram stain for *H. ducreyi* is low in comparison to that of culture or detection with nucleic acid amplification tests.

Antigen Detection

Commercial immunochemical techniques are available for the detection of *S. pneumoniae*, *Streptococcus dysgalactiae*, *H. influenzae*, and *N. meningitidis* directly from CSF and other body fluids. While these techniques provide a rapid identification of the pathogen, they lack sensitivity and specificity compared with Gram staining (50). Thus, the use of *H. influenzae* antigen detection is of limited clinical value and is generally discouraged. However, in certain clinical contexts, such as in resource-constrained regions when the prevalence of disease is high and routine culture is unreliable, antigen-based detection methods may prove useful.

Molecular Techniques

Nucleic acid amplification assays, most notably assays predicated on PCR, have been developed to detect *H. influenzae* directly in various clinical specimens, including CSF, plasma, serum, and whole blood (27, 51). These techniques can be multiplexed to detect other common bacterial causes of specific infectious disease entities, such as meningitis.

While publications cite variable detection sensitivities of these techniques, specificity is generally excellent (52–54).

Studies have demonstrated that the diagnostic accuracy of chancroid based on clinical presentation and patient history ranges from 33 to 80% (55) and that culture is ~75% sensitive (56, 57), making it an ideal target for molecular techniques. Molecular strategies have been developed to directly detect *H. ducreyi* from clinical specimens. Primers for these assays have been designed to amplify sequences from either the *H. ducreyi* 16S rRNA gene, the *rrs* (16S)-*rrl* (23S) ribosomal intergenic spacer region, an anonymous fragment of cloned *H. ducreyi* DNA, or the *groEL* gene, which encodes the *H. ducreyi* heat shock protein (56). One strategy includes a chloroform extraction followed by a one-tube nested PCR directed to the 16S rRNA gene, with longer outer primers for annealing at a higher temperature and shorter inner primers labeled with biotin and digoxigenin for binding with streptavidin and colorimetric detection (58). Another strategy is to target the *hhdA* gene; a protocol utilizing this target has been described by Chen and Ballard (59). The sensitivity of PCR directly from clinical specimens varies among assays between 83 and 95% compared to culture or clinical diagnosis (56, 58). The adaptations of molecular methods offer superior sensitivity for the diagnosis of chancroid; they are clearly advantageous in areas where the organism is endemic, particularly where testing by culture is difficult or impossible.

In addition to direct detection of *H. ducreyi* from clinical specimens, studies have also applied PCR to direct detection of *H. influenzae* and *H. parainfluenzae* from clinical specimens. In one study by Kuhn et al. (60), the authors evaluated three commercial PCR assays for detection of infectious agents associated with infective endocarditis. This study demonstrated superior performance of PCR compared with culture using both whole blood and valve tissue; the sensitivity of PCR reached 85%, compared with 45% for culture from both whole blood and heart valve tissue. However, while this study resulted in high sensitivity for PCR, the specificity of the PCR was low (40%) due to culture-negative specimens, likely as a result of antimicrobial treatment of patients prior to collection of diagnostic specimens (60). In a second study on meningitis, Wu et al. (61) demonstrated that the sensitivity of real-time PCR and Gram stain was less affected by the presence of antibiotics than culture and Gram stain for detection of *H. influenzae*, *N. meningitidis*, and *S. pneumoniae*. The study further demonstrated that the sensitivity of PCR and Gram stain exceeded that of culture (95.7, 98.25, and 81.3%, respectively) and resulted in identification of an additional 33 (of 451 total specimens) cases of meningitis that were culture negative (61). Other studies have evaluated molecular methods to detect *Haemophilus* spp. from respiratory specimens (62–64), demonstrating sensitivity of up to 95%, but did not correlate detection of *H. influenzae* with clinical disease.

The use of molecular methods for the identification of other *Haemophilus* spp. directly from clinical samples has proven difficult. The lack of both sensitivity and specificity has been problematic. In clinical specimens, small numbers of organisms may be present, leading to limitations in detection sensitivity. That is especially the case in patients with *Haemophilus* bacteremia (65). To achieve adequate sensitivity, large volumes of blood or CSF must be processed, creating laborious nucleic acid extraction and concentration processes with little clinical relevance. Another complicating factor is related to the influence of antimicrobial therapy. While culture frequently becomes negative following administration of the first appropriate dose of antimicrobials, patients can retain bacterial DNA in their blood or CSF for at least 2 weeks following clearance of the organism, leading to false-positive reactions, often making the interpretation of results challenging.

In certain other specimens, particularly specimens from the respiratory tract, commensal strains of *Haemophilus* spp. are often present, rendering positive results inconclusive. Further, the presence of other commensals or use of antimicrobials prior to screening may result in false-positive reactions, thus contributing to a lack of assay specificity. For these reasons, the use of molecular detection techniques is not currently advocated for the detection of *Haemophilus* spp. directly in clinical specimens until clinically significant thresholds for molecular quantification of organisms from respiratory specimens are achieved.

ISOLATION PROCEDURES

Media

Optimum recovery of *Haemophilus* spp. in culture requires the use of enriched media that support the growth of these fastidious bacteria. Media must contain at least 10 μg/ml of free X and V factors. High concentrations of both the X and the V factor are found in whole blood, most of it sequestered within erythrocytes. X factor is protoporphyrin IX and can be derived from whole blood or can be added to bacteriological media using crystalline hemin; X factor is readily available in standard blood agar. V factor is composed of nicotinamide complexed as NAD or NADP and is also readily available in blood. However, nicotinamide is not readily bioavailable because of its intracellular location and the presence of NAD-glycohydrolase enzymes in blood. For the growth of *Haemophilus* spp. on solid media, either crystalline hemin and NAD must be added to a final concentration of 10 μg/ml, or the blood used in the medium must be heated such that the red cells lyse and release free X factor and V factor into the medium. The latter can be accomplished by adding blood to the basal medium as it cools to 80°C after being autoclaved. This is referred to as "chocolatizing" blood. For optimal growth of *Haemophilus* spp., a concentration of 5% chocolatized sheep blood should be employed (1).

The optimum growth of *Haemophilus* spp., especially of more-fastidious species, such as *H. ducreyi* and *H. aegyptius*, requires, in addition to the X and V factors, supplementation of media with various other growth factors. Two commercially available supplements that supply these growth factor requirements are IsoVitaleX (BD) and Vitox (Remel). These growth factors contain glucose, cystine, glutamine, adenine, thiamine, vitamin B_{12}, guanine, iron, and aminobenzoic acid and provide adequate supplementation for the growth of *H. ducreyi* and *H. aegyptius*.

Enriched chocolate agar containing 5% lysed sheep red blood cells and supplemented with 1% IsoVitaleX or 1% Vitox represents one general-purpose medium that is commonly used in clinical laboratories to effectively propagate *Haemophilus* spp. (Fig. 3). Another medium that reliably supports the growth of *Haemophilus* spp. is Levinthal medium (66). Because of its transparency, Levinthal medium offers the added benefit of permitting the detection of colony iridescence, a property that is frequently associated with encapsulation (Fig. 4) (67, 68). One investigation found that a medium consisting of GC (BD or Remel) agar base, 5% heated sheep red blood cells, and 1% yeast autolysate provided the best growth of all *Haemophilus* spp. other than *H. ducreyi* (69).

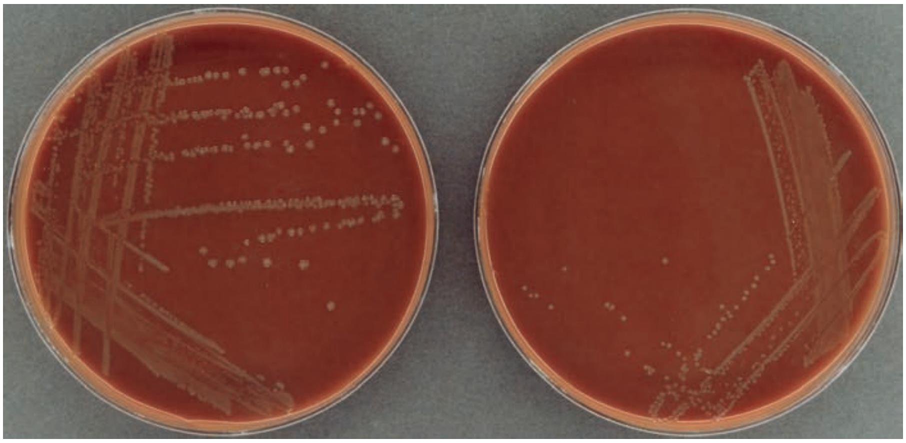

FIGURE 3 Colony morphologies of type b encapsulated (left) and nonencapsulated (right) strains of *Haemophilus influenzae* when propagated on enriched chocolate agar. doi:10.1128/9781555817381.ch36.f3

A significant challenge in the recovery of *Haemophilus* spp. from respiratory tract specimens is bacterial overgrowth due to the presence of other less fastidious commensal bacteria. Supplementation of media with some combination of bacitracin, vancomycin, and/or clindamycin serves to inhibit overgrowth with commensals, thus permitting the recovery of *Haemophilus* spp. (70). Use of such selective media is particularly relevant to the recovery of *Haemophilus* spp. from respiratory tract specimens from patients with cystic fibrosis, acute exacerbation of chronic bronchitis, conjunctivitis, and epiglottitis (Fig. 5) (71). Several versions of selective *Haemophilus* media are available commercially, including *Haemophilus* isolation agar (Remel) and *Haemophilus* isolation agar with bacitracin (BD). These media contain beef heart infusion agar with casein peptone to supply nutritional requirements, combined with horse blood to supply the X and V factors and to distinguish hemolytic species of *Haemophilus* from those that are not. Bacitracin is added to inhibit normal biotas, including *Neisseria* spp.

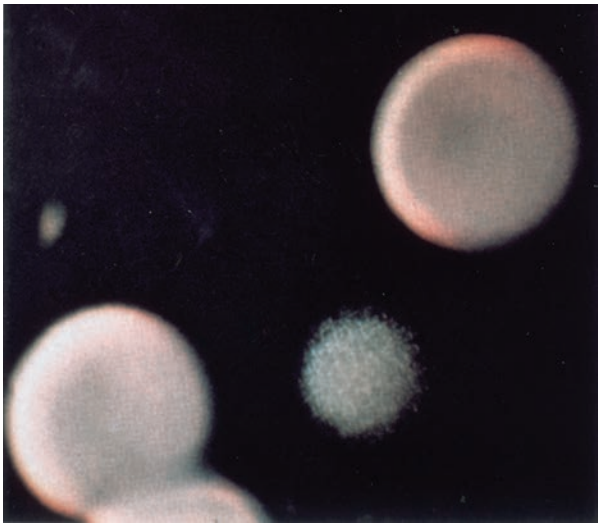

FIGURE 4 Colony morphologies of type b encapsulated (left and right) and nonencapsulated (center) strains of *Haemophilus influenzae* when propagated on Levinthal agar. Note the conspicuous iridescence apparent with the encapsulated strain. doi:10.1128/9781555817381.ch36.f4

The use of selective media is also helpful in recovering *H. ducreyi* from genital tract specimens (49). Selective media may include any of the following: GC agar base with 1% IsoVitaleX, 5% fetal bovine serum, 1% hemoglobin, and 3 µg vancomycin; GC agar base with 5% Fildes enrichment, 5% horse blood, and 3 µg vancomycin; 5% fresh rabbit blood agar with 3 µg vancomycin; or Mueller-Hinton agar with 5% chocolatized horse blood, 1% IsoVitaleX, and 3 µg vancomycin (49). Preferably, two different selective media are employed to ensure optimal sensitivity (49).

Growth of *Haemophilus* spp. may also be achieved on 5% sheep blood agar by use of the microsatellite phenomenon, although it is not recommended for routine clinical testing. With the microsatellite test, a single streak line of hemolysin-producing *Staphylococcus* spp. is placed on an agar surface previously inoculated with a specimen suspected of containing *Haemophilus* spp. The hemolysin produced by the *Staphylococcus* species lyses the erythrocytes immediately adjacent to the streak line in the medium, releasing sufficient concentrations of X factor (hemin) and V factor (NAD) into the medium to supply the growth factor requirements of *Haemophilus* spp. *Staphylococcus* also secretes NAD into the medium in proximity to the streak line. Colonies of *Haemophilus* thus appear in a narrow zone adjacent to the staphylococcal streak. This is referred to as "satelliting" growth (Fig. 6). Organisms other than staphylococci can also produce the satellite phenomenon with *Haemophilus*, e.g., enterococci and yeast.

Although *Haemophilus* spp. are not a common cause of bacteremia, special techniques are not necessary for their recovery from blood specimens with modern, continuously monitoring blood culture systems (7, 72). The broth medium used in such systems supports the growth of *Haemophilus* spp. because the blood specimen itself supplies adequate concentrations of both the X and V factors when the erythrocytes present in the specimen lyse as they come into contact with the blood culture broth. However, the common practice of using such systems for the culture of normally sterile body fluids, e.g., synovial, peritoneal, pericardial, and pleural fluid, may be problematic insofar as these specimens may not contain sufficient amounts of blood to supply the necessary levels of the X and V factors to support the growth of *Haemophilus* spp. In situations where *Haemophilus* spp. is strongly suspected in such specimens, blood culture bottles

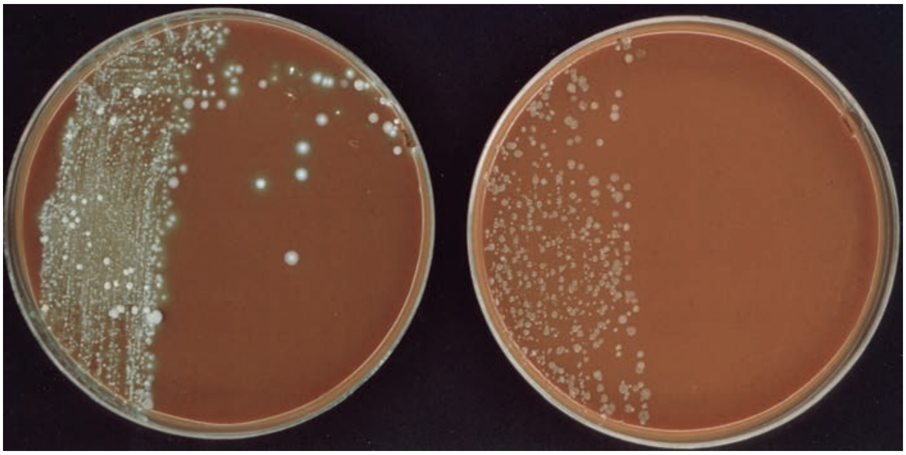

FIGURE 5 Colony growth from an expectorated-sputum specimen containing *Haemophilus influenzae* from a patient with cystic fibrosis propagated on enriched chocolate agar (left) and enriched chocolate agar containing bacitracin, clindamycin, and vancomycin (right).
doi:10.1128/9781555817381.ch36.f5

should be supplemented with at least 10 μg of both sterile hemin and NAD/ml prior to inoculation with clinical specimens. The use of commercial supplements such as Fildes enrichment (BD, Franklin Lakes, NJ) can also be used to aid in the propagation of *Haemophilus* spp.

Following inoculation, solid media should be incubated at 35 to 37°C in a moist atmosphere and in the presence of 5 to 7% CO_2. Under these conditions, most *Haemophilus* spp. grow within 24 to 48 h. When specimens for *H. ducreyi* and *H. aegyptius* are cultured, incubation may be necessary for up to 5 days to allow sufficient time for the growth of these fastidious organisms. Further, when technologists attempt to propagate *H. ducreyi*, plates should be incubated at slightly lower temperatures, i.e., 30 to 33°C in 5% CO_2 in a high-moisture environment. Use of lower incubation temperatures will improve the recovery of *H. ducreyi* in comparison to incubation temperatures of 35 to 37°C.

Colony Appearance

Colonies of *Haemophilus* spp. on suitable solid media, in general, are nonpigmented or slightly yellow and flat to convex, and have a diameter of 0.5 to 2 mm after 48 h of incubation. Certain species of *Haemophilus* produce beta-hemolysis (Table 1).

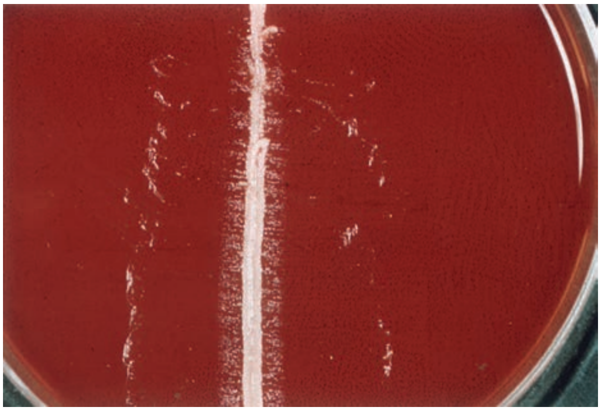

FIGURE 6 Satellite phenomenon observed when *Haemophilus influenzae* is propagated next to a streak of *Staphylococcus aureus* on a 5% sheep blood agar plate.
doi:10.1128/9781555817381.ch36.f6

Colonies of *H. influenzae* on chocolate agar are smooth, low, convex, grayish, and translucent. Encapsulated strains often have a mucoid appearance, while nonencapsulated strains produce smaller, buff colonies (Fig. 3). Most strains of *H. influenzae* produce indole, emitting a strong amine-like odor. Non-indole-producing strains emit a “mousy” odor. Colonies are 1 to 2 mm in diameter and often grow within 24 h. Colonies grown on clear media, such as Levinthal agar, demonstrate iridescence under obliquely transmitted light (67, 68). Iridescence is most conspicuous with young colonies and disappears with age. Iridescent colors may include yellow, red, green, or blue. Iridescence is more apparent with capsular type b strains; nonencapsulated strains typically demonstrate a blue-green color (Fig. 4).

Colonies of *H. aegyptius* reach a colony size of only ca. 0.5 mm after 48 h of growth. Colonies are low, convex, and translucent, with a smooth, entire surface. On semisolid agar media (which contains ~0.4% agar, compared with 1.5% for solid media), “comet-like” colonies are produced (5, 42).

Colonies of *H. parainfluenzae* are typically off-white to yellow and, like *H. influenzae*, 1 to 2 mm in diameter after 24 h of growth. The colony appearance is extremely varied, i.e., flat and smooth, granular with serrated edges, or heaped up and wrinkled. Colonies exhibiting the last morphology may be slid intact across the surface of the agar. The colony morphology of *H. parainfluenzae* may change as the colonies age.

Colonies of *H. haemolyticus* are translucent, smooth, and convex and do not form satellites around *Staphylococcus*. Colonies usually achieve a diameter of 0.5 to 1.5 mm after 24 h, with a clear zone of beta-hemolysis surrounding each colony when the organism is grown on blood agar; *H. haemolyticus* can lose its ability to cause hemolysis following serial subculture on bacteriological media. The growth properties and colony morphology of *H. parahaemolyticus* and *H. paraphrohaemolyticus* are similar to those of *H. haemolyticus*.

H. ducreyi grows poorly, regardless of the medium used, and frequently 3 to 5 days will pass before growth appears. Colonies growing on chocolate agar are small, flat, gray, and smooth. Larger colonies may be interspersed among small colonies but have the same morphology. Growth on blood agar is poor, with a slight beta-hemolysis surrounding the colonies. As with *H. parainfluenzae*, older colonies of *H. ducreyi* are cohesive and can be slid across the agar.

IDENTIFICATION

The identification and differentiation of *Haemophilus* spp. are achieved through determination of X and V factor requirements for growth; performance of the porphyrin test; assessment of hemolysis; determination of carbohydrate fermentation patterns; and production of indole, ornithine decarboxylase, urease, catalase, and β-galactosidase (Table 1). The pattern of X and V growth factor requirements and the porphyrin test provide sufficient information for the presumptive species identification of selected *Haemophilus* spp. Definitive species identification, however, requires assessment of the other phenotypic characteristics listed above (Table 1). Alternatively, sequencing of the 16s rRNA gene or matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) also provide definitive identification of these organisms.

X and V Factor Growth Requirements

X and V factor requirements for the growth of *Haemophilus* spp. may be determined by swab inoculation of a suspension of test organism equivalent in turbidity to a 0.5 McFarland standard across the entire surface of a 100-mm petri dish containing tryptic soy agar. Filter paper disks or strips impregnated with X factor, V factor, and the X and V factors (Remel or BD) are then placed on the agar surface, and the plate is incubated for 20 to 24 h at 35°C in an atmosphere of 5 to 7% CO_2. The pattern of satellite growth around individual disks or strips, in the absence of growth elsewhere on the plate, is used to define the growth factor requirements of the test strain (Fig. 7). Tryptic soy agar is the preferred medium for use when the X and V growth factor requirements for *Haemophilus* spp. are determined, as other media may yield erroneous results (73, 74). Alternatively, triplates (Haemophilus ID II; Remel) and quadplates (Haemophilus ID Quad; Remel) can be used to assess X and V growth factor requirements. When performing X and V factor studies, care should be taken to avoid carrying X factor along with the inoculum. This can result in erroneous identification of *H. influenzae* as *H. parainfluenzae*.

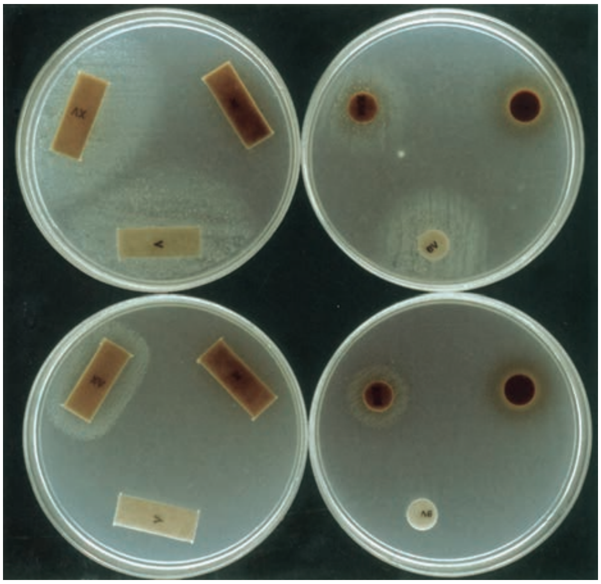

FIGURE 7 Use of X and V factor disks and strips in determining the growth factor requirements of *Haemophilus influenzae* (left disks) and *Haemophilus parainfluenzae* (right disks). doi:10.1128/9781555817381.ch36.f7

All of the X factor-requiring species of *Haemophilus*, most notably *H. influenzae*, lack the enzymes necessary to convert δ-aminolevulinic acid (ALA) into protoporphyrin, a metabolic intermediate in the biosynthesis of X factor (75). Thus, they require that X factor be supplied exogenously in order to support growth. By taking advantage of this observation, a rapid test, known as the porphyrin or ALA test, can be performed to quickly determine if a test organism requires X factor for growth (76). When positive, the porphyrin test indicates that the test organism is X factor independent; when negative, the porphyrin test indicates that the organism requires X factor. Since the vast majority of X factor-requiring *Haemophilus* spp. recovered in the clinical laboratory are *H. influenzae*, when the porphyrin test is performed with a clinical isolate and found to be negative, it can be inferred with a high likelihood that the organism is *H. influenzae*.

The porphyrin test is performed using commercially available ALA disks (Remel) or through preparation of liquid porphyrin medium (76). To prepare liquid porphyrin medium, 2 mM ALA and 0.8 mM $MgSO_4$ in 0.1 M phosphate buffer (pH 6.9) are aliquoted in glass tubes with 0.5 ml of porphyrin medium in each tube. Tubes can be stored at 4°C for several months or for years at −20°C. Tubes are inoculated with a loopful of freshly grown bacteria and incubated for 4 h at 35°C in ambient air (1). Following incubation, the tubes are examined for brick-red fluorescence with a device, such as a Wood's lamp, that emits a 360-nm-long-wave UV light (Fig. 8). Tubes with questionable results may be reincubated for up to 24 h. Alternatively, Kovács reagent (0.5 ml) can be added to the liquid porphyrin medium, and the tube can be shaken and observed for a red color in the lower water phase (1). A negative-control tube lacking ALA should also be inoculated when the porphyrin test is performed to rule out false-positive reactions due to the presence of indole. The porphyrin test has been shown in several studies to outperform

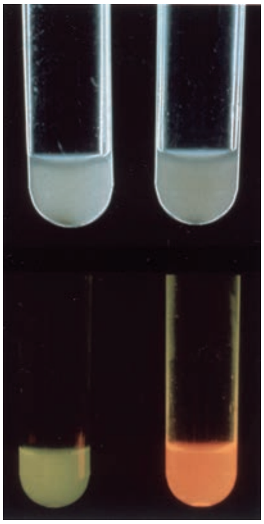

FIGURE 8 Positive (top) and negative (bottom) porphyrin tests. doi:10.1128/9781555817381.ch36.f8

growth factor-based methods for differentiation of *H. influenzae* from non-*H. influenzae* species (77).

Conventional Biochemical Tests

The list of biochemicals necessary for the differentiation of *Haemophilus* species is provided in Table 1. Carbohydrate fermentation is determined in phenol red broth containing 1% carbohydrate supplemented with 10-µg/ml NAD and hemin. Following heavy inoculation, fermentation tubes are incubated at 35°C without CO_2 for up to 1 week and examined periodically for a red-to-yellow color, indicating a positive reaction. Usually positive reactions become apparent within 24 h; however, *H. aegyptius* and *A. segnis* have been noted to demonstrate weak reactions following a 24-h incubation (1).

Indole production can reliably be determined with most strains of *H. influenzae* and *H. parainfluenzae* using a spot indole test (Remel or BD). However, reliable assessment of indole production by other *Haemophilus* spp. requires use of a solution of 0.1% L-tryptophan in 0.067 M phosphate buffer at pH 6.8. Following inoculation, the suspension is incubated for 4 h at 35°C in ambient air, 0.5 ml of Kovács reagent is added to the tube, and the tube is shaken and examined for the appearance of red color in the upper portion of the tube as an indication of a positive reaction (1, 5) (Fig. 9).

Production of ornithine decarboxylase is determined using ornithine decarboxylase medium (see chapter 19) or Moeller medium. A tube containing either medium is heavily inoculated, incubated for 4 to 24 h at 35°C in ambient air, and examined for a purple coloration as an indication that the organism produces ornithine decarboxylase (1, 5) (Fig. 9). Species of *Haemophilus* positive for ornithine decarboxylase include several biotypes of *H. influenzae* and *H. parainfluenzae*.

As was the case with indole production, in most instances, a spot urease test (Remel or BD) can be used to reliably detect urease production by most strains of *H. influenzae* and *H. parainfluenzae*. Determination of urease production with other species requires the use of urease medium containing 0.1 g KH_2PO_4, 0.1 g K_2HPO_4, 0.5 g NaCl, and 0.5 ml phenol red (1:500) dissolved in 100 ml of distilled water. The pH is adjusted to 7.0 with NaOH, and 10.4 ml of a 20% aqueous solution of urea is added. After inoculation, the tube is incubated for 4 h at 35°C in ambient air and examined for a pink-to-red coloration, indicating a positive reaction (1, 5) (Fig. 9).

Commercial Biochemical Identification Systems

Several commercial identification systems have been developed to identify *Haemophilus* spp. These systems employ a battery of conventional biochemical tests, frequently in a miniaturized form, with results available in shorter time periods than with conventional biochemical tests. The performance characteristics and identification accuracy of these commercial systems are extremely variable (69, 73, 77, 78). The RapID NH system (Remel) contains 11 biochemical reactions in a microwell tray. The reactions used for identification of *Haemophilus* include the production of urease, indole, ornithine decarboxylase, proline, and γ-glutamyl aminopeptidase; resazurin reduction; glucose and sucrose utilization; nitrate reduction; and phosphate hydrolysis. The kit uses phosphate hydrolysis and nitrate reduction reactions to identify an isolate as belonging to the genus *Haemophilus* and the remaining reactions to identify the isolate to the species level and to determine the biotype of *H. influenzae*. Although various results have been reported with the RapID NH system, when used properly, >95% of clinical isolates of *H. influenzae* should be correctly identified (73).

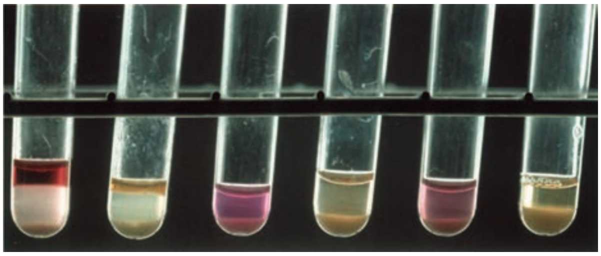

FIGURE 9 Conventional biochemicals depicting positive and negative reactions for indole, urease, and ornithine decarboxylase production (left to right) by *Haemophilus* spp. doi:10.1128/9781555817381.ch36.f9

The BBL Crystal *Neisseria/Haemophilus* ID system (BD) and API NH kit (bioMérieux, Marcy l'Etoile, France) provide miniaturized biochemical identification schemes in a microwell tray, with results available in ≤5 h. The Crystal system employs 29 different growth substrates and is predicated on measuring the substrate conversion chromogenically and fluorogenically after 5 h of incubation. The API NH kit consists of 12 dehydrated substrates and a well to detect penicillinase, and it permits the identification of *Haemophilus* spp., *Neisseria* spp., and *Moraxella* spp. The test is performed by inoculating each well with an organism suspension equivalent to a 4 McFarland turbidity standard prepared from 24-h colony growth and incubating the plate for 2 to 2.25 h at 35°C. In addition to testing for penicillinase production, it tests for the following biochemicals: glucose, fructose, maltose, saccharose, ornithine decarboxylase, urease, lipase, alkaline phosphatase, β-galactosidase, proline arylamidase, γ-glutamyl aminotransferase, and indole (79, 80). Independent studies evaluating the performances of the Crystal *Neisseria/Haemophilus* and API NH kits compared to accepted gold standards have not been published.

One instrument-based identification system has been developed for the species identification of *Haemophilus* spp., the *Neisseria-Haemophilus* identification cards for use with the VITEK 2 instrument (bioMérieux). This system is based on colorimetric detection of preformed enzyme complexes using chromogenic substrates. The database supporting these cards encompasses 27 taxa, including *Neisseria*, *Haemophilus*, *Actinobacillus*, *Campylobacter*, *Capnocytophaga*, *Cardiobacterium*, *Eikenella*, *Gardnerella*, *Kingella*, *Moraxella*, *Oligella*, and *Suttonella* species. Studies with the VITEK 2 system using both collections of well-characterized stock strains and clinical isolates have demonstrated identification accuracies of 90 to 95%, with results varying by species (78, 81). In one recent study, >95% of isolates of *H. influenzae*, *A. segnis*, *H. parahaemolyticus*, *H. parainfluenzae*, and *A. actinomycetemcomitans* were correctly identified, while none of the test strains of *H. haemolyticus* were correctly identified (81).

Mass Spectrometry

Studies evaluating MALDI-TOF MS largely demonstrate excellent performance for HACEK group organisms (i.e., *Haemophilus* spp., *Aggregatibacter* spp., *Cardiobacterium* spp., *Eikenella corrodens*, and *Kingella* spp.), including *Haemophilus* species (82, 83). Couturier et al. (82) demonstrated that >90 and >98% of *H. parainfluenzae* and *H. influenzae* strains, respectively, could be identified to the species level using MALDI-TOF. Similarly, van Veen et al. (83) demonstrated >98% identification of HACEK organisms to the species level. Branda et al. (84) recently evaluated the VITEK MS IVD (bioMérieux) and found 96 and 92% correct identification to the species level for *H. influenzae* and *H. parainfluenzae*, respectively.

Comparisons of the Bruker MALDI Biotyper (Bruker Daltonics, Billerica, MA) to the VITEK MS are limited; however, Frickmann et al. (85) compared the VITEK MS RUO (bioMérieux) with both the Biotyper and fluorescence *in situ* hybridization for identification of *H. influenzae*, *H. parainfluenzae*, and *H. parahaemolyticus*. With formic acid extraction, the Biotyper identified 100% of *H. influenzae* (50/50), 88% of *H. parainfluenzae* (22/25), and 100% of *H. parahaemolyticus* (2/2) strains to the species level. Results were not significantly different for the Biotyper without prior formic acid extraction, although identification of *H. parainfluenzae* dropped to 72% (18/25) and the Biotyper missed an additional *H. parahaemolyticus* identification. The VITEK MS RUO identified 82% of *H. influenzae* (41/50), 68% of *H. parainfluenzae* (17/25), and 50% of *H. parahaemolyticus* (1/2) strains. All identifications on the VITEK MS were performed without prior formic acid extraction (85). Neither system identified any of the seven *H. haemolyticus* strains analyzed (85), likely due to poor representation of the organism in the databases. Martiny et al. (86) compared the performance of the VITEK MS IVD to the Biotyper IVD and found 100% identification ($n = 30$) of *H. influenzae* by both systems. Evaluation of species other than *H. influenzae* in this study was limited by the lack of diversity of species included in the study (86).

Performance of MALDI-TOF on *Haemophilus* strains other than those discussed above and performance of MALDI-TOF for typing *H. influenzae* have not been evaluated, and further study is warranted.

Molecular Identification

Several molecular methods, including 16S rRNA gene sequencing, next-generation sequencing, PCR, microarrays, and fluorescence *in situ* hybridization, have been described in the literature as being effective tools for the species identification of *Haemophilus* spp. when performed on organisms recovered in culture (51, 87–89).

Molecular targets for the detection and identification of *Haemophilus* spp. are numerous. Previous studies have described the detection of *H. influenzae* using the *cap* locus (which includes the capsule *bexA*) (53, 63, 90, 91), the 16S rRNA gene (51, 63, 92, 93), the insertion-like sequence (IS*1016*) (91), the fumarate reductase iron-sulfur gene B (*frdB*) (94), the manganese-dependent superoxide dismutase (*sodA*) (95), and the outer membrane protein P6 gene (*ompP6*) (63, 92, 96). Many of these targets are then combined with PCR (real time or traditional), microarrays, or sequencing to identify the organism. Widely utilized for sequencing, 16S rRNA frequently resolves the identities of strains to the species level; however, identification of *H. influenzae*, *H. aegyptius*, and *H. influenzae* biogroup aegyptius can be problematic due to the high degree of homology in their sequences. In these instances, sequencing of other targets, such as *ropD*, should be considered, or the use of combined sequencing and biochemical studies can be used.

Application of highly multiplex disease-state panels such as the FilmArray (BioFire Diagnostics, Salt Lake City, UT) or Verigene (Nanosphere, Inc., Northbrook, IL) to positive blood cultures has demonstrated reduction in length of stay and enhanced antimicrobial stewardship (97). Application of these technologies to identification of *Haemophilus* spp. from positive blood cultures may offer a significant reduction in turnaround time; however, peer-reviewed studies evaluating these technologies in *Haemophilus* have not been published.

Next-generation sequencing has been applied to *Haemophilus* spp. in two recent studies without the need for prior culture. In a study by Salipante et al. (98), the authors conducted deep sequencing on 66 sputum specimens from cystic fibrosis patients and identified *H. influenzae* in 5 specimens. Of the 5 specimens that yielded *H. influenzae*, 4 could be detected only via sequencing and 1 could be detected only in culture (98). The discrepancy between the results of culture and sequencing illustrates the challenge of correlating cultures with sequencing. While the presence of an organism on culture definitively confirms the presence of a viable organism, the presence of DNA detected in a sequencing reaction may still represent an infectious process but can also represent a remnant of a previous infection. Attempts to resolve this dilemma are important since, at a cost of less than $75 in reagents per sample, sequencing has quickly become affordable for the routine clinical microbiology laboratory (98).

Problems in Identification

A significant challenge in the species identification of *H. influenzae*, *H. aegyptius*, and *H. influenzae* biogroup aegyptius is a lack of biochemical diversity and sequence divergence (1, 5, 7). Biochemical profiling and standard 16S rRNA gene sequencing fail to adequately distinguish these organisms (1, 5, 7), necessitating the use of alternate sequencing targets, such as those mentioned in the previous section, or a combination of sequencing and biochemical testing. This is problematic, since *H. aegyptius* lacks the potential to cause Brazilian purpuric fever, while strains of *H. influenzae* biogroup aegyptius cause the disease. Xylose fermentation by most *H. influenzae* isolates combined with the lower growth rate of *H. influenzae* biotype aegyptius and the ability of *H. aegyptius* to agglutinate human erythrocytes may be of some value in distinguishing these organisms (1, 7, 99).

TYPING SYSTEMS

Capsular Serotyping and Biotyping

The capsular antigen of *H. influenzae* is a principal virulence determinant of this organism. Six different capsular antigens have been recognized, each of which is characterized by a distinct carbohydrate chemical composition and given a letter designation from "a" to "f." Prior to the introduction of the pediatric HIB vaccine, capsular type b strains were recovered from human clinical material most often. However, today, at least among populations in which there is widespread use of the HIB vaccine, non-b encapsulated strains occur with nearly equal frequency. For this reason, it may be instructive to know the capsular serotypes of *H. influenzae* strains recovered from clinical specimens, especially those representative of invasive disease. This may be accomplished using both phenotypic and genotypic methods.

Capsular serotyping of *H. influenzae* is best accomplished by the use of a slide agglutination assay that employs polyclonal antisera specifically reactive with each of the six capsular antigens (5, 100, 101). It is advisable to perform serotyping as soon as possible after isolation of *H. influenzae*, as the amount of capsular antigen produced may diminish over time, especially with repeated subculture. A thick, homogenous suspension of test organism is prepared in saline, 1 to 2 drops are placed on a glass slide, and then a drop of type-specific antisera is added. The antisera is mixed with the organism suspension, and then the glass slide is rocked gently for ca. 1

min before being examined for the presence of clumping, an indication of a positive reaction. The reagents for performing slide agglutination serotyping of *H. influenzae* are commercially available in kit form from Remel and BD.

Alternatively, primary type-specific antibodies can be directly or indirectly detected with fluorescent molecules, and binding of the antibody to the homologous capsular antigen can be determined by fluorescence microscopy (102, 103). While these technologies remain viable, reagents are not commercially available.

Whether a slide agglutination test or fluorescent antibodies are used to determine the capsular serotype of an isolate of *H. influenzae*, positive- and negative-control strains should always be processed simultaneously with clinical isolates as a means of validating test results.

As noted above, based on three phenotypic properties, the production of indole, ornithine decarboxylase, and urease, strains of *H. influenzae* and *H. parainfluenzae* can be distinguished into multiple different biotypes (Table 2). Also, as outlined previously, at least with *H. influenzae*, certain biotypes have been found to have specific disease associations. Assessment of indole, ornithine decarboxylase, and urease production with clinical isolates of *H. influenzae* and *H. parainfluenzae* can be accomplished using the conventional methods described above (Fig. 9) or by use of commercially available miniaturized biochemical kit systems (11). In one recent study that compared the API NH strip kit (bioMérieux) with the RapID NH system (Remel) and the *Neisseria-Haemophilus* identification card (bioMérieux) as a means for determining the biotypes of a large collection of recent clinical isolates of both *H. influenzae* and *H. parainfluenzae*, the API NH kit yielded the most reliable results, correctly classifying the biotypes of >97% of the strains tested (80).

Typing by Molecular Methods

Molecular methods have the advantage of enhanced sensitivity and specificity (104) due to the use of standardized techniques and a lack of false-positive reactions observed with nonencapsulated strains in slide agglutination tests.

Capsular typing of *H. influenzae* can also be accomplished by use of various molecular methods. Most such assays rely on the amplification of genes in the *cap* locus, the outer membrane protein D gene (*glpQ*), the capsule-producing gene (*bexA*), the 16S rRNA gene, and the insertion-like sequence (105). One algorithm was used for detection of the *cap* genes to determine capsular serotypes a through f, while the capsule-producing gene, *bexA*, was used to separate strains that produce capsule from those that do not (105). Detection of the *ompP2* (outer membrane lipoprotein P2) gene was used as a control. Using this system, both a conventional PCR and a real-time PCR assay were found to be more sensitive than a slide agglutination test for serotyping *H. influenzae* (105). Similarly, Wroblewski et al. (106) described a two-step algorithm combining a two-plex real-time PCR with a five-plex serotype-specific real-time PCR (type a, *acsA*; type c, *ccsB*; type d, *dcsC*; type e, *ecsC*; and type f, *fcsA*). In this algorithm, the two-plex PCR containing primers targeting *bexA* (capsule transport gene) and *bcsB* (type b-specific gene) is used to screen isolates for NTHi and serotype b-specific strains. Strains that are positive for *bexA* but negative for *bcsB* are referred to the five-plex PCR for serotype-specific amplification (106). This method demonstated 100% concordance with slide agglutination (106).

As with the *Enterobacteriaceae*, pulsed-field gel electrophoresis (PFGE) is considered the gold standard for strain typing of *Haemophilus*. The method demonstrates excellent separation of clones but is laborious and time-consuming (107–109). Other molecular methods for typing have also been applied to *Haemophilus* species. Studies evaluating repetitive-element sequence-based PCR using intergenic dyad sequence (IDS)-specific primers (IDS-PCR) for nonencapsulated *Haemophilus* strains have been developed and demonstrate excellent separation of NTHi strains (108). In one study evaluating the performance of IDS-PCR with 69 NTHi isolates, the assay demonstrated 65 different banding patterns that were epidemiologically classified as fingerprints similar to those obtained by PFGE (108). Other typing technologies applied to *Haemophilus* with a high degree of separation include ribotyping, restriction fragment length polymorphism analysis, multilocus enzyme electrophoresis, randomly amplified polymorphic DNA profile analysis, and multilocus sequence typing (108). While all of these techniques have demonstrated excellent separation, many are laborious and time-consuming (multilocus enzyme electrophoresis, PFGE, and ribotyping), others produce overly complex banding patterns (restriction fragment length polymorphism analyses), and others lack reproducibility (randomly amplified polymorphic DNA profile analysis) (108). Multilocus sequence typing offers the advantage of superior discriminatory power because it combines sequence typing of seven housekeeping genes with results that can readily be compared between laboratories (7).

Next-generation sequencing offers the promise of detection of an organism, classification of antimicrobial resistance factors, and typing of the organism in a single assay. Furthermore, with turnaround time for sequencing and annotation of the sequence decreasing to <24 h, real-time identification of outbreaks will become the standard of care. A recent investigation by Power et al. (110) sequenced the genome of 85 strains of *H. influenzae* and aligned the sequences by Mauve alignment. The study demonstrated distinct clusters of strains associated with serotype and regions of significant variation in sequence, likely due to transformation events. While preliminary, this study demonstrates the principle of typing based on genome sequence and may result in significant improvement in our ability to resolve strains into sequence types with much greater precision (110).

A comprehensive review of the available typing methods for *Haemophilus* species has been provided by Harrison et al. (104).

SEROLOGIC TESTS

Antibody tests have been developed for the detection of *Haemophilus* antibodies; however, they are of little clinical value and are not readily available. Studies evaluating the performances of the enzyme-linked immunosorbent assay (111) and immunofluorescent assays (112) have been conducted; however, because immunity to *Haemophilus* is derived from an eclectic combination of antibodies against the *H. influenzae* capsule and membrane proteins, assays to detect a single class of antibody are of little value. Further complicating the use of *Haemophilus* serology is the individual variation in antibody level (many adults have undetectable antibody levels), avidity, and persistence (18).

ANTIMICROBIAL SUSCEPTIBILITIES

Resistance Rates

H. influenzae may produce one of two β-lactamases, TEM-1 and ROB-1. Both enzymes are plasmid associated, are extracellular, and are produced constitutively in large amounts (113). β-Lactamase-producing strains should be considered resistant to ampicillin and amoxicillin, as these drugs typically have MICs of ≥128 μg/ml against these strains (114).

The prevalence of β-lactamase-producing NTHi declined in the United States during the 1990s, possibly due to the increased use, beginning in the early 1990s, of non-β-lactam antimicrobials, such as the macrolides and fluoroquinolones, in the empiric management of infections such as otitis media, sinusitis, and bronchopulmonary infections, i.e., infections with which NTHi is most often associated (115). As a consequence of this therapeutic paradigm shift, β-lactams such as ampicillin and amoxicillin were used less often, with resulting diminished selective pressure on the emergence and persistence of β-lactamase-producing NTHi. This trend toward decreasing rates of β-lactamase-producing NTHi, however, seems paradoxically to have changed during the past 10 years, possibly as a result of the widespread use of pediatric pneumococcal conjugate antigen vaccines. As *S. pneumoniae* has become less common as a cause of acute otitis media, NTHi has reemerged as the most common cause of the infection, with a proportionate increase in rates of β-lactamase-mediated ampicillin resistance (116).

β-Lactamase-producing strains of *H. influenzae* remain susceptible to oral and parenteral cephalosporins and carbapenems (114, 115, 117, 118). They are also susceptible to combination agents in which a β-lactamase inhibitor, such as clavulanate, sulbactam, or tazobactam, is combined with a β-lactam agent (114, 115, 117, 118). Examples include amoxicillin-clavulanate, ampicillin-sulbactam, and piperacillin-tazobactam.

Strains of *H. influenzae* that fail to produce β-lactamase but for which the MICs of ampicillin and amoxicillin are elevated have been described (119). These strains, which are often referred to as β-lactamase negative and ampicillin resistant (BLNAR), have altered penicillin-binding proteins, which abrogates the binding of drugs such as ampicillin and amoxicillin to their cell wall targets, in turn resulting in elevated MICs (120, 121). The activities of cephalosporins are also diminished with such strains. If one uses an ampicillin or amoxicillin MIC of ≥4 μg/ml to define resistance within *Haemophilus* spp., as is recommended by the Clinical and Laboratory Standards Institute (CLSI) (122), the prevalence of BLNAR strains of *H. influenzae* remains at levels of <1% in the United States (115).

Among other antimicrobials that are relevant to the management of *Haemophilus* infections, with the exception of trimethoprim-sulfamethoxazole (TMP-SMX), resistance rates remain at levels of <1% (114, 115). These antimicrobials include both oral and parenteral cephalosporins, macrolides, fluoroquinolones, and tetracycline. TMP-SMX resistance rates approach 20% (114, 115). Currently, in the United States, ca. 27% of clinical isolates of NTHi produce β-lactamase (123).

Susceptibility Test Methods

β-Lactamase production by *Haemophilus* spp. can be determined rapidly with either a chromogenic cephalosporin spot test or an acidimetric penicillinase assay, as described in chapter 74 in this *Manual*. Because the β-lactamases of *H. influenzae* are extracellular, constitutive, and produced in large amounts, assuming the test is performed carefully and with adequate positive and negative controls, both methods yield reliable results.

Disk diffusion susceptibility tests can be performed using *Haemophilus* test medium (HTM) agar (118), with incubation of plates for 16 to 18 h at 35°C in 5 to 7% CO_2, as described by the CLSI (122). Zone diameter interpretive criteria have been developed for 39 different antimicrobial agents (124). The details of disk diffusion susceptibility tests are presented in chapter 71.

MICs can be determined with *Haemophilus* spp. by use of broth microdilution (BMD). The CLSI advocates the use of HTM broth (118) when determining MICs by the BMD method (125). Following inoculation, trays are incubated for 20 to 24 h in ambient air at 35°C prior to determination of MICs; MIC interpretive criteria have been developed for 43 different antimicrobial agents (124). The details of BMD MIC tests are presented in chapter 71. In circumstances in which HTM is not available or when equivocal results have been obtained with this medium, BMD MICs can be determined with *Haemophilus* spp. using Mueller-Hinton broth supplemented with 3 to 5% sterile lysed horse blood and 10 μg/ml NAD (126). The MIC interpretive criteria for *Haemophilus* promulgated by the CLSI for BMD tests in HTM can also be applied to MICs determined by BMD in medium containing lysed horse blood. Unfortunately, there exist almost no published data validating the use of the Etest method with *Haemophilus* spp., and therefore, use of this method is not recommended for susceptibility tests with this organism group.

Susceptibility tests with clinical isolates of *H. ducreyi* should not be attempted in routine clinical microbiology laboratories, as standardized susceptibility test methods of proven reliability for this organism have not yet been developed. Similarly, instrument-based susceptibility tests for other *Haemophilus* spp., including *H. influenzae*, have not been proven to be effective and are not recommended for testing this organism group.

Irrespective of the method used for performing susceptibility tests with *Haemophilus* spp., it is essential that adequate quality control is applied using two *H. influenzae* quality control strains, ATCC 49247 and ATCC 49766.

Susceptibility Testing Algorithm

Susceptibility testing with clinical isolates of *Haemophilus* spp. should be applied only to those strains known to be of clinical significance. Further, in the vast majority of instances, only a β-lactamase assay as a means for assessing the activities of ampicillin and amoxicillin need be performed. The prevalence of BLNAR strains of *H. influenzae* and their resistance to other agents that are commonly used to treat the types of infections with which *Haemophilus* spp. are associated are simply too low to justify routine testing. One exception might be resistance to TMP-SMX. This agent, however, is used almost exclusively for oral therapy of community-acquired respiratory tract infections that are invariably managed empirically without performance of laboratory studies aimed at elucidating the specific cause of an individual patient's infection. In other words, in settings where knowledge of the activity profile of TMP-SMX versus *H. influenzae* could be of value, rarely, if ever, is a patient isolate available for testing. In those rare circumstances when the assessment of the activities of agents other than ampicillin or amoxicillin is found to be warranted, either a disk diffusion susceptibility test or a BMD MIC test should be performed.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The genus *Haemophilus* is a diverse group of organisms that may exist as part of the normal bacterial biota of healthy humans or may be associated with significant disease. As a result, simple recovery of *Haemophilus* from a human clinical sample may not always indicate that the organism is clinically significant. In the following three circumstances, recovery of *Haemophilus* spp. in the laboratory is pathognomonic: (i) isolates from normally sterile sites, including blood cultures, are compatible clinically with illness; (ii) *H. ducreyi* is recovered from genital tract specimens obtained from patients with genital ulcers; and (iii) isolates of *H. aegyptius* from conjunctival specimens are obtained from patients with exudative inflammation of the conjunctiva.

Recovery of *Haemophilus* spp. from specimens that may be contaminated with commensal microbial biota represents a situation in which the clinical significance of the isolate must be questioned. This is often the case, for example, with isolates from respiratory tract sites. In such instances, the quantity of organism recovered, both the absolute quantity and the quantity of the isolate in comparison to quantities of other organisms recovered from the specimen, is of limited value in assessing clinical significance. It may be helpful to try to assess the quality of the specimen, as is possible, for example, with expectorated sputa and endotracheal aspirates. It may also be instructive to compare the results of a given culture with results obtained from previous and subsequent cultures from the same site. Generally speaking, repetitive recovery of the same organism from multiple specimens representative of a specific infectious disease process in an individual patient can be taken as an indication of clinical significance. And finally, it must be recognized that in some cases, it simply is not possible to know with certainty whether a given isolate of *Haemophilus* spp. is clinically significant. In such instances, active dialog with health care providers is encouraged.

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Escherichia, *Shigella*, and *Salmonella*

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ESCHERICHIA

Taxonomy

Members of the genus *Escherichia* are classified in the family *Enterobacteriaceae*, which is addressed in chapter 38 of this *Manual* (1–4). There are six species in this genus: *Escherichia albertii*, *Escherichia blattae*, *Escherichia coli*, *Escherichia fergusonii*, *Escherichia hermannii*, and *Escherichia vulneris*. The G+C content is 48 to 59 mol%, and the type species is *E. coli* (Migula 1895) (5). Average DNA relatedness between the type species and other *Escherichia* species, as assessed by DNA-DNA hybridization, ranges from 38 to 64%. As more genomes become sequenced, refinements to the classification of members of the family *Enterobacteriaceae* will be possible. A recent publication proposes to reclassify *E. blattae* in a newly formed genus as *Shimwellia blattae*, based on sequence analysis of the 16S rRNA gene and four protein-coding genes (6). *E. blattae* exhibits about 43% DNA relatedness to *E. coli* by DNA-DNA hybridization.

Members of the genus *Shigella* are phenotypically similar to *E. coli* and, with the exception of *Shigella boydii* serotype 13, would be considered the same species by DNA-DNA hybridization analysis (4) and whole-genome sequence analysis (7). Findings from phylogenetic studies with nucleotide sequences of internal fragments from 14 housekeeping genes show that *S. boydii* 13 strains cluster in a neighbor-joining tree with *E. albertii* (8).

Comparative genomic analysis has provided important insights into the structure and organization of bacterial genomes and mechanisms by which they have evolved. The number of genomes available for the *E. coli*/*Shigella* group continues to increase (9–15), with a recent study comparing the gene content of 186 genomes from a diverse selection of commensal and pathogenic strains (15). Findings from these studies showed that the *E. coli* genome varies in size from 4,639,221 base pairs for *E. coli* K-12 (14) to 5,945,000 base pairs for a Shiga toxin-producing strain of *E. coli* serotype O26:H11 (10). In a comparison of 17 genomes by Rasko et al. in 2008 (11), the number of genes per genome ranged from 4,238 to 5,589, with an average of 2,344 (46%) of these being conserved among all strains (core genome). When >50 complete genomes were studied in 2012 by Kaas et al. (15), the average number of genes (homolog gene clusters) per genome was 4,837; however, the number of genes representing the core genome fell to 1,702. In the 2012 study, the estimated number of genes representing the "pangenome" in a set of 186 genomes was 16,363, and this number continued to increase in a linear fashion as genomes were added (16). Rasko et al. (11) found that strains representative of a pathotype contained shared genes as well as unique genes. Enterohemorrhagic *E. coli* (EHEC) strains shared the largest number of genes, with 122 group-specific genes, while uropathogenic *E. coli* (UPEC) strains shared 45 to 56 genes; enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), and enterotoxigenic *E. coli* (ETEC) strains each shared 3 to 5 genes; and commensal strains shared 11 genes. The number of strain-specific genes varied widely and was not consistent within a pathotype (11).

Pathogenomic analysis of the numerous plasmids present within representative strains of each of the *E. coli* pathotypes and commensal *E. coli* has revealed considerable diversity and plasticity within these genetic elements (17). In contrast to the overall plasmid diversity, there is limited diversity in the virulence plasmids, which are restricted to a few plasmid backbones with conservation and linkage of their core components (17). These plasmids contain distinct regions for genetic exchange that appear to evolve via insertion sequence-mediated site-specific recombination. Many such plasmids have also acquired multidrug resistance-encoding islands. In addition to plasmids, bacteriophages play a major role in generating genome diversity by promoting homologous recombination and horizontal gene transfer between bacteria (18, 19). For example, the Sakai strain of *E. coli* O157:H7 contains 18 prophages and 6 prophage-like elements (16% of the total genome), which carry a wide range of virulence genes, including the genes for Shiga toxins 1 and 2 (20, 21). Although many of these prophages carry genetic defects, some are able to be induced and recombine with each other to generate new phages capable of transferring virulence genes to other bacteria (19). The dynamic nature of the Shiga toxin-converting phages has implications for diagnostic testing for Shiga toxin-producing *E. coli* (STEC). Since STEC strains can lose critical virulence genes, some researchers have proposed that multiple virulence-associated genes, as well as conserved genes, be used to diagnose infections by these bacteria (22, 23). This concept would also apply to other pathotypes of *E. coli*, as most of them carry critical virulence genes on mobile genetic elements.

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Description of the Genus

The genus *Escherichia* is composed of motile or nonmotile bacteria that conform to the definitions of the family *Enterobacteriaceae* (1, 2). Species in this genus are Gram-negative, oxidase-negative rods that grow well on MacConkey agar (MAC). When these organisms are motile, it is by peritrichous flagella. They can grow aerobically or anaerobically. All ferment D-glucose, and most produce gas from the fermentation of this substrate and other fermentable carbohydrates. Lactose is fermented by most strains of *E. coli*, but its fermentation may be delayed or absent in all or most strains of *E. albertii*, *E. blattae*, *E. fergusonii*, and *E. vulneris*. Typical phenotypic properties are listed in Table 1.

Epidemiology and Transmission

E. coli occurs naturally in the lower part of the intestines of humans and warm-blooded animals. In humans, it typically colonizes an infant's gastrointestinal tract within hours of birth and subsequently becomes the predominant facultative anaerobe in the human colonic microbiota, where it exists in a mutually beneficial relationship with its host. *E. coli* generally remains confined to the intestinal lumen; however, in a debilitated or immunosuppressed host or when bacteria are introduced to other tissues following trauma or surgical procedures, even commensal, "nonpathogenic" strains of *E. coli* can cause infection. *E. coli* is typically transmitted through ingestion of contaminated food and water, person-to-person contact, contact with animals, or contact with environments or fomites contaminated with fecal material. Convincing evidence of respiratory transmission has not been reported. Most strains of *E. coli* do not cause disease in healthy persons; however, there are specific pathogenic groups, addressed below, whose members are capable of causing disease in humans and animals. The infectious dose for diarrheagenic *E. coli* varies by strain and pathotype and ranges from 10 to 100 bacteria for *E. coli* O157:H7 (24) to 10^8 or more bacteria for ETEC (25).

Because *E. coli* is ubiquitous in human and animal feces, the presence of this species in water is considered an indicator of fecal contamination. It can be isolated from feces-contaminated foods or water but probably does not occur as a free-living organism in the environment.

E. fergusonii, *E. hermannii*, *E. vulneris*, and *E. albertii* have been isolated from a wide variety of human clinical specimens (stool, urine, sputum, blood, spinal fluid, peritoneal dialysis fluid, and wounds) (26–32). *E. fergusonii* and *E. albertii* have also been isolated from wild and domestic birds (33, 34). Recently *E. vulneris* strains with traits common to mammalian pathogens were found actively multiplying within legumes, raising the possibility that plants may represent a niche for transmission of clinically important strains (35, 36). In addition, *E. vulneris* was recovered in combination with other members of the *Enterobacteriaceae* from neonatal enteral feeding tubes, highlighting the potential importance of these devices as risk factors for neonatal infections (37). *E. blattae* occurs naturally in the hindgut of cockroaches and is not known to cause disease in humans.

Clinical Significance

Of the six *Escherichia* species, *E. coli* is the species usually isolated from human specimens and the one that we know the most about. We know little about the pathogenesis of the other *Escherichia* species; however, it is interesting to note that strains of *E. albertii*, which have been associated with diarrheal disease in children, contain the locus for enterocyte effacement (LEE) pathogenicity island that is also present in EPEC and EHEC (8, 30, 38). One restaurant-associated outbreak of *E. albertii* has been reported (39).

Extraintestinal *E. coli*

Pathogenic *E. coli* strains are broadly grouped into two categories—extraintestinal pathogenic *E. coli* and intestinal or diarrheagenic *E. coli*—depending on whether they cause disease outside or within the intestinal tract. The application of methods for phylogenetic grouping and DNA sequence typing has revealed extensive genetic substructure among strains within these broad groups and has facilitated studies to understand their evolution and transmission. For example, a recent study of *E. coli* causing community-associated and hospital-acquired urinary tract and bloodstream infections from across the world showed that a high proportion of infections were due to a globally disseminated, multidrug-resistant clone of *E. coli* subtyped as sequence type 131 (ST131) (40).

Commensal *E. coli* strains, which comprise the majority of the facultatively anaerobic intestinal microbiota in most humans and other mammals, typically do not cause disease. However, they can be opportunistic pathogens when certain conditions exist, such as the presence of a foreign body (e.g., urinary catheter), host compromise (e.g., local anatomical or functional abnormalities, such as urinary or bile tract obstruction or immunocompromise), or a breach in normally sterile sites causing the introduction of feces or high concentrations of mixed bacteria. Extraintestinal pathogenic *E. coli* strains carry a distinct set of virulence genes that enable them to cause disease outside the intestine. This category contains at least two well-recognized pathogenic groups or pathotypes—UPEC and meningitis/sepsis-associated *E. coli* (MNEC)—and a variety of disease-associated strains not yet classified into specific pathotypes.

UPEC

UPEC strains are a major cause of community-acquired urinary tract infections and possess a variety of chromosomally and plasmid-encoded virulence factors that are present in various combinations. Members of this group have a limited number of O antigens (six O groups cause 75% of urinary tract infections) and show combinations of traits, including expression of adhesins (P [Pap], type 1, and other fimbriae), toxins (hemolysin, cytotoxic necrotizing factor [CNF], and an autotransported protease [Sat]), or aerobactin, serum resistance, and encapsulation, that are epidemiologically associated with cystitis and acute pyelonephritis in individuals with normal urinary tracts. No single phenotypic profile defining UPEC has emerged. UPEC strains possess large and small pathogenicity islands containing blocks of genes not found in the chromosomes of fecal strains. For a review of the virulence genes and a proposed model of the pathogenesis of UPEC, see the work of Croxen and Finlay (41), Johnson and Russo (42), and Kaper et al. (43).

MNEC

MNEC strains are the most common Gram-negative organisms causing neonatal meningitis, which is associated with high morbidity and mortality. The MNEC pathotype comprises strains with a limited number of O antigens, and ~80% are positive for the K1 antigen. *E. coli* strains that cause meningitis are spread hematogenously. Levels of bacteremia correlate with the development of meningitis; levels of $>10^3$ CFU/ml of blood are significantly associated with the development of meningitis (44). After entering the blood, these bacteria invade brain microvascular endothelial cells through membrane-bound vacuoles. Within these

TABLE 1 Biochemical reactions of the six species of *Escherichia* and selected members of the family *Enterobacteriaceae*[a]

Species/biogroup	Indole production	Voges-Proskauer	Motility (35°C)	Yellow pigment	Lysine decarboxylase	Ornithine decarboxylase	Growth in KCN	Acetate utilization	Mucate utilization	D-Glucose, gas	Adonitol, acid	L-Arabinose, acid	D-Arabitol, acid	Cellobiose, acid	Dulcitol, acid	Lactose, acid	Sucrose, acid	D-Mannitol, acid	Raffinose, acid	L-Rhamnose, acid	D-Sorbitol, acid	D-Xylose
Escherichia albertii/ biogroup 1 (*n* = 5) (e.g., Albert 19982)	0	0	0	0	100	100	0	20	20	100	0	100	0	0	0	0	0	100	0	0	0	0
Escherichia albertii/ biogroup 2 (*n* = 10) (e.g., former *S. boydii* 13)	100	0	0	0	0	100	0	0	0	40	0	100	0	0	0	0	0	100	0	0	100	0
Escherichia blattae	0	0	0	0	100	100	0	0	50	100	0	100	0	0	0	0	0	0	0	100	0	100
Escherichia coli	98	0	95	0	90	65	0	90	95	95	5	99	5	2	60	95	50	98	50	80	94	95
Escherichia coli (inactive biotypes)	80	0	5	0	40	20	1	40	30	5	3	85	5	2	40	25	15	93	15	65	75	70
Escherichia fergusonii	98	0	93	0	95	100	0	96	0	95	98	98	100	96	60	0	0	98	0	92	0	96
Escherichia hermannii	99	0	99	98	6	100	94	78	97	97	0	100	8	97	19	45	45	100	40	97	0	100
Escherichia vulneris	0	0	100	50	85	0	15	30	78	97	0	100	0	100	0	15	8	100	99	93	1	100
Shigella boydii[b]	37	0	0	0	0	0	0	0	0	0	0	94	0	0	12	1	0	99	0	1	59	27
Shigella dysenteriae	40	0	0	0	0	0	0	0	0	0	0	45	0	0	4	0	0	0	0	30	29	3
Shigella flexneri	42	0	0	0	0	0	0	8	0	3	0	60	1	0	2	0	1	91	33	5	30	3
Shigella sonnei	0	0	0	0	0	98	0	0	10	0	0	95	0	5	0	2	1	99	3	77	1	1
Hafnia alvei	0	85	85	0	100	98	95	15	0	98	0	95	0	15	0	5	10	99	2	97	0	98
Hafnia alvei/biogroup 1	0	70	0	0	100	45	0	0	0	0	0	0	0	0	0	0	0	55	0	0	0	0
Salmonella serotype Paratyphi A	0	0	95	0	0	95	0	0	0	99	0	100	0	5	90	0	0	100	0	100	95	0
Salmonella serotype Choleraesuis	0	0	95	0	95	100	0	1	0	95	0	0	1	0	5	0	0	98	1	100	90	98
Yersinia ruckeri	0	10	0	0	50	100	15	0	0	5	0	5	0	5	0	0	0	100	5	0	50	0

[a] Values are percentages of isolates tested with positive test results within 1 or 2 days of incubation at 35 to 37°C. Reactions for isolates that become positive after 2 days are not considered. Data were compiled from findings published by Ewing et al. (247), Wathen-Grady et al. (248, 249), Ansaruzzaman et al. (250), Pryamukhina and Khomenko (251), and Farmer (252) and from unpublished findings from the reference laboratory at the CDC (1972 to 2005).

[b] Excludes strains previously identified as *S. boydii* 13.

vacuoles, the organisms control intracellular trafficking to avoid lysosomal fusion and to gain access to the central nervous system without causing apparent damage to the blood-brain barrier. Recent studies have identified several bacterial determinants (IbeA, IbeB, IbeC, AslA, CNF1, FimH, and OmpA) that contribute to the pathogensis of MNEC *in vitro*. For reviews, see the work of Kim et al. (45), Kaper et al. (43), and Croxen and Finlay (41).

Diarrheagenic *E. coli*

There are at least five categories of recognized diarrheagenic *E. coli*: STEC, which includes a subset of strains referred to as enterohemorrhagic *E. coli* for their ability to cause bloody diarrhea and hemorrhagic colitis; ETEC; EPEC; EAEC; and enteroinvasive *E. coli* (EIEC) (41, 43, 46). The clinical significance of several other groups of putative diarrheagenic *E. coli*, particularly diffusely adherent *E. coli* (DAEC), is unclear.

STEC

We refer to the STEC category of diarrheagenic *E. coli* according to the toxins that these organisms produce, i.e., as STEC rather than EHEC, because the essential genetic features that define organisms capable of causing hemorrhagic colitis and hemolytic-uremic syndrome (HUS) are not clear. *E. coli* serotypes O157:H7 and O157:nonmotile (O157:NM) (collectively called O157 STEC) produce one or more Shiga toxins, also called verocytotoxins, and are the most frequently identified diarrheagenic *E. coli* serotypes in North America and Europe. Each year, an estimated 63,000 cases of illness and 20 deaths are caused by O157 STEC and 112,000 cases with no deaths are caused by other STEC serotypes in the United States (47).

E. coli O157:H7 and other STEC serotypes cause illness that can present as mild nonbloody diarrhea, severe bloody diarrhea (hemorrhagic colitis), or HUS (reviewed in reference 48). Additional symptoms of *E. coli* O157:H7 infection include abdominal cramps and lack of a high fever. Among patients with O157 STEC diarrhea, 4% or more develop HUS (49), a condition characterized by microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. The fatality rate of HUS has declined in recent years due to improvements in case management.

O157 STEC is thought to cause at least 80% of cases of HUS in North America and is recognized as a common cause of bloody diarrhea in developed countries (49). In the United States, the rate of isolation of O157 STEC from fecal specimens is highest in the Northern Tier states, where it may approach the rates for common diarrheal pathogens. Many U.S. clinical laboratories do not routinely culture or otherwise test stools for O157 STEC; as a result, many illnesses are not detected (50). O157 STEC colonizes dairy and beef cattle, and therefore ground beef has caused more O157 STEC outbreaks than any other vehicle of transmission (49, 51). Other known vehicles of transmission include raw milk, sausage, roast beef, unchlorinated municipal water, apple cider, raw vegetables, and sprouts; these vehicles are typically exposed to water contaminated by bovine manure. O157 STEC spreads easily from person to person because the infectious dose is low (<200 CFU); outbreaks associated with person-to-person spread have occurred in schools, long-term care institutions, families, and day care facilities.

More than 150 non-O157 STEC serotypes have been isolated from persons with diarrhea or HUS (http://www.microbionet.com.au/frames/feature/vtec/brief01.html). In some countries, non-O157 STEC strains, particularly *E. coli* serotypes O111:NM and O26:H11, are more commonly isolated than O157 STEC strains, although most outbreaks and cases of HUS are attributed to the latter (serotypes characteristic of diarrheagenic *E. coli* pathotypes are presented in Table 2). In 2011, the largest known outbreak of hemorrhagic colitis occurred in Germany, with 3,800 cases, 800 of which resulted in the development of HUS, and 54 deaths. The outbreak was caused by a strain of *E. coli* serotype O104:H4 with virulence genes and a genome characteristic of EAEC, which had acquired a bacteriophage encoding Shiga toxin 2a (52). In the United States, *E. coli* O157:H7 is the most frequently isolated STEC serotype, but increasingly, non-O157 STEC strains are identified as causes of outbreaks and sporadic illness (53). At the Centers for Disease Control and Prevention's (CDC) *E. coli* Reference Laboratory, 71% of all non-O157 STEC isolates received between 2003 and 2008 belonged to six serogroups (O26, O103, O111, O121, O45, and O145) (54). Because most laboratory methods for the detection of O157 STEC do not detect non-O157 STEC, the numbers of infections with serotypes other than O157:H7 or O157:NM are probably underestimated.

ETEC

ETEC, which produces heat-labile *E. coli* enterotoxin (LT) and/or heat-stable *E. coli* enterotoxin (ST), is an important cause of diarrhea in developing countries, particularly among young children (46). ETEC also is a frequent cause of traveler's diarrhea. From 1975 to 2008, 33 U.S. outbreaks were reported to the CDC (C. A. Bopp, unpublished data; 55). ETEC is infrequently identified in the United States, but this may be attributable in part to the fact that few laboratories are capable of identifying this pathogen. ETEC strains, particularly those associated with outbreaks, tend to cluster in a few serotypes (Table 2).

The most prominent symptoms of ETEC illness are diarrhea and abdominal cramps, sometimes accompanied by nausea and headache, but usually with little vomiting or fever (55). Although ETEC is usually associated with relatively mild watery diarrhea, illness in some recent ETEC outbreaks has been notable for its prolonged duration (56).

EPEC

In the past, EPEC strains were defined as certain *E. coli* serotypes that were epidemiologically associated with infantile diarrhea but did not produce enterotoxins or Shiga toxins and were not invasive. The traditional EPEC serotypes are listed in Table 2; typically, these serotypes show a distinct pattern of localized adherence to HeLa and HEp-2 cells (57). These serotypes usually also demonstrate actin aggregation in the fluorescent actin stain test, which correlates with the attaching-and-effacing (A/E) lesion *in vivo* (46). Because of the lack of simple diagnostic methods for EPEC strains, few laboratories attempt to identify these organisms. Full EPEC pathogenicity requires two genetic elements: the EPEC adherence factor (EAF) plasmid, which encodes, most importantly, the bundle-forming pilus; and the chromosomal LEE, which mediates the A/E phenotype. The term "typical EPEC" has been suggested for those organisms harboring both the EAF plasmid and the LEE pathogenicity island (see below). Typical EPEC strains correspond to EPEC strains of the classical serotypes and are important causes of diarrhea in developing countries (46, 58); these organisms were implicated in highly lethal nursery outbreaks in the United States and the United Kingdom before 1970. The infection is currently rare in the industrialized world. More recently, atypical EPEC strains have been implicated as enteric pathogens in the United States, including in an outbreak of diarrheal disease (59, 60). These strains possess

TABLE 2 Frequently encountered serotypes of diarrheagenic *E. coli*[a]

ETEC	EPEC	EIEC	STEC			EAEC
O6:NM	**O55:NM**	O28:NM	O8:H19	**O104:H4**[b]	O128:H2	O3:H2
O6:H16	**O55:H6**	O29:NM	O22:H8	O104:H7	O128:H45	O15:H18
O8:H9	O55:H7	O112:NM	**O26:NM**	**O104:H21**	**O145:NM**	**O44:H18**
O15:H11	O86:NM	O124:NM	**O26:H11**	**O111:NM**	O146:H21	O51:H11
O20:NM	O86:H34	O124:H7	O28:H25	**O111:H2**[c]	O153:H2	O77:H18
O25:NM	**O111:NM**	**O124:H30**	**O45:H2**	**O111:H8**	O153:H25	O86:H2
O25:H42	**O111:H2**	O136:NM	O55:H7	**O113:H21**	O156:H25	O111ab:H21
O27:NM	O111:H12	**O143:NM**	O69:H11	**O118:H2**	**O157:NM**	O126:H27
O27:H7	O111:H21	O144:NM	O76:H19	O118:H12	**O157:H7**	O141:H49
O27:H20	**O114:NM**	O152:NM	O84:NM	O118:H16	O165:NM	ONT:H21
O49:NM	**O114:H2**	**O164:NM**	O88:H25	O119:NM	O165:H25	ONT:H33
O63:H12	**O119:H6**	O167:NM	O91:NM	O119:H4	O172:NM	
O78:H11	**O125:H21**	**ONT:NM**	O91:H14	O119:H25	O174:H21	
O78:H12	O126:NM		O91:H21	**O121:H19**	O174:H28	
O128:H7	O126:H27		O103:NM	O123:NM	O177:NM	
O148:H28	**O127:NM**		**O103:H2**	O123:H11	O178:H19	
O153:H45	**O127:H6**		O103:H11	O128:NM	O179:H8	
O159:NM	O127:H9		O103:H25			
O159:H4	O127:H21					
O159:H20	**O128:H2**					
O167:H5	O128:H7					
O169:NM	O128:H12					
O169:H41	**O142:H6**					
	O157:H45					

[a] Outbreak-related serotypes are shown in bold. NM, nonmotile; NT, not typeable.

[b] STEC/EAEC O104:H4 strain from large foodborne outbreak in Germany associated with fenugreek seeds (253).

[c] STEC/EAEC O111:H2 strain identified from patients associated with an outbreak of HUS in France (254).

a functional LEE apparatus but do not carry the EAF plasmid. The full role of these pathogens has yet to be elucidated, but they may be considered potential causes of diarrheal outbreaks when no other pathogens are identified.

Symptoms of often severe and prolonged nonbloody diarrhea, vomiting, and fever in infants or young toddlers are characteristic of EPEC illness (46). Infection with EPEC has been associated with chronic diarrhea; sequelae may include malabsorption, malnutrition, weight loss, and growth retardation.

EIEC

EIEC strains invade cells of the colon and produce a generally watery, but occasionally bloody, diarrhea by a pathogenic mechanism similar to that of *Shigella*. EIEC is rare in the United States and is less common than ETEC or EPEC in the developing world (46). EIEC strains, like ETEC and EPEC, are associated with a few characteristic serotypes (Table 2). Three large outbreaks of diarrhea caused by EIEC have been reported in the United States (46), and an outbreak of EIEC O96:H19 was recently detected in Italy using a commercial diagnostic PCR kit (61).

EAEC

EAEC, as originally defined by its specific pattern of aggregative adherence to HEp-2 cells in culture, has been associated with diarrhea in a variety of clinical settings, including endemic diarrhea in children of both impoverished and industrialized countries, epidemic diarrhea, diarrhea of travelers to developing countries, and persistent diarrhea among patients with HIV infection or AIDS (62). The pathogenicity of EAEC has been confirmed in volunteer studies (63) and by implication of EAEC in diarrhea outbreaks (64). Early studies frequently failed to find an association of EAEC with pediatric diarrhea, but this association has been strengthened by the use of molecular techniques that discriminate the true pathogens exhibiting the aggregative pattern (65, 66). The term "typical EAEC" describes organisms harboring virulence genes under the control of the global EAEC regulator AggR (66). Typical EAEC may be a common cause of pediatric diarrhea in U.S. infants (65) and should be considered a potential cause of foodborne outbreaks and diarrhea in HIV-infected and AIDS patients (62). EAEC diarrhea is accompanied by signs and symptoms of mild inflammation (abdominal pain and fever), but stools usually do not contain blood or fecal leukocytes (62).

In 2011, a typical EAEC strain lysogenized with a Shiga toxin-encoding phage caused a large outbreak of foodborne illness in Europe (67). The illness was clinically indistinguishable from that caused by *E. coli* O157:H7, although most of the cases and complications occurred in adults. Whether or not Shiga toxin-producing EAEC strains will become more common is unknown.

Putative Diarrheagenic *E. coli*

Several putative pathotypes have been described. Virulence has not been demonstrated clearly for any of these types by either volunteer studies or outbreak investigations. DAEC strains, which exhibit a characteristic diffuse pattern of adherence to HEp-2 cells, have been implicated as causes of diarrhea in some epidemiologic studies but not others (46), and a prototypical DAEC strain did not elicit diarrhea in adult volunteers (68). In several studies, DAEC infections were significantly associated with watery diarrhea among children 1 to 5 years of age but were not associated with illness among infants (69). DAEC may occur in industrialized countries (46). A complex signal transduction cascade has been suggested as the mechanism of DAEC pathogenesis (70).

CNF-producing *E. coli* strains produce a toxin that induces morphological alterations (multinucleation) and death in tissue cultures (71). Two forms have been described: CNF1 and CNF2. CNF1-producing strains were originally detected in infants with enteritis and later in humans with extraintestinal infections (71, 72). Most CNF1-producing strains are also hemolytic, although the toxin is distinct from hemolysin. CNF2-producing strains have been isolated from animals with diarrhea (73–75). The role of these strains in human diarrheal disease has not been determined definitively (46).

Cytolethal distending toxin-producing *E. coli* strains produce a heat-labile factor that induces cytotonic and cytotoxic changes in Chinese hamster ovary cells similar to those caused by LT (76). This factor does not affect Y-1 cells. The results of one study in Bangladesh suggested that cytolethal distending toxin-producing *E. coli* strains are associated with diarrhea (77), but other studies are needed to establish their status as etiologic agents.

Several diarrheal outbreaks have been linked to *E. coli* strains that do not belong to any of the established pathotypes. Some of these strains carry the gene encoding the enteroaggregative ST-like toxin (EAST1), which is related to the ETEC ST enterotoxin. Further studies are needed to prove the pathogenicity of these strains, but the EAST1 gene can be identified using molecular techniques (78).

Collection, Transport, and Storage of Specimens

Information on the collection, transport, and storage of specimens from extraintestinal sites is provided in chapter 18 of this *Manual*. Fecal specimens should be collected in the early stages of any enteric illness (preferably within 4 days of onset), when pathogens are usually present in the stool in highest numbers and before antimicrobial therapy has been started. Whole stools are usually the specimen of choice, but carefully collected rectal swabs with visible fecal staining may be preferable for diagnosis of *Shigella* (79–81). Collection of multiple specimens may enhance the recovery rate of *E. coli*, *Shigella*, and *Salmonella* (82).

Transport of fecal specimens to the laboratory in a timely fashion is critical, particularly for more delicate organisms such as *Shigella* (83). Ideally, fecal specimens should be examined as soon as they are received in the laboratory, but if not processed immediately, they should be either refrigerated or frozen at −70°C. Fecal specimens that will not be examined within 1 to 2 h of collection and all rectal swabs should be placed in cold transport medium and kept at 4°C (84). Transport and storage of fecal specimens at 4°C is very important for *Shigella* as well as *Campylobacter* spp. Manufacturers of commercial transport media, including the acceptable media listed below, commonly state that fecal specimens may be transported and stored at ambient temperature. For *Shigella* and *Campylobacter* spp., this is not advised because there are data showing that transport at ambient temperature may deleteriously affect recovery of these organisms (85).

Many of the commercially available transport media (e.g., Cary-Blair, Stuart, and Amies transport media) and buffered glycerol saline are satisfactory for *E. coli*, *Salmonella*, and *Shigella*. Although acceptable for the transport of the organisms addressed in this chapter, buffered glycerol saline should not be used for specimens that must also be tested for *Campylobacter* and *Vibrio* (85, 86).

Direct Examination

Microscopy

Gram stains of patient specimens from normally sterile body sites can provide a preliminary indication of which category of bacteria to cultivate from the specimen and if inflammatory cells or blood are present; however, *E. coli* and other *Escherichia* species cannot readily be distinguished from other Gram-negative rods by staining or microscopy methods.

Antigen Detection

Several commercial immunoassays are available to diagnose STEC infections by detecting Shiga toxin or the O157 antigen (lipopolysaccharide [LPS]) in fecal specimens (Table 3) (87, 88). These assays are usually more sensitive when performed on enriched specimens than on stool directly. Isolation and serotyping of STEC from fecal specimens that are positive by nonculture assays should always be attempted because serotype information is important for public health purposes and may also help in clinical decisions. Determination of the subtype of O157 STEC and the serotype of a non-O157 STEC isolate is valuable for outbreak investigations and surveillance purposes (see “Typing Systems” and “Identification” below).

Nucleic Acid Detection

At the time of publication, we are aware of four Food and Drug Administration (FDA)-cleared nucleic acid detection methods for the clinical diagnosis of STEC, *Salmonella*, and *Shigella*/EIEC. These include the xTAG Gastrointestinal Pathogen Panel (Luminex Molecular Diagnostics, Inc., Toronto, Ontario, Canada), ProGastro SSCS (Hologic Gen-Probe, Inc., San Diego, CA), FilmArray Gastrointestinal Panel (BioFire Diagnostics, Salt Lake City, UT), and BD MAX Enteric Bacterial Panel (Becton Dickinson and Co., Franklin Lakes, NJ). In addition to the pathogens listed above, the xTAG and FilmArray panels claim to be able to identify ETEC and *E. coli* O157, while the FilmArray panel also claims to detect EPEC and EAEC. Published findings from the initial evaluations of two of these assays have been promising, with reported overall sensitivities and specificities compared to conventional methods of ~94% and 98.5 to 99%, respectively, for the xTAG panel (89, 90) and 100% and 99 to 100%, respectively, for the ProGastro SSCS panel after resolution of discrepant results (91). Individual sensitivities and specificities of the xTAG panel for STEC, O157 STEC, ETEC, and *Shigella*/EIEC ranged from 93.7 to 100% and 97 to 100%, respectively, but were slightly lower for *Salmonella*, at 82.7 to 92% and 98.5 to 100%, respectively (89, 90). When the panel was cleared by the FDA, confirmation of positive xTAG results was recommended in the manufacturer’s product insert, and this recommendation was affirmed for *Salmonella* and *Entamoeba histolytica* by Wessels et al. (92). The individual sensitivities and specificities of the ProGastro SSCS panel for STEC, *Shigella*, and *Salmonella* were each 100% (91). Published peer-reviewed evaluations were not available for the FilmArray and BD MAX panels, which received FDA clearance in May 2014. Validation studies for gastrointestinal panels targeting diarrheagenic *E. coli* other than STEC will be a challenge to perform due to the cost and complexity of the phenotypic assays required to isolate or confirm these organisms and the lack of consensus within the scientific community for a genetic definition of certain pathotypes, e.g., EAEC and EPEC.

Overall, the gastrointestinal panels offer physicians a valuable diagnostic tool for managing the health of individual patients; however, strategies to support surveillance systems intended to protect the health of the public are urgently needed. According to guidelines published in 2009, all fecal specimens or enrichment broths positive for Shiga toxin

TABLE 3 Partial listing of commercial suppliers of reagents for detection of STEC[a]

- Antisera for tube agglutination
  - Difco Laboratories (Division of Becton Dickinson and Co., Sparks, MD)
    - O157 and H7 antisera
  - Statens Serum Institute, Copenhagen, Denmark
    - O157, H7, O26, O103, O111, O145, and other *E. coli* O and H antisera
  - Denka Seiken Co., Ltd., Tokyo, Japan
    - O157, H7, O26, O103, O111, O145, and other *E. coli* O and H antisera
- Latex slide agglutination reagents
  - Denka Seiken Co., Ltd., Tokyo, Japan
    - O157, O26, and O111 reagents
  - Oxoid Inc. (Division of Thermo Fisher Scientific Inc., Waltham, MA)
    - O157, O26, O91, O103, O111, and O145 reagents
  - ProLab Diagnostics, Inc., Richmond Hill, Ontario, Canada
    - O157, H7, O26, O45, O103, O111, O121, and O145 reagents
  - Remel, Inc., Lenexa, KS
    - O157 and H7 reagents
- Immunomagnetic beads
  - Invitrogen Corporation, Carlsbad, CA
    - Dynabeads anti-O157-, anti-O26-, anti-O103-, anti-O111-, and anti-O145-labeled beads
  - Denka Seiken Co., Ltd., Tokyo, Japan
    - Anti-O157-, anti-O26-, and anti-O111-labeled beads
- O157 immunoassays
  - Meridian Bioscience, Inc., Cincinnati, OH
    - For testing stool specimens or enrichment broths for O157 antigen
  - Denka Seiken Co., Ltd., Tokyo, Japan
    - For testing colony sweeps or individual colonies for O157, O111, or O26 antigens
- Shiga toxin immunoassays
  - Meridian Bioscience, Inc., Cincinnati, OH
    - For testing stool specimens, enrichment broths, colony sweeps, or individual colonies for Shiga toxin
  - Remel, Inc., Lenexa, KS
    - For testing stool specimens or enrichment broths for Shiga toxin
  - Denka Seiken Co., Ltd., Tokyo, Japan
    - For testing colony sweeps or individual colonies for Shiga toxin
  - Merck KGaA, Darmstadt, Germany
    - For testing stool specimens or individual colonies for Shiga toxin
  - Alere Inc., Waltham, MA
    - For direct stool testing and enrichment broths for Shiga toxin
- Chromogenic agars (for visual detection of O157:H7 colonies upon direct inoculation of agar plates)
  - Merck KGaA, Darmstadt, Germany
  - bioMérieux Inc., Hazelwood, MO
  - Biosynth International, Inc., Naperville, IL
  - Biolog, Inc., Hayward, CA
  - Becton Dickinson and Co., Franklin Lakes, NJ
    - Holds license to CHROMagar

[a] This table is not intended to be a comprehensive listing. The FDA has not cleared all of these reagents for use with clinical specimens. This table does not include reagents or tests specifically intended for examination of food, water, or environmental specimens. The online version of the *Bacteriological Analytical Manual* lists many tests for food specimens (http://www.fda.gov). Inclusion does not constitute endorsement by the CDC or ASM.

genes but from which O157 STEC strains are not recovered should be forwarded by the diagnostic laboratory to the appropriate public health laboratory to attempt to obtain a STEC isolate (93).

Isolation Procedures

Isolation Procedures for Extraintestinal *E. coli*

Isolation procedures for *E. coli* and other *Escherichia* species from sites outside the intestines are covered in chapter 18 in this *Manual*. Any *E. coli* strain isolated in large numbers (particularly >10^5 CFU/ml of urine) or from normally sterile body sites should be considered a potential pathogen. Although *E. coli* strains with particular virulence in the urinary tract cannot be easily distinguished on differential and selective plating media from organisms of lower virulence, they are commonly hemolytic on sheep blood agar and express one or more of several urinary tract adhesins. Several chromogenic media have been proposed for use in detecting UPEC; these have been compared in published studies (94, 95).

Isolation Procedures for Diarrheagenic *E. coli*

Isolation Procedures for STEC

Guidelines published in 2009 (93) recommend that all stools submitted for testing for routine enteric pathogens (*Salmonella*, *Shigella*, and *Campylobacter*) at clinical diagnostic laboratories and all patients with suspected HUS should be cultured for O157 STEC on selective and differential agar and assayed for non-O157 STEC with a test that detects the Shiga toxins or genes encoding these toxins. The recommendations to isolate O157 STEC and detect non-O157 STEC were extended to all stools from patients with acute, community-acquired diarrhea because selective testing strategies such as testing only bloody stools or specimens from children, limiting testing to summer months, or basing testing on the presence of indicators such as white blood cells will miss many STEC infections. The absence of blood in the stool does not negate the possibility of a STEC-associated diarrheal illness (96–98). In several studies, most STEC isolates, including both O157 and non-O157 strains, were from patients with apparently nonbloody diarrhea (93, 99–102). Also, STEC strains are isolated more frequently from children, but almost half of all isolates are obtained from persons >12 years of age (51, 102–104), so limiting STEC testing to children would miss many infections. Although STEC infections are more common in the summer months, sporadic cases and outbreaks do occur year-round (51, 53, 101). Leukocytes are often but not invariably observed in the stools of patients with STEC infection; thus, determination of white blood cells in stool should not be used as a criterion for STEC specimen selection (51, 105).

Enrichment. Although broth enrichment is widely used for the recovery of O157 STEC from foods, there is little evidence that it enhances isolation from human fecal specimens. However, immunomagnetic separation (IMS), a technique shown to increase the rate of isolation of O157 STEC from food specimens, has been adapted to culture of fecal specimens (106). IMS enhances the detection of O157 STEC from patients with HUS, patients presenting an extended period of time after the onset of illness (>5 days), asymptomatic carriers, or specimens that have been stored or transported improperly. IMS beads for O157, O111, and O26 are available commercially (Table 3), or laboratories may produce beads with other O-specific antibodies (107).

Plating media. Because O157 STEC strains ferment lactose, they are impossible to differentiate from other lactose-fermenting organisms on lactose-containing media. Most O157 STEC strains do not ferment the carbohydrate D-sorbitol overnight, in contrast to the ~80% of other *E. coli* strains that ferment sorbitol rapidly. Thus, sorbitol-containing selective media are often used for isolation of O157 STEC. Sorbitol-nonfermenting colonies are suspected (but not definitively known) to be *E. coli* O157:H7 (108). In some areas of central Europe, sorbitol-fermenting O157 STEC strains are commonly isolated from patients with HUS (109); these organisms are very rare in North America (Strockbine, unpublished data).

Specific culture media have been developed to exploit phenotypic and antibiotic resistance traits that are characteristic of STEC strains. Although sorbitol-containing MAC (SMAC) is widely used, cefixime-tellurite-containing SMAC (CT-SMAC) and CHROMAgar O157 have been shown to increase the sensitivity of culture for O157 STEC (110). It has been reported that some nonmotile strains of O157 STEC fail to grow on CT-SMAC (106). Several chromogenic agar media are available commercially to assist in rapid identification (Table 3); these media generally perform well for O157:H7 and for some non-O157 STEC strains (111–114).

Screening procedures for STEC strains. For the isolation of O157 STEC from SMAC, colorless (nonfermenting) colonies are tested with O157 antiserum or latex reagent (115) (Table 3). If the O157 latex reagent is used, it is important to test positive colonies with the latex control reagent to rule out nonspecific reactions. The manufacturers of these kits recommend that strains that react with both the antigen-specific and control latex reagents be heated and retested. However, in a study that followed this procedure, none of the nonspecifically reacting strains were subsequently identified as O157 STEC (116).

Unlike most other *E. coli* strains, O157 STEC strains do not express β-glucuronidase; therefore, the MUG reaction (4-methylumbelliferryl-β-D-glucuronide for detection of β-glucuronidase activity) is helpful for screening for O157 STEC (117). MUG-positive, urease-positive O157 STEC strains have been isolated in the United States but are still rare (118; Strockbine, unpublished data).

For the recovery of STEC strains from stool specimens that test positive for Shiga toxin, CT-SMAC, CHROMagar O157, or a similar selective agar for isolation of O157 STEC should be used. If sorbitol-nonfermenting colonies are negative with O157 latex, then sorbitol-fermenting colonies (because most non-O157 STEC strains ferment sorbitol) and a representative sample of sorbitol-nonfermenting colonies may be selected for Shiga toxin testing. Latex reagents and antisera (Table 3) for detecting certain non-O157 STEC serotypes are now available and could also be used to test colonies from Shiga toxin-positive specimens or to serogroup Shiga toxin-positive isolates.

Virtually all O157 STEC strains and 60 to 80% of non-O157 STEC strains produce a characteristic *E. coli* hemolysin, referred to as enterohemolysin (Ehly), which is distinct from the alpha-hemolysin produced by other *E. coli* strains (119). Washed sheep blood agar supplemented with calcium (WSBA-Ca) is used as a differential medium for the detection of enterohemolytic activity (119). Ehly-producing colonies can be differentiated from alpha-hemolysin-producing colonies on WSBA-Ca because the latter are visible after 3 to 4 h of incubation. After 3 to 4 h, colonies are marked for the appearance of alpha-hemolysin, and the plates are examined again after 18 to 24 h. Incorporation of mitomycin C into WSBA-Ca enhances the appearance of the Ehly hemolysis and increases the proportion of non-O157 STEC strains that exhibit this activity (120). Because some non-O157 STEC strains do not demonstrate the enterohemolytic phenotype, nonhemolytic colonies should also be screened for Shiga toxin (121).

Presumptive STEC isolates should be sent to a reference laboratory or a public health laboratory for further characterization.

Isolation Procedures for Other Diarrheagenic *E. coli*

Methods for the isolation of ETEC, EPEC, EIEC, EAEC, and putative diarrheagenic *E. coli* strains are generally available only in reference or research settings. Public health and reference laboratories usually examine specimens for these pathogens only when an outbreak has occurred and specimens are negative for routine bacterial pathogens. ETEC and EAEC should be considered possible etiologic agents of watery diarrhea when no other pathogen has been identified, especially for travelers. EPEC should be considered a possible pathogen in outbreaks of severe nonbloody diarrhea occurring in infants or young toddlers, particularly in nursery or day care settings (65). EIEC should be considered a possible etiologic agent in outbreaks of nonwatery diarrhea (bloody or nonbloody).

To capture *E. coli* for further testing, fecal specimens should be plated on a differential medium of low selectivity (e.g., MAC). Five to 20 colonies, mostly lactose fermenting but with a representative sample of nonfermenting colonies, should be selected and inoculated onto nonselective agar slants (such as L agar or nutrient agar). These colonies are then sent to a reference laboratory for testing or are screened for virulence-associated characteristics if assays are available. Strains can be kept frozen for long periods in L broth with 15 to 50% glycerol at −80°C. Arrangements for sending *E. coli* isolates from well-characterized outbreaks to the CDC for testing can be made through local and state health departments.

Screening procedures for ETEC, EPEC, EAEC, and EIEC strains. *E. coli* pathotypes other than STEC cannot be distinguished from other *E. coli* strains by phenotypic screening techniques. Many EIEC strains are nonmotile and fail to decarboxylate lysine; however, some EIEC strains are motile or lysine positive. Use of commercial antisera to the classical EPEC somatic (O) and capsular (K) antigens to detect EPEC is no longer recommended due to the number of false positives this approach yields.

Identification

Phenotypic Identification

With the exception of *E. albertii*, the commercial identification systems do a good job of identifying most *Escherichia* strains (122–124). Identification of *E. albertii* with these systems remains problematic because representative strains of this species are not yet included in commercial databases (125). Abbott and colleagues, who extensively characterized five strains of *E. albertii* by conventional phenotypic methods and by commercial identification panels, reported that *E. albertii* is an indole-negative species that ferments D-mannitol but not D-xylose (125). In their study, *E. albertii* strains were identified by commercial systems as *Hafnia alvei*, *Salmonella* or *Salmonella enterica* serotype Choleraesuis, *E. coli* (inactive or serotype O157:H7), or *Yersinia ruckeri*. Although some strains were clearly misidentified, the majority of the strains generated probability scores for the final identification that were unacceptable, or the identification was inconsistent with the source of the specimen (e.g., identification of the fish pathogen *Y. ruckeri* from a human specimen), which should have triggered additional phenotypic tests to establish a more reliable identification. The authors found that the most reliable clue to the possible presence of *E. albertii* was an unacceptable first-choice identification of *H. alvei* for an isolate that is both L-rhamnose and D-xylose negative.

Phenotypic tests that can help discriminate *E. albertii* strains from selected members of the *Enterobacteriaceae* family with similar phenotypic traits are shown in Table 1. Two biogroups of *E. albertii* are listed in Table 1. These correlate with two of the distinct clusters of strains identified in the *E. albertii* lineage by phylogenetic studies (8). Biogroup 1 comprises the five strains isolated from Bangladeshi children with diarrhea, while biogroup 2 comprises strains formerly identified as *S. boydii* 13. The strains in the two biogroups differ from each other in their abilities to produce indole from tryptophan, decarboxylate lysine, and ferment D-sorbitol. Antigenic relationships between members of the *E. albertii* lineage and other members of the *Enterobacteriaceae* family have been observed (e.g., *S. boydii* 7 and *E. coli* O28). A diagnostic PCR assay using three housekeeping genes was described by Hyma et al. (8) for *E. albertii*; this assay is independent of phenotypic or antigenic traits and should facilitate studies to learn about the diversity within the lineage, the natural habitat of the species, and its role in enteric disease.

The identification of presumptive O157 STEC isolates is necessary because other species may cross-react with O157 antiserum or latex reagents, including *Salmonella* O group N (O:30), *Yersinia enterocolitica* serotype O9, *Citrobacter freundii*, and *E. hermannii*. Additional phenotypic tests (cellobiose fermentation and growth in the presence of potassium cyanide [KCN]) may be necessary to differentiate *E. hermannii* from *E. coli*, but because *E. hermannii* is rarely detected in stool specimens, use of these tests is not cost-effective for most laboratories.

In recent years, matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) has been adopted in many clinical laboratories for identification. The method is rapid, is substantially less expensive than conventional culture methods, and has performed well in identifying *E. coli* to the species level (126–129). It holds promise for the identification of other *Escherichia* species (*E. hermannii*, *E. vulneris*, and *E. fergusonii*) to the species level, but not unexpectedly, it was unable to distinguish *Shigella* from *E. coli* (126, 127). Clark et al. (128) recently reported success using custom evaluation criteria (informative spectral peaks) as a screening tool to differentiate most *E. coli* pathotypes for subsequent testing.

In response to the growing problem of multidrug-resistant bacteria, labs are seeking rapid methods to screen bacteria to identify resistance mechanisms to inform and expedite the management of patients to control the spread of resistance. Several groups recently reported MS methods to rapidly test for antimicrobial resistance mechanisms. Jung et al. (130) described a MALDI-TOF MS assay to detect β-lactamase activity against aminopenicillins. The assay works by detecting a specific pattern of fragments generated from the digestion of the drug by the resistance-conferring enzyme. The assay took just 2.5 h to complete and had a sensitivity and specificity of 100 and 91.5%, respectively, when evaluated on a set of 100 isolates. Another assay using MALDI-TOF MS to detect class D carbapenemase OXA-48-like-producing *Enterobacteriaceae* was reported by Sauget et al. (131). This assay had a turnaround time of 90 min and a sensitivity and specificity of 98.9 and 97.8%, respectively, when tested on 372 strains. A third assay that uses liquid chromatography-tandem MS to detect carbapenemase KPC, NDM, OXA, IMP, and VIM activity produced against imipenem, meropenem, and ertapenem was described by Kulkarni et al. (132). The sensitivity and specificity of the assay were best with imipenem—96 and 95%, respectively, for a group of 402 Gram-negative bacteria—and the assay took only 75 min to complete. These assays offer valuable information quickly to facilitate interventions to limit the dissemination of resistant organisms and prevent outbreaks. Additional information on the use of MS to detect antibiotic resistance mechanisms is reviewed by Hrabak et al. (133).

Serotyping

The serologic classification of *E. coli* is generally based on the O antigen (somatic) and the H antigen (flagellar) (134). The O and H antigens of *E. coli* are stable and reliable strain characteristics, and although 181 O antigens and 56 H antigens have been described (a few of which are no longer recognized), the actual number of serotype combinations associated with diarrheal disease is limited (Table 2). Determination of the O and H serotypes of *E. coli* strains implicated in diarrheal disease is particularly useful in epidemiologic investigations (Table 2). Even though antisera for microtiter or tube agglutination tests are available from manufacturers, most laboratories do not attempt to perform complete *E. coli* serotyping because it is costly. For well-characterized outbreaks with no identified etiologic agent, arrangements may be made through state health departments to send *E. coli* isolates to the CDC for virulence testing and serotyping.

Serologic Confirmation of O157 STEC

Confirmation of *E. coli* O157:H7 requires identification of the H7 flagellar antigen. H7-specific antisera and latex reagents are commercially available (Table 3), but detection of the H7 flagellar antigen often requires multiple passages (115). Isolates that are nonmotile or negative for the H7 antigen should be tested for the production of Shiga toxins or the presence of Shiga toxin gene sequences.

Approximately 85% of O157 isolates from humans received by the CDC are serotype O157:H7, 12% are nonmotile, and 3% are H types other than H7 (Strockbine, unpublished data). *E. coli* O157:NM strains often produce Shiga toxin, and those that do produce Shiga toxin have the same *fliC* restriction profile as that observed for *E. coli* O157:H7 (135). No O157 strain from human illness with an H type other than H7 has been found to produce Shiga toxin (Strockbine, unpublished data).

Nucleic Acid-Based Methods

Accurate identification of bacterial isolates is important for directing patient care and management. Compared to traditional phenotypic approaches, which can be influenced by phenotypic variation or subjective interpretation, 16S rRNA gene sequencing is a more objective identification tool and has the potential to reduce laboratory errors. Some clinical laboratories have begun using molecular methods to aid in the identification of organisms that cannot be cultivated due to unusual growth characteristics or antibiotic treatment or cannot be classified by phenotypic methods (136–141). In one study, results obtained with 16S rRNA gene sequencing and the SmartGene IDNS (Zug, Switzerland) database and software compared favorably to those obtained by conventional phenotypic methods and were better than those obtained with a similar rRNA gene method employing a smaller database for a collection of 300 clinical isolates (137). The performance differences between the two 16S rRNA gene methods highlight the importance of the size and breadth of the database for successful classification. Difficulties in separating *E. coli* from *Shigella* with 16S rRNA gene sequencing should be expected. These are genetically the same species and have been maintained as separate taxa for medical expediency. The limited findings reported in the studies above and those reported by others (142) show that a small region of the 16S rRNA gene alone will not provide reliable separation of certain medically relevant members of the *Enterobacteriaceae* family (*E. coli*/*S. sonnei* [137] and *Escherichia*/*Shigella*/*Hafnia* [136]). The incorporation of virulence genes that define the *Shigella*/EIEC pathotype should help discriminate it from noninvasive pathotypes or communal *E. coli*. Another approach that has potential to improve microbial identification involves MS. The Ibis T5000 Biosensor System (Abbott Molecular, Des Plains, IL), which is currently used in nonclinical and research settings, uses multiple regions of the 16S and 23S rRNA genes plus several housekeeping genes to discriminate between species within 6 h (143). Validation studies are needed to assess the performance of this technology on clinical specimens.

Simple and cost-effective strategies to use whole-genome sequencing to differentiate bacterial species, determine antibiotic resistance potential, detect virulence determinants, and conduct pathogen surveillance for outbreak detection are developing rapidly. The application of this technology in the clinical microbiology setting is reviewed by Didelot et al. (144), who highlight its recent successful use to rapidly characterize the novel *E. coli* O104:H4 variant that caused the 2011 outbreak of bloody diarrhea in Germany and France (145).

Virulence Testing

Extraintestinal *E. coli*

Numerous virulence factors have been identified for extraintestinal *E. coli* (43), particularly the K1 antigen, but these are usually identified only in epidemiologic studies.

Diarrheagenic *E. coli*

Detection of diarrheagenic pathotypes is typically performed on *E. coli* colonies chosen from selective or nonselective media. If PCR techniques are used, a sweep of confluent growth from a MAC plate may be screened; if the PCR assay is positive, isolated colonies may then be picked and screened individually. Multiplex PCR assays are capable of simultaneously detecting multiple *E. coli* pathotypes (146).

STEC. Two distinct Shiga toxins, Stx1 and Stx2, also referred to as verocytotoxins 1 and 2, have been described in *E. coli*. STEC may produce either Stx1 or Stx2 or both toxins. The toxin produced by *Shigella dysenteriae* serotype 1 was recognized first and is designated Stx, while the toxins in *E. coli* include an Arabic number in association with "Stx." The Shiga toxins comprise a family of toxins that have similar biologic, genetic, and structural features. Stx and Stx1 are essentially identical. Subtypes of Stx1 and Stx2 have been identified, and a standardized nomenclature for the toxins and PCR protocols for their detection were recently published by Scheutz et al. (147). The toxin subtypes vary in their association with severe disease, with Stx2a, Stx2c, and Stx2d being associated with bloody diarrhea and HUS (148–150); however, host factors, dose, and other bacterial factors play a role in the severity of disease.

The production of Stx or the genes encoding Stx can be detected by a variety of biologic, immunologic, or nucleic acid-based assays (46, 151). FDA-approved diagnostic PCR assays for STEC that discriminate between the two toxin types are now available (see "Nucleic Acid Detection" above). Stx has also been directly detected in the blood of HUS patients by use of flow cytometry, even in the absence of serologic or microbiologic evidence of STEC infection (152).

STEC strains represent a spectrum of virulence potentials, ranging from the highly virulent O157:H7 serotype that has been responsible for the majority of outbreak cases to low-virulence serotypes that have been isolated only from nonhuman sources. The presence of additional virulence factors other than Stx correlates with disease potential. The most important of these virulence factors are the intimin adhesin and the type III secretion system encoded by the LEE pathogenicity island (43). The *eae* gene probe for intimin and the *hlyA* (*E-hlyA or ehxA*) gene probe for a plasmid-encoded hemolysin have been the most frequently employed methods to determine virulence potential, but probes for at least 25 different virulence-associated genes have been employed to characterize STEC strains (153, 154). STEC strains have been classified into five "seropathotypes" (A through E) based on the occurrence of serotypes in human disease, in outbreaks, and in severe disease (HUS or hemorrhagic colitis) and on possession of specific virulence genes (155).

ETEC. The ST and LT enterotoxins produced by ETEC may be detected by a variety of biologic, immunologic, and nucleic acid-based assays (46). Two distinct ST variants (STh and STp) have been identified in human strains. Strains that produce ST only or ST in combination with LT have caused most ETEC outbreaks in the United States (55).

Immunoassays for the identification of ST or LT in culture supernatants of ETEC strains are available from at least two commercial sources (Table 3). The ST EIA assay (Denka Seiken Co., Ltd., Tokyo, Japan; and Oxoid Ltd., Basingstoke, United Kingdom) is a competitive enzyme immunoassay for the detection of ST only (156). A reversed passive latex agglutination assay (VET-RPLA; Oxoid [a similar kit is available from Denka Seiken]) detects both cholera toxin and LT, which are highly related antigenically. The effectiveness of the VET-RPLA may be optimized by use of a culture medium designed for LT production, such as Biken's medium, rather than the medium recommended by the manufacturer (157).

EPEC. EPEC, EAEC, and DAEC can be detected by their characteristic patterns of adherence to HEp-2 or HeLa cells in culture (57). These patterns are also observed on formalin- or glutaraldehyde-fixed cells, obviating the need to prepare cells expressly for the assay (158).

EPEC strains are defined on the basis of the A/E histopathology produced on epithelial cells and the lack of Stx (reviewed in references 58 and 43). The A/E phenotype can be detected by tissue culture cell assays or by DNA probe or PCR tests for the *eae* gene, encoding intimin, or the LEE pathogenicity island. The EAF plasmid of typical EPEC (see above) is detected by use of fragment or oligonucleotide probes or PCR primers (46). Atypical EPEC strains possess only the A/E phenotype (LEE pathogenicity island) but do not possess the EAF plasmid. It is likely that only a subset of atypical EPEC strains comprise true human pathogens, although no tests can reliably identify pathogenic isolates.

EAEC. Several simple assays have been described as surrogates for the cell adherence test for identification of EAEC. These include a simple biofilm formation assay on polystyrene (159) and screening for the presence of a pellicle at the surface of broth media (160). EAEC can be identified more definitively by use of a specific DNA probe (the AA or CVD432 probe) (161), which is superior to tissue culture adherence assays in identifying pathogenic strains of EAEC (65). More recent data suggest that the AA probe corresponds to a putative virulence gene called *aatA* (162), which is under the control of a regulator termed AggR. AggR, in turn, controls several other virulence factors (66). Thus, the *aggR* gene (which defines typical EAEC) may represent a superior diagnostic target, although data to support this notion are not available.

EIEC. EIEC can be identified by various *in vivo* assays, immunoassays, and nucleic acid-based assays for invasiveness. EIEC can be detected along with *Shigella* with two commercial kits, one of which targets the invasion plasmid-associated gene *ipaH* (See "Nucleic Acid Detection" above). Cell culture invasion assays or DNA-based assays for other invasion-related factors are, for the most part, practical only in research settings (46). Plasmid DNA electrophoresis may be used to detect the large, 120- to 140-MDa plasmid associated with invasiveness, but this plasmid is easily lost when the isolate is subcultured. Because of shared invasiveness-related characteristics, these assays also detect *Shigella* strains.

DAEC. DAEC strains were initially defined on the basis of a diffuse adherence pattern to cultured epithelial cells, but this phenotype is not specific for enteric strains (70). Various DNA probes and PCR assays have been proposed for DAEC identification, as reviewed previously (46).

Typing Systems

Several methods for subtyping have been used for *E. coli* O157:H7 isolates. In particular, pulsed-field gel electrophoresis (PFGE) methods and multilocus variable-number tandem-repeat analysis (MLVA) methods are useful (46, 163). A national molecular subtyping network, PulseNet, was established in 1996 by the CDC to facilitate subtyping of bacterial foodborne pathogens, including *E. coli* O157:H7, *Shigella*, nontyphoidal *Salmonella* serotypes, and *Listeria monocytogenes* (164). Successful detection of outbreaks by this network of state and local public health laboratories is dependent on submission of isolates by clinical laboratories for confirmation and subtyping.

Determination of the serotype and the antimicrobial susceptibility pattern is usually adequate for defining outbreak strains of ETEC, EPEC, and EIEC. Plasmid typing or PFGE methods may also be helpful for distinguishing between sporadic isolates and outbreak strains, but neither method has been widely used for these groups of *E. coli*.

Serologic Tests

At present, serodiagnostic tests for diarrheagenic *E. coli* are valuable only for seroepidemiology surveys and are not useful for the diagnosis of sporadic infections. Assays that measure serum antibody response to LPS have been used to detect STEC infection in culture-negative HUS patients (46). Enzyme-linked immunosorbent assays have been described to detect saliva antibodies to LPS (165) and serum antibodies to the secreted EspB protein in HUS patients (166).

Antimicrobial Susceptibilities

Extraintestinal *E. coli*

In the past 20 years, *E. coli* strains producing CTX-M β-lactamases and AmpC β-lactamases have emerged as significant causes of extraintestinal infections in the United States and globally (35, 167–170). CTX-M-producing *E. coli* strains are often isolated from urinary tract infections, both health care and community acquired, and have also been detected in retail meat samples in the United States (35). Recently, a single pandemic clone of multiresistant *E. coli* ST131 has emerged as the predominant extended-spectrum β-lactamase (ESBL)-positive *E. coli* strain isolated from urinary tract infections and bacteremia in hospitalized patients and community-acquired infections in the elderly (40, 169–171). In addition to being resistant to fluoroquinolones, *E. coli* ST131 is often associated with the CTX-M-15 ESBL (169) and often possesses multiple virulence genes (*iha*, *sat*, and *iutA*).

AmpC β-lactamases are problematic for clinical laboratories because these enzymes can interfere with ESBL confirmatory tests, resulting in a false report of cephalosporin susceptibility (169, 170). The AmpC β-lactamase CMY-2 has the broadest geographic distribution and has been found in *E. coli* and other *Enterobacteriaceae* (reviewed by Jacoby [172]).

Carbapenemases are β-lactamases that confer resistance to the carbapenems, and the *Klebsiella pneumoniae* carbapenemase (KPC) is the most frequently encountered enzyme of this class in *E. coli* and other carbapenem-resistant *Enterobacteriaceae*. It is important to detect KPC and other carbapenemases in patients colonized with carbapenem-resistant *Enterobacteriaceae* so that isolation precautions may be instituted to prevent transmission in health care settings (173). Until laboratories can implement the carbapenem interpretive criteria published in the 2014 CLSI guidelines (174), performance of the modified Hodge test (MHT) as described in the guidelines (Table 3C) is advised. If the 2014 CLSI interpretive criteria are used, the MHT does not need to be performed other than for epidemiologic or infection control purposes and no change in the interpretation of carbapenem susceptibility test results is required for MHT-positive isolates. Results of the MHT should be reported to infection control or those requesting epidemiologic information.

When identifying carbapenemase-producing isolates for infection control or epidemiologic purposes, a targeted strategy for testing isolates can be used. Selection of isolates for testing in the MHT with the following characteristics is suggested: intermediate or resistant to one or more carbapenems when using the CLSI 2014 interpretive criteria (ertapenem nonsusceptibility is the most sensitive indicator of carbapenemase production) and resistant to one or more agents in cephalosporin subclass III (174).

Current automated susceptibility test systems may not be able to accurately detect ESBLs, AmpCs, and KPCs (175, 176). Issues surrounding the testing for these enzymes are reviewed by Thomson (177).

In January 2010, the CLSI published interpretative criteria for phenotypically assessing the susceptibility of the *Enterobacteriaceae* to the cephalosporins and aztreonam (178). Under these guidelines, lower breakpoints were recommended, thereby eliminating the need to perform routine ESBL tests and to edit the results on reports from susceptible to resistant for cephalosporins, aztreonam, or penicillins. No reduction in breakpoints was proposed at that time for cefuroxime (parenteral) and cefepime; however, in 2014, the CLSI substantially revised only the cefepime interpretive criteria (breakpoints) and introduced the "susceptible-dose dependent" (SDD) category to replace the "intermediate" category (174). The SDD interpretive category, which has been used successfully to guide antifungal therapy, is a new category for antibacterial susceptibility testing that implies that the susceptibility of an organism is dependent on the dosing regimen that is used in the patient. The CLSI recommends that the following comment be reported with the new cefepime breakpoints: "The interpretive criterion for susceptible is based on a dosage regimen of 1 gram every 12 hours. The interpretive criterion for susceptible-dose dependent is based on dosing regimens that result in higher cefepime exposure, either higher doses or more frequent doses or both, up to approved maximum dosing regimens." Educating physicians and other stakeholders about the meaning of the SDD category will be important to maximizing the useful life span of cefepime. Additional guidance for implementing the new criteria is discussed in the 2014 CLSI guidelines (174).

Until the FDA revises its breakpoints for manufacturers to update their instruments, laboratories using automated antimicrobial susceptibility devices are advised to consult with manufacturers for guidance on how to manually adjust the cefepime breakpoints. A validation study should be performed when altering manufacturers' settings, and guidelines for performing such a study are described by Clark et al. (179). Implementation of the revised breakpoints and SDD category currently applies only to cefepime susceptibility testing of members of the *Enterobacteriaceae*. The SDD category has been adopted due to the growing need to refine susceptibility reporting to maximize clinicians' use of available drugs.

The CLSI suggests that routine antimicrobial susceptibility testing for *Enterobacteriaceae* include ampicllin, cefazolin (MIC only), gentamicin, and tobramycin testing. Urinary isolates of *E. coli* may be tested against fosfomycin and other drugs used only for these infections (trimethoprim-sulfamethoxazole). For *E. coli* and other *Enterobacteriaceae* isolates recovered from cerebrospinal fluid (CSF), cefotaxime or ceftriaxone should be tested and reported in place of cefazolin. The following antimicrobial agents should not be routinely reported for *E. coli* or other *Enterobacteriaceae* isolated from CSF because they are not the drugs of choice and may not be effective for treating CSF infections: agents administered by the oral route only, first- and second-generation cephalosporins (except cefuroxime parenteral), cephamycins, clindamycin, macrolides, tetracyclines, and fluoroquinolones.

Diarrheagenic *E. coli*

STEC

Antimicrobial therapy for O157 STEC diarrhea or HUS is controversial: some publications have suggested that antibiotics increase the risk of HUS (48, 180), while a meta-analysis of published reports found no significantly increased risk (181). There is a lack of evidence to support routine antimicrobial susceptibility testing of STEC strains.

Until recently, *E. coli* O157:H7 isolates were almost uniformly susceptible to antimicrobial agents. However, since the early 1990s, O157 and other STEC strains have demonstrated slowly increasing levels of resistance to certain antibiotics, particularly streptomycin, sulfonamides, and tetracycline (http://www.cdc.gov/narms/index.html).

ETEC, EPEC, EAEC, and EIEC Strains and Other Diarrheagenic *E. coli* Strains

Treatment with an appropriate antibiotic can reduce the severity and duration of symptoms of ETEC infection (46). Antimicrobial resistance, particularly to tetracycline, is common among ETEC strains isolated from outbreaks in the United States (55). Antibiotic treatment may be helpful for diarrhea caused by EPEC (46). Most EPEC strains associated with outbreaks are resistant to multiple antimicrobial agents (58). EAEC strains are commonly resistant to most antibiotics, though these strains are typically susceptible to fluoroquinolones. Clinical studies have demonstrated the effectiveness of ciprofloxacin for travelers with diarrhea caused by EAEC (182). Little information about the efficacy of antimicrobial treatment or the prevalence of resistance is available for EIEC or other putative diarrheagenic *E. coli* strains, but determination of the antimicrobial susceptibility pattern may be helpful in establishing whether the isolates are associated with an outbreak.

Evaluation, Interpretation, and Reporting of Results

Extraintestinal *E. coli*

The final written report should include the final Gram stain result, the final identification as *E. coli*, and the antimicrobial susceptibility test results. Any supplemental tests used to detect resistance markers like KPCs should be highlighted. Laboratories should determine what resistance markers are important for institutional infection control practices.

Diarrheagenic *E. coli*

STEC

A presumptive diagnosis of an O157 STEC (isolate positive for O157 antigen) or a non-O157 STEC (isolate positive for Shiga toxin) infection should be reported to the clinician as soon as the laboratory obtains this result. The 2009 recommendations for clinical diagnosis of STEC include examples of how to word the final report for results from testing for Shiga toxin immunoassays or PCR for Shiga toxin genes as well as culture results (93; http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5812a1.htm [Table 2]). When O157 STEC is not found in a specimen, it is advisable to include a comment in reports stating that non-O157 STEC strains can cause diarrhea and HUS. Cases of STEC infection and HUS should be reported to public health authorities. O157 STEC isolates or specimens in which Shiga toxin or STEC genes are detected (e.g., stool, other primary specimens, and enrichment broths) should be forwarded to a local or state public health laboratory (93).

ETEC, EPEC, EAEC, and EIEC Strains

Generally, the ETEC, EPEC, EAEC, and EIEC classes of diarrheagenic *E. coli* are identified only during outbreak investigations. A laboratory reporting these results, which usually will be a retrospective diagnosis obtained by a reference laboratory, should provide an explanation of the clinical significance of these organisms and may refer the clinician to the reference laboratory for further information. All suspected outbreaks should be reported to public health authorities.

SHIGELLA

Taxonomy

Shigella is classified in the family *Enterobacteriaceae*, which is addressed in chapter 38 of this *Manual* (2, 3). There are four subgroups of *Shigella* that historically have been treated as species: subgroup A as *S. dysenteriae*, subgroup B as *Shigella flexneri*, subgroup C as *S. boydii*, and subgroup D as *Shigella sonnei*. From a genetic standpoint, the four species of *Shigella*, with the exception of *S. boydii* 13, and *E. coli* represent a single genomospecies (183–185). Using a genetic definition for species, the four species of *Shigella* would be regarded as serologically defined anaerogenic biotypes of *E. coli*. The current nomenclature for *Shigella* organisms is maintained largely for medical purposes because of the useful association of the genus epithet with the distinctive disease (shigellosis) caused by these organisms. The G+C content of the DNA is 49 to 53 mol%, and the type species for the genus is *S. dysenteriae* (Shiga 1898) (5).

S. boydii 13 strains were first described in 1952 and then added to the *Shigella* scheme in 1958 (186). Early findings from DNA-DNA hybridization showed that these strains represent a new species (185); however, it was not until recently that findings from phylogenetic studies showed that they cluster in a neighbor-joining tree with *E. albertii*, a newly described species of *Escherichia* associated with diarrheal disease in Bangladeshi children (8, 30).

Description of the Genus

The genus *Shigella* is composed of nonmotile bacteria that conform to the definition of the family *Enterobacteriaceae* (2, 187). Species in this genus are Gram-negative rods that grow well on MAC. All strains of *Shigella* spp. are nonmotile; do not decarboxylate lysine; do not utilize citrate, malonate, or sodium acetate (with exceptions for *S. flexneri*); and do not grow in KCN or produce H_2S. Compared with *Escherichia*, *Shigella* strains are less active in their use of carbohydrates (Table 4). All ferment D-glucose without the production of gas (a few exceptions produce gas, e.g., certain strains of *S. flexneri* serotype 6 and *S. boydii* serotype 14). *S. sonnei* strains ferment lactose and sucrose on extended incubation, but other species generally do not use these substrates in conventional medium. Salicin, adonitol, and *myo*-inositol are not fermented. There are numerous identical and reciprocal serologic reactions between *Shigella* and *E. coli* (188).

Epidemiology and Transmission

Humans and other large primates are the only natural reservoirs of *Shigella* bacteria. Most transmission is by person-to-person spread, but infection is also caused by ingestion of contaminated food or water. Shigellosis is most common in situations in which hygiene is compromised (e.g., child care centers and other institutional settings). In developing populations without running water and indoor plumbing, shigellosis can become endemic. Sexual transmission of *Shigella* among men who have sex with men also occurs.

In the United States, an estimated 495,000 cases of shigellosis occur each year, with 38 deaths (47). Up to 15% of all U.S. cases of shigellosis are related to international travel. Most infections in the United States and other developed countries are caused by *S. sonnei*; *S. flexneri* is the second-most-common subgroup (http://www.cdc.gov/nationalsurveillance/shigella-surveillance.html). In the developing world, the majority of endemic dysentery cases are caused by *S. flexneri*, with the balance of cases caused by subgroups that vary temporally and geographically (189–191). Epidemic dysentery is most commonly caused by *S. dysenteriae* 1, whose prevalence rises dramatically during outbreak periods and then falls as the epidemic resolves. Infection with *S. dysenteriae* 1 is associated with high rates of morbidity and mortality in developing countries, particularly when antimicrobial resistance or misdiagnosis delays appropriate treatment. In the United States and other developed countries, *S. sonnei* is endemic and causes large, protracted outbreaks in day care

TABLE 4 Differentiation of *E. coli* and *Shigella*

Result[a] of test with:	Test					
	Lysine decarboxylase	Motility	Gas from glucose	Acetate utilization	Mucate	Lactose
Shigella	−	−	−	−	−	−
Inactive *E. coli*[b]	d	−	−	d	d	d
E. coli	+	+	+	+	+	+

[a] Abbreviations and symbols: +, 90% or more positive within 2 days; −, no reaction (90% or more) in 7 days; d, different reactions [+, (+), +]. Adapted from Ewing (2).

[b] Nonmotile, anaerogenic biotypes sometimes referred to as Alkalescens-Dispar bioserotypes.

centers (192–194) and among men who have sex with men (195, 196). The protracted nature of these outbreaks is attributed to a large number of asymptomatically infected individuals in the population and the tendency for secondary spread (197). For most individuals, antibiotic treatment reduces the number of symptomatic days and the length of shedding (198); however, the decision to treat is nuanced and is influenced by a variety of factors, including severity of disease, immune status of the host, likelihood of transmission to others, preservation of the microbiome, and development of antibiotic resistance. Because resistance to ampicillin and trimethoprim-sulfamethoxazole is common in day care-associated *S. sonnei* infections, azithromycin has been recommended for treatment of pediatric shigellosis. The effectiveness of this recommendation will require careful monitoring in light of an outbreak in 2012 caused by a *S. sonnei* strain with reduced susceptibility to azithromycin (199).

Clinical Significance

Members of the genus *Shigella* have been recognized since the late 19th century as causative agents of bacillary dysentery. *Shigella* causes bloody diarrhea (dysentery) and nonbloody diarrhea. Shigellosis often begins with watery diarrhea accompanied by fever and abdominal cramps but may progress to classic dysentery with scant stools containing blood, mucus, and pus. Ulcerations, which are restricted to the large intestine and rectum, typically do not penetrate beyond the lamina propria. Bloodstream infections can occur but are rare. Appropriate antimicrobial therapy will decrease the duration, transmission, and severity of symptoms and is customarily prescribed based on the severity of illness or the need to protect close contacts. Patients in certain occupations (i.e., food handlers, child care providers, and health care workers) and children who attend day care often are required to have a documented negative stool culture following treatment. The infectious dose is low (1 to 100 organisms), and the incubation period is 1 to 4 days. Shigellae are shed in stools for several days to several weeks after illness, and persons who receive appropriate antimicrobial therapy will be culture negative at 72 h (198). All four subgroups of *Shigella* are capable of causing dysentery, but *S. dysenteriae* serotype 1 has been associated with a particularly severe form of illness thought to be related in part to its production of Shiga toxin. Infection can occasionally be asymptomatic, particularly infection with *S. sonnei* strains. Complications of shigellosis include HUS, which is associated with *S. dysenteriae* 1 infection, and reactive arthritis or Reiter's chronic arthritis syndrome, which is associated with *S. flexneri* infection (200). The identification of *Shigella* species is important for both clinical and epidemiologic purposes.

Collection, Transport, and Storage of Specimens

See "Collection, Transport, and Storage of Specimens" in the *Escherichia* section.

Direct Examination

Microscopy

Shigella cannot readily be distinguished from other Gram-negative rods by staining or microscopy methods.

Antigen Detection

Because there is no single somatic antigen common to all *Shigella* strains, antigen detection in clinical specimens is not practical and has not been validated, and no commercial FDA-approved kits are available.

Nucleic Acid Detection

There are four FDA-cleared nucleic acid detection methods for clinical diagnosis of *Shigella* infections. See "Nucleic Acid Detection" under "Direct Examination" in the *Escherichia* section for more information.

Isolation Procedures

Enrichment and Plating Media

There is no reliable enrichment medium for all *Shigella* isolates, but Gram-negative broth and Selenite broth (SEL) are frequently used. For the optimal isolation of *Shigella*, two different selective media should be used: a general purpose plating medium of low selectivity (e.g., MAC) and a more selective agar medium (e.g., xylose-lysine-deoxycholate agar [XLD]). Statens Serum Institut enteric medium, deoxycholate-citrate agar, and Hektoen enteric agar (HE) are suitable alternatives to XLD as media with moderate to high selectivities. Salmonella-shigella agar should be used with caution because it inhibits the growth of some strains of *S. dysenteriae* 1.

Screening Procedures

Shigella strains appear as lactose- or xylose-nonfermenting colonies on the isolation media described above. *S. dysenteriae* 1 colonies may be smaller on all of these media, and these strains generally grow best on media with low selectivities (e.g., MAC). *S. dysenteriae* 1 colonies on XLD agar are frequently very tiny, unlike those of other *Shigella* species. *S. sonnei* colonies often appear flattened and spread out on blood agar plates.

Suspect colonies may be screened phenotypically on Kligler iron agar (KIA) or triple sugar iron agar (TSI). *Shigella* species characteristically produce an alkaline slant because strains do not ferment lactose (or sucrose) and do not produce gas or H_2S. A few strains of *S. flexneri* 6 and a very few strains of *S. boydii* produce gas in KIA or TSI. The motility and lysine decarboxylase tests are characteristically negative for *Shigella* and can be used to further screen isolates before serologic testing (Table 4). Serologic testing should be performed as recommended by the manufacturer of the product being used. Due to the many antigenic cross-reactions between *E. coli* and *Shigella*, *Shigella* antisera are typically validated to be used in the identification of bacteria that biochemically conform to the definition of *Shigella*. For this reason, the initial screening of isolates for which there is no phenotypic evidence that they conform to the definition of *Shigella* is discouraged. Isolates that react appropriately with the screening tests should then be identified with a complete set of phenotypic tests, with automated systems or self-contained commercial kits being satisfactory, and should be tested with grouping antisera. Confirmation requires both phenotypic and serologic identification, and laboratories that do not perform both types of tests should send *Shigella* isolates to a reference laboratory for confirmation.

Identification

Phenotypic Identification

Shigellae are biopathotypes of *E. coli* that are identified conventionally using a combination of biochemical tests and O serogrouping (188). *Shigella* and inactive *E. coli* (anaerogenic or lactose-nonfermenting) strains are frequently difficult to distinguish by routine phenotypic tests.

See Table 4 for the phenotypic reactions characteristic of *Shigella* spp. Although *S. dysenteriae* and *S. sonnei* are phenotypically distinct, *S. flexneri* and *S. boydii* are often phenotypically indistinguishable, so serologic grouping is essential to identify them to the species (subgroup) level.

The ability of commercial methods to identify *Shigella* was assessed several years ago by O'Hara and Miller (122–124), who found that these systems correctly identified only 50 to 70% of the 10 *Shigella* strains in their test panel and misidentified several other members of the *Enterobacteriaceae* as *Shigella*. MALDI-TOF MS systems for the rapid identification of bacteria were similarly disappointing and could not correctly identify *Shigella* (they identified *Shigella* as *E. coli*) (126, 127). This is not surprising because the accurate identification of *Shigella* requires performing serogrouping in *Shigella*-specific antisera in addition to biochemical profiling. Molecular assays targeting *Shigella* virulence genes (e.g., *ipaH*, *ipaB*, *ipa*C, *inv*, and *ial*) have been very useful for detecting *Shigella*/EIEC and identifying potential new serotypes.

Serotyping

Serotyping is essential for the identification of *Shigella*. Three of the four subgroups, A (*S. dysenteriae*), B (*S. flexneri*), and C (*S. boydii*), are made up of a number of serotypes. Subgroup A has 15 serotypes; subgroup B has 8 serotypes (with serotypes 1 to 5 subdivided into 11 subserotypes); and subgroup C has 19 serotypes, numbered 1 through 20, with *S. boydii* 13 reclassified as *E. albertii*. Subgroup D (*S. sonnei*) is made up of a single serotype. Subgroups A and C are rare. Several provisional *Shigella* serotypes have also been described, which are held sub judice until findings from the characterization of representative isolates show them to be unique and of sufficient prevalence to merit inclusion in the *Shigella* scheme. Antisera for the identification of provisional serotypes are typically available only at reference laboratories.

Serotyping is typically performed by slide agglutination with polyvalent somatic (O) antigen grouping sera, followed, in some cases, by testing with monovalent antisera for specific serotype identification. Monovalent antiserum to *S. dysenteriae* 1 is required to identify this serotype and is not widely available. Because of the potentially serious nature of illness associated with this serotype, isolates that agglutinate in subgroup A reagent should be sent to a reference laboratory immediately for further serotyping.

Phenotypically typical *Shigella* isolates that agglutinate poorly or that do not agglutinate at all should be suspended in saline and heated in a water bath at 100°C for 15 to 30 min. After cooling, the antigen suspension should be tested in normal saline to determine if it is rough (agglutinates spontaneously). If the heated and cooled suspension is not rough, it may then be retested for agglutination in antisera.

Typing Systems

A variety of methods have been used to subtype *Shigella*, including colicin typing (particularly for *S. sonnei*), plasmid profiling, restriction fragment length polymorphism analysis, PFGE, and ribotyping (201). More recently, MLVA and a single nucleotide polymorphism typing scheme for the rapid and discriminatory typing of *Shigella* have been developed (202, 203). For an overview of the epidemiologic use of typing methods, see chapter 10.

Serologic Tests

Several serodiagnostic assays based on different antigens possessed by *Shigella* have been described (204, 205). These assays are practical only in research settings for seroepidemiology surveys and are not currently used for the diagnosis of infection in individual patients.

Antimicrobial Susceptibilities

Because of the widespread antimicrobial resistance among *Shigella* strains, all isolates should undergo susceptibility testing. The CLSI recommends that susceptibility results be reported routinely for only ampicillin, trimethoprim-sulfamethoxazole, and a fluoroquinolone and warns that *Shigella* should not be reported as susceptible to first- and second-generation cephalosporins, cephamycins, and aminoglycosides, because these drugs are often not effective clinically (206). Because of widespread resistance in the United States, ampicillin and trimethoprim-sulfamethoxazole, two safe drugs that were the most commonly prescribed for treating children with *S. sonnei* infections, are no longer options for empiric treatment. Macrolides, in particular azithromycin, are being used to treat these infections, but there are no interpretive criteria for antimicrobial susceptibility testing for *Shigella*, making it problematic to monitor for development of resistance (199).

Reporting of susceptibility results to the clinician is particularly important for *S. dysenteriae* 1 isolates. Infections caused by these strains are often acquired during international travel to areas where most strains are multidrug resistant (207). In many areas of Africa and Asia, *S. dysenteriae* 1 strains are resistant to all locally available antimicrobial agents, including nalidixic acid (190, 208), and fluoroquinolone-resistant strains have been reported in Asia (191, 209, 210).

Evaluation, Interpretation, and Reporting of Results

A preliminary report of suspected *Shigella* infection may be issued if serologic or biochemical screening tests are positive or consistent with *Shigella*. If serotyping results (O antigen determination) are available, these should also be reported, particularly if the isolate is *S. dysenteriae* 1. All *Shigella* isolates should be tested for antimicrobial susceptibility. Before issuing a final report, isolates should be confirmed by both biochemical and serologic methods, because the definition of *Shigella* is dependent on both the biochemical profile and O antigen expressed. Isolates, particularly those from individuals with dysentery-like illness, that are biochemically identified as *Shigella* but that are serologically negative may be new serotypes of *Shigella* and should be sent to a reference laboratory for further characterization. Isolates from sites other than the gastrointestinal tract that resemble *Shigella* should be scrutinized carefully for gas production and other differentiating characteristics because extraintestinal *Shigella* infections are rare. These isolates should be sent to a reference laboratory for confirmation because they are more likely to be anaerogenic *E. coli*, certain strains of which may cross-react with *Shigella* antiserum.

SALMONELLA

Taxonomy

Members of the genus *Salmonella* are classified in the family *Enterobacteriaceae* (2, 3). Species of this genus are motile, Gram-negative, facultative rods. Salmonellae are typically defined by their ability to use citrate as a sole carbon source and lysine as a nitrogen source and by the production of H_2S on triple sugar agar; exceptions to these traits are used to define species, subspecies, and some serotypes (2, 211).

The genus *Salmonella* is composed of two species, *Salmonella enterica* and *Salmonella bongori* (212, 213). *S. enterica* is subdivided into six subspecies: *S. enterica* subsp. *enterica*, often called subspecies I; *S. enterica* subsp. *salamae*, or subspecies II; *S. enterica* subsp. *arizonae*, or subspecies IIIa; *S. enterica* subsp. *diarizonae*, or subspecies IIIb; *S. enterica* subsp. *houtenae*, or subspecies IV; and *S. enterica* subsp. *indica*, or subspecies VI. The type species is *S. enterica* subsp. *enterica*. Subspecies IIIa and IIIb represent organisms originally described in the genus "*Arizona*"; subspecies IIIa contains the monophasic strains and subspecies IIIb the diphasic strains of "*Arizona*" (214). Despite their common history, subspecies IIIa and IIIb are more closely related to some of the other subspecies of *S. enterica* than they are to each other and thus should be considered separate entities (215, 216).

Genome analysis of the salmonellae has revealed a high degree of genetic variability. Serotypes within *S. enterica* subspecies I have been shown to differ by as much as 10% in gene content (i.e., presence or absence of whole genes) (217, 218). Recombination, particularly among strains of *S. enterica* subspecies I, likely contributes to this diversity (219). The use of whole-genome sequence analysis to characterize strains continues to expand, with >500 complete or partial sequences from *Salmonella* strains available as of the end of 2013 (http://www.ncbi.nlm.nih.gov/genome/browse/). Characterization of whole-genome sequences continues to increase our understanding of *Salmonella* biology and phylogeny. Analysis of genome sequences revealed two distinct lineages among *S. enterica* subsp. *enterica*; differences in gene content were noted that may contribute to differences in ecology or transmission (220, 221). The development of tools for the analysis of whole-genome sequences based on single nucleotide polymorphisms, core genes, or other markers has already begun and will undoubtedly lead to a revolution in epidemiologic strain typing (221–223).

Description of the Genus

Subspecies I strains are commonly isolated from humans and warm-blooded animals. Subspecies II, IIIa, IIIb, IV, and VI strains and *S. bongori* are usually isolated from cold-blooded animals and the environment. Non-subspecies I strains are typically considered rare human pathogens; they make up about 1 to 2% of *Salmonella* isolates reported to the U.S. National *Salmonella* Surveillance System (http://www.cdc.gov/nationalsurveillance/salmonella-surveillance.html). The phenotypic traits useful for identification of *Salmonella* are given in Table 5 and for subspecies differentiation are given in Table 6.

The nomenclature employed to describe the genus *Salmonella* was problematic for many years due to the use of multiple schemes in the literature and the historical practice of considering different serotypes of *Salmonella* to be different species. The publication of Judicial Opinion 80 in the *International Journal of Systematic and Evolutionary Microbiology* in 2005 (212) hopefully served to clarify nomenclatural issues regarding the genus *Salmonella*, and the conventions set forth in that opinion are used here. *Salmonella* history and nomenclature are reviewed at http://www.bacterio.net/salmonella.html.

TABLE 5 Phenotypic traits useful for differentiating *Salmonella* from other *Enterobacteriaceae* and identifying *Salmonella* serotypes Typhi and Paratyphi A[a]

Test	Reaction for:		
	Nontyphoidal *Salmonella* subsp. I	*Salmonella* serotype Typhi	*Salmonella* serotype Paratyphi A
TSI	K/A, g	K/A	K/A, g
H_2S (TSI)	+	$+^{weak}$	− or $+^{weak}$
Indole	−	−	−
Methyl red	+	+	+
Voges-Proskauer	−	−	−
Citrate (Simmons)	+	−	−
Urea	−	−	−
Lysine decarboxylase	+	+	−
Arginine dihydrolase	+	d	(+)
Ornithine decarboxylase	+	−	+
Motility	+	+	+
Mucate	+	−	−
Malonate	−	−	−
L(+)-Tartrate (*d*-tartrate[b])	+	+	−
Growth in KCN	−	−	−
Glucose	A, g	A	A, g
Lactose	−	−	−
Sucrose	−	−	−
Salicin	−	−	−
Dulcitol	A, g	−	A, $g^{2\ days}$
Inositol	d	−	−
Sorbitol	A, g	A	A, g
o-Nitrophenyl-β-D-galactopyranoside	−	−	−
Galacturonate	−	−	−

[a] Reactions after incubation at 37°C. Abbreviations and symbols: +, 90% (or more) of strains were positive within 2 days; (+), positive reaction after 3 or more days; −, no reaction (90% or more) in 7 days; A, acid; d, different reactions [+, (+), −]; g, gas; K, alkaline slant. Adapted with permission of the publisher from reference 2.

[b] Sodium potassium tartrate (2).

TABLE 6 Phenotypic traits useful for differentiating *Salmonella* species and subspecies[a]

Species or subspecies (no. of strains tested)	Reaction for:										
	Dulcitol	Lactose	ONPG	Salicin	Sorbitol	Galacturonate	Malonate	Mucate	Growth in KCN	Gelatin (strip)	L(+)-Tartrate (*d*-tartrate[j])
S. enterica I (650)	+	−	−	−	+	−	−	+	−	−	+
S. enterica II (146)	+	−	−[f]	−	+	+	+	+	−	+	−
S. enterica IIIa (120)	−	−[c]	−	−	+	−	+	+	−	+	−
S. enterica IIIb (155)	−	−[d]	−	−	+	+	+	−[i]	−	+	−
S. enterica IV (120)	−	−	−	+[h]	+	+	−	−	+	+	−
S. enterica VI (9)	d[b]	d[e]	d[g]	−	−	+	−	+	−	+	−
S. bongori (16)	+	−	+	−	+	+	−	+	+	−	−

[a] Reactions after incubation at 37°C. Abbreviations and symbols: +, 90% (or more) of strains were positive within 1 or 2 days; (+), positive reaction after 3 or more days; −, no reaction (90% or more) in 7 days; d, different reactions [+, (+), −]; ONPG, *o*-nitrophenyl-β-D-galactopyranoside. Adapted with permission of the publisher from reference 2.

[b] A total of 67% were positive.

[c] A total of 15% were positive.

[d] A total of 85% were positive.

[e] A total of 22% were positive.

[f] A total of 15% were positive.

[g] A total of 44% were positive.

[h] A total of 60% were positive.

[i] A total of 30% were positive.

[j] Sodium potassium tartrate (2).

Epidemiology and Transmission

Salmonella organisms are isolated most frequently from the intestines of humans and animals. Some serotypes are isolated only from humans (e.g., *Salmonella* serotype Typhi), while others (e.g., *Salmonella* serotype Gallinarum and *Salmonella* serotype IV 48:g,z51:− [formerly serotype Marina]) are strongly associated with certain animal hosts. Members of this genus can be isolated from feces-contaminated food or water but probably do not occur as free-living organisms in the environment. Historically, *Salmonella* has been considered a pathogen of meat and poultry products, but it has recently been associated with other food vehicles, such as fresh produce and manufactured products (224–226).

Salmonella Serotypes

Salmonella serotyping is a subtyping method based on the immunologic characterization of three surface structures: O antigen, which is the outermost portion of the LPS layer that covers the bacterial cell; H antigen, which is the filament portion of the bacterial flagella; and Vi antigen, which is a capsular polysaccharide present in specific serotypes. Serotyping of *Salmonella* is commonly performed to facilitate public health surveillance for *Salmonella* infections and to aid in the recognition of outbreaks. While molecular methods such as PFGE and MLVA have become the cornerstone of public health subtyping (227, 228), serotyping remains an important tool. The serotype of an isolate often correlates with a particular disease syndrome or food vehicle, making serotype data particularly useful in identifying cases and defining outbreaks. For example, *Salmonella* serotype Typhi causes typhoid fever, a more severe disease syndrome than those caused by most other *Salmonella* serotypes. *Salmonella* serotype Enteritidis is often associated with infections acquired from chicken or egg products (223). Furthermore, *Salmonella* serotyping is performed worldwide and has aided in the recognition of international outbreaks (229).

Clinical Significance

Strains of *Salmonella* are categorized as typhoidal and nontyphoidal, corresponding to the disease syndrome with which they are associated. Strains of nontyphoidal *Salmonella* usually cause intestinal infections (accompanied by diarrhea, fever, and abdominal cramps) that often last 1 week or longer (230). Less commonly, nontyphoidal *Salmonella* can cause extraintestinal infections (e.g., bacteremia, urinary tract infection, or osteomyelitis), especially in immunocompromised persons. Persons of all ages are affected, but the incidence is highest in infants and young children. *Salmonella* is ubiquitous in animal populations, and human illness is usually linked to foods. Salmonellosis is also transmitted by direct contact with animals, by water, and occasionally by human contact. Each year, an estimated 1 million cases of illness and 378 deaths are caused by nontyphoidal salmonellosis in the United States (47).

Typhoid fever, caused by *Salmonella* serotype Typhi, is a serious bloodstream infection common in the developing world. However, it is rare in the United States, where on average an estimated 1,800 cases, with <1 death, occur each year; >60% of U.S. cases are related to foreign travel (47). Typhoid fever typically presents with a sustained debilitating high fever and headache. Adults characteristically present without diarrhea. Illness is milder in young children, where it may manifest as nonspecific fever. Humans are the only reservoir for *Salmonella* serotype Typhi, indicating that this serotype is adapted to the human host; healthy carriers have been noted. Typhoid fever typically has a low infectious dose (<10^3 organisms) and a long, highly variable incubation period (1 to 6 weeks). It is transmitted through person-to-person contact or feces-contaminated food and water. Fatal complications of typhoid most commonly occur in the second or third week of illness.

A syndrome similar to typhoid fever is caused by "paratyphoidal" strains of *Salmonella*, i.e., *Salmonella* serotypes Paratyphi A, Paratyphi B, and Paratyphi C. Serotypes Paratyphi A and Paratyphi C are rare in the United States (http://www.cdc.gov/nationalsurveillance/salmonella-surveillance.html). Serotype Paratyphi B is a diverse serotype that is associated with both paratyphoid fever and gastroenteritis (231). The two pathovars are typically differentiated on the basis of the ability to ferment tartrate. Isolates causing paratyphoid fever, the systemic pathovar, are tartrate negative. Isolates associated with gastroenteritis, the enteric pathovar, are typically tartrate positive and are referred to as *Salmonella* Paratyphi B variant L(+)-tartrate + or *Salmonella* Paratyphi B variant Java. The systemic pathovar of *Salmonella* Paratyphi B is considered rare in the United States; however, the tartrate reaction is often not reported, making it impossible to distinguish between the two pathovars (http://www.cdc.gov/nationalsurveillance/salmonella-surveillance.html).

Salmonella serotypes Choleraesuis and Dublin are host adapted to pigs and cattle, respectively, causing serious disease in these two animal species. They rarely cause human infection, but such infections are typically severe, with spread to extraintestinal sites (232).

Collection, Transport, and Storage of Specimens

See "Collection, Transport, and Storage of Specimens" in the *Escherichia* section.

Direct Examination

Microscopy

Salmonella cannot be distinguished from other Gram-negative rods by microscopy or staining methods.

Antigen Detection

A number of commercial rapid diagnostic tests are available for the testing of foods, but to our knowledge, none are FDA approved for clinical specimens.

Nucleic Acid Detection

There are four FDA-cleared methods for nucleic acid detection of *Salmonella* in clinical specimens. All four detect multiple pathogens, including *Salmonella*. See "Nucleic Acid Detection" under "Direct Examination" in the *Escherichia* section for additional information.

Isolation Procedures

Enrichment

Maximal recovery of *Salmonella* from fecal specimens is obtained by using an enrichment broth, although isolation from acutely ill persons is usually possible by direct plating of specimens. Enrichment broths for *Salmonella* are usually highly selective and inhibit certain serotypes of *Salmonella*, particularly *Salmonella* serotype Typhi. The selective enrichment medium most widely used to isolate *Salmonella* from fecal specimens is SEL. SEL may also be used for the recovery of *Salmonella* serotype Typhi and for *Shigella*, although its value as enrichment for the latter has not been clearly established. Specimens that might contain organisms inhibited by selective enrichment medium should be plated directly or cultured in a nonselective enrichment broth (e.g., Gram-negative broth).

Plating Media

Many differential plating media, varying from slightly selective to highly selective, are available for isolation of *Salmonella* from fecal specimens. Media of low selectivity include MAC and eosin-methylene blue. Media of intermediate selectivity include XLD, deoxycholate-citrate agar, salmonella-shigella agar, HE, Statens Serum Institut enteric medium, and CHROMagar Salmonella. Highly selective media include bismuth sulfite agar, the preferred medium for the isolation of *Salmonella* serotype Typhi, and brilliant green agar. Bismuth sulfite agar, XLD, and HE all have H_2S indicator systems, which are helpful for the detection of lactose-fermenting *Salmonella*. Many laboratories use HE or XLD because these media may also be used for the isolation of *Shigella*.

In the developing world, typhoid fever is frequently diagnosed solely on clinical grounds, but isolation of the causative organism is necessary for a definitive diagnosis. *Salmonella* serotype Typhi is isolated more frequently from blood cultures than from fecal specimens. Blood cultures are positive for 80% of typhoid patients during the first week of fever but show decreasing positive results thereafter.

Screening Procedures

A latex agglutination kit has been described for *Salmonella* screening in SEL enrichment broth (Wellcolex Color *Salmonella*; Remel, Inc., Lenexa, KS) (233). This kit can also be used to screen individual colonies from primary plates. In using this kit, it should be kept in mind that it identifies only those *Salmonella* isolates belonging to the more common O serogroups and does not differentiate between O groups C_1 (O:7) and C_2 (O:8).

Suspect colonies may be inoculated onto a screening medium such as KIA or TSI. On KIA or TSI, most *Salmonella* strains produce an alkaline slant, indicating that only glucose is fermented, with gas and H_2S. On these media, *Salmonella* serotype Typhi isolates characteristically produce an alkaline slant but do not produce gas, and only a small amount of H_2S will be visible at the site of the stab and in the stab line. Lysine iron agar is also a useful screening medium because most *Salmonella* isolates, even those that ferment lactose, also decarboxylate lysine and produce H_2S. Alternately, isolates may be identified by a battery of phenotypic tests or by slide agglutination with antisera for *Salmonella* O groups. Isolates suspected of being *Salmonella* serotype Typhi should be tested serologically with *Salmonella* Vi and O group D antisera (see below); serotype Typhi can also be identified by its unique phenotypic profile (Table 5).

If the phenotypic traits for a particular isolate are not characteristic of *Salmonella* but *Salmonella* antigens are found, the cultures should be plated to obtain a pure culture, tested with a complete set of phenotypic tests, or forwarded to a reference laboratory.

Identification

Clinical laboratories may issue a preliminary report of *Salmonella* when an isolate is positive either with *Salmonella* O group antisera or by phenotypic identification methods. An isolate is confirmed as *Salmonella* when the specific O serogroup has been determined and phenotypic identification has been completed.

Phenotypic Identification

Suspect colonies from one of the differential plating media mentioned above can be identified phenotypically as *Salmonella* spp. by use of traditional media in tubes or commercial identification systems. Methods for phenotypic identification and specific commercial manual and automated identification systems are covered in chapter 4. The species and subspecies of *Salmonella* can be identified phenotypically, as indicated in Tables 5 and 6. However, *Salmonella* is a diverse group, and phenotypically atypical strains are not uncommon. Phenotypic identification is commonly combined with serogrouping or serotyping for culture confirmation. Perhaps due to strain diversity, commercial identifications systems sometime misidentify *Salmonella* (122–124). In particular, laboratories may overlook *Salmonella* serotype Paratyphi A because its atypical phenotypic profile (H_2S negative, lysine negative, and citrate negative; Table 5) can be confused with that of *E. coli*.

Identification Using MS

MS has emerged as a rapid, accurate means of identifying many bacteria, offering substantial cost and time savings over phenotypic methods. Several diagnostic systems based on MS are available in Europe (234) and either moving toward FDA approval or already FDA cleared in the United States. The ability of MS to identify bacteria is somewhat dependent on the bacterial taxon itself. For *Salmonella*, MALDI-TOF MS appears to easily identify an isolate as *Salmonella* (234, 235). MALDI-TOF MS was also shown to correctly discriminate serotype Typhi from other serotypes (236). Identifying *Salmonella* to the serotype level may be possible for at least some serotypes (237), although such applications are still in the research phase.

Serogrouping and Serotyping

O serogroup determination is adequate for confirmation of isolates as *Salmonella*. Full serotype determination is useful for public health surveillance but is beyond the scope of most routine clinical laboratories. The methods for serotyping described below are intended primarily for reference laboratories. *Salmonella* isolates are serotyped based on the antigenic properties of their O (somatic) antigens, H (flagellar) antigens, and Vi (capsular) antigens (238, 239). O antigen is a carbohydrate antigen and is the outermost component of LPS. It is a polymer of O subunits; each O subunit is typically composed of four to six sugars depending on the O antigen. O antigens are designated by numbers and are divided into O serogroups based on antigenic factors associated with the O subunit. Many of the common O groups were originally designated by letter and are still commonly referred to by letter (e.g., *Salmonella* serotype Typhimurium belongs to group O:4 or group B, and *Salmonella* serotype Enteritidis belongs to group O:9 or group D1). Additional O antigenic factors have been identified for specific O groups. They are typically associated with a side sugar that is added to the basic O subunit structure, and they are often variably present or variably expressed within O groups or within serotypes.

H antigen is a protein antigen called flagellin; multiple flagellin subunits make up the flagellar filament. The ends of flagellin are conserved and give the flagellum its characteristic filament structure. The antigenically variable portion of flagellin is the middle region, which is surface exposed. Salmonellae are unique among the enteric bacteria in that they commonly express two different flagellin antigens, although specific serotypes, such as *Salmonella* serotypes Typhi and Enteritidis, possess only one flagellar antigen. The two flagellar antigens are referred to as phase 1 and phase 2 antigens; monophasic and diphasic strains express one and two flagellar antigens, respectively. Individual flagellar antigens can be composed of multiple antigenic factors. For example, the phase 2 flagellar antigen of *Salmonella* serotype Typhimurium is antigen 1,2, which is composed of two antigenic factors, i.e., 1 and 2.

Serotypes are designated according to the conventions of the Kauffmann-White scheme (239). Many of the O and H antigenic types are found in multiple subspecies, and isolates from different subspecies can have the same antigenic profile. Thus, subspecies determination is an integral component of serotype determination for *Salmonella*. The serotypes for all *Salmonella* strains can be designated by antigenic formulae; additionally, serotypes belonging to subspecies I are given a name, which is typically related to the geographical place where the serotype was first isolated. The antigenic formulae of *Salmonella* serotypes are listed in the Kauffmann-White scheme and are expressed as follows: O antigen(s), Vi antigen (when present):phase 1 H antigen(s):phase 2 H antigen(s) (when present). For example, the antigenic formula for *Salmonella* serotype Typhimurium is 4,5,12:i:1,2. Serotype names for subspecies I serotypes are written in roman (not italicized) letters, and the first letter is a capital letter (for example, *Salmonella* serotype Typhimurium). Serotypes belonging to other subspecies are designated by their antigenic formulae following the subspecies name (for example, *S. enterica* subsp. *salamae* serotype 50:z:e,n,x or *Salmonella* serotype II 50:z:e,n,x).

The WHO Collaborating Centre for Reference and Research on *Salmonella*, which is located at the Pasteur Institute in Paris, France, maintains the Kauffmann-White scheme for the designation of *Salmonella* serotypes (239). Most common serotypes belong to O groups A, B, C1, C2, D1, and E1 (also known as groups O:2, O:4, O:7; O:8; O:9, and O:3,10, respectively).

Determination of O Antigens

O (heat-stable, somatic) antigens are typically identified by first testing the isolate in antisera that detect one or multiple antigenic factors corresponding to the O groups (O grouping antisera). Once the O group is determined, antisera that recognize single antigenic factors are used to confirm the O group and identify any additional antigenic factors that are associated with that O group (238). The approach most commonly used for determining O antigens is to initially test the isolates by slide agglutination in antisera against O groups A to E1 because ~95% of *Salmonella* isolates from human specimens belong to one of these O groups. If no agglutination occurs in antisera for these O groups, the isolate is tested in pools containing the remaining *Salmonella* O antisera, for groups O:11 through O:67.

Determination of H Antigens

H (flagellar) antigens are typically determined by tube or slide agglutination tests. Isolates are initially tested with antisera pools that recognize multiple antigenic factors, and then with antisera that recognize individual antigenic factors corresponding to the appropriate pool. Typically, the flagellar antigens in a diphasic strain are coordinately regulated so that only one antigenic type is expressed at a time in a single bacterial cell; however, both phases may be detected in the whole culture. When only one phase is detected (either phase 1 or phase 2), the strain should be inoculated into a semisolid medium to which sterile antiserum to the detected flagellar antigen has been added aseptically. Growth of the strain in this semisolid agar immobilizes cells expressing the detected antigen and allows the movement of bacteria expressing the antigen in the other phase through the semisolid medium. Cells are recovered away from the area of initial inoculation, and the strain is tested in appropriate H typing and single-factor antisera to complete the serotyping. A strain must be actively motile to ensure the good expression of H antigens; sometimes a strain must be passed through one or more tall tubes of semisolid agar to enhance motility before H antigens can be detected.

Detection of the Vi Antigen and Identification of *Salmonella* Serotype Typhi (9,12,[Vi]:d:−)

The Vi antigen, a heat-labile capsular polysaccharide, is useful for the identification of *Salmonella* serotype Typhi. It is also occasionally detected in *Salmonella* serotypes Dublin and Paratyphi C and some *Citrobacter* strains, so its detection does not constitute definitive evidence of *Salmonella* serotype Typhi. The Vi antigen is identified by slide agglutination with a specific antiserum.

If *Salmonella* serotype Typhi is suspected, the culture is first tested live (unheated) in O group D1 (O:9) antiserum and Vi antiserum via slide agglutination. The Vi capsular polysaccharide can mask the O antigens, blocking their reactivity with the O grouping antiserum. If only the Vi antiserum is positive, the bacterial suspension is heated in boiling water for 15 min to remove the capsule, cooled, and tested again in the same antisera. After being heated, *Salmonella* serotype Typhi isolates will be negative in the Vi antiserum but positive in the O group D1 antiserum. The Vi antigen of *Salmonella* serotype Typhi is more frequently detected in freshly isolated cultures than in subcultures. If the strain has a typical reaction for *Salmonella* serotype Typhi on TSI or KIA (see "Screening Procedures" above), is urease negative, and reacts in O group D or Vi antiserum, a presumptive report is made. The identity of the isolate is typically confirmed by phenotypic testing and determination of the H (flagellar) antigen. However, because serotype Typhi has a unique phenotypic profile (Table 5), it can and should be reported based on phenotype alone (i.e., identification of the O and H antigens is not required to identify an isolate as *Salmonella* serotype Typhi).

Molecular Methods for the Determination of Serotype

Given the specialized reagents and skills required to perform traditional serotyping and the continued utility of serotype data, it is not surprising that a plethora of molecular methods for determination of serotype have appeared in the literature (240). The methods fall into two general categories, methods based on the genes responsible for serotype and methods based on random genetic markers. Methods based on the genes responsible for serotype have the advantages of duplicating the Kauffmann-White scheme fairly closely and of having the potential to identify most described serotypes by identifying the complement of antigens that determine any given serotype. Methods based on random genetic markers can only identify serotypes for which a profile has already been determined; i.e., when a new profile is encountered, the serotype that it represents is unknown. To date, no single molecular method has risen to a level of widespread use, and it seems unlikely that any one molecular method will gain the worldwide acceptance that traditional serotyping enjoys. With the increasing availability of whole-genome sequencing, the ability to determine serotype by detecting the genes responsible for serotype directly in a whole-genome sequence may not be far off.

Notes on Serotype Designations

The *Salmonella* serotyping scheme can be confusing because some serotypes actually represent biotypes or pathotypes that must be further characterized for full "serotype" determination. *Salmonella* serotypes Paratyphi B and Paratyphi B variant L(+)-tartrate + (also known as variant Java) have the same antigenic formula (4,5,12:b:1,2) and are distinguished phenotypically by their tartrate reaction. Similarly, *Salmonella* serotypes Choleraesuis and Paratyphi C have the same antigenic formula (6,7:c:1,5) but different phenotypic profiles. Some *Citrobacter* and *E. coli* strains possess O, H, or Vi antigens that are related to those of *Salmonella* and cross-react in *Salmonella* antisera; phenotypic identification may be necessary to confirm that an isolate is *Salmonella*.

Typing Systems

PFGE is the current method of choice for subtyping *Salmonella*, since it is universally applicable and provides good strain discrimination for most serotypes. PulseNet, an international subtyping network that tracks *Salmonella*, is based on PFGE (164). MLVA is often used to complement PFGE by providing additional discrimination within PFGE types (228), e.g., for *Salmonella* serotype Enteritidis, which has limited diversity in PFGE analysis (241). Despite their utility in public health surveillance and epidemiologic investigations, PFGE and MLVA both have drawbacks. PFGE is fairly labor-intensive and challenging to standardize. MLVA has very high throughput, but typically a unique MLVA scheme must be developed for each serotype. Neither method provides information on phylogenetic relationships. The routine use of whole-genome sequencing as a subtyping method is on the horizon. It no doubt will have its own challenges, such as managing and analyzing the massive amounts of data, but it holds the promise of providing high levels of discrimination and detailed phylogenetic relationships.

Serologic Tests

Serodiagnostic tests are not used for routine diagnosis of *Salmonella* infections in the United States. Serodiagnosis can be helpful in areas where typhoid fever is endemic, but its lack of specificity limits its utility. The Widal test measures agglutinating antibodies to the O and H antigens of *Salmonella* serotype Typhi. It produces false-positive reactions, likely due to cross-reaction with antibodies produced during infections with other *Salmonella* serotypes, as well as false-negative reactions; it does not provide a definitive diagnosis of individual cases of infection. Two other rapid serodiagnostic tests have proved more useful than the Widal test for the serodiagnosis of typhoid fever (242): Tubex (IDL Biotech, Sollentuna, Sweden) and TyphiDot (Malaysian Bio-Diagnostics Research Sdn. Bhd., Kuala Lumpur, Malaysia). Neither of these tests is FDA approved, and TyphiDot is not available in the United States.

Antimicrobial Susceptibilities

Antimicrobial therapy is not recommended for uncomplicated *Salmonella* gastroenteritis, and routine susceptibility testing of fecal isolates is not warranted for treatment purposes. However, determination of antimicrobial resistance patterns is often valuable for surveillance purposes and may be performed periodically to monitor the development and spread of antimicrobial resistance among *Salmonella* isolates.

In contrast to the case for uncomplicated salmonellosis, treatment with the appropriate antimicrobial agent can be crucial for patients with invasive *Salmonella* and typhoidal infections, and the susceptibilities of these isolates should be reported as soon as possible (243). The untreated case mortality rate for typhoid fever is >10%; when patients with typhoid fever are treated with appropriate antibiotics, the rate should be <1%. However, increasing levels of resistance to one or more antimicrobial agents in *Salmonella* isolates, particularly *Salmonella* serotype Typhi, make selection of an appropriate antibiotic problematic. In particular, reduced susceptibilities to ciprofloxacin among *Salmonella* serotype Typhi isolates and increasing numbers of treatment failures are of concern (244, 245). In 2013, the CLSI recommended testing *Salmonella* isolates from extraintestinal infections and serotypes Typhi and Paratyphi A, B, and C for susceptibility to both nalidixic acid and ciprofloxacin; it also established separate interpretive criteria for *Salmonella* for fluoroquinolones with lower MIC breakpoints (206). In addition, susceptibility to chloramphenicol and a broad-spectrum cephalosporin should be tested and reported for extraintestinal isolates of *Salmonella*. *Salmonella* should not be reported as susceptible to first- and second-generation cephalosporins, cephamycins, and aminoglycosides because these drugs are not effective clinically.

Antimicrobial resistance varies by serotype in *Salmonella*; resistance to multiple antibiotics is a particular concern (246). In 2011, 9.1% of *Salmonella* isolates tested as part of the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) were resistant to three or more CLSI drug classes; in particular, 26, 27, and 30% of serotype Typhimurium, I 4,[5]12:i:−, and Heidelberg isolates, respectively, were resistant to three or more CLSI classes. Additional information regarding these and other antimicrobial-resistant strains can be found at the CDC's NARMS website, including interactive displays of susceptibility data by pathogen and serotype (http://www.cdc.gov/narms/).

Evaluation, Interpretation, and Reporting of Results

A preliminary report can be issued as soon as a presumptive identification of *Salmonella* is obtained. In most situations, a presumptive identification is based on phenotypic traits determined either by traditional or commercial systems, or by reactivity with *Salmonella* O grouping antisera. A confirmed identification requires both phenotypic identification and O group or serotype determination. Because national surveillance systems depend on the receipt of serotype information for *Salmonella* strains isolated in the United States, laboratories should follow the procedures recommended by their state health departments for submitting *Salmonella* isolates for further characterization, including complete serotyping. The antimicrobial susceptibility of typhoidal *Salmonella* strains and strains from normally sterile sites should be determined, and the strains should be forwarded to a reference or public health laboratory for complete phenotypic identification and serotyping.

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Klebsiella, Enterobacter, Citrobacter, Cronobacter, Serratia, Plesiomonas, and Other *Enterobacteriaceae*

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This chapter deals with a number of both major and less prominent members of the *Enterobacteriaceae* family. The genera *Klebsiella*, *Enterobacter*, *Citrobacter*, *Serratia*, *Plesiomonas*, and *Cronobacter* cause a range of infections in humans. These continue to warrant serious consideration since many of these infections are poorly characterized, are likely to be underreported, and have the capacity to resist antimicrobial agents. The Infectious Diseases Society of America recognizes *Klebsiella* and *Enterobacter* as members of the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) which exhibit antimicrobial resistance and present new challenges in their pathogenesis and transmission. Therefore, this chapter contains a wide breadth of information not only on the taxonomy, detection, and identication of these genera but also on the global issue of antimicrobial resistance.

TAXONOMY

Despite years of research, the taxonomic designation of strains into specific species within the *Enterobacteriaceae* has been a matter of much debate and change. This frequent reclassification of organisms with clinical significance can lead to confusion for both microbiologists and regulatory authorities responsible for the monitoring and control of such organisms. This situation is not unique to the *Enterobacteriaceae* but is very apparent due to their clinical importance. Many of the *Enterobacteriaceae* genera are composed of heterogeneous groups of bacterial species, most of which were originally described based on morphological and phenotypic information several decades ago.

In this context, improvements in DNA sequence-based analysis, including whole-genome sequencing, are expected to have a major influence on the taxonomy of this very important bacterial family. The advent of 16S rRNA gene sequencing and the subsequent polyphasic approach to bacterial taxonomy have resulted in some major taxonomic revisions (1, 2). A polyphasic approach is especially needed for the taxonomy of *Enterobacteriaceae*. Although sequence analysis of the 16S rRNA gene is commonly used in bacterial phylogenetic studies, it is often unreliable for closely related members of the *Enterobacteriaceae* due to microheterogeneities within multiple copies of the genes (3–5). Consequently, it can be impossible to assign an unknown isolate to a specific species. It can be expected that in the future, whole-genome sequence comparisons could be used to resolve some problems in *Enterobacteriaceae* classification and identification, though currently the number of sequenced genomes of species type strains is limited (6).

The current trend is to use combinations of single-copy housekeeping gene regions (alleles or loci) to produce a multilocus sequence analysis (MLSA). Such housekeeping genes must be present within the taxon under study, must occur as single-copy genes, and must be independent of each other (7). In parallel, a large number of international multilocus sequencing typing (MLST) schemes based typically on seven loci have been established and can be accessed via curated centralized databases such as http://www.pubMLST.org. As is covered later in this chapter, the application of MLST has led to the recognition of a number of definable sequence types of particular clinical significance, e.g., *Escherichia coli* ST131, methicillin-resistant *S. aureus* (MRSA) 15 ST22, and *Klebsiella pneumoniae* ST258.

MLSA of the loci from several housekeeping genes is able to give better phylogenetic resolution than the single 16S rRNA gene sequence analysis. The application of MLSA to taxonomic description of members of the *Enterobacteriaceae* frequently uses only four or five loci, such as *recN*, *rpoA*, *rpoB*, *thdF*, *infB*, *atpD*, and *gyrB* (8–11). On occasion three loci have been used, but this is not desirable. The use of such housekeeping genes can result in different, or more discriminatory, phylogenetic classifications than 16S RNA gene sequencing. However, these may not correspond with data generated by the "gold standard" of DNA-DNA hybridization (12).

Correlation between sequence differences for determining genus and species definition thresholds has been problematic in the *Enterobacteriaceae*, as well as genera outside this family. The use of whole-genome sequencing has led to the development of a few *in silico* alternatives to DNA-DNA hybridization, such as the average nucleotide identity (13). Although a universal cutoff value to delineate bacterial species does not exist, a DNA-DNA hybridization value of <70% and average nucleotide identity of <95% ± 0.5% have been applied in general.

A small number of additions or taxonomic changes within the *Enterobacteriaceae* have occurred since publication of the 10th edition of this *Manual*. Those of clinical relevance include new species designations in the genera *Cronobacter*, *Pantoea*, and *Hafnia*. There have also been some reclassifications of some *Enterobacter* species into the

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Cronobacter genus and into the new genera *Lelliottia*, *Pluralibacter*, and *Kosakonia*. There is also the description of a new genus named *Pseudocitrobacter*. Nevertheless, the *Pantoea* and *Enterobacter* genera remain polyphyletic, and it is likely that there will be further taxonomic changes in the future following further polyphasic analysis (14, 15).

Tables 1 and 2 cover all of the *Enterobacteriaceae* species discussed in this chapter. For completeness, Table 2 does include genera which are not isolated from human clinical specimens. These may not be of clinical significance and may be unfamiliar to clinical microbiologists. *Enterobacteriaceae* genera which are exclusively isolated from insects, plants, fish, marine animals, or birds are not included in the tables or text of this chapter; however, information on them is available elsewhere (16).

DESCRIPTION OF THE GENERA

Members belonging to the family *Enterobacteriaceae* are Gram-negative, facultative anaerobic rods or coccobacilli ranging from 0.3 to 1.0 μm wide to 0.6 to 6.0 μm long. They ferment glucose with the production of various end products depending upon species as determined using the methyl red and Voges-Proskauer tests. Typically *Enterobacteriaceae* are oxidase negative and non-spore forming. Growth parameters for the *Enterobacteriaceae* are on the order of temperatures of 18 to 47°C, a pH range of 4.0 to 8.0, and salt concentrations of 0 to 5%. Motility is variable and may be due to peritrichous or polar flagella. Of those organisms in Tables 1 and 2 which are isolated from human specimens, all *Klebsiella*, *Leminorella*, *Moellerella*, and *Tatumella* organisms are nonmotile. The majority of *Enterobacter asburiae* strains are nonmotile, with only a few motile strains reported (17). Some strains of *Serratia plymuthica* may not grow at 37°C, but most other members of the genera discussed in this chapter grow well between 25°C and 37°C. Strains from all genera may grow as mucoid or rough colonies. *Klebsiella*, *Raoultella*, and *Enterobacter* spp. are encapsulated, and there is cross-reactivity between the capsular antigens of *Klebsiella* and *Enterobacter aerogenes*. Pigment production is restricted to a few genera. Some strains of *Serratia marcescens* and most *Serratia rubidaea* and *S. plymuthica* strains produce a red pigment, prodigiosin, which may appear throughout the entire colony or only as a red center or margin. Yellow pigment-producing organisms include ~80% of strains in the *Cronobacter* genus, *Pantoea agglomerans*, *Leclercia adecarboxylata*, and *Photorhabdus asymbiotica*. Yellow pigment production may be enhanced by incubation at 25°C as opposed to 37°C. Weak pigment producers may be most easily noted by placing the agar plate growth on a bench in sunlight for a short period. *Serratia odorifera*, as indicated by its name, and some *Cedecea* spp. produce a pungent (potato-like) odor due to the production of alkylmethoxypyrazines (18). Species of *Proteus* and *Providencia* oxidatively deaminate α-amino acids, producing pyruvic acids. L-Phenylalanine deamination yields a green color when ferric chloride is added; however, deamination of *dl*-tryptophan produces the deep reddish brown pigment often seen in media inoculated with these organisms without the addition of ferric chloride (19). *Proteus* species also produce swarmer cells, i.e., elongated forms created when cells fail to septate or divide. These cells, which are profusely covered with flagella, act in concert to produce swarming motility on solid media (20). *Photorhabdus luminescens* and *P. asymbiotica* cultures are luminescent, giving a visible glow in a darkroom after 5 min.

There are some exceptions to the general description of the *Enterobacteriaceae*. *Serratia marcescens* subsp. *sakuensis* and *Serratia ureilytica* are the only reported spore-forming organisms in the *Enterobacteriaceae* (21, 22). *Plesiomonas shigelloides* strains are oxidase positive, do not produce gas from glucose (*Enterobacteriaceae* are variable), and are susceptible to O/129 (2,4-diamino-6,7-diisopropylpteridine), a vibriostatic agent. Auxotrophic strains of *Enterobacteriaceae* from clinical specimens are uncommon. However, cysteine-requiring urinary isolates of *Klebsiella pneumoniae*, which grow as pinpoint colonies on routine media, do occur. When encountered, these strains require supplementation of biochemical media or commercial identification systems with 0.63 mM cysteine for accurate identification. *Klebsiella granulomatis* is extremely fastidious and is culturable only by *in vitro* cell culture techniques not usually employed by routine clinical microbiology laboratories.

EPIDEMIOLOGY, TRANSMISSION, AND CLINICAL SIGNIFICANCE

The *Enterobacteriaceae* are widely distributed throughout the environment (Tables 1 and 2). Many species of the genera in Table 1 are commonly recognized pathogens, consistently ranking among the top 10 organisms seen in health care-associated infections (23–25). In the United States between 2009 and 2010, there were 69,475 health care-associated infections and 81,139 pathogens reported from 2,039 hospitals (26). Eighty percent of the isolates were in one of eight pathogen groups: *Staphylococcus aureus* (16%), *Enterococcus* spp. (14%), *Escherichia coli* (12%), coagulase-negative staphylococci (11%), *Candida* spp. (9%), *Klebsiella pneumoniae*-*Klebsiella oxytoca* (8%), *Pseudomonas aeruginosa* (8%), and *Enterobacter* spp. (5%). Nearly 20% of these eight pathogen groups were multidrug-resistant phenotypes: MRSA (8.5%); vancomycin-resistant *Enterococcus* (3%); extended-spectrum cephalosporin-resistant *K. pneumoniae*-*K. oxytoca* (2%), *E. coli* (2%), and *Enterobacter* spp. (2%); and carbapenem-resistant *P. aeruginosa* (2%), *K. pneumoniae*-*K. oxytoca* (<1%), *E. coli* (<1%), and *Enterobacter* spp. (<1%).

European data on infection rates for 2010–2011, which are similar to those above, can be obtained from the European Centres for Disease Prevention and Control (http://ecdc.europa.eu/en/publications); rates for individual European countries can be obtained from the appropriate regulatory health authority, such as Public Health England (http://www.hpa.org.uk/Publications/InfectiousDiseases).

Pediatric patient data collected in 2004 from three continents (North America, South America, and Europe) indicated that *Klebsiella* spp., *Enterobacter* spp., *Proteus mirabilis*, and *Serratia* spp. ranked 4th, 7th, 11th, and 12th, respectively, in the top 15 most frequently isolated organisms (23). Both *Klebsiella* and *Enterobacter* were more prevalent (3rd to 5th versus 10th) in North and South America than in Europe. In all geographic areas there was a 2-fold decrease in prevalence for both *Klebsiella* and *Enterobacter* species in children older than 1 year.

Klebsiella and *Raoultella*

Klebsiella is carried in the nasopharynx and the bowel; however, feces are arguably the most significant source of patient infections (27). *K. pneumoniae* bloodstream infection isolation rates are 1.5 times greater during the warmest months of the year (28). These rates most likely reflect increased fecal carriage in humans, which, in turn, is a reflection of increased organisms in the environment during warm months. This has important implications since colonized patients have a 4-fold-increased risk of infection over noncarriers. Similar isolation rate increases were not seen with *Enterobacter* or *Serratia*.

TABLE 1 Nomenclature, isolation source, and significance of selected genera of the family *Enterobacteriaceae*[a]

Current (previous) designation	Clinical data			Environmental data
	Frequency	Source(s)	Significance	
Averyella dalhousiensis	Unk	Wound, blood	2	Unk
Citrobacter				
C. amalonaticus	++	**Feces,** blood, wound, UT, RT	2	Unk, one isolate from an animal
C. braakii	+++	**Feces,** UT, wound	2	Similar to *C. freundii*
C. farmeri	++	**Feces,** UT, blood, wound, RT	2	Unk
C. freundii	++++	All sites; feces most common	1	Water, soil, fish, animals, food
C. koseri	++	All sites; CSF most common	1	Unk
C. rodentium	−			Pathogenic for mice
C. sedlakii	+	Feces, UT, blood, wound	3	Same as for *C. braakii*
C. werkmanii	+	**Feces,** blood, wound	3	Same as for *C. braakii*
C. youngae	++	**Feces,** UT, blood, wound	3	Same as for *C. braakii*
C. gillenii	+	**Feces,** UT, blood	3	Same as for *C. braakii*
C. murliniae	+	Feces, blood, UT, wound	3	Same as for *C. braakii*
Cronobacter				
C. dublinensis (*E. sakazakii*)	+	Wound, eye, blood	3	Milk powder factory, water
C. condimenti (*E. sakazakii*)	−			
C. malonaticus (*E. sakazakii*)	+	CSF, blood, wound, RT, ear	2	Ubiquitous; powdered infant formula
C. muytjensii (*E. sakazakii*)	+	Bone marrow, blood	2	Ubiquitous; powdered infant formula
C. sakazakii (*E. sakazakii*)	++	RT, wound, CSF, feces	1	Ubiquitous; powdered infant formula
C. turicensis (*E. sakazakii*)	+	Blood	2	Ubiquitous; powdered infant formula
C. universalis (*Cronobacter* genomospecies 1)	+	Wound	3	Ubiquitous; powdered infant formula
Enterobacter				
E. aerogenes	++++	All sites	1	Ubiquitous
E. asburiae	++	**UT,** RT, feces, wound, blood	2	Water
E. cancerogenus	++	**Wound,** RT, feces	2	Animals, water
E. cloacae subsp. *cloacae*	++++	All sites	1	Water, soil, sewage, meat
E. cloacae subsp. *dissolvens*	−			Diseased corn stalks
E. hormaechei subsp. *hormaechei*	++	RT, wound, blood	1	Unk; one isolate from a frog
E. hormaechei subsp. *oharae* and *E. hormaechei* subsp. *steigerwaltii*	++	All sites	1	Plants
E. kobei	+	Blood, RT, UT	2	Food
E. ludwigii	Unk	UT, RT, blood, feces	2	Food
E. oryziphilus	−			Rice endosphere
E. oryzendophyticus	−			Rice endosphere
Hafnia alvei DNA groups 1 and 2	++	Feces, blood, RT	2	Ubiquitous
Klebsiella				
K. granulomatis	++	Genital tract	1	None; restricted to humans
K. pneumoniae subsp. *pneumoniae*	++++	All sites, RT/UT most common	1	Ubiquitous
K. ozaenae	++	**Nasal discharge,** RT, UT, blood	1	None; restricted to humans
K. rhinoscleromatis	++	Nasal discharge	2	None; restricted to humans
K. oxytoca	+++	All sites, common in feces	2	Ubiquitous
K. singaporensis	−			Soil
K. variicola	++	Blood, urine	1	Plants
Kosakonia				
K. (*Enterobacter*) *arachidis*	−			Plants
K. (*Enterobacter*) *cowanii*	Unk	UT, RT, blood, wound	3	Unk
K. (*Enterobacter*) *oryzae*	−			Wild rice
K. (*Enterobacter*) *radicincitans*	−			Phyllosphere of winter wheat
Lelliotta				
L. aminigena (*E. amnigenus* biogroups 1 and 2)	−			Plants, water
L. nimipressuralis (*E. nimipressuralis*)	−			Diseased elms

(Continued on next page)

TABLE 1 *(Continued)*

Current (previous) designation	Clinical data			Environmental data
	Frequency	Source(s)	Significance	
Morganella				
M. morganii subsp. *morganii* and *M. morganii* subsp. *sibonii*	++	All sites	1	Unk; mammalian and reptile gastrointestinal tracts
M. psychrotolerans	−			Fish, causes scombroid poisoning
Pantoea				
P. agglomerans	+++	All sites	2	Plants
P. ananatis	−			Plants
P. brenneri	+	Urethra, blood		
P. conspicua	+	Blood		
P. eucrina	+	RT, spinal fluid, UT		
P. septica	++	Blood, stool, skin		
Photorhabdus				
P. asymbiotica subsp. *asymbiotica* and *P. asymbiotica* subsp. *australis*	++	Wound	2	Unk; possibly insects
P. luminescens/*P. temperata*	−			Nematodes infecting insects
Plesiomonas shigelloides	+++	**Feces,** blood	1	Aquatic habitats, animals
Pluralibacter				
P. gergoviae (*E. gergoviae*)	++	RT, UT, blood	2	Water, cosmetics
P. pyrinus (*E. pyrinus*)	−			Pear trees
Proteus				
P. hauseri	+	Unk	Unk	Unk; probably like *P. mirabilis*
P. mirabilis	++++	**UT,** blood, CSF	1	Animals, birds, fish, foods
P. myxofaciens	−			Gypsy moth
P. penneri	++	**UT,** blood, wound, feces, eye	1	Probably like *P. mirabilis*
P. vulgaris	+++	**UT,** wound, stool, RT	1	Probably like *P. mirabilis*
Providencia				
P. alcalifaciens	+++	All sites; UT/feces common	1	Mammals, water
P. burhodogranariea/*P. sneebia*	−			Both from fruit flies
P. heimbachae/*P. rustigianii*	+	Feces	3	Unk; penguins
P. rettgeri	+++	All sites; UT most common	1	Same as for *P. alcalifaciens*; insects
P. stuartii	+++	All sites; UT most common	1	Mammals
P. vermicola	−			Nematodes infecting insects
Raoultella				
R. ornithinolytica	+	Wound, UT, blood	2	Food
R. planticola and *R. terrigena*	Unk	Similar to *K. pneumoniae*	3	Plants, water
Serratia				
S. entomophila	−			New Zealand grass grub, water
S. ficaria	+	RT, wound	3	Fig wasps, figs, plants
S. fonticola	+	**Wound,** RT	3	Water, birds
S. liquefaciens complex (*S. liquefaciens* sensu stricto, *S. proteamaculans*, *S. grimesii*)	+++	RT, wound	2	Ubiquitous
S. marcescens subsp. *marcescens*	++++	All sites; RT most common	1	Ubiquitous
S. marcescens subsp. *marcescens* biogroup 1	+	**UT,** RT	2	Unk
S. marcescens subsp. *sakuensis*	−			Wastewater
S. odorifera biogroups 1 and 2	+	RT, wound, feces, blood, UT	2	Plants
S. plymuthica	+	RT	3	Water, plants, small mammals
S. rubidaea	+	RT, wound, blood, UT, feces	2	Water, plants
S. nematodiphila/*S. ureilytica*	−			Nematodes/water

[a] Abbreviations and symbols: ++++, frequent; +++, occasional; ++, rare; +, very rare; −, not yet isolated from humans; CSF, cerebrospinal fluid; RT, respiratory tract; Unk, unknown; UT, urinary tract; 1, major pathogenic species of humans; 2, proven cause of disease in rare instances; 3, isolated from humans, significance unknown. Bold indicates most common source. Data are from references 18, 21, 38, 44–46, 50, 51, 53, 67–69, 77, 81, 105, 110, and 181–204.

TABLE 2 Other members of the family *Enterobacteriaceae*[a]

Human pathogens or opportunists	Primarily environmental strains[b,c]	Nonhuman isolates[c]
Buttiauxella gaviniae (207)	*Budvicia aquatica* (206)	*Buttiauxella* species (205, 207)
Edwardsiella tarda	*Buttiauxella noackiae* (207)	*Erwinia* species (211, 212)
Ewingella americana	*Edwardsiella hoshinae* (208)	*Edwardsiella ictaluri* (213)
Cedecea davisae, *Cedecea lapagei*, *Cedecea neteri*, *Cedecea* genomospecies 3 and 5	*Pragia fontium* (209)	*Kluyvera intermedia* (196, 207)
Kluyvera ascorbata, *K. cryocrescens*, *K. georgiana*	*Trabulsiella guamensis* (210)	
Leclercia adecarboxylata		
Leminorella grimontii, *L. richardii*, *Leminorella* genomospecies 3		
Moellerella wisconsensis		
Rahnella aquatilis, *Rahnella* genomospecies 2 and 3 (48)		
Tatumella spp. (51)		
Yokenella regensburgei		

[a] References are given in parentheses. Genomospecies listed cannot be biochemically separated from other species within their genus and/or only a single strain exists.

[b] Rare human isolates of no, or questionable, significance.

[c] Environmental isolates; fish, marine, animal, or bird isolates; insect isolates or pathogens; plant isolates or phytopathogens.

K. pneumoniae, primarily strains with capsular type K1, has emerged as an important cause of community-acquired pyogenic liver abscess worldwide (29–32). The majority of patients with *Klebsiella* pyogenic liver abscess are Asian males, 50 to 60 years of age, who present primarily with a right-lobe, solitary, monomicrobial abscess. Studies by Brisse et al. (29) indicate that pyogenic liver abscess-associated K1 isolates belong to a clonal complex designated CC23^{K1}, whereas K1 strains associated with severe pneumonia isolated from the respiratory tract and bloodstream infections belong to a different complex, CC82^{K1}. *magA* (mucoviscosity-associated gene), which resides within the *cps* (capsular polysaccharide synthesis) operon in all K1 isolates, is not an indicator for primary liver abscess. Conversely, the gene *allS* (an activator of the allantoin regulon) has been detected, to date, only in K1 strains isolated from primary liver abscesses (29, 33). *allS* may confer an advantage on these strains because levels of allantoin, which can serve as a carbon source, are elevated in non-insulin-dependent diabetics (33). Primary liver abscess K1 strains also appear to be separable by conventional biochemical methods (see "Identification" below). Mouse lethality studies show that *Klebsiella* liver abscess isolates that possessed both the hypermucoviscosity phenotype and the *rmpA* (regulator of mucoid phenotype) gene, regardless of the K type, had a 50% lethal dose of $<10^2$ CFU, versus $>5 \times 10^7$ CFU for type K1 or K2 urine isolates that were not hypermucoviscous and without *rmpA* or aerobactin genes (33).

The hypervirulent (hypermucoviscous) clinical variant hvKP has become established in the Pacific Rim and is emerging in Western countries but is underrecognized. A hypermucoviscous phenotype is used as a laboratory surrogate marker for this variant. The propensity of hvKP strains for metastatic spread in noncompromised hosts is both a defining and an unusual trait. The hvKP1 strains are more resistant to complement and neutrophil-mediated bactericidal activity and can be more virulent in a rat subcutaneous abscess model than cKP1 to cKP4. The recognition of the hypermucoviscous phenotype (defined by a positive string test) is indicative that the clinical strain may be an hvKP strain, which is hypervirulent compared to cKP. An improvement in our understanding of the epidemiology and clinical spectrum of infection by hvKP is needed since it may be more prevalent than appreciated.

Klebsiella rhinoscleromatis and *Klebsiella ozaenae* have adapted to cause specific chronic infectious diseases, i.e., rhinoscleroma and atrophic rhinitis (ozena), respectively (29). Neither organism is isolated from the environment or the intestinal tract, and both have lost the ability to utilize substrates involved in plant product degradation pathways. However, isolates from the bloodstream, urinary tract, and other infection sites indicate that *K. ozaenae* is more diverse in its ability to cause disease than *K. rhinoscleromatis*. Atrophic rhinitis is restricted to the nose, but rhinoscleroma may spread to the trachea and larynx (32). Both of these tissue-destructive diseases occur more frequently in tropical areas of the world and are spread by person-to-person transmission, although prolonged contact with persons producing airborne nasal secretions is required. A retrospective study has updated the epidemiological and clinical features of rhinoscleroma (34).

Klebsiella granulomatis is the agent of donovanosis or granuloma inguinale, a disease characterized by chronic genital ulcers (29, 35). It also occurs predominantly in tropical countries and is thought to be sexually transmitted, with humans as the only known reservoir. *K. oxytoca* strains carrying a chromosomally encoded heat-labile cytotoxin have been increasingly recognized as a cause of antibiotic-associated hemorrhagic colitis (36, 37). Antibiotic-associated hemorrhagic colitis, associated with the use of β-lactam antibiotics, is self-limiting and resolves spontaneously with the withdrawal of the contributing antibiotic. It differs from *Clostridium difficile* disease in that there is no pseudomembrane formation and *K. oxytoca* antibiotic-associated hemorrhagic colitis stools are bloody. The majority of isolations reported to date for *Klebsiella variicola* are from sterile sites, mainly blood and urine (38, 39). Isolates of this organism (three of five from urine), identified by *rpoB* sequencing reported from a Brazilian study, provide increased evidence that this organism is a human pathogen (39).

Raoultella planticola and *Raoultella terrigena* share pathogenicity characteristics with *K. pneumoniae* and are difficult to distinguish from it biochemically without special tests. In European studies, 3.5% to 19% of clinical strains initially identified as *Klebsiella* were *R. planticola*, while in U.S. and Brazilian surveys of 436 and 122 strains, respectively, only 1 isolate in each was identified as *R. planticola*, indicating that the prevalences of these species may differ geographically (39–41). *Raoultella planticola* has rarely been documented as a cause of human infections. *Raoultella terrigena* is an aquatic and soil organism, and human infections are rare. The first reported human infection caused by this organism was in 2007; a 45-year-old patient developed endocarditis due to *R. terrigena* after liver transplantation (42).

Erwinia, Enterobacter, and *Pantoea*

Since the early 1970s, the *Erwinia herbicola-Enterobacter agglomerans* complex has been subjected to continuous reevaluation and taxonomic revisions. The *Erwinia* genus has been a depository for plant-associated members of the *Enterobacteriaceae*. Yet many *Erwinia* species were phenotypically similar or showed strong DNA-DNA hybridization to the species then classified as *Erwinia*: *Erwinia herbicola*, *Erwinia ananas*, *Erwinia uredovora*, *Erwinia milletiae*, *Erwinia dissolvens*, and the recently recognised *Lelliotta* (*Enterobacter*) *nimipressuralis* (43).

Enterobacter species are found in the natural environment in habitats such as water, sewage, vegetables, and soil. *Enterobacter cloacae* and *Enterobacter aerogenes* also occur in dairy products, meat, hospital environments, the skin, and the intestinal tracts of humans and animals. *Enterobacter hormaechei* and *Enterobacter ludwigii* are isolated from a variety of human sources, including blood (17, 44, 45). A nonhuman source for *Enterobacter ludwigii* has not been reported, but plants appear to be the natural habitat of *Enterobacter hormaechei*. The *Enterobacter* spp. are of increasing concern due to their increasing carriage of antibiotic resistance. Nosocomial *Enterobacter* colonization and infection are frequently associated with contaminated medical devices and instrumentation. However, *Enterobacter* spp. are commonly consumed in foods, and so endogenous sources should also be considered (46). The taxonomies of *Enterobacter cloacae* and *Enterobacter agglomerans* have been revised several times, and the groups represent polyphyletic complexes. The uncertain delineation and recognition of species within these complexes still remains, and this affects the reliability in our understanding of the ecology and epidemiology of a given *Enterobacter* species.

Species belonging to the genus of *Pantoea* are commonly isolated from plants, humans, and the natural environment. Their yellow pigmentation is due to their occurrence on plant surfaces to protect cells from photodamage. The genus *Pantoea* was originally designated for two groups of strains that had been assigned to the *Erwinia herbicola-Enterobacter agglomerans* complex, and also DNA groups I, II, IV, and V of Brenner et al. (47). This genus has undergone several taxonomic revisions since the 10th edition of this *Manual*. Currently, the genus *Pantoea* comprises almost two dozen validly published species. These include *Pantoea agglomerans*, *Pantoea dispersa*, *Pantoea ananatis*, and *Pantoea stewartii* (48, 49). More recently, the application of MLSA (four-locus scheme) has supported the designation of four additional *Pantoea* species: *Pantoea septica*, *Pantoea eucrina*, *Pantoea brenneri*, and *Pantoea conspicua* (50). In addition, *Pectobacterium cypripedii* has been transferred to the *Pantoea* genus as *Pantoea cypripedii* (50), while *Pantoea citrea*, *Pantoea punctata*, and *Pantoea terrea* have been transferred to the genus *Tatumella* (51). Rapid identification of species within the *Pantoea* genus has been problematic since they are phenotypically similar to closely related species of other *Enterobacteriaceae* genera. Many clinical isolates may be misassigned as *P. agglomerans* due to the reliance on commercially available biochemical test kits.

The majority of *Pantoea* species either are associated with plants or are plant pathogens. *Pantoea* species, especially *Pantoea agglomerans* and *Pantoea ananatis*, can also cause human infections (49, 52). *Pantoea agglomerans* is the species of *Pantoea* most commonly isolated from humans. Sporadic infections are associated with penetrating trauma by objects contaminated with soil or vegetation resulting in soft tissue infections, septic arthritis, or osteomyelitis, whereas health care-associated infections and outbreaks often involve contaminated intravenous fluid, parenteral nutrition, or other administered fluids. *Pantoea agglomerans* infections in children, most of whom have severe underlying conditions, are predominantly polymicrobic and therefore of questionable significance even when specimens are from sterile sites (49).

Cronobacter

The *Cronobacter* genus (formerly *Enterobacter sakazakii*) currently contains seven species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter univerisalis*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter dublinensis*, and *Cronobacter condimenti* (43, 53, 54). The *Cronobacter* genus used to include *Cronobacter helveticus*, *Cronobacter pulveris*, and *Cronobacter zurichensis*, which were formerly known as *Enterobacter helveticus*, *Enterobacter pulveris*, and *Enterobacter turicensis*. These have been taxonomically reassigned to *Franconibacter helveticus*, *Franconibacter pulveris*, and *Siccibacter turicensis*. Of these, *C. sakazakii* and *Cronobacter malonaticus* are the species most frequently isolated from clinical samples (55, 56), with *Cronobacter sakazakii* predominating in neonatal infections and *Cronobacter malonaticus* in adult infections. The others are primarily environmental in origin, although a few clinical cases have been documented (57). Although not common, *C. sakazakii* is associated with severe cases of neonatal meningitis and necrotizing enterocolitis with relatively high mortality rates in neonates. This species is the only member of the genus to utilize sialic acid (2-keto-3-deoxy-5-acetamido-D-glycero-D-galacto-nonulosonic acid). This may contribute to its propensity to cause neonatal and infant infections, since sialic acid is found in breast milk, infant formula, mucin, and gangliosides (58). Limited studies to date indicate that *C. malonaticus* may predominate the more numerous adult infections and may be carried in the laryngopharynx (59). Isolates of *Cronobacter* cannot be identified to the species level in clinical laboratories due to a lack of discriminatory biochemical tests. The most reliable and robust method is DNA sequence analysis of the *fusA* and *rpoB* genes, for which an open-access MLST database is available (http://pubMLST.org/cronobacter). The recognition and expansion of the *Cronobacter* genus have caused much confusion in the literature. Two common mistakes are (i) referring to all pre-2007 *E. sakazakii* isolates as *C. sakazakii* and (ii) the continued use of commercially available biochemical kits with supporting databases that both continue to use the name *Enterobacter sakazakii* and do not encompass all the species in the *Cronobacter* genus.

Lelliottia, Pluralibacter, and *Kosakonia*

The new genus *Lelliottia* has been formed and *Enterobacter nimipressuralis* and *Enterobacter amnigenus* have been reclassified as *Lelliottia nimipressuralis* and *Lelliottia amnigena*, respectively (43). *Enterobacter gergoviae* and *Enterobacter pyrinus* have been transferred to the new genus *Pluralibacter* as *Pluralibacter gergoviae* and *Pluralibacter pyrinus*, respectively. The new genus *Kosakonia* includes the former *Enterobacter cowanii*, *Enterobacter radicincitans*, *Enterobacter oryzae*, and *Enterobacter arachidis* as *Kosakonia cowanii*, *Kosakonia radicincitans*, *Kosakonia oryzae*, and *Kosakonia arachidis*, respectively (43).

Serratia

Serratia spp. are notorious health care-associated pathogens and colonizers. Transmission is predominately from person to person, but medical apparatuses, intravenous fluids, and other solutions are often implicated as well (46). Indwelling catheters, particularly for urinary tract infections, serve as a primary reservoir for transmission via hospital personnel. In children, the gastrointestinal tract is a common source of infections. Outbreaks transmitted by hand are often insidious, occurring over long periods, and may subside and peak a number of times before recognition and infection control efforts can contain them. Some species and biotypes of *Serratia* produce a nondiffusible red pigment, prodigiosin, or 2-methyl-3-amyl-6-methoxyprodigiosene. The pigment may be toxic to protozoa and therefore of ecological advantage in water and soil. Pigment production in *S. marcescens* appears to be a marker that the strain is environmental in origin and of low virulence (60). Community-acquired infections are rare, except for *S. marcescens* contact lens-induced acute red eye (61). Most of the other species of *Serratia* have also been isolated from humans, in whom they too are usually transients or cause opportunistic infections. Water is probably the principal habitat of *S. plymuthica*.

Serratia does not usually infect a healthy human being, but it frequently colonizes or infects hospitalized patients. *S. marcescens* is currently the only known nosocomial species of *Serratia*. Although *Serratia liquefaciens* and *Serratia rubidaea* are occasionally isolated from clinical specimens, their pathogenic role has not been established. *Serratia* infections are broad and similar to those by other opportunistic pathogens: respiratory and urinary tract infections, surgical wound infections, and septicemia in patients with intravenous catheterization. Meningitis, brain abscesses, and intra-abdominal infections are infrequent.

Citrobacter

Citrobacter organisms are responsible for diverse serious enteric diseases as well as extraintestinal disorders. *Citrobacter* infections include urinary tract infections, superficial wound infections, respiratory tract infections, intra-abdominal infections, bacteremia, endocarditis, infant meningitis (accompanied by brain abscess formation), and sepsis. The organisms are ubiquitous and can be isolated from soil, water, and food as well as the intestinal tracts of humans and animals. Citrobacters are primarily inhabitants of the intestinal tract, and their presence in the environment may reflect fecal excretion by humans and animals; the natural habitat of some newer *Citrobacter* species is unknown.

The *Citrobacter* genus contains 12 species, of which 3 are most commonly involved in hospital infections: *Citrobacter freundii*, *Citrobacter koseri*, and *Citrobacter braakii*. *Citrobacter* is an opportunistic pathogen which has been recognized as an important cause of nosocomial infection, especially in neonates and immunocompromised patients. Two important types of *Citrobacter* infection are healthcare associated and community acquired. Patients are usually older adults (≥65 years), more often males, and the urinary tract is the most common site of infection (62, 63). One-third to one-half of *Citrobacter* infections, including septicemias, are polymicrobic and are associated with higher mortality rates (18% to 50%) or longer hospital stays (62, 63). Meningitis is almost exclusively associated with *Citrobacter koseri* and involves children <2 months of age, with the highest onset rates recorded in neonates with a mean age of 7 days (46, 64). Brain abscesses occur in 75% of infected infants, and neurological defects are common sequelae in surviving infants. The most prominent risk factor is prior colonization; during outbreaks, colonization rates of 27% have been noted, versus a normal rate of <1% (65, 66). Person-to-person spread by hospital personnel and, less often, from mother to offspring is the most likely source of infection. Sampling of inanimate or environmental reservoirs in hospitals usually fails to yield *Citrobacter*. Other *Citrobacter* species, including *Citrobacter gillenii* and *Citrobacter murliniae*, have been found in human clinical, animal, food, and environmental specimens (67, 68). The role of many *Citrobacter* spp. in human infections is unclear because reports in the literature are insufficient to determine either clinical significance in humans or potential reservoirs for infection. *Citrobacter rodentium* causes murine colonic hyperplasia, which is self-limiting in adult mice but causes significant morbidity and mortality in infant mice in mouse colony outbreaks (69). The organism is used in mice as a model for enteropathogenic *E. coli* and enterohemorrhagic *E. coli* infections in humans.

Pseudocitrobacter

The new genus *Pseudocitrobacter* has been defined based on four fecal isolates from hospitalized patients and outpatients attending military hospitals in Pakistan (70). The strains were of particular importance for accurate identification, as three of them produced NDM-1 carbapenemase, and the fourth was phenotypically very similar. Initially the strains were phenotypically identified as *Citrobacter* species. However, 16S rRNA gene sequence comparison showed that the strains formed a separate cluster with only 97% to 98.3% shared identity with members of the genera *Citrobacter*, *Kluyvera*, *Pantoea*, *Enterobacter*, and *Raoultella*. MLSA based on the DNA and predicted amino acid sequence of only three loci (*recN*, *rpoA*, and *rpoB*) also demonstrated that the strains were in unique clusters from the remaining *Enterobacteriaceae*. The *thdF* gene could not be amplified for all strains. DNA-DNA hybridization supported the recognition of the new genus, which currently is composed of *Pseudocitrobacter faecalis* and *Pseudocitrobacter anthropi*; these species differ in their fermentation of raffinose and D-mannitol. The former species represent the strains which produced NDM-1.

Proteus, *Providencia*, and *Morganella*

Members of the genera *Proteus*, *Providencia*, and *Morganella* are widespread in the environment, are normal inhabitants of the gut, and are relatively common in clinical laboratories, especially *P. mirabilis*. In a large 6-year population-based survey of *Proteeae*, 85% (4,290 of 5,047) of isolates were community acquired, although providenciae were more likely to be acquired in nursing homes (71). Females (69%) and the elderly (median age, 70 years) were at the highest risk of infection, and the most common specimen sources were urine (86%), soft tissues (7%), blood (3%), miscellaneous fluids (2%), and the respiratory tract (2%). *P. mirabilis*, predominantly from urine, was the most frequently isolated agent (77%). Non-*P. mirabilis* species (primarily *Proteus vulgaris*) were isolated from wounds and soft tissue more often than urine. Of the *Providencia* species, *P. stuartii* was isolated twice as often as other species. *P. mirabilis*, *Proteus penneri*, *Morganella*, and *Providencia alcalifaciens* are seen in diarrheal stools with greater frequency than in normal stools, leading to speculation that they may cause diarrhea. Some strains of *P. alcalifaciens* are invasive in HEp-2 cell assays and elicit diarrhea in the RITARD (reversible intestinal-tie adult rabbit diarrhea) model, while other strains isolated in pure culture or in large numbers from diarrheal stools fail to invade cell lines (72, 73). However, a number of noninvasive *P. alcalifaciens* strains have been shown to be nonadherent to cell lines *in vitro*, which may provide insight to their inability to invade (74). Yoh et al. (75) used a specialized medium to isolate nine strains of *Providencia rettgeri* from 130 persons with traveler's diarrhea; eight of these strains were invasive in Caco-2 cells, indicating their potential for virulence in humans. Notably, vomiting was present in five *P. rettgeri* cases but was not seen in patients from whom other providenciae were isolated.

Hafnia

Hafnia organisms are commonly isolated from the gastrointestinal tracts of humans and animals (including mammals, birds, reptiles, fish, and insects) and from foods (including meat and dairy products) (76). They are associated with a range of animal infections and have been recovered from human clinical specimens during cases of bacteremia and gastroenteritis and respiratory tract infections. Their status as primary human or animal pathogens, however, remains controversial because these organisms are usually found together with other pathogens and/or opportunists at the site of infection (76).

Few systematic investigations regarding the ecological distribution of *Hafnia* have been published, although it is a common inhabitant of the gastrointestinal tracts of various animals (76). Isolation from consumables, especially meats, is not uncommon, and presumably its presence indicates prior contact of the item with feces. *Hafnia alvei* has been linked to gastrointestinal disease, although putative virulence characteristics have not been demonstrated (77, 78). However, a toxigenic strain producing a cytopathic effect on Vero cells indistinguishable from that of Shiga toxin, but not neutralized by anti-Shiga toxin antibody, has been reported (79). Although seen infrequently in extraintestinal disease, such infections occur both in healthy and in immunocompromised patients, with monomicrobial infection rates varying from 12 to 75%; the correlation with disease increases when the organism is isolated in pure culture and in high numbers (76). *Hafnia* appears to have a predilection for the biliary tree and may produce abscesses at the site of infection (80).

Until recently, *Hafnia alvei* has been the only recognized species in the genus. However, previous DNA-DNA hybridization studies and partial 16S rRNA gene sequencing have indicated that *Hafnia alvei* is genetically heterogeneous and consists of at least two DNA hybridization groups (HGs) (81). Previously published and new phenotypic data revealed that a combination of malonate assimilation and β-glucosidase activity enabled correct assignment of *Hafnia* isolates to one of the two HGs. *Hafnia alvei* corresponds to HG1 and includes the type strain ATCC 13337. The new species *Hafnia paralvei* is given for the former members of *H. alvei* HG2 (82, 83). *Hafnia* isolates can be unambiguously assigned to the correct species (*H. alvei* or *H. paralvei*) based upon the following five tests: β-glucosidase, malonate utilization, D-arabinose fermentation, salicin fermentation, and esculin hydrolysis. Both species can be frequently isolated from clinical laboratory samples (82). Although *H. alvei* strains are more likely to be toxigenic than *H. paralvei* isolates, both species produce a Vero cytolytic toxin. Other reported virulence characteristics include siderophores, resistance to complement-mediated lysis, and possession of type 1 and 3 fimbriae (84).

Plesiomonas shigelloides

Plesiomonas shigelloides is the only species of the genus *Plesiomonas* and is the only oxidase-positive member of the *Enterobacteriaceae* family (85). Freshwater and estuarine water are considered to be the natural environment of the organism, and it is a cause of waterborne infections. It is also found in a wide range of hosts, including amphibians, birds, fish, and animals (86). There is no evidence that the organism is the cause of diarrheal disease in any of these hosts, with the possible exception of cats. It is regarded as an emerging and significant enteric pathogen of water- and foodborne human infections and is a major a cause of traveler's diarrhea in Japan and China (85). Three major types of *P. shigelloides* gastroenteritis in humans occur: (i) a secretory, watery type; (ii) an invasive, dysentery-like type; and (iii) a subacute or chronic form, lasting between 2 weeks and 3 months (87–89). Outbreaks are generally related to consumption of seafood or untreated water. The pathogenicity of *P. shigelloides* is poorly understood. There are reports of several toxins being secreted *in vitro*: cholera-like toxin, thermostable and thermolabile toxins, β-hemolysins, and cytotoxin complex (90, 91). The somatic antigen may also play a role in pathogenicity, since the gene encoding the most common type, O17, shares almost complete identity with the form 1 (smooth) antigen gene of *Shigella sonnei* (92).

P. shigelloides is responsible for rare incidents of extraintestinal infections in humans, most notably, meningitis in neonates, bacteremia, sepsis, and septic shock with high fatality rates (85, 93). Wound infections associated with water contact are not encountered with plesiomonads despite their aquatic reservoir. *Plesiomonas* bacteremia, which is rare and usually polymicrobic, is generally community acquired, and major risk factors include biliary tract disease and advanced age (>75 years) (94).

Edwardsiella

The *Edwardsiella* genus includes *Edwardsiella tarda*, *Edwardsiella ictaluri*, *Edwardsiella piscicida*, and *Edwardsiella hoshinae*. These are primarily fish pathogens and cause economic losses to the aquaculture industries worldwide. *Edwardsiella tarda* is a broad-host-range pathogen that infects various species of fish and animals as well as humans. *Edwardsiella ictaluri* is highly host adapted to channel catfish and is not of clinical concern. The ability of *Edwardsiella hoshinae* to cause disease has not been established, and little is known regarding its habitats. *Edwardsiella tarda* is also associated with opportunistic infections in humans. These are most commonly gastroenteritis and wound infections and less frequently cases of septicemia, meningitis, and liver abscess. Most *Edwardsiella tarda* human infections are linked to contact with fish or turtles. Other than in tropical areas, there is only a low carriage rate in humans. The pathogenicity of *Edwardsiella tarda* is probably linked to the production of a cell-associated hemolysin and its ability to invade intestinal cells, as demonstrated with HEp-2 cells in tissue culture studies (95). Serious wound infections, including myonecrosis, have been reported to occur in immunocompetent individuals who have aquatic exposure. Systemic infections have been reported to occur patients with liver disease or iron overload conditions (96). Initial genomic studies indicate that *Edwardsiella tarda* can be split into two genotypes, with EdwGII strains being associated with human infections (97).

Miscellaneous *Enterobacteriaceae*

Miscellaneous members of *Enterobacteriaceae* that are infrequently encountered in clinical laboratories are primarily opportunistic pathogens in compromised patients or are present as transients or commensals in clinical specimens (46). For some, like *Cedecea*, *Leminorella*, *Moellerella*, and *Tatumella*, a reservoir has not been determined because they are rarely isolated from nonhuman sources, while *Ewingella*, *Leclercia*, and *Kluyvera* are found in a variety of foods, water, or animals (snails and slugs) and, like many enterics, appear to be ubiquitous in the environment (46, 98–104). Other genera isolated from humans have more specific natural habitats such as *Rahnella* (water) or *Yokenella* and *P. asymbiotica* (insects and infections from insect bites) (46, 105–107). There have been increased reports of clinically significant isolations of *L. adecarboxylata* and *Kluyvera* species, all of which cannot be covered here. *Kluyvera* infections occur in immunocompetent and immunosuppressed patients of all age groups (108, 109). The organism is isolated from a broad spectrum of sources, including blood, tissue, urine, cerebrospinal fluid, and peritoneal fluid, although urinary tract and bloodstream infections each account for approximately one-third of the infections. When strains are identified to the species level, *Kluyvera ascorbata* accounts for more than twice the number of *Kluyvera cryocrescens* infections. Originally considered to be an opportunistic pathogen, *L. adecarboxylata* is quite often isolated from polymicrobic infections in healthy patients without underlying disease (110). This suggests that the coinfecting agent(s) might alter the local tissue environment, allowing growth of *Leclercia*, or that a transfer of genetic factors occurs, enhancing its virulence. The isolation of *Leclercia* in pure culture from previously healthy persons (from a foot abscess and blood and wound caused by a hydrofluoric acid chemical injury) described in two reports would indicate that at least some strains may be pathogenic (110, 111). *Moellerella wisconsensis*, a rarely reported organism first isolated from stools of diarrheal patients, has been reported from an additional series of five patients with disease ranging from self-limited acute watery diarrhea without mucus to protracted diarrhea, lasting several weeks. No other common diarrheal bacterial pathogens or parasites were present in the stools, and specimens taken after clinical recovery were negative (112).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

The organisms covered in this chapter are, in general, readily isolated from clinical material, and the principles in chapter 18 of this *Manual* on specimen collection, handling, and processing are applicable. Most of these organisms survive in culture deeps for approximately a year. Long-term storage methods as recommended in chapter 11 work well for these organisms. *Plesiomonas* cells do not survive for more than 1 to 2 months when held at room temperature and should be maintained at −70°C. *Cronobacter* isolates do not survive well at 5°C. *Tatumella* strains have failed to survive longer than a year even at −70°C.

ISOLATION PROCEDURES

Few of the clinically relevant strains covered in this chapter present difficulties in isolation from sterile body sites. Isolation from nonsterile body or environmental sites may require specialized media such as CHROMagar Orientation (Becton Dickinson, Sparks, MD) and chromID CPS (bioMérieux, Hazelwood, MO), which perform similarly for the detection of urinary tract pathogens covered in this chapter and can reliably replace MacConkey and blood agars (113, 114). These media prevent swarming of *Proteus* and limit the spread of mucoid colonies, which reduces overgrowth of pathogenic colonies. Additionally, colonies on CHROMagar can be used to inoculate antimicrobial susceptibility tests directly without subculture. CHROMagar media can also be used for specimens from other nonsterile sites; when colony color was combined with indole, lysine, and ornithine decarboxylase tests and serology, 98.7% (466 of 472) of the above-listed organisms were correctly identified from nonurine samples (115).

Both of the diarrheal pathogens covered in this chapter are easily isolated. *E. tarda* is a lactose-negative, H_2S-positive organism, indistinguishable from *Salmonella* on enteric plating media (opaque or opaque with black centers). A positive indole reaction and failure to agglutinate in specific *Salmonella* antisera separate the two organisms. *Plesiomonas* produces non-lactose-, non-sucrose-fermenting colonies on enteric plating media. It does not grow on thiosulfate-citrate-bile salts-sucrose medium, but on cefsulodin-Irgasan-novobiocin medium, opaque colonies without a pink center (mannitol not fermented) are suspicious for plesiomonads. Two other oxidase-positive organisms, *Pseudomonas* and *Aeromonas*, grow on cefsulodin-Irgasan-novobiocin as well, although *Aeromonas* colonies have a pink center with an opaque apron. Inositol fermentation and a positive reaction in Moeller's lysine, arginine, and ornithine tests differentiate *Plesiomonas* from these agents as well as other organisms.

Other *Enterobacteriaceae* involved in opportunistic infections and that may be isolated from a variety of specimen types generally grow well on commonly used laboratory media. Some genera are lactose or sucrose fermenters and give the appearance of normal biota on enteric plating media, while others may produce H_2S and appear *Salmonella*-like. *Rahnella*, *Ewingella*, and *Tatumella* may require 48 h for growth. *Tatumella* also grows poorly on Mueller-Hinton agar, and a broth dilution method may be required for susceptibility testing. *K. granulomatis* does not grow on conventional laboratory media but has been grown in HEp-2 monolayers (116). Detection of Donovan bodies from tissue smears using the Giemsa or Wright stain is the method most commonly used to detect this organism. However, these pleomorphic, bipolar staining bodies shaped like a closed safety pin are not always present and are not reliable for diagnosis.

IDENTIFICATION

The biochemical tests most useful for separating members covered in this chapter are given in Tables 3 through 13. Correct identification to the species level is increasingly important in recognizing strains that are of high risk for carrying extended-spectrum β-lactamases (ESBLs), cephalosporinases, or carbapenemases.

It should be noted that identification problems arising from the use of commercial phenotyping systems vary with each genus. The percentage of correct identifications for many commercial systems increases significantly when additional biochemical tests are performed on organisms with "low-probability" identifications. Even when unusual enterobacteria covered in this chapter are included in commercial system databases, the number of strains available to use in challenge studies is very limited; therefore, the ability of these systems to accurately identify these organisms is really unknown. O'Hara (117) has published a comprehensive review on manual and automated systems for the identification of bacteria in clinical laboratories. It provides the component substrates used and the additional reagents that are needed, if any, as well as quality control, database content, accuracy of identification, important features of each automated identification system, and published evaluations, among other information, for each commercial system. It

TABLE 3 Separation of members of the genus *Citrobacter*[a]

Species	Indole	ODC	Malonate	Acid[b] from: Sucrose	Dulcitol	Melibiose	Adonitol
C. *amalonaticus*	+	+	–	–	–	–	–
C. *braakii*	V	+	–	–	V	V	–
C. *farmeri*	+	+	–	+	–	+	–
C. *freundii* (*sensu stricto*)	V	–	–	V	–	+	–
C. *koseri*	+	+	+	V	V	–	+
C. *rodentium*	–	+	+	–	–	–	–
C. *sedlakii*	V	+	+	–	++		–
C. *werkmanii*	–	–	+	–	–	–	–
C. *youngae*	V	–	–	V	+	–	–
C. *gillenii*	–	–	+	V	–	V	–
C. *murliniae*	+	–	–	V	+	V	–

[a]Abbreviations and symbols: ODC, ornithine decarboxylase; +, ≥85%; V, 15 to 84%; –, <15%.

[b]Fermentation reactions in commercial systems should be similar to reactions in conventional fermentation broths (1% carbohydrate in broth with indicator).

also emphasizes that the clinical laboratory should not rely solely on the identification provided by commercial systems but rather take into account colony morphology, susceptibility test data, and anatomic site of isolation.

Alternative methods of identification are based on DNA sequence and protein profiling. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry is increasingly being applied in clinical laboratories and has been evaluated in multicenter studies (118). These methods can be reliable provided the culture database used to generate the reference profiles used correctly identified strains initially. MALDI-TOF has been evaluated for the profiling of antibiotic-resistant *Enterobacteriaceae* from blood cultures (119). This can be an issue with genera that have undergone taxonomic reevaulations, such as *Cronobacter* (120).

Klebsiella and *Raoultella*

K. pneumoniae CC23^{K1} isolates associated with pyogenic liver abscess may be distinguished biochemically from CC82^{K1} and type K2 *K. pneumoniae* strains by their utilization of D-ribose, 3-hydroxybutyrate, D-tagatose, and dulcitol as sole carbon sources (78). *K. ozaenae* and *K. rhinoscleromatis* are slow-growing organisms and thus do poorly in commercial systems; however, they can be difficult to separate using conventional biochemicals as well. *R. planticola* and *R. terrigena* require temperature growth and carbon assimilation tests to separate them from *Klebsiella* species.

TABLE 4 Differentiation of *Pantoea agglomerans* and members of the genera *Enterobacter*, *Lelliotta*, *Pluralibacter*, and *Kosakonia*[a,b]

Species	LDC	ADH	ODC	VP	Acid[c] from: Sucrose	Adonitol	D-Sorbitol	L-Rhamnose	α-Methyl-D-glucoside	Esculin	Melibiose	Yellow pigment
Human species												
P. agglomerans	–	–	–	V	V	–	V	V	–	V	V	V
E. *aerogenes*	+	–	+	+	+	+	+	+	+	+	+	–
E. *amnigenus* biogroup 1	–	–	V	+	+	–	–	+	V	+	+	–
E. *asburiae*	–	V	+	–	+	–	+	–	+	+	–	–
E. *cancerogenus*	–	+	+	+	–	–	–	+	–	+	–	–
E. *cloacae* subsp. *cloacae*	–	+	+	+	+	V	+	+	V	V	+	–
E. *hormaechei* subsp. *hormaechei*	–	V	+	+	+	–	–	+	V	–	–	–
E. *kobei*	–	+	+	–	+	–	+	+	+	V	+	–
K. *cowanii*[d]	–	–	–	+	+	–	+	+	–	+	+	V
Pluralibacter gergoviae	+	–	+	+	+	–	–	+	–	+	+	–
Environmental species												
E. *cloacae* subsp. *dissolvens*	–	+	+	+	+	–	+	+	+	+	+	–
K. *radicincitans*	–	+	–	+	+	–	+	+	–	+	–	–
L. *amnigenus* biogroup 2	–	V	+	+	–	–	+	+	+	+	+	–
L. *nimipressuralis*	–	–	+	+	–	–	+	+	+	+	+	–
Pluralibacter pyrinus[e]	+	–	+	+	+	–	–	+	–	+	+	–

[a]See the text for *E. ludwigii*, *E. hormaechei* subsp. *steigerwaltii*, and *E. hormaechei* subsp. *oharae* identification.

[b]Abbreviations and symbols: LDC, lysine decarboxylase; ADH, arginine dihydrolase; ODC, ornithine decarboxylase; VP, Voges-Proskauer; +, ≥90%; V, 11 to 89%; –, ≤10%.

[c]See Table 3, footnote *b*.

[d]Separated from *P. agglomerans* by a negative malonate reaction and fermentation of D-sorbitol (31).

[e]Separated from *P. gergoviae* by positive reactions in potassium cyanide broth and *myo*-inositol.

TABLE 5 Separation of some members of the genera *Klebsiella* and *Raoultella*[a,b]

Species	Indole	ODC	VP	Malonate	ONPG	Growth at: 10°C	Growth at: 44°C	Acid[c] from D-melezitose
R. ornithinolytica	+	+	V	+	+	+	NA	NA
K. oxytoca	+	−	+	+	+	−	+	−
K. ozaenae	−	−	−	−	V	NA	NA	NA
K. pneumoniae[d]	−	−	+	+	+	−	+	−
R. planticola	V	−	+	+	+	+	−	−
R. terrigena	−	−	+	+	+	+	−	+
K. rhinoscleromatis	−	−	−	+	−	NA	NA	NA

[a] *K. singaporensis* biochemicals not available; only a single strain is known.

[b] Abbreviations and symbols: ODC, ornithine decarboxylase; VP, Voges-Proskauer; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; NA, not available; +, ≥90%; V, 11 to 89%; −, ≤10%.

[c] See Table 3, footnote *b*.

[d] A negative adonitol reaction may be an indication that the strain is *K. variicola*, but this must be confirmed with gene sequencing (*rpoB*) (39).

TABLE 6 Biochemical characterization of members of the genus *Serratia*[a,b]

Species	LDC	ODC	Mal	Acid[c] from: Arab	L-Rham	D-Xyl	Suc	Adon	D-Sorb	Cello	D-Arabitol	Red pigment	Odor
S. entomophila[d]	−	−	−	−	−	V	+	−	−	−	V	−	−
S. ficaria	−	−	−	+	V	+	+	−	+	+	+	−	V
S. fonticola	+	+	V	+	V	V	V	+	+	−	+	−	−
S. liquefaciens group	+	+	−	+	V	+	+	−	+	−	−	−	−
S. marcescens subsp. *marcescens*	+	+	−	−	−	−	+	V	+	−	−	V	−
S. marcescens biogroup 1	V	+	−	−	−	−	+	V	+	−	−	NA	−
S. odorifera biogroup 1	+	+	−	+	+	+	+	V	+	+	−	−	+
S. odorifera biogroup 2	+	−	−	+	+	+	−	V	+	+	−	−	+
S. plymuthica[e]	−	−	−	+	−	+	+	−	V	V	−	+	−
S. rubidaea	V	−	+	+	−	+	+	+	−	+	V	+	−

[a] *S. marcescens* subsp. *sakuensis* is reportedly a spore-forming organism. *S. ureilytica* is urea positive; only a single strain is known.

[b] Abbreviations and symbols: LDC, lysine decarboxylase; ODC, ornithine decarboxylase; Mal, malonate; L-Rham, L-rhamnose; D-Xyl, D-xylose; Suc, sucrose; Adon, adonitol; D-Sorb, D-sorbitol; Cello, cellobiose; +, ≥90%; V, 11 to 89%; −, ≤10%; NA, information not available.

[c] See Table 3, footnote *b*.

[d] Growth at 37°C but biochemical characterization optimal at 30°C.

[e] May fail to grow at 37°C.

TABLE 7 Separation of members of the genera *Proteus*, *Providencia*, and *Morganella*[a]

Organism	Indole	H_2S	Urea	ODC	Acid[b] from: Maltose	D-Adonitol	D-Arabitol	Trehalose	*myo*-Inositol
Proteus									
P. hauseri	+	V	+	−	+	−	−	+	−
P. mirabilis	−	+	+	+	−	−	−	+	−
P. penneri	−	V	+	−	+	−	−	V	−
P. vulgaris[c]	+	V	+	−	+	−	−	−	−
Providencia									
P. alcalifaciens	+	−	−	−	−	+	−	−	−
P. heimbachae	−	−	−	−	V	+	+	−	V
P. rettgeri	+	−	+	−	−	+	+	−	+
P. rustigianii	+	−	−	−	−	−	−	−	−
P. stuartii	+	−	V	−	−	−	−	+	+
Morganella									
M. morganii subsp. *morganii*	+	−[d]	+	+[e]	−	−	−	−	−
M. morganii subsp. *sibonii*	V	−[d]	+	+[e]	−	−	−	+	−

[a] Abbreviations and symbols: H_2S, hydrogen sulfide; ODC, ornithine decarboxylase; +, ≥90%; V, 11 to 89%; −, ≤10%.

[b] See Table 3, footnote *b*.

[c] *P. vulgaris* genomospecies 4, 5, and 6 cannot be differentiated phenotypically.

[d] Some members of some biogroups are H_2S positive.

[e] Some members of some biogroups are ornithine decarboxylase negative.

TABLE 8 Separation of *Cedecea* from selected *Enterobacter* and *Cronobacter* species (VP, ADH, and ODC variable or positive)[a]

Organism	Acid[b] from:					
	D-Sorbitol	Raffinose	L-Rhamnose	Melibiose	D-Arabitol	Sucrose
Cedecea davisae	−	−	−	−	−	+
Cedecea lapagei	−	−	−	+	+	−
C. sakazakii	−	+	+	+	−	+
E. cancerogenus	−	−	+	−	−	−
E. cloacae	+	+	+	+	V	−

[a] Abbreviations and symbols: VP, Voges-Proskauer; ADH, arginine dihydrolase; ODC, ornithine decarboxylase; +, ≥90%; V, 11 to 89%; −, ≤10%.

[b] See Table 3, footnote *b*.

R. terrigena can be separated from *R. planticola* by fermentation of β-gentibiose. *K. variicola* is not found in commercial systems, and information from additional isolates has shown that adonitol fermentation is of limited usefulness for its separation from *K. pneumoniae* (47, 87). At this point, sequencing of housekeeping genes (*rpoB* and others) is the only method of correctly identifying this organism. *phoE* and *scrA* (sucrose regulon) genes have been used to identify and to separate *K. granulomatis* (*phoE* positive and *scrA* negative) from other *Klebsiella* species (*phoE* and *scrA* positive) (121).

Enterobacter and *Pantoea*

Because of the genetic heterogeneity in several species, members of the genera *Enterobacter* and *Pantoea* appear to confound commercial systems more often than other genera (46). *E. ludwigii* and *E. hormaechei*, species previously residing within the *E. cloacae* complex, can be separated from *E. cloacae* by growth on 3-O-methyl-D-glucopyranose and putrescine and on 3-hydroxybutyrate, respectively (17, 45). By using commercially available tests, the three subspecies of *E. hormaechei* can be separated with adonitol, dulcitol, and D-sorbitol (*Enterobacter hormaechei* subsp. *hormaechei* tests negative, positive, and negative, respectively; *E. hormaechei* subsp. *steigerwaltii* tests positive, negative, and positive, respectively; and *E. hormaechei* subsp. *oharae* tests negative, negative, and positive, respectively) (17). *P. agglomerans* is very difficult to identify with either commercial systems or conventional biochemicals (117, 122). Yellow pigmented and lysine-, arginine-, and ornithine-negative organisms should raise suspicion that the strain is *P. agglomerans*; *Leclercia adecarboxylata* and *P. asymbiotica* also share these characteristics, but they can be separated from *P. agglomerans* by positive indole and negative D-mannitol reactions, respectively. *P. ananatis*, *P. dispersa*, and *Erwinia persicina* most closely resemble *P. agglomerans*, but *P. dispersa* can be separated from *P. agglomerans* by negative reactions for raffinose, salicin, and sucrose and *E. persicina* can be distinguished by negative reactions for maltose and D-xylose. *P. ananatis* is more difficult to differentiate, and all suspected isolates of this organism, as with other rare *Enterobacteriaceae*, should be sent to a reference laboratory for confirmation.

TABLE 9 Differentiation of *Kluyvera* from commonly seen indole-positive, VP-negative organisms[a]

Organism	Citrate	Urea	LDC	KCN
Kluyvera[b]	−	−	+	+
C. koseri	+	V	−	−
Morganella	−	+	−	+
Providencia	V	+	−	+
E. coli	−	−	+	−

[a] Abbreviations and symbols: VP, Voges-Proskauer; LDC, lysine decarboxylase; KCN, potassium cyanide; +, ≥90%; V, 11 to 89%; −, ≤10%. *Leclercia* and *E. tarda* are also indole positive and VP negative and can be found in Tables 10 and 12, respectively.

[b] Includes *K. intermedia* (*K. cochleae* and *Enterobacter intermedius* combined) (196).

Cronobacter

Cronobacter organisms form either mucoid colonies, smooth and irregularly round colonies, or rough and wrinkled colonies, which are difficult to remove from the agar surface. Biotyping was initially proposed as a means of identifying *Cronobacter* species but should no longer be followed given that DNA sequence-based (including whole-genome sequencing) studies have revealed that the biotyping scheme is in considerable variance with the known genus phylogeny and serves no useful purpose. Commercial kits have not been updated following the 2007 taxonomic revision and still use the former name of *Enterobacter sakazakii*. Therefore, these are of limited use for this genus. 16S ribosomal gene sequencing of the *Cronobacter* genus is not reliable for all species due to their close genetic relationships, in particular beween *Cronobacter sakazakii* and *Cronobacter malonaticus* (55, 57, 123). Instead, a number of molecular methods have been developed, the most useful being the sequencing of the housekeeping gene *fusA*, which forms part of the online MLST scheme (http://pubMLST.org/cronobacter) and the phylogeny of which matches the whole-genome phylogeny (55).

Serratia, *Citrobacter*, and *Proteae*

Serratia spp. are generally easily identified, except for the *S. liquefaciens* group; separation of members within this group can be achieved using a combination of API 50 CH (carbohydrate) and API ZYM (enzymatic) (bioMérieux, Hazelwood, MO) strips (18, 46). *Citrobacter* spp. may be included in databases individually or by subgroups (*C. braakii*-*C. freundii*-*C. sedlakii*, *C. werkmanii*-*C. youngae*, or *C. koseri*-*C. amalonaticus*); however, subgroup identification requires further biochemical testing by standard methodologies, and final species identification is delayed (46). A PYR (L-pyroglutamic acid, Oxoid PYR) disk, which detects pyrrolidonyl peptidase, may be useful for separating biochemically atypical strains of *Citrobacter* (positive) and *Salmonella* (negative) (124, 125). Gram-negative, oxidase-negative organisms that swarm on blood agar and appear flat with tapered edges on MacConkey agar may be reported as *Proteus*. Spot indole-negative and ampicillin-susceptible strains may be reported as *P. mirabilis*, while spot indole-positive, ampicillin-resistant strains are reported as *P. vulgaris* (126).

TABLE 10 Separation of LDC-, ODC-, and ADH-negative unusual *Enterobacteriaceae* found in clinical specimens[a]

Organism	Motility	Gas from glucose	KCN	VP	Acid[b] from:		
					Sucrose	L-Arabitol	Trehalose
Ewingella	V	−	−	+	−	−	+
Leclercia	+	+	+	−	+	+	+
Moellerella	+	+	V	−	−	+	+
Rahnella	−	+	−	+	+	+	+
Tatumella	−	−	−	−	+	−	+
Photorhabdus asymbiotica[c]	+	−	−	−	−	−	−

[a] Abbreviations and symbols: LDC, lysine decarboxylase; ODC, ornithine decarboxylase; ADH, arginine dihydrolase; KCN, potassium cyanide; VP, Voges-Proskauer; +, ≥90%; V, 11 to 89%; −, ≤10%. *Budvicia* is also LDC, ODC, and ADH negative and can be found in Table 12.

[b] See Table 3, footnote *b*.

[c] *Photorhabdus asymbiotica* subsp. *australis* can be separated from *P. asymbiotica* subsp. *asymbiotica* by fermentation of maltose and glycerol.

Proteus hauseri, previously a subgroup of *P. vulgaris*, can be differentiated from *P. vulgaris* by negative salicin/esculin and trehalose reactions (126). *Proteus* organisms that do not fit the above-listed criteria must be fully identified by commercial or conventional biochemical methods (126). *Proteus* species are identified with 95% to 100% accuracy by commercial systems, but *Providencia* identification rates vary from 79% to 100% (126). When *Providencia* spp. are misidentified, they are usually called *Morganella* or *Proteus*. Urea-positive *P. stuartii* may be misidentified as *P. rettgeri*, or the system may require additional tests for identification. Two-hour identification methods misidentify *Morganella morganii* subsp. *morganii* about 66% of the time.

Other *Enterobacteriaceae*

Averyella dalhousiensis is not in commercial system databases. It may be confused with *K. ascorbata* or misidentified as *Salmonella enterica* in commercial systems. It shares biochemical traits (positive for *o*-nitrophenyl-β-D-galactopyranoside, malonate, potassium cyanide, and fermentation of dulcitol and salicin) with *S. enterica* subsp. *salamiae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, and *S. enterica* subsp. *indica*. *Ewingella* and *Tatumella* are biochemically inactive, and the latter organism grows poorly *in vitro*. *Kluyvera* can be identified only to the genus level by commercial systems, but species determination requires an ascorbate test and Irgasan susceptibility and/or gas-liquid chromatography profiles (46). *P. asymbiotica* is not in most commercial databases.

Molecular Identification

Information on molecular identification techniques is available elsewhere (chapter 6). For laboratories using partial 16S rRNA (~500 bp) gene sequencing to identify members of the *Enterobacteriaceae*, the Clinical and Laboratory Standards Institute (CLSI) provides extremely useful information, including guidelines with suggested cutoff values for percent identity scores and identification algorithms (127). Specifically, Table 3 of the MM18-A guideline (127) provides information on the usefulness of 16S rRNA gene sequencing for various enteric groups, comments regarding relatedness within groups, alternative DNA targets, indications for identification to the species level, and recommendations for resolving species identification. Suggested cutoff values help the laboratory provide clinicians with practical, recognizable identifications of clinically significant organisms and allow the same organisms to be identified with consistency between laboratories. Appropriate cutoff values notwithstanding, accurate organism identification is ultimately dependent upon the availability of a reliable database of known sequences for comparison of the sequences generated for the unknown isolate. Both public and private databases are available, and each offers advantages (127). An evaluation of two commercially available reference sequence libraries, MicroSeq (Applied Biosystems, Foster City, CA) and SmartGene IDNS (SmartGene, Inc., Raleigh, NC), found that the second had a greater diversity of sequences for comparison and provided user-friendly software with enhancements such as the ability to add alternative gene target databases and to store and compare previous clinical sequences (128).

TABLE 11 Separation of *Yokenella* from *Hafnia*[a]

Organism	VP	Malonate	Citrate	Acid[b] from:	
				Melibiose	Glycerol
Yokenella	−	−	+	+	−
Hafnia alvei	+	+	−	−	+
H. paralvei	+	−	V	−	+

[a] Abbreviations and symbols: VP, Voges-Proskauer; +, ≥90%; V, 11 to 89%; −, ≤10%. Data are from reference 33.

[b] See Table 3, footnote *b*.

TYPING SYSTEMS

The ability to trace the spread of nosocomial pathogens in outbreaks caused by the *Enterobacteriaceae* has become a major responsibility for the laboratory. Chapters 9 and 10 of this *Manual* provide useful information on the molecular epidemiology of enteric outbreaks. Molecular techniques, including plasmid analysis, ribotyping, pulsed-field gel electrophoresis (PFGE), and various PCR methodologies, all appear to be satisfactorily discriminatory, with some working better for a specific genus or species than others (129). PCR techniques, particularly repetitive-element PCR methods for the *Enterobacteriaceae*, have proliferated at an astonishing pace and cannot be covered here more fully. To date, because economic constraints dictate the need for a single method that is applicable for a variety of organisms, PFGE remains the most universally accepted standardized technique and is considered the gold standard for typing of medically important bacteria, especially during outbreak investigations (129). The disadvantage of a long turnaround time (usually 4 days) has been partially overcome by a rapid PFGE protocol that is suitable for most enteric bacteria as well as other common clinical strains (130). Nevertheless, the method cannot determine that two strains are identical, only that the strains are indistinguishable, as only a small portion of the genome is analyzed.

As referred to above, MLST is a sequence-based typing method that is ideal for tracking certain clones or sequence types (STs) over time and to show common ancestry lineages

TABLE 12 Separation of *Enterobacteriaceae* that may be H_2S positive[a]

Organism(s)	LDC	ODC	Urea	Acid[b] from L-arabinose	Citrate	KCN	ONPG
Leminorella spp.	–	–	–	+	V[c]	–	–
Edwardsiella tarda	+	+	–	–	–	–	–
Budvicia aquatica[d]	–	–	+	V	–	–	+
Pragia fontium[d]	–	–	–	–	–	–	–
Trabulsiella guamensis[d]	+	+	–	+	V	+	+
Salmonella subgroup 1	+	+	–	+	+	–	–
Citrobacter	–	V	V	+	V	+	+
Proteus	–	V	+	–	V	+	–

[a] Abbreviations and symbols: LDC, lysine decarboxylase; ODC, ornithine decarboxylase; KCN, potassium cyanide; ONPG, *o*-nitrophenol-β-D-galactopyranoside; +, ≥90%; V, 11 to 89%; –, ≤10%.

[b] See Table 3, footnote *b*.

[c] *L. grimontii* is positive; *L. richardii* is negative.

[d] Found in clinical specimens but of questionable or no significance.

among bacteria (7, 131). MLST has been responsible for identifying certain successful international STs among *Klebsiella pneumoniae* isolates, i.e. ST16 with CTX-Ms (132) and ST258 with KPCs (133, 134), and neonatal meningitis *Cronobacter sakazakii* ST4 (55). There are three international open-access databases supporting an increasing range of organisms: http://pubMLST.org, http://www.mlst.net, and http://mlst.warwick.ac.uk/mlst/.

The high resolution obtained with whole-genome sequencing has the ability to rapidly identify single-nucleotide polymorphisms between isolates, and this has made it possible to reconstruct and redefine transmission pathways in outbreak situations (135). Snitkin and colleagues used whole-genome sequencing to gain insight into why an outbreak due to carbapenem-resistant *K. pneumoniae* progressed despite early implementation of infection control procedures (136). Integrated genomic and epidemiological analysis traced the outbreak to three independent transmissions from a single patient who was discharged 3 weeks before the next case became clinically apparent. They were also able to identify several unexpected transmission routes. It is therefore possible that the integration of genomic and epidemiological data can facilitate the control of nosocomial transmission in a real-time fashion, as can be demonstrated at http://epidemic.bio.ed.ac.uk/. The decreasing costs of next-generation sequencing and the increasing affordability of DNA sequencing equipment have already permitted the investigation of a number of outbreaks at the genome level (e.g., *E. coli* O104 in Germany and MRSA in the United Kingdom). It is reasonable to expect that this will become a trend in the future.

ANTIMICROBIAL SUSCEPTIBILITY

Increasing resistance in members of the *Enterobacteriaceae* (Table 13) has culminated in the emergence of pan-resistant strains of *K. pneumoniae* for which there are no therapeutic options (137, 138). While *K. pneumoniae* presents the most serious threat, *K. oxytoca*, *S. marcescens*, and *Enterobacter*, *Proteus*, *Morganella*, and *Citrobacter* spp. have all been reported to possess one or more Ambler class β-lactamases. The characteristics of β-lactamases are summarized in Table 13.

Most importantly, within several members of the family *Enterobacteriaceae* is the increasing recognition of isolates producing newer β-lactamases that consist of plasmid-mediated Amp C β-lactamases, ESBLs, and carbapenemases {e.g., class A (KPC types), class B (e.g., the metallo-β-lactamases [MBLs]), and class D oxacillinases (e.g., OXA-48-like)} (139). Frequently, plasmids encoding these enzymes also carry resistance genes for aminoglycosides, quinolones, and other antimicrobials (140).

The detection of antimicrobial-resistant organisms in laboratories is a critical step required for appropriate management of patients and infection prevention and control efforts. Although their clinical significance and the need to control intra- and interhospital transmission make detection of bacteria with newer β-lactamases a priority for the clinical laboratory, they are often unrecognized by routine susceptibility testing because of the difficulty in detecting these enzymes and the lack of standard techniques (141, 142). The methods for detection of bacteria that produce newer β-lactamases can be broadly divided into two groups. The first approach consists of phenotypic tests that essentially detect the ability of the enzymes to hydrolyze different cephalosporins and/or carbapenems. Another phenotypic type of approach is to use different inhibitors that inactivate different β-lactamases. Phenotypic methods, in general terms, are cost-effective and easy to perform and therefore have gained popularity among most clinical microbiology laboratories. The second approach consists of genotypic methods that use molecular techniques to detect the genes responsible for the production of the β-lactamases. Molecular methods are more expensive to perform than phenotypic tests and are mostly available in large referral or research laboratories. General antimicrobial susceptibility and specialized phenotypic testing procedures are discussed elsewhere in this *Manual* (chapters 70 to 72) and by Nordmann and Poirel (142), Patel et al. (143), and Sundin (144, 145).

Laboratory Detection of *Enterobacteriaceae* That Produce ESBLs

The CLSI in the United States before 2009 had published guidelines for ESBL detection in *Enterobacteriaceae* specifically for *E. coli*, *Klebsiella* spp., and *P. mirabilis* (127). The guidelines included an initial screening with 4 μg/ml of cefpodoxime (for *E. coli* and *Klebsiella* spp.) and 1 μg/ml each of cefpodoxime (for *P. mirabilis*), cefotaxime, ceftazidime, ceftriaxone, or aztreonam, followed by confirmatory tests using both cefotaxime and ceftazidime in combination with clavulanate (127). Automated systems using similar detection principles have proved to be popular in clinical laboratories, especially those in North America and certain European countries. These include Vitek 2 (bioMérieux, Durham, NC), Phoenix (Becton Dickinson), and MicroScan (Siemens Healthcare Systems, West Sacramento, CA) (146, 147). When ESBL-producing isolates are detected by automated systems, it is generally recommended

TABLE 13 Resistance mediators of the *Enterobacteriaceae*[a]

Resistance mediators	Ambler class[b]	Resistance to clavulanic acid	Substrates	Enzymes(s)	Location	Organism(s)	Testing procedures
Restricted-spectrum β-lactamases	A	S	Aminopenicillins, carboxypenicillins, narrow-spectrum cephalosporins	TEM-1 and -2, SHV-1	Plasmid or chromosome	*K. pneumoniae*, other genera	Cefpodoxime screen, double disk, BMD, Etest with CTX and CAZ ± clavulanic acid or CPD ± clavulanic acid, chromID agar, automated systems, PCR
ESBLs	A	S	Penicillins, narrow- and extended-spectrum cephalosporins, aztreonam	TEM-3, SHV-2 (other types, >130 and 50 types, respectively), CTX-M	Plasmid	*Klebsiella* spp., *S. marcescens*, *Enterobacter* spp., *Proteus* spp., *C. freundii*, *M. morganii*	
Cephalosporinases	C	R	Extended-spectrum β-lactams, cephamycins, aztreonam	AmpCs (CMY, MOX, FOX, DHA types)	Plasmid	*K. pneumoniae*	Same as above, plus induction test with CAZ and FOX
					Chromosome	*Enterobacter* spp., *C. freundii*, *S. marcescens*, *Proteus* spp., *M. morganii*	
Carbapenemases	A	S	All β-lactams, aztreonam	KPC type	Plasmid	*Klebsiella* spp., *Enterobacter* spp., *S. marcescens*, other genera	BMD, automated systems, Etest, disk diffusion with ETP, CHROMagar KPC, EDTA disk test, MHT, MER disk ± PBA, PCR
				GES	Plasmid	*K. pneumoniae*	
				SME, IMI, NMC	Chromosome	*Enterobacter* spp., *S. marcescens*	
	B	R	All β-lactams	IMP, VIM	Plasmid or chromosome	*K. pneumoniae*, *S. marcescens*, *Enterobacter* spp., *Citrobacter* spp.	Etest (IMI ± EDTA), disk approximation (IMI disk and Tris-EDTA disk), MHT, PCR
	D	R	Penicillins, carbapenems	OXA-48, OXA-181	Plasmid	*K. pneumoniae*	Screen: I or R to carbapenems with 2013 CLSI breakpoints, temocillin resistance; confirmation: MHT, Carba NP, molecular tests

[a] Abbreviations: S, susceptible; I, intermediate; R, resistant; BMD, broth microdilution; TEM, from patient's name Temoneira; SHV, sulfhydryl reagent variable; CTX-M, active on cefotaxime, isolated in Munich; CMY, active on cephamycins; MOX, active on moxalactam; FOX, active on cefoxitin; DHA, discovered at Dhahran; KPC, *K. pneumoniae* carbapenemase; GES, Guiana-extended spectrum; SME, *S. marcescens* enzyme; IMI, imipenem-hydrolyzing β-lactamase; NMC, not metalloenzyme carbapenemase; IMP, active on meropenem; VIM, Verona integron-encoded MBL; CTX, cefotaxime; CAZ, ceftazidime; CPD, cepoxidime; ETP, ertapenem; MER, meropenem. Data are from references 143, 145, 148, 149, 168, and 180.

Class A and C mediators are serine β-lactamases, and class B mediators are MBLs (named for serine or zinc, respectively, present at the active site involved in hydrolysis of the β-lactam ring).

that they be confirmed using a manual method (144–149). Etest ESBL strips and commercial disks from MAST Group Ltd. (Untited Kingdom) that contain cefpodoxime with different inhibitors have become available for the detection of *Enterobacteriaceae* that produce ESBLs and/or AmpC β-lactamases (146). If clinical laboratories adhere to the CLSI published guidelines for detecting ESBLs, these methods will show high sensitivity for detecting these enzymes in *E. coli*, *Klebsiella* spp., and *P. mirabilis* (147, 148). In 2010, the CLSI changed their approach to the detection of ESBLs in *E. coli*, *Klebsiella* spp., and *P. mirabilis* (127). The CLSI now recommends that clinical laboratories report the susceptibilities of the 3rd- and 4th-generation cephalosporins as tested using lower breakpoints and not to edit β-lactams (144, 145). The confirmation of ESBL testing is now optional but may still be useful for epidemiological or infection control purposes.

The phenotypic detection of ESBLs in bacteria other than *E. coli*, *Klebsiella*, and *P. mirabilis* remains a problematic and controversial issue (141, 146, 147). The reason for this is that inhibition of the ESBL by clavulanate might be masked by other types of β-lactamases (e.g., AmpCs) produced by bacteria such as *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp. Unfortunately, most of the tests described are technically demanding and difficult to interpret, and this has limited the widespread utilization of such methods to detect ESBLs in these bacteria. The methods include phenotypic tests that use cefepime with β-lactamase inhibitors (e.g., amoxicillin-clavulanic acid), including specific commercial Etests and MAST Group Ltd. disk combinations (143–145, 150, 151).

The molecular method most commonly used to detect ESBLs is the PCR amplification of the TEM and SHV genes, followed by sequencing (152). The situation is different for CTX-M enzymes in that the PCR amplification of CTX-M-specific products without sequencing usually provides sufficient evidence that *bla*$_{\text{CTX-M}}$ genes are responsible for ESBL phenotypes (151). There are also several commercial DNA microarray and real-time PCR methods available for the detection for the different types of ESBLs, including the SHV, TEM, and CTX-M types (152).

The simplest method for direct detection of ESBL-producing *Enterobacteriaceae* in patient screening samples is the use of selective culture media (e.g., MacConkey and Drigalski agar) supplemented with cefotaxime and/or ceftazidime at different concentrations. Commercially, such media are available as biplates, such as BLSE agar (AES Chemunex, Bruz, France) and EbSA ESBL agar (AlphaOmega B.V., The Netherlands) (153). Chromogenic media are the next generation of media and can be considered truly “rapid” culture-based methods, as they combine presumptive ESBL detection with organism identification. Currently available commercial chromogenic media for detection of ESBL producers include chromID ESBL (bioMérieux), Brilliance ESBL (Oxoid Ltd., Basingstoke, United Kingdom), and CHROMagar ESBL (CHROMagar, Paris, France) (153) (see also chapter 19). The sensitivity of chromogenic agar for the detection of ESBL-producing bacteria is high, while the specificity depends on which Gram-negative bacteria were used in the evaluation.

Laboratory Detection of *Enterobacteriaceae* That Produce AmpCs

Enterobacteriaceae with inducible AmpC cephalosporinases, such as *Enterobacter* spp., *Citrobacter freundii*, *Serratia marcescens*, *Morganella morganii*, and *Providencia stuartii*, can develop resistance to the oxyimino-cephalosporins (e.g., cefotaxime, ceftriaxone, and ceftazidime) and 7-α-methoxy-cephalosporins (cephamycins, e.g., cefoxitin and cefotetan) and monobactams (e.g., aztreonam) by overproducing their chromosomal AmpC β-lactamase (154). The use of β-lactams in patients infected with these bacteria can result in treatment failure (155). Since these enzymes are present on the chromosomes of the above-mentioned bacteria, their presences are not routinely tested for. The disk approximation tests with cefoxitin and ceftazidime have been successful for detection of Gram-negative bacteria with inducible chromosomal carbapenemases (156).

In *Klebsiella* spp., *Salmonella* spp., and *P. mirabilis*, which lack chromosomal β-lactamases, this type of resistance is usually mediated by plasmid-encoded or imported AmpC β-lactamases (157). Although it has been over a decade since plasmid-mediated AmpC β-lactamases were discovered, most clinical laboratories and physicians remain unaware of their clinical importance (139, 143). The fact that AmpC genes are now plasmid mediated in a variety of *Enterobacteriaceae* adds to the need for laboratories to recognize AmpC-positive isolates, as they are an even greater risk for transmission within the hospital. Their recognition is also complicated by the fact that reduced susceptibility to cefoxitin, which may be used as a screen for AmpC activity, may reflect a loss of permeability through the outer membrane (158). Incorrect reporting of a strain as AmpC positive may result in the unnecessary use of carbapenems and the concomitant risk of developing resistance to these drugs (158). Nonetheless, intermediate or reduced susceptibility to cefoxitin is still a useful indicator of AmpC presence, signaling the need for further testing (158). There are no published guidelines available from the CLSI for the detection of AmpC β-lactamases in *Klebsiella* spp., *E. coli*, *P. mirabilis*, or *Salmonella* spp. The best approach would be to screen for resistance to the cephamycins (e.g., cefoxitin and cefotetan) and then to perform inhibitor-based confirmation tests with AmpC inhibitors such as boronic acid (PBA) or cloxacillin (145, 159). An approach that screens for resistance to cefoxitin (>16 µg/ml), followed by disk confirmation tests using cefotetan with the inhibitor PBA, has become popular in certain clinical laboratories (165). Commercial Etest strips for AmpC confirmation have also become available and consist of cefotetan and cefotetan-cloxacillin combinations. MAST Group Ltd. and Rosco Diagnostica released commercial disks and Sensitabs containing cefpodoxime with different inhibitors for the detection of *Klebsiella* spp., *E. coli*, *P. mirabilis*, and *Salmonella* spp. that produce AmpC β-lactamases. Multiplex and real-time PCR methods have been described and are useful for the detection of the different types of plasmid-mediated AmpC β-lactamases (161, 162). There are also commercial real-time PCR and DNA microarray methods available for the detection of these enzymes.

Unfortunately, not all organisms producing plasmid-mediated AmpC- β-lactamases are resistant to the cephamycins, such as cefoxitin. One such enzyme, called ACC-1, originated from the chromosomal cephalosporinase of *H. alvei*. This enzyme is different from most other plasmid-mediated AmpC β-lactamases in that isolates with this enzyme are sensitive to the cephamycins but show decreased susceptibility to ceftazidime (149). Bacteria with ACC-1 are relatively rare and have mostly been reported from countries such as the United Kingdom, Germany, and France (154). Isolates (i.e., *E. coli*, *Klebsiella* spp., and *P. mirabilis*) that are intermediate to ceftazidime and sensitive to cefoxitin, combined with a negative ESBL confirmatory test, can be further tested with the cefoxitin double-disk test to confirm the presence of ACC-1 (149).

Laboratory Detection of *Enterobacteriaceae* That Produce Carbapenemases

The acquired carbapenemases that are present among *Enterobacteriaceae* can be divided into three classes (Table 13). These include the class A (i.e., KPC, SME, IMI, and NMC-A types), class B (MBLs, such as VIM, IPM, and NDM), and class D (oxacillinases, e.g., OXA-48-like) (154, 163). Current recommendations from the CLSI for the detection of *Enterobacteriaceae* that produce carbapenemases (164) can be summarized as follows:

> The carbapenem breakpoints (i.e. 0.5 µg/ml for ertapenem, and 1 µg/ml for meropenem, imipenem, doripenem) for *Enterobacteriaceae* will detect all clinically important resistance mechanisms (including the majority of carbapenemases). Some isolates that produce carbapenemases are categorized as susceptible with these breakpoints and should be reported as tested, i.e. the presence or absence of a carbapenemase does not in itself influence the categorisation of susceptibility. In many areas, carbapenemase detection and characterisation is recommended or mandatory for infection control purposes.

Several phenotypic confirmation tests have been described for the detection of carbapenemase-producing *Enterobacteriaceae*. These include bioassays that detect the ability of these enzymes to hydrolyze the carbapenems (e.g., modified Hodge test [MHT]) (128), indirect carbapenemase test (165), and Carba NP test (166) and inhibitor-based methods using metal chelators for MBLs (e.g., MBL Etest) and PBA for KPCs (167, 168). MAST Group Ltd. and Rosco Diagnostica released commercial disks and Sensitabs containing meropenem with different inhibitors for the detection of *Enterobacteriaceae* that produce carbapenemases (169). Nordmann and colleagues tested a panel of various carbapenemase-producing *Enterobacteriaceae* (*n* = 97) with Carba NP test II (168). This phenotypic test is based on the detection of the acidification resulting from imipenem hydrolysis, combined with tazobactam and EDTA as inhibitors. Their results suggest that the Carba NP test II is an easy and reliable technique with 100% sensitivity and specificity for detection and identification of carbapenemases among different members of the *Enterobacteriaceae* (170). Several in-house multiplex PCRs have been described that detect the different types of carbapenemases (171). There are also commercial real-time PCR and DNA microarray methods available for the detection of these enzymes in *Enterobacteriaceae* (152, 172).

Several selective commercial chromogenic media for screening of patients with carbapenem-resistant *Enterobacteriaceae* have also been introduced into clinical medicine and include chromID CARBA (bioMérieux), CHROMagar KPC (also available as a ready-to-use medium called Colorex KPC), Brilliance CRE (Oxoid), and Hardy CHROM carbapenemase (Hardy Diagnostics, Santa Maria, CA) (see also chapter 19). These CHROMagar media also combine presumptive carbapenem-resistant *Enterobacteriaceae* or carbapenemase detection with organism identification (153). The sensitivity and specificity of chromogenic agar for the detection of carbapenemase-producing bacteria are high, and several types of specimens (fecal, respiratory tract, urine, and wound) have been evaluated (153). The CDC has also published a procedure to screen for carbapenem-resistant *Enterobacteriaceae* (173). This consists of broth media (5 ml of Trypticase soy broth with a 10-µg meropenem disk or a 10-µg ertapenem disk). The supplies used for this procedure are readily available in most microbiology laboratories, and the sensitivity compares well to that of chromogenic agar. However, this method is labor-intensive, has lower specificity, and has a slower turnaround time (minimum of 2 days for negative specimens; longer for positive specimens).

Members of the *Enterobacteriaceae* (e.g., *S. marcescens* and *Enterobacter* spp.) can rarely produce chromosomal class A carbapenemases (e.g., SME, IMI-1, and NMC-A) (Table 13). These isolates are more of a curiosity than anything else and are not really considered infection control emergencies (174). However, if clinically indicated (e.g., a possible outbreak in an intensive care unit or ward), isolates should be referred to a reference laboratory for further analysis.

Resistance in Miscellaneous *Enterobacteriaceae*

Isolates of *P. mirabilis* are resistant to nitrofurantoin but susceptible to trimethoprim-sulfamethoxazole (SXT), ampicillin, amoxicillin, piperacillin, cephalosporins, aminoglycosides, and imipenem. Although most strains are susceptible to ciprofloxacin, resistance occurs with unrestricted use of the drug (175). *P. penneri* and *P. vulgaris* have resistance profiles similar to that of *Morganella*, although *P. penneri* is more resistant to penicillin than *P. vulgaris*. All three organisms are susceptible to broad-spectrum cephalosporins, cefoxitin, cefepime, aztreonam, aminoglycosides, and imipenem. They are resistant to piperacillin, amoxicillin, ampicillin, cefoperazone, cefuroxime, and cefazolin. *P. rettgeri* and *P. stuartii* are resistant to gentamicin and tobramycin but susceptible to amikacin. Urine isolates are susceptible to broad- and expanded-spectrum cephalosporins, ciprofloxacin, amoxicillin-clavulanic acid, imipenem, and SXT. *Providencia heimbachae*, although infrequently seen in humans, is resistant to tetracycline, most cephalosporins, gentamicin, and amikacin. Human isolates of *E. tarda* are susceptible to cephalosporins, aminoglycosides, imipenem, ciprofloxacin, aztreonam, and antibiotic–β-lactamase inhibitor combination agents (176). Isolates from fish and fish ponds may be more resistant because of the antibiotics used prophylactically in fish farming. Most strains of *E. tarda* produce β-lactamases, even though they are susceptible to β-lactams. *P. shigelloides* is resistant to ampicillin, carbenicillin, piperacillin, and ticarcillin and is variably resistant to most aminoglycosides and tetracycline (177). Cephalosporins, quinolones, carbapenems, and SXT show good activity against *P. shigelloides*.

Susceptibility results of uncommonly seen species of *Klebsiella*, *Enterobacter*, and *Serratia* are similar to those of conventional species within these genera (178). Susceptibilities for other *Enterobacteriaceae* vary from isolate to isolate, so no empirical guidelines are available for therapy prior to susceptibility testing of the suspected strain.

EVALUATION, INTREPRETATION, AND REPORTING OF RESULTS

When commercial systems identify species included in this chapter with a high level of accuracy (>90% probability), the identification is probably reliable. However, for organisms isolated from sterile sites that are identified with a probability of <90%, the isolate should be confirmed by conventional or molecular methods or sent to a reference laboratory using these techniques. In the interim, the isolate may be reported to the physician with a presumptive identification. Rare species that are identified with low probabilities should always be sent to a reference laboratory accompanied by a brief history.

The clinical laboratory acts as an early warning system, alerting the medical community to new resistance mechanisms present in clinically important bacteria. The detection of antimicrobial-resistant organisms in laboratories is a critical step required for appropriate management of patients and infection prevention and control efforts. An increasing number of patients are infected or colonized with *Enterobacteriaceae* possessing ESBLs and carbapenemases, and they serve as reservoirs for transmission of these enzymes within and between health care institutions. The ability to recognize these isolates is critical not only for patient care but also to prevent the emergence of panresistant strains.

At the very least, all isolates of *K. pneumoniae* should be screened for ESBLs and plasmid-mediated AmpC β-lactamases using the CLSI 2013 breakpoints for cefpodoxime, cefotaxime, ceftriaxone, ceftazidime, and aztreonam (for ESBLs) and cefoxitin (for AmpC β-lactamases) (164). The CLSI ESBL confirmation tests show a high sensitivity with *Klebsiella* spp. (165), and ESBL-positive isolates should be reported as resistant to all penicillins, expanded-spectrum cephalosporins, and aztreonam (166).

The presence of carbapenemases (especially KPC and NDM) in *Klebsiella* spp. and *Enterobacter* spp. is an infection control emergency, and clinical microbiology laboratories should be able to rapidly detect these enzymes among members of the *Enterobacteriaceae*. This is a critical step required for appropriate management of patients and infection prevention and control efforts. The initial screening with 0.5 μg/ml of ertapenem and 1 μg/ml of meropenem, imipenem, or doripenem, followed by the phenotypic confirmatory tests, provides a cost-effective approach for the rapid detection of carbapenemases. The MHT, when using meropenem as substrate, is able to reliably detect KPC- and OXA-48-producing isolates (179). Unfortunately, the MHT performs poorly in the detection of MBL-producing isolates (180). The MBL Etest using imipenem as a substrate is often difficult to interpret for detection of *Enterobacteriaceae* that produce MBLs (170). This is due to the fact that the MICs for imipenem are low. The MAST Group Ltd. and Rosco Diagnostica commercial disks and Sensitabs performed well for the detection of KPCs and NDMs but poorly for VIMs, IMPs, and OXA-48-like enzymes (170). The most accurate methods available for the detection of the different types of carbapenemases are the phenotypic Carba NP tests (142) and several in-house and commercial genotypic tests (173). It is important to remember that the screening process for ESBL- or carbapenemase-producing bacteria in patient specimens essentially looks for cephalosporin- and carbapenem-resistant bacteria, and additional tests are required to confirm the presence of ESBLs or carbapenemases.

Clinical microbiology urgently needs reliable, sensitive, and cost-effective phenotypic methods that will rapidly detect ESBLs, AmpC β-lactamases, and carbapenemases in all Gram-negative bacteria. These tests should also be manageable for all institutions regardless of size and capability.

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Yersinia[*]

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The pathogenic *Yersinia* species, *Y. pseudotuberculosis*, *Y. enterocolitica*, and *Y. pestis*, are zoonotic agents that cause disease in humans. Human clinical infections caused by *Y. pseudotuberculosis* and *Y. enterocolitica* occur after the ingestion of contaminated food or water and manifest primarily as mild gastroenteritis, whereas the etiologic agent of plague, *Y. pestis*, is transmitted to humans by the bite of an infected flea and results in life-threatening illness. Within the *Yersinia* genus, these three species are joined by 14 lesser-known *Yersinia* species which are largely considered environmental species and nonpathogenic to humans. Pathogenic *Yersinia* species share a highly conserved virulence plasmid and a chromosomal high-pathogenicity island (HPI) and show tropism for lymphoid tissue, where their ability to evade host innate immunity enables extracellular proliferation. Plague is a notorious disease, with strikingly high mortality rates of 40 to 100% if untreated; it is the cause of three major pandemics, including the Black Death of the 14th century, in which an estimated 17 to 28 million Europeans died. *Y. pestis* was weaponized by the United States, Japan, and the former USSR during and after World War II and remains a high-level biothreat agent (1). In 2012, *Y. pestis* was classified as a tier 1 agent, one of six bacterial agents considered to present the greatest risk of deliberate misuse with the most significant potential for mass casualties or devastating effects to the economy, critical infrastructure, or public confidence (www.gpo.gov/fdsys/pkg/FR-2012-10-05/html/2012-24389.htm).

TAXONOMY

Y. pseudotuberculosis (previously *Bacillus pseudotuberculosis*, *Bacterium pseudotuberculosis rodentium*, and *Pasteurella pseudotuberculosis*) was the first pathogenic *Yersinia* species described. Isolated by Malassez and Vignal in 1883, the bacterium derives its name from the tuberculosis-like granulomatous abscesses it causes in the spleens and livers of infected animals (2, 3). Alexander Yersin subsequently isolated *Y. pestis* (previously *Bacterium pestis*, *Bacillus pestis*, and *Pasteurella pestis*) from lymph nodes of plague victims during an investigation of a bubonic plague outbreak in Hong Kong in 1894 (4). In 1939, Schleifstein and Coleman described five cultures of *Y. enterocolitica* (previously *Bacterium enterocoliticum*) isolated from the intestines of patients with enteritis and also from facial lesions (2).

By 1944, phenotypic differences of *P. pestis* and *P. pseudotuberculosis* from other *Pasteurella* species prompted van Loghem to propose transfer of these two bacteria into a new genus, *Yersinia*, in honor of the French bacteriologist Yersin (2). Ten years later, *Yersinia* species were included in the family *Enterobacteriaceae*, and in 1964, *Y. enterocolitica* was added to the genus (5). On the basis of taxonomic studies, the diverse members of *Y. enterocolitica* were subsequently divided into an additional eight species, *Y. intermedia*, *Y. frederiksenii*, *Y. kristensenii*, *Y. aldovae*, *Y. rohdei*, *Y. mollaretii*, *Y. bercovieri*, and *Y. aleksiciae*. Similarly, isolates originally identified as *Y. pseudotuberculosis* have been assigned to an additional two species, *Y. pekkanenii* and *Y. similis*. Other *Yersinia* species include *Y. ruckeri* (serogroup 01, the agent of enteric red mouth disease in rainbow trout), *Y. entomophaga*, *Y. massiliensis*, and *Y. nurmii* (www.bacterio.net/xz/yersinia.html).

In the last 15 years, whole-genome sequences have been completed and assembled for the following *Yersinia* strains: 12 *Y. pestis* (including the three primary biotypes, orientalis, mediaevalis, and antiqua), 4 *Y. pseudotuberculosis*, and 3 *Y. enterocolitica* strains (http://www.ncbi.nlm.nih.gov/genome/?term=Yersinia). In addition, unassembled genomes for multiple strains of each of these three species are available in GenBank. Genome comparisons indicate that the sizes of completed *Yersinia* genomes range from 4.62 to 4.94 Mb, with G+C contents ranging from 47 to 47.7% (http://www.ncbi.nlm.nih.gov/genome/?term=Yersinia). All *Yersinia* genomes are comprised of a single chromosome; in addition, the pathogenic *Yersinia* genomes also consist of an ~70-kb virulence plasmid (termed pCD1 in *Y. pestis* and pYV in *Y. enterocolitica* and *Y. pseudotuberculosis*) (6). Two additional plasmids (pMT1 and pPla) make up *Y. pestis* genomes, while some *Y. pseudotuberculosis* strains harbor an additional plasmid (2, 7, 8).

Genome analyses reveal that *Y. pestis* is a clone of *Y. pseudotuberculosis* that emerged relatively recently, within the last 1,500 to 20,000 years, but that *Y. enterocolitica* and *Y. pseudotuberculosis* diverged within the last 200 million years (9–14). The high degree of genetic similarity (75% of the 3,974 predicted genes in *Y. pseudotuberculosis* show ≥97% identity to homologues in *Y. pestis*) is the reason for *Y. pestis* and *Y. pseudotuberculosis* being previously classified as one species; however, their profoundly distinct pathogenesis mechanisms,

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clinical presentations, and modes of transmission warrant their separation into two distinct species (2, 14).

Y. enterocolitica is the only *Yersinia* species that has been further subdivided into subspecies. The two subspecies, *Y. enterocolitica* subsp. *enterocolitica* and *Y. enterocolitica* subsp. *palearctica*, can be distinguished based on their 16S rRNA gene sequences (15).

DESCRIPTION OF THE AGENTS

Yersinia species, as members of the family *Enterobacteriaceae*, are Gram-negative, non-spore-forming bacilli that exhibit bipolar staining, particularly in primary specimens stained with Giemsa or Wayson's dye. The bacilli are smaller (0.5 to 0.8 µm in diameter and 1 to 3 µm in length) than other members of their family and tend to grow more slowly as well. Most *Yersinia* species are motile at temperatures less than 30°C due to peritrichous or paripolar flagella (Table 1). Interestingly, the pathogenic *Yersinia* spp. either repress (*Y. pseudotuberculosis* and *Y. enterocolitica*) or, through mutation, have lost the ability to express (*Y. pestis*) the flagellar apparatus in the mammalian host; by either mechanism, they effectively avoid stimulation of innate immune responses (16).

Yersinia species are facultative anaerobes that grow at temperatures ranging from 4 to 43°C. *Yersinia* species ferment glucose (with the production of acid but no gas), are catalase positive and oxidase negative, and display nonfastidious growth. Most strains grow on MacConkey, blood, and chocolate agars but may be outcompeted by other bacteria in clinical specimens from nonsterile sites or sources and especially from environmental samples (see Isolation Procedures below), as *Yersinia* species grow more slowly than most *Enterobacteriaceae*. *Yersinia* species exhibit poor growth in liquid media and do not form a turbid suspension (17).

The cell walls of *Yersinia* species are very similar to those of other members of the *Enterobacteriaceae* family, and lipopolysaccharide (LPS) is a major component of their outer membrane (18). The LPS of *Y. pseudotuberculosis* and *Y. enterocolitica* (smooth forms) is complete (lipid A–oligosaccharide core–O-antigen polysaccharide), and O-chain variation within these species has enabled serodiscrimination of close to 100 O groups (17–19). In contrast, through inactivation of the O-antigen gene cluster and the *pagP* gene, as well as loss of the *lpxL* gene, *Y. pestis* produces R (rough)-type LPS, which is limited to a short carbohydrate chain bound to lipid A (20).

Virulence genes, their environment-dependent expression control, and the complex mechanisms of their product action and coordination, which enable immune system evasion and disease progression, have been actively investigated and described. Comparison of orthologous genes between *Y. enterocolitica* (strain 8081), *Y. pestis* (strain CO92), and *Y. pseudotuberculosis* (strain IP32953) identified a shared core set of 2,747 coding sequences, as well as a number of coding sequences unique to *Y. enterocolitica* (~29%), *Y. pseudotuberculosis* (~9%), or *Y. pestis* (~11%) (13, 14). In comparison to *Y. pseudotuberculosis* and *Y. enterocolitica*, *Y. pestis* contains a larger number of pseudogenes, genes that were inactivated because they were not needed by this species, which does not survive outside insect or mammalian hosts (14, 21).

A key feature of *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* biogroup 1B is the ability to scavenge iron from the host by a siderophore called yersiniabactin (Ybt) (13, 22, 23). The *ybt* genes are chromosomally located and clustered on an HPI located in a 102-kb chromosomal region termed the *pgm* (pigmentation) locus. Other chromosomal virulence genes include *yst*, encoding a heat-stable toxin unique to enteropathogenic *Y. enterocolitica* strains and absent in *Y. pseudotuberculosis*, and *invA*, an epithelial cell adhesin gene common to *Y. enterocolitica* and *Y. pseudotuberculosis* (3, 21). Invasin facilitates efficient binding to intestinal mucosal cells and translocation from the lumen to Peyer's patches; an insertion element within the *invA* gene of *Y. pestis* renders it dysfunctional (3).

The pathogenic *Yersinia* species possess a common and genetically conserved 68- to 75-kb virulence plasmid (termed pCD1 in *Y. pestis* and pYV in *Y. enterocolitica* and *Y. pseudotuberculosis*). This plasmid carries the low-calcium response genes, components of the type 3 secretion system (TTSS), V antigen, and the associated effectors or *Yersinia* outer membrane proteins (Yops) (24–26). The TTSS forms a needle structure on the surfaces of pathogenic *Yersinia* species, with V antigen at the tip, and interacts with target cells (macrophages, dendritic cells, and granulocytes/neutrophils) to enable injection of Yops, which effectively interfere with phagocytosis and other innate host cell responses as well as the adaptive inflammatory cascade, ultimately resulting in target cell apoptosis (27, 28). YadA is also encoded on the *Yersinia* virulence plasmid. In *Y. enterocolitica* and *Y. pseudotuberculosis*, this adhesion protein facilitates pathogen binding to M cells of the intestinal mucosa, signal-induced internalization, and subsequent translocation to the Peyer's patches and mesenteric lymph nodes (3). *Y. pestis* YadA is inactivated by point mutation through a base deletion resulting in a frameshift and a truncated product (3).

Two *Y. pestis* unique plasmids (~110 and 10 kb) encode proteins important for the organism's utilization of a flea vector and its ability to cause acute disease in infected mammals. The 110-kb plasmid (pMT1) encodes the murine toxin (Ymt) and the fraction 1 antigen (F1) (7, 24). Ymt, expressed only at temperatures below those of mammalian systems, is a phospholipase whose activity is necessary for pathogen survival within the harsh environment of the flea digestive tract during blood meal digestion (29). In contrast to Ymt, F1 is expressed only at temperatures >33°C and during mammalian infection. F1 forms a capsule-like structure on the bacterial surface with antiphagocytic properties that enable dissemination and replication of the bacteria (7). Plasminogen activator (Pla), encoded by the 10-kb plasmid, is a surface protease that activates mammalian plasminogen and degrades complement; it is essential for dissemination after subcutaneous or intradermal inoculation (experimental proxies for flea bite transmission) in mouse models of bubonic disease (7, 24).

Whole-genome shotgun sequencing of eight nonpathogenic *Yersinia* species, *Y. aldovae*, *Y. bercovieri*, *Y. frederiksenii*, *Y. kristensenii*, *Y. intermedia*, *Y. mollaretii*, *Y. rohdei*, and *Y. ruckeri*, identified no orthologs of genes found in the virulence-associated plasmids pYV, pMT1, and pPla or in the HPI of pathogenic *Yersinia*. Only 16 additional clusters of chromosomal proteins were found to be absent from all eight nonpathogens and common to pathogenic *Yersinia* species (*Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*). Acquisition of mobile elements therefore appears to be the defining feature of *Yersinia* spp. virulent for humans (6).

EPIDEMIOLOGY AND TRANSMISSION

Plague is an acute, often fatal disease caused by *Y. pestis*. It exists in natural enzootic cycles between wild rodents and

TABLE 1 Biochemical reactivities and characteristics of *Yersinia* species[a]

Yersinia species	Reaction or characteristic[b]																
	Motility	Ornithine decarboxylase	Urease	VP test	Citrate	Indole	Rhamnose	Sucrose	Cellobiose	Sorbose	Sorbitol	Melibiose	Raffinose	Esculin	L-Arabinose	Inositol	D-Xylose
Y. pestis	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+
Y. pseudotuberculosis	+	−	+	−	−	−	(+)	−	−	−	−	+	−	+	−	−	+
Y. enterocolitica	+	+	+	V	−	V	−	+	+	+	+	−	−	−	+	+	V
Y. frederiksenii	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+
Y. kristensenii	+	+	+	−	−	+	−	−	+	+	+	−	−	−	+	(+)	+
Y. ruckeri	V	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Y. mollaretii	+	+	+	−	+	−	−	+	+	+	+	−	−	(+)	+	(+)	+
Y. bercovieri	+	+	+	−	−	−	−	+	+	−	+	−	−	−	+	−	+
Y. rohdei	+	+	+	−	+	−	−	+	+	−	+	+	+	−	+	−	+
Y. aldovae	+	+	+	+	+	−	+	−	−	−	+	−	−	−	+	+	+
Y. intermedia	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y. aleksiciae	+	+	+	−	−	−	−	−	+	−	+	−	−	−	+	(+)	+
Y. pekkanenii	−	−	+	−	−	−	−	−	+	−	−	−	−	−	+	−	+
Y. similis	+	−	+	−	+	−	+	−	−	−	−	−	−	+	+	−	+
Y. entomophaga	+	+	+	ND	+	+	−	+	ND	ND	+	−	ND	+	+	−	+
Y. massiliensis	+	+	+	−	+	+	−	(+)	+	+	+	−	−	+	+	+	+
Y. nurmii	+	+	−	+	+	−	−	+	(+)	−	−	−	−	−	−	−	−

[a] From references 17 and 110 to 113.

[b] Incubation was at 25 to 28°C. VP, Voges-Proskauer; V, variable; ND, not determined; −, negative; +, positive; (+) weakly positive.

their fleas. The most common mode of transmission to humans is by the bite of infected fleas. Less frequently, infection is the result of handling infected animals, directly contacting infectious body fluids or tissues, or inhaling infectious respiratory droplets. Most cases of human plague are sporadic and occur in rural areas. Epidemics occur occasionally when the disease spreads from wild rodents into populations of rats (genus *Rattus*) that live near human populations. Human risk is greatest when epizootics cause high mortality in these commensal rat populations, thereby forcing fleas to seek alternate hosts, including humans. Although many different species of fleas parasitize rodents and can transmit infection to them, transmission of plague is classically associated with the rat flea (*Xenopsylla cheopis*) (8). *Y. pestis* can survive and multiply in the midgut (stomach) and proventriculus (a valve that connects the esophagus to the midgut) of the flea (30, 31). Bacterial replication results in blockage of the proventriculus, which causes the flea to starve and triggers the infected flea to bite hosts in repeated attempts to acquire a blood meal, thereby increasing chances for disease transmission (30, 31).

Between 2004 and 2009, 12,503 human cases and 843 deaths were reported from 16 different countries (32). Of these cases, 97.6% were reported from Africa, 1.2% from Asia, and 1.1% from the Americas (32). In the United States between 1900 and 2010, 999 plague cases were reported. In recent decades, on average 7 human plague cases have been reported each year in the United States (range, 1 to 17 cases per year), with 50% of cases occurring in people ages 12 to 45 years (www.cdc.gov/plague). Plague occurs in rural and semirural areas of the western United States and has been found in wild animal populations in all of the 17 contiguous states west of the 100th meridian. More than 80% of human cases occur in New Mexico, Arizona, and Colorado, and approximately 10% occur in California (33–35).

Domestic dogs and cats may serve as carriers of *Y. pestis*-infected fleas into human dwellings. A study in New Mexico found that plague victims were significantly more likely than controls to have allowed pets to sleep on their beds (36). Cats are also highly susceptible to plague, acquiring infection by flea bite or ingestion of infected rodents, and develop all of the clinical forms of disease, including pneumonic plague. Infected cats serve as direct sources of human infection via aerosol transmission of organisms, leading to primary pneumonic plague, or via inoculation of organisms through scratches or bites (34). Dogs are most often resistant to clinical infection and develop antibody titers upon exposure.

Hunting in areas where plague is endemic is associated with human infections; transmission may occur during skinning and handling of animals or ingestion of undercooked meat. Although many animal species are susceptible to plague infection and thus pose a risk factor associated with hunting, documented cases of transmission in the United States are most frequently associated with rabbits, squirrels, prairie dogs, and pet cats (37).

Human-to-human transmission can occur with the pneumonic form of disease and therefore poses a special public health threat (38). Most transmissions are to unprotected/untrained family members and health care providers through close-contact inhalation of particles from infected persons who are coughing bloody sputum. In over 60 separate cases of pneumonic plague in the United States since 1924, there have been no human-to-human transmissions (1). Analysis of primary pneumonic cases in the United States indicates that the majority of cases fail to transmit, even in the absence of antimicrobial treatment or prophylaxis (39), consistent with the fact that outbreaks during the preantibiotic era were controlled quickly and effectively with routine measures. Nevertheless, the potential for sustained outbreaks exists due to superspreading events. Control measures included social distancing, isolation, quarantine, and simple barrier precautions (39).

Y. enterocolitica is a heterogeneous species with worldwide distribution and both pathogenic (human and animal) and nonpathogenic members (2). Incidence is higher in temperate and cold countries. Organisms are found in the gastrointestinal tracts of many animal species, most commonly swine, rodents, and dogs. Food products, particularly unpasteurized milk and raw and undercooked meats, have been found to contain these organisms. Transmission occurs primarily via the fecal-oral route; organisms are ingested via food or water contaminated by feces of infected animals. The species is divided into six biogroups—1A, 1B, 2, 3, 4, and 5—that are biochemically differentiated (Table 2) (40, 41). Group 1A (which lacks the 68- to 75-kb pYV virulence plasmid) is considered nonpathogenic, groups 2 through 5 (which lack the chromosomal HPI) are weakly pathogenic in mice, and group 1B (with pYV and the HPI) is highly pathogenic and lethal to mice (42). *Y. enterocolitica* bioserotypes most often associated with human illness are 1B/O:8, 2/O:5,27, 2/O:9, 3/O:3, and 4/O:3, with bioserotype 4/O:3 now predominating as the most common type in the United States and Europe (43). Group 1B is generally restricted to North America and Japan, whereas group 3 is predominantly isolated in Japan and China (43). Serogroup typing, based on reactivity to O-antigen polysaccharides, has also been developed, and over 70 serotypes are characterized (44). Bioserotypes have been shown to be geographically focused, and only a small subset is associated with disease in animals or humans (43).

Y. pseudotuberculosis is found in the environment (soil and water) and in a diverse group of wild and domesticated animal species. The organism has a wide distribution in countries with cold climates; it is most common in northern Europe and Asia, including Japan. *Y. pseudotuberculosis* is not frequently isolated in the United States; FoodNet reported that the average annual incidence of *Y. pseudotuberculosis* infections was only 0.04 case per 1,000,000 persons from 1996 to 2007 (45). *Y. pseudotuberculosis* serotypes associated with human illness include O:1 and O:3, frequently isolated in Europe, and O:4 and O:5, isolated in Japan (43). The main reservoirs for the organism are rodents, rabbits, and wild birds. The mode of transmission of *Y. pseudotuberculosis* is via ingestion of contaminated food (vegetables and meat) or water contaminated by animal reservoirs or via contact with infected animals.

CLINICAL SIGNIFICANCE

Plague, one of nine quarantinable diseases in the United States, is a severe febrile illness and, without prompt and appropriate antibiotic treatment, is often fatal. There are three major forms of plague: bubonic, septicemic, and pneumonic (1). Bubonic plague, the most common clinical presentation, accounts for 80 to 90% of cases and is due to the bite of an infected flea (35). After a 2- to 6-day incubation period, a sudden onset of fever, headache, chills, and weakness and one or more swollen, tender, and painful lymph nodes (buboes) occur. The case fatality rate for untreated bubonic plague is 50 to 60% (35). Septicemic plague occurs when the organisms are introduced directly into the bloodstream without localizing in regional lymph nodes or

TABLE 2 Reactions of biotypes of Y. *enterocolitica*[a] after incubation at 25°C for 48 h

Test	Reaction[b] for biotype:					
	1A	1B	2	3	4	5
Lipase (Tween esterase)	+	+	−	−	−	−
Esculin	+	−	−	−	−	−
Salicin	+	−	−	−	−	−
Indole	+	+	(+)	−	−	−
Xylose	+	+	+	+	−	d
Trehalose	+	+	+	+	+	−
$NO_3 \rightarrow NO_2$	+	+	+	+	+	−
DNase	−	−	−	−	+	+
Pyrazinamidase[c]	+	−	−	−	−	−

[a] Modified from reference 41 with permission of the publisher, S. Karger AG, Basel, Switzerland.

[b] +, ≥90% of strains are positive; d, 11 to 89% of strains are positive; −, ≥90% of strains are negative; (+), weakly positive reaction.

[c] According to Kandolo and Wauters (114).

when the bacteria are directly introduced into the bloodstream via a cut or wound. Patients develop fever, chills, extreme weakness, abdominal pain, shock, and possibly bleeding into the skin and other organs. This form of the disease is rapidly fatal. Septicemic plague can also occur secondarily to bubonic plague that is not treated.

Pneumonic plague can be a secondary complication of bubonic or septicemic plague or can be the primary infection following direct inhalation of aerosolized organisms. After an incubation period of 1 to 3 days, symptoms include fever, headache, weakness, and a rapidly developing pneumonia with shortness of breath, chest pain, cough, and sometimes bloody or watery mucus (1). The pneumonia may cause respiratory failure and shock. Pneumonic plague is the most serious form of the disease and is the only form that can be spread from person to person (by infectious droplets).

In the preantibiotic era (1900 through 1941), mortality among those infected with all forms of plague in the United States was 66%. Antibiotics greatly reduced mortality, and by 1990 to 2010, overall mortality had decreased to 11% (http://www.cdc.gov/plague/faq/index.html). Plague can still be fatal despite effective antibiotics, though the risk of death is lower for bubonic plague cases than for septicemic or pneumonic plague cases. Mortality among untreated pneumonic cases is essentially 100%, and even among treated cases, mortality often exceeds 50%. In the United States between 1947 and 1977, approximately 50% of primary septicemic cases were fatal (35). It is hard to assess the mortality rate of plague in developing countries, as relatively few cases are reliably diagnosed and reported to health authorities. The WHO cites a global mortality rate of 6.7% for the time period of 2004 to 2009; however, mortality is likely higher in some areas where plague is endemic (32).

The most common form of disease due to Y. *enterocolitica* is gastroenteritis associated with consumption of contaminated food or water. It is not unusual to isolate Y. *enterocolitica* from raw meats, including beef, lamb, pork, and chicken. The organism has also been found as a contaminant of cooked, prepackaged deli meat and unpasteurized milk products. The majority of strains isolated from human food sources are of the nonpathogenic serotypes. Carriage of the pathogenic serotypes of Y. *enterocolitica* is more common in swine; therefore, consumption of raw or undercooked pork, such as chitterlings, is the main risk factor for gastroenteritis (46–48). Severity of disease is related to the serotype and can range from self-limited gastroenteritis to terminal ileitis and mesenteric lymphadenitis, often misdiagnosed as appendicitis (49). Young children most commonly develop gastroenteritis and present with fever, watery diarrhea (occasionally bloody and severe), and abdominal pain following consumption of food contaminated by Y. *enterocolitica*. Although symptoms typically resolve within approximately 7 days, patients can carry the organism in their gastrointestinal tracts for as long as several months. Organisms can migrate out of the gut via the lymphatics into local lymph nodes. An uncommon complication of gastroenteritis is septicemia. Persons at high risk for septicemia include the elderly and immunocompromised patients, particularly those with underlying metabolic diseases that are associated with iron overload, cancer, liver disease, and steroid therapy.

The production of urease allows Y. *enterocolitica* to survive in the stomach and colonize the small intestine of the human host. Pathogenic strains produce a heat-stable enterotoxin, Yst, as well as Yop effectors that enable them to resist the normal phagocytic and complement killing process that takes place in Peyer's patches (13, 14). Y. *enterocolitica* is a common cause of transfusion-related infections due to contaminated red blood cells. Since the organism is able to survive and multiply at refrigeration temperatures, donated blood contaminated with small numbers of organisms from an asymptomatic person can transmit infection to the transfused patient (50, 51). Reactive arthritis is an uncommon sequela of diarrhea due to Y. *enterocolitica* (49). Patients at increased risk include those who are carriers of the HLA-B27 allele and those with immunologic disorders. Symptoms appear several days to months after the onset of diarrhea and may persist for months. Other less common diseases associated with Y. *enterocolitica* infection include inflammatory bowel disease, most commonly associated with serotype O:3 (52), and autoimmune thyroid disorders, such as Graves' disease and Hashimoto's thyroiditis (53). Both Y. *enterocolitica* and Y. *pseudotuberculosis* have been isolated from patients with Crohn's disease, although a causal relationship has not been proven (54).

Y. *pseudotuberculosis* usually produces a self-limiting disease, particularly in children and young adults. Rarely, Y. *pseudotuberculosis* can cause mesenteric lymphadenitis and terminal ileitis which clinically mimics appendicitis and septicemia and generally occurs in immunocompromised patients (diabetics and those with liver cirrhosis or iron overload) (55, 56). Long-term sequelae of Y. *pseudotuberculosis* infection include erythema nodosum, Reiter's syndrome, and nephritis. Y. *pseudotuberculosis* has also been implicated in outbreaks of gastroenteritis/pseudoappendicitis associated with consumption of contaminated lettuce and carrots (57–59). In addition to causing gastrointestinal illness, *Y. pseudotuberculosis* is the causative agent of Far East scarlet-like fever in Japan and Russia (60, 61).

The lack of classic virulence markers, i.e., the 70-kb virulence plasmid and the HPI, in the other *Yersinia* species (Table 1) has led to their general classification as nonpathogenic. Nonetheless, several of them (*Y. intermedia*, *Y. frederiksenii*, and *Y. kristensenii*) have been isolated from stool specimens of up to 20% of diarrhea patients for whom etiologic agents were not determined (62). Possible predisposing correlates of infection include corticosteroid, acid suppressant, and antibiotic use and an immunocompromised host status (62, 63).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

In the United States, *Y. pestis* is classified as a tier 1 select agent. To receive or possess *Y. pestis* strains, laboratories must be registered with the federal Select Agent Program (www.selectagents.gov). Clinical laboratories are exempt from the registration requirement of the Select Agent Program as pertains to conducting diagnostic testing for select agents. Diagnostic testing for select agents may be performed by clinical laboratories as long as the laboratory destroys or transfers any confirmed select agent within 7 days of identification. Laboratories identifying an organism as *Y. pestis* are required to report this finding immediately; laboratories need to complete form 4 and submit it to the Select Agent Program. If the organism is to be transferred following identification, then the laboratory must also complete form 2 and obtain transfer approval from the Select Agent Program. Select agent report forms, contact information, laboratory registration information, and pertinent citations of the U.S. Federal Code may be found at www.selectagents.gov.

All procedures for *Y. pestis* should be done in a facility with a biosafety level of at least 2 (BSL-2), and clinical laboratories should be aware of the sentinel-level clinical microbiology laboratory guidelines as outlined by the American Society for Microbiology (64; www.asm.org/index.php/issues/sentinel-laboratory-guidelines). All patient specimens should be handled in a BSL-2 facility; technicians should wear gloves and gowns and work in a biosafety cabinet (BSC). Subcultures should be performed in a class II BSC. Due to the severity of disease caused by *Y. pestis*, processes which increase the risk of creating an aerosol, such as liquid culture manipulation, should be performed under BSL-3 conditions. *Y. pestis* is a documented laboratory hazard. Laboratory-acquired cases of plague, several of which have been fatal, have been reported in the United States (39, 65, 66).

Y. pestis strains lacking either the *pgm* locus (a 102-kb chromosome region encoding yersiniabactin iron transport, the Hms biofilm, and other systems) or the 60- to 85-kb virulence plasmid (which encodes the TTSS and its effector Yop proteins) are exempt from select agent regulations in the United States (www.selectagents.gov) and can be handled under BSL-2 conditions. It is important to note, however, that *pgm* mutants of *Y. pestis* are still capable of causing disease, and in 2009, a fatal laboratory-acquired infection by an attenuated *pgm* mutant *Y. pestis* strain was described (66). *Y. pestis* strains lacking the virulence plasmid are considered avirulent (67).

For *Y. pestis*, preferred specimens for laboratory diagnosis are dependent on the clinical presentation; lymph node aspirates and blood are recommended in bubonic presentation, blood for septicemic presentation, and respiratory samples (e.g., sputum) and blood for pneumonic presentation. As bubonic plague patients may shed organisms into the blood intermittently, obtaining multiple sets of blood cultures over a 24-h period can increase the sensitivity of detection from this sample source.

Cary-Blair medium and swabs offer an excellent means of transport for the preservation of viable organisms if samples cannot be cultured immediately. Tissue samples from autopsy specimens, lymph node, spleen, liver, and lung can be utilized for testing. For animals suspected of having died from *Y. pestis*, blood remnants or tissue specimens can be collected for testing. Acute- and convalescent-phase sera can be collected from animals and humans to test for antibody to *Y. pestis*. Specimens should be sent to the laboratory immediately, and if a delay in transit of more than 2 h is expected, the sample should be transported at 2 to 8°C. Sera may be stored at 2 to 8°C for up to 14 days. If testing is delayed for a longer period, serum samples may be frozen.

The appropriate specimens for culture of *Y. enterocolitica* and *Y. pseudotuberculosis* as well as other *Yersinia* species are stool, blood, or lymph nodes, depending on the disease form suspected. If food is suspected as the source of an outbreak, the local health department should be involved in the processing of such specimens. Maintain food at 4°C, and transport it as soon as possible. Swabs should be transported to the laboratory at 4°C in Cary-Blair, Amies, or Stuart's medium. Stool specimens can also be placed in transport media and should be maintained at 4°C if transport is expected to take longer than 2 to 4 h. Enrichment broths for recovery of *Y. enterocolitica* from surface waters have been evaluated, but the ability to recover clinically important strains from this source is uncertain (68).

DIRECT EXAMINATION

Microscopy

Yersinia species are small (1 to 2 μm by 0.5 μm) Gram-negative bacilli that appear either as single cells when slides are prepared from agar plates or as pairs or short chains when slides are prepared from liquid medium. Although not normally performed in the microbiology laboratory, Wright, Giemsa, or Wayson staining of peripheral blood or tissue may reveal the bipolar staining characteristics of *Y. pestis* (Fig. 1A) (www.asm.org/index.php/issues/sentinel-laboratory-guidelines). Bipolar staining, however, is not a unique feature of *Yersinia* species and may not always be evident.

A rapid and specific staining method for the detection of *Y. pestis* in specimens includes the use of a fluorescently labeled antibody (available from the CDC) to the capsular F1 antigen (69). Cultures, lesions, tissues (lymph node, liver, spleen, and lung) or aspirates may be rapidly assessed by this approach (Fig. 1B). As the F1 antigen is expressed only at temperatures above 33°C, this staining procedure cannot be performed on environmental samples (fleas, food, etc.) or cultures that have been maintained at <33°C. Tissue biopsy specimens fixed in formalin can also be tested via an immunohistochemical (IHC) stain that utilizes a monoclonal antibody to the F1 capsular antigen of *Y. pestis* (70).

Antigen Detection

A capture enzyme-linked immunosorbent assay for detection of *Y. pestis* F1 antigen in bubo aspirates and serum shows high sensitivity (100% with buboes and 90% with sera; limit of detection, 4 ng/ml) and specificity (approximately 99%) (71). This antigen detection approach has been modified to a lateral-flow dipstick assay and successfully used under field conditions to diagnose plague infections in humans and

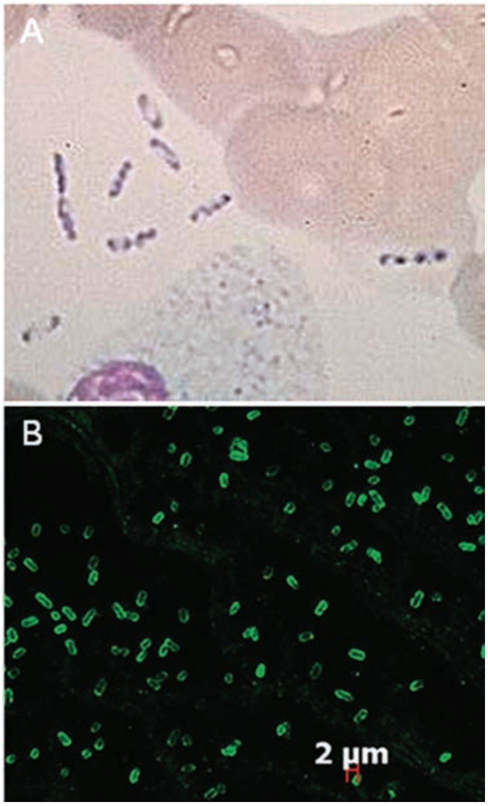

FIGURE 1 (A) Giemsa stain of a blood smear from a patient with *Y. pestis* infection. Note the bipolar staining of "closed safety pin"-shaped cells. (B) Direct fluorescent-antibody (F1 conjugate) staining of *Y. pestis* cells grown on sheep blood agar at 37°C. doi:10.1128/9781555817381.ch39.f1

animals in Madagascar (72). F1 antigen capture-based immunochromatographic dipsticks, namely, *Yersinia pestis* (F1) Smart II test strips and Plague BioThreat Alert test strips, are commercially available in the United States and have been analyzed using purified F1 antigen, *Y. pestis* organisms, and whole-blood samples from mice experimentally infected with *Y. pestis* (73). To date, neither of these tests is FDA cleared for use in diagnosing plague in patients or for the identification of *Y. pestis* isolates.

Nucleic Acid Detection

PCR assays for the direct detection of *Yersinia* nucleic acids in patient specimens have been developed to increase diagnostic sensitivity. For *Y. pestis*, most real-time PCR assays are directed against the plasminogen activator gene (*pla*) or *caf1* gene, which encodes the F1 antigen; both targets are located on high-copy-number *Y. pestis*-specific plasmids. Of note, a *pla* gene with high homology to the *Y. pestis pla* gene (98.8% similarity) was identified in rats, suggesting that if PCR assays targeting the *pla* gene are used for diagnosis of plague, they should not be used as stand-alone assays (74). To date, validation of *Y. pestis* PCR assays using human clinical samples is limited. Real-time (5′ nuclease) PCR assays directed against both *caf1* and *pla* were tested using lymph node aspirates from plague patients, and diagnostic sensitivity was found to be higher than for both culture and an F1 antigen immunochromatographic test (75). Real-time PCR assays for diagnosis of plague are available in LRN reference laboratories in the United States.

Genome sequencing of *Y. pestis* and *Y. pseudotuberculosis* has allowed the identification of unique chromosomal genes in *Y. pestis* that allow for development of *Y. pestis*-specific PCR assays targeting chromosomal regions (11, 12). A multiplex real-time PCR assay for identification of *Y. pestis* and *Y. pseudotuberculosis* has been developed based on the *Y. pestis* chromosomal gene *ypo*2088, the plasmid gene *pla*, and the *wzz* gene, common to both *Y. pestis* and *Y. pseudotuberculosis* (76). Due to the high degree of genetic relatedness between *Y. pestis* and *Y. pseudotuberculosis*, 16S rRNA PCR and sequencing cannot distinguish these two species; their 16S rRNA sequences are essentially identical (77).

PCR has also been used as a sensitive method to detect small numbers of *Y. enterocolitica* in foodstuff (78), as well as in stored red blood cells to prevent transfusion reactions (79). Use of multiplex PCR assays containing primer sets for the detection of up to five different virulence genes allows for the distinction between pathogenic and nonpathogenic serotypes and specifically identifies the presence of *Y. enterocolitica* serotype O:3 (78, 80). PCR methods have also been developed for identification of *Y. pseudotuberculosis* from *Y. enterocolitica* and include O genotyping as well as virulence typing (78, 80).

ISOLATION PROCEDURES

Yersinia species grow on most routine media, including blood, chocolate, and MacConkey agars incubated at 25 and 35°C for 24 to 48 h in ambient air. Incubation of *Y. pseudotuberculosis* and *Y. enterocolitica* cultures at 25°C is recommended due to the instability of the virulence plasmid at higher temperatures. Eosin-methylene blue, xylose-lysine-deoxycholate agar, and Hektoen enteric agars do not provide any advantage in the isolation of *Y. enterocolitica* and the differentiation of enteropathogenic *Yersinia* species from other organisms in the normal stool biota. Because *Yersinia* species grow more slowly than most *Enterobacteriaceae*, selective medium is recommended for culturing *Yersinia* species from nonsterile sites. There are various selective media for the recovery of *Y. enterocolitica*, including cefsulodin-Irgasan-novobiocin (CIN) agar, which inhibits the growth of many other *Enterobacteriaceae*, CHROMagar *Yersinia*, and salmonella-shigella deoxycholate calcium chloride agar (81, 82). CIN agar has been found to provide better rates of recovery of *Yersinia* than either MacConkey or salmonella-shigella agar incubated at room temperature. Growth of many strains of *Y. pseudotuberculosis* can be inhibited on CIN agar, and therefore MacConkey agar is preferred for isolation. The use of pectin agar has also been described for the isolation of *Y. enterocolitica* from stool and for its differentiation from other *Enterobacteriaceae*. Although this medium was more sensitive than other currently used selective media and inhibitory to other enterics except *Klebsiella oxytoca* (which demonstrates a similar colony morphology), the medium is currently not commercially available (83). CHROMagar *Yersinia* has been shown to be as sensitive as CIN agar for the recovery of *Yersinia* and more specific for the isolation of pathogenic *Y. enterocolitica*, which may be useful for direct isolation of pathogenic strains from stool (82). However, as with CIN agar, *Y. pseudotuberculosis* can be inhibited on CHROMagar, and therefore MacConkey agar is preferred for isolation.

Recovery of *Y. enterocolitica* from food is more difficult than recovery from human clinical specimens, and samples are usually referred to a public health laboratory. Food must be enriched with saline (or a selective broth, such as modified Rappaport broth [MRB], containing magnesium chloride, malachite green, and carbenicillin) at cold temperatures for approximately 21 days (2 to 4 days in MRB) (84).

On sheep blood agar incubated at 25°C, well-isolated single colonies of *Y. enterocolitica* and *Y. pseudotuberculosis* are about 0.5 to 1.0 mm and 3 to 4 mm in size after 24 and 72 h of growth, respectively (Fig. 2). *Y. enterocolitica* colonies have a bull's-eye appearance, i.e., a red center on CIN agar.

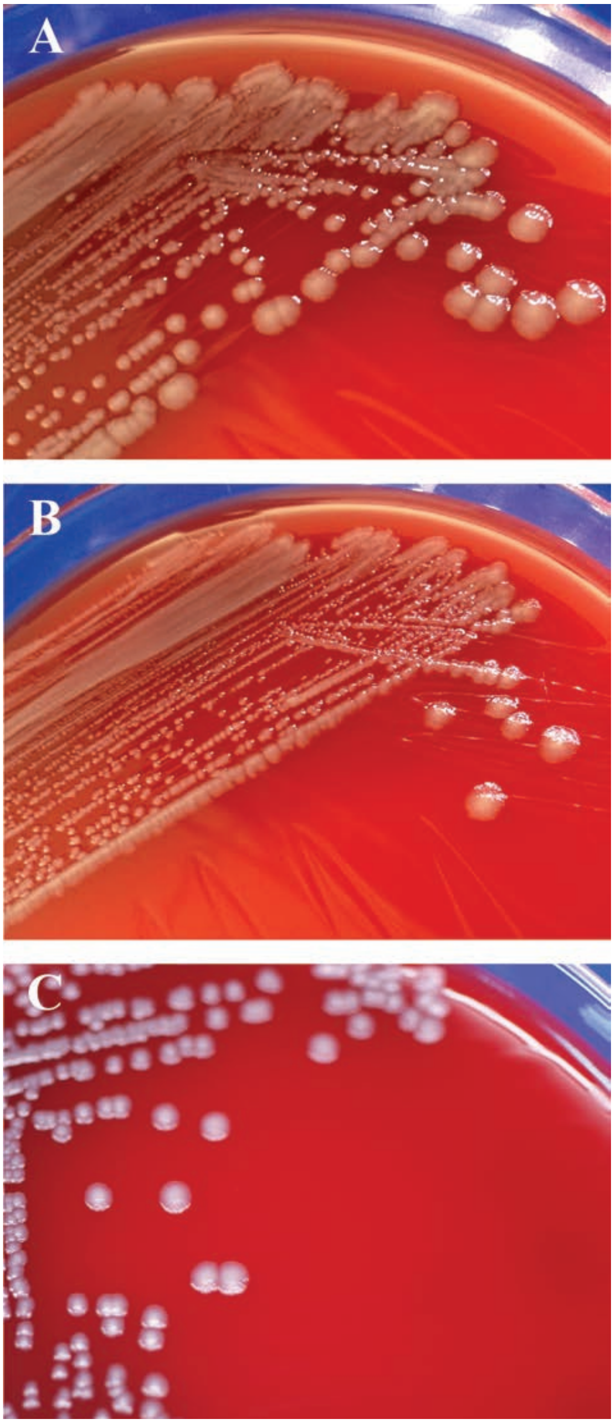

FIGURE 2 *Y. enterocolitica* (A) and *Y. pseudotuberculosis* (B) were grown on sheep blood agar at 25°C for 72 h, and *Y. pestis* was grown on sheep blood agar at 28°C for 72 h (C). doi:10.1128/9781555817381.ch39.f2

Other members of the *Enterobacteriaceae* family which grow on CIN agar, such as *Serratia*, *Morganella*, and *Citrobacter* species, produce colonies similar in appearance to those of *Y. enterocolitica* but larger.

Y. pestis can be isolated from sterile specimens (blood and lymph node aspirates) on sheep blood agar (SBA). Although *Y. pestis* grows faster at 28°C, this may not be readily observable in clinical laboratories. On agar plates, *Y. pestis* colonies are typically the same size at 28°C as at 37°C (or only slightly larger). Therefore, it is recommended that technicians in clinical laboratories look only for isolates that grow at both temperatures (28°C and 37°C) as opposed to looking for better growth at 28°C. For isolation of *Y. pestis* from nonsterile sources, including sputum samples, CIN agar should be used, as *Y. pestis* will rapidly be outcompeted by the growth of other organisms. CIN agar plates should be incubated at 25 to 28°C, which also aids in the isolation of *Y. pestis*, as the growth of other bacteria will be slowed at the lower temperature. *Y. pestis* will grow in blood culture bottles; whole blood should be directly inoculated into blood culture bottles, with subsequent culture of blood culture-positive specimens on agar. Cultures from suspected plague patients should be incubated for 5 to 7 days.

Well-isolated *Y. pestis* colonies are gray-white to opaque and ~1 to 1.5 mm in diameter, with irregular edges, after 48 h of incubation on SBA and 3 to 4 mm in size after 72 h of incubation (Fig. 2). No hemolysis is seen on blood agar. Viewed with a dissecting microscope, the colonies are raised and have irregular edges, with a "hammered copper" appearance. After 48 to 72 h, a fried-egg appearance is observed, with the center of the colonies being more opaque (Fig. 2). *Y. pestis* grows more slowly on MacConkey agar than on SBA, and colonies are only pinpoint in size as well as colorless (non-lactose fermenters) after 48 h of growth. On CIN agar, *Y. pestis* colonies have a red center. The growth of *Y. pestis* is slowed on CIN agar compared to on SBA. Organisms growing in broth appear in clumps along the side of the tube in flocculent or stalactite-like formations if the tube is not shaken. After 24 h of incubation, the clumps settle to the bottom of the tube.

IDENTIFICATION

Yersinia species are catalase positive and oxidase negative and ferment glucose, as do all other members of the family *Enterobacteriaceae*. *Y. enterocolitica* and *Y. pseudotuberculosis* can be presumptively identified by reactions on triple sugar iron (TSI) and lysine iron agar slants. *Y. enterocolitica* produces a yellow color in the entire TSI tube without gas production, and *Y. pseudotuberculosis*, like *Shigella*, produces an alkaline slant and an acid butt. Both species are lysine decarboxylase negative and therefore produce a yellow butt in lysine iron agar slants. *Yersinia* species are included in the databases of some automated biochemical identification systems; however, most databases were established with only a few *Yersinia* isolates tested. Automated systems may not adequately identify *Yersinia* species due in part to their slow growth and biochemical inactivity. API 20E was shown to have the highest sensitivity and specificity for the identification of *Y. enterocolitica* and *Y. pseudotuberculosis* (85, 86).

Identification of suspected *Y. pestis* in clinical laboratories is based on the identification of small Gram-negative bacilli (0.5 by 1 to 2 μm), which are catalase positive and oxidase and urease negative, and display the following growth characteristics after 48 h: 1 to 1.5 mm gray-white irregular colonies on blood agar after growth at both 28°C and 37°C and non-lactose-fermenting, pinpoint colonies on MacConkey agar. See the *Sentinel Level Clinical Microbiology Laboratory Guidelines* (64) on the American Society for Microbiology website (http://clinmicro.asm.org/index.php/bench-work-resources/guidelines/sentinel-guidelines) for *Y. pestis*-specific information, pictures, and flow charts. If isolates with the above-named *Y. pestis* characteristics are identified, clinical laboratories should notify the local public health laboratory and immediately refer the isolate for confirmatory testing. Rapid diagnosis and treatment are essential to reduce mortality associated with *Y. pestis* infection.

Automated biochemical systems are not recommended for the identification of *Y. pestis* due to its slow growth and the limited *Y. pestis* databases available with many of these systems. In 2010 to 2011, the misidentification of two *Y. pestis* human isolates by multiple automated systems was described (87). The two isolates were tested at several different commercial laboratories and misidentified by multiple automated systems; misidentifications included *Acinetobacter lwoffii* by the MicroScan test, *Pseudomonas luteola* and *Y. pseudotuberculosis* by the Vitek-2 test, and *Y. pseudotuberculosis* by the Phoenix test. As a result, the isolates were not correctly identified until 2 to 4 weeks after the patients presented with illness (87). Data are currently unavailable in the literature with respect to the accuracy of the Biolog Microbial ID system in a clinical laboratory setting.

Because of the limitations in accurately identifying *Y. pestis* isolates by automated biochemical identification systems in clinical laboratories, a high level of suspicion by clinical microbiologists is essential for accurate and timely diagnosis of plague. Potential indications of misidentification by automated systems include a low probability or confidence value and/or an identification of a pathogen inconsistent with the clinical or microbiology picture. Results from automated identification systems should be analyzed critically in the clinical laboratory, taking into consideration the reliability of these systems for accurate identification of slow-growing and/or rare organisms (87).

Isolates can be identified as *Y. pestis* in reference laboratories via specific bacteriophage lysis as well as molecular and antigen detection methods, including PCR, sequencing and direct fluorescent-antibody assay (DFA) (Fig. 1B). Bacteriophage lysis is the confirmatory method for identifying *Y. pestis* isolates in the United States; reagents and testing are available in LRN reference laboratories. 16S rRNA sequencing should not be used as the sole diagnostic test to identify *Y. pestis* isolates due to our inability to distinguish *Y. pestis* from *Y. pseudotuberculosis*.

Y. enterocolitica and *Y. pseudotuberculosis* can most easily be differentiated from *Y. pestis* by urease activity (highly positive at 25°C; positive at 37°C) and motility (25°C); *Y. pestis* is negative for both of these characteristics (Table 1). Because of the risk for aerosol exposure to *Y. pestis*, the slide motility test is not recommended; motility agar should be utilized to distinguish enteropathogenic *Yersinia* from *Y. pestis*. *Y. enterocolitica* can be differentiated from other *Yersinia* species since it typically does not utilize citrate or pyrazinamidase (except in 1A strains), and it does not ferment mucate or rhamnose; however, it does ferment sucrose (Table 1). *Y. pseudotuberculosis* may be differentiated from other *Yersinia* species (non-*Y. pestis*) by a negative ornithine decarboxylase test. Identification of other *Yersinia* species can be performed by biochemical analysis (Table 1) (40, 88).

A number of methods other than the more traditionally based culture techniques have shown potential for the future identification of *Yersinia* species, but to date, commercially available instruments and existing databases in clinical laboratory settings for the identification of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* have not been evaluated. These methods include matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) (MALDI Biotyper system; Bruker) and fatty acid analysis (Sherlock Microbial ID system; MIDI, Inc.) (89, 90–92). Both methods are attractive for two reasons: identification is rapid, and the cost is low (<$1 U.S./identification). As with other identification systems, a limitation is that they require a database of well-characterized reference strains to avert misidentifications. Additionally, as *Y. pestis* and *Y. pseudotuberculosis* strains are quite similar in major cellular fatty acids, fatty acid methyl esther (FAME) analysis cannot differentiate by simply comparing fatty acid profiles. A recent study, however, has shown that *Y. pestis* can be differentiated from *Y. pseudotuberculosis* by plotting the ratios of the 14:0/18:0 versus 18:1ω7c/18:0, 3-OH-14:0/18:0 versus 18:1ω7c/18:0, 16:1ω7c/18:0 versus 18:1ω7c/18:0, 12:0/18:0 versus 18:1ω7c/18:0, and 12:0 ALDE/18:0 versus 16:1ω7c/18:0 fatty acids (92).

A culture-independent multiplex bead-based assay, xTAG gastrointestinal pathogen panel (Luminex Corporation), has been developed to identify the most common gastrointestinal pathogens directly from stool, including *Yersinia enterocolitica* (93). Only a few isolates were included in the evaluation; thus, the ability of this assay to accurately identify *Yersinia enterocolitica* isolates (including all pathogenic biotypes) in a clinical laboratory setting is unknown. In the future, it is likely that many other culture-independent methods will be developed in order to reduce the cost and turnaround time and improve the quality of patient care; however, there are important implications to consider with respect to preserving cultures (94, 95). Culture provides conclusive diagnosis of infection and is invaluable for public health surveillance systems, including PulseNet. Cultures are also essential for identifying and characterizing new or emerging species, monitoring antimicrobial resistance, and investigating outbreaks. It is therefore important that we continue to retain the valuable information gained from cultures as we shift into the era of culture-independent diagnostics.

TYPING SYSTEMS

Once isolates have been identified as *Yersinia* spp., biochemical, serological, and molecular typing methods can be used to further differentiate within a given *Yersinia* species; typing is particularly useful for epidemiologic analyses to assess the pathogenesis and geographic distribution of different strains. *Y. enterocolitica* has six biogroups, 1A, 1B, 2, 3, 4, and 5, which can be differentiated based on reactivity to esculin, indole, D-xylose, trehalose, pyrazinamidase, β-D-glucosidase, and lipase. Although the issue is controversial, biogroup 1A is thought to be nonpathogenic, whereas biotypes 1B and 2 through 5 are pathogenic. Strains belonging to biotype 1A can be differentiated from the others by salicin and pyrazinamidase positivity (Table 2) (96). Serotyping may also help to determine the pathogenicity of the isolate, since only a small number of the >70 known serotypes are pathogenic; however, antisera are not readily available. Serotyping of pathogenic *Y. enterocolitica* may be performed at the CDC (Atlanta, GA) upon request. Other methods that have been evaluated to determine the pathogenicity of *Y. enterocolitica* are based on the presence of the virulence plasmid and include autoagglutination, calcium-dependent growth at 37°C, and pigmentation on Congo red. Selective media containing

Congo red as well as PCR assays have been evaluated for differentiating virulent from avirulent strains but are currently being used only in research laboratories (79).

Three classic biotypes (orientalis, mediaevalis, and antiqua) of *Y. pestis* have been defined based on differences in the strains' abilities to ferment glycerol and reduce nitrate. Although these three biotypes have historical significance and display differing geographic distributions, all appear to cause virtually identical signs and symptoms in humans; thus, biotyping is not clinically significant for *Y. pestis*. In the Americas, only the orientalis biotype (glycerol negative, nitrate positive) occurs. Whole-genome sequencing of *Y. pestis* strains to identify single nucleotide polymorphisms and screening of global isolates have defined a *Y. pestis* phylogenetic tree containing four distinct branches (97, 98).

For distinguishing individual strains within *Yersinia* spp., molecular typing methods, including pulsed-field gel electrophoresis (PFGE) and multilocus variable-number tandem-repeat analysis (MLVA), have been used. PFGE was found to be a more useful tool than ribotyping for typing of pathogenic isolates of *Y. enterocolitica* (78). A PFGE method for typing of *Y. pestis* isolates is available through the CDC PulseNet website and has been used in case investigations to epidemiologically link isolates and identify the source of human infections (70). Similarly, MLVA has also been used on *Y. pestis* strains to identify the source of human plague infections (99).

SEROLOGIC TESTS

Serology can play a role in the diagnosis of plague, particularly when a culture is not recovered or for retrospective diagnosis or epidemiologic studies in areas where plague is endemic. Most patients infected with *Y. pestis* seroconvert 1 to 2 weeks following the onset of symptoms. The most commonly used antigen in serologic assays for *Y. pestis* is the capsular F1 antigen, which is specific for *Y. pestis*, highly immunogenic, and produced in large amounts during mammalian infections. Studies have shown a persistence of anti-F1 antibodies in plague patients; antibody to F1 was shown in 88% and 69.5% of patients infected within the previous 5 and 10 years, respectively (100). Serologic methods include passive hemagglutination, which can be used to test both human and animal samples. A 4-fold rise in serum antibody titer to F1 antigen between acute- and convalescent-phase serum samples is confirmatory for plague. Reagents for passive hemagglutination are available only in some reference laboratories. Commercial reagents for the serodiagnosis of plague are not available or FDA approved in the United States.

Serology can be used as an adjunct in the diagnosis of disease due to *Y. enterocolitica* or *Y. pseudotuberculosis*. Antibody is detectable within the first week of illness and returns to normal levels 3 to 6 months later. The specificities of serologic assays range from 82 to 95% due to cross-reactivity between the two species and also with *Brucella* and *Vibrio* species, as well as *Borrelia burgdorferi*, *Chlamydia pneumoniae*, and some *Escherichia coli* serogroups. Another disadvantage of using serology for diagnosis is that antibodies to *Y. enterocolitica* O antigens are often found in healthy subjects due to the frequency of exposure to nonpathogenic serotypes.

Antibody to Yops that are present only in virulent strains of *Y. enterocolitica* may be more helpful. In a small study of healthy blood donors, immunoglobulin M (IgM) antibody to Yops was 97% specific for acute infection (101). Testing of blood donors for anti-Yop IgA in New Zealand, where a high incidence of *Y. enterocolitica* gastroenteritis is reported, showed promise in preventing transfusion-related infections (102). The presence of IgG and IgA antibodies to *Y. enterocolitica* Yops is also used as an aid in the diagnosis of autoimmune disorders that occur postinfection, such as reactive arthritis, erythema nodosum, Graves' disease, and Hashimoto's thyroiditis (53). IgM-, IgA-, and IgG-specific antibody reactivity against Yops in Western immunoblot formats has been correlated with clinical presentation and sequelae (103).

ANTIMICROBIAL SUSCEPTIBILITIES

Due to the severity of illness caused by *Y. pestis*, antimicrobial treatment is essential and should be initiated as soon as plague is suspected to ensure the highest chance of recovery. Streptomycin, gentamicin, tetracyclines, and levofloxacin are FDA approved for the treatment of plague. Chloramphenicol is recommended for the treatment of plague meningitis. Treatment guidance is available on the CDC website (http://www.cdc.gov/plague/healthcare/clinicians.html). Antimicrobial susceptibility testing of *Y. pestis* is not performed in clinical microbiology laboratories because of safety concerns in working with this organism and because resistance to antibiotics used for clinical treatment of plague has only very rarely been reported. The Clinical and Laboratory Standards Institute (www.clsi.org) has published interpretative criteria and quality control limits for broth microdilution testing of *Y. pestis* using Mueller-Hinton medium (104).

Antibiotic resistance among isolates of *Y. pestis* has never been documented in the United States and only very rarely elsewhere (105). No resistance was observed in 392 *Y. pestis* isolates from 17 countries (214 were from the United States and isolated from 1971 to 2009) against eight antimicrobials (gentamicin, streptomycin, tetracycline, doxycycline, ciprofloxacin, levofloxacin, chloramphenicol, and trimethoprim-sulfamethoxazole) used for treatment or prophylaxis of plague (105). Treatment failure due to antibiotic resistance in *Y. pestis* has also never been documented. An analysis of *Y. pestis* isolates from 10 fatal plague cases where patients had been treated with gentamicin, tetracycline, chloramphenicol, doxycycline, or streptomycin demonstrated no resistance to these antimicrobials (105). An isolate of *Y. pestis* from a 1995 plague case in Madagascar was found to be multidrug resistant, with resistance to streptomycin, sulfonamides, tetracycline, and chloramphenicol conferred by a multidrug resistance plasmid (106). Routine susceptibility testing of patient isolates in this region of endemicity has failed to identify any further evidence of multidrug resistance during the last 18 years (105–107). These surveillance data are consistent with the lack of a selective advantage for *Y. pestis* to acquire and maintain multidrug resistance plasmids in natural cycles.

Most cases of *Y. enterocolitica* gastroenteritis do not require treatment; however, treatment is necessary in cases of systemic disease, especially in immunosuppressed patients. Treatment options include trimethoprim-sulfamethoxazole and a fluoroquinolone. *Y. enterocolitica* produces two different β-lactamases, one of which is a class A constitutive enzyme and the other of which is an inducible class C enzyme that is not inhibited by β-lactamase inhibitors. The presence of one or both of these enzymes varies depending on the biogroup (108). Although the β-lactamase confers resistance to penicillin on *Y. enterocolitica*, the organism remains uniformly susceptible to the extended-spectrum cephalosporins (109). Resistance to fluoroquinolones is due to either a mutation in the *gyrA* gene or efflux mechanisms. In a study conducted in Spain, 23% of *Y. enterocolitica* strains isolated from patients with gastroenteritis were nalidixic acid resistant. All resistant isolates had a mutation in *gyrA*, and some were resistant based on an efflux mechanism as well (108). *Y. enterocolitica* strains are susceptible *in vitro* to aminoglycosides, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, and extended-spectrum cephalosporins.

Y. pseudotuberculosis is susceptible to ampicillin, tetracycline, chloramphenicol, cephalosporins, and aminoglycosides. Although self-limiting infections due to *Y. pseudotuberculosis* are not usually treated, patients with septicemia should be treated with ampicillin, streptomycin, or tetracycline. *Y. aldovae* and *Y. ruckeri* are also susceptible to penicillin. *Y. frederiksenii*, *Y. intermedia*, and *Y. rohdei* produce a β-lactamase similar to that of *Y. enterocolitica*, which is expressed at different levels in different strains (10).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Isolation of a small Gram-negative bacillus (0.5 by 1 to 2 μm) that grows slowly at both 28°C and 37°C on blood agar (pinpoint, gray-white colonies after 24 h) and that is a non-lactose fermenter on MacConkey agar (pinpoint colonies after 48 h), oxidase negative, catalase positive, and urease negative should be strongly suspected of being *Y. pestis* and immediately referred to a reference laboratory. A positive test result for a primary clinical specimen via detection of F1 antigen, DFA, or IHC staining provides only a presumptive diagnosis of *Y. pestis*. A single positive serum sample is also considered presumptive for plague. Confirmation of *Y. pestis* infection includes (i) identification of a culture as *Y. pestis* and/or (ii) a 4-fold difference in serum antibody titers to *Y. pestis* F1 antigen in acute- and convalescent-phase serum samples. Isolation of *Y. pestis* from any human specimen is clinically significant and warrants further investigation. The sources most likely to yield *Y. pestis* are respiratory specimens, bubo (lymph node) aspirate, and blood. Due to the lack of accuracy of commercial systems for the identification of *Y. pestis*, the potential use of *Y. pestis* as a bioweapon, and the seriousness of the disease, all suspected *Y. pestis* isolates should be sent to a local public health laboratory for confirmation.

In the United States, plague is a nationally notifiable disease, and *Y. pestis* is also classified as a tier 1 select agent. Laboratories identifying an organism as *Y. pestis* are required to report this finding immediately; laboratories need to complete form 4 and submit it to the Select Agent Program. Report forms, contact information, laboratory registration information, and pertinent citations of the U.S. Federal Code may be found at http://www.selectagents.gov.

Isolation of *Y. enterocolitica* or *Y. pseudotuberculosis* from stool culture is not sufficient for causal evidence of disease, since nonpathogenic serotypes may be normal stool biota. However, no readily available methods except those using routine biochemicals, which are not usually maintained in routine clinical laboratories, are available for differentiation of pathogenic serotypes. Isolation of either species in pure culture from a symptomatic patient with no other diagnosis should be considered suspect. Isolation of either species from blood or other normally sterile sites should be considered significant. Reporting of enteropathogenic *Yersinia* is not nationally notifiable, so the actual incidence of disease may be underreported.

It has not been shown to be cost-effective to screen all stools for *Y. enterocolitica* by using CIN agar. Isolation rates vary based on geographic locations, with the highest incidence in temperate regions, so the decision to routinely rule out these organisms in stool cultures should be evaluated in individual laboratories after consultation with the infectious disease physicians.

Although the *Yersinia* species besides *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* are not considered human pathogens, they have been isolated from the gastrointestinal tracts of symptomatic patients with no other diagnosis. It has been recommended that the presence of these *Yersinia* species in pure culture be reported. These organisms may be underrecognized pathogens (62).

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Aeromonas[*]

AMY J. HORNEMAN

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TAXONOMY

The genus *Aeromonas* resides within the family *Aeromonadaceae* (1) and the newly proposed order *Aeromonadales*, ord. nov., along with the genera *Oceanimonas* and *Tolumonas* (2). *Aeromonas* is the only one of these three genera that is pathogenic for humans. The use of frequent reclassifications and constant amended or extended descriptions within *Aeromonas* taxonomy can often be initially puzzling to microbiologists not working with these organisms on a daily basis. However, information in this chapter should clarify the identification and significance of those species most often associated with human disease (Table 1). DNA hybridization group numbers, which no longer serve a meaningful purpose, and synonymous species designations for *Aeromonas veronii* bv. sobria (*Aeromonas ichthiosmia*) and *Aeromonas trota* (*Aeromonas enteropelogenes*) (3) are not included for simplicity. Likewise, *Aeromonas* sp. DNA hybridization group 11, which is made up of *Aeromonas eucrenophila*/*Aeromonas encheleia*-like organisms, is not addressed (4). *Aeromonas culicicola*, a species proposed in 2002 and isolated from mosquitoes, has subsequently been shown to be a later subjective synonym of *Aeromonas veronii* (5).

Newly proposed *Aeromonas* species and subspecies since the publication of the previous edition of this *Manual* include *Aeromonas diversa*, sp. nov., isolated from wounds (6); *Aeromonas fluvialis*, sp. nov., isolated from a Spanish river (7); *Aeromonas taiwanensis*, sp. nov., and *Aeromonas sanarellii*, sp. nov., both isolated from wounds (8); *Aeromonas rivuli*, sp. nov., isolated from a karst water rivulet (9); *Aeromonas cavernicola*, sp. nov., isolated from fresh water of a brook in a cavern (10); and *Aeromonas australiensis*, sp. nov., isolated from Australian irrigation water (11). The past controversy surrounding the proposal of *Aeromonas aquariorum* in 2008, which is purported to be both phenotypically and genetically identical to *Aeromonas hydrophila* subsp. *dhakensis* (first proposed in 2002), has been resolved. The original first author of the 2008 paper on *Aeromonas aquariorum*, sp. nov., recently coauthored a formal synonymization of *A. aquariorum* and *A. hydrophila* subsp. *dhakensis* with a concomitant reclassification of both as *A. dhakensis*, sp. nov., comb. nov. (12). This is significant, since there are numerous references in the clinical literature to this "arabinose-negative" *A. hydrophila*-like group of organisms being associated with serious wound infections, sepsis, and diarrhea and being misidentified as *A. hydrophila* (13–15).

Clinically significant strains formerly referred to as *Aeromonas sobria* are, in fact, *Aeromonas veronii* bv. sobria (esculin hydrolysis and ornithine decarboxylase negative and arginine dihydrolase positive) and should be reported as such. Nearly all rapid identification databases, excepting API 20E strips (bioMérieux, Inc., Durham, NC), have converted their *A. sobria* identifications to *A. veronii* bv. sobria. This is especially important because of *A. veronii* bv. sobria's association with more-severe, extraintestinal infections, such as septicemia, meningitis following leech therapy, and disseminated intravascular gas production (16, 17). It usually is not necessary to definitively separate members of the *A. hydrophila* complex (*A. hydrophila*, *A. dhakensis*, sp. nov., comb. nov., *Aeromonas bestiarum*, and *Aeromonas salmonicida*) or the *Aeromonas caviae* complex (*A. caviae*, *Aeromonas media*, and *Aeromonas eucrenophila*), especially when they are isolated from feces (see "Evaluation, Interpretation, and Reporting of Results" below).

The type strain *Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966 was the first aeromonad to be completely sequenced, annotated, published, and deposited in GenBank (as accession number CP000462) (18). This was followed by the complete genome sequence of *Aeromonas salmonicida* subsp. *salmonicida* A449, an agent of furunculosis (a bacterial septicemia of salmonid fish), which was deposited in GenBank as accession number NC 00938 (19). Additional species whose genome sequences have been deposited in GenBank include *A. veronii*, *A. caviae*, and *A. aquariorum* (now reclassified as *A. dhakensis*, sp. nov., comb. nov.).

DESCRIPTION OF THE GENUS

Members of the genus *Aeromonas* are Gram-negative facultative anaerobes that are straight, coccobacillary to bacillary cells with rounded ends (0.3 to 1.0 μm in diameter and 1.0 to 3.5 μm in length). They can occur singly, in pairs, or, rarely, in short chains. Most species are motile by a single, polar flagellum with a 1.7-μm wavelength, but peritrichous flagella may be formed on solid media in young cultures, and lateral flagella occur in some species. Aeromonads are usually oxidase positive and catalase positive and are generally resistant to 150 μg of the vibriostatic agent 2,4-diamino-6,7-diisopro-

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[*] This chapter contains information presented in chapter 38 by Amy J. Horneman and Afsar Ali in the 10th edition of this *Manual*.

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TABLE 1 Members of the genus *Aeromonas*[a]

Organism	Human isolation (extraintestinal/fecal)	Human pathogen (extraintestinal/fecal)	Frequency in humans	Pathogenic for animals, fish, and reptiles
A. hydrophila complex				
A. hydrophila subsp. *hydrophila*	Yes	Yes	Common	Yes
A. hydrophila subsp. *ranae*	No	No	Rare	Yes
A. dhakensis,[b] sp. nov., comb. nov.	Yes	Yes	Common	No
A. bestiarum	No/yes	—/no	Rare	Yes
A. salmonicida[c]	No/yes	No/no	Rare	Yes
subsp. *salmonicida*				Yes
subsp. *achromogenes*				Yes
subsp. *masoucida*				Yes
subsp. *smithia*				Yes
subsp. *pectinolytica*				No
A. caviae complex				
A. caviae	Yes	Yes	Common	Yes
A. media	No/yes	—/yes	Rare	No
A. eucrenophila	Yes	No/—	Very rare	No
A. veronii complex				
A. veronii bv. sobria	Yes	Yes	Common	Yes
A. veronii bv. veronii	Yes	Yes	Rare	No
A. jandaei	Yes	Yes/unknown	Rare	No
A. trota	Yes	Yes/yes	Rare	No
A. schubertii	Yes/no	Yes/—	Rare	No
A. diversa	Yes/no	Yes/—	Rare	No
A. encheleia	Yes/no	No/—	One case	No
Miscellaneous *Aeromonas* species				
A. sobria	Neither	—	—	No
A. allosaccharophila	No/yes	—/no	Very rare	Yes
A. popoffii	Yes	Yes	Very rare	No
A. simiae	No	No	—	No
A. molluscorum	No	No	—	No
A. bivalvium	No	No	—	No
A. tecta	Yes	No	Rare	No
A. piscicola	No	No	—	Yes
A. fluvialis	No	No	—	No
A. taiwanensis	Yes/no	Yes/no	Rare	No
A. sanarellii	Yes/no	Yes/no	Rare	No
A. rivuli	Neither	—	—	No
A. cavernicola	Neither	—	—	No
A. australiensis	Neither	—	—	No

[a] —, not applicable.

[b] *A. dhakensis* was formerly known as *A. hydrophila* subsp. *dhakensis* and *A. aquariorum*, sp. nov. (12).

[c] There are motile strains of *A. salmonicida* that grow at 37°C and resemble clinical *A. hydrophila* strains that have been isolated from human feces; these can be distinguished using the tests in Table 3.

pylpteridine (O/129). They are chemoorganotrophic, displaying oxidative and fermentative metabolism of glucose. Acid, and often acid with gas, is produced from many carbohydrates, especially glucose, and nitrate is reduced to nitrite. A variety of exoenzymes, such as arylamidases, amylase, DNase, esterases, peptidases, proteases, chitinase, chondroitinase, and hemolysins, are produced. The main cellular fatty acids produced are hexadecanoic acid (16:0), hexadecenoic acid (16:1), and octadecenoic acid (18:1). Human (mesophilic) strains grow between 10 and 42°C, but occasional isolates may be more active in some biochemical assays at 22 to 25°C. Psychrophilic strains from fish and the environment (*Aeromonas popoffii* and *A. salmonicida*) seldom grow above 37°C and preferentially grow at 22 to 25°C. In brain heart infusion broth at 28°C, growth occurs between pH 4.5 and 9.0 and at salt concentrations between 0 and 4%. The mol% G+C content of the DNA is 57 to 63%.

EPIDEMIOLOGY AND TRANSMISSION

Aeromonads are inhabitants of aquatic ecosystems, such as groundwater, reservoirs, and clean or polluted lakes and rivers, worldwide. *Aeromonas* may also be found in marine environments but only in brackish water or water with a low saline content. Most *Aeromonas* species, particularly those associated with human infections, are found in a wide variety of fresh produce, meat (beef, poultry, and pork), and dairy products (raw milk and ice cream) (20). *A. veronii* bv. sobria is a symbiont in the gut of medicinal leeches, where it may grow as a pure culture (21). Infections in frogs, pigs, cattle, birds, and marine animals have also been reported (20).

Most clinical infections with aeromonads are related to an exposure to some type of aquatic source, whether the clinical specimen is feces or extraintestinal, and, to a lesser extent, to the ingestion of foods. The majority of studies have found a seasonal relationship between the recovery of aeromonads from specimens and the warmer months of the year (22). This is not surprising, since the optimal temperatures for the growth of mesophilic aeromonads are those occurring in the warmer months. This therefore increases the likelihood of recreational human exposure to these bacteria, thereby resulting in an increased risk of colonization and/or infections with these indigenous aquatic microorganisms.

Since *Aeromonas* is not a reportable condition in the United States or in most other countries, the true incidence of *Aeromonas* infections worldwide is not known. Estimates from England/Wales and the United States for septicemia with aeromonads in 2004 revealed an incidence of 1.5 per million population (23). However, any estimates of incidence would most likely be an underestimation, particularly as relates to exposure through drinking water. A large 2010 study of the distribution of virulence factors and molecular fingerprinting of 227 *Aeromonas* isolates from water and clinical samples provided strong evidence of water-to-human transmission (24).

CLINICAL SIGNIFICANCE

Aeromonas gastroenteritis ranges from an acute watery diarrhea (most common form) to dysenteric illness to chronic illness. Stools from acute watery diarrhea are loose (take the shape of their container), and erythrocytes and fecal leukocytes are absent. Accompanying symptoms include abdominal pain (60 to 70% of patients), fever and vomiting (20 to 40%), and nausea (40%) (25). Infections are usually self-limiting, but children may require hospitalization due to dehydration. *A. caviae* is the most common species associated with these infections, and *A. caviae* infection can even mimic inflammatory bowel disease in children (26). *A. veronii* bv. sobria strains may be associated with rare cholera-like disease characterized by abdominal pain (60%) and fever and nausea (20%) (20). In cases of dysenteric diarrhea resembling shigellosis, patients suffer from severe abdominal pain and have bloody stools containing mucus and polymorphonuclear leukocytes. About 10 to 15% of patients with either cholera-like or dysenteric diarrhea are coinfected with another enteric pathogen(s).

A comprehensive Bangladesh study found that the presence of loose stools or severe watery diarrhea was associated with *Aeromonas* strains possessing an *alt* gene (for a heat-labile cytotonic enterotoxin) alone or both *alt* and *ast* (for a heat-stable cytotonic enterotoxin), respectively (27). A large traveler's diarrhea study in Spain found the predominant species to be *A. veronii* bv. veronii and *A. caviae* (28). A third large study in India found seven different species among hospitalized patients with diarrhea, with *A. caviae* predominating, followed by *A. hydrophila* and *A. veronii* bv. sobria, along with the presence of the *alt* and *ast* genes as well as the *act* gene, which encodes a well-established cytotoxic enterotoxin often present in clinical aeromonad isolates (29).

Finally, in a large acute diarrheal outbreak in Brazil that involved 2,170 cases, *Aeromonas* was the species that was recovered in 19.5% of those cases (30). Although most diarrheal cases are generally self-limited, a combination of supportive therapy and antimicrobials is often indicated in the pediatric, geriatric, and immunocompromised populations (25). A 2007 article gives a nice summary of the latest data and theories related to the association of *Aeromonas* with diarrhea (31).

Complications from *Aeromonas* diarrheal disease include hemolytic-uremic syndrome (32, 33) and kidney disease requiring kidney transplantation (34). These more severe infections are usually associated with *A. hydrophila* or *A. veronii* bv. sobria. Also, nonresolvable, intermittent diarrhea can occur months after the initial infection and may persist for months or several years.

Aeromonas can also be isolated from a variety of extraintestinal sites, although blood and wounds are the most common sources. *Aeromonas* septicemia occurs rarely in immunocompetent hosts; most cases are in patients with liver disease and hematological malignancies and can be accompanied by necrotizing fasciitis (35, 36). However, a 2011 report describes three cases of bacteremia in pregnant women at the Thailand/Myanmar border (37).

The species more commonly isolated from patients with septicemia are *A. hydrophila*, *A. veronii* bv. sobria, and *Aeromonas jandaei*. Wound infections are usually preceded by traumatic injury that occurs in contact with water, where the predominant species are *A. hydrophila* subsp. *hydrophila* and, now, *A. dhakensis* sp. nov., comb. nov. (formerly *A. aquariorum*). These infections range from uncomplicated cases of cellulitis to myonecrotic infections with a poor prognosis (38, 39). Two such scenarios are the reported outbreaks of wound infections with *A. hydrophila* associated with mud football (40) and wound infections among both the 2004 Asian tsunami survivors (41) and the 2005 Hurricane Katrina survivors in New Orleans, LA (42). Surveys indicate that only 17 to 52% of *Aeromonas* wound infections are monomicrobic (25). Use of medicinal leeches postoperatively to enhance blood flow to surgical sites has resulted in wound infection rates of 20%, primarily with *A. veronii* bv. sobria (21, 43). Most alarming are three recent reports of ciprofloxacin-resistant *Aeromonas* strains in patients after receiving leech therapy (44–46).

Other extraintestinal infections include ocular, respiratory, surgical, and urinary tract infections, meningitis, osteomyelitis, cholecystitis, pneumonia, endocarditis, peritonitis, portal pyemia, pancreatic abscess, and spa bath folliculitis (47–55). A few such examples were the isolation of *A. caviae* from a patient with keratitis associated with contact lens wear (56) and isolation of *A. caviae* and *A. popoffii* from separate cases of urinary tract infection (57, 58). A very recent case of serious cellulitis in a polymicrobic leg infection involved two different strains of *A. hydrophila* with different virulence patterns and antimicrobial resistance profiles, and the authors proposed an *A. hydrophila* wound pathotype based on comparative genomic and functional analyses of virulence genes (59).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Aeromonads survive well in specimens, and any of the widely used transport media are acceptable for transport (Amies, Cary-Blair, modified Stuart's, and buffered glycerol in saline), with Cary-Blair generally considered to be the best (see chapter 18). Feces are always preferable to rectal swabs for isolation of enteric pathogens, and stools should be collected in the acute phase of disease. Most strains grow equally well at room temperature (20 to 25°C) and incubator temperature (35 to 37°C). Because isolates being kept for long-term storage do not survive well at room or refrigerator temperature in the laboratory for long periods (>1 month), placing aeromonads in media, such as Trypticase soy broth with 30% glycerol, and deep-freezing them at −80°C is recommended for their long-term storage.

DIRECT EXAMINATION

The direct microscopic examination of wound or skin/superficial specimens or positive blood culture specimens is somewhat unremarkable in that aeromonads appear as straight, Gram-negative bacilli, with or without the presence of white cells, which is not unlike the presentation of a similar infection with either enterics or pseudomonads.

Although various DNA probe and multiplex PCR methods have been described for the possible identification of aeromonads (specifically, identification of genes for enterotoxins) from either water, food, or a veterinary source (60), there are no widely recognized antigen detection and/or nucleic acid detection methods available for accurate species identification from clinical specimens.

ISOLATION PROCEDURES

Aeromonads generally grow well on a variety of enteric differential and selective agars, although sucrose- and/or lactose-fermenting strains usually resemble nonpathogens on these media. Blood agar with 20 µg of ampicillin per ml had previously been considered useful for isolating all *Aeromonas* species; however, a substantial percentage (15 to 57%) of *A. caviae* isolates are resistant to ampicillin, and certain species, like *A. trota,* are intrinsically susceptible to ampicillin (61, 62). In fact, a recent environmental sampling study to detect aeromonads showed that when ampicillin is used as a selective agent, a significant portion (17.3%) of the aeromonad population, in at least some environments, cannot be isolated using such media (63). Therefore, laboratories should use caution when medium with ampicillin is used in the setup of stool specimens for detecting the presence of all clinically relevant aeromonad species as bacterial enteropathogens.

Modified cefsulodin-irgasan-novobiocin (CIN) (4 µg of cefsulodin per ml, versus 15 µg/ml in unmodified CIN) is also an excellent isolation medium for aeromonads. On this medium, *Aeromonas* colonies have a pink center with an uneven, clear apron and are indistinguishable from *Yersinia enterocolitica* morphologically. One can incubate CIN at 25°C to enhance the recovery of *Yersinia* cells and still be able to recover *Aeromonas* within 24 h at this temperature.

Aeromonas agar, available from Lab-M (http://www.labm.com), is a relatively new alternative medium to CIN agar that uses D-xylose (which aeromonads do not ferment) as a differential characteristic (64).

Since most clinically relevant species are beta-hemolytic, including an increasing number of *A. caviae* strains, beta-hemolytic colonies on blood agar should be screened with oxidase and a spot indole test. Any colonies positive by both tests should be characterized further, although occasional indole-negative *A. caviae* strains do occur, and nearly all known *Aeromonas schubertii* isolates (which are generally associated with severe aquatic wounds) are indole negative (65). Thiosulfate-citrate-bile salts-sucrose medium is usually inhibitory to aeromonads. Enrichment in alkaline peptone water enhances the recovery of *Aeromonas* spp. from populations that are generally expected to shed low numbers of organisms (carriers, convalescent-phase patients, and those with subclinical infections). For patients with acute diarrhea, enrichment is probably unnecessary (66).

IDENTIFICATION

Aeromonas spp. are most easily confused in the laboratory with other oxidase-positive fermenters, i.e., *Vibrio* and *Plesiomonas* spp. *Plesiomonas* is easily differentiated from *Aeromonas* by positive reactions in Moeller's lysine, ornithine, and arginine tests and by fermentation of *m*-inositol. Vibrios may be more difficult to distinguish from aeromonads (67), which is particularly true for *Vibrio fluvialis* and *A. caviae,* and in laboratories where the sole means of identification is a rapid miniaturized system (68, 69). Resistance to an O/129 vibriostatic agent (150 µg), string test negativity (with 0.5% sodium desoxycholate), and the inability to grow in salt concentrations of ≥6% usually indicate the genus *Aeromonas*. *Vibrio cholerae* O139, a cholera toxin-positive, non-salt-requiring, O/129 vibriostatic-agent-resistant vibrio, is a major exception to this rule. However, the decarboxylase pattern (positive for lysine and ornithine), a negative reaction for arginine dihydrolase, no production of gas from glucose, and no fermentation of salicin separate this organism from most aeromonads. Unfortunately, strains of ornithine decarboxylase-positive *A. veronii* bv. veronii will often yield an excellent to very good identification for *V. cholerae* with the rapid identification API-20-E strip (bioMérieux, Inc.), and serotyping and/or additional testing is required to resolve the issue. *A. veronii* bv. veronii is string test negative, O/129 resistant, and able to produce gas from glucose fermentation, does not require additional salt for growth, and is inhibited on thiosulfate-citrate-bile salts-sucrose agar. *V. cholerae* strains have the opposite reactions. Once it has been determined that you have a glucose-fermenting, oxidase-positive, motile Gram-negative rod that is resistant to O/129, a small number of biochemical tests can be used for separating *Aeromonas* species into the three major species complexes (Table 2). If warranted, even more discriminatory results for separating members of each complex can be found in bold text in Table 3 (65), which should

TABLE 2 Biochemical identification of *Aeromonas* to the complex level

Test	No. of strains identified as belonging to[a]:		
	A. hydrophila complex (*A. hydrophila, A. bestiarum, A. salmonicida)*[b]	*A. caviae* complex (*A. caviae, A. media, A. eucrenophila)*	*A. veronii* complex (*A. veronii* HG8,[c] *A. jandaei, A. schubertii, A. trota)*
Esculin	87 (92, 81, 85)	71 (76, 55, 78)	0
Voges-Proskauer	75 (88, 63, 62)	0	54 (88, 87, 17, 0)
Glucose (gas)	81 (92, 69, 77)	16 (0, 0, 78)	87 (92, 100, 0, 69)
L-Arabinose	93 (84, 100, 100)	96 (100, 100, 78)	4 (12, 0, 0, 0)

[a] The first number is the overall percentage of strains that were positive for each complex for a given trait; the numbers in parentheses are percentages of strains positive for each species listed within that complex. Data are derived and modified from Table 5 in reference 65 and reprinted with permission.

[b] *Aeromonas dhakensis*, sp. nov., comb. nov. (formerly *Aeromonas aquariorum*/*Aeromonas hydrophila* subsp. *dhakensis*), is the newest member of this complex and has positive reactions in esculin, Voges-Proskauer, and glucose (gas) tests but negative reactions for utilization of L-arabinose and L-fucose, negative fermentation of L-arabinose, and positive utilization of urocanic acid (96).

[c] Biovar sobria (DNA hybridization group 8). The separation of *A. veronii* bv. veronii (DNA hybridization group 10) from *A. veronii* bv. sobria is achieved with *A. veronii* bv. veronii having positive reactions for ornithine decarboxylase and esculin hydrolysis and a negative reaction for arginine dihydrolase.

TABLE 3 Tests useful in the separation of members within the *Aeromonas* species complexes

Test	Result[a]									
	A. hydrophila complex			*A. caviae* complex			*A. veronii* complex			
	A. hydrophila[b]	*A. bestiarum*	*A. salmonicida*	*A. caviae*	*A. media*	*A. eucrenophila*	*A. veronii*[c]	*A. jandaei*	*A. schubertii*	*A. trota*
Utilization of:										
Citrate	**+ (92)**	**V (38)**	**+ (85)**	**+ (88)**	**V (82)**	**– (0)**	V (52)	+ (87)	V (58)	+ (94)
DL-Lactate	**V (84)**	**– (0)**	**– (0)**	**+ (96)**	**V (56)**	**– (0)**	−(0)	− (7)	V (58)	+ (88)
Urocanic acid	**V (16)**	**+ (94)**	**+ (100)**	**+ (100)**	**+ (100)**	**– (0)**	− (0)	− (7)	− (0)	V (75)
Gluconate oxidation	**V (64)**	**– (13)**	**– (0)**	− (0)	− (0)	− (0)	V (60)	V (60)	− (0)	− (0)
Gas from D-glucose	+ (92)	V (69)	V (77)	**– (0)**	**– (0)**	**V (78)**	**+ (92)**	**+ (100)**	**– (0)**	**V (69)**
PZA test	V (24)	V (50)	V (31)	**+ (88)**	**V (18)**	**+ (100)**	ND	ND	ND	ND
Indole test	+ (96)	+ (100)	+ (100)	V (84)	+ (100)	+ (89)	**+ (100)**	**+ (100)**	**V (17)**	**+ (100)**
Voges-Proskauer test	+ (92)	V (63)	V (62)	− (0)	− (0)	− (0)	**+ (92)**	**+ (87)**	**V (17)**	**– (0)**
Lipase (corn oil) test	+ (100)	+ (88)	+ (92)	V (76)	V (82)	+ (89)	**+ (92)**	**+ (100)**	**+ (100)**	**– (0)**
Acid from:										
Cellobiose	**– (4)**	**V (38)**	**V (69)**	+ (100)	+ (100)	V (56)	**V (20)**	**V (20)**	**– (0)**	**+ (100)**
Lactose	**V (64)**	**– (13)**	**+ (92)**	V (60)	V (64)	− (11)	− (12)	− (0)	− (0)	− (0)
L-Rhamnose	**V (24)**	**V (69)**	**– (0)**	− (0)	− (0)	V (22)	− (0)	− (0)	−(0)	− (0)
D-Sorbitol	**– (0)**	**– (0)**	**+ (85)**	− (4)	− (0)	− (0)	−(0)	− (0)	− (0)	− (0)
Glucose-1-phosphate	ND	ND	ND	**– (4)**	**+ (100)**	**+ (100)**	ND	ND	ND	ND
Glucose-6-phosphate	ND	ND	ND	**– (4)**	**+ (100)**	**+ (100)**	ND	ND	ND	ND
Lactulose	ND	ND	ND	**V (68)**	**V (55)**	**– (0)**	ND	ND	ND	ND
D-Mannose	+ (100)	+ (100)	+ (100)	**V (32)**	**+ (100)**	**+ (100)**	ND	+ (100)	+ (92)	+ (100)
Glycerol	+ (96)	+ (100)	+ (100)	V (68)	V (55)	− (11)	**+ (100)**	**+ (100)**	**– (0)**	**+ (94)**
D-Mannitol	+ (96)	+(100)	+ (100)	+ (100)	+ (100)	+ (100)	**+ (100)**	**+ (100)**	**– (0)**	**V (69)**
Sucrose	+ (100)	+ (94)	+ (100)	+ (100)	+ (100)	V (33)	**+ (100)**	**– (0)**	**– (0)**	**V (19)**
Amp^r	+ (100)	+ (94)	+ (85)	+ (100)	V (73)	+ (100)	**+ (100)**	**+ (93)**	**+ (92)**	**– (6)**

[a] Data were compiled from Tables 2, 6, 7, and 8 in reference 65 and reprinted with permission. +, ≥85% of the strains positive; −, <15% positive; V, 15 to 85% positive (results at 48 h). Numbers in parentheses indicate percentages of strains positive for the test at the final day of reading, which for gluconate was 2 days; DL-lactate and urocanic acid, 3 days; citrate, 4 days; carbohydrates, indole, and lipase, 7 days; pyrazinamidase (PZA), 2 days; Amp^r (resistance to 10 μg of ampicillin), 1 day; Voges-Proskauer, 3 days. ND, not done. For each of the three *Aeromonas* species complexes, the discriminatory reactions between the species within each complex are presented in bold type.

[b] *Aeromonas dhakensis*, sp. nov., comb. nov. (formerly *A. aquariorum*/*A. hydrophila* subsp. *dhakensis*) is considered to have positive reactions in the esculin, Voges-Proskauer, and glucose (gas) tests but negative reactions for utilization of L-arabinose and L-fucose, negative fermentation of L-arabinose, and positive utilization of urocanic acid (96).

[c] Biovar sobria (DNA hybridization group 8). The separation of *A. veronii* bv. veronii (DNA hybridization group 10) from *A. veronii* bv. sobria is achieved with *A. veronii* bv. veronii having positive reactions for ornithine decarboxylase and esculin hydrolysis and a negative reaction for arginine dihydrolase.

replace earlier published results for species identification (70).

A recent comparison study on the accuracy of six commercial systems for identifying 87 clinical *Aeromonas* isolates to the correct species highlighted the outdated databases and taxonomy, weak algorithms, and impractical tests with only 67.8 to 91.9% accuracy (71). However, this same study reported the ability to accurately identify 95.5% of their 87 clinical isolates to the correct species level using the seven conventional phenotypic tests within the simple dichotomous key Aerokey II (72). The use of Aerokey II or of the discriminatory biochemicals in Table 2 is recommended for isolates from sterile body sites or serious extraintestinal infections.

Other Identification Methods

Both the sequencing of a single housekeeping gene, the 16S rRNA gene (73), and the development of an extended method using 16S ribosomal DNA (restriction fragment length polymorphism) analysis (74) were initially promising as methods to identify aeromonads to the species level. However, data on the intragenomic heterogeneity within the 16S rRNA gene in *Aeromonas* strains suggest caution in using this single gene for anything beyond genus-level identification (75). Therefore, the use of other housekeeping genes as multiple molecular markers, such as *gyrB* and *rpoD* (76), *dnaJ* (77), and *cpn60* (78), or an even broader approach like multilocus (seven gene loci) phylogenetic analysis (MLPA), appears to be a credible avenue for accurate species identification (79). Extensive studies by Chopra et al. have delineated several DNA probes for the detection of a number of possible virulence-related factors. This was the result of the public release of the *Aeromonas hydrophila* ATCC 7966^T genome sequence and comparative work with the diarrheal *A. hydrophila* strain SSU (80). These include, but are not limited to, the discovery of a new hemolysin and the presence of a functional type VI secretion system, a cold shock exoribonuclease R (VacB), and a surface-associated enolase.

One promising new method of species identification is matrix-assisted laser desorption ionization–time of flight (MALDI-TOF), which was first described for *Aeromonas* species identification in 2007 (81), with further validation in 2011 (82), and resulted in a 2012 rapid MALDI-TOF mass spectrometry (MS) *Aeromonas* database for clinical and environmental isolates (83).

SEROLOGIC TESTS

Most serologic assays that have been used to detect antibodies to *Aeromonas* (tube agglutination, immunoblotting, and enzyme-linked immunoassay) have low sensitivity and specificity and are not considered reliable.

TABLE 4 *Aeromonas* species susceptibilities

Susceptibility[a]	Antibiotic agent
Resistant	Ampicillin (except *A. trota* [100% susceptible], *A. caviae* [35% susceptible][b])
Variable	Ticarcillin or piperacillin (except *A. veronii* bv. veronii [100% resistant], *A. trota* [100% susceptible])
	Cephalothin
	Cefazolin
	Cefoxitin (except *A. veronii* bv. veronii [100% susceptible])
	Cefuroxime
	Ceftriaxone
	Cefotaxime
Susceptible	Ciprofloxacin[c]
	Gentamicin
	Amikacin
	Tobramycin (*A. veronii* bv. veronii [42% resistant])
	Imipenem (*A. jandaei* [65% resistant], *A. veronii* bv. veronii [67% resistant])
	Trimethoprim-sulfamethoxazole

[a] Resistant or susceptible, ≥90% of all isolates were resistant or susceptible; variable, 10 to 90% of isolates were susceptible (65).

[b] Data for *A. caviae* susceptibility are found in references 57 and 62.

[c] Data for resistance to nalidixic acid and pipemidic acid in 26 and 20% of *A. caviae* and *A. hydrophila* strains, respectively, and 88% of *A. veronii* clinical strains suggest possible future resistance to fluoroquinolones (87). References 44, 45, and 46 denote emerging ciprofloxacin resistance associated with leech therapy.

ANTIMICROBIAL SUSCEPTIBILITIES

Two of the earliest articles on *Aeromonas* antimicrobial susceptibilities (84, 85) included only strains well characterized to the species level and expanded previously known susceptibility information on aeromonads isolated less frequently from clinical specimens. A general antimicrobial susceptibility profile for *Aeromonas* derived from both of these investigations as well as other studies (20, 86, 87) is given in Table 4. There are Clinical and Laboratory Standards Institute (CLSI) testing guidelines for the major clinical *Aeromonas* species that relate to antimicrobial dilution and disk susceptibility testing in document M45-A2 for infrequently isolated or fastidious bacteria (88).

Ciprofloxacin, commonly used to treat Gram-negative infections, was initially reported as active against all species of *Aeromonas*, with little or no resistance reported in studies in the United States and most of Europe (84, 85). However, 2 to 3% of *A. caviae*, *A. hydrophila*, and *A. veronii* bv. sobria strains in Asia were reported to be ciprofloxacin resistant as early as 1996 (86). As previously reported above, there are increased reports of ciprofloxacin-resistant strains associated with the use of leech therapy after surgery (44–46).

Aeromonas species can express three chromosomal β-lactam-induced β-lactamases, including a group 1 molecular class C cephalosporinase, a group 2d molecular class D penicillinase, and a group 3 molecular class B metallo-β-lactamase (carbapenemase). Further, the presence of these β-lactamases in *Aeromonas*, in particular the carbapenemase, may not be detected by conventional susceptibility methods. CphA, one of several enzymes responsible for resistance to carbapenems, hydrolyzes nitrocefin poorly or not at all, indicating that the nitrocefin test is not reliable for detecting carbapenemases (89).

A 2004 case of *A. hydrophila* necrotizing fasciitis with probable *in vivo* transfer of a TEM-24 plasmid-borne extended-spectrum β-lactamase (ESBL) gene from *Enterobacter aerogenes* and a 2009 report on the development of imipenem resistance in an *Aeromonas veronii* bv. sobria clinical isolate recovered from a patient with cholangitis warrant concern among physicians as to the possible emergence of multidrug resistance within this genus (90, 91). Antimicrobial susceptibility testing of local isolates is necessary for the detection of species-related patterns, because susceptibilities may differ from one geographic area to another. This was very apparent in a study on the *in vitro* activities of tigecycline, a novel glycylcycline antimicrobial agent, against clinical isolates of *Aeromonas*, *Salmonella*, and *Vibrio* in Taiwan. It was found that 200 of 201 *Aeromonas* isolates were susceptible to tigecycline, with the MIC for 1 *A. caviae* isolate being 4 μg/ml, and past references to species-related patterns that varied with geographic areas were confirmed (92).

Aeromonas caviae strains carrying New Delhi metallo-beta-lactamase 1 (NDM-1) on the chromosome have been isolated from the New Delhi aquatic environment (93), and an examination of 51 consecutive *Aeromonas* blood isolates in Taiwan for the distribution of *cphA* MBL genes found the distribution of these genes to be species specific among *A. hydrophila*, *A. veronii*, and *A. dhakensis*, sp. nov., comb. nov. (formerly *A. aquariorum*) (94). The complexity of beta-lactamases among clinical *Aeromonas* isolates can result in resistance to a broad spectrum of beta-lactams, and therefore both conventional *in vitro* susceptibility testing and additional testing for ESBL production and carbapenemases using the modified Hodge test are strongly suggested, with the caveat that not all resistance mechanisms are detected with these additional phenotypic tests (95).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Regardless of the site of isolation (intestinal or extraintestinal), aeromonads should be identified as belonging either to the *A. hydrophila* or *A. caviae* complex or to the *A. veronii* complex and not "*A. sobria*," which is now *A. veronii* bv. sobria, a member of the *A. veronii* complex. For routine isolates recovered from uncomplicated cases of gastroenteritis, this level of identification may be sufficient. Although there is very strong evidence that some aeromonads are gastrointestinal pathogens, there is no convincing evidence, at present, that all fecal isolates of *Aeromonas* are involved in diarrheal disease. Thus, the significance of the recovery of aeromonads from stool specimens should be interpreted cautiously and must rely on both laboratory information and clinical interpretation. Because of this, the relative quantity of *Aeromonas* organisms recovered on enteric media (few colonies, moderate growth, or predominant organism) should be reported in conjunction with the *Aeromonas* complex or species identification. For complicated cases of diarrhea, e.g., prolonged bloody diarrhea in pediatric patients or chronic gastroenteritis of >1 month's duration or in cancer patients with positive fecal cultures (in whom *Aeromonas* tends to disseminate), a definitive species identification is warranted.

For extraintestinal isolates (from blood or wounds), the same general rules should apply to species identification of aeromonads. Although it is clear that both the *in vitro* and *in vivo* pathogenic potentials of *Aeromonas* species and strains vary considerably, for the present time, there are no universal markers or indicators available that dictate when isolates should be definitively identified to the species level. Thus, for extraintestinal isolates, identification of aeromonads beyond complexes should be reserved (i) for strains isolated from sterile body sites (blood and cerebrospinal fluid) and serious wound infections (cellulitis and necrotizing fasciitis), (ii) for strains exhibiting unusual resistance patterns associated with nosocomial outbreaks, and (iii) for publications describing traditional species associated with new disease processes or newly described species isolated from new anatomic sites.

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Vibrio and Related Organisms*

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TAXONOMY

The family *Vibrionaceae* is presently composed of six genera (*Vibrio*, *Photobacterium*, *Salinivibrio*, *Enterovibrio*, *Grimontia*, and *Aliivibrio*) and over 110 species with standing in bacterial nomenclature (http://www.bacterio.cict.fr/). *Vibrio* is the type genus for the family, and *Vibrio cholerae*, which includes strains that cause pandemic cholera, is the type species (1). Pathogenic species for humans can be found in three genera: *Vibrio* (10 species), *Photobacterium*, and *Grimontia* (one species each). Phylogenetic investigations indicate that multiple clades (separate or distinct groups in a phylogenetic sense) exist within *Vibrio*, which has led to the reclassification of some species into different genera. *Photobacterium damselae* is currently the accepted taxon for *Vibrio damsela*, and most scientific and medical publications now report infections associated with this taxon as *P. damselae*.

DESCRIPTION OF THE *VIBRIONACEAE*

The *Vibrionaceae* involved in clinical specimens are Gram-negative, facultatively anaerobic, straight, curved, or comma-shaped rods, 0.5 to 0.8 µm in width and 1.4 to 2.6 µm in length, that are catalase and oxidase positive (except *Vibrio metschnikovii*) (1). Most species are motile by means of sheathed monotrichous or multitrichous polar flagella when grown in liquid media. Strains of some species, such as *Vibrio parahaemolyticus* and *Vibrio alginolyticus*, swarm on solid media by production of numerous lateral flagella (1, 2). All *Vibrionaceae* require Na^+ for growth, with the minimal concentration for optimum growth ranging from 0.029 to 4.1% NaCl (1). They also ferment D-glucose but rarely produce gas, reduce nitrate to nitrite (except *Vibrio metschnikovii*), or grow on thiosulfate-citrate-bile salts-sucrose (TCBS) medium. The G+C content of the DNA is 38 to 51 mol% (1). Key properties or characteristics useful in separating clinically significant *Vibrionaceae* from phylogenetically related or phenotypically similar species are listed in Table 1.

EPIDEMIOLOGY, TRANSMISSION, AND CLINICAL SIGNIFICANCE

The genera covered in this chapter are ubiquitous in aquatic environments, primarily marine waters. Vibrios, such as *V. cholerae* and *V. mimicus*, that require minimal amounts of Na^+ for growth can be found in freshwater rivers and lakes as well as estuarine and marine environments. *Vibrionaceae* are commonly isolated from a variety of bivalves and crustaceans, and like other genera found in marine environments, their concentrations peak during the warmer months of the year. In aquatic environments, vibrios can persist in a free-living state or in association with phytoplankton and zooplankton (3–5). In the environment, vibrios may enter a state referred to as viable but nonculturable, in which cells retain basic metabolic processes even though they fail to grow on standard laboratory media (6). Because the viability of these cells is still in question, the term "active but nonculturable" has been proposed (4). Recent studies have shown that in mixed populations of nonculturable and culturable cells of *V. cholerae*, the latter appear to be the main contributors to human infections (4).

The *Vibrionaceae* can be isolated from a wide variety of intestinal and extraintestinal human illnesses. These illnesses include diarrhea, soft tissue disease (cellulitis and necrotizing fasciitis), septicemia, and eye and ear infections (1). In some cases of gastroenteritis and extraintestinal infections, it may be difficult to determine if a positive vibrio culture represents true infection or merely colonization because of its widespread occurrence in marine and estuarine waters. Ten *Vibrio* species (with perhaps two additional ones described in subsequent paragraphs) and one species each of *Photobacterium* and *Grimontia* occur in clinical specimens and are listed in Table 2 (1). Extraintestinal *Vibrio* infections are frequently associated with traumatic injuries or inapparent exposure to estuarine or marine waters. Primary septicemia may occur after ingestion of raw seafood (oysters) or as a secondary bacteremia subsequent to a wound infection.

V. cholerae

V. cholerae is divided into more than 200 serogroups, of which only *V. cholerae* serogroups O1 and O139 cause epidemic and pandemic cholera (7, 8). Cholera is associated with poor sanitation, and direct contact with contaminated surface water for drinking, bathing, cooking, and irrigation is a major risk factor (13).

V. cholerae O1

In 2011, the WHO reported over 580,000 cases of cholera worldwide, with more than 7,800 deaths (http://www.who.int/gho/epidemic_diseases/cholera/cases/en). From 1996 through 2009, most cholera cases occurred in Africa (9),

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TABLE 1 Properties of the genus *Vibrio* and its relatives and differentiation from other organisms that are phenotypically similar[a]

Test or property	Result for species of:					
	Vibrio	*Grimontia*	*Photobacterium*	*Aeromonas*	*Plesiomonas*	*Enterobacteriaceae*
Causes or is associated with intestinal or extraintestinal infections in humans	+	+	+	+	+	+
Oxidase reaction	+	+	+	+	+	−
Na^+ is required for or stimulates growth	+	+	+	−	−	−
Growth on TCBS agar	+	V[b]	+	−	−	−
Sensitivity to vibriostatic compound O/129[c]	+[d]	+	+	−	+	−
Lipase production	+	−	V	+	−	V
D-Mannitol fermentation	+	−	−	+	−	V

[a] These are general properties of the genera and/or family, but there are exceptions. The properties of *Vibrio* apply to the species that occur in human clinical specimens and may not apply to all nonclinical species. +, most strains positive; −, most strains negative; V, variable reaction among the species/strains.

[b] Strains of *Grimontia hollisae* typically grow poorly or not at all on TCBS agar; those that grow may have a reduced plating efficiency.

[c] O/129, 2,4-diamino-6,7-diisopropylpteridine phosphate (commercially available, 150-μg disks).

[d] Resistance to O/129 has become common in *Vibrio cholerae* strains isolated from India and Bangladesh; *V. cholerae* O139 strains are resistant.

but since the start of the large outbreak in Haiti in October of 2010, more than 60% of cholera cases have been reported from the Americas. A total of 604,634 cases of infection and 7,436 deaths occurred over the first 2 years of the epidemic (10). The majority of *V. cholerae* O1 infections are asymptomatic, and these undetected infections may serve as an ongoing reservoir in regions where cholera is endemic (11). Severe infections of cholera typically result in copious watery diarrhea, with fluid loss reaching 200 ml/kg of body weight/day. If untreated, the patient becomes prostrate with symptoms of severe dehydration, electrolyte imbalance, painful muscle cramps, watery eyes, loss of skin elasticity, and anuria. Dehydration subsequently leads to hypovolemic shock, acidosis, circulatory collapse, and death, even in previously healthy adults (12). In the United States, occasional cases of cholera are seen in travelers returning from regions of the world where cholera is highly endemic. Traditional therapy consists of fluid replacement by oral rehydration and/or intravenous fluids.

The unique ability of *V. cholerae* serogroup O1 to cause this fulminant form of diarrhea is due to the presence of virulence cassette regions and pathogenicity islands on the bacterial chromosome. These regions contain a number of key determinants, including the *ctxAB* genes, encoding cholera enterotoxin, which is responsible for the large excretion of fluids and electrolytes into the lumen, and the TCP gene cluster, which includes *tcpA*, the gene encoding the major protein subunit of the toxin-coregulated pilus (responsible for attachment to the gastrointestinal epithelium) (13). *Vibrio cholerae* O1 strains are divided into two serotypes, Inaba and Ogawa (a third serotype, Hikojima, is very rarely isolated), and two biotypes, El Tor and classical. The classical biotype caused the sixth pandemic, while the El Tor biotype, which was first isolated in 1905, is the cause of the ongoing seventh pandemic that began in 1961 (8). Recently, new variants of *V. cholerae* O1 have emerged in Africa and Asia (14, 15). These atypical or hybrid El Tor strains may be more virulent than most El Tor strains based upon projected case fatality rates (16). Hybrid strains differ from prototypical El Tor isolates in that they share some characteristics with the classical biotype (e.g., an El Tor strain may have the classical *ctxB* gene on its chromosome) (17). These hybrid strains appear to have evolved via lateral gene transfer and recombination events (14).

V. cholerae O139

In 1992, a new serogroup of *V. cholerae*, O139 (synonym, *V. cholerae* O139 Bengal), emerged throughout Asia (18). This serogroup probably resulted from the lateral transfer of a novel somatic antigen and capsule from an unknown bacterium to an El Tor strain (18). O139 and O1 strains carry similar virulence factors, including the *ctx* and *tcpA* genes (19). Clinical diseases due to O1 and O139 *V. cholerae* strains are also strikingly similar, except that adults are more frequently affected with O139, since previous infection with O1 cholerae is not protective (20). In 2002, O139 reemerged in Bangladesh, causing an estimated 30,000 cases of cholera, primarily in older patients (20). To date, no cases of *V. cholerae* O139 infection have been identified in Africa, and in 2011, China was the only country which reported O139 infections (21).

Unlike O1 strains, *V. cholerae* non-O1 strains (non-O1, non-O139) rarely produce cholera toxin. While the diarrhea produced is watery and severe disease is reported, infections are usually milder than typical cholera. Non-O1 strains of *V. cholerae* that carry the cholera toxin genes, such as strains of serogroups O75 and O141, have caused sporadic cases of cholera-like disease in the United States and elsewhere (22, 23). Risk factors for infection appear to be similar to those for other vibrios and include consumption of oysters and other seafood. In 2011, there was a small outbreak of serogroup O75 infections linked to consumption of raw or lightly cooked oysters from Apalachicola Bay, FL. The diarrheal symptoms were mild, and no cases required rehydration or hospitalization (24).

Non-O1 vibrios, in contrast to O1 *V. cholerae*, are associated with extraintestinal infections, such as septicemia and epidural brain abscess (25, 26). Persons at increased risk of developing non-O1 bacteremia include those with liver disease/cirrhosis or hematologic malignancies (26). The case fatality rate in these patients ranges from 10% to 65% (26–28). Strains have also been isolated from ears, wounds, the respiratory tract, and urine (29).

V. mimicus

V. mimicus, another nonhalophilic species, is phenotypically similar to *V. cholerae* except that it does not ferment sucrose. Human infections are uncommon, but it is recovered from patients with diarrhea, in which it may or may not have a causal role and is usually associated with consumption of

TABLE 2 Phenotypic test results and other properties of the 12 *Vibrionaceae* species that occur in human clinical specimens[a]

Test	% of strains positive[b]												
	P. shigelloides	*V. cholerae*	*V. mimicus*	*V. metschnikovii*[d]	*V. cincinnatiensis*	*G. hollisae*	*P. damselae*	*V. fluvialis*	*V. furnissii*	*V. alginolyticus*	*V. parahaemolyticus*	*V. vulnificus*[e]	*V. harveyi*
Indole (HIB)[c]	100	99	98	20	8	97	0	13	11	85	98	97	100
VP[c]	0	75	9	96	0	0	95	0	0	95	0	0	50
Moeller's													
Arginine[c]	98	0	0	60	0	0	95	93	100	0	0	0	0
Lysine[c]	99	99	100	35	57	0	50	0	0	99	100	99	100
Ornithine[c]	95	99	99	0	0	0	0	0	0	50	95	55	0
Motility	95	99	98	74	86	0	25	70	89	99	99	99	0
Gelatin hydrolysis[c] (22°C)	0	90	65	65	0	0	6	85	86	90	95	75	0
D-Glucose, gas production	0	0	0	0	0	0	10	0	100	0	0	0	0
Acid production from:													
L-Arabinose	0	0	1	0	100	97	0	93	100	1	80	0	0
Cellobiose	0	8	0	9	100	0	0	30	11	3	5	99	50
Lactose	80	7	21	50	0	0	0	3	0	0	1	85	0
myo-Inositol	95	0	0	40	100	0	0	0	0	0	0	0	0
Salicin	0	1	0	9	100	0	0	0	0	4	1	95	0
Sucrose	0	100	0	100	100	0	5	100	100	99	1	15	50
ONPG	90	94	90	50	86	0	0	40	35	0	5	75	0
Salt tolerance (growth in nutrient broth with):													
0% NaCl	100	100	100	0	0	0	0	0	0	0	0	0	0
6% NaCl	NA	53	49	78	100	83	95	96	100	100	99	65	100
O/129 susceptibility[f]	100	99	95	90	25	40	90	31	0	19	20	98	100

[a] Abbreviations: HIB, heart infusion broth; VP, Voges-Proskauer; ONPG, *o*-nitrophenyl-β-D-galactopyranoside; NaCl, sodium chloride; NA, not available.

[b] Percentage of strains positive after 48 h of incubation at 36°C unless otherwise indicated. Most positive reactions occur within 24 h.

[c] One percent NaCl is added to specific tests.

[d] This organism is oxidase negative and does not reduce nitrate to nitrite.

[e] Biogroup 1 strains.

[f] Zone of inhibition present (disk content, 150 μg). Data for *Plesiomonas* are from the Microbial Diseases Laboratory.

uncooked seafood, particularly raw oysters. Very few strains carry the *ctxAB* genes and can produce cholera-like symptoms. Symptoms generally include abundant watery diarrhea, vomiting, and severe dehydration. Most descriptions of *V. mimicus* gastroenteritis involve individual case reports, but a large-scale foodborne outbreak of gastroenteritis was reported in Thailand in 2004 (30). In that outbreak, over 300 persons were ill, and rectal swabs collected from 24 patients all yielded *V. mimicus*. Presumptive causes of this outbreak included freshwater fish, seafood, and seafood soup. In 2010, a small outbreak in Washington state was associated with consumption of crayfish and was attributed to a *V. mimicus* strain encoding cholera toxin (31).

V. parahaemolyticus

V. parahaemolyticus is the leading cause of bacterial foodborne intestinal infections in Asia and is almost invariably associated with the consumption of raw fish or shellfish (32). In Japan, 50 to 70% of the cases of foodborne diarrhea are due to *V. parahaemolyticus*. In the United States, it is the *Vibrio* species most frequently isolated from clinical specimens and is associated primarily with watery diarrhea but also may be isolated from blood, wounds, or other extraintestinal sites (28). Symptoms of *V. parahaemolyticus*-associated gastroenteritis often include nausea, vomiting, abdominal cramps, low-grade fever, and chills. Fatalities are extremely rare but can occur in cases of severe dehydration. Rehydration is usually the only treatment needed, but antimicrobial therapy may be beneficial in some instances. A recent outbreak of gastroenteritis involving 22 passengers aboard a cruise ship was linked to Alaskan oysters (33). A now widely dispersed clone of *V. parahaemolyticus*, serotype O3:K6, emerged worldwide in 1997 (34). Strains of this serotype caused an unusually high proportion of *V. parahaemolyticus* foodborne disease outbreaks in Taiwan from 1996 to 1999, which suggests that there is something unusual regarding its ecology, epidemiology, or virulence. This clone, with several of its serovariants (O4:K68, O1:K25, and O4:K12), has continued to spread throughout Asia and to the United States, Canada, Mexico, Russia, France, Italy, Brazil, Chile, Peru, and Mozambique (34). The serologic variants that have arisen since O3:K6 do not appear to have the same capacity to spread or a propensity for causing severe infections requiring hospitalization (34).

V. vulnificus

V. vulnificus causes primary septicemia and wound infection and is responsible for more than 90% of deaths due to vibrios in the United States yearly. Primary septicemia has a fatality rate exceeding 50%, even with hospitalization, and occurs predominantly in men over 50 years old (35, 36). Patients usually have predisposing conditions, such as liver disease, immunosuppression, increased serum iron, or other chronic diseases (35, 36). CDC data indicate that >95% of patients consumed raw oysters within 7 days of their infection. Patients typically present with a sudden onset of fever and chills, vomiting, diarrhea, and abdominal pain. Secondary skin lesions often appear, progressing to bulla formation and necrosis. Endotoxic shock often occurs and can rapidly lead to death. Both blood cultures and biopsy samples (scrapings) from skin lesions are usually positive. *V. vulnificus* also causes severe wound infections, usually after trauma and exposure to marine animals or the marine environment (35, 36). Wound infections may progress to cellulitis with extensive necrosis (often requiring surgical debridement), myositis, and necrotizing fasciitis that may mimic gas gangrene, as well as to secondary septicemia. The fatality rate for wound infections ranges from 20 to 30%. Three biogroups have now been defined for *V. vulnificus* (35, 36). Most infections in the United States are due to biogroup 1; biogroup 2 has been isolated principally from diseased eels and also isolated from one human wound infection. *V. vulnificus* biogroup 3 was described in 1999 and has been limited to wound and blood-borne disease in Israeli patients exposed to live tilapia grown in aquaculture.

V. alginolyticus

V. alginolyticus is very common in the marine ecosystem and is the fourth most commonly isolated *Vibrio* species in the United States. It is most frequently isolated from ear specimens and wound infections following seawater exposure. A woman was diagnosed with a *V. alginolyticus* infection after sea bathing in the British Isles and applying an unsterile seaweed dressing to a preexisting wound (37). *V. alginolyticus* has also been isolated from ocular infections and from infrequent cases of monomicrobial or polymicrobial bacteremia, mostly in immunocompromised persons (38, 39). It is occasionally isolated from diarrheal stool, but there is no evidence that it actually causes diarrhea (40).

Photobacterium damselae (formerly *V. damsela*)

P. damselae is an aggressive pathogen that causes serious life-threatening illnesses. Fatality rates for *P. damselae* are unknown, but many reports in the literature describe fatal infections, suggesting a fairly high attributable mortality rate (41). Disease syndromes associated with this bacterium include soft tissue infections (cellulitis and necrotizing fasciitis) and bacteremia. Most wound infections develop as indolent processes that progress to more-severe disease within a matter of hours, and vibrios are often not suspected during the initial diagnosis. In addition to antibiotics, medical intervention, including irrigation, fasciotomy, debridement, and sometimes amputation, is often required.

Typically, *P. damselae* wound infections occur in fishermen and result from penetrating traumas caused by fish fins, fish hooks, or harpoons (41–43). More recently, however, other sources of infection have been reported for this organism. These include a case of cellulitis in a healthy teenage surfer who sustained a laceration to his hand from his surfboard, a 30-month-old child with sickle cell anemia who developed bacteremia after handling fish and then scratching an open wound on her buttock, and a urinary tract infection in a pregnant female with increased frequency of urination and dysuria who had sexual intercourse in the Caribbean Sea 1 week prior to infection (36, 44, 45).

Vibrio fluvialis and *Vibrio furnissii*

V. fluvialis appears to cause sporadic cases of diarrhea worldwide, with severe cases of gastroenteritis sometimes linked to bacteremia or associated with cholera-like symptoms (46, 47). Based upon published reports, there appears to be a small but increasing incidence of extraintestinal *V. fluvialis* infections. These include acute infectious and continuous ambulatory peritoneal dialysis-associated peritonitis, soft tissue infections (cellulitis) associated with cerebritis, and bacteremia (47–50). For many of these systemic infections, some of which have poor outcomes, vibrios are not initially suspected as a cause. They are eventually associated with seafood consumption or exposure to seawater. *V. furnissii* is rarely isolated from human clinical specimens, but when it is recovered, it is usually from fecal specimens of patients with diarrhea (51). A case of bacteremia caused by *V. furnissii* following ingestion of seafood was recently described (52).

Miscellaneous Vibrios and *Vibrio*-Like Organisms

Vibrio harveyi is an important pathogen of marine fish and invertebrate species. *Vibrio carchariae* is another name formerly used for this organism before it was determined that it was a later synonym. *V. harveyi* is now the accepted name based on priority. To date, there are only two published case reports of human infection attributed to *V. harveyi*. The first report was of a wound infection resulting from a shark bite (53). The second report involved a 9-year-old boy with anaplastic large-cell lymphoma and central-line sepsis who, after completing chemotherapy and autologous stem cell transplantation, developed a febrile episode after swimming in the Mediterranean Sea (54). There is an anecdotal report of two isolates from blood and gallbladder (histories unavailable) that were retrospectively identified by *rpoB* sequencing (55). *Grimontia hollisae* is a halophilic, vibrio-like species associated primarily with moderate to severe cases of diarrhea, sometimes involving hypovolemic shock (56). Most recorded cases of infection involve a history of consumption of seafood, such as oysters. *V. metschnikovii* is frequently isolated from freshwater and brackish and marine waters. It was first reported to cause peritonitis and bacteremia in a patient with an inflamed gallbladder. Since then, it has been isolated from additional patients with bacteremia and, rarely, from wound infections; it has also been reported from cases of cholecystitis, diarrhea, and pneumonia (57, 58). *Vibrio cincinnatiensis* was first reported from a patient with bacteremia and meningitis. Subsequent isolates have been from the stool of a person with diarrhea, from aborted bovine fetuses, and from mussels (59). CDC data indicate that two additional *Vibrio* species are occasionally isolated from human clinical specimens. One species, provisionally named "*Vibrio metecus*" (60), is closely related to *V. cholerae*. Like its close relative, it is sucrose positive and nonhalophilic. *Vibrio navarrensis* is a halophilic species that was first isolated from sewage (61). The CDC used *rpoA* sequencing and phylogenetic analysis (62) to identify 15 human isolates collected since 1983 as *V. navarrensis*. Both species have been isolated from cases of gastroenteritis and extraintestinal infections, but their causative role requires additional investigation.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Pertinent clinical histories (when known) should accompany specimens to alert the laboratory to include appropriate isolation media for the *Vibrionaceae* in their stool workup; this is especially important in areas where the isolation of vibrios is infrequent (63). Helpful information includes history of travel, consumption of seafood, activity associated with marine or brackish water (or wounds associated with such exposure), and hobbies associated with aquaria.

Vibrionaceae, like other enteric organisms, are particularly susceptible to drying, so stool specimens that cannot be inoculated onto plating media within 2 to 4 h should be placed in a transport medium. Cary-Blair or any noninhibitory transport medium is acceptable for vibrios. For specimens collected in the field, if necessary, liquid stool may be placed on strips of blotting paper or gauze and then inserted in airtight plastic bags with a few drops of saline to maintain moisture and sent to the laboratory. Detailed information on the collection and transport of specimens for vibrio isolation is available elsewhere (64). Special methods for the collection and processing of extraintestinal specimens (blood, wounds, etc.) for vibrio isolation are not required, as vibrios are, as a rule, isolated in pure culture from these sites and the concentration of salt in primary plating media is usually sufficient for their recovery. Upon isolation, however, salt may need to be added to subsequent media to attain growth of salt-requiring vibrios. *Vibrionaceae* may die within weeks *in vitro*, even in moist environments at room temperature, and should be maintained at −70°C in tryptic soy broth with 15% glycerol as directed in chapter 11.

DIRECT EXAMINATION

Direct microscopic detection of vibrios in stool is not routinely recommended, since it may not be possible to distinguish pathogenic vibrios from other members of the enteric microbiota.

Direct Detection of *V. cholerae* O1 in Stool

Direct detection of *V. cholerae* from stool is typically conducted only in laboratories in areas where cholera is epidemic or endemic or in field situations where laboratory services are unavailable and rapid diagnosis is required. One of the oldest assays, the microscopic immobilization test, detects loss of motility of *V. cholerae* O1 organisms by the addition of O1 antibody and can be used to detect *V. cholerae* O139 by using O139 antibody. Two membrane antigen rapid tests, the Smart Cholera O1 (sensitivity 83%, specificity 88%) and Bengal Smart O139 (sensitivity 100%, specificity 97%) tests, are available from New Horizons Diagnostics Corp., Columbia, MD. Two rapid immunochromatographic dipstick assays which detect the presence of *V. cholerae* O1 or O139 antigens in stool are commercially available from Span Diagnostics (Surat, India) and Standard Diagnostics (Kyonggi-do, South Korea). The Span Crystal Vc for O1 and O139 demonstrated a sensitivity of 92% and a specificity of 73% (65). For the SD Bioline Cholera Ag O1/O139 test (Alere Inc., Waltham, MA), the manufacturer claims a sensitivity of 95.4% for the O1 antigen and 99.2% for O139, with respective specificities of 94.1% and 98%.

Molecular Detection in Clinical Specimens

Publications on molecular methodologies for detection of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* in stool can be found in the literature starting in the 1990s and are too numerous to cover here. Most of the advantages of PCR-based assays over culture methods apply to vibrios and include the ability to freeze stools for epidemiological studies for delayed testing. Also, in areas where vibrios are rarely isolated and laboratory experience or resources are limited, molecular methods may be more reliable and may provide a shorter turnaround time. Inhibitors in stool may affect the analytical sensitivity of an assay of PCR but can be overcome by dilution of the sample. Current PCR assays for *V. cholerae* use a multiplex PCR that includes primers for the *ctxA* gene, *rfb* genes (O antigens O1 and O139, allowing differentiation of serotypes), and *tcpA* genes (specific for the El Tor and classical biotypes, useful for epidemiological purposes) (66, 67). For clinical strains of *V. parahaemolyticus*, the thermostable direct hemolysin, a major virulence determinant, is a common target for detection, but it is not universally present in strains isolated from human infections (68, 69). In areas where *V. cholerae* is seldom isolated or in noncoastal areas where *V. parahaemolyticus* is an infrequent pathogen, the use of PCR is impractical and costly by comparison to alkaline peptone water and TCBS medium. The multiplexed Luminex xTAG gastrointestinal pathogen panel was evaluated for detection of *V. cholerae* from three stool specimens, with 100% sensitivity and specificity (70); however, the study did not include closely related species, such as *V. mimicus*, as negative controls.

ISOLATION PROCEDURES

Vibrionaceae associated with human disease can be isolated from routine enteric media, but recovery is enhanced when specific media are used. On MacConkey or salmonella-shigella (SS) agar, vibrios (with the exception of the rapidly lactose-fermenting strains of *V. vulnificus*) present as colorless colonies. Some strains of *V. cholerae* are inhibited on MacConkey and SS agar. On sucrose-containing media, such as Hektoen agar and xylose-lysine-deoxycholate, sucrose-positive vibrios associated with human disease, such as *V. cholerae*, *V. fluvialis*, *V. alginolyticus*, and some strains of *V. vulnificus*, cannot be differentiated from strains of other enteric bacteria that rapidly ferment sucrose. The addition of a blood agar plate allows colonies to be screened for oxidase, which may improve the recovery of vibrios as well as colonies of *Aeromonas* spp. and *Plesiomonas shigelloides*. *G. hollisae* may grow poorly or not at all on any enteric isolation medium, including TCBS medium; it is probably most reliably isolated from blood agar.

TCBS agar is formulated specifically for the isolation of vibrios (1). Both powdered formulations and prepared plates are readily available from a number of commercial sources. Inclusion of sucrose allows for preliminary differentiation of *Vibrio* species, with *V. cholerae*, *V. fluvialis*, and *V. alginolyticus* producing yellow colonies, while *V. parahaemolyticus*, *V. mimicus*, and most strains of *V. vulnificus* produce green colonies (sucrose not fermented). It should be noted that yellow colonies may convert to green if plates are examined after more than 24 h or are refrigerated after incubation. Oxidase testing is unreliable when performed directly on colonies growing on this medium. Growth from a non-sugar-containing medium, such as blood or nutrient agar, should be used for oxidase testing.

A chromogenic agar, CHROMagar Vibrio (CHROMagar Microbiology, Paris, France), has been developed primarily for the recovery of *V. parahaemolyticus* from seafood and supports the growth of other vibrios as well (71). *Vibrio* colonies on this medium range in color from milk white to pale blue to violet. Other members of the enteric biota usually do not grow, with the exception of *Proteus mirabilis* and *Providencia rettgeri*, which also produce milk white colonies. Marine agar (BD Biosciences, Sparks, MD), which does not contain any inhibitory or selective ingredients, may be more appropriate for isolation of vibrios from the environment, especially salt-requiring vibrios, because of its high salt content.

It is common for pure cultures of vibrios to produce multiple colony morphologies (as many as five) on any medium, but this phenomenon is most readily noticeable on nonselective media, such as blood or heart infusion agars. Variations in morphology include smooth, rough, convex, flat, spreading, and compact cells in various combinations. Occasionally, *V. cholerae* produces rugose (extremely wrinkled) colonies on non-carbohydrate-containing media (72). Like their smooth counterparts, they are fully virulent for humans (73). Rugosity, which is due to production of a unique extracellular polysaccharide, confers biofilm formation and resistance to chlorine, acid pH, and serum killing (72, 73). Although it is believed to enhance survival in aquatic environments, to date it has been demonstrated only *in vitro*. Isolates of the classical biotype of *V. cholerae* also possess the *vps* (vibrio polysaccharide synthesis) gene cluster, which encodes this phenotype, but rugose variants have been demonstrated only from El Tor strains (73).

In acute diarrheal disease, stool enrichment is generally not required; however, when enrichment is necessary, alkaline peptone water (1% NaCl, pH 8.5) is the most commonly used enrichment broth for human specimens. It should be incubated at 36°C and subcultured at 18 h on TCBS medium. Occasionally, vibrios are recovered only after a shorter incubation (6 h), and for these specimens, longer incubation times fail to yield a vibrio, probably due to overgrowth by other organisms (S. Abbott, personal observation).

IDENTIFICATION

Conventional Phenotypic Tests

Phenotypic properties that separate members of the *Vibrionaceae* from the *Enterobacteriaceae* (including *Plesiomonas shigelloides*) and the *Aeromonadaceae* are found in Table 1, and phenotypic profiles of the 12 species that occur in human clinical specimens are given in Table 2. Generally, 0 to 10% of species are positive for the following: H_2S in triple sugar iron, urea (except *V. parahaemolyticus*, 15%), phenylalanine deaminase (except *V. vulnificus* biogroup 1, 35%), malonate, mucate, yellow pigment production, and fermentation of D-adonitol, dulcitol, melibiose (except *V. vulnificus* biogroup 1, 40%), raffinose, L-rhamnose (except *V. furnissii*, 45%), D-sorbitol (except *V. metschnikovii*, 45%), α-methyl-β-D-glucoside, and D-xylose (except *V. cincinnatiensis*, 57 and 43%, respectively). Variable reactions are seen with methyl red, growth in potassium cyanide broth, D-galactose, glycerol, sodium acetate, DNase at 25°C, and lipase. All species are 99 to 100% positive for growth in 1% NaCl and fermentation of maltose (except *G. hollisae*, 0%) and D-mannose (except *V. cholerae*, 78%, and *V. harveyi*, 50%). Many commercial standard tube tests have sufficient salt to support growth without salt supplementation (0.5 to 1%), but some tests—such as the Voges-Proskauer test, Moeller's test for decarboxylases and dihydrolase, and the nitrate broth test—may contain no or insufficient NaCl to support the growth of some NaCl-requiring strains, and these tests' media should always have salt added to them (to a final concentration of 1%).

It should be noted that many *V. cholerae* O1 strains from Bangladesh and surrounding areas and all strains of *V. cholerae* O139 are resistant to both 10- and 150-μg disks of the vibriostatic compound O/129 (Remel, Lenexa, KS), a test historically used to distinguish vibrios (Table 1). In areas of the world where cholera is uncommon, complete phenotypic testing should be performed and all cultures identified as *V. cholerae* should be sent to public health laboratories for further characterization, including O1 and O139 agglutination, PCR tests for the presence of the *ctxA* gene, and other molecular typing or standard testing to determine the biotype variant. Prototypical as well as hybrid El Tor biotypes can be differentiated by a number of phenotypic tests, including hemolysis of sheep erythrocytes, production of acetylmethylcarbinol (Voges-Proskauer test), and resistance to polymyxin B, and positivity for the prototypical El Tor biotype (14). Except in their serogroup and O/129 reactions, *V. cholerae* O139 strains are phenotypically similar to *V. cholerae* O1 El Tor strains. Strains of *V. cholerae* that fail to agglutinate in either O1 or O139 antiserum are reported as *V. cholerae* non-O1. Serogrouping for non-O1 strains is available in only a limited number of reference laboratories, which typically use only some of many possible sera. *V. cholerae* and *V. mimicus* are separated from other species by growth in media lacking NaCl (Difco, BD Biosciences; nutrient broth is the only broth that accurately determines NaCl requirement). Strains of *V. parahaemolyticus*, *V. alginolyticus*, and *P. damselae* may be urea positive. Most vibrios isolated from humans produce a buff or tan pigment; however, strains of *V. parahaemolyticus* may produce a dark-brown pigment. *G. hollisae* generally grows poorly, especially in Moeller's decarboxylase and dihydrolase broths, even after salt supplementation, and produces extremely large zones of inhibition, often necessitating the use of two plates when disk antimicrobial susceptibility testing is performed. *V. metschnikovii* is distinctive because it is an oxidase- and nitrate-negative vibrio. *V. fluvialis* and *V. furnissii* are frequently confused with *Aeromonas caviae*, especially as some strains are weakly halophilic and only moderately susceptible to O/129, and because some strains of *A. caviae* grow on TCBS agar. *V. furnissii* is the only vibrio isolated from humans that is positive for gas production from D-glucose. Rapid, correct identification of *V. vulnificus* strains is critical because of the mortality associated with this organism. Some strains of *V. vulnificus* are sucrose positive, which may add to the confusion in identifying it.

Commercial Systems

There are no recent studies evaluating commercial systems' abilities to identify organisms covered in this chapter; however, based on individual case reports, identification of these organisms by commercial systems remains problematic (74–76). One evaluation that compared six commercial systems (77) indicated that when tested only against those species listed in their databases, API 20E (bioMérieux Inc., Durham, NC), Crystal E/NF (BD Biosciences), MicroScan Neg ID type 2 and type 3 (Siemens Healthcare Systems, West Sacramento, CA), and Vitek GNI+ and ID-GNB cards (bioMérieux) correctly identified only 63 to 81% of these organisms to the species level. Correct identifications of the three most commonly isolated species of *Vibrio* from clinical samples varied. For *V. cholerae*, API 20E gave the least (50%) and Crystal E/NF the most (97%) accurate identification. For *V. parahaemolyticus*, Rapid Neg ID3 fared the worst (40%) and API 20E and GNI+ the best (97% each), while for *V. vulnificus* biogroup 1 strains, GNI+ (50%) and Crystal E/NF (97%) gave the lowest and highest rates, respectively (64). Only Crystal E/NF was able to correctly identify ≥90% of *V. cholerae* or *V. vulnificus* strains, and only API 20E and the two Vitek cards correctly identified ≥90% of *V. parahaemolyticus* strains. In another study using only *V. vulnificus* biotype 3 strains, the MicroScan (98%) and Phoenix (90%) systems did the best in identifying 51 well-characterized isolates to the correct species, while the identification rate with Vitek (13.7%) was much less satisfactory (73). Croci et al. (74) found that for the identification of *V. parahaemolyticus*, API 20NE exhibited greater sensitivity than API 20E (20 versus 16 of 27 specimens) but that API 20E was more specific (100% versus 82%). In another study of a mixture of 111 clinical and environmental *V. vulnificus* strains, API 20E, API 20NE, and Biolog (Biolog, Inc., Hayward, CA) correctly identified 60, 0, and 85% of strains, respectively.

Manufacturers' instructions should be checked prior to testing of salt-requiring vibrios to determine if salt supplementation is required. A recent publication indicated that API 20E gave incorrect identifications for clinical isolates of *V. parahaemolyticus* if 2.0% NaCl was used for the diluent, versus 0.85% (74). For clinical isolates of *V. alginolyticus*, either concentration yielded identifications with high probabilities, but for *V. vulnificus*, the 0.85% diluent failed to identify 1 of 2 isolates, whereas 2.0% NaCl identified both strains with ≥94% probabilities.

Molecular Methods

Molecular identification of vibrios is commonplace in surveys and in research studies. However, it is not commonly employed in clinical laboratories for routine identification because vibrios are relatively rare pathogens in noncoastal areas or in regions where cholera is not endemic. The use of 16S rRNA gene sequencing alone is less than ideal for *Vibrionaceae* identification, as interspecies sequence differences can be very small and polymorphism has been shown to be fairly common in 16S rRNA genes (78). Tarr et al. (55) developed a multiplex PCR assay, using primers directed at *V. cholerae sodB*, *V. mimicus sodB*, *V. parahaemolyticus flaE*, and *V. vulnificus hsp* genes, that correctly identified 109 isolates and found an additional 4 strains of *V. parahaemolyticus* that either had not been identified to the species level (*n* = 3) or had been identified incorrectly as *V. alginolyticus* (*n* = 1). Additionally, *rpoB* gene sequencing was used to identify 12 of 15 isolates not previously identified to the species level by phenotypic methods. In other studies, the *toxR* gene (*V. parahaemolyticus*), *vvhA* gene (hemolysin, *V. vulnificus*), *vcg* genes (virulence-correlated gene, *V. cholerae*), *wbe* and *wbf* genes (for the O1 and O139 serotypes, respectively), and *tcpA* genes (specific for the El Tor and classical biotypes) have been used for identification (67, 74, 76, 79). Matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) promises to be a valuable new tool for identification of closely related bacterial species, with a rapid turnaround time and modest test cost. MALDI-TOF MS was shown to be a rapid and reproducible method of identification for *V. parahaemolyticus* (80). MALDI-TOF MS produced phylogenetic classification results comparable to those of *rpoB* sequencing for *Vibrio* spp. (81).

V. cholerae and *V. parahaemolyticus* Toxin Detection

Historically, reference laboratories detected cholera toxin by fluid accumulation in animal assays or detection of a cytopathic effect in Y1 adrenal or Chinese hamster ovary cell cultures. A reverse passive latex agglutination (VET-RPLA) assay (Denka Seiken) which detects both cholera toxin and the heat-labile toxin of *Escherichia coli* is commercially available. The majority of human strains of *V. parahaemolyticus* produce a thermostable direct hemolysin encoded by two genes, *tdh* and *tdh2x*. These toxins are rarely produced by environmental strains of *V. parahaemolyticus* but have been detected in *V. cholerae* non-O1, *V. mimicus*, and *G. hollisae* strains. Like cholera toxin, thermostable direct hemolysin can be detected by a commercial latex assay (KAP-RPLA; Denka Seiken), but there are no commercial products that detect the related thermostable hemolysin seen in *V. parahaemolyticus* strains. PCR assays for cholera toxin genes and both *V. parahaemolyticus* hemolysins have been developed but are not commercially available (82).

TYPING SYSTEMS

Serotyping

Serotyping is a widely utilized subtyping procedure. Typing schemes have been described for a number of species, including *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*; however, commercial typing sera are available only for

V. cholerae and *V. parahaemolyticus* (83, 84). *V. cholerae* O1 (polyclonal, Inaba and Ogawa) and O139 antisera are available from BD Biosciences, Denka Seiken, Remel, and New Horizons. *V. parahaemolyticus* antisera (11 O groups and 9 polyvalent and 65 monovalent K groups) are available from Denka Seiken.

Molecular Typing of Vibrios

Pulsed-field gel electrophoresis using NotI and SfiI enzymes is a well-standardized approach to molecular typing of vibrios for surveillance purposes (79, 85, 86). Ribotyping appears to be less discriminatory (76). Multilocus sequence typing (MLST) schemes are available for the three most commonly isolated species (*V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*) (http://www.pubmlst.org). Kotetishvili et al. (87) reported that MLST was more discriminatory than pulsed-field gel electrophoresis for *V. cholerae*. MLST and repetitive extragenic palindromic PCR (REP-PCR) were similar to each other in resolving the population structure of *V. parahaemolyticus* strains in the northwestern United States (88). Likewise, for *V. vulnificus*, REP-PCR is an effective subtyping method (35). Genotyping systems based on nucleotide sequence polymorphisms in several genes (16S rRNA gene, *vvhA*, and *vcg*) were originally thought to distinguish clinical from environmental isolates, but these methods do not clearly differentiate pathogenic from nonpathogenic strains (76). The subtyping methods currently in use are likely to be replaced by whole-genome sequencing and *in silico* analysis of traditional epidemiological markers in the near future.

SEROLOGIC TESTS

Reagents for the serodiagnosis of cholera are available only in specialized reference laboratories, but titration of acute- and convalescent-phase sera in agglutination, vibriocidal, or antitoxin tests is considered reliable (89).

ANTIMICROBIAL SUSCEPTIBILITIES

Generally, for most *V. cholerae* and *V. parahaemolyticus* gastrointestinal infections, treatment by rehydration is recommended over antimicrobial therapy and has the added benefit of reducing the risk of antibiotic resistance. Antimicrobial therapy, however, can reduce the duration of diarrhea, the duration of shedding of the organism, and the volume of rehydration fluids needed for recovery, and patients are often treated before culture results are known (23). Clinical and Laboratory Standards Institute (CLSI) document M45-A2 (96) includes susceptibility testing guidelines for non-cholera vibrios; the CLSI interpretive guidelines are limited to ampicillin, tetracyclines, folate pathway inhibitors, and chloramphenicol for *V. cholerae* (87, 88). *In vitro* susceptibility surveys show *V. cholerae* strains to be susceptible (>90%) to aminoglycosides, azithromycin, fluoroquinolones, extended-spectrum cephalosporins, carbapenems, and monobactams (90, 91). However, a conjugative transposon designated the SXT element and carrying resistance to sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin emerged first in *V. cholerae* O139 strains in India and is now seen in O1, non-O1, and O139 cholera strains and *V. fluvialis* (92). Ahmed et al. (92) have reported on a *V. fluvialis* isolate with the SXT element and a novel aminoglycoside acetyltransferase gene encoding resistance to gentamicin; resistance to ampicillin, furazolidone, and nalidixic acid was also reported. Multidrug-resistant strains of *V. fluvialis* have also been noted among other strains from India (45, 93). *V. parahaemolyticus* is generally susceptible to most antibiotics used for traveler's diarrhea (69). The fluoroquinolones alone or the synergistic combination of ciprofloxacin and cefotaxime shows excellent *in vitro* activity against *V. vulnificus* strains (94, 95).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Isolation of *V. cholerae* O1 or O139 should be reported immediately to the attending physician because of the severe dehydration that cholera can produce. The case should also be phoned to public health authorities and the isolate sent to a public health laboratory for confirmation and toxin testing.

When vibrios are isolated from blood or cerebrospinal fluid (bacteremia and meningitis are associated with high mortality rates) or wound infections which cause extensive tissue damage (*V. vulnificus* and *P. damselae*), the results should also be phoned immediately to the attending physician so that rapid and appropriate antibiotic therapy can be initiated. This is especially true for *V. vulnificus* infections, which have a high mortality rate without rapid, appropriate intervention. The clinical significance of *Vibrio* strains (*V. mimicus*, *V. alginolyticus*, *G. hollisae*, *V. harveyi*, and *V. metschnikovii*) in other specimens, particularly stool, may be more difficult to determine and requires prompt consultation with the attending physician to better understand the clinical context. Most physicians are not familiar with many *Vibrionaceae* species, and a phone consultation would be mutually beneficial to the clinician and laboratory provider. Information helpful to the physician includes the presence or absence of other pathogens and the relative amount of growth (pure or almost pure culture) of the vibrio. *Vibrionaceae* isolates should also be submitted to public health laboratories, as they are monitored under the CDC's Cholera and Other *Vibrio* Illness Surveillance (COVIS) system; they may also be needed for subtyping.

Vibrionaceae species that are known to cause diarrhea should be considered clinically significant, particularly if they are present in large numbers and no other potential pathogens are present. Isolation of vibrios from stool in small numbers may reflect only transitory colonization; however, species such as *V. cholerae*, *V. mimicus*, and *V. parahaemolyticus* have documented virulence factors that correlate with their ability to cause intestinal infections. Laboratory tests helpful in determining pathogenic potential are available primarily only in reference laboratories. *Vibrionaceae* isolation from sites such as the ears may represent infection, transient colonization, or merely their presence after exposure to seawater. Again, isolation of *Vibrionaceae* requires prompt consultation with the clinician to better understand the clinical context, which can help direct the need for further laboratory investigations.

Finally, readers should also be cautioned that misidentification of *Vibrio* species and their relatives can be a problem in the literature unless investigators used methods that are very sensitive in differentiating all of the species in the family *Vibrionaceae* (1).

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*Pseudomonas**

NIELS HØIBY, OANA CIOFU, AND THOMAS BJARNSHOLT

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TAXONOMY

Pseudomonas is a large and complex genus of Gram-negative bacteria of importance, as it includes species with both clinical and environmental implications. Many species are saprophytic or pathogenic for plants. The metabolic versatility of species within this genus allows many to degrade low-molecular-weight organic and aromatic compounds (1) and environmental pollutants (2–4). The genus *Pseudomonas* first proposed by Migula in 1894 (5) has undergone many taxonomic revisions since the seminal study of Stanier et al. (1966) (6) as methodologies of species identification continue to improve, and it comprised five unrelated groups, as determined by rRNA-DNA hybridization studies in the early 1970s by Palleroni (7). The genus *Pseudomonas* (*sensu stricto*) is rRNA homology group I (8) in the gamma subclass of the *Proteobacteria* (9). The other rRNA homology groups are II, *Burkholderia* and *Ralstonia* spp.; III, *Comamonas*, *Acidovorax*, *Delftia*, and *Hydrogenophaga* spp.; IV, *Brevundimonas* spp.; and V, *Stenotrophomonas* spp. (1). The genus *Pseudomonas* is reduced to include only those species belonging to rRNA group I, and of the currently listed species, 12 are of clinical relevance to humans. These 12 clinically relevant species can be divided into two distinct groups based on their ability to produce fluorescent pigments. The fluorescent group comprises *P. aeruginosa*, *P. fluorescens*, *P. putida*, *P. veronii*, *P. monteilii*, and *P. mosselii*; its members produce pyoverdin, a water-soluble, yellow-green or yellow-brown pigment that fluoresces blue-green when exposed to UV light. Many *P. aeruginosa* strains also produce the blue pigment pyocyanin. When pyoverdin combines with the blue, water-soluble phenazine pigment pyocyanin, the bright green color characteristic of *P. aeruginosa* is created. This characteristic separates *P. aeruginosa* from other members of the fluorescent group. *P. aeruginosa* can also produce other diffusible pigments, such as pyomelanin (brown-black) and pyorubin (red). The six nonfluorescent species are *P. stutzeri*, *P. mendocina*, *P. alcaligenes*, *P. pseudoalcaligenes*, *P. luteola*, and *P. oryzihabitans*.

Pseudomonas spp. are extremely versatile, being able to survive in a broad range of habitats (7) where most of their cells grow as biofilms attached to surfaces, which become slimy. Although the number of free-floating planktonic cells may be very low, the total number of *P. aeruginosa* cells (biofilms and planktonic) may be very high even with minimal nutrients. In health care settings, it can be found in sinks, showers, and respirator equipment and in the water of dentists' water-cooled drilling equipment.

Several of the clinically relevant *Pseudomonas* species demonstrate marked heterogeneity and have been subdivided into biovars or genomovars. Genomovars are genetically distinct groups that warrant species designation but lack phenotypically defining characteristics, and they are determined by DNA-DNA reassociation experiments, 16S rRNA gene sequencing in combination with chemotaxonomic total fatty acid analysis, and total protein pattern analysis (10). Much work in genome sequencing is occurring; several strains of *P. aeruginosa* and other *Pseudomonas* species have been sequenced (http://www.pseudomonas.com).

The highest level of genetic diversity of any species known is found in *P. stutzeri* (11), as established by multilocus enzyme electrophoresis. *P. stutzeri* has at least nine genomovars, with clinical isolates being found in genomovars 1 and 2. There are currently no consistent phenotypic differences to justify splitting *P. stutzeri* into unique species (10).

P. fluorescens was originally divided into biotypes A, B, C, D, E, F, and G (biotypes A to E are also referred to as biovars I, II, III, IV, and V). Biotype B was reclassified as *Pseudomonas marginalis*. Biotypes D and E (*Pseudomonas chlororaphis* and *Pseudomonas aureofaciens*) have now been combined into the single species *P. chlororaphis*, which is no longer considered a member of the fluorescens group.

Pseudomonas putida consists of biovars A and B. Biovar A should be regarded as the "typical" *P. putida* (12), while biovar B may have a closer affinity with *P. fluorescens*. More biovars of *P. putida* are warranted (12).

P. stutzeri, *P. fluorescens*, and *P. putida* are of interest in plant, marine, soil, and biotechnical sciences. They are of limited importance in clinical medicine. As polyphasic taxonomy continues to advance, more changes will doubtlessly arise; the clinical laboratory must keep abreast of such changes in order to differentiate these isolates from the more clinically important *Pseudomonas* species.

DESCRIPTION OF THE AGENT

Pseudomonas spp. are aerobic, non-spore-forming, Gram-negative rods that are straight or slightly curved and are 0.5 to 1.0 by 1.5 to 5.0 μm depending on the growth phase.

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*This chapter contains information presented by Deborah A. Henry and David P. Speert in chapter 40 of the 10th edition of this *Manual*.

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They are usually motile, with one or several polar flagella. They possess a strictly aerobic respiratory metabolism with oxygen as the terminal electron acceptor; in some species, e.g., *P. aeruginosa*, nitrate can be used as an alternative electron acceptor that allows anaerobic growth, and some species can use arginine fermentation for anaerobic growth (13). This is of pathogenic relevance, since anaerobic conditions are found in sputum in the bronchi and in mucus in the paranasal sinuses in cystic fibrosis (CF) patients with chronic infections in these two locations (14–16). Most species of clinical interest are oxidase positive (except *P. luteola* and *P. oryzihabitans*). *Pseudomonas* spp. are catalase positive and are chemolithotrophs.

EPIDEMIOLOGY AND TRANSMISSION

Endogenous versus Exogenous Infection

Endogenous infection can only occur in those whose colonization resistance has been perturbed: bacteremia secondary to gastrointestinal colonization in neutropenic hosts and pneumonia in individuals who have required endotracheal intubation (ventilator-associated pneumonia) (17). Exogenous infection likely occurs in patients with CF, as their initial infecting isolates usually resemble environmental morphotypes, although patient-to-patient spread has been demonstrated in CF centers in several countries (see below). Most other infections caused by *P. aeruginosa* are probably acquired exogenously, such as in burn wound infection, conjunctivitis and keratitis, urinary tract infection, otitis externa, folliculitis, and osteomyelitis, e.g., in the diabetic foot.

Exposure to Inanimate Reservoirs

Although efforts to prevent colonization with *P. aeruginosa* have been made, none has proven uniformly successful. Strict infection control procedures and the practice of compulsory hand hygiene are most effective at preventing patient-to-patient spread, particularly in hospitals.

Special Considerations for Patients with CF

P. aeruginosa is the predominant respiratory tract pathogen in patients with CF (18). Several studies have each demonstrated a common clone in particular groups of patients who have received their care at the same center (19, 20); the most likely explanation for this finding is patient-to-patient spread (21). Most patients tend to carry a unique strain during the course of infection (22), so one assumes that the infection was acquired from a contaminated environmental source or from other CF patients. Evidence has been presented that the paranasal sinuses are the initial focus for lung infection (23, 24). In chronic respiratory infections in CF, isolates of *P. aeruginosa* from patients displayed a high prevalence of DNA mismatch repair system-deficient hypermutable strains on isogenic backgrounds for each patient (25).

Species Other than *P. aeruginosa*

Pseudomonas species other than *P. aeruginosa* are usually acquired from the environment, and some species (*P. fluorescens* and *P. syringae*) are actually used as biocontrol products—as also some *Burkholderia* species—against soil-borne crop diseases (26, 27).

CLINICAL SIGNIFICANCE

Normal Host Defenses against *P. aeruginosa*

Polymorphonuclear neutrophilic leukocytes (28) are the most important defense against invasive, systemic *P. aeruginosa* infection. Individuals with intact host defenses are not at risk for serious infection with *P. aeruginosa*. *P. aeruginosa* rarely colonizes the pharynx, gut, or skin of healthy persons. An exception is the outer ear of swimmers and divers who spend many hours in the water. When *P. aeruginosa* is recovered from a normally sterile body site, such as blood, pleural fluid, or joint space, it usually constitutes a true infection. However, pseudoinfection (29) should sometimes be considered if the patients are neither severely ill nor at enhanced risk of such infection. A search for the source of the cluster should include culture of the antiseptic used for skin preparation for venipuncture or similar procedures.

P. aeruginosa is able to colonize wounds and mucosal surfaces, such as the oropharynx of patients receiving intensive care and broad-spectrum antibiotics or the endotracheal tubes or the tracheostomies of patients receiving mechanical ventilation, causing ventilator-associated pneumonia.

Infection in Patients with Neutropenia

Patients with neutropenia (neutrophil count of $<0.5 \times 10^9$ per liter) are at risk for invasive disease if the neutropenia is prolonged (≥10 days). Patients at greatest risk are adults undergoing induction chemotherapy for acute leukemia or marrow ablation for autologous or allogeneic bone marrow transplantation (30–33).

Infection in Patients with CF

P. aeruginosa is the predominant respiratory tract pathogen in patients with CF due to the reduced volume of the periciliary fluid in the respiratory tract, which leads to delayed clearance of aspirated microbes (34). The focus of the lung infection seems to be the paranasal sinuses (23, 24). Once infection is established, it usually persists as a biofilm (36), and the bacteria undergo a transition to the adapted "chronic CF phenotype" due to mutations in the global regulator genes (*mucA*, *algT* [= *algU*], *rpoN*, and *lasR*), consisting of the following: (i) a rough lipopolysaccharide (LPS) (37), in which the O polysaccharide is incompletely expressed, rendering the bacteria susceptible to the bactericidal effect of human serum; (ii) mucoid colonial morphology causing biofilm mode of growth *in vivo* (38), resulting from the exuberant production of a mucoid exopolysaccharide composed of O-acetylated guluronic and mannuronic acids; (iii) nonmotility (38) in which the bacteria lack normal functional flagellar function; and (iv) hypoexpression of various exotoxins due to mutations in the *lasR* gene of the quorum-sensing system, which regulates the production of these virulence factors—especially the rhamnolipid, which destroys polymorphonuclear leukocytes—and other exoproducts (25, 39, 40).

Transition of *P. aeruginosa* from nonmucoid to mucoid in the CF lung is usually associated with an accelerated decline in pulmonary function and an adverse prognosis (41), because of the capacity of the mucoid exopolysaccharide to interfere with normal host phagocytic defenses (42) and to facilitate the formation of biofilms. Another colonial form, small-colony variants (previously known as dwarf colonies), which are very resistant to antibiotics, is also found in CF sputum (43, 44). Different colony morphologies of *P. aeruginosa* from sputum of a patient with CF are presented in Fig. 1. Furthermore, CF patients receive frequent courses of anti-*Pseudomonas* antimicrobial therapy, leading to occurrence of multiresistant strains (18, 45).

Ventilator-Associated and Nosocomial Pneumonia

The normal respiratory tract is well protected against infection by means of mucociliary clearance of inhaled particles

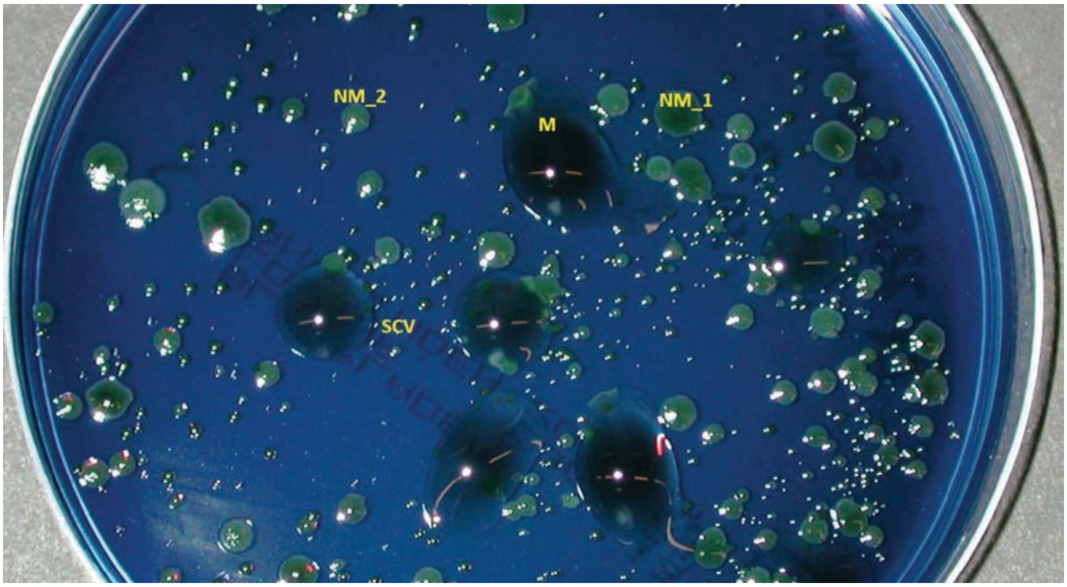

FIGURE 1 Different colony morphologies of *P. aeruginosa* from CF sputum cultivated on a modified Conradi-Drigalski agar selective for Gram-negative rods ("blue plate"; Statens Serum Institut). M, mucoid; NM_1, nonmucoid colony 1 (dark green); NM_2, nonmucoid colony 2 (light green); SCV, small colony variant. doi:10.1128/9781555817381.ch42.f1

and potentially infectious agents. Placement of an endotracheal tube or a tracheostomy for mechanical ventilation allows upper respiratory tract microbes to gain access to the lower respiratory tract, where infection can be established. Adults receiving mechanical ventilation are at high risk for developing *P. aeruginosa* ventilator-associated pneumonia (46), particularly after or during treatment with broad-spectrum antimicrobial agents. Ventilator-associated pneumonia—which is a biofilm infection—is seen in 10 to 30% of mechanically ventilated patients, and the pathogenesis is aspiration of bacteria from the biofilm on the tube (47, 48). Initial empiric therapy, until an etiologic agent is identified, should include a drug effective against *P. aeruginosa*.

Burn Wound Infections, Chronic Wound Infections, and Diabetic Foot Infections

Thermal burns of the skin abrogate an essential component of the body's defense against infection, the physical barrier of the intact skin (49). The resulting damaged tissue is a rich culture medium and is at great risk for colonization and infection by *P. aeruginosa*, which is transmitted to the burn wound by the initial hydrotherapy; such infections have been one of the leading causes of morbidity and mortality in victims of burns. Topical therapy is designed to prevent *P. aeruginosa* and other pathogens from causing infection. Infections of burn wounds with *P. aeruginosa* typically occur about 1 week after the injury. The extent of the burn has a profound influence on risk of infection and prognosis.

P. aeruginosa can cause chronic ulcers, and here they are located as biofilm aggregates below the surface of the ulcer (50). Biopsies, rather than swabs of the surface, are required to detect *P. aeruginosa* in the ulcers. *P. aeruginosa* is the most common Gram-negative rod found in moderate to severe diabetic foot infections, which sometimes spread to the bones of the foot and give rise to osteomyelitis (51, 52). Practical guidelines have been published for diagnosis and treatment of these infections (53).

Folliculitis, Otitis Externa, and Infectious Keratitis

Since *P. aeruginosa* can survive at temperatures as high as 42°C, it has the propensity to cause infection in people who are exposed to heated water for extended periods. Hot tub users are at risk of *P. aeruginosa* folliculitis (54), a condition that is self-limited for healthy hosts and resolves rapidly. People who spend extended periods swimming or diving are at risk of external ear infections ("swimmer's ear"), another self-limiting condition in immunocompetent people that responds readily to therapy with topical antimicrobial agents (55).

The cornea is relatively resistant to infection except when its integrity has been broken. Users of contact lenses are at risk of *P. aeruginosa* keratitis, especially if hygiene is poor or lenses are used for extended periods, possibly due to contamination during daily cleaning of the lenses (56).

Other Infections with *P. aeruginosa*

P. aeruginosa can cause meningitis (usually following trauma or surgery), malignant otitis externa in which the cartilage of the ear is invaded (57), sepsis and meningitis in newborns (58), endocarditis or osteomyelitis in users of intravenous drugs (59), chronic pneumonia in people with underlying lung disease such as bronchiectasis (60), and urinary tract infections in patients with complex urinary tract abnormalities (61, 62). Each of these presentations is unusual and is superimposed on some abrogation of normal host defenses.

Infections with *Pseudomonas* Species Other than *P. aeruginosa*

Healthy individuals are resistant to serious infections by all *Pseudomonas* species, including *P. aeruginosa*. However, immunocompromised hosts are occasionally infected with one of the many non-aeruginosa species, including (but not limited to) *P. fluorescens*, *P. putida*, *P. stutzeri*, *P. oryzihabitans*, *P. luteola*, *P. alcaligenes*, *P. mendocina*, and *P. veronii*. Several of these species have been recovered from the respiratory secretions of patients with CF, but their role in the pathogenesis of lung disease has not been determined. Some of these species have the capacity, like *P. aeruginosa*, to grow in hostile environments, such as antiseptic solutions; they can therefore be the cause of pseudobacteremia. Because of their low virulence, infections due to these species are often iatrogenic and are associated with the administration of contaminated solutions, medicines, and blood products or the presence of indwelling catheters (63–67).

P. fluorescens and *P. putida* have the ability to grow at 4°C, and *P. fluorescens* and various other *Pseudomonas* species have been implicated in outbreaks of pseudobacteremia (67–69).

P. stutzeri is an unusual cause of human infection. It can cause bacteremia in immunosuppressed persons (70), meningitis in HIV-infected individuals (71), pneumonia in alcoholics (72), and osteomyelitis (73). Iatrogenic infections due to *P. stutzeri* include endophthalmitis following cataract surgery (74) and bacteremia in hemodialysis patients as a result of contaminated dialysis fluid (75). *P. stutzeri* has also been recovered from wounds, the respiratory tract of intubated patients, and the urinary tract, although its pathogenic role in those settings is unclear (76).

P. oryzihabitans is being recognized increasingly as a cause of bacteremia in immunocompromised patients with central venous access devices. Synthetic bath sponges can be a source of bacteremia with this organism in patients with Hickman catheters (65). This organism has also been reported to cause peritonitis in patients undergoing chronic ambulatory peritoneal dialysis and in patients with cellulitis, abscesses, wound infections, and meningitis following neurosurgical procedures (63).

P. luteola is a rare cause of infections in humans. There have been case reports of a variety of different infections, including bacteremia, cellulitis, osteomyelitis, peritonitis, endocarditis, and postsurgical meningitis (66, 77).

Other *Pseudomonas* species are found even less frequently in human infection. *P. alcaligenes* has been associated with catheter-related endocarditis in a bone marrow transplant recipient (78). *P. mendocina* has been isolated from patients with endocarditis (79). *P. veronii* has been reported to be associated with an intestinal inflammatory pseudotumor (80). *P. monteilii* has been recovered from stool, bile, placenta, bronchial aspirates, pleural fluid, and urine, but its clinical significance is uncertain (81, 82), *P. mosselii* has also been isolated from various specimens, but the clinical significance is not known (83).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Pseudomonas spp. are able to survive in diverse environments and through a wide temperature range. Some species prefer incubation temperatures lower than 25°C, while *P. aeruginosa* can grow at temperatures up to 42°C. These organisms are easily recovered from clinical specimens using standard collection, transport, and storage techniques as outlined in chapter 18. Samples of *Pseudomonas* spp. can be refrigerated at 2 to 8°C for up to 4 weeks. Organisms can be kept in long-term storage at −80°C using standard laboratory freezing protocols.

DIRECT EXAMINATION

Microscopy

Gram stain morphology cannot easily distinguish *Pseudomonas* spp. from other nonfermenting bacilli, although they are usually thinner than *Enterobacteriaceae*. Among the pseudomonads, there is some variation in Gram stain morphology. Certain strains of *P. putida* can appear elongated. Organisms from older cultures may appear slightly pleomorphic. Flagellar stains reveal one or more polar flagella. *P. aeruginosa* has a single polar flagellum (monotrich), which gives a diagnostically significant different swimming motility compared with peritrichous bacteria, as can be observed microscopically on wet mounted slides of, e.g., positive blood cultures.

Mucoid phenotypes may be distinguished on direct examination by the presence of clusters or long filaments of short, Gram-negative bacilli surrounded by light pink-staining material (alginate) (Fig. 2A to E). The reason for the mucoid phenotype is mutations in the *mucA* gene. It is important to note this on direct examination, as the organisms may grow very slowly (84).

Simultaneously, nonmucoid phenotypes (revertants frequently due to additional mutations in the *algT* gene) and small-colony variants may be observed on the culture plate (Fig. 1). They always belong to the same clone and represent adaptation to different niches in the respiratory tract (38). The presence of these mucoid forms, which represent biofilms in the lungs, should be documented on clinical reports, and antibiotic susceptibility testing should be performed on the different phenotypes and reported to the clinician, although the biofilm mode of growth makes the mucoid phenotype resistant to antibiotic treatment in spite of *in vitro* susceptibility of the planktonic bacteria. The reason is that antibiotic therapy can still suppress the spread of the infection and maintain the pulmonary function (18). Many strains from chronically infected CF patients are mutator strains with mutations rate >20 times that of the reference strain PAO1, and they rapidly become multiresistant to antibiotics (25, 85, 86). Because *Pseudomonas* spp. may be colonizers, their isolation does not always link them to clinical disease. However, their presence intracellularly in polymorphonuclear cells is clinically significant and should be documented and direct further workup.

Nucleic Acid Detection and Mass Spectrometry Detection

P. aeruginosa and other *Pseudomonas* species are ordinarily detected by culture techniques; these methods are particularly important for determining antimicrobial susceptibility, as these organisms have a high degree of intrinsic and acquired resistance (87, 88). However, situations exist in which rapid, non-culture-dependent methods such as PCR amplification of the 16S rRNA gene followed by sequencing (turnaround time, 5 to 6 h) or fluorescence *in situ* hybridization (FISH) of fluorescent probes to 16S rRNA (turnaround time, 1 to 2 h) can be instituted, such as for rapid identification of Gram-negative rods in tissue biopsies evaluating the sputum of patients with CF or in blood cultures for precise localization of the planktonic or biofilm-growing bacteria (36, 89–92). One example of this is the peptide nucleic acid (PNA) FISH method (AdvanDx, Woburn, MA) (93) (Fig. 2F). For blood cultures and pure samples, matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) is the fastest method to date (94, 95). PCR amplification of the 16S rRNA gene followed by sequencing or MALDI-TOF MS can be particularly valuable in identification of non-aeruginosa *Pseudomonas* species.

ISOLATION PROCEDURES

Pseudomonas species have very simple nutritional requirements and grow well on standard broth and solid laboratory

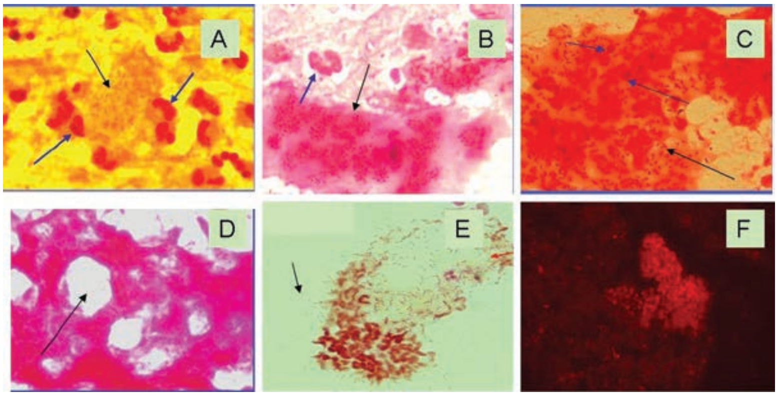

FIGURE 2 Diversity of *P. aeruginosa* biofilms in sputum (15). Sputum samples from 77 CF patients chronically infected with *P. aeruginosa* were examined by Gram stain and PNA FISH. The exclusive presence of *P. aeruginosa* was verified by standard culturing and *P. aeruginosa*-specific PNA FISH (F). A great diversity in the organization of *P. aeruginosa* was detected; both compact biofilm-forming microcolonies (A, B, C, and F: blue arrows pointing at leukocytes in A and B, and black arrows pointing at bacterial aggregates in A, B, and C) and nonadhered planktonic bacteria (E, arrow) were observed. The organization of the polymorphonuclear leukocytes was also diverse, with these cells surrounding the biofilms (A), distant from the biofilms (B), and in a very few cases appearing inside the biofilms (C). The biofilm microcolonies were mostly very compact; however, some samples were perforated with "holes" mimicking water-filled channels visible in some mature *in vitro* biofilms (D, arrow pointing at hole). Magnification, ×1,000.
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media such as tryptic soy agar with 5% sheep blood, chocolate agar, and MacConkey agar, which are recommended to isolate *Pseudomonas* spp. from clinical specimens. MacConkey agar is also a differential medium helpful in identifying different strains of *Pseudomonas* spp., including mucoid strains of *P. aeruginosa* from CF patients. Multiple selective media containing inhibitors such as acetamide, nitrofurantoin, phenanthroline, 9-chloro-9-[4(diethyamino)phenyl]-9,10-dihydro-10-phenylacridine hydrochloride (C-390), and cetrimide have been used in the past for the isolation and presumptive identification of *P. aeruginosa* from clinical and environmental samples. Cetrimide and a combination of phenanthroline with C-390 are the most commonly used selective agents. Inhibition of some strains of *P. aeruginosa* from sputum specimens from CF patients has been reported using a selective agar containing cetrimide (200 mg/liter) and nalidixic acid (15 mg/liter), and some strains are auxotrophic, emphasizing the need to use both selective and nonselective rich media for recovery of bacteria from these patients, such as MacConkey agar, horse or sheep blood agar, and chocolate agar. This range of media is required since CF patients may simultaneously harbor other CF pathogens such as *Staphylococcus aureus*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, or *Burkholderia* spp., in addition to *P. aeruginosa*. The plates should be incubated for 48 to 72 h since some isolates grow slowly. Some of the non-aeruginosa pseudomonads, like *P. fluorescens*, *P. putida*, and *P. oryzihabitans*, may grow better at the lower temperatures of 28 to 30°C. Good growth is usually achieved after 24 to 48 h of incubation. For cultures from CF patients, it is recommended that solid medium plates be held at 35 to 37°C for 5 days since some strains from chronic infections grow very slowly (84).

IDENTIFICATION

Identification of fluorescent and nonfluorescent pseudomonads has become less difficult using MALDI-TOF MS technology, which has replaced conventional phenotypic identification systems in many European clinical microbiology laboratories. However, the latter methods are still used in smaller laboratories and in many non-European countries, and these methods also give information about the physiological and biochemical capabilities of the bacteria, which may sometimes be important for the pathogenesis of infections.

Fluorescent Group

Members of the fluorescent pseudomonad group produce the pigment pyoverdin, which fluoresces under short-wavelength UV light. King's medium B facilitates the detection of pyoverdin (96). Most strains of *P. aeruginosa*—except those from CF patients with chronic lung infection—produce the blue pigment pyocyanin. King's medium A facilitates the detection of pyocyanin, and extraction of pyocyanin with chloroform results in a bluish color (96). This organism may also produce other water-soluble pigments such as pyorubrin (red) or pyomelanin (brown-black). Conditions of iron limitation enhance pigment production, as these pigments act as siderophores in iron uptake systems of the bacteria. Non-dye-containing media enhance visualization of pigments.

P. aeruginosa

Most *P. aeruginosa* organisms are easily recognizable on primary isolation media on the basis of characteristic colonial morphology, production of diffusible pigments, and a grapelike odor. Cultures may exhibit a corn tortilla-like odor. Colonies are usually flat and spreading and have a serrated edge and a metallic sheen that is often associated with autolysis of the colonies (97). Other morphologies exist, including smooth, mucoid, and dwarf (small-colony variants) (44, 86, 98, 99). Mucoid colonial variants are particularly prevalent in respiratory tract specimens from CF patients and are a hallmark of chronic infection (also in non-CF patients) in contrast to intermittent colonization (36, 100) (Fig. 1).

P. aeruginosa is distinct from the rest of the clinically relevant fluorescent pseudomonads in its ability to grow at 42°C. In addition to pigment production, other tests that confirm its identification are positive oxidase and arginine tests and an alkaline over no-change reaction in the triple sugar iron test. Microbiologists must be aware of certain variations in the phenotypes of *P. aeruginosa*. Isolates lacking oxidase activity have occasionally been reported, but they exhibit the other characteristic features. Prior antibiotic therapy with agents that affect protein synthesis may cause the aberrant phenotype. Mucoid isolates of *P. aeruginosa* from CF patients may undergo several phenotypic changes, including slow growth, loss of motility, and loss of pigment production. Small-colony variants may require prolonged incubation, lack motility, be hyperpiliated, adhere to agar surfaces, and show autoaggregative properties in liquid medium (99).

P. fluorescens and *P. putida*

P. fluorescens and *P. putida* do not possess distinctive colony morphology or odor. Their inability to reduce nitrates to nitrogen gas and their ability to produce acid from xylose distinguish these two species from the other fluorescent pseudomonads. *P. fluorescens* can be differentiated from *P. putida* by its ability to grow at 4°C and to hydrolyze gelatin; *P. putida* can do neither. *P. fluorescens* isolates may require 4 to 7 days of incubation for accurate detection of gelatin hydrolysis. According to the package insert for API 20NE (version 7.0; bioMérieux, Inc., Durham, NC), only 39% of *P. fluorescens* isolates hydrolyze gelatin in 24 to 48 h.

P. veronii, *P. monteilii*, and *P. mosselii*

P. veronii can reduce nitrates to nitrogen gas but is unable to hydrolyze acetamide. The type strain of *P. veronii* (LMG 17761) is negative for acid from lactose and maltose and does not grow at 36°C. *P. monteilii* can be distinguished from the other members of the fluorescent group by its inability to reduce nitrates to nitrites or nitrogen gas, hydrolyze gelatin, or produce acid from xylose. *P. mosselii* can reduce nitrates neither to nitrites nor to nitrogen gas, nor can it produce acid from xylose, but most isolates (92%) can hydrolyze gelatin (Table 1).

Other fluorescent pseudomonads are rarely encountered in clinical specimens. Many of these isolates are negative for arginine dihydrolase activity. Identification as "*Pseudomonas* species, not *aeruginosa*" and susceptibility testing of the isolates, when appropriate, are sufficient in most circumstances. When necessary, these isolates can be referred to reference laboratories.

Nonfluorescent Group

P. stutzeri and *P. mendocina*

Most *P. stutzeri* isolates are easily recognized on primary isolation media by their distinctive dry, wrinkled colony morphology, similar to the morphology of *Burkholderia pseudomallei*. *P. stutzeri* can be distinguished from the latter species by its lack of arginine dihydrolase activity and inability to produce acid from lactose. *P. stutzeri* colonies can pit or adhere to the agar and are buff to brown. The adherence can make removal of colonies from agar medium difficult. Because of the difficulty in making suspensions of specific turbidity, commercial susceptibility systems may not work well with this organism. Not all isolates of *P. stutzeri* produce wrinkled colonies; such strains can be distinguished from other pseudomonads by their ability to hydrolyze starch, a unique reaction for this species.

P. mendocina colonies are smooth, nonwrinkled, and flat, producing a brownish yellow pigment. Key biochemical characteristics of this species include the ability to reduce nitrates to nitrogen gas, positive arginine dihydrolase activity, and inability to hydrolyze acetamide or starch.

P. alcaligenes and *P. pseudoalcaligenes*

P. alcaligenes and *P. pseudoalcaligenes* have rarely been encountered in clinical samples (78) and do not have a distinctive colony morphology. Compared to other pseudomonads, they are biochemically inert. Characteristics that distinguish them from other biochemically inert Gram-negative rods are a positive oxidase reaction, motility due to a polar flagellum, and growth on MacConkey agar. *P. alcaligenes* is distinguished from *P. pseudoalcaligenes* by its inability to oxidize fructose. Although growth at 42°C was thought to be a distinguishing feature between them, further studies now indicate that growth at 41°C (and probably 42°C) is also present in most strains of *P. alcaligenes*. These organisms are difficult to identify by many commercial systems, and for most clinical situations, they can simply be referred to as "*Pseudomonas* spp., not *aeruginosa*." If the clinical situation dictates a definitive identification, assistance from reference laboratories should be sought.

P. luteola and *P. oryzihabitans*

P. luteola and *P. oryzihabitans* can be distinguished from other pseudomonads by their negative oxidase reaction and production of an intracellular, nondiffusible yellow pigment. Both organisms typically exhibit rough, wrinkled, adherent colonies or, more rarely, smooth colonies. *P. luteola* can be differentiated from *P. oryzihabitans* on the basis of its ability to hydrolyze *o*-nitrophenyl-β-D-galactopyranoside and esculin.

Use of Commercial Identification Systems

Commercial identification systems rather than conventional biochemical tests increasingly are used in many laboratories to identify *Pseudomonas* spp. Commercial products can be divided into manual and automated systems. The more frequently used manual systems are the API 20NE (bioMérieux), Crystal E/NF (Becton Dickinson, Franklin Lakes, NJ), and RapID NF Plus (Innovative Diagnostic Systems, Norcross, GA), whereas the OmniLog Phenotype MicroArray system (Biolog, Inc., Hayward, CA), which gives a very comprehensive description of the metabolic capabilities of bacteria, is mostly used in research laboratories. The manual systems usually provide accurate identification of *P. aeruginosa*, including mucoid isolates as well as other *Pseudomonas* species, and are preferred over automated systems for isolates from CF patients.

Automated systems are commonly used in many medium to large clinical laboratories. As *P. aeruginosa* is easily identified by a few conventional biochemical tests, it is often not necessary to use a more expensive commercial system. Several of the automated systems are not very accurate and may require additional testing for non-*P. aeruginosa* species; thus, their labor-, cost-, and time-saving benefits are lost. Automated systems can identify *P. aeruginosa* from non-CF sources with 90 to 100% accuracy (101, 102), but some systems may require additional tests to achieve these results (103, 104). Most reviews focus on the evaluation of *P. aeruginosa*, with only a few, if any, other *Pseudomonas* species represented in the organisms being tested. When other *Pseudomonas* species were included, the VITEK 2 GN panel (bioMérieux) performed well (102, 105), while other systems often relied on

TABLE 1 Characteristics of *Pseudomonas* species found in clinical specimens[a]

Test	*P. aeruginosa* (*n* = 201)	*P. fluorescens* (*n* = 155)	*P. putida* (*n* = 16)	*P. veronii* (*n* = 8)	*P. monteilii* (*n* = 10)	*P. mosselii* (*n* =12)	*P. stutzeri* (*n* = 28)	*P. mendocina* (*n* = 4)	*P. pseudoalcaligenes* (*n* = 34)	*P. alcaligenes* (*n* = 26)	*P. luteola* (*n* = 34)	*P. oryzihabitans* (*n* = 36)
Oxidase	99	97	100	100	100	100	100	100	100	96	0	0
Growth												
MacConkey agar	100	100	100	ND[c]	ND	ND	100	100	100	96	100	100
Cetrimide	94	89	81 (6)	ND	90	100	4	75 (25)	56 (18)	15	0	25 (28)
6% NaCl	65	43	100	ND	0	100[e]	80 (16)	100	62 (6)	41	74	62
42°C	100	0	0	0	0	0	69	100	94	V[f]	94	33
Nitrate reduction	98	19	0	100	0	0	100	100	100	54	62	6
Gas from nitrate	93	3	0	100	0	0	100	100	0	0	0	0
Pyoverdin	65	96	93	100	100	100	0	0	0	0	0	0
Pyocyanin	97[g]	0	0	0	0	0	0	0	0	0	0	0
Arginine dihydrolase	100	97	100	100	100	100	0[d]	100	78	12	100	14
Lysine decarboxylase	0	0	0	ND	0	0	0	0	0	0	0	7
Ornithine decarboxylase	0	0	0	ND	0	0	0	0	0	0	0	3
Hydrolysis												
Urea	48 (9)	21 (31)	31 (44)	25	50	ND	33 (22)	50	3 (6)	0	26 (38)	77
Gelatin (7-day incubation)	82	100	0	13	0	92	0	0	0	0	61	17
Acetamide	100	6 (12)	0	0	0	ND	0	0	ND	ND	ND	ND
Esculin	0	0	0	ND	0	0	0	0	0	0	100	0
Starch	0	0	0	ND	0	8	100	0	0	0	0	0
Acid from[b]:												
Glucose	97	100	100	100	100	100	96 (4)	100	9	0	100	100
Fructose	ND	ND	ND	100	100	100	ND	ND	79 (21)	0	ND	ND
Xylose	90	100	100	100	0	0	93 (7)	75 (25)	18 (12)	0	100	100
Lactose	<1	24	25 (13)	ND	0	0	0	0	0	0	3 (24)	14 (22)
Sucrose	0	48	0	100	0	17	0	0	0	0	12	25
Maltose	<1	2	31	ND	0	17	100	0	0	0	100	97
Mannitol	70	53	25	ND	0	75	89 (4)	0	0	0	76 (18)	100
Simmons citrate	95	93	94 (6)	ND	100	100	82 (14)	100	26 (9)	57 (8)	100	97
No. of flagella	1	>1	>1	1	ND	1	1	1	1	1	>1	1

[a] Results are given as percentage of positive strains; percentages in parentheses represent strains with delayed reactions. Data are from references 12, 82, and 83.
[b] Oxidative-fermentative basal medium with 1% carbohydrate.
[c] ND, no data.
[d] *P. stutzeri*-like organisms (formerly CDC group 3b) are arginine dihydrolase positive.
[e] Growth at 3 to 5% NaCl but not at 7% NaCl.
[f] V, variable; many strains can grow at 41°C: See comment in text under identification.
[g] King's medium A(96).

additional testing to obtain an identification (18, 103, 104, 106). Hence, it is wise to consider carefully the clinical significance, colonial morphology, and other key features before accepting results from automated systems.

Identification of *Pseudomonas* species, especially those isolated from CF patients, is not always optimal with rapid systems. The MicroScan system (Negative Combo 15) (Cruinn Diagnostics, Dublin, Ireland), when incubated for 20 to 24 h according to the manufacturer's method, performed poorly for CF isolates, with only 57% of nonmucoid and 40% of mucoid *P. aeruginosa* isolates correctly identified (107). Extended incubation for 48 h improved accuracy to 86 and 83%, respectively. Misidentified species were most commonly either *Alcaligenes* spp. or *P. fluorescens*/*P. putida*. For *P. aeruginosa* from non-CF samples, the overall accuracy has been reported as 94% (107). Other automated systems have not been evaluated to date specifically for the identification of CF isolates, so caution in interpreting results is advised. The importance of non-aeruginosa *Pseudomonas* species as the cause of significant infection has not been established in most cases. The need to pursue species identification beyond the ruling out of *P. aeruginosa* will depend on the individual institution's requirements.

TYPING SYSTEMS

Phenotypic Typing Methods

Historically, typing of *P. aeruginosa* for epidemiological purposes has relied on phenotypic characteristics of the bacteria; examples include pyocin, phage, O, and flagellum typing. The most widely used method was based on differences in LPS O polysaccharide (LPS serotyping) and used either polyclonal or monoclonal antibodies. *P. aeruginosa* produces two species of LPS: the common A-band, with only shorter-chain polysaccharides, which most strains produce; and the high-molecular-weight, type-specific B-band. During chronic infection in CF patients, the B-band LPS disappears and is replaced by the A-band and the strains then become polyagglutinable (108). These phenotypic methods have therefore largely been replaced by genotypic methods except in studies of the efficacy of *P. aeruginosa* vaccines in, e.g., minks, which are susceptible to acute pneumonia caused by *P. aeruginosa* (109).

Genotypic Typing Methods

Several genotypic methods have been developed over the past 2 decades for typing *P. aeruginosa* for epidemiological purposes (110). These are briefly described in the order in which they were developed. Each is useful, even for typing isolates from patients with CF, but they are not available in most clinical diagnostic laboratories.

RFLP

Restriction fragment length polymorphism (RFLP) relies on the genetic diversity at a specific site within the bacterial genome. Such diversity exists upstream of the gene for exotoxin A (*exoA*) in *P. aeruginosa*. In a study of different typing methods, *exoA* RFLP proved superior to all phenotypic methods for typing *P. aeruginosa*. This method was also the first to demonstrate convincingly that patients with CF were usually each infected with a unique strain that was usually present durably (without eradication or replacement) for extended periods. Pilin gene RFLP has demonstrated that individual CF patients are durably infected with the same strain despite changes in pilin protein expression. The disadvantages of RFLP are its relatively weak discriminatory power (compared to that of newer methods), its cumbersome nature, and its predominant use of radioactive probes.

PFGE

Pulsed-field gel electrophoresis (PFGE) has long been considered the gold standard for bacterial typing, as it provides a view of the entire genome. The banding pattern is unique to each strain (or clone) and can be used for any bacterial species, and the phylogenetic relatedness can be shown by dendrograms using commercial software (111).

PCR-Based Typing Methods

Several different PCR-based methods have been used for typing *P. aeruginosa*. They are directed at known elements within the genome or against random but relatively frequently encoded sequences. The latter, random amplified polymorphic DNA analysis, has proved quite robust for typing *P. aeruginosa*, but it must be run consistently on the same equipment to yield reproducible results. Data from random amplified polymorphic DNA analysis usually are highly consistent with those from PFGE. PCR-amplified products can be digested with restriction enzymes to yield more discriminatory data.

Multilocus Sequence Typing

Multilocus sequence typing based on allelic variation in seven housekeeping genes (*ascA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*) has also been employed for typing *P. aeruginosa*, and it is as discriminatory as PFGE (112). It also allows for allele-based clustering techniques such as eBURST, which arrange isolates into clonal complexes based on the number of shared alleles.

Single-Nucleotide Polymorphism Typing

Single-nucleotide polymorphism typing by means of AT biochips (Clondiag Chip Technologies, Jena, Germany) was developed by L. Wiehlmann and B. Tümmler (Hannover Medical School, Hannover, Germany). This method allows simultaneous detection of 13 single-nucleotide polymorphisms in highly conserved genomic regions such as *oriC*, *citS*, *ampC*, *oprI*, *fliC*, *oprL*, and *alkB2*, and in addition, the AT chip allows detection of several gene islands, pathogenicity factors, and variable genes, providing some insight into the repertoire of the accessory genome of a given isolate (113). This method has been shown to be especially useful in studying the epidemiology and evolution of chronic *P. aeruginosa* lung infection in CF (114–116).

SEROLOGIC TESTS

Serologic tests are useful for discrimination between intermittent colonization and chronic infection and for evaluation of the effect of treatment and prognosis in CF patients with *P. aeruginosa*. Several methods have been published, and two approved commercial tests are available: the Pseudomonas-CF-IgG ELISA Kit (Statens Serum Institut, Copenhagen, Denmark), which measures antibodies against sonicates from 17 O groups of *P. aeruginosa*; and the Anti-*Pseudomonas aeruginosa* EIA (Mediagnost, Reutlingen, Germany), which detects serum antibodies against three *P. aeruginosa* antigens (alkaline phosphatase, elastase, and exotoxin A) (100, 117–119). The Pseudomonas-CF-IgG ELISA Kit can also be modified to measure secretory IgA in, e.g., saliva or mucosal secretions for diagnosis of chronic *P. aeruginosa* rhinosinusitis (120).

ANTIMICROBIAL SUSCEPTIBILITIES

P. aeruginosa possesses intrinsic resistance to many antibiotic classes and has the ability to develop resistance by mutations in different chromosomal loci or by horizontal acquisition of resistance genes carried on plasmids, transposons, or integrons. The various mechanisms of resistance, substrate specificities, and geographic distributions are discussed below. The frequent acquisition of antimicrobial resistance in *P. aeruginosa* limits the utility of antimicrobial susceptibility patterns (antibiogram) as a tool in epidemiological typing. Only the resistance mechanisms of planktonic-growing bacteria are discussed, since biofilm-growing *P. aeruginosa* organisms are physiologically resistant to the concentrations of antibiotics that can be obtained by systemic administration (18), but may be susceptible to local application (121). The routine methods for measuring of susceptibility of planktonic-growing bacteria—dilution or diffusion methods—are not relevant for biofilm-growing bacteria and do not give any guidance for the clinical choice of antibiotic therapy against biofilm infections (45). Although biofilm-growing *P. aeruginosa* organisms are much more resistant to antibiotics than planktonic-growing cells, the pharmacokinetics and pharmacodynamics of β-lactams, aminoglycosides, ciprofloxacin, and colistin follow the same general rules (122, 123).

The adaptive tolerance of biofilms depends on the presence of the antimicrobial agent and is mediated by a transient induction of resistance mechanisms, rendering the bacterial population temporarily refractory to their action. Transient tolerance does not require genetic changes; it is channeled through regulatory networks and is switched off when antibiotic exposure stops (for an extensive review of adaptive resistance, see reference 124). These types of tolerance are not due to mutations and are reversible.

Induction of β-lactamase transcription in response to the presence of a β-lactam antibiotic is an example of a specific adaptive tolerance response that impairs the penetration of the β-lactam molecules through the biofilm layers (125). Upregulation of efflux pumps is a nonspecific mechanism that has been shown to affect the tolerance to several classes of antibiotics, including macrolides such as azithromycin (126); the antimicrobial peptide colistin (127); as well as biocides such as EDTA, SDS, and chlorhexidine. Upregulation of efflux pumps in biofilms might be triggered not only by the presence of antibiotics but also as part of the bacterial stress response to, for example, oxidative and nitrosative stress (128), representing a mechanism for elimination of toxic compounds that are accumulated in biofilms (129) (for a detailed description of efflux pump regulation, see reference 124).

Mechanisms of Resistance

Intrinsic Resistance

Intrinsic resistance is mediated through multiple mechanisms. *P. aeruginosa* has an inducible chromosomal AmpC β-lactamase that renders it resistant to ampicillin, amoxicillin, amoxicillin-clavulanate, and first- and second-generation cephalosporins, as well as cefotaxime and ceftriaxone. Although impermeability was originally thought to be responsible for resistance to other antibiotic classes, efflux pump systems have been identified as a more prevalent intrinsic mechanism of resistance.

Multidrug efflux systems of the resistance-nodulation-division family exist in *P. aeruginosa* that can result in expulsion of β-lactams, chloramphenicol, fluoroquinolones, macrolides, novobiocin, sulfonamides, tetracycline, trimethoprim, and aminoglycosides (124). Sequencing of the *P. aeruginosa* genome indicates that a high proportion of genes, including regulatory genes, are involved in the efflux of organic compounds, accounting for this organism's ability to adapt to diverse environments and to resist most antimicrobial agents. Efflux systems also export virulence determinants in *P. aeruginosa*, enhancing their toxicity to the host.

Acquired Resistance

Various antibiotics overcome the intrinsic resistance of *P. aeruginosa* and are active against this organism. These include extended-spectrum penicillins (piperacillin and ticarcillin), certain third- and fourth-generation cephaloporins (ceftazidime and cefepime), carbapenems (imipenem, meropenem, and doripenem), monobactams (aztreonam), fluoroquinolones (ciprofloxacin and levofloxacin), aminoglycosides (gentamicin, tobramycin, and amikacin), and colistin. Unfortunately, mutational resistance to all the antipseudomonal antibiotics can develop.

Efflux Pumps

Although multidrug efflux pump systems play a significant role in the intrinsic resistance of *P. aeruginosa*, they also are critical to the development of multidrug resistance. The overexpression of efflux systems is frequently associated with a loss of fitness and virulence properties. For this reason, microorganisms need to ensure that genes encoding efflux pumps are expressed only when necessary, and therefore, their expression is tightly regulated by both local and global regulators. MexAB-OprM is expressed constitutively in all strains of *P. aeruginosa*. Upregulation or a mutation in the *mexR* repressor gene (*nalB* mutant) results in efflux pump overproduction and significant increase in the MICs of multiple antibiotics, including quinolones, penicillins, cephalosporins, aztreonam, and meropenem (low-level resistance MIC, 8 to 32 μg/ml), but not imipenem. Upregulation of efflux pumps (MexCD-OprJ and MexXY-OprM) is an important determinant of resistance to quinolones and aminoglycosides, respectively, in *P. aeruginosa* from CF patients (130, 131). Interestingly, *mexXY* genes are induced by oxidative stress (see "Adaptive Resistance" below), which is present in the CF lung (128).

Impermeability Mutations

Impermeability mutations may result in resistance to carbapenem, aminoglycosides, colistin, and quinolones. They are important in carbapenem resistance and result from the loss of the OprD porin, a protein that forms a narrow transmembrane channel permeable to carbapenems.

β-Lactamases

Mutations in the regulatory mechanisms of the chromosomally encoded AmpC β-lactamase (132) lead to constitutive expression of high-level enzymes, rendering *P. aeruginosa* resistant to ureidopenicillins and cephalosporins. The acquisition of β-lactamases (133) is not as common for *P. aeruginosa* as it is for *Enterobacteriaceae*. Nevertheless, β-lactamases are being recognized increasingly and are very diverse in this organism. Genes for these enzymes are encoded on plasmids, transposons, or integrons, making their further dissemination likely. They confer resistance predominantly to antipseudomonal penicillins, ceftazidime, cefipime, and aztreonam but not carbapenems. Their activity is inhibited poorly by clavulanic acid or tazobactam.

Carbapenemases

With the exception of GES-2, all carbapenemases in *P. aeruginosa* belong to Ambler class B, commonly referred to as metalloenzymes. Metalloenzymes are not inhibited by clavulanic acid but are susceptible to inhibition by divalent ion chelators such as EDTA. They hydrolyze all β-lactam antibiotics, except aztreonam, and are associated with high-level (MIC, >32 µg/ml) carbapenem resistance. Underreporting of carbapenem resistance may occur, as expression of the carbapenemases varies, resulting in a wide range of MICs (2 to 128 µg/ml) that may go undetected in clinical laboratories that rely only on automated systems.

Genes for these enzymes are plasmid mediated and are located on mobile gene cassettes inserted in variable regions of integrons, resulting in enhanced potential for expression and dissemination. Of concern is the close proximity of these genes to those for aminoglycoside resistance (88).

Carbapenemases are spreading throughout Asia, Europe, and the Americas (134–139). The plasmid-mediated IMP family of enzymes was first described in Japan. The VIM family was first described in Italy. Enzymes from both of these carbapenemase families have been found in *Pseudomonas* spp. (138, 140, 141). New Delhi metallo-β-lactamase-1, first described in 2008 in *Enterobacteriaceae* from the Indian subcontinent, has been identified in *P. aeruginosa* strains from central Europe (142).

Aminoglycoside-Modifying Enzymes

Although impermeability mutations can result in aminoglycoside resistance, especially in CF and intensive care patients, drug inactivation by plasmid-encoded or chromosomally encoded enzymes is the most common mechanism for resistance worldwide to this class of antimicrobials (143). Aminoglycoside-modifying enzymes have been detected in *P. aeruginosa* for more than 30 years; these result in various combinations of resistance to gentamicin, tobramycin, and/or amikacin. *P. aeruginosa* isolates, especially those from Europe and Latin America, increasingly carry multiple modifying enzymes resulting in broad-spectrum aminoglycoside resistance. These enzymes are often encoded on transposons and/or integrons that carry resistance determinants for other classes of antibiotics such as sulfonamides, β-lactams, and chloramphenicol. Multiresistance genes for both aminoglycosides and extended-spectrum β-lactamases and metalloenzymes are of particular concern (143). Aminoglycoside-modifying enzymes can occur together with impermeability mutations, resulting in broad-spectrum aminoglycoside resistance. Broad-spectrum aminoglycoside resistance due to a gene (*rmtA*) encoding a 16S rRNA methylase has been described (144).

Transmissible Quinolone Resistance

The discovery of a plasmid-borne quinolone resistance determinant (*qnr*) in Gram-negative organisms is of significance for several reasons: (i) it has been transferred by conjugation to multiple organisms, including *P. aeruginosa*; (ii) it is associated with high-level quinolone resistance (up to 250-fold increase in MICs); (iii) it appears to be associated with integrons that carry determinants for resistance to β-lactams and aminoglycosides; and (iv) it expands the spectrum of high-level plasmid-mediated resistance to quinolones.

Adaptive Resistance

Adaptive resistance is inducible and depends on the presence of either an antibiotic or another environmental stimulus. A number of triggering factors are now recognized to induce this type of resistance, including antibiotics, oxidative stress (also antibiotic induced [145]), anaerobiosis, cations, and carbon sources. These triggering factors modulate the expression of many genes, leading to effects on efflux pumps, the cell envelope, and enzymes (128). The importance of adaptive resistance in *P. aeruginosa* is consistent with the large repertoire of regulatory genes (9.4% of all genes) in its genome. An important feature of adaptive resistance is that once the inducing factor or condition is removed, the organism reverts to its wild-type susceptibility (124). Polycationic antimicrobials such as aminoglycosides, polymyxins, and cationic antimicrobial peptides pass across the outer membrane by self-promoted uptake, which involves the interaction of the polycations with divalent cation-binding sites on LPS to competitively displace these cations, causing local disruption that enables the passage of the polycation across the membrane. The *arnBCADTEF* operon mediates the addition of 4-aminoarabinose to lipid A of LPS, which blocks self-promoted uptake, leading to resistance. Adaptive resistance to polymyxins and host cationic antimicrobial peptides can be mediated by low concentrations of divalent cations (Mg^{2+} and Ca^{2+}), leading to the activation of two-component regulatory systems such as PhoPQ, PmrAB, and ParRS, and consequent induction of the *arn* operon and adaptive resistance in the presence of the antipseudomonal drug colistin. The adaptive resistance is, as previously shown, of particular concern where *P. aeruginosa* grows as biofilms. Mutations in the different two-component systems that regulate the adaptive resistance to colistin can cause acquired resistance to colistin and have been described in *P. aeruginosa* from CF patients (22, 146, 147).

Multidrug Resistance

Worldwide, despite some geographic variability, antimicrobial resistance, including multidrug (three or more antimicrobial classes) resistance to *P. aeruginosa*, is widespread and increasing (148, 149). According to the European antimicrobial resistance surveillance report from 2011, high percentages of resistance in *P. aeruginosa* isolates were reported, especially by countries in southern and eastern Europe; 34.7% of the *P. aeruginosa* isolates were resistant to one or more of the five considered antimicrobial classes (fluoroquinolones, piperacillin-tazobactam, ceftazidime, carbapenems, and aminoglycosides). Combined resistance was common; 15.3% of the isolates were resistant to at least three antimicrobial classes and 4.6% of the isolates were resistant to all five antimicrobial classes.

The percentage of isolates with combined resistance to at least three of the five classes varied considerably between countries, from <1% in some countries to >50% in other countries. The genetic background of the multidrug- or pan-drug-resistant *P. aeruginosa* has been shown to be a combination of AmpC hyperproduction, OprD inactivation, target mutations conferring high-level fluoroquinolone resistance, mutations involved in efflux pump overexpression, and the production of a class 1 integron harboring aminoglycoside-hydrolyzing enzymes (150).

ANTIMICROBIAL SUSCEPTIBILITIES

Worldwide, susceptibility methods vary in terms of choice of media, inoculum preparation, antimicrobial disk content, breakpoints, and interpretation of those breakpoints. Even when these variables are taken into consideration, susceptibility testing of *P. aeruginosa* remains challenging given the multiple mechanisms of resistance, both intrinsic and acquired, which are frequently expressed concurrently, often at low levels.

In clinical laboratories, susceptibility testing for *Pseudomonas* species may be performed by disk diffusion, agar or broth dilution, Etest (bioMérieux), or automated susceptibility systems using broth microdilution, which have been shown to perform very well using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (151). Disk diffusion tests perform satisfactorily for most clinical isolates of *P. aeruginosa* (152). Limitations to this method include the lack of a quantitative result (MIC) and the potential to miss low-level resistance. Etest has been shown to correlate well with agar dilution for isolates from CF (153, 154) and non-CF (155) patients. Breakpoint interpretation for disk diffusion zones and MICs is standardized by national committees in several European countries such as the British Society for Antimicrobial Chemotherapy and by international committees such as EUCAST (http://www.eucast.org) in Europe and the Clinical and Laboratory Standards Institute (CLSI) (http://www.clsi.org/) in North America and elsewhere, and since 2013 the United States Committee on Antimicrobial Susceptibility Testing (USCAST) has initiated similar work in the United States (http://www.uscast.org). Although some harmonization has started, differences between these organizations still exist, so susceptibility results should be reported according to an individual institution's operating procedures. For EUCAST, the clinical MIC breakpoints for susceptible *P. aeruginosa* strains are as follows: piperacillin and piperacillin-tazobactam, ≤16 µg/ml; ticarcillin and ticarcillin-clavulanate, ≤16 µg/ml; ceftazidime, ≤8 µg/ml; imipenem, ≤4 µg/ml; meropenem, ≤2 µg/ml; doripenem, ≤1 µg/ml; aztreonam, ≤1 µg/ml; ciprofloxacin, ≤0.5 µg/ml; levofloxacin, <1 µg/ml; gentamicin, netilmicin, and tobramycin, ≤4 µg/ml; and amikacin, <8 µg/ml.

Good correlation with reference methods has been reported for most automated systems (106, 156, 157) when testing *Pseudomonas* isolates from non-CF patients. Results evaluating the performance of various automated systems must be interpreted with caution, as the number of isolates tested is often limited, especially for non-*P. aeruginosa* strains. Whereas most *P. aeruginosa* isolates grow well on agar media, growth of some isolates in broth is variable and may pose difficulties for laboratories that rely solely on automated systems. Alternatively, a liquid medium improves the detection of the efflux resistance phenotype, which may not be detected using solid-medium-based testing (158). This may account for some of the discrepancies reported when comparing different susceptibility testing methods.

Several antibiotics pose specific challenges to susceptibility testing. Carbapenem susceptibility testing results are difficult to interpret due to several factors, including rapid imipenem degradation, variable levels of efflux pump expression, and unstable impermeability mutations. Carbapenemase is especially challenging, as it is associated with a wide range of MICs and lacks a simple test for detection. Susceptibility testing of imipenem with and without EDTA (disks or Etest strips) may be used but has been associated with falsely resistant results. Reproducibility of carbapenem resistance results using various susceptibility test methods is poor, and it is recommended that initial carbapenem resistance be confirmed by a second antimicrobial susceptibility test method (159). A novel phenotypic method has been developed for carbapenemase detection (Carba NP), which is based on *in vitro* hydrolysis of imipenem by a bacterial lysate, which is detected by the changes in pH values using the indicator phenol red (red to yellow-orange). A recent evaluation confirmed the 100% specificity of the test but reported a sensitivity of 72.5% (160). Although still restricted to reference laboratories, there are PCR-based methods for detection of carbapenemase production (141, 161, 162).

Colistin is being used more in the treatment of multidrug-resistant *P. aeruginosa*. Disk diffusion testing does not correlate well with MIC results, possibly due to the adaptive resistance mechanism described above (163). Susceptibility testing of colistin should be performed by a MIC method such as agar dilution, Etest, or broth microdilution. Prolonged incubation (for 48 h) is recommended for broth microdilution (164).

Isolates of *P. aeruginosa* from CF patients pose specific difficulties for microbiology laboratories. Isolates from these patients often exhibit mixed morphotypes, including mucoid phenotypes, small-colony variants, and bacterial microcolonies in biofilms. Susceptibility testing is complicated by several factors, including lack of correlation between susceptibility results and clinical response (165), different susceptibility patterns within a morphotype (166), lack of reproducibility of susceptibility tests, undercalling resistance, and presence of hypermutable strains (86). Mucoid and nonmucoid phenotypes of *P. aeruginosa* are often coisolated from patients with CF. Mucoid isolates tend to be more susceptible and have lower β-lactamase activity than nonmucoid isolates (167). One explanation may be that these isolates are protected from selective antibiotic pressure. Selective antibiotic pressure, notably from inhalational tobramycin or colistin therapy, gives rise to small-colony variants of *P. aeruginosa* with properties of increased antimicrobial resistance, autoaggregative growth behavior, and enhanced ability to form biofilms (44, 98, 168). In turn, bacterial cells in biofilms adapt into symbiotic bacterial communities in which the mucoid alginate-producing bacterial cells provide physical protection to the biofilm, while the highly antibiotic-resistant nonmucoid cells protect against antibiotic killing (167). Increased ability of biofilm bacteria to acquire resistance phenotypes (169) and selection of hypermutable strains following antimicrobial therapy (86, 170) may further explain the lack of eradication of *P. aeruginosa* from chronically infected CF patients. Since bacteria found in biofilms exhibit MICs 100- to 1,000-fold higher than those for free-living bacteria, routine susceptibility testing of planktonic bacteria (171) may underestimate resistance and may contribute to treatment failures. In a study of 597 CF isolates (152), both disk diffusion and Etest were found to be generally acceptable as routine susceptibility testing methods. However, poor correlation was found with diffusion testing of mucoid isolates for piperacillin, piperacillin-tazobactam, and meropenem. Underreporting of resistance was more frequent with disk diffusion than with Etest, especially when testing ceftazidime, piperacillin, and piperacillin-tazobactam.

Hypermutable strains may be detected using either disk diffusion or Etest methods by the presence of resistant mutant subpopulations within the inhibition zones of three or more antibiotics (172).

Mucoid isolates pose a specific challenge for automated systems (153, 158, 173). Overestimating susceptibility may occur, as mucoid isolates often demonstrate insufficient growth at 24 h. Automated systems that allow for longer incubation may be preferable. On the other hand, overcalling resistance may result from the presence of large amounts of exopolysaccharide, resulting in turbidity without adequate bacterial growth. These limitations have led many microbiologists who routinely work with mucoid isolates of *P. aeruginosa* to avoid using automatic methods for mucoid isolate testing. Using phenotypically different colonies directly from sputum cultures, or from subcultures of isolated colonies, has been shown to correlate well with disk diffusion and MIC susceptibility methods and may provide clinically useful susceptibility data with significant time and cost savings. However, the correlation appears to be better for susceptible strains than for resistant strains. Direct sputum susceptibility testing using the Etest method has been suggested as an alternative to morphotype testing in assessing the *in vivo* situation by evaluating bacterial population susceptibility as well as potential interactions with other organisms, including commensal microbes (174).

Other methods have been recommended in an attempt to better predict susceptibility results. Biofilm susceptibility assays have been developed that confirm that minimal biofilm inhibitory concentrations are much higher than conventionally determined MICs for multiple antibiotics (171). These values should guide pharmacokinetics/pharmacodynamics-based antibiotic treatment. A multicenter, randomized, double-blind, controlled clinical trial looked at whether using the results of testing of antibiotics against *P. aeruginosa* grown as a biofilm rather than in liquid improved outcomes in people with CF with lung infections (175). There was no difference in the reduction of sputum bacteria or in the improvement in lung function between the two groups. However, further studies including larger numbers of CF patients and younger patients with less advanced lung disease should be conducted in the future. Synergy testing using microtiter checkerboard, time-kill test, broth macrodilution breakpoint combination sensitivity test, or Etest methods (174, 176, 177) has been used to assess the activities of antibiotic combinations *in vitro* in order to predict *in vivo* synergistic activity. This testing is labor-intensive, time-consuming, and difficult to reproduce, and it remains controversial, as very few clinical data exist demonstrating correlation with prediction of outcomes.

Pseudomonas species other than *P. aeruginosa* are generally susceptible to most antipseudomonal antibiotics as well as to trimethoprim-sulfamethoxazole (except most *P. fluorescens/putida* isolates), a property that differentiates them from *P. aeruginosa*. *P. fluorescens*, *P. putida*, and *P. oryzihabitans* may be more resistant to aztreonam and ticarcillin-clavulanate. *P. stutzeri* is usually very susceptible to all antipseudomonal agents (35).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

P. aeruginosa may be associated with colonization or clinically significant infections. Interpretation of the Gram stain often directs the further workup of this organism. The presence of small clusters of Gram-negative organisms surrounded by amorphous material is indicative of biofilm formation compatible with a chronic infection. This finding should be reported to physicians, and incubation should be prolonged, as these isolates usually exhibit slower growth characteristics. The presence of these organisms intracellularly in polymorphonuclear cells is a strong indication of true infection rather than colonization. Isolation of *P. aeruginosa* from sterile body sites should always be interpreted as indicative of probable infection. Isolation in mixed culture requires correlation with the direct smear, other organisms isolated, and clinical history. Isolates from sites of chronic infection, such as CF respiratory sites, often exhibit multiple morphotypes that can make identification difficult. Molecular methods increasingly are finding a role in the identification of this organism, especially for epidemiological studies. Susceptibility testing of this organism is difficult, especially for mucoid isolates, due to increasing resistance, lack of reproducibility of results, and lack of clinical correlation. Piperacillin and piperacillin-tazobactam results obtained from automated systems may be unreliable for *Pseudomonas* spp., and in particular for mucoid isolates, and results should be confirmed by disk diffusion or Etest systems. A basic understanding of the multiple mechanisms of resistance, both intrinsic and acquired, is essential to interpret susceptibility testing results and give therapeutic recommendations to physicians. Other *Pseudomonas* species are infrequently isolated in the laboratory and are usually not clinically significant. Clinical correlation and correlation with the Gram stain are essential before further workup is undertaken.

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Burkholderia, *Stenotrophomonas*, *Ralstonia*, *Cupriavidus*, *Pandoraea*, *Brevundimonas*, *Comamonas*, *Delftia*, and *Acidovorax*

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TAXONOMY

Many of the bacteria discussed in this chapter formerly belonged to the genus *Pseudomonas*; others were described and named after the taxonomic dissection of this genus to conform to a modern phylogeny-based taxonomy. In this reclassification process, the name *Pseudomonas* was confined to rRNA homology group I organisms because they included the type species, *Pseudomonas aeruginosa* (see chapter 42).

rRNA group II pseudomonads belong to the class of the *Betaproteobacteria* and were reclassified into the genera *Burkholderia* and *Ralstonia* (1, 2). They form a remarkable group of primary and opportunistic human, animal, and plant pathogens, as well as environmental species with a considerable potential for biological control, remediation, and plant growth promotion. During the past 2 decades, the interest in several peculiar characteristics of these organisms led to the discovery and description of a multitude of novel species. The genus *Burkholderia* now contains >70 validly named species, many of which have been isolated from soil and water samples. Some other *Burkholderia*-like species were found to represent a distinct phylogenetic lineage with a position intermediate between those of the genera *Burkholderia* and *Ralstonia* and were classified in the novel genus *Pandoraea* (3).

Comparative 16S rRNA gene sequence analysis, further supported by phenotypic differences, indicated that two distinct sublineages existed within the genus *Ralstonia* (4). It was proposed that species of the *Ralstonia eutropha* lineage be classified in a novel genus named *Wautersia*, whereas the name *Ralstonia* was preserved for the lineage comprising *Ralstonia pickettii*, the type species. Shortly thereafter, it became known (5) that *Wautersia eutropha*, the type species of the genus *Wautersia*, was a junior synonym of *Cupriavidus necator*, the type (and only) species of the genus *Cupriavidus*, an environmental organism that was validly named in 1987, i.e., long before 16S rRNA gene sequence studies were performed routinely (6). To conform to the *International Code of Nomenclature of Bacteria* (7), the name *Wautersia* had to be replaced by *Cupriavidus* and all species of the genus *Wautersia* became species of the genus *Cupriavidus*.

Several *Burkholderia* species have been isolated from human clinical samples, but only *B. cepacia* complex, *B. gladioli* (including strains previously classified as *B. cocovenenans* [8]), *B. mallei*, and *B. pseudomallei* are generally recognized as human or animal pathogens. Polyphasic taxonomic studies revealed that *B. cepacia*-like bacteria belong to at least 18 distinct genomic species, referred to collectively as the *B. cepacia* complex (9, 10). Ongoing surveys of the diversity of *B. cepacia*-like bacteria recovered from specimens from cystic fibrosis (CF) patients and other specimens revealed the presence of several additional groups in the *B. cepacia* complex that cannot be assigned to one of the established species within this complex by using traditional or molecular identification approaches (11). Further polyphasic taxonomic analyses are needed to determine if these groups represent additional novel species within the *B. cepacia* complex or if they represent new variants of established species. All *B. cepacia* complex species have been recovered from human clinical samples. Within this group, however, *B. multivorans* and *B. cenocepacia* are the most common opportunistic pathogens in CF patients (11).

Apart from the *B. cepacia* complex species, *B. gladioli*, *B. mallei*, and *B. pseudomallei*, the genus *Burkholderia* now comprises another 50 validly named species. Most of these organisms are not associated with human disease. Species that rarely have been reported as associated with human infections include *B. fungorum*, *B. glumae*, *B. thailandensis*, *B. tropica*, *B. endofungorum*, and *B. rhizoxinica* (12–16). A complete overview of validly named species can be obtained through Internet sites such as http://www.bacterio.net/.

There are now five species in the genus *Ralstonia*. The human pathogens include *R. pickettii*, *R. mannitolilytica* (previously known as *R. pickettii* biovar 3/'*thomasii*') (17), and *R. insidiosa* (18). Other validly named *Ralstonia* species are now classified as *Cupriavidus* species (5). The latter genus consists of *C. pauculus* (previously known as Centers for Disease Control group IVc-2) (19), *C. gilardii* (20), *C. respiraculi* (21), *C. taiwanensis* (22), and nine additional species, which thus far have only been recovered from environmental samples.

The genus *Pandoraea* presently comprises five species that have been isolated from human clinical specimens—these are *P. apista*, *P. pulmonicola*, *P. pnomenusa*, *P. sputorum*,

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and *P. norimbergensis*—but also several unnamed strains, each representing a distinct additional *Pandoraea* species (3). *P. faecigallinarum* was isolated from chicken feces; other, more recently named *Pandoraea* species are of environmental origin only (23, 24).

Organisms in the *Pseudomonas* rRNA homology group III also belong to the *Betaproteobacteria* and are now classified in the family *Comamonadaceae*, which includes the genera *Comamonas*, *Delftia*, and *Acidovorax* (25, 26). The genus *Comamonas* was created in 1985 and included a single species, *C. terrigena*. Shortly thereafter, *Pseudomonas acidovorans* and *Pseudomonas testosteroni* were reclassified as members of the genus *Comamonas*; the former was, however, again reclassified as *Delftia acidovorans* (27). *C. terrigena* encompassed three strain clusters with human clinical isolates (28), which are now known as *C. terrigena*, *C. aquatica*, and *C. kerstersii* (29). The genus *Comamonas* at present totals 16 validly named species, which are primarily of environmental origin. The genus *Delftia* comprises another 3 validly named species of environmental origin (30). The genus *Acidovorax* comprises 15 species. It includes several well-known plant-pathogenic bacteria and environmental species (31). Only *A. delafieldii*, *A. temperans*, and more recently *A. wautersii* have been isolated from human clinical samples (32).

Pseudomonas species of the rRNA homology group IV belong to the *Alphaproteobacteria* and are now classified in the genus *Brevundimonas*. The latter consists of 24 validly named species, most of which are of environmental origin. *B. diminuta*, *B. vesicularis*, and *B. vancanneytii* have been isolated from human clinical samples (33, 34).

Finally, *Pseudomonas maltophilia* represented *Pseudomonas* rRNA homology group V. Based on genotypic and phenotypic characteristics, its transfer to the genus *Xanthomonas*, a member of the *Gammaproteobacteria*, and subsequently to the novel genus *Stenotrophomonas* was proposed (35, 36). *S. maltophilia* is a well-known opportunistic pathogen (37) and is considered taxonomically heterogeneous (38); 10 other *Stenotrophomonas* species are exclusively known as environmental bacteria.

DESCRIPTION OF THE AGENTS

Burkholderia, *Ralstonia*, *Cupriavidus*, *Pandoraea*, *Brevundimonas*, *Comamonas*, *Delftia*, and *Acidovorax* spp. are aerobic, non-spore-forming, straight or slightly curved, Gram-negative rods. They are 1 to 5 µm in length and 0.5 to 1.0 µm in width (39). *Stenotrophomonas* spp. are straight rods and tend to be slightly smaller than members of the other genera (0.7 to 1.8 µm in length and 0.4 to 0.7 µm in width) (39). With the exception of *B. mallei*, these organisms are motile due to the presence of one or more polar flagella (40). These bacteria are catalase positive, and most are either weakly or strongly oxidase positive. All grow on MacConkey agar, except for certain strains of *B. vesicularis*, and appear to be nonfermenters. The majority of species degrade glucose oxidatively, and most degrade nitrate to either nitrite or nitrogen gas. Certain species have distinctive colony morphologies or pigmentation. They are nutritionally quite versatile, with different species being able to utilize a variety of simple and complex carbohydrates, alcohols, and amino acids as carbon sources. Certain species can multiply at 4°C, but most are mesophilic, with optimal growth temperatures of between 30 and 37°C (40). For some genera, growth at higher temperatures (i.e., 42°C) can be useful for species identification.

EPIDEMIOLOGY AND TRANSMISSION

Burkholderia, *Ralstonia*, *Cupriavidus*, *Pandoraea*, *Comamonas*, *Delftia*, *Acidovorax*, *Brevundimonas*, and *Stenotrophomonas* spp. are environmental organisms found in water, soil, the rhizosphere, and in and on plants including fruits and vegetables. They have a worldwide distribution. Members of these genera are widely recognized as phytopathogens, and many species were first described in that context. Because of their ability to survive in aqueous environments, these organisms have become particularly problematic as opportunistic nosocomial pathogens in hospitals and health care settings.

The natural distribution of *B. cepacia* complex species is being intensively studied because of interest in their biotechnological properties and their pathogenicity in persons with CF (41). *B. cepacia* complex bacteria often have antifungal, antinematodal, or plant growth-promoting properties, which make them attractive as biological pesticides and fertilizers (42). Because of their nutritional versatility, *B. cepacia* complex bacteria also have applications for bioremediation of contaminated soils. These species are infrequently recovered from sites such as sinks, swimming pools, and showers (43). However, some species within the complex, especially *B. ambifaria*, *B. anthina*, and *B. pyrrocinia*, can be readily recovered from certain soil samples (44), provided that appropriate growth conditions are used to inhibit the growth of vast numbers of other environmental bacteria. Of particular note, *B. multivorans*, which is among the most common *B. cepacia* complex species recovered from CF patients, has been only infrequently recovered from environmental sources. Studies of a variety of foodstuffs and bottled water have shown that *B. cepacia* complex bacteria have been found in unpasteurized dairy products (45). Due to their intrinsic resistance to antibiotics and disinfectants, *B. cepacia* complex bacteria are also notorious contaminants of pharmaceutical preparations (46, 47). Numerous health care-associated outbreaks of *Burkholderia* infection due to contaminated medical devices and products, including mouthwashes, ultrasound gels, skin antiseptics, and medications, have been described (41).

Genotypic and conventional epidemiologic investigations provide compelling evidence for interpatient transmission of shared or "epidemic" *B. cepacia* complex strains among persons with CF (41). One such strain, referred to as the ET12 (for electrophoretic type 12) lineage, is common among CF patients in eastern Canada and the United Kingdom (48, 49). This organism is a *B. cenocepacia* strain that is characterized by the presence of a distinctive cable-like pilus and an associated adhesin that mediates adherence to respiratory epithelium. *B. cenocepacia* strain PHDC dominates among infected CF patients in the mid-Atlantic region of the United States and has been identified in agricultural soil as well as in CF patients in several European countries (50–52). *B. cenocepacia* "Midwest clone" is common among CF patients in the midwestern United States (53).

B. pseudomallei and *B. thailandensis* are found in soil and surface water primarily in tropical and subtropical areas. Both species have been isolated in the rice-growing regions of northeast Thailand, western Cambodia, Laos, and southern and central Vietnam (54–56). In northern Australia, associations have been made between *B. pseudomallei* and native grasses in undisturbed land and the presence of livestock animals, lower soil pH, and different combinations of soil texture and color in environmentally disturbed sites (57, 58). Recent environmental studies have identified *B. thailandensis*-like organisms from Australia (59).

The known endemic distribution of *B. pseudomallei* is being expanded beyond the traditional regions of endemicity for melioidosis in Southeast Asia and northern Australia, with recent case reports of the disease from the Americas, Madagascar, several countries in Africa, Mauritius, India, and elsewhere in south Asia, China, and Taiwan. To what extent this reflects a true expansion of endemicity rather than unmasking of the long-standing presence of the bacterium remains unclear (60–62). What is apparent is that *B. pseudomallei* can occasionally persist in temperate environments after introduction via animals infected with melioidosis (63).

S. maltophilia can cause nosocomial infections; however, the sources of hospital-acquired infection are poorly understood. Inappropriate use of hand-moisturizing lotion instead of soap by a health care worker was associated with infection among bone marrow transplant recipients in one center (64), while in another, contamination of faucet aerators in intensive care unit sinks was implicated as the source of *S. maltophilia* infection (65).

Unlike that of certain *B. cepacia* complex strains, evidence for person-to-person transmission of *B. gladioli*, *B. pseudomallei*, *B. mallei*, *S. maltophilia*, and the other species discussed in this chapter is lacking.

CLINICAL SIGNIFICANCE

B. cepacia Complex and *B. gladioli*

B. cepacia has been long recognized as an occasional opportunistic human pathogen, capable of causing a variety of infections, including bacteremia, urinary tract infection, septic arthritis, peritonitis, and pneumonia in persons with underlying illness. Persons with chronic granulomatous disease (CGD) and CF are particularly susceptible to infection (66). *B. cepacia* also has a history as a nosocomial pathogen, causing infections associated with contaminated hospital equipment, medications, and disinfectants including povidone-iodine and benzalkonium chloride (41). Nosocomial outbreaks of respiratory tract infections in patients on mechanical ventilation in intensive care units have been attributed to contamination of nebulizers or nebulized medications such as albuterol. Contamination of blood culture systems or disinfectants resulting in pseudobacteremia has been described following the isolation of *B. cepacia* from the blood of multiple patients over a short period. Early reports of infection in CF described patients with acute pulmonary deterioration and sepsis (referred to as cepacia syndrome) or chronic respiratory tract infections associated with an accelerated decline in lung function (67). Clinical outcome studies consistently identified *B. cepacia* infection as a significant independent risk factor for morbidity and mortality in CF (68).

The recognition that several closely related species can be distinguished from among organisms previously identified as *B. cepacia* has stimulated interest in the clinical significance of each of these species (69). Approximately 3% of CF patients in the United States are infected with *B. cepacia* complex species, although rates of infection vary from 0 to 20% among CF treatment centers (70). Rates of infection increase with increasing patient age; approximately 5 to 7% of adults with CF are infected (70). Many strains exhibit broad-spectrum antimicrobial resistance (see below), and pulmonary infection is generally refractory to therapy. Furthermore, due to the poor postoperative prognosis associated with *B. cepacia* complex, most CF treatment centers consider infection to be an absolute contraindication for lung transplantation, which at present offers the only therapeutic option for successful intermediate-term survival of persons with end-stage pulmonary disease (71). Thus, respiratory tract infection by these species is a cause of great concern to CF patients and their caregivers.

Although 17 of the 18 species of the *B. cepacia* complex have been recovered from persons with CF (the exception is *B. ubonensis*), the distribution of species in this patient population is quite disproportionate. In the United States, *B. multivorans* and *B. cenocepacia* together account for ~80% of *B. cepacia* complex infections, with *B. vietnamiensis*, *B. cepacia*, and *B. dolosa* together accounting for an additional 15% of infections (11, 41). The remaining *B. cepacia* complex species are much less frequently recovered from human sources. These species include *B. stabilis*, *B. ambifaria*, *B. anthina*, *B. pyrrocinia*, *B. contaminans*, *B. seminalis*, *B. diffusa*, *B. metallica*, *B. arboris*, *B. latens*, *B. lata*, and *B. pseudomultivorans* (10, 41). Some CF patients are infected with bacteria that, based on genetic analyses, are most appropriately included in the *B. cepacia* complex but cannot be assigned to one of the currently named species. These strains most likely represent additional novel species within the complex. Finally, the incidences of *B. multivorans* and *B. cenocepacia* infection in CF appear to have shifted during the last few years. Whereas *B. cenocepacia* previously accounted for the majority of *Burkholderia* infections in CF (11), currently, ~3 times as many CF patients in the United States become infected with *B. multivorans* as with *B. cenocepacia* (41). In the United Kingdom, the recovery of *B. multivorans* from CF patients now also exceeds that of *B. cenocepacia* (72).

Available data suggest that *B. cepacia* complex species also vary with respect to their virulence levels and clinical impacts in CF. Studies in lung transplant recipients, for example, indicate that rates of postoperative mortality are greater for persons infected preoperatively with *B. cenocepacia* than for patients infected with other *B. cepacia* complex species (73, 74). Carefully conducted multivariate analyses of posttransplant outcomes are less definitive, however (75). Thus, although it is almost certainly true that *B. cenocepacia* is the species most frequently associated with cepacia syndrome, it remains to be shown whether this species, in general, is disproportionately associated with poor outcome; case reports document fatal infection associated with other *B. cepacia* complex species, including *B. multivorans*, *B. stabilis*, and *B. dolosa* (76, 77). Although a positive correlation between species frequency and poor clinical outcome seems likely, firm conclusions regarding the relative virulence of *B. cepacia* complex species must await more definitive study. Evidence also suggests that certain strains within species are relatively more virulent in human infection. The *B. cenocepacia* ET12 epidemic strain and the *B. dolosa* SLC6 strain, in particular, appear to be relatively more virulent in CF patients (78, 79). Again, however, further comparative outcome studies are needed before firm conclusions about relative virulence of specific strains can be drawn.

B. gladioli is most notable as a plant pathogen, but is also well recognized to be capable of causing infection in persons with CF or CGD and, occasionally, other immunocompromised patients (80). Anecdotal reports describe acute pulmonary deterioration and recurrent soft tissue abscesses, as well as severe post-lung transplantation infections due to *B. gladioli* in CF patients (75, 81). A more complete appreciation of the epidemiology and clinical significance of *B. gladioli* infection in CF has been confounded by difficulty with accurate identification of this species, which typically is capable of growth on selective media used to isolate

B. cepacia complex species and is frequently misidentified as a member of the *B. cepacia* complex by commercial test systems (82). Genetic analysis of *Burkholderia* isolates recovered from CF patients indicates that *B. gladioli* is much more commonly involved in infection in this patient population than are most *B. cepacia* complex species, with the exception of *B. multivorans* and *B. cenocepacia* (41).

B. pseudomallei and *B. mallei*

B. pseudomallei is the causative agent of the human and animal disease melioidosis, which is endemic in Southeast Asia and tropical northern Australia and is being increasingly recognized on the Indian subcontinent and in Central and South America (60, 61, 83, 84). In locations where the disease is endemic, infection is seasonal, with up to 85% of cases occurring during the monsoon wet season. Severe weather events and environmental disturbances have been associated with melioidosis clusters in Australia (85), and the Asian tsunami of 2004 resulted in cases across the affected region (86, 87). As travel to Southeast Asia and northern Australia has become more frequent, reports of melioidosis in travelers returning to Europe and the United States are becoming more common (63). Melioidosis is an especially important potential travel-related illness for those with CF, and persistent colonization of airways with *B. pseudomallei* can occur in CF despite prolonged therapy, with repeated disease flares and deteriorating lung function (88, 89). Infection with this organism should be considered in the differential diagnosis of any individual with a fever of unknown origin or a tuberculosis-like illness who has a history of travel to a region where *B. pseudomallei* infection is endemic.

B. pseudomallei is acquired from the environment by inoculation through cut or abraded skin, inhalation, aspiration, or ingestion (90). Zoonotic disease is described but is exceedingly uncommon, as are person-to-person transmission and laboratory-acquired infection (83). The association of severe weather events with respiratory infection and high mortality rates has been attributed to a shift from percutaneous inoculation to inhalation (91). This idea supports the potential of *B. pseudomallei* as a bioterrorism agent; its isolation from patients who do not give a history of travel to an area where melioidosis is endemic should be immediately reported to local or state public health authorities. For further details, see chapter 14 or http://www.bt.cdc.gov.

The majority of persons exposed to *B. pseudomallei* do not develop clinical infection, with rates of seropositivity in the general population as high as 80% in some locations (92). Latent infection with subsequent reactivation is well recognized, with a recent description of disease onset in the United States, where melioidosis is not endemic, 62 years after presumed infection in Thailand (93). Such cases often achieve notoriety but are rare, and the vast majority of cases of melioidosis are from recent infection, with an incubation period of 1 to 21 days (mean, 9 days) (84). Risk factors for clinical disease following infection with *B. pseudomallei* include diabetes, hazardous alcohol use, chronic renal disease, chronic lung disease, malignancy, and immunosuppressive therapy (94–96). Twenty to 36% of cases have no identified risk factor, and mortality in this group is usually low. Disease in children is also uncommon, although parotid abscesses are well recognized as an important manifestation of melioidosis in children in Thailand and Cambodia (97). Overall rates of mortality from melioidosis vary from 10 to 15% in centers where state-of-the-art intensive care therapy is available to >50% in locations with poor resources (83, 84). Fifty percent of cases present with pneumonia, which can be part of a fatal septicemia or a less severe unilateral infection indistinguishable from other community-acquired pneumonias or a chronic illness mimicking tuberculosis (98). Chronic melioidosis, defined as illness present for >2 months, occurs in only 10% of cases. Overall, 50% of cases are bacteremic; the presence of >100 CFU/ml of blood and a blood culture showing growth in the first 24 h of incubation are markers for high mortality. At the mild end of the clinical spectrum of melioidosis is presentation with a skin ulcer or abscess without systemic illness (99). Other common presentations with or without bacteremia are genitourinary infections, septic arthritis, and osteomyelitis (83, 84, 96). Prostatic abscesses are especially common (100). Abscesses can also occur in spleen, liver, kidneys, and adrenal glands. Parotitis, lymphadenitis, sinusitis, orchitis, myositis (especially psoas abscess), mycotic aneurysms, and pericardial and mediastinal collections have all been described. Lesions can be frankly purulent and may include microabscesses or granulomas or a combination of these features. Clinical meningitis is rare, but melioidosis encephalomyelitis syndrome (96) and brain abscesses have also been reported. The one presentation that has yet to be described is *B. pseudomallei* endocarditis.

B. mallei is the etiologic agent of glanders, a highly communicable disease of livestock, particularly horses, mules, and donkeys. It can be transmitted to humans and is also identified as a potential agent of bioterrorism. Unlike *B. pseudomallei*, *B. mallei* is a host-adapted pathogen that does not persist in the environment outside its host. Glanders has been eradicated from most countries, but enzootic foci persist in the Middle East, Asia, Africa, and South and Central America. The only human case of glanders in the past 50 years in the United States was a recent laboratory-acquired case in a biodefense scientist (101). Like melioidosis, human glanders can be acute or chronic, with the clinical presentation and course depending on the mode of infection, the inoculation dose, and host risk factors. Respiratory inoculation can result in pneumonia with potential for dissemination to internal organs and septicemia. Cutaneous inoculation can result in skin nodules and regional lymphadenitis, also with potential for disseminated disease. Involvement of nodes, both mediastinal and peripheral, is much more common in glanders than in melioidosis, often with suppurative abscesses in untreated cases.

S. maltophilia

S. maltophilia, although typically not pathogenic for healthy persons, is a well-known opportunistic human pathogen. It is among the most common causes of wound infection due to trauma involving agricultural machinery (102). It is also an important nosocomial pathogen associated with substantial morbidity and mortality, particularly in debilitated or immunocompromised patients and patients requiring ventilatory support in intensive care units (103). The incidence of human infection appears to have increased in recent years, and a variety of clinical syndromes have been described, including bacteremia, pneumonia, urinary tract infection, ocular infection, endocarditis, meningitis, soft tissue and wound infection, mastoiditis, epididymitis, cholangitis, osteochondritis, bursitis, and peritonitis (104, 105). Septicemia can be accompanied by ecthyma gangrenosa, a skin lesion more commonly associated with *P. aeruginosa* and *Vibrio* spp.

The incidence of *S. maltophilia* respiratory tract infection in persons with CF also appears to be increasing (106); however, the unreliability of historical data limits firm conclusions. Approximately 14% of CF patients included in the

Cystic Fibrosis Foundation's patient registry were reported to be culture positive for *S. maltophilia* in 2011 (70). In large, multicenter clinical trials, however, *S. maltophilia* was found in a larger proportion of CF patients, being second only to *P. aeruginosa* in frequency of isolation from study subjects. Infection or colonization was most frequently transient, with 30% of subjects having at least one sputum culture positive for *S. maltophilia* during the course of 6 months (107). Several case-control studies have drawn conflicting conclusions regarding the role that *S. maltophilia* plays in contributing to pulmonary decline in CF (108).

Ralstonia **and** ***Cupriavidus*** **spp.**

As described above, several former *Ralstonia* species are now assigned to the genus *Cupriavidus* (5). Among the species in these two genera, *R. pickettii* is best known with respect to human infection. Older reports describe this species as being recovered from a variety of clinical specimens and as causing various infections including bacteremia, meningitis, endocarditis, and osteomyelitis (109). *R. pickettii* also has been identified in pseudobacteremias and nosocomial outbreaks due to contamination of intravenous medications, "sterile" water, saline, chlorhexidine solutions, respiratory therapy solutions, and intravenous catheters (110, 111). This species has also been recovered from the respiratory tract of persons with CF. However, *R. pickettii* is easily confused with *Pseudomonas fluorescens* and members of the *B. cepacia* complex based on phenotype (17, 112). Furthermore, several *R. pickettii*-like species are also now known to be involved in human infection, particularly in CF (113). Thus, the role of *R. pickettii* as a human pathogen is difficult to assess based on historical data.

R. mannitolilytica (formerly known as *R. pickettii* biovar 3/'*thomasii*') was recently described as causing nosocomial outbreaks and a case of recurrent meningitis (17). This species accounts for the majority of *Ralstonia* infections in CF patients, being found in more than twice as many CF patients as those infected with *R. pickettii* (113). *R. insidiosa* and *C. respiraculi* are species that are also recovered from persons with CF (18, 21). *C. gilardii* has been recovered from cerebrospinal fluid (20), and cases of *C. pauculus* bacteremia, peritonitis, and tenosynovitis have been reported (19). Both of these species may be found in sputa from patients with CF (113). Although *Cupriavidus metallidurans* and *Cupriavidus basilensis* are not known to cause other human infection, they too have been recovered recently from sputum cultures from patients with CF (113). Despite these observations, the roles of *Ralstonia* and *Cupriavidus* species in human infection, particularly in persons with CF, require further elucidation.

Other Genera

In general, *Brevundimonas*, *Comamonas*, *Delftia*, *Acidovorax*, and *Pandoraea* spp. infrequently cause human infection. Interest in these species focuses primarily on their roles as plant pathogens or in studies of microbial biodiversity and biodegradation.

Brevundimonas spp. are occasionally recovered from clinical specimens (114). *B. vesicularis* bacteremia in patients with various underlying illnesses has been reported (115), and the organism has been recognized in cervical specimens because of its ability to produce bright orange colonies on Thayer-Martin agar. *B. diminuta* has been recovered from blood, urine, and pleural fluid from patients with cancer (116).

Among the *Comamonas* species, *C. testosteroni* has been implicated most often in human infection, with reports describing endocarditis, meningitis, and catheter-associated bacteremia due to this species (117–119). *D. acidovorans* has similarly been reported to cause infection, being identified in cases of bacteremia, endocarditis, ocular infection, and suppurative otitis (120). *Acidovorax* spp. have been isolated from a variety of clinical sources, including blood from a patient with hematological malignancy (121). *Acidovorax* spp., *C. testosteroni*, and *D. acidovorans* have also been recovered from sputa of persons with CF (122; J. J. LiPuma, unpublished data); however, the roles of these species in contributing to lung disease in CF have not been established.

In addition to causing infection in CF patients (123, 124), *Pandoraea* spp. have been recovered from blood and from patients with chronic obstructive pulmonary disease or CGD (125). Although the roles of these species in contributing to poor outcomes in persons with underlying diseases are unclear, a recent report describes sepsis, multiple organ failure, and death in a patient who underwent lung transplantation for sarcoidosis (126).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

The genera described in this chapter include organisms that can survive in a variety of hostile environments and at temperatures found in clinical settings. Therefore, standard collection, transport, and storage techniques as outlined in chapter 18 are sufficient to ensure the recovery of these organisms from clinical specimens. Recovery of *B. pseudomallei* for the diagnosis of melioidosis is increased by the additional collection of throat and rectal swabs into selective media (see "Isolation Procedures" below) and by collecting larger-than-standard volumes of cerebrospinal fluid for culture in suspected neurological melioidosis (127).

DIRECT EXAMINATION

Members of these genera have similar morphologies and, with the exception of *B. pseudomallei*, are not easily distinguished from each other on the basis of Gram staining. *B. pseudomallei* may appear as small, Gram-negative bacilli with bipolar staining, making the cells resemble safety pins (Fig. 1). This may increase the index of suspicion for the presence of *B. pseudomallei*, but the sensitivity and specificity of this appearance are not high enough to be relied on for a presumptive clinical diagnosis.

Although PCR-based assays have been described for the identification of *B. cepacia* complex species, *B. pseudomallei*, *B. gladioli*, several *Ralstonia* and *Cupriavidus* species, *Pandoraea* species, and *S. maltophilia* following culture and isolation (see "Identification" below), the use of PCR for direct detection of these species in clinical specimens remains a research tool (128, 129). Studies of CF sputum samples have indicated that some specimens may be PCR positive but culture negative for certain *B. cepacia* complex species, raising important questions about the natural history of infection in CF. However, the sensitivities and specificities of such PCR assays for the intended target species are difficult to determine in the absence of reliable gold standards. Assays employing real-time PCR or culture-independent strategies employing next-generation bacterial genome sequencing are likely to provide alternative approaches to direct detection of these species in clinical specimens in the near future.

Because septicemia with *B. pseudomallei* is frequently fatal and death often occurs in the first few days after presentation to hospital prior to the availability of culture results, several rapid direct-detection methods have been developed, including urinary antigen detection using latex agglutination and enzyme immunoassay as well as direct fluorescent-antibody

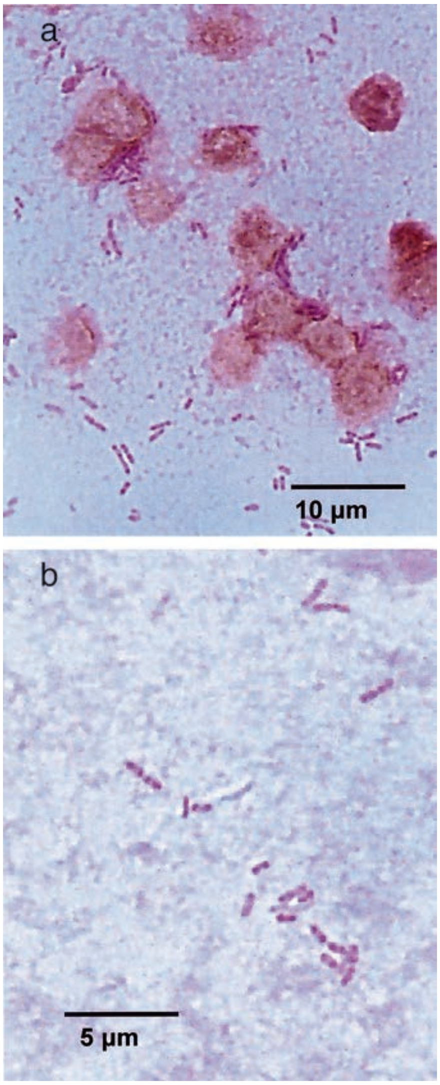

FIGURE 1 (a) Gram stain of *B. pseudomallei* in a blood culture; (b) Gram stain of *B. pseudomallei* from a colony on blood agar. doi:10.1128/9781555817381.ch43.f1

(DFA) staining (130–132). Antibodies raised against heat-killed whole cells of *B. pseudomallei* have been used to prepare a reagent for DFA staining. When this DFA reagent was used to stain clinical specimens from patients with suspected melioidosis, using a rapid assay that took 10 min to prepare and read, it showed a sensitivity of 66% and specificity of 99.5% (132). A DFA assay has also been described based on a monoclonal antibody specific to *B. pseudomallei* and Alexa Fluor 488 (133). The reagents described in the literature are largely prepared in-house and are not available commercially.

Evaluation of a conventional PCR assay targeting the 16S rRNA gene for the detection of *B. pseudomallei* in clinical specimens indicated that the assay was sensitive but lacked specificity, resulting in positive predictive values of only 70% (134). Real-time PCR assays targeting *B. pseudomallei* genes encoding 16S rRNA, flagellin (*fliC*), ribosomal protein subunit S21 (*rpsU*), or type III secretion system (TTS) genes have been developed and been shown to have high sensitivities and specificities with pure bacterial cultures (135–137). Two clinical evaluations of real-time PCR have met with mixed results, with a sensitivity of 91% in one study using an assay targeting a gene in the TSS1 cluster (129) and a considerably lower sensitivity of 61% in a second study that used an assay targeting the 16S rRNA gene (138). Sensitivity of PCR is highest for pus and other body fluids but is low for blood, most likely reflecting a bacterial copy number effect. Various methods have been assessed for optimal DNA extraction from clinical samples (139), and comparisons have been made between the published real-time PCR targets, with the TTS1-*orf2* assay found to be superior in detecting *B. pseudomallei* directly from clinical specimens (140). Loop-mediated isothermal amplification is easy and quick to perform and needs minimal equipment, with amplification being achieved at a fixed temperature in a water bath or heating block. This procedure has been developed and evaluated for the detection of *B. pseudomallei* and diagnosis of melioidosis (141). The assay was sensitive and specific for the laboratory detection of *B. pseudomallei* and had 100% specificity when applied to clinical samples but had a very low diagnostic sensitivity (44%). At present, molecular assays are not sufficiently sensitive to replace conventional culture.

ISOLATION PROCEDURES

The species discussed in this chapter grow well on standard laboratory media such as 5% sheep blood and chocolate agars. Such media can be used to recover the organisms from sterile fluid or tissue where a mixed biota is not anticipated (see chapter 18). All species that have been reported to be recovered from blood, including *B. pseudomallei*, grow in broth-based blood culture systems within the standard 5-day incubation period, so that special blood culture techniques such as lysis-centrifugation and extended incubation periods are not required. The use of selective media facilitates the isolation of these organisms from specimens with mixed microbiota. With the exception of *B. vesicularis*, MacConkey agar can be used to isolate most species of these genera.

Burkholderia species grow on MacConkey agar (Fig. 2a), but the use of specific selective media with the ability to inhibit *P. aeruginosa* is preferred for the isolation of *B. cepacia* complex and *B. pseudomallei*. Several selective media have been described, and some are commercially available. A multicenter comparison of three media, PC (for *Pseudomonas cepacia*) agar (BD Diagnostics, Franklin Lakes, NJ) (142), OFPBL (for oxidation-fermentation base–polymyxin B–bacitracin–lactose) agar (BD Diagnostics) (143), and BCSA (for *B. cepacia* selective agar; Hardy Diagnostics, Santa Maria, CA) (25), showed that BCSA was superior, being both more sensitive (more *B. cepacia* isolates were recovered) and more specific (fewer other types of organisms grew) than PC or OFPBL agar (25, 144). The sensitivities of TB-T (for trypan blue-tetracycline), PC-AT (for *Pseudomonas cepacia* azelaic acid), and BCSA (25, 144) were also compared with those of three commercial media, i.e., *B. cepacia* media from MAST Diagnostics (Bootle, United Kingdom), Lab M Ltd. (Heywood, United Kingdom), and Oxoid Ltd. (Basingstoke, United Kingdom), through the analysis of 142 clinical and environmental isolates representing all species within the *B. cepacia* complex (145). BCSA and MAST *B. cepacia* medium supported the growth of *B. cepacia* complex isolates most efficiently. The latter two media were also compared in a study to evaluate the sensitivities and specificities for the isolation of *B. cepacia* complex species from sputum specimens from CF patients

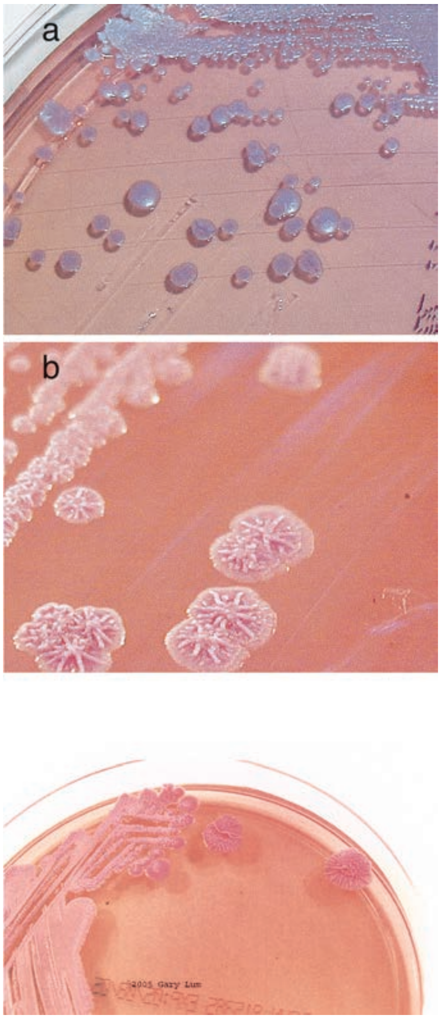

FIGURE 2 (a) *B. pseudomallei* colonies on MacConkey agar; (b) *B. pseudomallei* colonies on blood agar; (c) *B. pseudomallei* colonies on Ashdown medium agar.
doi:10.1128/9781555817381.ch43.f2

(146). BCSA was reported as being as sensitive as MAST agar but more selective.

Ashdown medium is effective for the isolation of *B. pseudomallei* (Fig. 2); crystal violet and gentamicin act as selective agents. It has been shown to be superior to MacConkey agar or MacConkey agar supplemented with colistin for the recovery of *B. pseudomallei* from clinical specimens containing mixed bacterial microbiota, such as throat, rectal, and sputum specimens (147). A more recently described selective agar, BPSA (for *B. pseudomallei* selective agar), was reported to improve the recovery of *B. pseudomallei* over that with other media (148); however, this medium is not commercially available. BPSA was more inhibitory to *P. aeruginosa* and *B. cepacia* complex species and made recognition of *Burkholderia* species easier due to their distinctive colony morphology. A clinical comparison of BPSA, Ashdown medium, and *B. cepacia* medium demonstrated equivalent sensitivity for all three media, but the selectivity of BPSA was lower than that for the other two media (149). *B. cepacia* medium is widely used and represents a good alternative when Ashdown medium is not available. An enrichment broth consisting of Ashdown medium supplemented with 50 mg of colistin is superior to standard enrichment broth such as tryptic soy broth and increases recovery of *B. pseudomallei* from clinical specimens taken from colonized sites compared with plating on Ashdown medium alone (127). Selective broth cultures should be subcultured to Ashdown medium after 48 h of incubation in air at 37°C, and all inoculated plates should be incubated at 37°C in air and examined daily for 4 days before being discarded, since some colonies become apparent to the naked eye only after extended incubation.

The use of selective media increases the isolation rates of *S. maltophilia* from clinical and environmental samples (150). Denton et al. (150) studied the sensitivity of a selective medium incorporating vancomycin, imipenem, and amphotericin B as selective agents (VIA medium) for isolating *S. maltophilia* from sputum samples collected from children with CF. This study compared the use of VIA medium to an existing in-house method that utilized an imipenem disk placed upon bacitracin-chocolate agar (BC medium) and reported an improved detection using VIA as a selective medium.

IDENTIFICATION

B. cepacia Complex and *B. gladioli*

Accurate identification of *B. cepacia* complex species can present a challenge (151). Historically, many commercial bacterial identification systems have not been able to reliably distinguish among the 18 species of the *B. cepacia* complex and often failed to differentiate these species from other closely related species such as *B. gladioli* and *Ralstonia*, *Cupriavidus*, and *Pandoraea* spp. (82, 112, 152). This has presented a significant problem for CF patients and their caregivers, as detailed in “Clinical Significance” above. The identification of *B. cepacia* complex species from CF sputum culture has a dramatic impact on patient management and is a cause of considerable anxiety for patients with CF (71, 153). Consequently, when *Burkholderia*, *Ralstonia*, *Cupriavidus*, or *Pandoraea* species are tentatively identified in a patient with CF by using a commercial system, the identity of the isolate should be confirmed by molecular techniques. To aid clinical microbiologists in the United States, the CF Foundation has established a *B. cepacia* reference laboratory, which uses a combination of phenotypic and genotypic methods (described below) to confirm the identity of suspected *B. cepacia* complex isolates (154). Further information concerning this and other reference laboratories with special expertise in *Burkholderia* can be found on the CF Foundation website (http://www.cff.org).

B. cepacia complex species may require 3 days of incubation before colonies are seen on selective media. On MacConkey or Mueller-Hinton agar, these colonies may be punctate and tenacious, and on blood agar or selective medium such as BCSA, PC agar, or OFPBL agar, the colonies are smooth and slightly raised; occasional isolates are mucoid. On MacConkey agar, colonies of the *B. cepacia* complex frequently become dark pink to red due to oxidation of lactose after extended incubation (4 to 7 days). Most clinical isolates are nonpigmented, but on iron-containing media such as a triple sugar iron slant, many strains produce a bright yellow pigment. *B. cepacia* complex species have a characteristic dirt-like odor.

The species of the *B. cepacia* complex are phenotypically very similar, making their differentiation, even with an extended panel of biochemical tests, rather difficult (Table 1)

(112). Further, isolates within these species show considerable phenotypic variability, which is likely due to their unusually large genomes rich in insertion sequences and mobile elements such as plasmids, transposons, and bacteriophages (155). These features can contribute to genetic plasticity and diversity, which, when differentially expressed in isolates, result in variable biochemical phenotypic profiles. Most strains are weakly oxidase positive, although some strains of *B. contaminans*, *B. lata*, and *B. pyrrocinia* are oxidase negative. *B. multivorans*, *B. pseudomultivorans*, *B. stabilis*, and *B. dolosa* rarely oxidize sucrose. *B. stabilis* is ornithine decarboxylase positive, as are most *B. cenocepacia* strains, but is distinctive in that more than two-thirds of strains are *o*-nitrophenyl-β-D-galactopyranoside (ONPG) negative. *B. stabilis*, *B. lata*, and most *B. ambifaria* strains show poor growth at 42°C. *B. dolosa* is usually lysine decarboxylase negative, whereas only approximately half of *B. multivorans* strains are negative. Other *B. cepacia* complex species are usually lysine decarboxylase positive. *B. vietnamiensis* and most *B. anthina* strains do not oxidize adonitol. *B. anthina* strains show a distinctive creamy morphology on BCSA, which also turns pink (i.e., alkaline) despite the ability of this species to utilize sucrose (156).

Phenotypic differentiation of *B. cepacia* complex species from *B. gladioli* and *Pandoraea* spp. is also difficult (Table 1). Cellular fatty acid analysis is unable to differentiate *B. cepacia* complex species from *B. gladioli* (157). However, in contrast to *B. cepacia* complex species, most *B. gladioli* strains are oxidase negative, and whereas most *B. cepacia* complex strains oxidize maltose and lactose, *B. gladioli* typically oxidizes neither. *Pandoraea* spp. do not oxidize maltose, lactose, xylose, sucrose, or adonitol, and most are ONPG negative. *B. cepacia* complex species also may be difficult to differentiate from *Ralstonia* and *Cupriavidus* species. However, several of the latter species show a fast and strong oxidase reaction, whereas *B. cepacia* complex species produce a slow, weak-positive oxidase test. Further, in contrast to most *B. cepacia* complex species, *Ralstonia* and *Cupriavidus* are lysine decarboxylase negative and most often ONPG negative.

The challenge in differentiating *B. cepacia* complex species prompted the development of molecular genetic diagnostic tests capable of identifying these species individually and distinguishing them (as a group) from biochemically similar species. DNA sequence differences in 16S and 23S rRNA genes have been used to develop species-specific PCR

TABLE 1 Characteristics of the *B. cepacia* complex, *B. gladioli*, and *Pandoraea* spp.[a]

Test	*B. cepacia* complex																			
	B. ambifaria	*B. anthina*	*B. arboris*	*B. cenocepacia*	*B. cepacia*	*B. contaminans*	*B. diffusa*	*B. dolosa*	*B. lata*	*B. latens*	*B. metallica*	*B. multivorans*	*B. pseudomultivorans*	*B. pyrrocinia*	*B. seminalis*	*B. stabilis*	*B. ubonensis*	*B. vietnamiensis*	*B. gladioli*	*Pandoraea* spp.
Oxidase[b]	+	+	+	+	+	V	+	+	V	+	+	+	+	V	+	+	+	+	V	V
Growth:																				
MacConkey[c]	+	+	+	V	V	+	+	+	+	+	+	+	+	+	+	+	+	V	+	+
BCSA[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	V
42°C[c]	V	V	V	V	V	V	V	+	−	+	+	+	+	V	+	−	V	+	−	V
Yellow pigment[c]	V	−	V	−	V	V	−	−	V	−	V	−	−	V	V	−	−	−	V	−
Brown pigment[c]	−	−	−	V	−	−	−	−	−	−	−	−	−	−	−	−	−	−	V	−
Hemolysis[d]	V β	−	V β	−	−	V	−	−	−	−	−	−	−	V β	−	−	−	V β		
Acid from[e]:																				
Maltose[c]	+	+	+	V	V	V	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Lactose[b]	+	+	V	+	+	+	+	+	+	+	+	+	+	+	+	V	+	+	−	−
D-Xylose[c]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	V	+	V	+	−
Sucrose[b]	+	V	V	+	V	+	V	−	V	+	V	−	−	V	V	−	+	+	−	−
Adonitol[c]	+	V	+	V	V	+	V	+	V	+	+	+	V	+	+	+	−	−	+	−
Nitrate reduction[c,g]	V	V	V	V	−	V	+	+	V	−	−	+	V	V	−	−	V	V	V	V
Lysine decarboxylase[b]	+	V	V	+	+	+	+	−	+	+	+	V	+	+	V	+	−	+	−	−
Ornithine decarboxylase[c]	−	−	+	V	V	−	−	−	V	−	−	−	−	V	V	+	−	−	−	−
Esculin hydrolase[c,g]	V	−	−	V	V	V	−	−	V	−	+	−	−	−	V	−	−	−	V	−
Gelatinase[c,g]	+	−	+	V	V	+	V	−	V	V	+	−	−	V	+	+	+	−	V	−
PNPG or ONPG[b,f]	+	V	+	+	+	+	+	+	V	+	+	+	+	+	+	V	−	+	+	V

[a] Abbreviations and symbols: +, >90% of isolates are positive; V, 10 to 90% are positive; −, <10% of isolates are positive.

[b] Number of strains of each species tested: *B. ambifaria*, 51; *B. anthina*, 24; *B. arboris*, 16; *B. cenocepacia*, 928; *B. cepacia*, 181; *B. contaminans*, 54; *B. diffusa*, 16; *B. dolosa*, 57; *B. lata*, 25; *B. latens*, 6; *B. metallica*, 7; *B. multivorans*, 715; *B. pseudomultivorans*, 11; *B. pyrrocinia*, 85; *B. seminalis*, 19; *B. stabilis*, 73; *B. ubonensis*, 2; *B. vietnamiensis*, 145; *B. gladioli*, 280; and *Pandoraea* spp., 75. Data from references 112, 156, and 162 and from LiPuma (unpublished) and D. A. Henry (unpublished data).

[c] Number of strains of each species tested: *B. ambifaria*, 18; *B. anthina*, 16; *B. arboris*, 13; *B. cenocepacia*, 139; *B. cepacia*, 23; *B. contaminans*, 7; *B. diffusa*, 6; *B. dolosa*, 12; *B. lata*, 11; *B. latens*, 6; *B. metallica*, 3; *B. multivorans*, 109; *B. pseudomultivorans*, 11; *B. pyrrocinia*, 5; *B. seminalis*, 13; *B. stabilis*, 27; *B. ubonensis*, 2; *B. vietnamiensis*, 36; *B. gladioli*, 27; and *Pandoraea* spp., 9. Data from references 112, 156, and 162 and from Henry (unpublished).

[d] Hemolysis of sheep blood; β, beta-hemolysis.

[e] Oxidation test results were recorded after 2 to 7 days of incubation.

[f] PNPG, *p*-nitrophenyl-β-D-glucoside.

[g] Results presented are from the API 20 NE test strip.

assays for the identification of several *B. cepacia* complex species (154, 158), as well as *B. gladioli* (159). *B. multivorans*, *B. vietnamiensis*, and *B. dolosa* can be reliably identified with 16S rRNA-targeted assays, but insufficient sequence variation in rRNA genes exists to enable reliable separation of the remaining *B. cepacia* complex species. Fortunately, species-specific sequence variation does exist in the *recA* gene, and PCR assays targeting this locus enable the reliable identification of the *B. cepacia* complex species most commonly recovered from human specimens (160–164). Other 16S rRNA- and *recA*-based PCR assays identify all *Burkholderia* spp. (i.e., at the genus level) or all species within the *B. cepacia* complex (i.e., as a group) (154, 161, 165).

Another molecular genetic approach to identifying *B. cepacia* complex species involves restriction fragment length polymorphism (RFLP) analysis of either 16S rRNA or *recA* genes (161). Again, insufficient sequence variation in the 16S rRNA gene limits the use of RFLP analysis of this locus, even when multiple restriction enzymes are used (166). In contrast, *recA* RFLP analysis has proved quite useful in reliably distinguishing all species within the *B. cepacia* complex (161–165).

Although a multilocus sequence typing (MLST) scheme for the *B. cepacia* complex was developed primarily as a tool to study the epidemiology and population genetic structure of these species (167), it offers another approach to differentiate species within this group. This scheme was subsequently modified to allow typing of all species within the genus *Burkholderia* (168). Other genomic approaches, including amplified fragment length polymorphism typing, ribotyping, and whole-cell protein profiling, have been proposed for the differentiation of *B. cepacia* complex species (169–171). However, these methods are time-consuming and expensive and require an extensive validated database before isolates can be reliably identified. These limitations render them impractical for use in a routine diagnostic laboratory. Cellular fatty acid methyl ester analysis is useful for identification of *Burkholderia* strains at the genus level but is not reliable for identification of individual *B. cepacia* complex species and does not differentiate *B. gladioli* (19).

More recently, several studies have reported good results in using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) for the identification of *Burkholderia* spp. (172–181). This method relies on a comparison between the mass spectrum of an isolate and the mass spectra in available databases. The discriminatory power of MALDI-TOF MS varies depending on the species and the breadth of the database used (i.e., some bacterial taxa are underrepresented in databases). In general, studies of MALDI-TOF MS for identification of *Burkholderia* (and the other species discussed in this chapter) have examined relatively small numbers of isolates, and/or not all species in the *B. cepacia* complex have been included. For example, Desai et al. (180) found that when compared with genotype-based methods, MALDI-TOF MS correctly identified to the genus level 29 isolates belonging to six *B. cepacia* complex species; 24 of these were correctly identified to the species level. As MALDI-TOF MS spectra library databases are enhanced with additional species reference strains and greater numbers of isolates, it is expected that this method will provide a reliable alternative to genotype-based analyses. The seemingly ever-expanding number of novel *Burkholderia* species identified among "*B. cepacia*-like" isolates recovered from human specimens (e.g., the recent description of *B. pseudomultivorans*) will continue to challenge phenotype-based analyses, however.

B. pseudomallei and *B. mallei*

B. pseudomallei colonies on blood agar are typically small, smooth, and creamy in the first 48 h. On further incubation, this appearance may change to dry, wrinkled colonies. *B. pseudomallei* on Ashdown agar grows as very small (pinpoint) colonies by 18 h, which are usually purple, flat, dry, and wrinkled ("cornflower head") after 48 h of incubation (Fig. 2c). The organism is motile, indole negative, oxidase positive, and resistant to colistin and gentamicin, features that aid identification. Other typical biochemical reactions are shown in Table 2. *B. pseudomallei* produces a distinctive musty or earthy odor, but sniffing of open plates should never be undertaken on safety grounds. *B. thailandensis* may be indistinguishable from *B. pseudomallei* by these simple criteria but can be distinguished based on arabinose assimilation, since *B. thailandensis* can utilize L-arabinose as the sole carbon source but neither *B. pseudomallei* nor *B. mallei* can do so (182). *B. thailandensis* is found in clinical samples extremely rarely, and cases reported of human *B. thailandensis* infection in which the bacterial species was fully verified amount to a single patient (183). *B. mallei* is also an extreme rarity in clinical specimens from humans but could be confused with *B. pseudomallei* and *B. thailandensis*. Two differentiating tests in the event that *B. mallei* is suspected or needs to be ruled out are (i) that *B. mallei* is nonmotile and *B. pseudomallei* and *B. thailandensis* are motile and (ii) that *B. mallei* is susceptible to gentamicin while the other two species are inherently resistant. The latter represents a catch for the inexperienced microbiologist, since Ashdown medium normally contains gentamicin and thus fails to support the growth of *B. mallei*. Cellular fatty acid profiles may be useful for differentiating *B. pseudomallei* from other genera, but reports vary on its utility in differentiating *B. thailandensis* and *B. pseudomallei* (184) or other pathogenic *Burkholderia* species including *B. mallei*, *B. cepacia* complex species, and *B. gladioli*.

Multiple evaluations have been performed to determine the accuracy of API 20NE for the identification of *B. pseudomallei*, with the reported percentage of isolates identified correctly ranging from 37 to 99% (183, 185–189). One possible reason for this interstudy variability is that *B. pseudomallei* is phenotypically distinct in different geographic areas and/or between clinical isolates and those from the environment. A study using a large collection ($n = 800$) of *B. pseudomallei* isolates obtained from clinical cases, the environment, and animals from seven Asian countries and northern Australia reported that the API 20NE correctly identified 99% of *B. pseudomallei* isolates (185). This supports the use of API 20NE for the identification of *B. pseudomallei*. API 20NE is unable to identify *B. mallei* or *B. thailandensis* (185). The automated VITEK 1 system provides accurate identification of *B. pseudomallei* (189). Two evaluations of the VITEK 2 colorimetric GN card system have reported an accuracy of ~80% for *B. pseudomallei*, the most common incorrect identification being the *B. cepacia* group (186, 190). The accuracy of VITEK 2 has been reported to be affected by the medium on which *B. pseudomallei* is grown prior to testing, with culture on Columbia horse blood agar being associated with the highest rate of accuracy (190). A recent study has also shown geographic variation in the accuracy of VITEK 2 (191). An evaluation of MALDI-TOF MS for the identification of *Burkholderia* spp. reported accurate identification of *B. pseudomallei* but misidentification of *B. thailandensis* as *B. pseudomallei* (176). This was considered to be due to limitations of the reference spectra

TABLE 2 Characteristics of *B. mallei*, *B. pseudomallei*, and *B. thailandensis*[a]

Test	*B. mallei*	*B. pseudomallei*	*B. thailandensis*
Oxidase	v	+	+
Growth:			
MacConkey	+	+	+
42°C	−	+	+
Nitrate reduction	+	+	+
Gas from nitrate	−	+	+
Arginine dihydrolase	+	+	+
Lysine decarboxylase	−	−	−
Ornithine decarboxylase	−	−	−
Hydrolysis:			
Urea	v	v	v
Citrate	−	v	v
Gelatin	−	v	v
Esculin	−	v	v
Acid from:			
Glucose	+	+	+
Xylose	v	+	+
Lactose	v	+	+
Sucrose	−	v	v
Maltose	−	+	+
Mannitol	−	+	+
Arabinose	ND	−	+
Motility	0	100%	100%
No. of flagella	0	≥2	≥2

[a] Abbreviations and symbols: +, ≥90% of isolates are positive; v, 10 to 90% are positive; −, <10% of isolates are positive; 0, none are motile; 100%, all are motile; ND, not determined. Data from reference 198.

database. In a subsequent study, whole-cell MALDI-TOF MS was evaluated for the identification of *B. mallei* and *B. pseudomallei* after a library of reference spectra was generated (175). Both species could be identified provided the dedicated subset of the reference spectra library was used. MALDI-TOF MS requires additional evaluation to determine its accuracy in clinical practice and also with *B. pseudomallei* from diverse geographic locations. Because of the difficulty associated with accurate laboratory identification, referral to a reference laboratory is advised when isolation of *B. pseudomallei* or *B. mallei* is suspected. This is especially important with the advent of emerging bioterrorism legislation in many countries (see chapter 14).

B. pseudomallei must be differentiated from *Pseudomonas stutzeri* and *B. cepacia* complex species in clinical specimens. *P. stutzeri* appears very similar to *B. pseudomallei* after a few days of incubation, and both *B. pseudomallei* and *B. cepacia* complex species may be isolated from persons with CF (88, 89). Whereas *B. pseudomallei* produces gas from nitrate and is arginine dihydrolase positive, most *B. cepacia* complex isolates are negative for both characteristics. *P. stutzeri* is negative for arginine dihydrolase, oxidation-fermentation glucose, and gelatin hydrolysis. *P. stutzeri* also has only one flagellum, and *B. pseudomallei* has more than one.

Ralstonia **and** ***Cupriavidus*** **spp.**

Although *R. pickettii* was considered to be the *Ralstonia* species most frequently isolated from clinical specimens, the recent recognition that several other *Ralstonia* and *Cupriavidus* species can be identified from among *R. pickettii*-like isolates limits previous observations. As is the case with *B. cepacia* complex species, *Ralstonia* and *Cupriavidus* species are phenotypically similar, requiring rather extensive biochemical testing to reliably differentiate them; species-level identification with standard biochemical testing is difficult (Table 3). These species may grow slowly on primary isolation media, requiring ≥72 h of incubation before colonies are visible. They are lysine decarboxylase negative and generally catalase positive, although catalase-negative *R. pickettii* strains have been described (19, 20). Most species show a fast and strong oxidase reaction; however, the intensity of the oxidase reaction varies for *R. mannitolilytica*, *R. pickettii*, and *C. gilardii*, with some strains showing a weakly positive reaction (19, 20; LiPuma, unpublished). *R. pickettii*, *R. mannitolilytica*, and *R. insidiosa* grow on BCSA; most *Cupriavidus* strains do not, but growth is strain dependent. These species do not produce acid from sucrose. Most *R. mannitolilytica* strains acidify lactose, whereas most strains from other species do not. *R. insidiosa*, *C. respiraculi*, *C. gilardii*, and *C. paucula* are differentiated from *R. pickettii* and *R. mannitolilytica* in failing to acidify glucose. *Cupriavidus* species have a characteristic cellular fatty acid profile different from that of other *Ralstonia* species (17, 20). The main fatty acid components of these species are C16:0, C16:1 w7c, and C18:1 w7c (each accounting for 20 to 30% of the overall fatty acid content); in addition, C14:0, C14:0 3OH, and C17:0 cyclo are always present (each accounting for 5 to 10% of the overall fatty acid content) (21, 192).

Molecular genetic tests have proved quite helpful in differentiating these species. A 16S rRNA-directed PCR assay reliably identifies all *Ralstonia* and *Cupriavidus* species (as a group), allowing their differentiation from the phenotypically similar species in the genera *Burkholderia* and *Pandoraea* (113). Species-specific 16S rRNA-based PCR assays have also been developed; these enable the accurate identification of *R. pickettii*, *R. mannitolilytica*, *R. insidiosa*, and *C. respiraculi*

TABLE 3 Characteristics of *Ralstonia* and *Cupriavidus* spp.[a]

Test	*R. pickettii*	*R. mannitolilytica*	*R. insidiosa*	*C. respiraculi*	*C. gilardii*	*C. pauculus*
Catalase	v	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Growth:						
BCSA	+	+	+	−	−	v
42°C	v	+	ND	ND	+	v
Colistin resistance	+	+	ND	ND	ND	−
Nitrate reduction	+	−	+	v	−	−
Tween 80 hydrolysis	+	+	ND	ND	−	+
Urease	+	+	V	−	−	+
Lysine decarboxylase	−	−	−	−	−	−
ONPG	−	v	v	−	v	ND
Acid from:						
L-Arabinose	+	+	ND	ND	−	−
D-Arabitol	−	+	ND	ND	ND	−
Glucose	+	+	−	−	−	−
Inositol	−	−	ND	ND	ND	−
Lactose	v	+	v	−	−	−
Maltose	v	+	ND	ND	ND	−
Mannitol	−	+	ND	ND	ND	−
Sucrose	−	−	−	−	−	−
Xylose	+	+	ND	ND	−	−
Motility	+	+	ND	ND	+	+
Flagella	1 polar	1 polar	ND	ND	1 polar	Peritrichous

[a] Abbreviations and symbols: +, ≥90% of isolates are positive; v, 10 to 90% are positive; −, <10% of isolates are positive; ND, not determined. Data from references 17–20 and from LiPuma (unpublished).

(21, 113, 193). MALDI-TOF MS has been reported to provide accurate identification of small numbers of certain *Ralstonia* species (172, 194).

Pandoraea spp.

Overall, the biochemical profiles of *Pandoraea* strains are similar to those of *Burkholderia* and *Ralstonia* strains isolated from clinical specimens (Table 1) (3, 112, 125). The lack of saccharolytic activity is indicative of *Pandoraea* but is also seen with some *Ralstonia* species. Definitive identification of putative *Pandoraea* isolates requires molecular confirmation. Coenye et al. (195) described 16S rDNA-based PCR assays for the identification of these bacteria. A PCR assay was developed for the identification of *Pandoraea* isolates to the genus level. PCR assays for the identification of *P. apista* and *P. pulmonicola* (as a group), *P. pnomenusa*, *P. sputorum*, and *P. norimbergensis* were also developed. *Pandoraea* strains can be differentiated from *Burkholderia* and *Ralstonia* strains by their specific 16S rRNA gene restriction profile (112, 196) and can be identified at the species level through MspI restriction analysis of the *gyrB* gene (197). A quantitative comparison of the whole-cell fatty acid profiles of the members of these three genera allows the differentiation of *Pandoraea* strains from the others (20, 125). However, with the use of the commercially available microbial identification system database (Microbial ID, Inc., Newark, DE), these organisms are mostly identified with low identification scores as *Burkholderia* or *Ralstonia* species (20, 112) due to a lack of discriminatory fatty acids. Individual strains of *Pandoraea* species have been reported to be accurately identified by MALDI-TOF MS (172, 194).

S. maltophilia

Key features for identifying *S. maltophilia* include oxidation of glucose and maltose with a more intense reaction with the latter, positive reactions for DNase and lysine decarboxylase, and a tuft of polar flagella (Table 4) (198). Although most strains were previously believed to be oxidase negative, testing of large numbers of isolates recovered from human specimens and referred to a reference laboratory indicates that as many as 20% may be oxidase positive (199). This proportion may be higher than expected since strains with an "atypical" phenotype may be preferentially referred for analysis. Detection of extracellular DNase activity by *S. maltophilia* is a key to differentiating this species from most other glucose-oxidizing, Gram-negative bacilli. It can be detected on tube-based or plated DNase medium with a methyl green indicator. Care must be taken when interpreting the DNase reaction, since one report documented the misidentification of *S. maltophilia* as *B. cepacia* partially based on false-negative DNase reactions that were finalized with 48 h of incubation rather than 72 h (200). Selected isolates of *Flavobacterium* and *Shewanella* spp. may also be DNase positive (see chapter 44). On sheep blood agar, colonies appear rough and lavender-green and have an ammonia-like odor. *S. maltophilia* has a characteristic cellular fatty acid profile with large amounts (>30%) of 13-methyl tetradecanoic acid ($C_{15:0\ iso}$) and lesser amounts (>10%) of 12-methyl tetradecanoic acid ($C_{15:0\ anteiso}$) and *cis*-9-hexadecenoic acid ($C_{16:1\ cis9}$) (198). To overcome the problems associated with definitive identification of *S. maltophilia*, Whitby et al. (201) developed a species-specific PCR assay targeting the 23S rRNA gene and reported sensitivity and specificity of 100%. This PCR test was used as a standard to evaluate the identification of *S. maltophilia* using the API 20NE strip and the VITEK 2 ID-GNB card (202). Both systems showed good reliability compared to PCR. A multiplex PCR assay to identify *P. aeruginosa*, *B. cepacia* complex species, and *S. maltophilia* directly in sputum and oropharyngeal specimens from CF patients has been reported, but only a very limited number of *S. maltophilia* isolates were examined. MALDI-TOF MS appears to be an acceptable alternative for identification of *S. maltophilia* (172, 194); however, Desai et al. (180) found

TABLE 4 Characteristics of *Acidovorax*, *Brevundimonas*, *Delftia*, *Comamonas*, and *Stenotrophomonas* spp. found in clinical specimens[a]

Test	A. *delafieldii* (*n* = 2)	A. *facillis* (*n* = 2)	A. *temperans* (*n* = 2)	B. *diminuta* (*n* = 68)	B. *vesicularis* (*n* = 94)	D. *acidovorans* (*n* = 69)	*Comamonas* spp. (*n* = 28)	S. *maltophilia* (*n* = 228)
Oxidase	100	100	100	100	98	100	100	20[b]
Growth:								
MacConkey	100	0	100	100	43	100	100	100
Cetrimide	0	0	0	0	0	4	0	2
6.0% NaCl	0	0	0	21	23	6	0	22
42°C	50	0	100	38	19	29	68	48
Nitrate reduction	100	100	100	3	5	99	96	39
Gas from nitrate	0	0	100	0	0	0	0	0
Pigment	Yellow, soluble	None	Yellow, soluble	Brown-tan, soluble	52% yellow-orange, insoluble[e]	26% fluorescent, 44% yellow-tan, soluble	27% yellow-brown, soluble	Brown-tan, soluble
Arginine dihydrolase	100	100	0	0	0	0	0	0
Lysine decarboxylase	0	0	0	0	0	0	0	93
Ornithine decarboxylase	0	0	0	0	0	0	0	0
Indole	0	0	0	0	0	0	0	0
Hemolysis	0	0	0	0	0	0	0	1
Hydrolysis:								
Urea	100	100	50	13	2	0	7	3
Citrate	100	0	0	1	1	94	47	34
Gelatin	0	100	0	68	25	11	0	93
Esculin	0	0	0	5	88	0	0	39
Acid from:								
Glucose[c]	100	100	100	21	87	0	0	85
Xylose	85	100	0	0	27	0	0	35
Lactose	0	0	0	0	0	0	0	60
Sucrose	0	0	0	0	0	0	0	63
Maltose	0	0	0	0	94	0	0	100
Mannitol	50	100	50	0	0	100	0	0
H_2S[d]	100	100	100	34	49	57	0	95
Motility	100	100	100	100	100	100	100	100
No. of flagella	1–2	1–2	1–2	1–2	1–2	>2	>2	>2

[a] Values are the percentages of strains positive. Data from reference 198.

[b] 793 isolates tested by using BactiDrop Oxidase (Remel, Dartford, United Kingdom) (LiPuma, unpublished).

[c] Oxidation-fermentation basal medium with 1% carbohydrate.

[d] Lead acetate paper.

[e] Pigment observed on Thayer-Martin agar.

that some strains were incorrectly identified as *Pseudomonas* species by MALDI-TOF MS. As is the case with several nonfermenting opportunistic species, an incomplete understanding of *Stenotrophomonas* taxonomy challenges accurate identification of clinical and environmental isolates.

Acidovorax, *Brevundimonas*, *Delftia*, and *Comamonas* spp.

Characteristics of *Acidovorax*, *Brevundimonas*, *Delftia*, and *Comamonas* are given in Table 4.

Acidovorax species, rarely encountered in clinical and environmental samples, are straight to slightly curved, Gram-negative bacilli that occur either singly or in short chains. They are oxidase positive and nonpigmented and have a single polar flagellum. Urease activity varies among strains (198).

B. diminuta and *B. vesicularis*, infrequently encountered in clinical and environmental samples, have growth requirements for specific vitamins, including pantothenate, biotin, and cyanocobalamin. An additional growth requirement for *B. diminuta* is cysteine. Most strains of *B. diminuta* grow on MacConkey agar, while only ~25% of *B. vesicularis* strains do so. On primary isolation media, *B. diminuta* colonies are chalk white whereas many strains of *B. vesicularis* are characterized by an orange intracellular pigment. These organisms are oxidase positive, have a single polar flagellum, and weakly oxidize glucose (*B. vesicularis* more so than *B. diminuta*), and the vast majority fail to reduce nitrate to nitrite. The most reliable method for differentiating these two species is the test for esculin hydrolysis. Almost all strains of *B. vesicularis* (88%) are reported to hydrolyze this substrate, while *B. diminuta* strains rarely do (5%) (Table 4) (198).

Comamonas spp. are straight to slightly curved, Gram-negative bacilli that occur singly or in pairs. The organisms are catalase and oxidase positive and have a single tuft of polar flagella. All human clinical *Comamonas* species reduce nitrate to nitrite. Phenotypic differentiation of *C. terrigena* from *C. testosteroni* is difficult, and as a result, isolates are typically reported as *Comamonas* spp. (Table 4).

D. acidovorans is phenotypically similar to *Comamonas*. Key characteristics of the species include abilities to oxidize fructose and mannitol. One-quarter of the strains produce a fluorescent pigment, while approximately one-half of the strains may produce a soluble yellow to tan one (26, 198).

TYPING SYSTEMS

Several molecular genetic methods are available to assess the relatedness of isolates of these genera during nosocomial or community outbreak investigations. These methods are preferred over phenotypically based systems, which are less discriminatory and reproducible. Analysis of whole-genome macrorestriction profiles with pulsed-field gel electrophoresis (PFGE) has gained acceptance as a preferred genotyping method and has proved useful in numerous studies of *Burkholderia*, *Ralstonia*, and *S. maltophilia* (111, 203). The endonucleases XbaI and SpeI are most frequently used and typically yield a dozen or more DNA fragments for analysis. Care must be taken in interpreting PFGE profiles of *Burkholderia* species, however. These species have unusually large and dynamic multichromosome genomes that are prone to large-scale alterations in content and arrangement (204). Consequently, epidemiologically irrelevant genomic polymorphisms may arise in the short term and confound outbreak investigations (203). Ribotyping, which relies on polymorphisms in and around rRNA operons, has been used to investigate the epidemiology of *B. cepacia* complex and *B. pseudomallei* (205–207). Both PFGE and ribotyping are relatively time-consuming and expensive to perform and are therefore not particularly well suited for routine analysis by clinical microbiology laboratories. A variety of PCR-based methods, including randomly amplified polymorphic DNA typing and repetitive-sequence PCR typing, offer attractive alternatives for genotyping *S. maltophilia* and *Burkholderia*, *Ralstonia*, and *Pandoraea* spp. (50, 122, 196, 208, 209). These methods are inexpensive and can provide rapid, reliable results. MLST, which assesses DNA sequence variation at several chromosomal loci, has been developed for numerous species, including the *B. cepacia* complex, *B. pseudomallei*, and *B. mallei* (167, 210, 211). A modification of the scheme developed for the *B. cepacia* complex enables MLST analysis of all species within the genus (168). This genotyping strategy provides robust, reproducible, and portable results and is quickly becoming the preferred method for investigating bacterial epidemiology, evolution, and population structure. Both repetitive-sequence PCR using a BOX A1R primer and multilocus variable-number tandem repeat analysis have been developed for *B. pseudomallei* to exclude a clonal outbreak (212, 213). Typing methods have not been reported for *Brevundimonas*, *Delftia*, *Comamonas*, or *Acidovorax* spp.

SEROLOGIC TESTS

Of the organisms discussed in this chapter, *B. pseudomallei* is the only one for which serologic tests have been used clinically to diagnose the infection. The indirect hemagglutination assay, although not available commercially, is the most widely used test. It is performed by using a prepared antigen from strains of *B. pseudomallei* sensitized to sheep cells and includes unsensitized cells as a control. This assay can be adapted to a microtiter plate test system. The serologic tests currently in use have limited value for the diagnosis of melioidosis in persons who have lived in regions where melioidosis is endemic because the healthy indigenous population is often seropositive (56, 92, 214). Serologic testing is potentially useful in persons who do not normally reside in regions endemic for melioidosis, including returning travelers and laboratory workers following accidental laboratory exposure to *B. pseudomallei* (215). The interpretation of the indirect hemagglutination assay or other serologic assays is complicated by the fact that there are no validated guidelines, and different cutoff points have been used to define seroconversion following exposure and acute infection. Testing should be performed whenever possible on paired samples. Seroconversion with the development of detectable antibodies to *B. pseudomallei* in the second sample is supportive of exposure. A 4-fold rise in titer is commonly used to diagnose a range of infectious diseases, but this has not been validated for melioidosis and any reproducible rise between two samples should be viewed as possible evidence of exposure. A single high titer in persons from a nonendemic region with a relevant travel history who presents late after a putative exposure event and for whom paired sera may be less relevant is also suggestive of exposure. Some individuals with culture-proven melioidosis do not have detectable antibodies (216), and so a negative serologic test does not rule out exposure or melioidosis. Given the complexity of this situation, experts in the field should be consulted when serology is used to diagnose melioidosis. Several evaluations of a commercial rapid immunochromatographic test kit (Pan-Bio, Windsor, Queensland, Australia) for the detection of IgG and IgM antibodies to *B. pseudomallei* have been performed (217–219), but this test is not currently available.

ANTIMICROBIAL SUSCEPTIBILITIES

Specific susceptibility testing interpretative criteria are not available for all of the species discussed in this chapter. For some species, such as the *B. cepacia* complex and *S. maltophilia*, interpretive criteria for disk diffusion testing are available for only a limited number of antibiotics. In general, MIC microbroth microdilution tests or Etests are preferred for this group of organisms.

B. cepacia complex species are among the most antimicrobial-resistant bacteria encountered in the clinical laboratory. These species are intrinsically resistant to aminoglycoside and polymyxin antibiotics and are often resistant to β-lactam antibiotics due to inducible chromosomal β-lactamases and altered penicillin-binding proteins (220). Antibiotic efflux pumps may mediate resistance to chloramphenicol, fluoroquinolones, and trimethoprim (221). Clinical strains may be susceptible to only a handful of agents, including trimethoprim-sulfamethoxazole (TMP-SMX), ceftazidime, chloramphenicol, minocycline, imipenem, meropenem, and some fluoroquinolones (222, 223). The glycylcycline antibiotic tigecycline shows highly variable activity *in vitro* (224). The relatively high MIC observed for some strains and the potential for discoloration of permanent teeth in children younger than 7 years of age limit the use of tigecycline in CF patients. Clinical and Laboratory Standards Institute (CLSI) interpretative criteria for disk diffusion susceptibility testing are available for ceftazidime, meropenem, minocycline, and TMP-SMX (225). Because isolates that are initially susceptible may become resistant during the course of therapy, susceptibility testing of repeat isolates may be warranted. Furthermore, strains recovered from patients with CF who have received repeated courses of antibiotic therapy are frequently resistant to all currently available antimicrobial agents (222). Combinations of antimicrobial agents may provide synergistic activity against resistant strains; however, antagonism with combinations is also observed *in vitro* (222).

B. pseudomallei is intrinsically resistant to penicillins, aminoglycosides, and macrolides. Susceptibility testing should be performed to the antimicrobial agents commonly used to treat melioidosis, which are ceftazidime, imipenem or meropenem, amoxicillin-clavulanate, doxycycline, and

TMP-SMX (226). *B. pseudomallei* is usually susceptible to all of these agents with the exception of TMP-SMX, reported rates of resistance for which are in the order of 2% in Australia (227, 228) and 13 to 16% in northeast Thailand (229, 230). Disk diffusion testing of TMP-SMX overestimates resistance and is unreliable (228–230); acceptable alternatives include Etest, broth microdilution, and agar dilution (226). Fluoroquinolones are associated with a high rate of therapeutic failure (231) and should not be included in the test panel. Recent studies indicate that tigecycline has good activity against *B. pseudomallei in vitro* and is effective when combined with other agents in an animal model of *B. pseudomallei* infection (232, 233), but there are no clinical trial data to support its use.

Current trends in the management of melioidosis involve an initial 10- to 14-day intensive therapy phase with ceftazidime or meropenem, followed by eradication therapy with TMP-SMX for at least 3 months (83, 84, 234, 235). A recent trial from Thailand supported dropping the traditional practice of adding doxycycline to TMP-SMX for the eradication phase of therapy (236). In Australia, TMP-SMX is added to ceftazidime or meropenem during the intensive phase for neurological, prostatic, cutaneous, and bone and joint melioidosis. Amoxicillin-clavulanate is recommended for eradication therapy in pregnancy and is an alternative to TMP-SMX in children (237). In critically ill patients requiring intensive care, meropenem or imipenem may be superior to ceftazidime, and granulocyte colony-stimulating factor is being used in some centers, although a study from Thailand showed no benefit (227, 238–240). From a molecular genotyping study of cases of recurrent melioidosis, relapse following antimicrobial therapy occurred in 9.7% of patients and a new infection occurred in 3.4% (95).

Because of the potential role of *B. mallei* as a bioterrorism agent, studies have been done recently to determine the activities of a variety of agents against this species. *B. mallei* has a susceptibility profile similar to that of *B. pseudomallei*, except that *B. mallei* is susceptible to aminoglycosides and newer macrolides such as clarithromycin and azithromycin, whereas *B. pseudomallei* is resistant (241). Current recommended treatment and duration of therapy for glanders are the same as those for melioidosis.

Guidelines on the management of accidental laboratory exposure to *B. pseudomallei* and *B. mallei* have been published (215, 242).

S. maltophilia is intrinsically resistant to many classes of antibiotics. Resistance can also develop rapidly during infection (243). Resistance to β-lactam agents is mediated by at least two β-lactamases, one of which is zinc dependent and resistant to β-lactamase inhibitors and confers resistance to imipenem. Aminoglycoside and quinolone resistance results from mutations in outer membrane proteins. In a study of isolates recovered from patients with CF, doxycycline was the most active agent *in vitro* (244). TMP-SMX is usually active and is often used in combination with ticarcillin-clavulanate, minocycline, or piperacillin-tazobactam (244). Other combinations that may be effective include ciprofloxacin paired with ticarcillin-clavulanate, ciprofloxacin and piperacillin-tazobactam, or doxycycline and ticarcillin-clavulanate. Tigecycline is reported to have good activity *in vitro* (224). CLSI interpretive criteria for disk diffusion susceptibility testing are available for minocycline, levofloxacin, and TMP-SMX (225). Many U.S. laboratories comment only on the activity of TMP-SMX but will test additional antibiotics such as minocycline, ceftazidime, ticarcillin-clavulanate, and ciprofloxacin or levofloxacin upon request.

In general, *C. testosteroni* is susceptible to extended- and broad-spectrum cephalosporins, carbapenems, quinolones, and TMP-SMX (245). *D. acidovorans* is frequently resistant to the aminoglycosides.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The species discussed in this chapter are found in the natural environment and may occasionally contaminate clinical specimens. Nevertheless, they are increasingly recognized as nosocomial and opportunistic pathogens, especially in certain patient populations, such as persons with CF. They are also frequently misidentified by commercial microbial identification systems. Therefore, their recovery in the clinical laboratory must be given careful consideration. In particular, species of the *B. cepacia* complex are not reliably differentiated by phenotypic analyses, and their recovery from persons with CF has serious consequences with respect to patient management and psychosocial well-being (69). Identification of these species should be confirmed by genotypic analyses at a reference laboratory and should promptly be reported to the CF care team. Recovery of *B. pseudomallei* and *B. mallei* in any context should always be considered to reflect clinical disease. Identification of these species should be confirmed by a reference laboratory with experience with these species. Care must be given to ensure that culture handling and shipping comply with current biosafety regulations (see chapter 18). Identification of these species must be reported to public health officials due to the potential of these species as agents of bioterrorism (see chapter 14). The relevance of the recovery of the other genera described in this chapter, outside the context of CF, is less clear and should be interpreted with caution.

Interpretive criteria for disk diffusion antimicrobial susceptibility testing of most of these species are lacking; MIC broth microdilution and the Etest are therefore the preferred methodologies for susceptibility testing. For multiresistant strains, consideration could be given to testing in reference laboratories for synergy with double or triple combinations of antimicrobial agents (222). It is important to note, however, that neither checkerboard MIC broth microdilution testing nor multiple combination bactericidal antibiotic testing is standardized at present.

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Acinetobacter, *Chryseobacterium*, *Moraxella*, and Other Nonfermentative Gram-Negative Rods*

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TAXONOMY

The organisms covered in this chapter belong to a group of taxonomically and phylogenetically diverse, Gram-negative, nonfermentative rods and coccobacilli. Still, several of the genera dealt with belong to the same family; i.e., *Acinetobacter*, *Moraxella*, *Oligella*, and *Psychrobacter* belong to the family *Moraxellaceae* (*Gammaproteobacteria*) (1), and *Balneatrix*, *Bergeyella*, *Chryseobacterium*, *Elizabethkingia*, *Empedobacter*, *Myroides*, *Sphingobacterium*, *Wautersiella*, and *Weeksella* belong to the family *Flavobacteriaceae* (*Bacteroidetes*) (2).

DESCRIPTION OF THE AGENTS

The species dealt with in this chapter all share the common phenotypic features of being catalase positive and failing to acidify the butt of Kligler iron agar (KIA) or triple sugar iron (TSI) agar or of oxidative-fermentative media, indicating their inability to metabolize carbohydrates by the fermentative pathway. These organisms grow significantly better under aerobic than under anaerobic conditions, and many, i.e., those species that can use only oxygen as the final electron acceptor in the respiratory pathway, fail to grow anaerobically at all.

EPIDEMIOLOGY AND TRANSMISSION

Most of the organisms described in this chapter are found in the environment, i.e., soil and water. For methylobacteria, tap water has been implicated as a possible agent of transmission in hospital environments, and methods for monitoring water systems for methylobacteria have been described previously (3). No person-to-person spread has been documented for the species covered in this chapter, except for *Acinetobacter* and *Moraxella catarrhalis*.

CLINICAL SIGNIFICANCE

Although for almost all of the species in this chapter, case reports, e.g., of meningitis and endocarditis, can be found, their clinical importance is mostly restricted to that of opportunistic pathogens, except, e.g., for *Elizabethkingia meningoseptica*, *Moraxella lacunata* (eye infections), *M. catarrhalis* (respiratory tract infections), and species of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex.

A. baumannii ventilator-associated pneumonia and bloodstream infections have been documented to be associated with a high degree of mortality and morbidity (4). Particular manifestations of *A. baumannii* are its implication in severely war-wounded soldiers (5), from which stems its popular designation "*Iraqibacter*," and in victims of natural disasters (6).

The clinical impact of infections with *A. baumannii* is a continuous source of debate (7, 8). Indeed, although severe infections with *A. baumannii* have been documented, colonization is much more frequent than infection, and differentiation between these conditions can be difficult. Still, although uncommon, community-acquired infections with *A. baumannii* occur. In particular, community-acquired pneumonia with *A. baumannii* is increasingly reported from tropical areas, like Southeast Asia and tropical Australia (9, 10).

The clinical role of the closely related species *Acinetobacter pittii* and *Acinetobacter nosocomialis* resembles that of *A. baumannii*, although, compared to these two species, *A. baumannii* is more often associated with multidrug resistance and epidemic spread in hospitals and possibly also with higher mortality among patients with systemic infections (11, 12).

Other *Acinetobacter* species occasionally associated with human infections are listed in Table 1. *A. johnsonii*, *A. lwoffii*, and *A. radioresistens* seem to be common inhabitants of human skin (13). *A. lwoffii* was a frequent species in clinical specimens during an 8-year study in a university hospital, where it was isolated mainly from blood or intravascular lines (14). Several species, *A. ursingii* in particular, have been found to cause bloodstream infections in hospitalized patients (15–18), while *A. junii* and *A. soli* have also been implicated in outbreaks of neonatal infections (19, 20). *A. parvus* is regularly isolated from blood cultures (18, 21), but is often overlooked because of its small colonies and misidentification by API 20NE as *A. lwoffii* (our unpublished data). Many of the infections with these species are related to intravascular catheters or have another iatrogenic

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*This chapter contains information presented by Mario Vaneechoutte, Lenie Dijkshoorn, Alexandr Nemec, Peter Kämpfer, and Georges Wauters in chapter 42 of the 10th edition of this *Manual*.

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TABLE 1 Oxidase-negative, indole-negative, nonfermentative, Gram-negative rods: the genus *Acinetobacter*[a]

Characteristic	*A. baumannii* (25)[b,c]	*A. calcoaceticus* (11)[c]	*A. nosocomialis* (20)[c]	*A. pittii* (20)[c]	*A. beijerinckii* (16)	*A. bereziniae* (16)	*A. guillouiae* (17)	*A. gyllenbergii* (9)	*A. haemolyticus* (17)	*A. junii* (15)	*A. johnsonii* (20)	*A. lwoffii* (14)	*A. parvus* (10)	*A. radioresistens* (12)	*A. schindleri* (22)	*A. soli* (8)	*A. ursingii* (30)
Growth at:																	
44°C	100[d]	0	95	10	0	0	0	0	0	0	0	0	0	0	0	0	0
41°C	100	9	100	100	0	0	0	0	94	87	0	7	0	100	100	88	0
37°C	100	100	100	100	100	100[e]	0[e]	100	100	100	25[f]	100	90	100	100	100	100
Acidification of D-glucose	100	91	100	95	0	88	0	0	82	0	0	7	0	0	0	100	0
Assimilation of:																	
Adipate	88	100	95	100	0	63	100	100	0	0	0	79	0	92	38	100	100
β-Alanine	100	91	85	90	0	100	94	100	0	0	0	0	0	0	0	100	0
4-Aminobutyrate	100	100	100	100	100	100	88	/[g]	100	87	75	79	0	100	0	100	0
L-Arginine	100	100	100	100	0	0	0	100	88	93	70	0	0	83	0	100	0
L-Aspartate	100	100	100	100	100	100	100	0	18	27	75	0	0	0	0	100	97[f]
Azelate	88	100	95	100	0	63	100	100	0	0	0	100	0	100	64	100	100
Citrate (Simmons)	100	100	100	100	100	100	100	100	82	80	85	14	0	0	59	100	97
D-Glucose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0
Glutarate	96	91	95	90	0	100	100	/[g]	0	0	0	0	0	100	95	88	97
Histamine	0	0	0	0	0	63	65	0	0	0	0	0	0	0	0	0	0
L-Histidine	96	100	100	100	100	94	94	100	100	93	0	0	0	0	0	100	0
DL-Lactate	100	100	100	100	0	100	100	100	0	93	100	93	0	100	100	100	100
Malonate	88	100	20	95	100	0	18	78	0	0	50	7	0	100	0	100	0
Phenylacetate	84	100	85	75	0	25	65	100	0	0	0	71	0	100	0	100	0
Gelatinase	0	0	0	0	13	0	0	100	88	0	0	0	0	0	0	0	0
Hemolysis of sheep blood	0	0	0	0	100	0	0	100	100	47	25[f]	0	0	0	0	75	0

[a] All data were provided by one of the authors (A. Nemec) using standardized tests described in detail previously (67).

[b] Numbers in parentheses after organism names are numbers of strains tested.

[c] Species of the *A. calcoaceticus-A. baumannii* complex (65).

[d] The numbers are percentages of strains positive in a given test. Carbon assimilation tests were evaluated after 6 days of incubation at 30°C.

[e] Growth tested at 38 instead of 37°C.

[f] Weak growth of most strains with positive reactions.

[g] Unreproducible or delayed growth of most strains.

origin (15, 22), and their course is generally benign. For various other named or yet-unnamed *Acinetobacter* species, although recovered from clinical specimens (23, 24), a possible role in infection has not been documented.

Moraxella species are rare agents of infections (conjunctivitis, keratitis, meningitis, septicemia, endocarditis, arthritis, and otolaryngologic infections) (25–27), but *M. catarrhalis* has been reported to cause sinusitis and otitis media by contiguous spread of the organisms from a colonizing focus in the respiratory tract (25). However, isolation of *M. catarrhalis* from the upper respiratory tract (i.e., a throat culture) of children with otitis media or sinusitis does not provide evidence that the isolate is the cause of these infections, because *M. catarrhalis* is present frequently as a commensal of the upper respiratory tract in children (28). Isolates from sinus aspirates and middle ear specimens obtained by tympanocentesis should be identified and reported. Similarly, little is known about the pathogenesis of lower respiratory tract infection in adults with chronic lung diseases, although a clear pathogenic role may be assigned to this species because *M. catarrhalis* is not a frequent commensal of the upper respiratory tract in adults (28) and because examination of Gram-stained smears of sputum specimens from patients with exacerbations of bronchitis and pneumonia due to *M. catarrhalis* usually reveals an abundance of leukocytes, the presence of many Gram-negative diplococci as the exclusive or predominant bacterial cell type, and the presence of intracellular Gram-negative diplococci. Such specimens may yield *M. catarrhalis* in virtually pure culture, and the organism should be identified and reported. Furthermore, *M. nonliquefaciens* (29, 30) and *M. osloensis* (31, 32) are the two species most frequently isolated, approximately in equal numbers, from nonrespiratory clinical material, especially blood cultures from patients at risk. *M. canis* has been isolated from dog bite wounds (33) and from debilitated patients (27). *M. lacunata* has been involved in eye infections and in infective endocarditis (34, 35).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Standard methods for collection, transport, and storage of specimens as detailed in chapters 11 and 18 are satisfactory for this group of organisms. The only fastidious species handled in this chapter are *Asaia* species, *Granulibacter bethesdensis*, *Methylobacterium* species, and some *Moraxella* species; these should be cultured on media containing blood.

DIRECT EXAMINATION

There are no characteristics available that can help to recognize the species dealt with in this chapter by means of direct microscopic examination of the samples. On Gram stain, organisms appear as Gram-negative rods, coccobacilli, or diplococci. Neither direct antigen tests nor molecular genetic tests to use directly on clinical materials have been developed.

ISOLATION PROCEDURES

Initial incubation should be at 35 to 37°C, although some strains, among them many of the pink-pigmented species, grow better at or below 30°C and may be detected only on plates left at room temperature. In such cases, all tests should be carried out at room temperature. In fact, some of the commercial kits, such as the API 20NE, are designed to be incubated at 30°C.

Growth on certain selective primary media, e.g., MacConkey agar, is variable and may be influenced by lot-to-lot variations in the composition of media. Gram-negative, nonfermentative bacteria (GNF) that grow on MacConkey agar generally form colorless colonies, although some form lavender or purple colonies due to uptake of crystal violet contained in the agar medium. Selective media have been described for *Acinetobacter* spp. (36) and for *Moraxella* spp. (37).

IDENTIFICATION

This chapter provides an overview in Fig. 1, which provides a key to the five large groups that can be distinguished among the species described in this chapter. This key is based only on colony color (pink or not) and the presence or absence of oxidase, of benzyl arginine aminopeptidase (trypsin) activity, and of the production of indole.

Figure 1 refers to Tables 1 and 3 to 6, which provide further keys to identify the species of these five groups on the basis of biochemical reactions. Results for enzymatic reactions can be read within hours or up to 2 days of incubation, whereas results of carbon source assimilation tests (*Acinetobacter*) and acid production from carbohydrates are read after up to 6 and 7 days, respectively.

For each group of closely related species, we present their taxonomic history (explaining the use of other names in the past and the taxonomic changes introduced since the previous edition), address the clinical importance of the species, and describe the phenotypic data that are useful to differentiate this group from other groups and to differentiate the species within this group. When relevant, antibiotic susceptibility characteristics and treatment options are discussed immediately; otherwise, they are discussed at the end of each section for the five large groups in this chapter.

Although several of the genera discussed in this chapter comprise many more species than the ones addressed here, we focus on those species that can be isolated from clinical samples.

Classical Biochemical Identification Schemes Presented in This Chapter

For all the species in this chapter, except those of the genus *Acinetobacter*, the biochemical tests listed have been carried

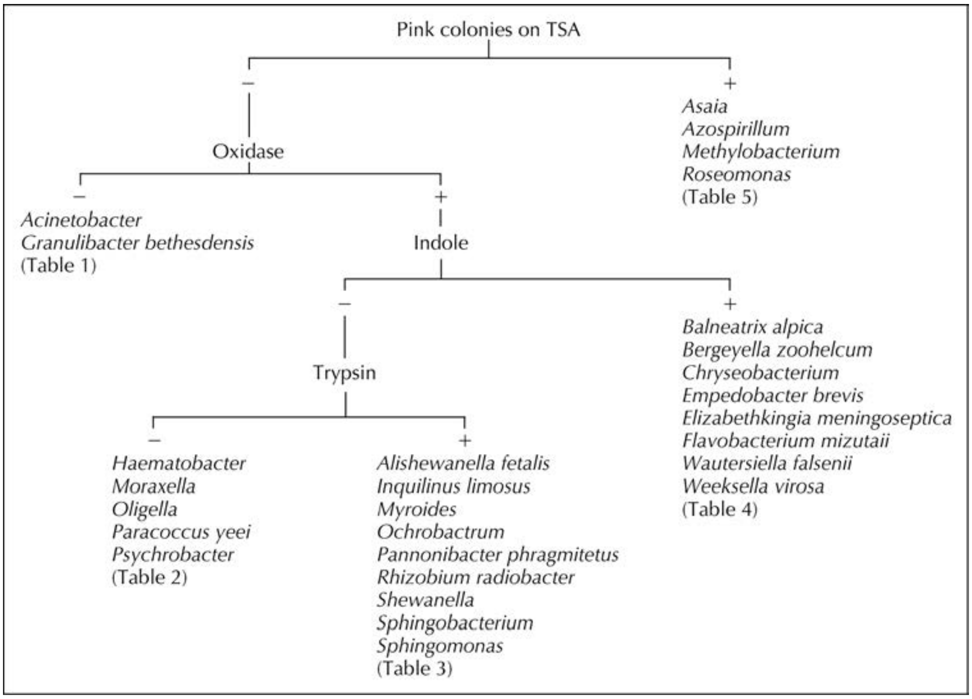
Pink colonies on TSA
–
+
Asaia
Azospirillum
Methylobacterium
Roseomonas
(Table 5)
Oxidase
–
Acinetobacter
Granulibacter bethesdensis
(Table 1)
+
Indole
–
+
Balneatrix alpica
Bergeyella zoohelcum
Chryseobacterium
Empedobacter brevis
Elizabethkingia meningoseptica
Flavobacterium mizutaii
Wautersiella falsenii
Weeksella virosa
(Table 4)
Trypsin
–
Haematobacter
Moraxella
Oligella
Paracoccus yeei
Psychrobacter
(Table 2)
+
Alishewanella fetalis
Inquilinus limosus
Myroides
Ochrobactrum
Pannonibacter phragmitetus
Rhizobium radiobacter
Shewanella
Sphingobacterium
Sphingomonas
(Table 3)

FIGURE 1 Identification of miscellaneous GNF. The organisms covered in this chapter belong to a group of taxonomically diverse, Gram-negative, nonfermentative rods and coccobacilli. They all share the common phenotypic features of failing to acidify the butt of KIA or TSI agar or of oxidative-fermentative media, indicating their inability to metabolize carbohydrates by the fermentative pathway. These characteristics are shared with those of the species of the emended genus *Pseudomonas* (chapter 42) and those of the species of genera that previously were named as *Pseudomonas* (chapter 43). *a*, G. *bethesdensis* grows slowly and poorly on SBA. *A. parvus* forms small colonies as well, but these are already visible after 24 h of incubation.
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out by one of us (G. Wauters), according to standardized protocols, described in detail in chapter 33. The limited number of tests that have been used to discriminate between the species dealt with in this chapter have been selected because they can be carried out easily and quickly, because they mostly yield uniform results per group or species, and because they are highly discriminatory. For the genus *Acinetobacter*, data based on standardized physiological and nutritional tests were provided by one of the authors (A. Nemec) (see footnotes to Table 1).

Automated, Commercially Available Phenotypic Identification Systems

Traditional diagnostic systems, e.g., those based on oxidation-fermentation media, aerobic low-peptone media, or buffered single substrates, have now been replaced in many laboratories by commercial kits or automated systems like the VITEK 2 (bioMérieux, Marcy l'Etoile, France) and the Phoenix (BD Diagnostic Systems, Sparks, MD). The ability of commercial kits to identify this group of GNF is variable and often results in identification to the genus or group level only, necessitating the use of supplemental biochemical testing for species identification. O'Hara and Miller (38), using the VITEK 2 ID-GNB identification card, reported that of 103 glucose-fermenting and nonfermenting, nonenteric strains, 88 (85.4%) were correctly identified at probability levels ranging from excellent to good and that 10 (9.7%) were correctly identified at a low level of discrimination, for a total of 95.1% accuracy within this group. Bosshard et al. (39) compared 16S rRNA gene sequencing for the identification of clinically relevant isolates of GNF (non-*Pseudomonas aeruginosa*) with two commercially available identification systems (API 20NE and VITEK 2 fluorescent card; bioMérieux). By 16S rRNA gene sequence analysis, 92% of the isolates were assigned to species level and 8% to genus level. Using API 20NE, 54% of the isolates were assigned to species level, 7% were assigned to genus level, and 39% of the isolates could not be discriminated at any taxonomic level. The respective numbers for VITEK 2 were 53, 1, and 46%. Fifteen percent and 43% of the isolates corresponded to species not included in the API 20NE and VITEK 2 databases, respectively. Altogether, commercial identification systems can be useful for identification of organisms commonly found in clinical specimens, like *Enterobacteriaceae*. However, for rare organisms the performance of these systems can be poor.

Chemotaxonomic Methods

The fatty acid profiles for the most common species of GNF have been published (40).

Matrix-Assisted Laser Desorption Ionization–Time-of-Flight Mass Spectrometry

Few developments in clinical bacteriology have had as rapid and profound an impact on identification of microorganisms as matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) (41, 42). Conventional identification methods rely on biochemical properties and require sometimes lengthy incubation procedures, whereas MALDI-TOF MS can identify bacteria within minutes directly from colonies grown on culture plates. Two commercial platforms, Bruker Biotyper (Bruker Daltonics, Billerica, MA) and VITEK MS (bioMérieux), are available, and although the initial cost of mass spectrometry equipment and maintenance costs are relatively high, the additional identification costs per isolate are minimal.

Studies on the reliability and accuracy of MALDI-TOF MS in identifying species dealt with in this chapter are summarized in Table 2. MALDI-TOF MS appears to be a promising tool for identification of these species, with a reported 80.4% correct species identifications and 92.3% correct genus identifications (43, 44), with most difficulties in obtaining a correct identification due to a missing spectrum in the database at that time. These problems can be overcome by creating new databases or by adding missing spectra (43, 44).

The accuracy of both MALDI-TOF MS systems was found to be similar for identifying GNF (45), and both systems demonstrated performance superior to that of conventional methods (45). Mellmann and coworkers (46) reported 98.75% interlaboratory reproducibility among eight laboratories in a study of MALDI-TOF MS for identifying 480 GNF isolates. Sedo et al. (47) successfully applied MALDI-TOF MS for identification of selected *Acinetobacter* species, but could not differentiate between different strains belonging to the same species. Besides bacterial identification, MALDI-TOF MS is also being explored for the phenotypic detection of certain antibiotic resistance mechanisms, e.g., the detection of carbapenemase enzymatic activity in *A. baumannii* strains (48), and for typing.

Schaller and coworkers (49) found MALDI-TOF MS to be a rapid and robust tool for typing the two subpopulations and three 16S rRNA types of *M. catarrhalis* described previously. Mencacci and coworkers (50) explored the potential utility of MALDI-TOF MS to detect nosocomial spread of multidrug-resistant *A. baumannii* outbreaks in comparison with the repetitive sequence-based PCR DiversiLab system (bioMérieux) and suggested that it shows promise in routine clinical microbiology.

DNA Sequence-Based Methods

Sequence-based methods involving rRNA (16S, 16S-23S spacer, or 23S) and housekeeping genes, such as those encoding RNA polymerase subunit B (*rpoB*), gyrase subunit B (*gyrB*), or the recombination A protein (*recA*), have become standard techniques to identify bacteria in general (51) and have contributed to the better delineation of several of these groups and the discovery and description of new species. Because these are generally applicable methods, their application for species of this chapter is not outlined in detail. Other sequence-based methods, based on DNA array hybridization, have been used for some species of these groups (52). DNA sequence-based fingerprinting methods like amplified ribosomal DNA (rRNA gene) restriction analysis (ARDRA) (53, 54) and tDNA PCR (55, 56) have been applied for the identification of species of several groups as well. These fingerprinting approaches are also generally applicable, but they require reference fingerprint libraries and are often poorly exchangeable between different electrophoresis platforms and laboratories.

IDENTIFICATION OF THE FIVE GENOTYPIC GROUPS

Oxidase-Negative GNF

Acinetobacter

Members of the genus *Acinetobacter* are strictly aerobic, oxidase-negative, catalase-positive, coccobacillary bacteria.

TABLE 2 Accuracy of MALDI-TOF MS compared to other identification methods[a]

Species	MALDI-TOF MS result	Reference(s)
Acinetobacter baumannii (141)[b,c,d,e,f]	*A. baumannii* (135),[g,h] *Acinetobacter* sp. (6)[g,i]	42, 126, 201–203
A. beijerinckii (3)[e,j]	*A. beijerinckii* (2), *A. tjernbergiae* (1)	47, 201
A. berezinae (18)[e,j]	*A. berezinae* (11),[k] *A.* genomic species 3 (7)	47, 201
A. calcoaceticus (11)[b,d,e]	*A. calcoaceticus*, *Acinetobacter* sp. (8),[g] *A.* genomic species 3 (2)	42, 201, 202
A. genomic species 3 (8)[e,j]	*A.* genomic species 3 (8)	201
A. genomic species 13TU (3)[e,j]	*Acinetobacter* sp., *A.baumannii* (2)	201
A. genomic species 14BJ (2)[d,e,j]	*A.* genomic species 3, *A. pittii*[l]	42, 201
A. genomic species 17[d,j]	*Acinetobacter* sp.	42
A. guillouiae (11)[e]	*A. guillouiae* (11)	47
A. haemolyticus (3)[d]	*A. haemolyticus* (3)	42, 47
A. johnsonii (5)[b,f]	*A. johnsonii* (2),[g,h] *A. junii*,[g] *Acinetobacter* sp.[g,h]	126, 202–203
A. junii (7)[d,e,f]	*A. junii* (7)[g,h]	42, 126, 201
A. lwoffii (8)[c,d,f]	*A. lwoffii* (6),[g,h,i] *Acinetobacter* sp.,[g] *Ochrobactrum anthropi*[g]	42, 126
A. nosocomialis (18)[d]	*A. nosocomialis* (18)[m]	42
A. pittii (16)[d]	*A. pittii* (16)	42
A. radioresistens (4)[b,d,f,j]	*A. radioresistens* (3),[g,h] misidentification[n]	42, 126, 202
A. schindleri[f]	*A. schindleri*[g,h]	126
A. soli[e,j]	*A. baylyi*	201
A. ursingii (2)[f]	*A. ursingii*,[g,h] *Acinetobacter* sp.[g,h]	126
Acinetobacter sp.[b]	*A. junii*/*A. haemolyticus*[g]	202
Chryseobacterium gleum[b]	No identification[g,o]	203
C. indologenes (6)[b,f]	*C. indologenes* (5),[g] no identification (1)[k,o]	44, 45, 203
Chryseobacterium spp. (1)[b]	*C. indologenes* (1)[g]	203
Elizabethkingia meningoseptica (3)[b,f]	*E. meningoseptica* (2),[g,h] *E. miricola* (1)[k]	44, 45, 126
Moraxella catarrhalis (18)[b]	*M. catarrhalis* (18)[g]	202
Ochrobactrum anthropi (29)[b,f]	*O. anthropi* (6),[g,k] *Ochrobactrum* sp. (21),[g,k] *O. tritici* (2)[g,k]	203; Wauters (unpublished)
O. intermedium (9)[b,f]	*O. intermedium* (9)[g,k]	Wauters (unpublished)
Ochrobactrum spp.[b]	*Ochrobactrum anthropi*[g]	203
Oligella spp.[b]	*Oligella urethralis*[g]	203
Psychrobacter phenylpyruvicus[f,j]	No identification[g]	126
Rhizobium radiobacter (3)[b,f]	*R. radiobacter* (3)[g,h]	126, 203
Roseomonas mucosa[f,j]	Misidentification[g,h]	126
Shewanella algae[f,j]	No identification[g,h]	126
Sphingobacterium spiritivorum[b]	*S. spiritivorum*[g]	203
No identification (5)[b,f]	*Acinetobacter* sp. (2),[k] *Wohlfahrtiimonas chitiniclastica* (3)[g]	44; Wauters (unpublished)

[a] All identifications obtained with the Bruker MALDI-TOF MS system, except for those of reference 45, which compared the Bruker and VITEK systems. Numbers in parentheses after organism names are numbers of strains tested.

[b] Identification by conventional methods, with discrepant results resolved by 16S rRNA gene sequencing.

[c] Conventional biochemical identification.

[d] Identification by ARDRA (61), rRNA intergenic spacer (ITS), *recA* sequencing, and/or *bla*$_{\text{OXA-51}}$ PCR.

[e] Identification by *bla*$_{\text{OXA-51}}$ PCR and/or *rpoB* gene sequencing.

[f] Identification by 16S rRNA gene sequencing.

[g] Direct transfer method.

[h] Identification also taking into account the rule of thumb that the difference between the first two species listed is larger than 0.2 if the first log score is <1.7.

[i] Ethanol/formic acid extraction.

[j] Not present in current database or in database at time of research.

[k] Using own MALDI-TOF MS database.

[l] *Acinetobacter* gen. sp. 14BJ by ARDRA and *recA* sequencing, but *A. pittii* by ITS sequencing and MALDI-TOF MS.

[m] After including *A. nosocomialis* in database.

[n] Identified to the wrong genus level.

[o] Log score of <1.7.

They are Gram negative but may be difficult to destain. Most strains do not reduce nitrate to nitrite in a laboratory test, owing to the lack of a dissimilative nitrate reductase. Tween 80 esterase activity is frequently present, while hemolysis and gelatinase production varies. Swimming motility is negative, but "twitching motility" on soft agar may occur. Individual cell sizes are 0.9 to 1.6 µm in diameter and 1.5 to 2.5 µm in length. In the stationary phase, the cells are usually coccoid and occur in pairs. Most strains grow at between 20 and 35°C. Medically relevant species commonly grow well at 37°C, although some of them (*A. johnsonii* and *A. guillouiae*) show reduced or no *in vitro* growth at this temperature.

The organisms can form a pellicle (biofilm) on the surface of fluid media. They grow well on complex media, including blood agar, nutrient agar, and mostly on MacConkey agar. After 24 h of incubation, colonies are 1 to 2 mm in diameter (except for *A. parvus*, with markedly smaller colonies), colorless to beige, domed, and smooth to mucoid (Fig. 2). Colonies on MacConkey agar can become pink. Many strains can use a wide variety of organic compounds as single sources of carbon and energy. Selective enrichment can be

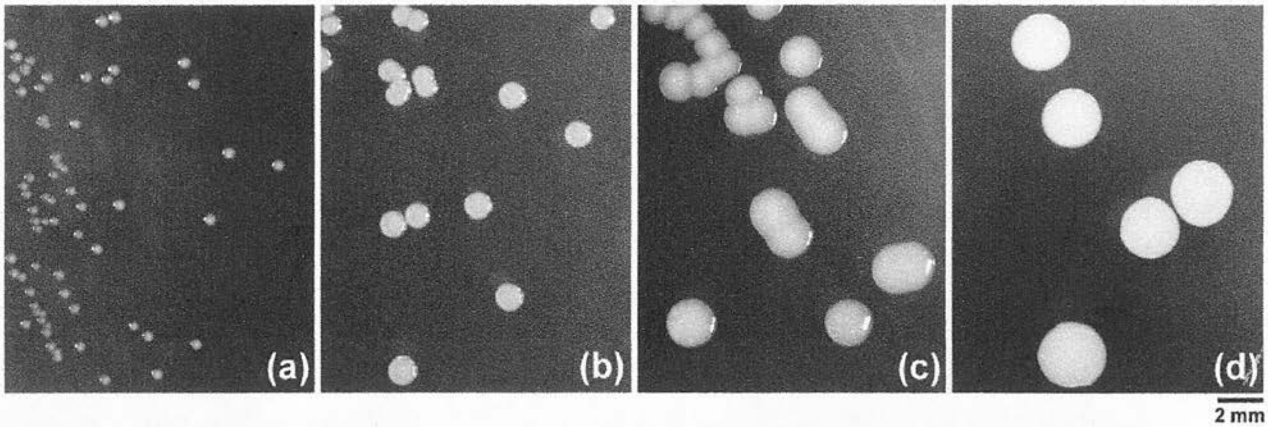

FIGURE 2 Differences in the size of the colonies formed by different *Acinetobacter* species isolated from human clinical specimens. The strains were grown on TSA (Oxoid) at 30°C for 24 h. (a) *A. parvus* NIPH 384^T; (b) *A. ursingii* NIPH 137^T; (c) *A. schindleri* NIPH 1034^T; (d) *A. baumannii* ATCC 19606^T. doi:10.1128/9781555817381.ch44.f2

obtained in mineral media with acetate as the carbon source and ammonium salt as the nitrogen source with shaking incubation at 30°C (36). General features of *Acinetobacter* have been reviewed previously (57, 58).

Members of the genus *Acinetobacter* are widespread in nature and have been found in soil, water, sewage, and food, as well as in human and animal specimens. Some of the *Acinetobacter* species have been predominantly cultured from humans (e.g., *A. baumannii*, *A. nosocomialis*, and *A. ursingii*), while others inhabit diverse ecosystems (*A. johnsonii* and *A. guillouiae*) or are confined to specific niches (*A. nectaris* and *A. boissieri*) (59).

The ecology of most clinically relevant *Acinetobacter* species is still poorly resolved. *A. baumannii*, *A. pittii*, *A. nosocomialis*, and *A. ursingii* have been mainly recovered from clinical specimens in hospitals. Human skin carrier rates of *A. baumannii* outside hospitals are low (13), but higher rates (also for *A. pittii* and *A. nosocomialis*) have been found in tropical areas (60). *A. baumannii*, including multidrug-resistant strains, has been isolated from sick animals (61) and from hospitalized horses (62), but an animal or environmental reservoir has not been found. *A. baumannii* is, due to its role as a prominent nosocomial pathogen, the species for which the epidemiology has been studied most intensively. Epidemic strains of this species can survive well in the environment, as they have been found on equipment and on environmental surfaces and materials (63), usually in the vicinity of colonized patients. Multiple sites of the skin and mucosae of patients can be colonized, and colonization may last days to weeks (64).

Species Diversity and Identification

The taxonomy of the genus *Acinetobacter* has recently been updated with valid names for several medically relevant genomic species known previously under provisional designations, i.e., *A. pittii* (65), *A. bereziniae* (66), *A. guillouiae* (66), and *A. nosocomialis* (65) (former genomic species 3, 10, 11, and 13TU, respectively), and with new medically relevant species, i.e., *A. beijerinckii* (67), *A. gyllenbergii* (67), *A. parvus* (21), and *A. soli* (20, 68). Some of the recently proposed species names have been shown to be synonymous with already existing ones: *A. grimontii* with *A. junii* (69) and "*A. septicus*" with *A. ursingii* (70).

At present, the genus *Acinetobacter* comprises 31 distinct species with valid names (http://www.bacterio.net/acinetobacter.html) and a number of taxa that include either genomic species delineated by DNA-DNA hybridization (7, 57) or species with effectively (but not validly) published names. Recent studies have shown that nearly all *Acinetobacter* isolates from colonized or infected patients belong to one of these species (14, 17, 18). This indicates that the species diversity of acinetobacters found in the human body environment is well covered by the current classification. Validly named species found in human clinical specimens are listed in Table 1.

Surprisingly, and in spite of its name (derived from the Greek *akineto*, meaning "motionless" or "nonmotile") and its lack of flagella, *A. baumannii* spreads rapidly over certain surfaces. This is likely the result of twitching motility, a form of surface translocation described before for the genus *Acinetobacter* (71). As shown for *A. baumannii* ATCC 17978, cultured at 24°C, white (low-intensity) light, and in particular its blue component, is a stimulus governing several metabolic processes, inhibiting motility and the formation of biofilms and pellicles (72). Light regulation is lost at 37°C in *A. baumannii* (72).

Further studies showed that light regulation is not restricted to *A. baumannii*, but widespread within the genus *Acinetobacter* (73). In fact, blue light modulates motility and biofilm formation in many species of the genus. In the *Acinetobacter* species studied thus far, except *A. baumannii*, blue light contributes to the decision between motility and sessility and also may facilitate acclimation to different environments. For *A. baumannii*, both motility and sessility (biofilm formation) were inhibited by blue light and only at low temperature (72).

Identification of *Acinetobacter* species by commercial identification systems based on biochemical and physiological properties is problematic. This stems from the small number of relevant characters included in these systems and/or from the insufficient quality of reference data in the identification matrices. *A. baumannii* and the other species of the *A. calcoaceticus*-*A. baumannii* complex (i.e., *A. pittii*, *A. nosocomialis*, and *A. calcoaceticus*, the latter a primarily soil-dwelling organism) are generally not differentiated by these systems, while non-glucose-oxidizing species such as *A. ursingii* and *A. schindleri* can be misidentified (15). We compared VITEK 2 and Phoenix for the ability to identify

76 isolates of 16 clinical *Acinetobacter* species and found that only 19 isolates were correctly identified by VITEK 2 and 5 by Phoenix (M. Vaneechoutte, unpublished data). Nonetheless, the diagnostic systems can be useful for genus-level identification and, when supplemented with testing growth ability at 44°C, also for presumptive differentiation between *A. baumannii* and *A. nosocomialis* (Table 1).

More reliable phenotypic identification of *Acinetobacter* species can be achieved using physiological and nutritional (mainly carbon source assimilation) tests based on the modified system of Bouvet and Grimont (67). Table 1 presents an update of this system aimed to differentiate validly named species of clinical relevance. Assimilation tests were carried out using a basal liquid medium supplemented with 0.1% (wt/vol) carbon source (67). Growth was evaluated after 4, 6, and 10 days of culture at 30°C by means of visual comparison between inoculated tubes containing carbon sources and control tubes containing only inoculated basal medium. However, the species of the *A. calcoaceticus*-*A. baumannii* complex are not clearly distinguished from each other by this approach. In addition, the need for in-house preparation of most of the tests precludes the use of this identification scheme in routine diagnostics.

The most precise identification of *Acinetobacter* species can be achieved by a number of genotypic methods (57), with those based on sequencing particular genes being the current standard. Common targets for this purpose are the *rpoB* gene (67), currently the best-studied single-gene taxonomic marker for *Acinetobacter*; the 16S rRNA gene sequence (74); and the 16S-23S rRNA gene spacer region (75). PCR detection of the $bla_{\text{OXA-51}}$-like gene, which is intrinsic to *A. baumannii*, enables rapid identification of this species (76). The whole-genome sequences of >100 *Acinetobacter* strains reflecting the currently known breadth of *Acinetobacter* diversity have been recently published at the NCBI website (http://www.ncbi.nlm.nih.gov/bioproject/183623), and the availability of such a comprehensive set of genomic data may further facilitate efficacious identification.

Genotyping and Epidemiology

A number of molecular methods have been described for differentiation between isolates of the same species and study of the epidemiology of acinetobacters, in particular that of *A. baumannii*. Standardized random amplification PCR fingerprinting was useful for typing within one laboratory, and its (interlaboratory) reproducibility has been evaluated (77). Macrorestriction analysis with pulsed-field gel electrophoresis allowed for 95% intra- and 89% interlaboratory reproducibility (78). Amplified fragment length polymorphism fingerprinting also enables genotyping of strains (57, 63), and its robustness makes it suited for setting up a local database for longitudinal studies. Typing based on the variable number of tandem repeats has allowed for discrimination between genotypically related but epidemiologically distinct strains, i.e., those belonging to a single type based on several typing methods including macrorestriction analysis and PCR fingerprinting (79, 80). Sequence analysis of the OXA-51-like gene has been proposed as a useful typing method, based on the correlation between particular OXA-51-like enzymes and epidemic clones of *A. baumannii* (81).

Multilocus sequence typing (MLST) is the standard for global and long-term epidemiological and population studies. Two MLST schemes are currently available for *A. baumannii*: the scheme proposed by Bartual et al. (82) (http://pubmlst.org/abaumannii/) and that proposed by Diancourt et al. (83) (http://www.pasteur.fr/recherche/genopole/PF8/mlst/). Both schemes are based on the internal fragments of seven housekeeping genes, and they share three genes. The two schemes yield compatible results, although a higher resolution has been reported for the scheme of Bartual et al. (82) by Karah et al. (84).

Resistance to multiple antimicrobials and hospital outbreaks have been associated with several international lineages of *A. baumannii*, particularly with the so-called European (or international) clones I and II (7, 83, 85). The earliest known strain of clone I was isolated in 1977 (86), and this lineage prevailed among outbreak and multidrug-resistant *Acinetobacter* strains in some European countries in the 1980s and 1990s (87). Currently, strains belonging to or related to clone II seem to dominate in the current global population of multidrug-resistant *A. baumannii*, although clone I and other lineages can be common or even prevail in some regions (84, 88–90). Allocation of isolates to international lineages or the identification of novel clones can be achieved by both MLST schemes. European clones I and II correspond, respectively, to MLST-based clonal complexes CC1 and CC2 in the scheme of Diancourt et al. (83) and to CC109 and CC92 in that of Bartual et al. (82), according to Karah et al. (84). Multiplex PCR, targeting the *ompA*, *csuE*, and $bla_{\text{OXA-51-like}}$ gene sequences, provides rapid assignment to the main clonal lineages (91).

Antimicrobial Susceptibilities

A. baumannii has an extraordinary capacity to acquire or develop resistance to virtually all antibiotics used in the treatment of *Acinetobacter* infections (7, 8). With the growing proportion of *A. baumannii* strains resistant to carbapenems, a last therapeutic option is disappearing, although these strains mostly remain susceptible to colistin. Even though multidrug resistance is mainly confined to *A. baumannii*, strains of other clinically relevant species, such as *A. pittii*, *A. nosocomialis*, and *A. ursingii*, can also be resistant to multiple antibiotics, including carbapenems (92, 93). Resistance mechanisms in *A. baumannii* comprise all currently known mechanisms, including enzymatic breakdown, modification of target sites, active efflux, and decreased influx of antibiotics. The known resistance mechanisms have been reviewed previously (94). Recent genomic studies have also shed light on the genetic organization of resistance determinants in multidrug-resistant *A. baumannii*. Large clusters of horizontally transferred genes, conferring resistance to multiple antibiotics and interspersed with mobile genetic elements (resistance islands), were found to be integrated at a specific genomic site in a number of strains (87, 95). Despite the high intraclonal variability of the resistance islands, these structures seem to have evolved independently in two main *A. baumannii* clones (87, 96).

In vitro determination of antimicrobial susceptibility can be achieved by disk diffusion, agar dilution, or broth microdilution, as recommended by the Clinical and Laboratory Standards Institute (CLSI) (97), or by Etest. The panel of tested antibiotics should cover the spectrum of agents with potential against *A. baumannii*, including third- or fourth-generation cephalosporins, sulbactam, antipseudomonal penicillins combined with β-lactamase inhibitors, carbapenems, aminoglycosides, fluoroquinolones, polymyxins, and tetracyclines (98). Of note, susceptibility to colistin should not be tested by disk diffusion due to poor diffusion of this compound in agar. Etest and broth microdilution for determination of the MIC for this drug have been compared and showed a good concordance in the MIC range of 0.25 to 1 mg/liter (99).

Granulibacter bethesdensis

G. bethesdensis (*Acetobacteraceae*, *Alphaproteobacteria*) (100) is a Gram-negative, aerobic, coccobacillary to rod-shaped bacterium, the only species of a new sublineage within the acetic acid bacteria in the family *Acetobacteraceae*. This fastidious organism grows poorly and slowly on sheep blood agar (SBA) at an optimum temperature of 35 to 37°C and an optimum pH of 5.0 to 6.5. It produces a yellow pigment, oxidizes lactate and weakly acetate to carbon dioxide and water, acidifies ethanol, and can use methanol as a sole carbon source, all characteristics that distinguish it from other acetic acid bacteria. The DNA base composition is 59.1 mol% G+C. It was isolated first from three patients with chronic granulomatous disease (100) and more recently from an additional patient with chronic granulomatous disease (101).

Oxidase-Positive, Indole-Negative, Trypsin-Negative GNF

Haematobacter

Three *Haematobacter* species (*Rhodobacteraceae*, *Alphaproteobacteria*) have been described, i.e., *H. massiliensis* (formerly *Rhodobacter massiliensis*), *H. missouriensis*, and *Haematobacter* genomic species 1 (Table 3) (102). These species cannot easily be differentiated phenotypically, and even the 16S rRNA gene sequences are closely related. *Haematobacter* species were described as asaccharolytic, but when low-peptone phenol red agar is used (see chapter 33), *H. missouriensis* is clearly saccharolytic, producing acid from glucose and xylose and sometimes from mannitol, whereas *H. massiliensis* strains do not acidify carbohydrates. Acid is produced from ethylene glycol by all species. All the species are strongly urease and phenylalanine deaminase positive. Arginine dihydrolase is also positive but sometimes delayed. Asaccharolytic *Haematobacter* strains resemble *Psychrobacter phenylpyruvicus* but can be differentiated by the lack of tributyrine esterase, the lack of growth improvement by Tween 80, and the presence of arginine dihydrolase. Differences from *Psychrobacter faecalis*, *Psychrobacter pulmonis*, and related species are the lack of tributyrine and Tween 80 esterase, the lack of nitrate reductase, and a positive arginine dihydrolase test.

Strains received at the Centers for Disease Control and Prevention (CDC) have been mainly isolated from patients with septicemia.

Haematobacter strains have low MICs for amoxicillin, fluoroquinolones, aminoglycosides, and carbapenems but variable MICs for cephalosporins, monobactams, and piperacillin.

Moraxella

The genus *Moraxella* comprises ~20 species that have been validly named. *M. catarrhalis*, *M. osloensis*, *M. nonliquefaciens*, and *M. lincolnii* are part of the normal microbiome of the human respiratory tract. Animal species include *M. bovis*, isolated from healthy cattle and other animals, including horses; *M. boevrei* and *M. caprae* (goats); *M. canis* (dogs,

TABLE 3 Oxidase-positive, indole-negative, trypsin-negative, coccoid GNF[a]

Characteristic	*Haematobacter massiliensis* (10)	*Haematobacter missouriensis* (3)	*Moraxella atlantae* (7)	*Moraxella canis* (5)	*Moraxella catarrhalis* (7)	*Moraxella lacunata* (5)	*Moraxella lincolnii* (2)	*Moraxella nonliquefaciens* (16)	*Moraxella osloensis* (28)	*Oligella ureolytica* (3)	*Oligella urethralis* (7)	*Paracoccus yeei* (14)	*Psychrobacter faecalis* (19)	*Psychrobacter phenylpyruvicus*[b] (5)	*Psychrobacter pulmonis* (8)	*Psychrobacter sanguinis*[b] (15)	*Wohlfahrtiimonas chitiniclastica* (3)
Motility (flagella)	0	0	0	0	0	0	0	0	0	67 (Pt)	0	0	0	0	0	0	0
Growth on MacConkey agar	90	100	100	60	0	0	0	0	50	0	57	100	100	0	(50)	0	100
Alkalinizaton of acetate	100	100	0	100	0	60	0	0	100	100	100	100	100	100	100	87	(100^{w})
Susceptibility to desferrioxamine	0	0	71	100	100	100	0	100	0	100	100	0	0	0	0	0	100
Acidification of:																	
Glucose	0	100	0	0	0	0	0	0	0	0	0	100	100	0	0	0	100
Mannitol	0	66	0	0	0	0	0	0	0	0	0	(50)	0	0	0	0	0
Xylose	0	100	0	0	0	0	0	0	0	0	0	100	100	0	0	0	(100^{w})
Ethylene glycol	100	100	0	100	0	20	0	0	100	100	100	100	100	100	100	87	100
Gelatinase	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0	0
Tween 80 esterase	0	0	0	0	0	100	0	0	4	0	0	0	100	100	100	100	0
Tributyrate esterase	0	0	0	100	100	100	0	100	100	0	0	100	100	100	100	100	0
Alkaline phosphatase	0	0	100	0	0	80	0	0	79	0	0	0	0	0	0	0	0
Phenylalanine deaminase	100	100	0	0	0	0	0	0	0	0	100^{w}	0	10	100	0	33	100
Pyrrolidonyl aminopeptidase	0	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Urease	100	100	0	0	0	0	0	0	0	100	0	100	0	100	0	100	0
Nitrate reductase	0	0	0	80	100	100	0	100	18	100	0	100	100	20	100	33	0
Nitrite reductase	0	0	0	20	100	0	0	0	0	100	100	0	100	0	100	0	0
Arginine dihydrolase	100	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a] Numbers in parentheses after organism names are numbers of strains tested. Values are percentages; those in parentheses represent delayed positivity. Abbreviations: Pt, peritrichous; W, weakly positive.

[b] Growth markedly promoted by Tween 80. *P. phenylpyruvicus* grows in 12% NaCl tryptic soy broth supplemented with 0.1% Tween 80, in contrast to *P. sanguinis*.

cats, and camels); M. *caviae* (guinea pigs); M. *cuniculi* (rabbits); and M. *ovis* and M. *oblonga* (sheep).

Both M. *catarrhalis* and M. *canis* grow well on SBA and even on tryptic soy agar (TSA), and their colonies may reach >1 mm in diameter after 24 h of incubation. Colonies of M. *catarrhalis* grow well on both blood and chocolate agars, and some strains also grow well on modified Thayer-Martin and other selective media. Colonies are generally gray to white, opaque, and smooth and measure about 1 to 3 mm after 24 h of incubation. Characteristically, the colonies may be nudged intact across the plate with a bacteriological loop like a "hockey puck" and can be removed from the agar entirely, being very consistent. Most M. *canis* colonies resemble those of the *Enterobacteriaceae* (large, smooth colonies) and may produce a brown pigment when grown on starch-containing Mueller-Hinton agar (33). Some strains may also produce very slimy colonies resembling colonies of *Klebsiella pneumoniae* (33). M. *nonliquefaciens* forms smooth, translucent to semiopaque colonies 0.1 to 0.5 mm in diameter after 24 h and 1 mm in diameter after 48 h of growth on SBA plates. Occasionally, these colonies spread and pit the agar. The colonial morphologies of M. *lincolnii* (103), M. *osloensis*, and P. *phenylpyruvicus* (formerly M. *phenylpyruvica*) are similar, but pitting is rare. On the other hand, pitting is common with M. *lacunata*, whose colonies are smaller and form dark haloes on chocolate agar. Rod-shaped *Moraxella* species, especially M. *atlantae* and M. *lincolnii*, are more fastidious and display smaller colonies on SBA, <1 mm in diameter after 24 h. Colonies of M. *atlantae* are small (usually 0.5 mm in diameter) and show pitting and spreading (104). The growth of M. *atlantae* is stimulated by bile salts, which explains its growth on MacConkey agar. M. *nonliquefaciens* and M. *osloensis* produce colonies that are somewhat larger than those of M. *atlantae* and that are rarely pitting. Colonies of M. *nonliquefaciens* may be mucoid. A selective medium, acetazolamide agar, inhibiting growth of neisseriae when incubated in ambient atmosphere, has been described for M. *catarrhalis* (37).

Moraxella species are coccoid or coccobacillary organisms (plump rods), occurring predominantly in pairs and sometimes in short chains, that tend to resist decolorization in the Gram stain (104). M. *canis* and M. *catarrhalis* are *Neisseria*-like diplococci, and they can easily be distinguished from other moraxellae or other coccoid species by performing a Gram stain on cells cultured in the vicinity of a penicillin disk: cells of M. *canis* and M. *catarrhalis* remain spherical diplococci of 0.5 to 1.5 µm in diameter, although of irregular size, whereas coccobacilli show obviously rod-shaped and filamentous cells.

Moraxella species are asaccharolytic and strongly oxidase positive. M. *catarrhalis* and M. *canis* are also strongly catalase positive, and most strains reduce nitrate and nitrite. M. *catarrhalis* and M. *canis* may be easily distinguished from the commensal *Neisseria* species, which are also frequently isolated from respiratory clinical specimens, by the ability of the former to produce DNase and butyrate esterase (tributyrine test). Rapid butyrate esterase tests have been described (105), and the indoxyl-butyrate hydrolysis spot test is commercially available (Remel, Inc., Lenexa, KS). Butyrate esterase is, however, also present in some other *Moraxella* species. M. *canis* acidifies ethylene glycol and alkalinizes acetate, in contrast to M. *catarrhalis*. There are few biochemical differences between M. *catarrhalis* and M. *nonliquefaciens*, which are differentiated from each other mainly on the basis of morphological characteristics and by nitrite reductase and DNase activity of M. *catarrhalis*.

M. *atlantae* is the only *Moraxella* species to be positive for pyrrolidonyl aminopeptidase (106). M. *lacunata* is the only proteolytic species with gelatinase activity. Using the plate method (see chapter 33), gelatin hydrolysis occurs usually within 2 to 4 days. A more rapid and almost equally specific test to differentiate M. *lacunata* from other moraxellae is the detection of Tween 80 esterase activity, which is often positive within 2 days, whereas all other species, except for very rare M. *osloensis* strains, remain negative. This species should also be distinguished from *Psychrobacter* species, which are also Tween 80 esterase positive, but *P. phenylpyruvicus* and *P. sanguinis* are urease positive and other *Psychrobacter* species exhibit luxuriant growth on plain agar, like TSA, even at 25°C.

M. *lincolnii* is biochemically quite inactive.

M. *osloensis* alkalinizes acetate, acidifies ethylene glycol, and is resistant to desferrioxamine (250-µg disk). M. *nonliquefaciens* has opposite properties to those of M. *osloensis* and is, in addition, always nitrate reductase positive.

Most *Moraxella* species are susceptible to penicillin and its derivatives, cephalosporins, tetracyclines, quinolones, and aminoglycosides (107, 108). Production of β-lactamase has been only rarely reported for *Moraxella* species other than M. *catarrhalis*, of which most isolates produce an inducible, cell-associated β-lactamase (37). Isolates of M. *catarrhalis* are generally susceptible to amoxicillin-clavulanate, expanded-spectrum and broad-spectrum cephalosporins (i.e., cefuroxime, cefotaxime, ceftriaxone, cefpodoxime, ceftibuten, and the oral agents cefixime and cefaclor), macrolides (e.g., azithromycin, clarithromycin, and erythromycin), tetracyclines, rifampin, and fluoroquinolones.

Oligella urethralis and Oligella ureolytica

The genus *Oligella* comprises two species, O. *ureolytica* (formerly CDC group IVe) and O. *urethralis* (formerly *Moraxella urethralis* and CDC group M-4) (109), which have both been isolated chiefly from the human urinary tract and have been reported to cause urosepsis (110).

Colonies of O. *urethralis* are smaller than those of M. *osloensis* and are opaque to whitish. Colonies of O. *ureolytica* are slow growing on blood agar, appearing as pinpoint colonies after 24 h but large colonies after 3 days of incubation. Colonies are white and opaque, with entire borders, and are nonhemolytic.

O. *ureolytica* and O. *urethralis* are small, asaccharolytic coccobacilli that rapidly acidify ethylene glycol and are susceptible to desferrioxamine. Most strains of O. *ureolytica* are motile by peritrichous flagella, and all are strongly urease positive (with the urease reaction often turning positive within minutes after inoculation) and reduce nitrate. O. *urethralis* strains are nonmotile and urease and nitrate reductase negative, but they reduce nitrite and are weakly phenylalanine deaminase positive. *Bordetella bronchiseptica* and *Cupriavidus pauculus* are also rapidly urease positive but are desferrioxamine resistant.

O. *urethralis* and M. *osloensis* have biochemical similarities, e.g., accumulation of poly-β-hydroxybutyric acid and failure to hydrolyze urea, but can be differentiated on the basis of nitrite reduction and alkalinization of formate, itaconate, proline, and threonine, all positive for O. *urethralis* (111). Moreover, O. *urethralis* is susceptible to desferrioxamine and tributyrate esterase is negative, in contrast to M. *osloensis*.

O. *urethralis* is generally susceptible to most antibiotics, including penicillin, while O. *ureolytica* exhibits variable susceptibility patterns (107).

Paracoccus yeei

The genus *Paracoccus* (*Rhodobacteraceae*, *Alphaproteobacteria*) comprises ~25 species, of which only *P. yeei* is of some clinical importance. Daneshvar et al. (112) proposed the name *P. yeeii*, later changed to *P. yeei*, for the former CDC group EO-2.

Colonies are large and mucoid, with a pale yellow pigmentation. *P. yeei* organisms are coccoid cells, showing many diplococci and a few very short rods. Microscopically, *P. yeei* is characterized by distinctive O-shaped cells (Fig. 3) upon Gram stain examination due to the presence of vacuolated or peripherally stained cells. The species is saccharolytic and urease positive.

P. yeei has been isolated from various human wound infections (112).

Psychrobacter

The genus *Psychrobacter* (113) comprises 34 species, of which only a few are clinically important. Apart from *P. phenylpyruvicus*, the *Psychrobacter* strains isolated from clinical material were considered until recently as belonging to the species *Psychrobacter immobilis*. In a recent study (114), it was shown that almost all the strains formerly identified as *P. immobilis* belong in fact to the species *P. faecalis* and *P. pulmonis*, isolated first from pigeons and lambs, respectively (115, 116). *P. immobilis* itself is apparently rarely isolated, if at all, from humans.

P. faecalis and *P. pulmonis* are coccoid, Gram-negative rods growing on TSA with large, creamy colonies. *P. faecalis* is saccharolytic and acidifies glucose and xylose, while *P. pulmonis* is asaccharolytic. Both species produce acid from ethylene glycol. They are Tween 80 esterase, tributyrate esterase, and nitrate reductase positive and, unlike the type strain of *P. immobilis*, urease negative and nitrite reductase positive. Colonies may resemble those of *Haematobacter*, but the latter lack nitrate reductase, Tween 80 esterase, and tributyrin esterase and are strongly urease positive, arginine dihydrolase positive, and phenylalanine deaminase positive.

Few cases of infection have been reported to be caused by *P. immobilis*, but in light of the data reported here, these might concern infections with one of the other *Psychrobacter* species.

P. phenylpyruvicus and *P. sanguinis* are phenotypically very similar, although genetically clearly distinct. *P. sanguinis* (117) seems to occur more frequently in clinical samples than *P. phenylpyruvicus*, and the majority of older *P. phenylpyruvicus* collection strains are in fact *P. sanguinis*. A salient feature is the dramatic effect of Tween 80 in promoting growth of both species, resulting in colonies 2 to 3 times larger on Tween 80 agar compared to blood agar. Since these species grow very poorly or not at all on usual media, like TSA, supplementation with 0.1% Tween 80 is recommended when blood agar cannot be used, as for susceptibility testing to desferrioxamine or for testing growth abilities in broth at different temperatures.

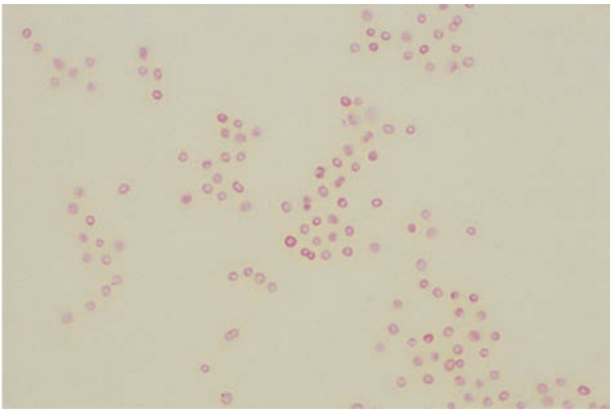

FIGURE 3 Gram stain of *P. yeei*, showing characteristic doughnut-shaped morphology.
doi:10.1128/9781555817381.ch44.f3

P. phenylpyruvicus grows in 12% NaCl tryptic soy broth supplemented with 0.1% Tween 80, while *P. sanguinis* does not. Phenylalanine deaminase is always positive in *P. phenylpyruvicus* but only in one-third of *P. sanguinis* strains.

Most *Psychrobacter* species are resistant to penicillin but susceptible to most other antibiotics (107).

Wohlfahrtiimonas chitiniclastica

W. chitiniclastica is a Gram-negative, nonfermentative rod living in the larvae of some parasitic flies. It is closely related to the "Gilardi rod group 1" and to *Ignatzschineria larvae* (118, 119). The type strain of *W. chitiniclastica* was first classified as "Gilardi rod group 1."

W. chitiniclastica may be associated with human myiasis, sometimes resulting in bacteremia (120) or sepsis (118). Fifteen human strains of "Gilardi rod group 1" were isolated from wounds and blood (121).

The organisms are short, coccoid rods, growing on current media with flat, slightly spreading colonies. Some carbohydrates, like glucose, xylose, and fructose, are weakly and slowly acidified. This species exhibits a very strong phenylalanine deaminase reaction. Chitinase activity is present as in *I. larvae*, which is, however, urease positive. *W. chitiniclastica* is resistant to penicillin and susceptible to many other antibiotics.

Oxidase-Positive, Indole-Negative, Trypsin-Positive GNF

Alishewanella fetalis

A. fetalis (*Alteromonadaceae*, *Gammaproteobacteria*) (Table 4) is a Gram-negative rod that grows at temperatures between 25 and 41°C, with optimum growth at 37°C. *A. fetalis* can withstand NaCl concentrations of up to 8% but not 10%, which helps differentiate this species from *Shewanella algae*, which can grow in 10% NaCl (122). Also, in contrast to *Shewanella* species, it does not produce H_2S in the butt of TSI and KIA. The type strain tested by us acidifies glucose and does not hydrolyze esculin.

A. fetalis has been isolated from a human fetus at autopsy, although its association with clinical infection is unknown (122).

Inquilinus limosus

I. limosus is a rod-shaped, Gram-negative bacterium that measures 1.5 to 2 μm in width by 3.5 μm in length and grows at 35 and 41°C but poorly at 25°C. Colonies are nonpigmented and very mucoid and grow on ordinary media such as TSA. Some strains are motile by one or two polar flagella, but motility is difficult to demonstrate due to the mucoid character of the colonies. In opposition to the original description (123), the species is saccharolytic, acidifying glucose, mannitol, xylose, and other carbohydrates. In contrast, ethylene glycol is not acidified. β-Galactosidase, pyrrolidonyl aminopeptidase, and trypsin are positive, but alkaline phosphatase is negative. Esculin is hydrolyzed. All strains are positive for catalase, β-glucosidase, proline

TABLE 4 Oxidase-positive, indole-negative, trypsin-positive GNF[a]

Characteristic	*Alishewanella fetalis* (1)	*Inquilinus limosus* (3)	*Myroides odoratimimus* (21)	*Myroides odoratus* (4)	*Ochrobactrum anthropi* (29)	*Ochrobactrum grignonense* (2)	*Ochrobactrum haematophilum* (3)	*Ochrobactrum intermedium* (9)	*Ochrobactrum pseudintermedium* (1)	*Ochrobactrum pseudogrignonense* (2)	*Pannonibacter phragmitetus* (7)	*Pseudochrobactrum asaccharolyticum* (3)	*Rhizobium radiobacter* (22)	*Shewanella algae*[b] (10)	*Shewanella putrefaciens*[b] (2)	*Sphingobacterium multivorum* (7)	*Sphingobacterium spiritivorum* (4)	*Sphingobacterium thalpophilum* (3)	*Sphingomonas* spp. (15)
Motility	0	33	0	0	100	100	100	100	100	100	100	0	100	100	100	0	0	0	66
Flagella	0	P, 1	0	0	Pt, L	Pt, L	Pt, L	Pt, L	Pt, L	Pt, L	Pt, L	0	Pt, L	P, 1	P, 1	0	0	0	P, 1
Production of flexirubin pigments	0	0	95	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Growth on MacConkey agar	100	(100)	100	75	100	100	100	100	100	100	100	100	100	100	100	100	(50)	100	0
Growth at 41°C	ND	ND	ND	ND	0	0	100	100	100	0	100	0	0	ND	ND	ND	ND	ND	ND
Growth at 45°C	ND	ND	ND	ND	0	0	0	0	100	0	0	0	0	ND	ND	ND	ND	ND	ND
Acidification of:																			
Glucose	100	100	0	0	100	100	100	100	100	100	100	(100)	100	0	50	100	100	100	87 (13)
Mannitol	0	100	0	0	59	50	33	33	0	50	28	0	100	0	0	0	100	0	0
Xylose	0	100	0	0	100	100	100	100	100	100	100	(100)	100	0	0	100	100	100	87
Ethylene glycol	0	0	0	0	100	100	100	100	100	100	86	86	100	100	100	0	100	0	93
Esculin hydrolysis	0	100	0	0	0	0	0	0	0	0	100	0	100	0	0	100	100	100	100
Gelatinase	100	0	100	100	0	0	0	0	0	0	0	0	0	100	100	0	0	0	0
Urease	100	(33)	100	100	100	0	100	(44)	0	(100)	100	0	100	0	0	100	100	(100)	0
Nitrate reductase	100	0	0	0	100	100	0	100	100	100	100	0	100	100	100	0	0	100	7
Nitrite reductase	0	0	100	100	100	100	0	100	100	100	100	0	64	100	100	0	0	0	0
β-Galactosidase (ONPG[c])	0	100	0	0	0	0	0	0	0	0	100	0	100	0	0	100	75	100	100
Pyrrolinodyl aminopeptidase	0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	27
Production of H_2S (on KIA)	0	0	0	0	0	0	0	0	0	0	0	0	0	100	100	0	0	0	0
Alkaline phosphatase	100	0	100	100	0	0	0	0	0	0	0	0	0	100	100	100	100	100	100
Requirement for NaCl	0	0	0	0	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0
Phenylalanine deaminase	ND	ND	ND	ND	100	100	100	100	100	100	0	100	100	ND	ND	ND	ND	ND	ND
Tributyrine esterase (<1 h)	ND	ND	ND	ND	0	0	0	0	0	0	100	0	0	ND	ND	ND	ND	ND	ND
Susceptibility to:																			
Colistin	100	0	0	0	100	50	100	0	0	0	0	0	73	60	100	0	0	0	7
Desferrioxamine	100	0	0	100	0	100	33	0	0	100	100	100	18	0	0	0	0	0	7
Netilmicin	ND	ND	ND	ND	100	100	100	0	100	100	100	66	0	ND	ND	ND	ND	ND	ND
Tetracyline	ND	ND	ND	ND	100	100	100	100	0	100	100	100	100	ND	ND	ND	ND	ND	ND

[a] Numbers in parentheses after organism names are numbers of strains tested. Values are percentages; those in parentheses represent delayed positivity. Abbreviations: L, lateral; ND: no data; P, polar; Pt, peritrichous.

[b] Both *Shewanella* species are ornithine decarboxylase positive.

[c] ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

aminopeptidase, and pyrrolidonyl aminopeptidase and negative for lysine, arginine, ornithine, denitrification, and indole production (123).

I. limosus can be distinguished from *Sphingobacterium* spp. by its lack of alkaline phosphatase activity and by its acidification of mannitol. *Sphingobacterium spiritivorum* also produces acid from mannitol but acidifies ethylene glycol, unlike *Inquilinus*. *I. limosus* differs from *Rhizobium radiobacter* and *Pannonibacter phragmitetus* by the absence of acid production from ethylene glycol (positive in most of the *P. phragmitetus* strains) and by the lack of nitrate reductase activity.

Identifying the species is difficult because it is not contained in the databases of commercial identification kits and its mucoid appearance may lead to confusion with mucoid *P. aeruginosa* strains (124). Isolates can be recovered on colistin-containing *Burkholderia cepacia* selective media but are inhibited on *B. cepacia* selective agar, which also contains gentamicin (125).

All isolates are reported to be resistant to penicillins and cephalosporins, kanamycin, tobramycin, colistin, doxycycline, and trimethoprim-sulfamethoxazole and susceptible to imipenem and ciprofloxacin (124, 125).

I. limosus has been isolated from soil samples and from respiratory secretions of cystic fibrosis patients (124, 125, 127, 128). The clinical impact of chronic colonization with *I. limosus* remains unclear. Chiron et al. (125) reported that for one patient, *I. limosus* was the only potential pathogen recovered from the sputum, whereby acquisition of this bacterium was followed by a worsening of his lung function.

Myroides odoratimimus and *Myroides odoratus*

The genus *Myroides* includes two species, *M. odoratimimus* and *M. odoratus*, formerly *Flavobacterium odoratum* (129), which can be isolated from clinical samples. Cells are thin, middle-sized (0.5 μm in diameter and 1 to 2 μm long), nonmotile rods, but both species display a gliding motility. The spreading colonies develop a typical fruity smell, similar to the odor of *Alcaligenes faecalis*. The yellow pigment, although less pronounced than that of some *Chryseobacterium* species, is of the flexirubin type.

Myroides species grow on most media, including MacConkey agar. Growth occurs at 18 to 37°C but usually not at 41°C. *Myroides* species are asaccharolytic, urease positive, and nitrate reductase negative, but nitrite is reduced. *M. odoratus* can be differentiated by its susceptibility to desferrioxamine, while *M. odoratimimus* is resistant. The species also differ by their cellular fatty acid patterns, with *M. odoratimimus* having significant amounts of C13:0*i* and C15:0 (129).

Organisms identified as *M. odoratus* have been reported mostly from urine but have also been found in wound, sputum, blood, and ear specimens (130, 131). Clinical infection with *Myroides* species is exceedingly rare. In our experience, based on 25 clinical isolates, *M. odoratimimus* is 4 to 5 times more frequently isolated from clinical material than *M. odoratus*.

Most strains are resistant to penicillins, cephalosporins, aminoglycosides, aztreonam, and carbapenems (131).

Ochrobactrum anthropi and *Ochrobactrum intermedium*

Ochrobactrum, *Pseudochrobactrum*, *Rhizobium*, and *Pannonibacter* have many morphological and biochemical characteristics in common, and therefore additional characteristics for these taxa are provided in Table 4.

The genus *Ochrobactrum* comprises at present 17 species. Two species, i.e., *O. anthropi* (132) and *O. intermedium* (133), are currently isolated from humans, but recently new species, i.e., *O. haematophilum* (134), *O. pseudogrignonense* (134), and *O. pseudintermedium* (135), have been recovered from clinical samples. *O. anthropi* (132) comprises the so-called urease-positive *Achromobacter* species, formerly designated CDC group Vd (biotypes 1 and 2), and *Achromobacter* groups A, C, and D.

Colonies on SBA are small, ~1 mm after overnight incubation, but grow large and creamy after 2 days and appear smooth, circular, and clearly delineated. *Ochrobactrum* species are medium-length, Gram-negative rods and motile by peritrichous flagella, although most cells have only one or two very long lateral flagella.

Ochrobactrum species are strongly trypsin and pyrrolidonyl aminopeptidase positive and are saccharolytic, with rapid acidification of glucose and xylose. Acidification of mannitol is irregular and often delayed positive. Ethylene glycol is acidified. Urease is positive in most species; phenylalanine deaminase is always positive. Nitrate reduction and nitrite reduction are positive, except in *O. haematophilum*. *O. intermedium* can be distinguished from *O. anthropi* by colistin resistance and growth at 41°C. Urease is often negative or delayed in this species. *O. pseudintermedium* is the only species growing at 45°C. Resistance to colistin, tetracycline, and aminoglycosides may have diagnostic value for distinction between *O. intermedium* and *O. pseudintermedium*. Among aminoglycosides, netilmicin provides the most clear-cut results.

Ochrobactrum species (*Brucellaceae*, *Alphaproteobacteria*) are closely related to *Brucella* species, with *O. intermedium* occupying a phylogenetic position that is intermediate between *O. anthropi* and *Brucella* (133).

O. anthropi has been isolated from various environmental and human sources, predominantly from patients with catheter-related bacteremia (132, 136) and rarely with other infections (137). One hospital outbreak in transplant patients has been described (138). A few cases of *O. intermedium* infections have been reported (see, e.g., reference 139), but because of the close phenotypic similarity of *O. anthropi* and *O. intermedium*, it is possible that certain infections considered to be caused by *O. anthropi* were actually caused by *O. intermedium*.

O. anthropi strains are usually resistant to β-lactams, such as broad-spectrum penicillins, broad-spectrum cephalosporins, aztreonam, and amoxicillin-clavulanate, but are usually susceptible to aminoglycosides, fluoroquinolones, imipenem, tetracycline, and trimethoprim-sulfamethoxazole (136).

Pannonibacter phragmitetus

P. phragmitetus, of the family *Rhodobacteraceae* (*Alphaproteobacteria*), has been shown to be identical to the strains formerly designated *Achromobacter* groups B and E (140). The species resembles most strongly *R. radiobacter*, but saccharolytic activity is somewhat weaker and not as extended. Unlike *Rhizobium*, *Ochrobactrum*, and *Pseudochrobactrum*, which all have a strong phenylalanine deaminase activity, *P. phragmitetus* does not produce phenylpyruvic acid from phenylalanine. Another easy and reliable differential test is the hydrolysis of tributyrin, which is positive within 30 min for *P. phragmitetus*, whereas *R. radiobacter* strains are positive only after several hours' to overnight incubation or remain negative. *Ochrobactrum* species and *Pseudochrobactrum* species do not hydrolyze tributyrin. Moreover, *P. phragmitetus* grows at 41°C while *R. radiobacter* does not.

Cases of septicemia due to *Achromobacter* group B have been reported (141).

Pseudochrobactrum

The genus *Pseudochrobactrum* was described in 2006 (142) and comprises two species, i.e., *P. saccharolyticum* and *P. asaccharolyticum*. The type strain of *P. asaccharolyticum* was isolated from a knee aspirate. Two more human strains were isolated from Belgian clinical samples: a wound and an eye swab (our unpublished data). Both species are very similar to *Ochrobactrum* species, but cells are nonmotile. *P. asaccharolyticum* slowly acidifies glucose and xylose, while *P. saccharolyticum* is more saccharolytic. Both species are susceptible to desferrioxamine and do not produce urease. Nitrate reduction and nitrite reduction are negative. The clinical significance of *Pseudochrobactrum* species remains to be assessed.

Rhizobium radiobacter

The former genus *Agrobacterium* contained several species of plant pathogens occurring worldwide in soils. Four distinct species of *Agrobacterium* were recognized: *A. radiobacter* (formerly *A. tumefaciens* and CDC group Vd-3), *A. rhizogenes* (subsequently transferred to the genus *Sphingomonas* as *Sphingomona rosa*), *A. vitis*, and *A. rubi*. More recently, an emended description of the genus *Rhizobium* (*Rhizobiaceae*, *Alphaproteobacteria*) was proposed to include all species of *Agrobacterium* (143), of which only *R. radiobacter* is clinically important.

Colonies of *R. radiobacter* are circular, convex, smooth, and nonpigmented to light beige on SBA, with a diameter of 2 mm at 48 h. Colonies may appear wet and become extremely mucoid and pink on MacConkey agar with prolonged incubation. *R. radiobacter* cells measure 0.6 to 1.0 by 1.5 to 3.0 µm and occur singly and in pairs.

R. radiobacter grows optimally at 25 to 28°C. It grows at 35°C as well, but not at 41°C. *R. radiobacter* is phenotypically very similar to the *Ochrobactrum* species, although phylogenetically separate. *R. radiobacter* differs clearly from *Ochrobactrum* species by a positive β-galactosidase test and by the production of ketolactonate, which is, however, not routinely tested. *R. radiobacter* has broad saccharolytic activity, including mannitol and raffinose.

R. radiobacter has been most frequently isolated from blood, followed by peritoneal dialysate, urine, and ascitic fluid (144). The species has also been isolated from the airways of patients with cystic fibrosis (123). The few cases are from patients with transcutaneous catheters or implanted biomedical prostheses, and effective treatment often requires removal of the device. Most strains are susceptible to broad-spectrum cephalosporins, carbapenems, tetracyclines, and gentamicin but not to tobramycin (144, 145). Testing of individual isolates is recommended for clinically significant cases.

Shewanella algae and *Shewanella putrefaciens*

The organisms formerly called *Pseudomonas putrefaciens*, *Alteromonas putrefaciens*, *Achromobacter putrefaciens*, and CDC group Ib have been placed in the genus *Shewanella*, which comprises >50 species. *S. putrefaciens* was described with two CDC biotypes. CDC biotype 1 was later described as *S. putrefaciens sensu stricto*, whereas CDC biotype 2 was subsequently assigned to a new species, *S. alga* (146), later corrected to *S. algae*.

Colonies of *Shewanella* species on SBA are convex, circular, smooth, and occasionally mucoid; produce a brown to tan soluble pigment; and cause green discoloration of the medium. Cells are long, short, or filamentous, reminiscent of *Myroides*. Motility is due to a single polar flagellum.

Most strains of both *Shewanella* species produce H_2S in KIA and TSI agar, a unique feature among clinically relevant GNF. Both are also ornithine decarboxylase positive and have strong alkaline phosphatase, strong trypsin, and strong pyrrolidonyl aminopeptidase activities. *S. algae* is halophilic, asaccharolytic, and requires NaCl for growth, with growth occurring already on TSA plus 0.5% NaCl. *S. putrefaciens* does not require NaCl for growth and is saccharolytic, producing acid from maltose and sucrose, and irregularly and weakly from glucose.

Khashe and Janda (147) have reported that *S. algae* is the predominant human clinical isolate (77%), while *S. putrefaciens* represents the majority of nonhuman isolates (89%). Although infrequently isolated in the clinical laboratory, *S. putrefaciens* and *S. algae* have been recovered from a wide variety of clinical specimens and are associated with a broad range of human infections (148), including skin and soft tissue infections, otitis media, ocular infection, osteomyelitis, peritonitis, and septicemia. The habitat for *S. algae* is saline, whereas *S. putrefaciens* has been isolated mostly from fish, poultry, and meats as well as from freshwater and marine samples.

Shewanella species are generally susceptible to most antimicrobial agents effective against Gram-negative rods, except penicillin and cephalothin (107, 145). The mean MICs of *S. algae* for penicillin, ampicillin, and tetracycline are higher than the corresponding MICs of *S. putrefaciens* (147).

Sphingobacterium, Indole-Negative Species

A total of 15 species have been described as belonging to the genus *Sphingobacterium*. Based on 16S rRNA gene sequence data, the indole-producing *Sphingobacterium mizutaii* also belongs to this genus, and as a consequence, the description of the genus *Sphingobacterium* as indole negative was emended to indole variable (149).

In summary, most species of this genus do not produce indole, but *S. mizutaii* is indole positive and is therefore dealt with among the indole-positive GNF in Table 5.

The species of the genus *Sphingobacterium* encountered in clinical material include *S. multivorum* (formerly *Flavobacterium multivorum* and CDC group IIk-2), *S. spiritivorum* (including the species formerly designated as *Flavobacterium spiritivorum*, *Flavobacterium yabuuchiae*, and CDC group IIk-3), *S. thalpophilum*, and *S. mizutaii* (150, 151).

Colonies are yellowish. *Sphingobacterium* species are middle-sized, nonmotile, Gram-negative rods. All species are strongly saccharolytic; i.e., glucose, xylose, and other sugars are acidified. No acid is produced from mannitol, except by *S. spiritivorum*, which is also the only species to produce acid from ethylene glycol. *S. thalpophilum* can be distinguished from other *Sphingobacterium* species by its nitrate reductase and its growth at 41°C.

S. multivorum and *S. spiritivorum* can be distinguished from *Sphingomonas paucimobilis* (formerly CDC group IIk-1) because they are nonmotile, urease positive, and resistant to polymyxin. Many strains of other *Sphingomonas* species are also colistin resistant.

S. multivorum is the most common human species. It has been isolated from various clinical specimens but has only rarely been associated with serious infections (peritonitis and sepsis) (152, 153). Blood and urine have been the most common sources for the isolation of *S. spiritivorum* (154). *S. thalpophilum* has been recovered from wounds, blood, eyes, abscesses, and an abdominal incision (40).

Sphingobacterium species are generally resistant to aminoglycosides and polymyxin B while susceptible *in vitro* to the quinolones and trimethoprim-sulfamethoxazole. Susceptibility to β-lactam antibiotics is variable, requiring testing of individual isolates (108).

Sphingomonas Species

On the basis of 16S rRNA gene sequence and the presence of unique sphingoglycolipid and ubiquinone types, the genus *Sphingomonas* (*Sphingomonadaceae*, *Alphaproteobacteria*) was created for organisms formerly known as *Pseudomonas paucimobilis* and CDC group IIk-1 (155, 156). The original genus *Sphingomonas* can be divided into four phylogenetic groups, each representing a different genus (157), whereby the emended genus *Sphingomonas* contains at least 12 species, of which only *S. paucimobilis* and *S. parapaucimobilis* are thought to be clinically important. However, recent 16S rRNA gene sequencing of 12 strains of clinical origin (Wauters, unpublished) revealed that several named and unnamed *Sphingomonas* species were present, but no *S. paucimobilis* and only two *S. parapaucimobilis* isolates. Because many phenotypic characteristics are shared by these species, routine laboratories best report them as *Sphingomonas* species.

Sphingomonas colonies are slow growing on blood agar medium, with small colonies appearing after 24 h of incubation. Growth occurs at 37°C but not at 41°C, with optimum growth at 30°C. Almost all strains produce a yellow, insoluble pigment, different from flexirubin pigments, as can be established by the KOH test (2). Few strains are nonpigmented or develop a pale yellow color after several days. Older colonies demonstrate a deep yellow (mustard color) pigment.

Sphingomonas species are medium to long, motile rods with a single polar flagellum. Motility occurs at 18 to 22°C but not at 37°C. However, few cells are actively motile in broth culture, thus making motility a difficult characteristic to demonstrate.

Oxidase is only weakly positive or even absent. All the strains are saccharolytic, but some acidify glucose only weakly and slowly. Urease is always negative, and nitrate reduction is only very rarely positive. Esculin is hydrolyzed, and β-galactosidase and alkaline phosphatase are positive. The yellow pigment of some strains may hamper a correct

TABLE 5 Oxidase-positive, indole-positive GNF[a]

Characteristic	*Balneatrix alpica* (1)	*Bergeyella zoohelcum* (10)	*Chryseobacterium anthropi*[b] (11)	*Chryseobacterium gleum* (11)	*Chryseobacterium hominis*[b] (14)	*Chryseobacterium indologenes* (15)	*Chryseobacterium treverense*[b] (5)	*Elizabethkingia meningoseptica* (17)	*Empedobacter brevis* (7)	*Sphingobacterium mizutaii* (5)	*Wautersiella falsenii* (53)	*Weeksella virosa* (10)
Motility	100	0	0	0	0	0	0	0	0	0	0	0
Beta-hemolysis (after 3 days on SBA)	0	0	0	0	0	100	0	0	0	0	0	0
Production of flexirubin pigments	0	0	0	100	0	74	0	0	0	0	0	0
Production of other pigments[c]	PY	–	–/PS	–	–/PY	–	PY	–/PY/PS	PY	PY	–/PY	–
Growth on MacConkey agar	0	0	0	100	0	67	0	83	100	0	100	0
Growth at 41°C	0	0	0	100	0	0	0	47	0	0	0	100
Acidification of:												
Glucose	100	0	100	100	100	100	100	100	100	100	100	0
Mannitol	100	0	0	0	0	0	0	100	0	0	0	0
Xylose	0	0	0	27	0	0	0	0	0	100	0	0
L-Arabinose	0	0	0	100	0	0	0	0	0	80	0	0
Maltose	100	0	100	100	100	100	100	100	100	100	100	0
Sucrose	0	0	0	0	28	20	0	0	0	100	0	0
Ethylene glycol	0	0	0	100	100	0	0	100	0	0	47	0
Esculin hydrolysis	0	0	0	100	100	100	20	100	0	100	53	0
Gelatinase	0	100	100	100	100	100	0	100	100	0	53/(47)	100
Urease	0	100	0	73	0	0	0	0[d]	0	0	100	0
Nitrate reductase	100	0	0	73	65	26	60	0	0	0	0	0
Nitrite reductase	0	0	0	54	65	13	100	88	0	80	34	0
β-Galactosidase (ONPG[e])	0	0	0	0	0	0	0	100	0	100	19	0
Benzyl-arginine aminopeptidase (trypsin)	0	100	100	100	100	100	80	100	100	100	100	100
Pyrrolidonyl aminopeptidase	0	0	91	100	100	100	80	100	100	100	100	100
Susceptibility to:												
Colistin/polymyxin	100	0	0	0	14	0	0	0	0	0	0	100
Desferrioxamine	100	100	73	0	0	0	100	0	0	0	15	100

[a] Numbers in parentheses after organism names are numbers of strains tested. Values are percentages; those in parentheses represent delayed positivity.
[b] Alkalinization of acetate is positive for *C. anthropi* and *C. hominis* and negative for *C. treverense*.
[c] Abbreviations and symbols: –, none; PS, pale salmon-pink; PY, pale yellow.
[d] *E. miricola* is urease positive (data from reference 170).
[e] ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

reading of the yellow color shift when nitrophenyl compounds of the latter substrates are used.

Members of this genus are known as decomposers of aromatic compounds and are being developed for use in bioremediation.

Sphingomonas species are widely distributed in the environment, including water. *S. paucimobilis* has been isolated from a variety of clinical specimens, including blood, cerebrospinal fluid (CSF), peritoneal fluid, urine, wounds, the vagina, and the cervix, as well as from the hospital environment (158, 159). *S. parapaucimobilis* clinical isolates have been obtained from sputum, urine, and the vagina (156).

Most strains are resistant to colistin, but all are susceptible to vancomycin, which is exceptional for Gram-negative, nonfermenting rods. This is elsewhere only found in *Chryseobacterium* and related genera like *Elizabethkingia* and *Empedobacter*. Most *Sphingomonas* strains are susceptible to tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, and aminoglycosides. Susceptibility to other antimicrobial agents, including fluoroquinolones, varies (107, 158).

Oxidase-Positive, Indole-Positive GNF

The natural habitats of most oxidase-positive, indole-positive GNF (Table 5) are soil, plants, and food and water sources, including those in hospitals. Clinically relevant species include *Balneatrix alpica*, *Bergeyella zoohelcum*, *Chryseobacterium* species, *E. meningoseptica*, *Empedobacter brevis*, *S. mizutaii*, *Wautersiella falsenii*, and *Weeksella virosa*. All are indole, trypsin, pyrrolidonyl aminopeptidase, and alkaline phosphatase positive, except for *B. zoohelcum*, which is pyrrolidonyl aminopeptidase negative, and *B. alpica*, which is both trypsin and pyrrolidonyl aminopeptidase negative. Table 5 presents an overview of the characteristics useful to differentiate among these species.

Balneatrix alpica

B. alpica produces colonies that are 2 to 3 mm in diameter, convex, and smooth. The center of the colonies is pale yellow after 2 to 3 days and pale brown after 4 days. *B. alpica* is a straight or curved, Gram-negative rod. It is the only motile species among the clinically relevant indole-positive GNF. Cells have one or two polar flagella.

The species is strictly aerobic and saccharolytic. Both trypsin and pyrrolidonyl aminopeptidase are negative, unlike with other indole-positive GNF. Growth occurs at 20 to 46°C on ordinary media such as TSA but not on MacConkey agar. It acidifies fructose, glucose, glycerol, inositol, maltose, mannitol, mannose, sorbitol, and xylose. *B. alpica* is nitrate reductase and weakly gelatinase positive. It is similar to *E. meningoseptica* but can be differentiated from this species by its motility and nitrate reductase and by the absence of β-galactosidase.

B. alpica was first isolated in 1987 during an outbreak of pneumonia and meningitis among persons who attended a hot (37°C) spring spa in southern France (160). Isolates from eight patients were recovered from blood, CSF, and sputum, and one was recovered from water. This species is only rarely isolated from human clinical specimens.

Bergeyella zoohelcum

B. zoohelcum comprises former CDC group IIj strains (161).

B. zoohelcum and *W. virosa* are morphologically and biochemically similar organisms with cells measuring 0.6 by 2 to 3 µm, with parallel sides and rounded ends. *B. zoohelcum* colonies are sticky and tan to yellow.

Both species fail to grow on MacConkey agar and are nonsaccharolytic. Both species are susceptible to desferrioxamine and have the unusual feature of being susceptible to penicillin, a feature that allows them to be easily differentiated from the related genera *Chryseobacterium* and *Sphingobacterium*. *B. zoohelcum* can be differentiated from *W. virosa* because it is pyrrolidonyl aminopeptidase negative, strongly urease positive, and resistant to colistin.

B. zoohelcum is isolated mainly from wounds caused by animal (mostly dog) bites and can lead to meningitis and septicemia (161, 162). Both *B. zoohelcum* and *W. virosa* are susceptible to most antibiotics. However, at present no specific antibiotic treatment is recommended, and antimicrobial susceptibility testing should be performed on significant clinical isolates.

Chryseobacterium

CDC group IIb comprises the species *Chryseobacterium indologenes*, *Chryseobacterium gleum*, and other strains, which probably represent several unnamed taxa.

Strains included in CDC group IIb are nonmotile rods. Cells of *C. indologenes* are similar to those of *Chryseobacterium anthropi*, *Chryseobacterium hominis*, *E. meningoseptica*, and *S. mizutaii*; i.e., they are thinner in their central than in their peripheral portions and include filamentous forms.

CDC group IIb strains are oxidase and catalase positive, produce flexirubin pigments (2), are moderately saccharolytic, and are esculin and gelatin hydrolysis positive. *C. indologenes* and *C. gleum* can easily be differentiated from each other by four characteristics: *C. indologenes* displays a broad beta-hemolysis area within 3 days of incubation at 37°C on SBA, is always arabinose negative, does not acidify ethylene glycol, and does not grow at 41°C. *C. gleum* exhibits pronounced alpha-hemolysis, resembling viridans discoloration; always acidifies ethylene glycol; is arabinose positive or delayed positive; and grows at 41°C.

Beta-hemolysis is absent or very rare in other strains of CDC group IIb and is therefore almost specific for the

TABLE 6 Pink-pigmented GNF[a]

Characteristic	*Asaia* spp. (2)	*Azopsirillum* spp. (3)	*Methylobacterium* spp. (4)	*Roseomonas cervicalis* (2)	*Roseomonas gilardii* subsp. *gilardii* and *Roseomonas gilardii* subsp. *rosea* (5)	*Roseomonas mucosa* (9)	*Roseomonas* genomic species 4 (1)	*Roseomonas* genomic species 5 (4)
Oxidase	0	100	0	100	100^{w}	11^{w}	100	100^{w}
Acidification of:								
Glucose	100	0	(25)	0	(20)	100	(100)	0
Fructose	100	(100)^{w}	0	50	60/(40)	100	100	(100)
Mannitol	100	0	0	0	(80)	100	0	0
Xylose	100	100	100	(100)	0	0	0	0
Arabinose	100	100	100	100	100	100	0	0
Ethylene glycol	100	100	100	100	100	100	0	0
Urease	0	100	100	100	100	100	100	100
Nitrate reductase	0	100	0	0	0	0	100	0
Esculin hydrolysis	0	100	0	0	0	0	0	0
Trypsin	100	0	100	0	0	0	0	0
Pyrrolidonyl aminopeptidase	0	100	0	0	100	100	0	0
β-Galactosidase (ONPG[b])	0	100	0	0	0	0	0	0
Susceptibility to:								
Colistin	0	0	0	0	40	45	0	25
Desferrioxamine	0	0	0	0	0	0	100	0

[a] Numbers in parentheses after organism names are numbers of strains tested. Values are percentages; those in parentheses represent delayed positivity. Abbreviation: W, weakly positive reaction.

[b] ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

identification of *C. indologenes*, while the profile of *C. gleum* may be shared by other strains of this group. It should be noted that some *C. indologenes* strains do not produce flexirubin.

Among CDC group IIb species, *C. indologenes* is usually considered most frequently isolated from clinical samples, although it rarely has clinical significance (145). Nosocomial infections due to *C. indologenes* have been linked to the use of indwelling devices during hospital stays (163). More recently, *C. indologenes* has also been associated with the cause of neonatal meningitis (164, 165).

Still, the frequency of *C. indologenes* as reported in the literature should be interpreted with caution, because until recently and without molecular biology, *C. indologenes* could almost not be distinguished routinely from other CDC group IIb strains. We have recently examined 21 CDC group IIb strains both phenotypically and by 16S rRNA gene sequencing and found 9 *C. indologenes* isolates, 5 *C. gleum* isolates, and 7 isolates belonging to unnamed *Chryseobacterium* species.

The production of novel types of metallo-β-lactamases from *C. indologenes* has been studied in detail (166).

C. anthropi represents part of the strains formerly designated as CDC group IIe (167). Most strains display very sticky colonies, which are nonpigmented but may develop a slightly salmon-pinkish, rarely yellowish color after a few days. In contrast to *C. hominis*, the species is negative for esculin hydrolysis and acidification of ethylene glycol. In addition, many strains are susceptible to desferrioxamine. Most clinical isolates used for the description of the species were from wounds and blood cultures (167).

C. hominis includes the strains formerly included in CDC group IIc and most of the strains of CDC group IIh (168). This species does not produce flexirubin pigments, but some strains exhibit a slightly yellowish pigmentation. Colonies are often mucoid. *C. hominis* can be differentiated from *C. gleum* by the absence of flexirubin pigments and the lack of acid production from arabinose. *C. indologenes* strains lacking flexirubin pigments may resemble *C. hominis*, but the latter is never beta-hemolytic and always acidifies ethylene glycol.

Many strains have been isolated from blood. Others have been isolated from dialysis fluid, pus, the eye, infraorbital drain, and aortic valve, but their clinical significance remains to be assessed (168).

Chryseobacterium treverense has been described recently (169). Part of the strains formerly designated CDC group IIe belong to this species. Although described as indole negative, *C. treverense* strains produce indole that should be detected using the method recommended for GNF (see chapter 33). The species is phenotypically related to *C. anthropi* and *C. hominis* but can be distinguished by a negative (or very weak) gelatin hydrolysis and the absence of acetate alkalinization.

Elizabethkingia meningoseptica and *Elizabethkingia miricola*

Colonies of *E. meningoseptica*, formerly *Chryseobacterium meningosepticum* (170), are smooth and fairly large, either nonpigmented or producing a pale yellow or slightly salmon-pinkish pigment after 2 or 3 days. Characteristic features are acid production from mannitol and β-galactosidase activity. Gelatin and esculin hydrolysis are positive. *Elizabethkingia* and *Chryseobacterium* species can be differentiated as well on the basis of 16S rRNA gene sequence analysis (167, 170).

E. meningoseptica has been reported to be associated with (neonatal) meningitis (171), nosocomial outbreaks (see, e.g., references 172 and 173), and different types of infection (e.g., adult pneumonia and septicemia [145, 172] and infections reported in dialysis units [174]). A clinical case (sepsis) of *E. miricola* was reported only once.

Empedobacter brevis

E. brevis (175) colonies are yellowish pigmented but do not produce flexirubin. *E. brevis* can be differentiated from *C. indologenes*, *C. gleum*, other CDC group IIb strains, and *C. hominis* by its lack of esculin hydrolysis. Growth on MacConkey agar and stronger gelatinase activity are useful to distinguish it from *C. anthropi*. The species is rarely recovered from clinical material.

Sphingobacterium mizutaii

S. mizutaii, previously known as *Flavobacterium mizutaii* (151) but originally described as *Sphingobacterum mizutae* (176), is the only indole-positive *Sphingobacterium* species (149). It is saccharolytic—producing acid from a large number of carbohydrates, including xylose—similar to other *Sphingobacterium* species, from which it can be distinguished by its indole production, its failure to grow on MacConkey agar, and its usual lack of urease activity (40).

S. mizutaii can be distinguished from *Chryseobacterium* and *Empedobacter* species by its lack of gelatin hydrolysis and of flexirubin production. *S. mizutaii* produces acid from xylose but not from ethylene glycol, allowing differentiation from other indole-positive species. The phenotypic profile of *S. mizutaii* is similar to that of the strains described as *Chryseobacterium* CDC group IIi. Furthermore, 16S rRNA gene sequencing confirms that most CDC group IIi strains actually belong to the species *S. mizutaii* (149).

S. mizutaii has been described as an indole-negative species (151), but in our hands all strains tested, including the type strain, produce as much indole as the *Chryseobacterium* strains, using the method recommended for GNF (see chapter 33). *S. mizutaii* has been isolated from blood, CSF, and wound specimens (40).

Wautersiella falsenii

W. falsenii is closely related to *E. brevis*, from which it differs by its urease activity. Two genomovars have been described (177): genomovar 1 is always esculin positive and β-galactosidase negative, whereas 90% of the genomovar 2 strains are esculin negative and 63% are β-galactosidase positive.

W. falsenii was described as belonging to a separate genus from *Empedobacter*, based on comparison of its 16S rRNA gene sequence with an *E. brevis* EMBL sequence of poor quality (our unpublished data). A high-quality sequence of the rRNA gene of the type strain of *E. brevis* indicates that *W. falsenii* probably has to be renamed as *Empedobacter falsenii*.

W. falsenii is much more frequently isolated from clinical samples than *E. brevis* (177, 178). Its clinical significance remains to be assessed.

Weeksella virosa

W. virosa colonies are mucoid and adherent to the agar, reminiscent of the sticky colonies of *B. zoohelcum*. Colonies are not pigmented after 24 h of incubation but may become yellowish tan to brown after 2 or 3 days. The cellular morphology of *W. virosa* is dealt with above in the discussion of *B. zoohelcum*. *W. virosa* can be differentiated from *B. zoohelcum* because it is urease negative and polymyxin B and colistin susceptible, whereas *B. zoohelcum* is rapid urease positive and polymyxin B and colistin resistant. *W. virosa* comprises former CDC group IIf strains (179). *W. virosa* is isolated mainly from urine and vaginal samples (179), in contrast to *B. zoohelcum*, which is isolated mostly from animal bites.

The appropriate choice of effective antimicrobial agents for the treatment of chryseobacterial infections is difficult (173). *Chryseobacterium* species and *E. meningoseptica* are inherently resistant to many antimicrobial agents commonly used to treated infections caused by Gram-negative bacteria (aminoglycosides, β-lactam antibiotics, tetracyclines, and chloramphenicol) but are often susceptible to agents generally used for treating infections caused by Gram-positive bacteria (rifampin, clindamycin, erythromycin, trimethoprim-sulfamethoxazole, and vancomycin) (107, 108, 145). Although early investigators recommended vancomycin for treating serious infection with *E. meningoseptica*, subsequent studies showed greater *in vitro* activity of minocycline, rifampin, trimethoprim-sulfamethoxazole, and quinolones (108, 172). Among the quinolones, levofloxacin is more active than ciprofloxacin and ofloxacin (108). *C. indologenes* is reported to be uniformly resistant to cephalothin, cefotaxime, ceftriaxone, aztreonam, aminoglycosides, erythromycin, clindamycin, vancomycin, and teicoplanin, while susceptibility to piperacillin, cefoperazone, ceftazidime, imipenem, quinolones, minocycline, and trimethoprim-sulfamethoxazole is variable, requiring testing of individual isolates (108). Several studies reported that administration of quinolone, minocycline, trimethoprim-sulfamethoxazole, or rifampin and treatment of local infection improve the clinical outcome of patients with *E. meningoseptica* infections. The choice of appropriate antimicrobial therapy is further complicated by the fact that MIC breakpoints for resistance and susceptibility of chryseobacteria have not been established by the CLSI and the results of disk diffusion testing are unreliable in predicting antimicrobial susceptibility of *Chryseobacterium* species (180). The Etest is a possible alternative to the standard agar dilution method for testing cefotaxime, ceftazidime, amikacin, minocycline, ofloxacin, and ciprofloxacin but not piperacillin (181). Definitive therapy for clinically significant isolates should be guided by individual susceptibility patterns determined by an MIC method.

Pink-Pigmented GNF

It should be noted that colonies of *C. anthropi* and *E. meningoseptica* can be lightly salmon colored on some media after several days of incubation, but this is not to be confused with the clearly pink colonies of the taxa discussed here (Table 6).

Asaia

Asaia is a genus of the family *Acetobacteraceae* (*Alphaproteobacteria*), with some clinically relevant members, such as *A. bogorensis* (182), *A. lannensis* (183), and *A. siamensis* (184). The natural habitats of *Asaia* species are reported to be the flowers of the orchid tree, plumbago, and fermented glutinous rice, all originating in hot tropical climates, particularly in Indonesia and Thailand.

Growth of *Asaia* species is scant to moderate on SBA. Colonies are pale pink. In opposition to *Methylobacterium*, colonies are not dark under UV light. *Asaia* species are small to middle-sized, Gram-negative rods, usually motile by one or two polar or lateral flagella. The species are oxidase negative and strongly saccharolytic; i.e., glucose, mannitol, xylose, and L-arabinose are acidified very rapidly, often within 1 h on low-peptone phenol red agar. Acid is also produced from ethylene glycol. Furthermore, *Asaia* species are biochemically rather inert, except for benzyl arginine aminopeptidase (trypsin) activity. *Asaia* species can be distinguished from *Methylobacterium* species by cell morphology, a stronger saccharolytic activity, and acid production from mannitol.

A. bogorensis has been reported as a cause of peritonitis in a patient on automated peritoneal dialysis (185) and as a cause of bacteremia after intravenous drug abuse (186). *A. lannensis* has been identified as the cause of nosocomial infections in pediatric patients with idiopathic dilated cardiomyopathy (183).

Asaia species have been reported to be resistant to ceftazidime, meropenem, imipenem, trimethoprim, amikacin, vancomycin, aztreonam, penicillin, and ampicillin by disk diffusion (187). The *A. bogorensis* strain reported by Snyder et al. (185) was susceptible to aminoglycosides (amikacin, tobramycin, and gentamicin) and resistant to ceftazidime and meropenem by disk diffusion.

Azospirillum

The former *Roseomonas* genomic species 3 (*Roseomonas fauriae*) and genomic species 6 have been transferred to the genus *Azospirillum* (*Rhodospirillaceae*, *Alphaproteobacteria*), a genus of nitrogen-fixing plant symbionts that is in a different order of bacteria (188). Some strains of this genus may occasionally be isolated from clinical material (189).

Colonies are pale pink and resemble those of *Roseomonas*. Cells are somewhat more rod shaped than *Roseomonas* and are motile by one or two polar flagella. Oxidase is positive and urea is strongly positive, as in *Roseomonas* species. β-Galactosidase activity and esculin hydrolysis allow differentiation of *Azospirillum* from other pink-pigmented species.

Methylobacterium

The genus *Methylobacterium*, of the family *Methylobacteriaceae* (*Alphaproteobacteria*), currently consists of 20 named species plus additional unassigned biovars, recognized on the basis of carbon assimilation type, electrophoretic type, and DNA-DNA homology grouping (190). *Methylobacterium* species are isolated mostly from vegetation but may also occasionally be found in the hospital environment. *M. mesophilicum* (formerly *Pseudomonas mesophilica*, *Pseudomonas extorquens*, and *Vibrio extorquens*) and *M. zatmanii* have been the two most commonly reported species isolated from clinical samples.

Colonies of *Methylobacterium* species are small, dry, and coral pink. Under UV light, *Methylobacterium* colonies appear dark due to absorption of UV light (189). Growth is fastidious on ordinary media such as TSA, producing 1-mm-diameter colonies after 4 to 5 days on SBA, modified Thayer-Martin, buffered charcoal-yeast extract, and Middlebrook 7H11 agars, with the best growth occurring on Sabouraud agar and usually no growth on MacConkey agar. Optimum growth occurs between 25 and 30°C. They are able to utilize methanol as a sole source of carbon and energy, although this characteristic may be lost on subculture. Cells are pleomorphic, vacuolated rods that stain poorly and may resist decolorization (Fig. 4). Motility by one polar flagellum is difficult to demonstrate. In the description of the genus (191), methylobacteria were reported to be oxidase positive, but the strains tested by us with the dimethyl-paraphenylenediamine reagent were all oxidase negative. Saccharolytic activity is weaker than in *Asaia* spp., no acid is produced from mannitol, and acid is produced irregularly from glucose.

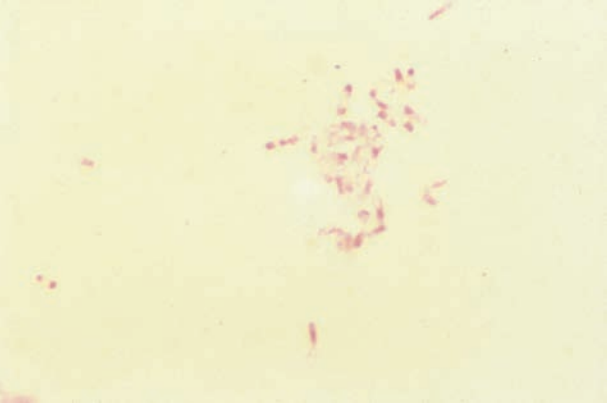

FIGURE 4 Gram stain of *Methylobacterium*, showing pleomorphic, Gram-negative rods with vacuoles. doi:10.1128/9781555817381.ch44.f4

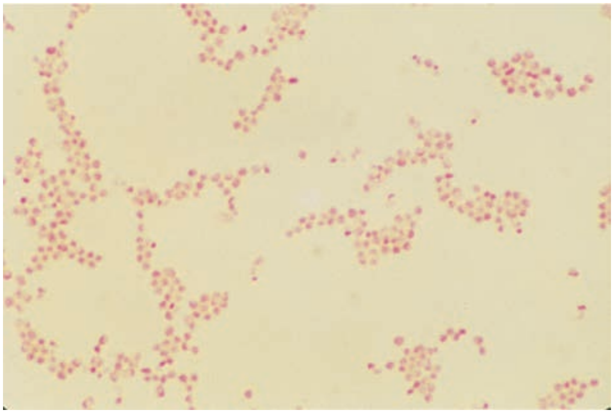

FIGURE 5 Gram stain of *Roseomonas*, showing Gram-negative, coccoid organisms. doi:10.1128/9781555817381.ch44.f5

Arabinose, xylose, and ethylene glycol are acidified. Urea and starch are hydrolyzed.

Methylobacterium species have been reported to cause septicemia, continuous ambulatory peritoneal dialysis-related peritonitis, skin ulcers, synovitis, and other infections often in immunocompromised patients (192–195). Tap water has been implicated as a possible agent of transmission in hospital environments, and methods for monitoring water systems for methylobacteria have been described previously (3).

Active drugs include aminoglycosides and trimethoprim-sulfamethoxazole, whereas β-lactam drugs show variable activity (196). These species are best tested for susceptibility by agar or broth dilution at 30°C for 48 h (30).

Roseomonas

The original description of the genus *Roseomonas* (*Acetobacteraceae*, *Alphaproteobacteria*) included three named species, *R. gilardii* (genomic species 1), *R. cervicalis* (genomic species 2), and *R. fauriae* (genomic species 3), and three unnamed species, *Roseomonas* genomic species 4, 5, and 6 (189). *Roseomonas* genomic species 3 and 6 have been transferred to the genus *Azospirillum*. More recently, Han et al. (188) proposed a new species, *R. mucosa*, and a new subspecies, *R. gilardii* subsp. *rosea* (to differentiate from *R. gilardii* subsp. *gilardii*).

The following *Roseomonas* species can be isolated from clinical samples: *R. gilardii* subsp. *gilardii*, *R. gilardii* subsp. *rosea*, *R. mucosa*, *R. cervicalis*, *Roseomonas* genomic species 4, and *Roseomonas* genomic species 5.

Colonies are mucoid and runny and grow larger than those of *Asaia* and *Methylobacterium*. Pigmentation varies from pale pink to coral pink. *Roseomonas* cells are nonvacuolated, coccoid, plump rods, mostly in pairs and short chains (Fig. 5) and usually motile by one or two polar flagella, but motility is often difficult to demonstrate. Genomic species 5 is nonmotile. Growth occurs at 37°C on ordinary media like SBA, and mostly on MacConkey agar, but the best growth is observed on Sabouraud agar. Oxidase is dependent on the species and often weak. Saccharolytic activity is also species dependent. All *Roseomonas* species strongly hydrolyze urea but not esculin. They are trypsin and β-galactosidase negative. Phenotypic distinction among the different species is based on oxidase, acid production from carbohydrates, and pyrrolidonyl aminopeptidase and nitrate reductase activities.

R. mucosa acidifies rapidly arabinose, mannitol, and fructose. Glucose is acidified within 1 to 3 days. Oxidase is negative, and pyrrolidonyl aminopeptidase is positive. *R. gilardii* subsp. *gilardii* and *R. gilardii* subsp. *rosea* exhibit a weak oxidase reaction and are pyrrolidonyl aminopeptidase positive. The two subspecies cannot be differentiated by current phenotypic tests. They are less saccharolytic than *R. mucosa* and produce acid from arabinose and fructose but only irregularly and slowly from mannitol and rarely from glucose. *R. cervicalis* is strongly oxidase positive but has no pyrrolidonyl aminopeptidase activity. Only arabinose and fructose are positive within 2 days and xylose within 3 or 4 days. The single strain of genomic species 4 examined is also oxidase positive and pyrrolidonyl aminopeptidase negative, and its saccharolytic activity is limited to acid production from glucose and fructose. It is the only species displaying nitrate reduction. Genomic species 5 is weakly oxidase positive and pyrrolidonyl aminopeptidase negative. It is the least saccharolytic species, with only a delayed acid production from fructose. Ethylene glycol is acidified by all species except by genomic species 4 and 5.

Roseomonas species are uncommon isolates from humans, but they are nevertheless the most frequently isolated pink GNF. Clinical isolates have been recovered from blood, wounds, exudates, abscesses, genitourinary sites, continuous ambulatory peritoneal dialysis fluid, and bone (197–200). A total of 35 cases of opportunistic infections with *Roseomonas* species were reviewed (199). In multiple-case reports, about 60% of the isolates recovered have been from blood, with about 20% from wounds, exudates, and abscesses and about 10% from genitourinary sites (197, 199).

Dé et al. (197) summarized susceptibility data from three published reports on a combined 80 strains of *Roseomonas*. All strains were susceptible to amikacin (100%); frequently susceptible to imipenem (99%), ciprofloxacin (90%), and ticarcillin (83%); less susceptible to ceftriaxone (38%), trimethoprim-sulfamethoxazole (30%), and ampicillin (13%); and rarely susceptible to ceftazidime (5%). All strains were resistant to cefepime. In catheter-related infections, eradication of the organism has proven difficult unless the infected catheter is removed.

ANTIMICROBIAL SUSCEPTIBILITIES

Decisions about performing susceptibility testing are complicated by the fact that the CLSI interpretive guidelines for disk diffusion testing of GNF are limited to *Pseudomonas* species, *B. cepacia*, *Stenotrophomonas maltophilia*, and *Acinetobacter* species and therefore, except for *Acinetobacter* species, do not include the organisms covered in this chapter. Furthermore, results obtained with, e.g., *Acinetobacter* species by using disk diffusion do not correlate with results obtained by conventional MIC methods. In general, laboratories should try to avoid performing susceptibility testing on the organisms included in this chapter. When clinical necessity dictates that susceptibility testing be performed, an overnight MIC method, e.g., Etest (bioMérieux) on Mueller-Hinton agar (181), is recommended.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Although certain GNF can on occasion be frank pathogens, e.g., *Burkholderia pseudomallei*, *E. meningoseptica*, and *P. aeruginosa*, they are generally considered to be of low virulence and often occur in mixed infections, making it difficult to determine when to work up cultures and when to perform susceptibility studies. *E. meningoseptica* in neonatal meningitis, *M. lacunata* in eye infections, and *M. catarrhalis* in respiratory tract infections should be reported as significant pathogens. Direct Gram stain interpretation of clinical specimens may be of limited importance, because these organisms often occur in mixed infections and because their clinical importance has to be interpreted taking into account the considerations discussed below. Decisions regarding the significance of GNF in a clinical specimen must take into account the clinical condition of the patient and the source of the specimen submitted for culture. In general, the recovery of a GNF in pure culture from a normally sterile site warrants identification and susceptibility testing, whereas predominant growth of a GNF from a nonsterile specimen, such as an endotracheal culture from a patient with no clinical signs or symptoms of pneumonia, would not be worked up further. Identification of GNF may also be important to document nosocomial outbreaks linked to hospital environment or medical devices. Because many GNF exhibit multiple-antibiotic resistance, patients who are on antibiotics often become colonized with GNF. GNF species isolated in mixed cultures can usually be reported by descriptive identification, e.g., "growth of *P. aeruginosa* and two varieties of GNF not further identified."

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Bordetella and Related Genera

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TAXONOMY

The genera *Bordetella*, *Achromobacter*, *Alcaligenes*, *Kerstersia*, *Advenella*, and *Paenalcaligenes* belong to the family *Alcaligenaceae* (order *Burkholderiales* in the β-subclass of the *Proteobacteria*) (1). Other members of this family include *Azohydromonas*, *Brackiella*, *Candidimonas*, *Castellaniella*, *Derxia*, *Oligella*, *Parapusillimonas*, *Pelistega*, *Pigmentiphaga*, *Pusillimonas*, and *Taylorella* (List of Prokaryotic names with Standing in Nomenclature [http://www.bacterio.net/]).

The genus *Bordetella* contains eight species: *Bordetella avium*, *B. bronchiseptica*, *B. hinzii*, *B. holmesii*, *B. parapertussis*, *B. pertussis* (the type species), *B. petrii*, and *B. trematum* (2–7). Other putative species, such as "*B. ansorpii*" and other strains similar to *B. trematum* (8, 9), have been described but not validly named. *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* could be considered a single species, but chemotaxonomic differences and differences in host range and pathogenesis (3, 10) support the status as separate species. Analysis of their genome sequences revealed that *B. parapertussis* and *B. pertussis* are independent derivatives of a *B. bronchiseptica*-like ancestor (10). The taxonomy of the genus *Achromobacter* is closely intertwined with the genus *Alcaligenes*. The genus *Alcaligenes* is now limited to *Alcaligenes aquatilis* and *A. faecalis* (the type species) (11, 12), while the genus *Achromobacter* consists of 16 species: *Achromobacter aegrifaciens*, *A. animicus*, *A. anxifer*, *A. denitrificans*, *A. dolens*, *A. insolitus*, *A. insuavis*, *A. marplatensis*, *A. mucicolens*, *A. piechaudii*, *A. pulmonis*, *A. ruhlandii*, *A. sediminum*, *A. spanius*, *A. spiritinus*, and *A. xylosoxidans* (the type species) (5, 11, 13–17, 153). In addition, this genus contains six yet-unnamed genogroups (designated genogroups 1, 3, 8, 9, 12, and 13) (18). The genus *Kerstersia* was proposed for a set of strains phenotypically resembling *A. faecalis*; this genus contains two species (*Kerstersia gyiorum* and *K. similis*) (5, 11). Similarly, the genus *Advenella* was created to harbor a number of *Alcaligenes*-like strains; these strains either belong to *Advenella incenata* or one of several unnamed genomic species (19) or were previously described as *Tetrathiobacter* species (20). Finally, the genus *Paenalcaligenes* (with the species *Paenalcaligenes hominis* and *P. suwonensis*) was created for single strains isolated from human blood (21) and spent mushroom compost (154), respectively.

DESCRIPTION OF THE GENERA

Bordetella

Bordetellae are small (1- to 2-µm) Gram-negative, nonsporulating coccoid rods (22). *B. pertussis* and *B. parapertussis* have smaller genomes than many of the other members of the genus (4.086 Mbp for *B. pertussis*, 4.774 Mbp for *B. parapertussis*, 5.338 Mbp for *B. bronchiseptica*, and 5.287 Mbp for *B. petrii*, but 3.732 Mbp for *B. avium* and around 3.8 Mbp for *B. holmesii*). Insertion sequences are mainly found in the genomes of *B. pertussis* (e.g., IS*481* and IS*1002*), *B. parapertussis* (e.g., IS*1001*), and *B. holmesii* (e.g., IS*481* and IS*1001-like*) and less so in *B. bronchiseptica* (10, 23, 24). A comparison between the species also revealed that only around 50% of the genome is conserved in all species and that multiple mechanisms, such as gain or loss of genes, point mutations, and horizontal gene transfer, may play a role in the development of the species (25).

Bordetellae are catalase positive and oxidize amino acids, but no carbohydrates can be fermented. Some species possess peritrichous flagella and are motile. Bordetellae are able to grow in simple synthetic media under aerobic conditions (except *B. petrii*; see below). However, *B. pertussis* and *B. parapertussis* are sensitive to toxic substances and metabolites present in many microbiological media and need special transport conditions, special culture media, and prolonged incubation. The other species are less sensitive and can be isolated by routine microbiological procedures.

B. petrii is the most versatile *Bordetella* species; it can grow aerobically and anaerobically and was initially found as a free-living environmental bacterium (24). All other Bordetellae are found only in warm-blooded animal und humans. *B. avium* is a pathogen for poultry and has only once been isolated from humans (8). *B. bronchiseptica* can cause respiratory infections in many animal species and, infrequently, also in humans. *B. holmesii* has recently been recognized as a relevant pathogen mostly in immunosuppressed but also in immunocompetent patients and may cause a variety of systemic diseases in various organ systems. "*B. ansorpii*," *B. hinzii*, *B. petrii*, and *B. trematum* are rarely found in human infections and mainly cause symptomatic diseases in immunocompromised patients. *B. parapertussis* is found in sheep and humans, and *B. pertussis* is thought to be a strictly human pathogen (Table 1).

Bordetellae express many virulence factors that are controlled by a complex virulence expression system operating

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TABLE 1 Members of the genus *Bordetella*

Species	Host or source	Transmission	Disease(s)
B. pertussis	Humans	Droplets	Pertussis
B. parapertussis	Humans	Droplets	Parapertussis (pertussis-like disease)
	Sheep	Unknown	Respiratory disease
B. holmesii	Humans	Droplets	Respiratory disease
	Humans	Unknown	Systemic infection (immunocompromised hosts)
B. bronchiseptica	Animals	Droplets (?)	Respiratory disease
	Humans	Droplets (?)	Respiratory disease
			Systemic infection (immunocompromised hosts)
B. hinzii	Poultry	Droplets	Respiratory disease
	Humans	Unknown	Cholangitis, arthritis
B. trematum	Humans	Unknown	Wound infection, otitis
B. petrii	Environment		
	Humans	Unknown	Osteomyelitis, mastoiditis
"*B. ansorpii*"	Humans	Unknown	Epidermal cyst, systemic infection (immunocompromised hosts)
B. avium	Poultry	Droplets	Respiratory disease
	Humans	Unknown	Respiratory disease

in response to environmental conditions (*BvgAS*) (3, 26), changing between mode X (respiratory tract infection), mode I (intermediate, possibly relevant for transmission), and mode C (starvation). Virulence factors of bordetellae can be classified as adhesins, autotransporters (e.g., filamentous hemagglutinin, fimbriae, pertactin), and toxins (e.g., pertussis toxin [PT], adenylate cyclase hemolysin, and lipopolysaccharide) (3). Only *B. pertussis* produces PT encoded by the *ptx* gene, whereas *B. parapertussis* and *B. bronchiseptica* contain the *ptx* gene but normally lack the promoter (3). PT has ADP-ribosyltransferase activity and ribosylates G(i) proteins (3), thereby suppressing chemotaxis, oxidative responses, and the overall activity of neutrophils and macrophages. Filamentous hemagglutinin (FHA) is a large (220-kDa) surface-associated and secreted protein and mediates the adhesion of the bordetellae to the ciliated epithelium of the upper respiratory tract. FHA is produced by *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* (3). Fimbria type 2 (FIM2) and FIM3 are important factors in colonizing the respiratory mucosa. Isolates of *B. pertussis* can display FIM2, FIM3, or both on their surfaces. Adenylate cyclase hemolysin is a hemolysin with enzymatic activity (27). *B. pertussis* also produces a lipopolysaccharide without a repetitive O-antigenic chain. Pertactin is a 68- to 70-kDa surface protein that mediates eukaryotic cell binding *in vitro* (3), is involved in cell attachment, and is highly immunogenic (28). In recent years, isolates not expressing pertactin have been found in many countries at increasing frequencies, and the clinical presentations were similar with pertactin-positive and pertactin-negative strains (29–31).

Achromobacter

Achromobacter species are Gram-negative, nonsporulating, small bacilli. Most species are motile, but motility is weak or absent in *A. pulmonis* and is strain dependent in *A. insuavis*. *Achromobacter* species are strictly aerobic and nonfermentative, although strains of some species are able to grow anaerobically with nitrate as an electron acceptor (15, 17). All *Achromobacter* species are oxidase and catalase positive, but none of them exhibit urease, DNase, lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase, or gelatinase activity (13, 15–17). They grow well on simple media, and on nutrient agar colonies are flat or slightly convex with smooth margins and range from white to light brown (13). Under laboratory conditions growth occurs between 30 and 37°C and in the presence of 0% to 1.5% NaCl (13, 15, 32, 33). Several *Achromobacter* species contain the Q-8 ubiquinone system (1). Predominant fatty acids are $C_{16:0}$, $C_{17:0}$ cyc, and $C_{18:0\ \omega 7c}$ (13, 15, 16). Although detailed information about the natural habitat of these organisms is lacking, soil and water are considered primary sources of infection (1, 17).

Alcaligenes, *Kerstersia*, *Advenella*, and *Paenalcaligenes*

Following many taxonomic revisions, the genus *Alcaligenes* is now limited to *A. faecalis* and *A. aquatilis*. Within *A. faecalis*, three subspecies (*A. faecalis* subsp. *faecalis*, *A. faecalis* subsp. *parafaecalis*, and *A. faecalis* subsp. *phenolicus*) have been described (34, 35). *A. faecalis* subsp. *parafaecalis* and *A. faecalis* subsp. *phenolicus* are represented by a single environmental isolate each, and *A. aquatilis* strains have been recovered only from lake sediments (12). Some *A. faecalis* strains produce a characteristic fruity odor and/or cause a greenish discoloration of blood agar medium; these strains were previously referred to as "*A. odorans*" (36). *Alcaligenes* species are Gram-negative, strictly aerobic rods or coccobacilli that possess oxidase and catalase activities (1). Cells are motile by means of 1 to 12 peritrichous flagella. The optimum growth temperature is between 20 and 37°C. They grow well on simple media, and colonies on nutrient agar are generally nonpigmented. Predominant fatty acids in *Alcaligenes* species are $C_{16:0}$ and $C_{17:0}$ cyc (12, 36).

Cells of *Kerstersia* and *Advenella* species are Gram negative, small (1- to 2-μm), and rod-shaped or coccoid and occur alone, in pairs, or in short chains. Motility is strain dependent. They grow well on simple media. On nutrient agar, colonies are flat or slightly convex with smooth margins and range from white to light brown. They are strictly aerobic and nonfermentative. All isolates studied so far are catalase positive, while none of them exhibit β-galactosidase activity (11, 19). *Kerstersia* strains can grow between 28 and 42°C; growth also occurs with up to 4.5% NaCl. Predominant fatty acids in *Kerstersia* species are $C_{16:0}$ and $C_{17:0}$ cyc (11). *Advenella* strains can grow between 30 and 37°C and at NaCl concentrations between 0 and 3%. Predominant

TABLE 2 Possible targets for detection of *Bordetella* DNA by real-time PCR

Target	Organism in which target is present	~Copy no./genome (reference)	Comments
IS*481*	*B. pertussis*	~200	
	B. holmesii	~10	
	B. bronchiseptica	<5	<1% of isolates
IS*1001*	*B. parapertussis*	~20	
	B. bronchiseptica	<5	~50% of isolates
IS*1002*	*B. pertussis*	~10	
	B. parapertussis	<5	
hIS*1001*	*B. holmesii*	5–20 (155)	
ptxP	*B. pertussis*	1	
recA	*B. holmesii*	1	

fatty acids in *Advenella* species are $C_{18:1\ \omega7c}$, $C_{16:0}$, and $C_{16:1\ \omega7c}$ (19).

P. hominis cells are Gram-negative, small (1- to 2-µm) rods. They grow well on nutrient agar at 25 to 30°C and are oxidase positive. Sugars and sugar-related compounds are not utilized. Predominant fatty acids are $C_{16:0}$, summed feature 3 (containing $C_{16:1\ \omega7c}$ and/or iso-$C_{15:0}$ 2-OH), $C_{17:0}$ cyc, and $C_{18:0\ \omega7c}$ (21).

EPIDEMIOLOGY AND TRANSMISSION

B. pertussis and *B. parapertussis*

B. pertussis and *B. parapertussis* are transmitted by droplets; for *B. pertussis* in susceptible contacts the transmission rate may be close to 90%, and the infectious dose is only around 100 CFU (3). The bacteria attach to the ciliated cells of the nasopharynx and then colonize the upper respiratory tract. *B. pertussis* and *B. parapertussis* are the causes of pertussis, or whooping cough. Infections by *B. parapertussis* tend to take a milder clinical course with a shorter duration of coughing and less vomiting and whooping (3, 37–40). Worldwide in 2012, around 200,000 cases and 90,000 deaths were notified to WHO (http://www.who.int/immunization_monitoring/diseases/pertussis/en/index.html), and it is assumed that the disease is grossly underreported. *B. pertussis* also continues to circulate in populations where high vaccination coverage of infants and children is achieved (40, 41), because the protection after natural infection and vaccination wanes after several years (38, 41). In vaccinating countries, cases of pertussis are now mainly observed in neonates, unvaccinated young infants, older schoolchildren, adolescents, and adults (38, 40–43). A permanent carrier state is not found with pertussis, although in outbreak situations asymptomatic transient carriage of *Bordetella* DNA detected by PCR has been observed (3, 39). In vaccinated European populations, the basic reproduction number was estimated to be around 6 (44); transmission mainly occurs from adolescents and adults to infants or among older vaccinated children, adolescents, and adults (3, 40). Neonates and young infants are at greatest risk of being infected by their parents, although casual contacts may be important (45). In various countries, such as Australia, Brazil, the United States, and the United Kingdom, significant increases in notified cases have occurred in recent years, and various factors, e.g., insufficient vaccine coverage, changes in vaccination calendar, more effective diagnostics, changes in circulating strains, decreased longevity of protection after vaccination, and differences in priming, have been hypothesized to be responsible for the outbreaks (46).

Other *Bordetella* Species, *Achromobacter*, *Alcaligenes*, *Kerstersia*, and *Advenella*

Among the bordetellae, *B. holmesii* (7) is increasingly found mainly by PCRs primarily using IS*481* as a target, which shows homogeneity in *B. pertussis*, *B. holmesii*, and other bordetellae (Tables 2 and 3) (47, 48).

Data on epidemiology and transmission are limited to *Achromobacter* infections in cystic fibrosis (CF) patients. Persistent infections with *A. xylosoxidans* can occur, as genotypically identical isolates are recovered from the respiratory tract over prolonged periods (49, 50). There have been several reports of multiple CF patients being colonized or infected by the same *A. xylosoxidans* isolate (50–52). However, no large-scale outbreaks caused by the same strain and involving multiple treatment centers have been identified, and epidemiological studies revealed that there are many different *A. xylosoxidans* strains infecting CF patients. The "Danish epidemic strain" was identified as *A. ruhlandii* (53). This strain has infected multiple patients in two Danish CF centers, and cross-infection was documented to also occur by indirect patient-to-patient contact (54).

TABLE 3 Useful characteristics for differentiating *Bordetella* species[a]

Test	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*	*B. avium*	"*B. ansorpii*"	*B. hinzii*	*B. holmesii*	*B. petrii*	*B. trematum*
Growth on:									
RL medium	+ (3–4 days)	+ (2–3 days)	+ (1–2 days)	ND	ND	ND	ND	ND	ND
Columbia agar	−	V	+	+	+	+	+	+	+
MacConkey agar	−	−	+	+	+	+	(+)	+	+
Oxidase	+	−	+	+	+	+	−	+	−
Motility	−	−	+	−	V	+	−	−	+
Pigment	−	Brown	−	−	−	−	−	Yellow	Yellow

[a] Symbols and abbreviations: +, ≥90% of the strains are positive; −, ≤10% of the strains are positive; (+), variable growth; V, 10 to 89% of the strains are positive; ND, not determined.

CLINICAL SIGNIFICANCE

B. pertussis and *B. parapertussis*

After an incubation period of 7 to 10 days (range, 4 to 28 days), the primary infection starts with rhinorrhea, sneezing, and nonspecific coughs (catarrhal phase). The typical clinical symptoms of pertussis are found in primary infections of nonvaccinated children, with coughing spasms, whooping, and vomiting (paroxysmal phase) (38, 55). Cases in neonates and unvaccinated young infants often present with apnea as the only symptom (3, 41, 56). In older schoolchildren, adolescents, and adults, the symptoms can vary widely. Adult pertussis is associated with a long illness, and the persistent cough is often paroxysmal and has a mean duration of approximately 6 weeks. It is frequently accompanied by choking, vomiting, and whooping (40). The CDC clinical case definition for pertussis (http://www.cdc.gov/vaccines/pubs/surv-manual/chpt10-pertussis.html) requires 14 days of coughing with paroxysms, whooping, or vomiting. The disease is most dangerous in infants, and most hospitalizations and deaths occur in this age group. Fatal cases of the disease may go undetected in young infants (41, 57).

Pertussis-like symptoms may also be caused by adenoviruses, respiratory syncytial virus, human parainfluenza viruses, influenza viruses, *Mycoplasma pneumoniae*, and other agents (41, 58).

Other *Bordetella* species

Due to its more frequent diagnosis, *B. holmesii* (7) is suspected to be a pathogen that may cause respiratory symptoms in adolescents and adults (47, 48) and inflammation in various organs in immunocompromised patients. *B. bronchiseptica* (59, 60), *B.petrii*, *B.avium*, "*B.ansorpii*," and *B. hinzii* (61–64) are rarely isolated from respiratory materials from patients with respiratory symptoms. In many cases, patients are systemically or locally immunocompromised. As with other Gram-negative nonfermentative bacilli, cases of bacteremia, septicemia, and other manifestations have been described.

B. trematum (4, 65) has been isolated from people working with poultry, and "*B. ansorpii*" (9, 66) is another rare course of septicemia. *B. petrii* (67, 68) and possibly other environmental bordetellae (69) are rarely found in clinical material, and *B. avium* has so far been isolated from respiratory material from humans only once (8) (Table 1).

Achromobacter

All *Achromobacter* species have been recovered from clinical samples or from the hospital environment. *A. xylosoxidans* (previously known as *A. xylosoxidans* subsp. *xylosoxidans*) is an opportunistic human pathogen capable of causing a wide range of infections, such as bacteremia, meningitis, pneumonia, and peritonitis (70–74). It has also been involved in nosocomial infections attributed to contaminated disinfectants, dialysis fluids, saline solution, and water (75). *A. piechaudii* has been isolated from various clinical samples, including pharyngeal swab, nose, wound, blood, and chronic ear discharge samples (76). There is a single report of recurrent *A. piechaudii* bacteremia associated with an intravenous catheter in an immunocompromised patient (77). *A. denitrificans* (previously known as *A. xylosoxidans* subsp. *denitrificans*) has been recovered from many clinical specimens, such as urine, proctoscopy specimens, prostate secretions, the buccal cavity, pleural fluid, and eye swabs (36), but there are no detailed reports about its clinical significance. *A. insolitus* (in a leg wound and in urine) and *A. spanius* (in blood) have been found in clinical material, but their significance is unclear (11). Strains from the recently described species *A. aegrifaciens*, *A. animicus*, *A. anxifer*, *A. dolens*, *A. insuavis*, *A. marplatensis*, *A. mucicolens*, *A. pulmonis*, and *A. spiritinus* have mainly been recovered from sputum of CF patients, but their clinical significance also remains unclear (15, 16).

Achromobacter spp. are frequently associated with CF. *A. xylosoxidans* has been reported from CF patients since 1985, and prevalence rates in CF vary from 3 to 18% (67, 78–80). However, recent studies based on multilocus sequence analysis indicate that patients with CF are often infected with *Achromobacter* species other than *A. xylosoxidans* (53, 81, 82). For example, among 341 *Achromobacter*-infected CF patients in the United States, 42% were infected with *A. xylosoxidans*, 23.5% with *A. ruhlandii*, and 17% with *A. dolens* (16, 82). A similar prevalence of *A. xylosoxidans* (38%) was reported for Danish *Achromobacter*-infected CF patients (83). *A. xylosoxidans* infections in CF patients do not seem to have a significant impact on lung function (51, 79, 84). Infection with the *A. ruhlandii* Danish epidemic strain can lead to deterioration of lung function (54).

Alcaligenes, *Kerstersia*, *Advenella*, and *Paenalcaligenes*

A. faecalis strains have been isolated from a wide range of clinical samples (36), but the accuracy of the identification (especially in older reports) is difficult to assess. *A. faecalis* was found in cases of bacteremia following surgery or cancer treatment, from ocular infections, from a pancreatic abscess, from infections following bone fractures, from urine, and from ear discharge (73, 85, 86). There are sporadic reports on the recovery of *A. faecalis* in sputum of CF patients (87). *K. gyiorum* was isolated from human feces, leg wounds, sputum, and chronic ear infections (11, 88, 89), while *K. similis* was isolated from leg wounds and a neck abscess (11, 32). *A. incenata* has been recovered from human sputum (including sputum from CF patients) and blood, and several unnamed *Advenella* species were isolated from similar sources (19). The sole *P. hominis* strain described so far was recovered from the blood of an 85-year-old man in Sweden (21).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

B. pertussis and *B. parapertussis*

Sampling for culture and PCR is difficult, and it influences the sensitivity markedly. Nasopharyngeal aspirates are adequate for infants and young children; for culture, they are more sensitive than swabs. Nasopharyngeal swabs taken from older children, adolescents, and adults by trained personnel provide valid specimens from these age groups and are better than throat swabs. Nasopharyngeal swabs should be taken by gently inserting the swab into the nasopharynx under the inferior nasal choana, and the nose of the patient should be slightly bent upwards (http://www.pasteur.fr/recherche/unites/film_cnr/prelev.swf). If possible, two nasopharyngeal swabs should be taken, one from each nostril. Swabs should be small and made of Dacron or rayon. Flocked nylon swabs, which are more convenient for the patient, may also be used, but they have not been validated for *B. pertussis* PCR or culture. Calcium alginate swabs and swabs with aluminium shafts should not be used for PCR. Samples should be taken before antibiotic treatment is started (90).

For nasopharyngeal aspirates or swabs, transport time is critical, and a transport medium protecting the bacteria from drying is required. Usual bacteriological transport media, such as Casamino Acids or Amies medium with charcoal, may be used, but transport time should not exceed 48 h. Half-strength Regan-Lowe (RL) charcoal-blood medium may also be used for transport, and it serves as enrichment for *B. pertussis* (41).

For PCR, swabs can be transported dry at ambient temperature. The use of microbiological transport media, such as Amies medium with charcoal, did not interfere with PCR (90). Other respiratory samples, such as sputum samples or throat washes, are less suitable and have not been validated (3).

Other *Bordetella* species, *Achromobacter*, *Alcaligenes*, *Kerstersia*, *Advenella*, and *Paenalcaligenes*

For other bordetellae, normal microbiological transport media seem suitable for transport. Similarly, *Alcaligenes*, *Kersteria*, *Advenella*, and *Paenalcaligenes* species can survive in a wide range of environments and at various temperatures. Standard collection, transport, and storage techniques are sufficient to ensure recovery of these organisms from clinical specimens, contaminated nosocomial sources, and the environment.

DIRECT DETECTION METHODS

PCR

PCR is 2- to 6-fold more sensitive than culture depending on age, vaccination status, and duration of symptoms of the patients, and real-time PCR formats offer a result within several hours instead of several days (90–92). Block-based and real-time PCRs seem to have comparable sensitivities (90, 93, 94). The sensitivity of PCR decreases with the duration of cough; however, due to its higher sensitivity, it may be a useful tool for diagnosis up to 4 to 6 weeks of coughing (90, 92). In outbreak situations and after household contacts, a positive PCR may also be obtained for patients with very mild or no symptoms (95). DNA extraction is necessary to limit inhibition of PCR (90). Commercially available extraction kits seem to be comparable and appropriate (90), but no head-to-head comparison has been done. Some of these kits are FDA cleared and/or CE marked for this purpose. Multicopy targets for detection are IS*481* (copy number, ~200 per cell in *B. pertussis*) (90, 92), which is present in *B. pertussis*, *B. holmesii*, and some *B. bronchiseptica* strains; IS*1001* (copy number, ~20 per cell in *B. parapertussis*) (96), which is present in *B. parapertussis*; and IS*1002*, which is present in *B. pertussis* and *B. parapertussis*. The combined use of the three ISs has been suggested to distinguish clinically relevant *Bordetella* spp. (97) and to distinguish *B. holmesii* (98). In current isolates, the number of ISs remained stable compared to those of the sequenced type strains (96). Monogenic targets include the PT promoter (*ptx*A-Pr), the PT gene, the *recA* gene from *B. holmesii*, and the FHA and porin genes. Targets are summarized in Table 2 (90, 91, 92, 97–100).

Tests detecting single-copy genes were, however, consistently less sensitive than IS*481*-based PCRs. Sequence-specific detection can be done by fluorescence resonance energy transfer hybridization probes, TaqMan probes, and molecular beacons and also by non-sequence-specific formats using SYBR green I (90). For specificity reasons, most laboratories use sequence-specific formats, and duplex or triplex PCRs for clinically relevant *Bordetella* spp. have been developed. Multiplex PCRs for the detection of various respiratory agents, including *Bordetella* spp., are also available but are mostly based on IS*481* as the sole target. External quality control programs have been implemented in the United States and in European countries (93). Apart from contamination problems in laboratories, pseudo-outbreaks of pertussis have also been reported on the basis of swabs contaminated with *Bordetella* DNA from clinic surfaces (101).

Direct Fluorescent-Antibody Staining

Direct fluorescent-antibody staining on nasopharyngeal swabs or nasopharyngeal aspirates is rapid and simple, but it lacks sensitivity and specificity and is not recommended (3). Other stains have no role in pertussis diagnosis.

ISOLATION PROCEDURES

B. pertussis and *B. parapertussis*

Although culture is thought to be almost 100% specific, PCR hast mostly replaced it in routine diagnosis (90). Detailed information on cultural procedures is contained in previous editions of this *Manual* (102). In brief, various culture media, such as RL medium, Bordet-Gengou (BG) medium, and Stainer-Scholte medium, can be used for culturing *B. pertussis* and *B. parapertussis*. Most media are supplemented with cephalexin to suppress concomitant bacteria. Incubation time should be at least 1 week at 35 to 37°C or ambient atmosphere (Table 4). The sensitivity of culture depends on the duration of symptoms, the age, and the vaccination status, and it varies between ~60% in young unvaccinated infants with few days' duration of symptoms and <1% in adolescents and adults with more than 3 weeks of coughing (102).

Other *Bordetella species, Achromobacter*, *Alcaligenes*, *Kerstersia*, *Advenella*, and *Paenalcaligenes*

Bordetella spp. other than *B. pertussis* or *B. parapertussis* from clinical material are encountered as Gram-negative nonfermentative bacilli in the laboratory. Table 4 gives some information about their growth characteristics and some biochemical reactions that may be used.

Achromobacter species can be isolated from clinical samples by the use of simple media and a selective enteric medium such as MacConkey agar (36). It has been reported that a minority of *A. xylosoxidans* isolates grow on *B. cepacia* selective oxidative-fermentative-polymyxin B-bacitracin-

TABLE 4 Possible results of *Bordetella* PCR and suggested interpretation

PCR result[a]					Organism name to use in report
IS481	*IS1001*	*IS1002*	*ptxP*	*recA*	
Pos	Neg	Pos	Pos	Neg	*B. pertussis*
Pos	Neg	Pos	Neg	Neg	*B. pertussis*
Pos	Neg	Neg	Neg	Neg	*Bordetella* spp.
Neg	Pos	Pos	Neg	Neg	*B. parapertussis*
Neg	Pos	Neg	Neg	Neg	*Bordetella* spp.
Pos	Neg	Neg	Neg	Pos	*B. holmesii*

[a] Pos, positive; Neg, negative.

lactose agar or *Pseudomonas cepacia* agar (30% and 20%, respectively) but do not grow on *Burkholderia cepacia* selective agar (103). Data from the Cystic Fibrosis Foundation *Burkholderia cepacia* Research Laboratory and Repository (J. J. LiPuma, unpublished data) and a previous study (33) indicate that the majority of *A. xylosoxidans* strains (~60%) grow on *Burkholderia cepacia* selective agar. Results obtained in a small-scale study suggest that particular selective media, such as Gram-negative selective agar, may increase the recovery of *A. xylosoxidans* from the sputa of CF patients (104).

IDENTIFICATION

B. pertussis and *B. parapertussis*

B. pertussis colonies become visible after 3 to 7 days of incubation, and *B. parapertussis* colonies are visible already after 2 to 3 days. On RL medium colonies are very small, round, and domed, and they appear silvery. *B. parapertussis* colonies are larger and less shiny. The minute colonies on Bordet-Gengou medium have a small zone of beta-hemolysis. *B. pertussis* is a small coccoid Gram-negative rod which is catalase and oxidase positive (*B. parapertussis* is oxidase negative). Their identity can best be confirmed by agglutination with specific antibodies. Basic biochemical characteristics are given in Table 4. Due to the fastidious growth of *B. pertussis* and *B. parapertussis*, commercial systems for Gram-negative rods are not reliable for identifying these species. 16S rRNA sequencing (59) and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (80) can effectively be applied for identification.

Isolates of *B. pertussis* and, less so, *B. parapertussis* should not be subcultured because they undergo a phase change as described above. If strains have to be stored, this should be done from primary clinical isolates, and these should be frozen in glycerol at −70°C or in a sucrose-bovine serum albumin medium. Biosafety level 2 is strongly recommended or obligatory.

Other Bordetellae

Colony morphology is not discriminative, and the bacteria are small coccoid rods that are catalase positive. In most instances, a biochemical system for identification of Gram-negative bacilli is used, such as API20-NE, Vitek (bioMérieux, Marcy-l'Etoile, France), MicroScan (Siemens, Eschborn, Germany), or Phoenix (Becton Dickinson, Franklin Lakes, NJ) (105). These systems were validated for nonfermentative rods, among which also a few *Bordetella* spp. were evaluated. Overall, the specificity of these biochemical systems for these bacteria is not very high, and for the API20-NE and Vitek II systems, it has been proposed that 16S rRNA gene sequencing be performed if the results of the biochemical identification are not reported as "excellent" or "very good" (105). 16S rRNA gene sequencing or ribotyping offers more reliable identification results, as does MALDI-TOF MS, and either method is available in many reference laboratories (80, 106–108). Apart from *B. bronchiseptica*, isolation of other bordetellae from clinical material is a rare event, and their identification should be confirmed by a reference laboratory to avoid misidentification (109).

Achromobacter

Achromobacter species typically show very limited action on carbohydrates (15, 16), which hampers the accurate identification at the genus level based on biochemical characteristics, and 16S rRNA gene sequence analysis is recommended for this purpose. Likewise, the increased taxonomic diversity and considerable intraspecies diversity observed in this genus make species-level differentiation based on phenotypic characteristics virtually impossible. A PCR assay (based on the 16S rRNA gene) was developed for *A. xylosoxidans*, but positive results may also be obtained with strains of other *Achromobacter* species as well as with some *Bordetella* species (82, 110). MALDI-TOF MS has successfully been used to identify *A. xylosoxidans* (80, 111) and reportedly allows the separation of *A. xylosoxidans*, *A. ruhlandii*, *A. insolitus*, and *A. spanius* (112), although in another study species-level identification of *Achromobacter* spp. with MALDI-TOF MS turned out to be not straightforward (113). In addition, Desai et al. (114) noted that strains identified as *A. xylosoxidans* by a phenotypic test were commonly identified as *A. ruhlandii* by MALDI-TOF MS. It should be noted that the studies referenced above were published prior to the recent description of additional diversity within the genus *Achromobacter*, and their applicability to differentiate all *Achromobacter* species will need to be reinvestigated. All established *Achromobacter* species can be separated using the concatenated *nusA*, *eno*, *rpoB*, *gltB*, *lepA*, *nuoL*, and *nrdA* sequences (15, 16, 18). A recent study on a large number of *Achromobacter* strains indicated that most species can even be distinguished based on sequence analysis of the *nrdA* gene alone (115). An alternative multilocus sequence analysis scheme using *atpD*, *icd*, *recA*, *rpoB*, and *tyrB* sequences has also been described previously (116).

Alcaligenes, Kerstersia, Advenella, and *Paenalcaligenes*

Also for *Alcaligenes*, *Advenella*, *Kerstersia*, and *Paenalcaligenes*, 16S rRNA gene sequence analysis is recommended for accurate identification at the genus level. Members of the genus *Advenella* can be separated from related species by their inability to assimilate phenyl acetate. *Kerstersia* strains are oxidase negative. *K. gyiorum* and *K. similis* can be distinguished based on oxidation of D-galacturonic acid and D-glucuronic acid (positive in *K. gyiorum* and negative in *K. similis*) and D-serine (negative in *K. gyiorum* and positive in *K. similis*). A distinguishing characteristic of *A. faecalis* isolates is that they reduce nitrite but not nitrate. *P. hominis* cannot assimilate citrate.

TYPING SYSTEMS

B. pertussis and *B. parapertussis* show limited genomic heterogeneity (37), but some polymorphic regions, such as the toxin gene (*ptx*), the toxin promoter (*ptxP*), and the pertactin gene (*prn*), have undergone changes the cause of which is not totally clear (117, 118). The expression of fimbriae undergoes temporal changes, possibly influenced by vaccine coverage (118). Nucleic acids of circulating isolates of *B. pertussis* have been typed by a vast array of methods, such as conventional and next-generation sequencing (31, 118), pulsed-field gel electrophoresis, variable number of tandem repeats (also called multilocus variable-number tandem repeat analysis [MLVA]) (119, 120), restriction fragment length polymorphism (90), single nucleotide polymorphism typing (121), real-time PCR (122), and others (106). Other methods use the proteome as the distinguishing parameter (123). Depending on the problem in question, different typing methods may be employed, and the results can mostly not be compared directly. Similar typing methods have also been used for isolates of *B. holmesii* (124, 125).

Typing of *A. xylosoxidans* isolates with pulsed-field gel electrophoresis of fragments obtained after digestion with

XbaI, SpeI, or DraI has been used in several studies (51, 53, 126) and is reported to have a high discriminatory power. Randomly amplified polymorphic DNA PCR (70) and PCR with ERIC or REP primers (52) have also been used. The discriminatory power of ribotyping (using the Riboprinter microbial characterization system) was rather low (127). Selective restriction fragment amplification (flourescent amplified fragment length polymorphism, using EcoRI and MseI) was also successfully applied to *A. xylosoxidans* (18). Two multilocus sequencing typing schemes have recently been proposed and can be used to type all named *Achromobacter* species (18, 116).

SEROLOGICAL TESTS

B. pertussis and *B. parapertussis*

Pertussis in older vaccinated children, in adolescents, and in adults is mostly diagnosed by serological tests. Enzyme-linked immunosorbent assays or bead-based assays to quantify anti-PT antibody levels are recommended diagnostic techniques (128–130) and can be performed with paired (acute and convalescent phase) or single serum samples (128, 129). Paired-sample serology is an optimal method of diagnosis (131), but single-sample serology also provides good sensitivity and specificity to determine cases in older children, adolescents, and adults (128, 132, 133). A WHO reference preparation for human pertussis serology is available (134), and quantitative results in pertussis serology should be reported in international units per milliliter. For single-sample serology, IgG anti-PT antibodies below 40 IU/ml are not indicative of recent contact, whereas levels above >100 IU/ml can be used as an indicator of recent contact with the bacteria (129, 135–137). IgG anti-PT antibodies are primarily measured, and IgA anti-PT may serve as an additional test for equivocal results (>40 IU/ml and <100 IU/ml). Measuring IgM antibodies is not recommended (129), nor are other methods such as immunoblotting, complement fixation, or agglutination (129). Serology is unable to detect infection with *B. parapertussis*, and it cannot distinguish between vaccine- and infection-induced immunological responses (symptomatic or asymptomatic infection) (129, 135). Commercial assays are of very variable quality and are in need of further standardization (138). Pertussis serology cannot be reliably interpreted for 1 year after vaccination with acellular vaccines (129). External quality control programs for pertussis serology are available (139).

ANTIMICROBIAL SUSCEPTIBILITIES

B. pertussis and *B. parapertussis*

B. pertussis and *B. parapertussis* are susceptible *in vitro* to a range of antibiotics, including some penicillins, macrolides, ketolides, quinolones, tetracyclines, chloramphenicol, and trimethoprim-sulfamethoxazole, whereas they are resistant to most oral cephalosporins (3). However, in contrast to the case with other bacterial diseases, the exact relationship between the pharmacokinetics and pharmacodynamics of these antibiotics and the *in vitro* susceptibility of the organism is unknown. Furthermore, the effect on the symptoms of pertussis is not well documented (41, 140).

Methods for antibiotic susceptibility testing of *B. pertussis* and *B. parapertussis* are not standardized. If testing is done, the methods include broth macro- and microdilution, agar dilution methods, breakpoint methods, and the Etest, whereas the disk diffusion method is mostly not feasible (41). Erythromycin resistance may be evaluated by disk diffusion method, using charcoal-horse blood (RL) medium without antibiotics and employing a 15-μg erythromycin disk. Few isolates with erythromycin resistance have been found in the United States and in other countries; they have existed in strain collections from the 1960s, and no data so far suggest that this resistance is spreading (41), although it may increase in some countries (141). Erythromycin resistance is mediated by a mutation in the macrolide binding domain of the 23S rRNA. Routine antibiotic susceptibility testing of *B. pertussis* isolates is not recommended and should be done only when special clinical or epidemiological circumstances are found (41). Continued surveillance of *B. pertussis* isolates should also include antimicrobial susceptibility testing.

Other Bordetellae

B. bronchiseptica possesses a β-lactamase (142) and is resistant to many penicillins and cephalosporins, and mostly to trimethoprim-sulfamethoxazole (143). A study of canine and feline isolates (144) showed that most isolates are susceptible to amoxicillin-clavulanic acid, to tetracycline, to gentamicin, and to a quinolone. *B. avium* was resistant to cefuroxime, trimethoprim-sulfamethoxazole, and tetracycline and sensitive to ampicillin, mezlocillin, and gentamicin (143). A human *B. hinzii* isolate was susceptible to amoxicillin, gentamicin, and meropenem but resistant to cefuroxime, ceftriaxone, and ciprofloxacin (62). A human "*B. ansorpii*" isolate was resistant to aztreonam, cefuroxime, and ceftriaxone and susceptible to amoxicillin, gentamicin, and ciprofloxacin (69). Antimicrobial susceptibility testing of these *Bordetella* isolates should be reported in accordance to other infrequently isolated and fastidious nonfermentative Gram-negative rods, and *in vitro* resistance does not necessarily reflect clinical ineffectiveness.

Achromobacter

A. xylosoxidans isolates (145) were sensitive only to imipenem, piperacillin, ticarcillin-clavulanic acid, ceftazidime, and trimethoprim-sulfamethoxazole. Aminoglycosides, expanded-spectrum cephalosporins other than ceftazidime, and quinolones showed no activity. The majority of the strains were resistant to conventional tobramycin concentration, but 41% of the strains were inhibited by the higher tobramycin concentrations achievable by aerosol delivery of the antibiotic. Similarly, 92% of strains were inhibited by high doses of colistin (100 μg/ml). Little synergistic activity was measured for combinations of antibiotics, and additive activity was noted with chloramphenicol-minocycline, ciprofloxacin-imipenem, and ciprofloxacin-meropenem (146). *A. xylosoxidans* was resistant against azithromycin and clarithromycin, and only modest synergistic and/or additive activities were observed when azithromycin was combined with meropenem or trimethoprim-sulfamethoxazole (147). These data correlate well with those obtained in many smaller clinical studies and case reports (49, 51, 52, 148). Antimicrobial susceptibility data for other *Achromobacter* species are rare, but *A. spanius* and *A. insolitus* are resistant to most quinolones, macrolides, and cephalosporins tested (19), while a blood isolate of *A. piechaudii* was resistant to ampicillin, cefpodoxime, and gentamicin but susceptible to all other antibiotics tested (149). More than 90% of *Achromobacter* isolates recovered from Danish CF patients were resistant to amikacin, ciprofloxacin, and tobramycin, and particularly high MICs were recorded for aztreonam (>256 μg/ml) and fosfomycin (>1,024 μg/ml) (150). The lowest MICs were observed for meropenem (94% of isolates were susceptible), piperacillin-tazobactam (all isolates were susceptible), and trimethoprim-sulfamethoxazole. Colistin (MIC_{90} = 8 μg/ml) and ceftazidime (MIC_{90} = 24 μg/ml), two antibiotics commonly used for inhalation therapy, exhibited good activity (150).

Alcaligenes, Kerstersia, Advenella, and *Paenalcaligenes*

A. faecalis is more susceptible to antibiotics than *A. xylosoxidans*. Most *A. faecalis* strains are resistant to amoxicillin, ticarcillin, aztreonam, kanamycin, gentamicin, and nalidixic acid while being susceptible to the combination of amoxicillin or ticarcillin with clavulanic acid, to various cephalosporins, and to ciprofloxacin (151). Most *Kerstersia* isolates are susceptible to ciprofloxacin and cefotaxime (11), although two recently described isolates were ciprofloxacin resistant (89). Recent clinical isolates were found to be susceptible to cefepime, gentamicin, and trimethoprim-sulfamethoxazole (88, 89). Antimicrobial susceptibility in *Advenella* spp. and *P. hominis* has not yet been studied.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The usefulness of diagnostic methods in pertussis and parapertussis cases is dependent on the duration of symptoms (Fig. 1). Due to its sensitivity and speed, real-time PCR is the preferred method for the direct detection of *B. pertussis* and *B. parapertussis* in the United States (152) and elsewhere. Suggestions for the interpretation and reporting of real-time PCR results are given in Table 3.

If culture is performed, the isolation of *B. pertussis* and *B. parapertussis* implies an infection, although the sensitivity is sufficiently high only for neonates and unvaccinated infants. Routine antimicrobial sensitivity testing of isolates is not necessary. Culture remains necessary, especially for surveillance of circulating *B. pertussis* and *B. parapertussis* isolates.

Serological diagnosis of pertussis is usually based on single-sample serology. Results cannot be correctly interpreted for about 1 year after vaccination with acellular pertussis vaccines. IgG anti-PT levels of ≥100 are indicative of recent contact. In adolescent and adult populations, IgG anti-PT levels of <40 IU/ml may be interpreted as not indicative of recent infection (129). Except in Massachusetts, serology is not accepted as a confirmation of cases in the United States. Many European countries with statutory notification and laboratory confirmation accept serology as a proof of infection.

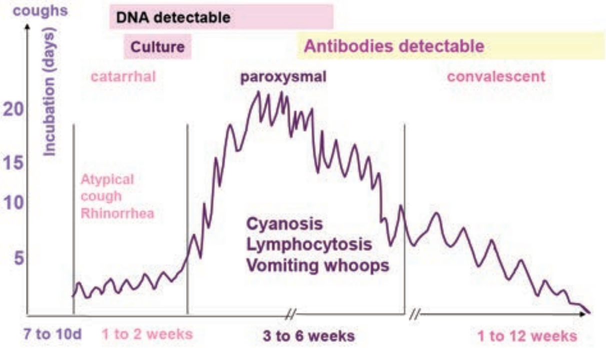

FIGURE 1 Schematic graph of symptoms and diagnostic procedures in pertussis. d, days. (Courtesy of Nicole Guiso, Institut Pasteur, Paris, France.) doi:10.1128/9781555817381.ch45.f1

Similar to the case with other rarely isolated Gram-negative nonenteric rods, the clinical relevance of isolation of *Achromobacter* spp., *Alcaligenes* spp., *Kerstersia* spp., *Advenella* spp., and *P. hominis* from clinical material should be discussed on a case-by-case basis between the microbiology laboratory and the clinician. Antimicrobial testing of these species should be performed and can be helpful in guiding therapeutic decisions.

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Francisella

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Francisella tularensis is the causative agent of tularemia, an acute and fatal illness in animals and humans. Human infections caused by *F. tularensis* occur via arthropod bite, handling of infected animals, inhalation of infectious aerosols, and ingestion of contaminated food or water. *F. tularensis* is highly infectious, with only 10 organisms sufficient to cause illness, and has a notorious reputation for causing laboratory-acquired infections. Within the *Francisella* genus, *F. tularensis* is joined by six lesser-known *Francisella* species which are largely considered environmental species and nonpathogenic or opportunistic pathogens of humans. Whereas *F. tularensis* causes tularemia in animals and humans, *F. noatunensis* and *F. halioticida* infect and cause death in fish and abalone, respectively. *F. novicida* and *F. philomiragia* are associated with salt or brackish water and are only infrequent causes of opportunistic infections in compromised individuals. *F. tularensis* was examined by multiple countries for its possible use as both an offensive and retaliatory biological weapon in the years leading up to and following World War II. In 2012, *F. tularensis* was classified as a Tier 1 agent, one of six bacterial agents considered to present the greatest risk of deliberate misuse with the most significant potential for mass casualties or devastating effects to the economy, critical infrastructure, or public confidence (100).

TAXONOMY

The family *Francisellaceae*, a member of the gamma subclass of proteobacteria, consists of the genus *Francisella*. Several publications have reviewed the history and descriptions of members of the genus *Francisella* (1–3). Originally thought to be a fairly small genus, comprised of only three species (*Francisella tularensis*, *F. novicida*, and *F. philomiragia*), in the last decade it has become clear that other *Francisella* spp. are widespread in the environment. The genus is currently comprised of *Francisella tularensis*, *Francisella novicida*, *Francisella philomiragia*, *Francisella noatunensis*, *Francisella halioticida*, *Francisella hispanensis*, and *Francisella guangzhouensis* as well as unclassified *Francisella* spp. (http://www.bacterio.net/francisella.html).

F. tularensis, the species of main clinical significance, is the causative agent of tularemia, a zoonosis affecting humans and a wide range of animals. The bacterium was first isolated from squirrels suffering a "plague-like" illness in Tulare County, CA. Presently, three subspecies of *F. tularensis*, *Francisella tularensis* subsp. *tularensis* (type A), *F. tularensis* subsp. *holarctica* (type B), and *F. tularensis* subsp. *mediasiatica*, are recognized, with ≥99.8% and ≥99.2% shared identities between their 16S rRNA genes and 1.1 Mb of genome sequence, respectively (1, 4).

F. novicida and *F. philomiragia*, both originally isolated from saltwater sources (5–8), have been *classified* as species distinct from *F. tularensis* on the basis of serologic, virulence, and biochemical differences (7, 9). Between *F. philomiragia* and *F. tularensis*, genome reassociation studies show 39% average DNA relatedness, while sequencing indicates >98.3% shared identity in their 16S rRNA gene sequences (5, 10). A high degree of genetic similarity exists between *F. novicida* and *F. tularensis* as evidenced by DNA hybridization studies (>70% DNA relatedness) (5, 11), and on this basis *F. novicida* was proposed as a fourth subspecies of *F. tularensis* (5, 11). Recent whole-genome comparisons, however, provide evidence that *F. novicida* and *F. tularensis* evolved as two distinct population lineages, consistent with *F. novicida* and *F. tularensis* being two separate species (4, 12). Clinical and ecological differences between *F. tularensis* and *F. novicida* also indicate the two represent distinct species (12) (see "Epidemiology and Transmission" and "Clinical Significance" below).

Only one isolate of *F. hispanensis* has been described to date (11, 13). In DNA hybridization experiments, *F. hispanensis* showed 52%, 57 to 61%, and 39% identities with *F. novicida*, *F. tularensis*, and *F. philomiragia*, respectively (11). *F. noatunensis*, *F. halioticida*, and *F. guangzhouensis* have been linked only to environmental sources, recovered from a number of saltwater fish species, giant abalone, and air-conditioning systems, respectively (14–17). Mean reassociation values of 68% between *F. noatunensis* and *F. philomiragia* (16), 49.2 to 59.6% between *F. noatunensis* and *F. halioticida*, and 61% between *F. philomiragia* and *F. halioticida* are observed by DNA hybridization (16, 18).

Additional *Francisellaceae* members, isolated in culture, including *Francisella* spp. recovered from seawater (18) and a *Francisella* sp. isolated from two different patients in the United States in 2005 and 2006, have been described (19). Preliminary characterization data indicate that these *Francisella* spp. are distinct from *F. tularensis*, *F. novicida*, and *F. philomiragia* but await final assignment into the genus. The genome of the tick endosymbiont, *Wolbachia persica*, has recently been sequenced, thereby confirming its placement in the *Francisella* genus (1, 3). Putative *Francisellaceae* family members identified on the basis of 16S rRNA gene sequencing include *Francisella*-like endosymbionts (FLEs) in *Amblyomma maculatum* and multiple *Dermacentor* and *Ornithodoros* tick species (1, 20).

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DESCRIPTION OF THE AGENTS

The genus *Francisella* comprises tiny Gram-negative coccobacilli, which range in size from 0.2 to 1.7 μm and can be distinguished from similar genera by several features (Table 1). Members of the genus take up Gram's counterstain (safranin) poorly; they are strict aerobes, weakly catalase positive, urease negative, nonmotile, and non-spore forming, and they metabolize limited number of carbohydrates (Table 2). Only a few sugars (glucose, maltose, sucrose, and glycerol) are utilized by most members of the genus. Acid is produced without gas. Unique cellular fatty acids are associated with the genus (Table 1) (1, 5, 21). For most species, *in vitro* growth is enhanced by cysteine supplementation. Cultured members of the genus share >97% sequence identity within their 16S rRNA genes (1, 14, 19).

A few key differences separate species of the *Francisella* genus, including oxidase production, growth in nutrient broth with 6% NaCl, optimum growth temperatures, and sugar utilization (Table 2). Additionally, levels of virulence differ in mammals, with *F. tularensis* being the most virulent. In laboratory mice, a 100% lethal dose (LD$_{100}$) of only 1 to 10 cells is observed for *F. tularensis*, compared to an LD$_{100}$ of >10^4 for *F. novicida* (9, 22). Levels of virulence also differ between *F. tularensis* subspecies; *F. tularensis* subsp. *tularensis* displays an LD$_{100}$ of <10 when inoculated subcutaneously into rabbits, compared to an LD$_{50}$ of ~10^3 for *F. tularensis* subsp. *holarctica* (23). No mortality occurs in mice inoculated with 10^8 cells of *F. philomiragia* (23) or 10^7 cells of *F. noatunensis* (24). Similarly, *F. guangzhouensis* was found to be avirulent in mice (17).

In the last 15 years, >40 whole-genome sequences have been completed for *Francisella* species, including *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, *F. tularensis* subsp. *mediasiatica*, *F. novicida*, *F. philomiragia*, *F. hispanensis*, *F. noatunensis*, and *Wolbachia persica* (3). The size of *Francisella* genomes is ~1.9 Mb, with a G+C content of ~32% (http://www.ncbi.nlm.nih.gov/genome/?term=Francisella). All *Franicsella* genomes are comprised of a single chromosome. Plasmids are unique to some environmental *Francisella* spp., including *F. novicida* and *F. philomiragia*, and are not conserved across all members of a given species (http://www.ncbi.nlm.nih.gov/genome/?term=Francisella). Whole-genome analyses indicate that *F. tularensis*, the causative agent of tularemia, contains only seven unique genes in comparison to other members of the *Francisella* genus (3). These genes encode proteins predicted to be involved in exopolysaccharide and lipopolysaccharide synthesis as well as a hypothetical membrane protein (3).

EPIDEMIOLOGY AND TRANSMISSION

F. tularensis has been recovered only from the Northern Hemisphere, with the distribution of the subspecies varying throughout this region (1, 2, 25) (Table 3). Infections caused by *F. tularensis* subsp. *tularensis* occur only in North America, whereas *F. tularensis* subsp. *holarctica* has a much wider distribution, causing disease throughout the Northern Hemi-

TABLE 1 Differentiation of *Francisella* from similar Gram-negative genera[a]

Test	*F. tularensis*	*Brucella* spp.	*Bartonella* spp.[b]	*Acinetobacter* spp.	*Haemophilus* spp.[c]	*Yersinia pestis*	*Pasteurella multocida*
Oxidase	–	+	–	–	V	–	+
Urease	–	+	–	–	V	–	–
Gram stain morphology	Very tiny ccb	Tiny ccb	Thin rod	Broad ccb	Small ccb	Small rod	Small ccb
Specimen source	Ulcer, wound, blood, lymph node aspirates, respiratory	Blood, bone marrow	Blood, bone marrow, lymph node	V	Blood, cerebrospinal fluid, other	Blood, lymph node aspirates, respiratory	Wound, blood, respiratory
X and/or V factor requirement	–	–	–[d]	–	+	–	–
Cysteine enhancement	+	–	–	–	–	–	–
Motility	–	–	–	–	–	–	–
Major CFA[e]	10:0, 14:0, 16:0, 3-OH-16:0, 18:1ω9c, 3-OH-18:0	16:0, 18:1ω7c, 18:0, 19:0cyc[f]	16:0, 17:0, 18:1ω7c	2-OH-12:0, 3-OH-12:0, 16:1ω7c, 16:0, 18:1ω9c	14:0, 3-OH-14:0, 16:1ω7c, 16:0	16:0, 17:0, 3-OH-14:0, 16:1ω7c, 18:1ω7c	16:1, 16:0; 14:0, 18:2, 3-OH-14:0

[a] +, greater than or equal to 90% positive; −, less than or equal to 10% positive; V, variable (11 to 89% positive); ccb, coccobacilli.

[b] Does not include *Bartonella bacilliformis*, which is the only motile species.

[c] *Haemophilus* spp. requiring X and V factors or V factor only.

[d] While not required, X factor (hemin) enhances growth for many strains.

[e] The number before the colon indicates the number of carbons; the number after the colon is the number of double bonds, "ω" indicates the location of the double bond counting from the hydrocarbon end of the carbon chain, "OH" indicates a hydroxy group at the 2 or 3 position from the carboxyl end, "c" indicates the *cis* isomer, and "cyc" indicates a cyclopropane ring structure. Hydroxy acids listed are at least 2% of the total cellular fatty acid (CFA) composition; all others are at least 10%.

[f] *B. canis* lacks 19:0cyc.

TABLE 2 Characteristics of *Francisella* spp.[a]

Characteristic	*F. tularensis* subsp. *tularensis*	*F. tularensis* subsp. *holarctica*	*F. tularensis* subsp. *mediasiatica*	*F. novicida*	*F. philomiragia*	*F. noatunensis*	*F. hispanensis*	*F. halioticida*	*F. guangzhouensis*
Gram stain (culture), safranin counterstain	Faintly staining, pleomorphic, single, rarely chained, Gram-negative tiny coccobacilli	As for *F. tularensis* subsp. *tularensis*	As for *F. tularensis* subsp. *tularensis*	As for *F. tularensis* subsp. *tularensis*	As for *F. tularensis* subsp. *tularensis*	Faintly staining, Gram-negative coccobacilli	Faintly staining, Gram-negative pleomorphic coccobacilli	Gram-negative coccobacilli	Gram-negative coccobacilli
Cell size (µm)	0.2–0.7 × 0.2	0.2–0.7 × 0.2	0.2–0.7 × 0.2	0.7 × 1.7	0.7 × 1.7	NT	0.5 × 1.5	0.5–1.0	NT
Growth on blood agar	–	–	–	+	+	–	+	NT; requires 70% artificial seawater for growth on Eugon agar	Delayed; weak
Cysteine heart blood agar, 48 h	1- to 2-mm diam, raised, smooth colonies with entire margins; colonies display green tint and opalescent sheen	As for *F. tularensis* subsp. *tularensis*	NT	As for *F. tularensis* subsp. *tularensis* but 2–4 mm in diam	Colonies are >4 mm in diam, creamy white-gray, mucoid, and smooth, with a purple-tinted opalescent sheen	Colonies are low convex, white, slightly translucent and mucoid and ~1 mm in diam after 4 days incubation at 22°C.	Colonies are convex, pale white to gray, 3- to 4-mm diameter	Colonies observed 10–14 days at 20°C were grayish to white, circular, slightly convex with entire margins	Greenish gray mucoid-opalescent colonies (~2 mm)
Growth in NB (6% NaCl)	–	–	–	+	+	–	NT	+	NT
Optimal growth temperature	35–37°C	35–37°C	35–37°C	35–37°C	25 or 37°C	22°C; no growth at 37°C	37°C	20°C; no growth at 37°C	25-28 °C
Catalase	Weakly +	Weakly +	Weakly +	Weakly +	Weakly +	Weakly +	Weakly +	+	+
Oxidase	–	–	–	–	+	–	+	–	–
Acid from:									
Glucose[b]	+	+	–	+	Weakly +	V	+	–	+
Maltose	+	+	–	V	+	–	–	NT	+
Sucrose	–	–	+	+	V	–	+	–	+
Glycerol	+	–	+	+	–	NT	+	NT	–
Citrulline ureidase	+	–	+	+	NT	NT	NT	NT	NT
Relative virulence (mice)	High	Intermediate	Intermediate	Low	None	None	NT	NT	None

[a] V, variable or slow reaction; NT, not tested; NB, nutrient broth.

[b] Delayed or variable reaction. *F. tularensis* subsp. *mediasiatica* does not ferment glucose (1).

TABLE 3 Epidemiology of *Francisella* spp. affecting humans[a]

Francisella sp.	Infection source(s)	Geographic distribution (documented cases)	Disease (humans)
F. tularensis subsp. *tularensis* (type A)	Animals (primarily rabbits and cats), ticks, deerflies	North America (United States and Canada)	Tularemia (all forms); severe disease; A1b strains most virulent
F. tularensis subsp. *holarctica* (type B)	Animals (primarily rodents), ticks (mosquitoes in Europe)	Northern Hemisphere	Tularemia (all forms); moderate to severe disease
F. novicida	Salt water	United States, Thailand, Australia	Very rare; mild illness (primarily compromised patients)
F. philomiragia	Salt water	United States, Turkey, Switzerland, Australia	Very rare; mild illness (primarily compromised patients)
F. hispanensis	Unknown	Spain	Very rare
Unclassified *Francisella* sp.	Unknown	United States	Very rare

[a] Human infections documented in the published literature.

sphere. *F. tularensis* subsp. *mediasiatica* has been found only in regions of central Asia. More recently, the geographic distributions of *F. tularensis* subsp. *holarctica* and *F. tularensis* subsp. *tularensis* subpopulations have also been shown to differ. Subpopulations of *F. tularensis* subsp. *holarctica* within North America are distinct from subpopulations throughout the rest of the Northern Hemisphere, except Scandinavia (26). The *F. tularensis* subsp. *tularensis* A1a and A1b subpopulations predominate in the eastern half of the United States, whereas the A2 subpopulation appears to be restricted to the western United States (26, 27).

Despite the broad geographic distribution of *F. tularensis*, overall disease occurrence is suspected to be low (28). Foci of endemicity have long been documented in regions of Russia and Kazakhstan, as well as Finland, Sweden, and the United States, where incidence may be higher (2). Annual cases are usually reported from most countries in Eastern Europe. Several outbreaks of tularemia have occurred over the last two decades in Spain, Sweden, Turkey, and Norway (29–33).

In the United States, the incidence of tularemia has declined to 100 to 200 cases each year since the 1940s, when several thousand cases were reported annually (34). All states except Hawaii have reported cases. A total of 1,208 human cases from 44 states were reported to the Centers for Disease Control and Prevention (CDC) between 2000 and 2010. Four states, Arkansas, Missouri, Oklahoma, and Massachusetts, accounted for 48% of these cases (http://www.cdc.gov/tularemia/statistics). Tularemia in humans is predominantly a rural disease; urban cases are rare. Most cases of tularemia occur in the summer months, with patients acquiring infection from tick or deerfly bites or from contact with infected animals (e.g., by skinning rabbits) (27, 34). Inhalation of an infective aerosol during landscaping and contact with infected cats are also important risk factors (27, 35). Tularemia cases are largely sporadic; outbreaks of tularemia occur rarely in the United States and may often follow tularemia epizootics in animals (30). An outbreak of pneumonic tularemia associated with landscaping activities occurred on Martha's Vineyard in 2000 (35), and since that time, Martha's Vineyard has recorded yearly cases (36). A more recent outbreak in the United States occurred in Utah in 2007 and was attributed to deerfly bites (37). From 1964 to 2004, 316 isolates recovered from human cases in the United States (39 different states) were submitted to the CDC; of these, 66% were *F. tularensis* subsp. *tularensis* and 34% were *F. tularensis* subsp. *holarctica* (38).

F. tularensis is a zoonotic pathogen, capable of infecting a large variety of animal species. The bacterium has been isolated from vertebrates, including mammals, amphibians, and birds, as well as from invertebrate arthropods (39–41). However, *F. tularensis* is most commonly associated with rodents and other mammals, particularly hares and rabbits. In both vertebrates and invertebrates, infection with *F. tularensis* can be lethal.

In contrast to *F. tularensis*, *F. novicida* and *F. philomiragia* do not appear to circulate in animal or arthropod hosts. Only a single isolation of *F. philomiragia* from an animal host (muskrat) has been documented (6). Arthropods infected with *F. novicida* or *F. philomiragia* have never been identified. Transmission of *F. novicida* and *F. philomiragia* to humans appears to be associated with proximity to or contact with near-drowning in saltwater sources (8, 42).

Most isolates of *F. novicida* are from North America, with a report of *F. novicida*-like organisms in Thailand and Australia (43, 44). Similarly, most isolates of *F. philomiragia* have been from North America, with two single incidences reported from Central and Eastern Europe and one from the Southern Hemisphere (Australia) (5, 8, 44–46).

F. noatunensis been isolated from both the Northern and Southern Hemispheres (14, 15), whereas *F. hispanensis*, *F. halioticida*, and *F. guangzhouensis* have been recovered only in Spain, Japan, and China, respectively (11, 13, 16, 17).

CLINICAL SIGNIFICANCE

F. tularensis

Globally, tularemia is caused by two *F. tularensis* subspecies, *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B). The disease has been known historically by a number of synonyms, such as rabbit fever, deerfly fever, market men's disease, glandular type of tick fever, Ohara's or yato-byo disease, and water rat trappers' disease, attesting to the variety of clinical presentations, the infectious agent's ubiquitous presence in nature, and the means by which humans may acquire the infection.

The clinical spectrum of tularemia depends on the mode of transmission, the virulence of the infecting strain, the immune status of the host, and timely diagnosis and treatment (47). Tularemia can be misdiagnosed since the disease is rare and its symptoms are not unique; a sudden onset of chills, fever, headache, and generalized malaise characterize the onset of illness. The differential diagnosis includes a wide range of infectious diseases, such as cat scratch fever, mycobacterial infections, anthrax, brucellosis, legionellosis, and plague (47).

Patients may present with any one of the clinical forms of tularemia: ulceroglandular, glandular, oculoglandular, oropharyngeal, typhoidal, and pneumonic (47). Upon infection, the period for disease onset is typically 3 to 5 days but can range from 1 to 21 days (28, 47). Disease presentation (i.e., signs and symptoms) correlates with the route of bacterial entry, with all forms of tularemia accompanied by fever (47). The most common form is ulceroglandular disease (45 to 80% of the reported cases), where the portal of entry is via an infective arthropod bite or other inoculation through the skin barrier. Glandular tularemia is similar to ulceroglandular disease but lacks the ulcerated site of infection. Oculoglandular tularemia occurs when the conjunctiva is the initial site of infection, usually as a result of the mechanical transfer of organisms from an infectious source to the eye by the fingers. Oropharyngeal tularemia occurs from ingestion of contaminated water or food and is associated with pharyngeal lymphadenopathy. Pneumonic tularemia occurs by direct inhalation of the organism and is considered the most severe form of the disease. Typhoidal tularemia is the most difficult form to recognize because there is no identified portal of entry and localized signs are absent. If untreated, bacterial dissemination from the primary sites of infection can lead to secondary clinical presentations, such as sepsis.

The severity of infection can range from mild and self-limiting to fatal and is largely dependent on the infecting strain. Little to no tularemia-related mortality is reported in Europe and Asia, where only *F. tularensis* subsp. *holarctica* causes tularemia. In comparison, mortality in the United States ranges between 2.3% (those diagnosed by culture and serology) and 9% (culture-confirmed cases only) (38, 48), with tularemia caused by the more virulent *F. tularensis* subsp. *tularensis* as well as *F. tularensis* subsp. *holarctica*. In the last decade, molecular epidemiologic studies have demonstrated that among culture-confirmed human cases in the United States, infections due to A1b strains of *F. tularensis* subsp. *tularensis* result in a significantly higher rate of mortality (24%) than infections caused by *F. tularensis* subsp. *tularensis* A1a (4%) and A2 (0%) strains or *F. tularensis* subsp. *holarctica* (7%) (27). Virulence comparisons in mice corroborate these findings and show that A1b strains are more virulent than other *F. tularensis* subsp. *tularensis* strains (49).

The third subspecies of *F. tularensis*, *F. tularensis* subsp. *mediasiatica*, has only been isolated from regions of Central Asia, and a description of human illness due to this subspecies is lacking in the literature. Experimental studies with rabbits indicate virulence comparable to that of *F. tularensis* subsp. *holarctica* (25).

F. novicida and *F. philomiragia*

Human infections caused by *F. novicida* and *F. philomiragia* are very rare, with these organisms primarily infecting patients with underlying compromising conditions. Fewer than 20 cases of *F. philomiragia* infection have been described since the discovery of this species in 1974 (5, 8, 44–46). All but one case have involved a host with an impaired physical barrier to infection (near drowning) or an impaired immunologic defense system (chronic granulomatous disease or myeloproliferative disease). The drowning and water exposure cases were associated with salt water and brackish water, in contrast to *F. tularensis* infections, which are associated with freshwater sources. Fewer than 10 cases of *F. novicida* infections have been reported worldwide (5, 8, 42–44, 50, 51). Healthy individuals presented with mild illness (regional lymphadenopathy without fever), whereas patients with compromising conditions, primarily underlying liver disease, presented with fevers; in the latter cases, *F. novicida* was isolated from patient blood samples.

F. hispanensis

Only one human case due to infection with *F. hispanensis* has been reported in the literature (11, 13). A previously healthy, immunocompetent patient presented with fever, myalgia, diarrhea, and lower left back pain; the organism was isolated from both blood and urine of this patient.

F. noatunensis and *F. halioticida*

F. noatunensis and *F. halioticida* have never been associated with human infection, and the risk of human infection is considered very low. *F. noatunensis* is the significant cause of disease in farmed and wild fish, whereas *F. halioticida* has been associated with mortality in shellfish (giant farmed abalone) (52).

Another *Francisella* Species

An unclassified *Francisella* sp. was isolated from the blood and cerebrospinal fluid of two different patients in the United States in 2005 and 2006 (19). No other cases with this species have been reported.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

In the United States, *F. tularensis* is classified as a Tier 1 select agent. To receive or possess *F. tularensis* strains, laboratories must be registered with the Federal Select Agent Program (http://www.selectagents.gov). Clinical laboratories are exempt from the registration requirement with the Select Agent Program as pertains to conducting diagnostic testing for select agents. Both Tier 1 and non-Tier testing for select agents may be performed by clinical laboratories as long as the laboratory destroys or transfers any confirmed select agent (including clinical samples the isolate was derived from) with 7 days of identification. Laboratories identifying an organism as *F. tularensis* are required to report this finding immediately; laboratories need to complete Form 4 and submit it to the Select Agent Program. If the organism is to be transferred following identification, then the laboratory must also complete Form 2 and obtain transfer approval from the Select Agent Program. Select agent report forms, contact information, laboratory registration information, and pertinent citations of the U.S. Federal Code may be found at http://www.selectagents.gov.

Personnel handling diagnostic cultures of *F. tularensis* are at considerable risk for infection. Due to the extremely low infectious dose for *F. tularensis*, tularemia has been one of the most commonly reported laboratory-associated bacterial infections (53, 54). Even though the use of biological safety cabinets and prophylactic antibiotic therapy (as well as vaccination, where available) provides safeguards for laboratory workers, these precautions have not fully eliminated laboratory exposures or modified practices in the clinical laboratory to minimize risks (55). All patient specimens should be handled and processed in biosafety level 3 (BSL3) or BSL2 with BSL3 precautions, wearing gloves and lab coats or gowns and working in a class II biosafety cabinet, and clinical laboratories should be aware of the sentinel-level clinical microbiology laboratory guidelines as outlined by the American Society for Microbiology (http://www.asm.org/index.php/issues/sentinel-laboratory-guidelines). Aerosol inhalation during manipulation of cultures presents the greatest risk to laboratory workers, due to the high concentration of organisms. BSL3 practices, containment equipment, and facilities are recommended for all manipulations of suspicious cultures (56). Preparatory work on cultures for automated identification systems should be performed at BSL3. Laboratories may want to consider developing policies that encourage physicians to alert the laboratory if a diagnosis of tularemia is expected.

The choice of specimen for diagnostic testing is generally dependent on the form of clinical illness: ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic, or typhoidal tularemia (47). Whole blood is an acceptable specimen for all clinical forms of tularemia, although this sample may be negative, particularly if the disease is in the early stages of progression. Serum for antibody detection is a standard specimen taken for diagnosis of all forms of illness. The first specimen should be collected as early in the course of infection as possible, followed by a second specimen taken in the convalescent period (at least 14 days apart and preferably 3 to 4 weeks after onset of symptoms). Pharyngeal swabs, bronchial or tracheal washes or aspirates, sputum, transthoracic lung aspirates, and pleural fluid are appropriate specimens for pneumonic, typhoidal, or oropharyngeal tularemia. Swabs of visible lesions or affected areas have been used most commonly for ulceroglandular and oculoglandular tularemia. Aspirates from lymph nodes or lesions can be used for diagnosis of ulceroglandular, glandular, and oropharyngeal tularemia. Necropsied materials from animals that are appropriate for testing include samples from visible abscesses as well as samples from lymph node, lung, liver, and spleen tissues and bone marrow.

For specimens to be tested by culture, it is important where possible to decontaminate the surface area prior to specimen collection, since contamination of the sample with normal flora could interfere with the interpretation of culture results. To minimize loss in viability, specimens should be delivered to the laboratory within 24 h and preferably within 2 h. In general, if transport is >24 h, specimens should be stored chilled (2 to 8°C) in an appropriate medium until processed in the laboratory. Freezing of samples, unless in a preservative environment, such as tissue specimens or glycerol-containing solutions, is not recommended because of the lysis of live bacteria upon thawing. Swabs should be placed in Amies agar with charcoal, a commercial transport system designed for anaerobic and aerobic pathogens (Becton, Dickinson and Company, Franklin Lakes, NJ). *F. tularensis* should remain viable for 7 days at ambient room temperature when stored in Amies medium (57). Stuart medium, designed for transporting gonococcal specimens, and saline are inadequate for keeping *F. tularensis* viable during transport (57). For serum samples, separation from blood should take place as soon as possible, preferably within 24 h. Sera may be stored at 2 to 8°C for up to 10 days. If testing is delayed for a long period, serum samples may be frozen. Guanidine isothiocyanate-containing buffer, which preserves *F. tularensis* DNA for up to 1 month, is recommended if there is a delay in PCR testing of samples (57). Arthropods may be stored intact in 2% NaCl for culture analysis or in ethanol for molecular testing.

DIRECT EXAMINATION

Microscopy

Fresh clinical specimens (ulcer and wound swabs, tissues, and aspirates) where the concentration of organisms might be expected to be high can be directly examined by microscopy by Gram straining, direct fluorescent-antibody (DFA) binding, or immunohistochemistry (IHC). Under microscopic examination of Gram-stained specimens, *Francisella* cells (single and pleomorphic) appear tiny and counterstain so faintly with safranin that they can easily be missed (Fig. 1). Basic fuchsin counterstains *F. tularensis* better than safranin. Due to the small size of *F. tularensis*, Gram staining of clinical specimens is usually of little diagnostic value. *F. tularensis* should be included in the clinical laboratory differential if very small, faintly staining Gram-negative coccobacilli are seen. *F. tularensis* cells are smaller than *Haemophilus*.

Antigen Detection

A rapid and specific staining method (DFA) for detection of *F. tularensis* in specimens includes the use of a fluorescein isothiocyanate-labeled hyperimmune rabbit polyclonal antibody directed against whole, killed *F. tularensis* cells. This staining method can be used to presumptively identify *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* in clinical specimens (47). Cultures, lesions, tissues, or aspirates may be rapidly assessed by this approach. This DFA reagent does not react well or at all with *F. novicida* or *F. philomiragia*. IHC staining using a monoclonal antibody directed against the lipopolysaccharide has been used successfully to visualize *F. tularensis* in formalin-fixed tissues (58). Neither the DFA reagent nor the IHC reagent is commercially available. DFA testing of specimens is provided by reference laboratories (Laboratory Response Network [LRN]) in the United States.

Nucleic Acid Detection

Because of the relative rarity of human tularemia, evaluation of molecular diagnostics with clinical specimens has been challenging. PCR-based diagnostic methods have been used most commonly for diagnosis of ulceroglandular tularemia, the most prevalent clinical form. DNA detection by conventional PCR directed at the *tul4* gene (unique to *Francisella*

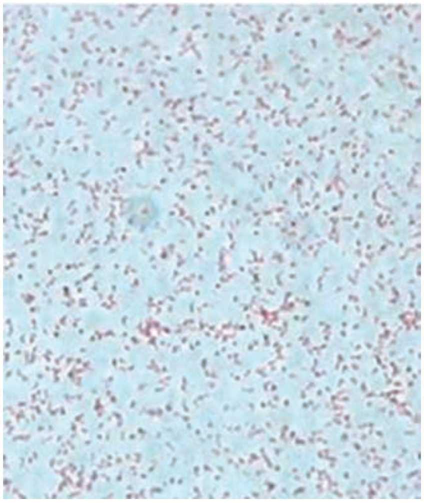

FIGURE 1 Gram stain of *F. tularensis*. Magnification, ~×810. doi:10.1128/9781555817381.ch46.f1

spp.) has been successfully and widely applied for diagnosis of ulceroglandular tularemia (47, 57, 59). The *tul4* PCR assay displays a sensitivity of 75% when applied to wound specimens from patients with ulceroglandular tularemia and was shown to be more sensitive than culture (sensitivity of 62%) (57). Real-time PCR assays using the TaqMan 5′ nuclease assay have been applied to detect *F. tularensis* DNA in a variety of clinical specimens, including ulcer specimens, aspirates, pharyngeal swabs, lymph node specimens, bronchial washes, and pleural fluid (60–63). These assays target multiple *Francisella* genes (*ISFtu2* element, *iglC*, *tul4*, and *fopA* genes). Real-time PCR assays for diagnosis of tularemia are available in LRN reference laboratories in the United States.

A limitation of most PCR-based diagnostics for *F. tularensis* is the inability to discriminate *F. tularensis* from *F. novicida*, due to their high degree of genetic relatedness. While this may not be significant for patient management, the correct identification of species has biothreat, epidemiologic, and public health importance. A recent study evaluated 38 published PCR assays against the current known diversity of the *Francisella* genus, and it found that most nucleic acid assays are not specific for only a single *Francisella* species (64).

Detection of *F. tularensis* in ectoparasites is based primarily on PCR methods. Of note, FLEs, present in a wide range of tick species, have been shown to cross-react with molecular targets used for the detection of *F. tularensis*, leading to false-positive results (20, 65, 66). If molecular assays are to be used for screening environmental samples (water or soil) for *F. tularensis*, it is important to evaluate the assays for cross-reactivity with other *Francisellaceae* members, particularly *F. novicida*, present in these sample types (18, 67). 16S rRNA gene sequencing can be used to discriminate *F. tularensis* from FLEs or environmental *Francisella* spp. other than *F. novicida* (65, 67).

Isolation Procedures

F. tularensis is slow growing and fastidious; it is auxotrophic for cysteine and consequently requires an exogenous source of cysteine in order to grow well on artificial medium. *F. tularensis* grows on several media common to clinical laboratories, including chocolate agar (CA) (Fig. 2A), buffered charcoal-yeast extract agar (BCYE), and Thayer-Martin agar. It also grows in thioglycolate broth, although growth in broth is not recommended in clinical laboratories due to the increased risk of exposure via aerosolization. When supplemented with 1 to 2% IsoVitaleX (which contains cysteine and other supplements), general bacteriological media (tryptic soy broth and Mueller-Hinton broth) can also support the growth of *F. tularensis*. The organism grows slowly (60-min generation time); good growth, therefore, is obtained by prolonged incubation (48 h or longer). Suspicious cultures should be incubated at 35 to 37°C aerobically and observed daily for up to 14 days; CO_2 neither impedes nor is required for growth. A specialized medium, cysteine heart agar supplemented with 9% chocolatized sheep blood (CHAB), is often used for the growth of *F. tularensis* in reference laboratories (47). *F. tularensis* grows well on CHAB, as this is a high-nutrient medium. Additionally, *F. tularensis* displays distinctive colony morphology on CHAB which can aid in identification (Fig. 2B). *F. tularensis* grows poorly or not at all on general bacteriological media, such as sheep blood agar. Nutritionally enriched specimens, such as blood or tissue, provide an intrinsic source of cysteine that may initially permit *F. tularensis* growth on general bacteriological media. Upon subculture, the fastidious nature of *F. tularensis* will become evident as the exogenous compounds are depleted, leading to the loss of the bacterium's viability unless the subculture is propagated on cysteine-supplemented medium.

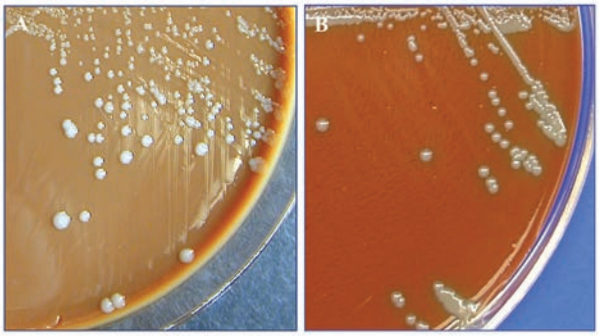

FIGURE 2 *F. tularensis* on CA (A) and CHAB (B) after 72 and 48 h of growth, respectively. (Panel A is courtesy of the CDC Public Health Image Library [Larry Stauffer, Oregon State Public Health Laboratory].) doi:10.1128/9781555817381.ch46.f2

Clinical samples from normally sterile sites can be plated on nonselective agars that support the growth of *F. tularensis*. Care should be taken not to permit laboratory contamination of *F. tularensis* isolates by environmental bacteria such as *Staphylococcus*, as these bacteria rapidly outcompete and inhibit the growth of *F. tularensis* on nonselective media (68). Clinical specimens obtained from nonsterile sites or autopsy and environmental sources should be plated on antibiotic-containing media. Incorporation of an antibiotic supplement (7.5 mg/liter of colistin, 2.5 mg/liter of amphotericin, 0.5 mg/liter of lincomycin, 4.0 mg/liter of trimethoprim, and 10 mg/liter of ampicillin) into the medium has been demonstrated to prevent other organisms from overwhelming *F. tularensis* (68). Commercially available antibiotic-containing media that support the growth of *F. tularensis* include improved Thayer-Martin agar (Remel, Lenexa, KS), BCYE selective agar containing polymyxin B, anisomycin, and vancomycin (Remel or Becton, Dickinson), and cysteine heart agar containing antibiotics (polymyxin B and penicillin) (Remel).

Specimens for culture should be taken on the basis of clinical presentation and before administration of antibiotics. Fresh clinical material that is likely to contain high concentrations of *F. tularensis* organisms, such as ulcer and wound specimens and lymphoid tissue (liver, spleen, or affected lymph node tissue), is inoculated directly onto an agar plate by using a sample-laden swab or bacteriological loop. Larger inocula are necessary for the recovery of *F. tularensis* from specimens that contain a lower concentration of the organisms, such as aspirates of pharyngeal washes, bronchial wash fluids, pleural fluids, and environmental samples. Whole blood should be directly inoculated into blood culture bottles (47), with subsequent culture of blood culture-positive specimens on agar. Blood culture bottles should be incubated for 5 to 7 days to allow for growth of *F. tularensis*.

IDENTIFICATION

Because of the rarity as well as the sporadic nature of most tularemia cases, the organism is often not easily identified when it is cultured. The isolation of a very tiny (individual cells may be difficult to discern), poorly counterstaining (by safranin), slow-growing Gram-negative coccobacillus (Fig. 1) that produces 1- to 2-mm gray to gray-white colonies on CA after 48 h, scant to no growth on blood agar, and no growth on MacConkey agar should raise suspicion for *F. tularensis*. *F. tularensis* is oxidase negative, weakly catalase positive, β-lactamase positive, X and V or satellite growth negative, and urease negative. Gram stain, oxidase, XV or satellite, and urease tests can help differentiate *F. tularensis* from other similar Gram-negative organisms, including *Brucella* spp., *Haemophilus influenzae*, *Bartonella* spp., *Yersinia pestis*, *Pasteurella multocida*, and *Acinetobacter* spp. (Table 1). If *F. tularensis* is suspected based on these results, the isolate should be sent to a reference laboratory that can confirm (or rule out) its identification as *F. tularensis*. In the United States, all states have at least one reference laboratory that is part of the LRN. LRN laboratories are able to confirm the identification of bacterial select agents, including *F. tularensis*. See also http://www.bt.cdc.gov/lrn.

The colony morphology of *F. tularensis* is most distinctive when the organism is grown on CHAB; after 48 h at 37°C, *F. tularensis* colonies are 1 to 2 mm, raised, shiny, green, and opalescent (Fig. 2B). On CA, BCYE, and Thayer-Martin agar after 48 h at 37°C, *F. tularensis* colonies are ~1 mm, white to gray, and smooth and opaque and have an entire margin (Fig. 2A). *F. novicida* and *F. philomiragia* grow more robustly than *F. tularensis* (Table 2), and colonies of *F. philomiragia* are mucoid.

Isolates can be identified as *F. tularensis* using antigen or molecular detection methods, including slide agglutination, DFA staining (Fig. 3), PCR, or sequencing. The slide agglutination test identifies a suspicious culture by mixing commercially available (Becton, Dickinson and Company) polyclonal rabbit anti-*F. tularensis* antibody with suspicious cultures. DFA staining can also be used to identify *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* isolates (47). Care should be taken to ensure that prepared smears are not too thick, as this can interfere with the performance and interpretation of the test. In general, antigen-based identification methods work optimally when fresh cultures (24 h) are tested. If a culture older than 24 h is to be tested by antigen detection methods, a fresh subculture should be prepared. Polyclonal antibodies to whole killed *F. tularensis*, used in both the DFA and slide agglutination tests, generally react poorly or not at all with *F. novicida* due to differences in the O antigens of the lipopolysaccharides of the two organisms (47, 69). Thus, this method for identification of *F. tularensis* isolates is preferred, due to this specificity.

PCR methods targeting *F. tularensis*-specific genes can also be used for the identification of suspicious cultures (59, 61). As described earlier in this chapter, it is important to consider that many *F. tularensis* PCR assays cross-react with *F. novicida*. Therefore, it may be important to rule out *F. novicida* as the cause of infection, particularly in areas where tularemia has not been previously reported. 16S rRNA gene sequencing, using universal 16S rRNA gene primers or *Francisella*-specific 16S rRNA gene primers, can identify the organism as a *Francisella* species (10, 29, 70). Due to the high degree of genetic relatedness between *F. tularensis* and *F. novicida*, 16S rRNA PCR and sequencing do not readily distinguish these two species. *F. novicida* isolates have been misidentified as *F. tularensis* in clinical laboratories by 16S sequencing (42).

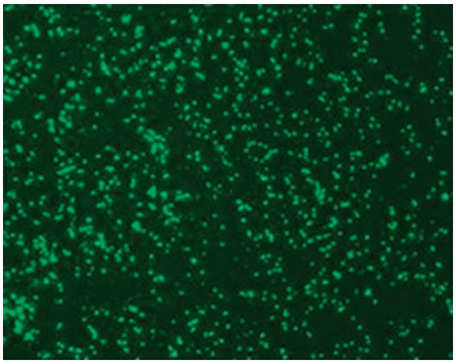

FIGURE 3 DFA staining of a culture of *F. tularensis*. Magnification, ~×490. doi:10.1128/9781555817381.ch46.f3

Members of the *Francisella* genus have a unique fatty acid composition, high in saturated even-chain acids ($C_{10:0}$, $C_{14:0}$, and $C_{16:0}$) and two long-chain hydroxyl acids (3-OH-$C_{16:0}$ and 3-OH-$C_{18:0}$) (71), which can be used to identify the organism as belonging to the *Francisella* genus (Table 1) (1, 5, 21). Bacterial identification systems that rely on fatty acid profiles have been used to identify *F. tularensis*; however, it is important to note that commercial systems do not include *F. novicida* or many other *Francisella* spp. in their databases. Indeed, misidentification of *F. novicida* as *F. tularensis*, in a clinical laboratory setting, using these systems has been described (42).

Commercial biochemical identification systems available in clinical laboratories are not recommended for the diagnosis of *F. tularensis* because of the high probability of misidentification and the potential for generating infectious aerosols (47). Misidentification is largely due to a lack of biochemical reactivity by *F. tularensis* and a limited number of strains used in the development of databases. Because of the limitations in identifying *F. tularensis* isolates using automated systems in clinical laboratories, a high level of suspicion by clinical microbiologists is essential for accurate and timely diagnosis of tularemia. Results from automated identification systems should be analyzed critically in the clinical laboratory, taking into consideration the reliability of these systems for accurate identification of slow-growing and/or rare organisms. Potential indications of misidentification by automated systems include a low probability or confidence value and/or an identification of a pathogen inconsistent with the clinical or microbiology picture.

The utility of matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) combined with dedicated bioinformatics and statistical methods for identification and differentiation of *Francisella* strains was recently demonstrated (72). Forty-four strains of *F. tularensis* were accurately identified to the subspecies level as well as differentiated from six strains of *F. philomiragia* (72). Thus, this method shows promise as a method for future identification of *F. tularensis* in the clinical laboratory. At the current time, however, documented use of MALDI-TOF MS with commercially available databases in clinical laboratory settings is lacking.

TYPING SYSTEMS

Once an isolate has been identified as a *Francisella* sp., supplemental tests can be used for additional characterization, including typing of species, subspecies, and strain. Oxidase can be used to differentiate *F. philomiragia* from *F. novicida* and *F. tularensis* (Table 2). Glycerol fermentation and citrulline ureidase activity distinguish *F. tularensis* subsp. *tularensis* from *F. tularensis* subsp. *holarctica* (Table 2); conventional assays for these biochemical tests have been described (73). The 96-well automated MicroLog MicroStation system with GN2 microplates (Biolog Inc., Hayward, CA) can also be used to assess the glycerol fermentation of *F. tularensis* (47). The genus can be typed by sequence analysis of the 16S rRNA gene (10, 70). PCR methods (both conventional and real-time) can type isolates at the level of genus, species, subspecies (*F. tularensis* subsp. *tularensis* or *F. tularensis* subsp. *holarctica*), and subclade (*F. tularensis* subsp. *tularensis* subclades A1 and A2) (47, 74). Pulsed-field gel electrophoresis can differentiate the three *F. tularensis* subsp. *tularensis* subpopulations, A1a, A1b, and A2 (27). For discrimination of individual strains, an optimized, multiplex multilocus variable-number tandem-repeat assay for *F. tularensis*, based on 11 different repeats in the genome, can be used (75, 76).

SEROLOGIC TESTS

A confirmatory diagnosis of tularemia can be established serologically by demonstrating a 4-fold or significant change in specific antibody titers to or response between acute- and convalescent-phase sera to *F. tularensis* antigen (47). A single positive antibody titer for a patient with no history of tularemia vaccination is a presumptive diagnosis of tularemia. As antibody responses to *F. tularensis* generally require 10 to 20 days to develop postinfection, serologic testing is of limited use in clinical management of patients with acute illness. IgM, IgA, and IgG antibodies may appear simultaneously (77, 78), and antibodies to *F. tularensis* can persist for more than 10 years (79, 80). IgM responses have been shown to be sustained for long periods and are therefore not indicative of early or recent infection (77, 79).

Agglutination testing, either by the tube agglutination (TA) or the microagglutination (MA) method, is a standard serologic test for determining the presence of antibodies in patients with tularemia (47, 79, 81). Formalin-killed antigen (prepared from *F. tularensis* subsp. *holarctica* strain LVS) is commercially available from Becton, Dickinson, and is also prepared within reference laboratories worldwide. In the United States, a single specimen with a TA titer of ≥1:160 or an MA titer of ≥1:128 is considered positive. Formalin-killed *F. tularensis* whole-cell antigen may display low-level cross-reactivity with *Brucella* antibodies (82, 83). No cross-reactivity of *F. novicida* or *F. philomiragia* sera has been observed with *F. tularensis*-killed cells (9). Enzyme-linked immunosorbent assays (ELISAs) have been adopted for use in the parts of Europe where tularemia is endemic (47, 79, 84, 85). The lipopolysaccharide and/or outer membrane fraction remains the primary ELISA antigen used in test applications. Antigenic differences between *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* have not been identified for use in serology assays. Thus, serology assays do not distinguish the infecting subspecies. This is of the most importance in North America, where both *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* cause tularemia.

F. tularensis organisms are intracellular bacteria and are capable of eliciting both humoral and cell-mediated immunity (77). The latter response has been known to remain strong 25 years after infection (86). Host T cells retain proliferative responses to unique *F. tularensis* membrane proteins, with concomitant increases in interferon and interleukin-2 levels (77, 86–88). Tests for measuring the cell-mediated immune response are specialized and are not routinely used for diagnosis of tularemia (89).

ANTIMICROBIAL SUSCEPTIBILITIES

F. tularensis infections are treatable with narrow-spectrum antibiotics. Naturally occurring resistance in *F. tularensis* to aminoglycosides, tetracyclines, and fluoroquinolones has never been shown (47, 90–92). The risk for development of antibiotic resistance in clinical disease is low, as tularemia is an end-stage disease and also is not transmitted from person to person (47). Treatment failures in tularemia patients correlate with a delay in the initiation of antibiotics with respect to symptom onset, as opposed to development of resistance (60, 93, 94). Lymph node suppuration, which is nonresponsive to all classes of antibiotics and requires surgical drainage, can develop in these cases. Considerably longer recovery times are observed in these cases and can be greater than 70 days (94).

β-Lactam antibiotics are not used for treatment of tularemia, as *F. tularensis* strains encode a class A β-lactamase, FTU-1 (95). β-Lactamase resistance appears to be limited to penicillins; *in vitro* MIC determinations indicate that FTU-1 does not confer resistance to other β-lactams, including first- and second-generation cephalosporins (95). Although third-generation cephalosporins have been shown to be active against *F. tularensis in vitro*, clinical experience indicates that ceftriaxone is not effective for treatment of tularemia (96). Macrolides are also not recommended for treatment of tularemia, as type B strains from Central and Eastern Europe and Asia are naturally resistant to erythromycin and other macrolides (97).

Antimicrobial susceptibility testing of *F. tularensis* is not performed in clinical microbiology laboratories because of safety concerns in working with this organism and because resistance to antibiotics used for clinical treatment of tularemia has never been reported (90). The Clinical and Laboratory Standards Institute (CLSI) has published interpretative criteria and quality control limits for broth microdilution of *F. tularensis* using Mueller-Hinton medium supplemented with 2% IsoVitaleX (98, 99).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Serology is a common method for laboratory confirmation of *F. tularensis* infection, due largely the organism's slow growth and fastidious nature. Nonetheless, culture provides a conclusive diagnosis of infection and whenever possible should be attempted using appropriate biosafety measures. A very tiny Gram-negative bacterium that grows slowly, shows fastidious growth characteristics, and is oxidase negative, weakly catalase positive, urease negative, X/V or satellite negative, and β-lactamase positive should be strongly suspected as *F. tularensis* and referred to a reference laboratory. Confirmation of *F. tularensis* infections includes (i) identification of a culture as *F. tularensis* and/or (ii) a 4-fold difference in titers in acute- and convalescent-phase serum samples, with one of the paired samples having a positive titer. A positive test result for a primary clinical specimen using antigen or molecular detection methods, including DFA staining, IHC staining, or PCR, provides only a presumptive diagnosis of *F. tularensis*. A single positive serum sample is also considered presumptive for tularemia. For all cases presumed to be tularemia, it is necessary to verify that the patients' symptoms are compatible with tularemia.

In the United States, tularemia is a nationally notifiable disease and *F. tularensis* is also classified as a select agent. Identification of a bacterial select agent requires isolation of the organism in culture and confirmation. Laboratories identifying an organism as *F. tularensis* are required to report this finding immediately; laboratories need to complete Form 4 and submit it to the Select Agent Program. Report forms, contact information, laboratory registration information, and pertinent citations of the U.S. Federal Code may be found at http://www.selectagents.gov.

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Brucella[*]

GEORGE F. ARAJ

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Brucella spp. are common zoonoses among domestic animals and among wildlife, including novel species of marine mammals. *Brucella* spp. also cause infections in humans and can mimic other infectious and noninfectious diseases, posing challenges to physicians in reaching a diagnosis. The remittent/undulant fever of brucellosis was first confused with other diseases, such as malaria and typhoid fever, and has had many synonyms pertaining mainly to the geographic locations where the disease occurred: Mediterranean fever, Malta fever, Gibraltar fever, and Cyprus fever (1, 2). Over the last decade, there has been renewed interest in this organism due to its inclusion in the potential biological weapons lists of most authorities (3, 4).

TAXONOMY AND GENOME

Brucellaceae is a family of phylogenetically closely related free-living soil organisms composed of *Brucella*, *Ochrobactrum*, and *Mycoplana* spp. The *Brucellaceae* are part of the order *Rhizobiales*, which includes other genera involved in human disease: *Bartonella*, *Afipia*, *Methylobacterium*, and *Roseomonas* (5, 6).

The taxonomy of *Brucella* spp. remains to be clarified. Studies indicate that terrestrial *Brucella* spp. are homogeneous species harboring >90% interspecies homology by DNA-DNA hybridization studies, identical 16S rRNA gene sequences, and >98% sequence homology by comparative genomics. Because of these findings, a suggestion was made to consider *Brucella* a monospecific genus and the different species as biovars of *Brucella melitensis* (7).

The average size of the genome is 2.37×10^9 Da, with a DNA G +C content of 58 to 59 mol%. Currently, the genus *Brucella* encompasses 10 recognized species: 6 terrestrial, 3 marine, and 1 proposed species of unknown origin (*Brucella inopinata* sp. nov.) from a breast implant (8–11). The six terrestrial *Brucella* species are *B. melitensis* (three biovars) (preferred hosts are goats, sheep, and camels), *B. abortus* (seven biovars) (cattle and buffaloes), *B. suis* (five biovars) (swine and a range of wild animals), *B. canis* (dogs), *B. ovis* (rams), and *B. neotomae* (desert and wood rats). The three identified marine species, *B. delphini*, *B. pinnipedialis*, and *B. ceti*, were recovered from marine mammals (e.g. seals, whales, and dolphins) and were found to differ phenotypically from the six terrestrial species by their patterns of substrate-mediated metabolic activity. Though preferred or predominant hosts are recognized for *Brucella* spp., cross-infection of other mammalian species, including humans, may occur (12).

DESCRIPTION OF THE GENUS

Brucella spp. are facultative, intracellular, small (0.5- to 1.5-mm), Gram-negative coccobacilli that lack capsules, flagella, endospores, or native plasmids. They are aerobic (some prefer CO_2 for their growth), do not ferment sugars, and are positive in a few oxidative metabolic tests. *Brucella* spp. can grow on a wide range of culture media, and colonies appear after 24 to 48 h of incubation as mostly smooth colonies, but rough variants can occur (12, 13).

ANTIGENIC COMPONENTS

Several antigenic determinants of *Brucella*, related mainly to lipopolysaccharide (LPS) and protein antigens, have been characterized. The LPS is the major antigen that dominates the antibody response. LPS of rough strains is very similar to LPS of smooth strains. Based on their O side chain, smooth strains were reported to be composed of two antigenic epitopes: A (*B. abortus*) and M (*B. melitensis*). The smooth-strain LPS has been reported to be responsible for observed cross-reactions in both the agglutination and complement fixation tests between smooth species of *Brucella* and *Yersinia enterocolitica* O:9, *Escherichia hermannii*, *Escherichia coli* O:157, *Salmonella enterica* serovar O:30, *Stenotrophomonas maltophilia*, *Vibrio cholerae* O:1, and *Francisella tularensis*. Cross-reaction has been attributed to the similarities of the O-specific side chains of the LPS molecules of these organisms (reference 14; refer also to chapter 46 in this *Manual*).

The characterized protein antigens include outer and inner membrane, cytoplasmic, and periplasmic antigens. Some are recognized by the immune system during infection and are potentially useful in diagnostic tests (15). For example, Omp25 is an outer membrane structural protein that is highly conserved in all brucellae and is associated with both LPS and peptidoglycan. In addition, some proteins, such as ribosomal proteins (e.g., L7/L12) and fusion proteins, demonstrate a protective effect against *Brucella* based on antibody and cell-mediated responses (16). These molecules may be useful in potential vaccines.

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VIRULENCE FACTORS, PATHOGENIC MECHANISMS, AND IMMUNE RESPONSE

The exact pathophysiologic aspects of infection remain to be defined, especially since this "stealth pathogen" can infect and survive without inducing a massive inflammatory response (17–20). The incubation period is variable but generally is 1 to 4 weeks. The intracellular location and survival of the organism contribute to its virulence and pathogenesis. Virulence determinants include urease to avoid stomach stress through oral passage and *Brucella*-containing vacuoles (BCV), which enable escape from immune system recognition and provide an acidic environment to hamper antibiotic activity. A *Brucella* LPS cell component (containing a poly *N*-formyl perosamine O chain and a CuZn superoxide dismutase) and outer membrane protein 25 (OMP25) help the bacteria survive within mononuclear phagocytes (21). Also, *Brucella* LPS poorly induces gamma interferon and tumor necrosis factor alpha (TNF-α), both of which are essential for T-helper 1 (Th1)-type-cell-mediated immunity for the elimination of the organism (22).

Briefly, once the brucellae enter the body by various routes, they are encountered by polymorphonuclear and mononuclear phagocytes, to which lectin facilitates attachment, and lipid rafts help in avoidance of defense mechanisms. After entry into the cell, the first step entails the establishment of an intracellular replication niche, and *Brucella* LPS and a periplasmic cyclic β-glucan are essential for this step. *Brucella* spp. escape from phagocytic killing by inhibiting the phagosome-lysosome fusion and hence evade the immune system. These factors together with the BCV aid in its survival, propagation, and persistence within macrophages and other cells. As brucellae remain within macrophages, they are transported through regional lymph nodes into the circulatory system and subsequently seeded throughout the body, with tropism for the reticuloendothelial system, resulting in different clinical phases of disease (18).

The overall inflammatory process results in a slow degradation of *Brucella* cell wall components by the polymorphonuclear leukocytes and can lead to granuloma formation, which is more often associated with *B. abortus* than *B. melitensis* (18).

Protective immunity, though not long term, is conferred by antibodies to LPS and T-cell-mediated macrophage activation, triggered by protein antigens. Lymphocytes are the main stimulant of the immune response. The Th1 response stimulates IgG2a production, which is involved mostly in protection against intracellular pathogens through cell-mediated immunity, and is critical for the clearance of *Brucella* infection. The Th2 response stimulates the production of IgG1 and is mainly responsible for protection against extracellular pathogens through the humoral immune response (23, 24).

Recently, the production of cytokines, chemokines, and matrix-metalloproteinases has been associated with induced osteoclastogenesis in brucella arthritis and osteomyelitis, and the OMP19 lipoprotein, together with TNF-α, were reported to be associated with astrocyte apoptosis in neurobrucellosis (23). Though receiving close attention, the exact nature of the immune response and the protective antigens/factors involved in this disease are still being investigated, and the pathogenic mechanisms of reinfection remain unknown (25).

EPIDEMIOLOGY AND TRANSMISSION

Animals are generally asymptomatic carriers of *Brucella* spp. The major symptoms appear during infectious abortion of the animal placenta and fetus. Invaded fetal tissue may contain up to 10^{10} infectious bacteria per gram of tissue and fluid (12). Although *Brucella* can be killed by pasteurization, exposure to UV light, acidity, or many antiseptics and disinfectants, it can survive for long periods under various conditions, e.g., 10 weeks in soil, 11 weeks in aborted fetuses, 17 weeks in bovine stool, around 3 weeks in milk and ice cream, and several months in fresh goat cheese (12, 26). *Brucella* can infect both humans and animals. In terms of the total numbers of infected human cases, *B. melitensis* dominates the world arena (especially in the Mediterranean and Arabian Gulf countries). However, *B. abortus* and *B. suis* supersede it in certain geographic locations in Europe. *B. canis* has also been reported to cause human diseases, while *B. ovis* and *B. neotomae* have not (12, 27–29). *Brucella* spp. associated with marine animals have been reported to cause disease in humans (30, 31). The epidemiologies of human brucellosis differ between areas of endemicity and nonendemicity in terms of age, sex, season, and risk factors. In regions of endemicity, such as the eastern Mediterranean Basin, Middle East, the Arabian Peninsula, Mexico, Central and South America, Southern Europe, Central Asia, and the Indian subcontinent, the disease occurs among the general population. In the general population, levels of infection are almost equal among adults and children of both sexes and mostly due to ingestion of unpasteurized goat, sheep, cow, and camel milk or its products (e.g., soft cheese, ice cream) (2, 27–29, 32–34).

In areas where the disease is not endemic, infection is seen predominantly among adult males, acquired occupationally by transmission through direct skin contact (e.g., through cuts and abrasions) with infected animal parts, inhalation of aerosolized infected particles, and accidental inoculation (e.g., sprays or aerosols inoculated into the eye, mouth, and nose). These infections occur mostly among dairy industry professionals, veterinarians, abattoir workers, and clinical and research microbiology staff (35, 36). Human-to-human transmission may occur, although very rarely. Few cases of neonatal brucellosis have been reported, and the isolation of *Brucella* from human milk may explain its pathogenesis (37, 38).

Laboratory-acquired infection (LAI) is an important source of transmission. *Brucella* has a very low infectious dose ($\leq 10^2$ organisms), and personnel should adhere to strict safety precautions, especially when handling cultures suspected of containing the organisms in clinical, research, and production laboratories (36, 39, 40). Most cases of laboratory-acquired disease result from mishandling and misidentification of the organism (40). The frequent failure of clinical laboratories to correctly identify isolates as *Brucella* species is particularly worrisome from the perspective of laboratory safety and potential use as a bioweapon. *B. melitensis*, *B. abortus*, and *B. suis* are category B select agents (4).

CLINICAL CATEGORIES OF HUMAN BRUCELLOSIS

The clinical categories of human brucellosis are based on arbitrary criteria. In 1956, Spink based them on the duration of symptoms (acute, <2 months; subacute, 2 to 12 months; chronic, >12 months) (41). Subsequently, others based them primarily on extent of clinical manifestations (e.g., subclinical, localized, chronic, and active, with or without localized disease, including bacteremic and serological classifications) (29, 42). To date, no uniform definition has been adopted.

The incubation period is variable but usually ranges between 1 and 4 weeks. The disease onset is insidious and can present with a wide and diverse range of nonspecific clinical signs and symptoms, such as fever, sweats, arthralgias, myalgia, fatigue, loss of appetite, weight loss, hepatomegaly, and splenomegaly. Complications can involve many organs and tissues with signs of focal disease. The routine hematology and biochemical profiles are usually within normal limits, with some elevation in erythrocyte sedimentation rate and liver function tests. Thus, to the unaware physician, the diagnosis of brucellosis can be a dilemma and may protract for weeks and, in some complicated cases, for years (27, 29, 43–47). Increased business and leisure travel to countries where the disease is endemic has led to diagnostic challenges in areas where brucellosis is uncommon, especially when the presentation is unusual (29, 48). Overall, the mortality is very low, but morbidity is high. Previously thought to be uncommon, brucellosis in childhood now seems to be as prevalent as and presents in a manner similar to that in adults in areas of endemicity (33, 49).

Because of these nonspecific clinical features, human brucellosis can imitate a variety of diseases and, thus, has been labeled "the disease of mistakes." For example, it can be misdiagnosed and confused with other diseases, such as typhoid fever, rheumatic fever, tuberculosis, malaria, infectious mononucleosis, endocarditis, histoplasmosis, ankylosing spondylitis, pyelitis, cholecystitis, thrombophlebitis, chronic fatigue syndrome, collagen vascular diseases, autoimmune diseases, and tumors (27, 29, 46, 47, 50).

COMPLICATIONS

The most commonly encountered focal complications are osteoarticular (10 to 70%) (mostly joints), genital in both males (6 to 8%) and females (2 to 5%), neurological (3 to 5%), cardiac (1 to 3%), pulmonary (1 to 2%), and renal (<1%). Mortality is very low (<1%) and is almost exclusively due to cardiac complications (27, 29, 42, 43, 45, 47, 50–53).

Osteoarticular complications occur mostly as arthritis (10 to 70%) and rarely as osteomyelitis (<1%). The joints most frequently involved are, in descending order, sacroiliac, knee, hip, vertebra, ankle, and multiple other joints. Generally, *Brucella* arthritis can be misdiagnosed as rheumatoid arthritis, rheumatic fever, tuberculosis, and systemic lupus erythematosus.

Neurobrucellosis (3 to 5% of cases) can affect both adults and children with diverse presentations, including fever, headache, meningeal signs, coma, or paresis. Depression, psychosis, and mental fatigue are not uncommon complaints (33, 54–58). Cerebrospinal fluid (CSF) analysis, of both adults and children, is nonspecific, and results can overlap those of other central nervous system diseases (such as mycobacterial, viral, syphilitic, or fungal infections) or noninfectious diseases (such as psychiatric problems, multiple sclerosis, and cancer) (55, 56, 59). The yield of *Brucella* culture from CSF is low (5 to 30%). Therefore, the use of *Brucella* serology tests, especially indirect Coombs' test, Brucellacapt, or enzyme-linked immunosorbent assay (ELISA), on CSF specimens is essential to diagnose neurobrucellosis (56, 60). With appropriate treatment, the prognosis is usually good for acute presentations and varies in settings of chronic disease.

Genital complications in males (6 to 8% of cases) are mostly orchitis or epididymo-orchitis (27, 43). In females, rare complications reported include abortion, cervicitis, salpingitis, tubo-ovarian abscess, and ovarian dermoid cyst (27, 43, 61–63).

Relapse is considered one of the most important features of brucellosis and its complications. The exact factors associated with relapse remain to be determined, but some were noted to include the use of less-effective antibiotic therapy, a positive blood culture during the initial presentation, and ≤10 days' duration of symptoms before initiation of treatment (27, 43, 45, 64, 65).

COLLECTION, HANDLING, STORAGE, AND TRANSPORT OF SPECIMENS

Specimens for the laboratory investigation of cases of brucellosis may be sent for culture, serology, and/or molecular testing. Culture can be performed on a wide range of specimen types, including blood (at least two sets), bone marrow, CSF, pleural and synovial fluids, urine, abscess specimens, and tissue specimens. Adequate volumes should be secured prior to initiation of antimicrobial therapy.

Blood (serum) and, when relevant, CSF specimens are used for serologic testing. Molecular testing, though usually for research purposes, can be performed on blood (serum or whole blood), CSF, and bone marrow specimens.

The guidelines for proper specimen collection, handling, transport, and processing are generally similar to those reported for blood cultures and other specimens submitted for bacterial culture (refer to chapter 18 in this *Manual*). If delay in delivery to the lab is anticipated, specimens can be held in the refrigerator. To avoid/minimize laboratory exposures to the pathogen, specimens from patients suspected of having the disease should be labeled appropriately and referred to a reference laboratory, with the label specifying that the laboratory should rule out brucellosis (66).

DIRECT DETECTION

To circumvent the limitations of routine culture and serodiagnostic tests for human brucellosis, in-house-developed conventional PCR and reverse transcription (RT)-PCR assays can directly detect *Brucella* from clinical specimens. Several *Brucella*-specific gene targets have been used, including BCS P31 (encodes a 31-kDa cell surface protein) and BP26 (encodes a 26-kDa periplasmic protein), 16S rRNA, and the insertion sequence IS*711*. The sensitivities of these assays are quite varied, ranging from 50% to 100%. This variation might be related to different DNA extraction methods, detection formats, and different types of specimens (67–74). The ribosomal 16S-23S ITS region constitutes a suitable target in clinical specimens and in formalin-fixed, paraffin-embedded, archived tissue, as well as for identification of isolates from culture to the species level (69).

Molecular assays constitute a useful adjunct and have promising potential for the diagnosis of human brucellosis in a clinical laboratory setting. Their routine incorporation in the diagnosis of human brucellosis remains in need of further optimization, standardization, and improvement (69, 74, 75).

CULTURE

Culture is considered the gold standard in the laboratory diagnosis of brucellosis. Recovery of isolates can also be helpful for epidemiologic tracing and antimicrobial susceptibility testing. Conventional methods require long incubation times (6 weeks) and generally have varied yields, being higher among patients with acute brucellosis (40% to 90%)

than among patients in the chronic, focal, and complicated stages (5% to 20%) (67, 68, 76). When positive, culture provides the definitive diagnosis. Bone marrow cultures result in a 15- to 20%-higher yield than peripheral blood cultures. The conventional standard medium for the nonautomated blood culture broth has been the biphasic Ruiz-Castañeda bottle. The growth of the pathogen takes between 7 and 35 days to become positive, and the bottles should be held for 6 weeks, with frequent visual inspection (every 3 days) and terminal subculture before the specimen is discarded as negative.

Automated continuously monitored blood culture systems, such as Bactec (BD Diagnostics, Sparks, MD) and BacT/Alert (bioMérieux, Durham, NC), result in higher yields than the conventional culture method and expedite the detection of bacterial growth (the majority of bacteria are recovered within 1 week). There is no need to incubate bottles longer than 10 to 14 days (68). The lysis centrifugation system showed improved and faster yields than nonautomated blood culture systems. However, due to the need for centrifugation and manipulation before direct plating, the system may entail exposure and contamination hazards. Rarely, some patients with brucellosis have a positive blood culture in the absence of positive serology (29, 76).

Recovery of *Brucella* from other clinical material, such as bone marrow, CSF, joint fluid, homogenate of tissues, and bones, in addition to blood specimens, can be achieved by inoculation of specimens into broth media (such as those used for blood cultures) in addition to plated media (blood and chocolate agar). Plated media are incubated at 37°C, preferably under 5 to 10% CO_2, for up to 10 days prior to being reported as negative (67, 68).

IDENTIFICATION

Clinical microbiology laboratories should identify colonies suspected of being *Brucella* spp. on the basis of a few morphologic, biochemical, and serologic tests. All manipulations of *Brucella* cultures should be done in a biological safety cabinet. In these setups, the colonies are generally recovered directly from inoculated clinical specimens or as a result of subculture from broth media (e.g., biphasic Ruiz-Castañeda medium and blood culture medium showing signs of growth) on blood (Fig. 1A) and chocolate agar. Colonies can grow on other media as well (e.g., Mueller-Hinton agar [Fig. 1B] and MacConkey agar [can show variable growth]). Thayer-Martin or Martin-Lewis medium can be used to isolate *Brucella* spp. from contaminated specimens. Generally, colonies are visible after 24 to 48 h of aerobic incubation or incubation with 5 to 10% CO_2 at 37°C, and there is no need to keep the plates more than 72 to 96 h before discarding them as negative. The colonies are 1 to 2 mm in diameter, entire, smooth, and glistening. Rough variants can occur with *B. canis* colonies. The presumptive identification of *Brucella* spp. from these colonies entails demonstrating small Gram-negative coccobacilli (0.5 to 0.7 mm in diameter and 0.6 to 1.5 mm in length) (Fig. 2). Biochemical reactions show positive oxidase, catalase, and urease tests, as well as a positive slide agglutination reaction with specific *B. abortus* and/or *B. melitensis* antisera (12, 13).

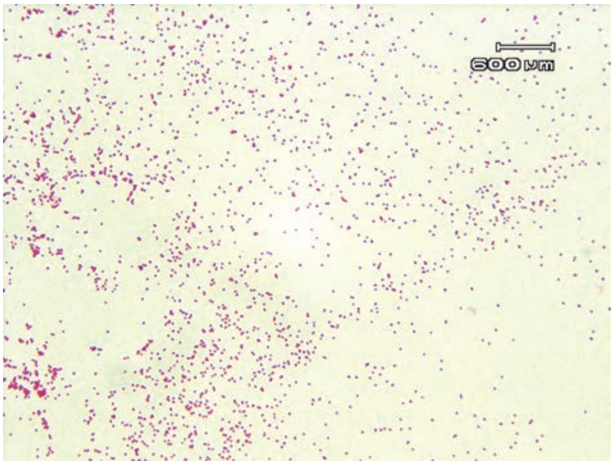

FIGURE 2 Gram stain of *B. melitensis*.
doi:1128/9781555817381.ch47.f2

Once these tests are performed and completed, the clinical laboratory may report the organism as presumptively *Brucella* spp. Further characterization and identification to the species level of the pathogen involve extensive testing not routinely performed in most clinical laboratories (13, 14). In the United States, Laboratory Response Network (LRN) reference laboratories are able to confirm and identify *Brucella* to the species level, and these laboratories can provide guidance and additional pertinent information. See also http://www.bt.cdc.gov/lrn/.

When definitive identification is indicated, conventional and molecular characterizations (e.g., based on DNA target sequencing) can be helpful. Conventional classification/identification to the species level of *Brucella* spp. can be determined from results of certain reactions, such as dye inhibition (thionine, fuchsin, safranin), CO_2 requirement,

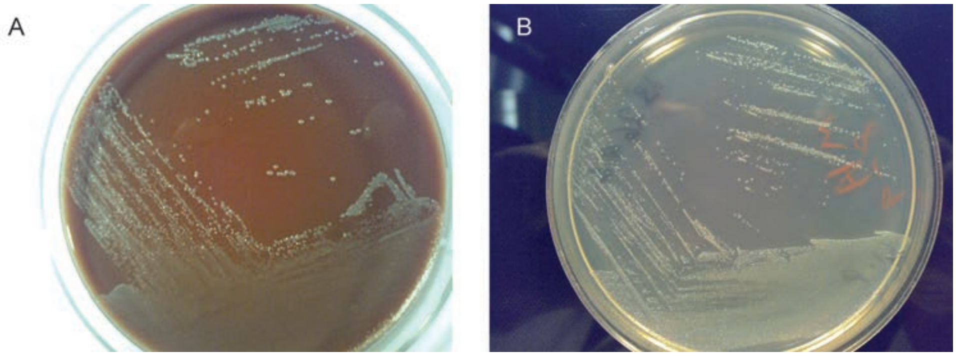

FIGURE 1 *B. melitensis* on blood agar (A) and Mueller-Hinton agar (B) after 48 h of growth.
doi:1128/9781555817381.ch47.f1

Tbilisi phage lysis, oxidative metabolic tests (glutamic acid, ornithine, ribose, and lysine), and reaction to monospecific sera. *Brucella* can be subtyped into biovars using multilocus enzyme electrophoresis, pulsed-field gel electrophoresis, randomly amplified polymorphic DNA analysis, enterobacterial repetitive intergenic consensus sequence PCR, repetitive intergenic palindromic sequence PCR, amplified fragment length polymorphism analysis, monolocus (such as *omp2a* and *omp2b*) sequence analysis, or multilocus sequence typing (11, 77, 78). A multiplex PCR microarray assay was also successfully developed to detect and differentiate *Brucella* spp. (79).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) for the rapid identification of microorganisms offers a very promising rapid approach to the identification of *Brucella* not only to the genus level but also to the species and subspecies levels, upon securing the biosecurity-relevant library in the database (80, 81). Rapid and reliable identification is important not only for clinical diagnosis but also for epidemiologic tracing of the source and in prognostic aspects; e.g., *B. melitensis* causes most severe forms of brucellosis.

TYPING SYSTEMS

One of the most highly discriminatory methodologies for epidemiological subtyping of isolates belonging to monomorphic bacterial species is multilocus variable-number tandem-repeat analysis (MLVA). In *Brucella*, MLVA schemes with 21 loci (MLVA-21) and MLVA-16, which use a combination of repeat markers distributed across the *Brucella* genome, were able to distinguish isolates of *Brucella* spp. of widespread temporal and geographical origins or of very close origins (11, 77, 78). A *Brucella* MLVA database is hosted at http://mlva.u-psud.fr/brucella/ and contains data derived from more than 500 animal and human *Brucella* isolates. Molecular subtyping methods, especially the promising MLVA, may potentially be useful not only for epidemiological trace back purposes or outbreaks but also for distinguishing relapses from reinfection, thereby influencing clinical therapeutic decisions. Other methodologies, like microarray-based assays based on single nucleotide polymorphisms (SNPs), and gene sequencing can also provide highly discriminatory results (10, 82).

SEROLOGIC TESTS

Serologic assays are the most commonly relied upon tests in the laboratory diagnosis of brucellosis (68). Serological results are optimally interpreted in the context of the evolution of antibody responses after infection with *Brucella* spp. IgM first appears, followed by the appearance of IgG within 10 to 14 days. The general evolution of these and other immunoglobulins depends on the response to treatment; in recovery, a gradual and slow decline in titers is observed, while persistent titers alert the clinician to a poor response to treatment due to focal complications, chronic infections, or relapse (83–85). Persistence, (i.e., detection of antibodies, mostly IgG and some IgM, for a very prolonged time [months and sometime years]) is observed in 15 to 20% of asymptomatic patients who have undergone treatment and cure. The explanation for this remains elusive (27).

Several antigens are used for serologic diagnostic assays, generally obtained from *B. melitensis* and *B. abortus*. Whole-cell preparations are the antigens used in the agglutination and the indirect fluorescent-antibody (IFA) tests, while sonic extracts, purified LPS, or protein extracts of *Brucella* are used mainly in ELISAs (13, 67, 68). Detection of antibodies against infections due to *B. canis* and *B. ovis* require using major outer membrane protein antigens because these strains exist in a rough colony form and do not share cross-reacting antigens with the other *Brucella* spp. (86). Since there is no standardized reference antigen, it is important to note that the source of the antigen, commercial or otherwise, can influence the test results (68).

A wide range of in-house serologic tests and formats have been used for investigating patients with brucellosis (Table 1). These include direct agglutination tests in tubes, e.g., the serum agglutination test (SAT), and on slides, e.g., the Rose Bengal test, indirect Coombs' test, Brucellacapt

TABLE 1 Tests commonly used in the laboratory diagnosis of brucellosis

Test	General comment(s)
Direct microscopy	Very low sensitivity; not useful routinely; uses differential or immunospecific stains
Culture	If positive, provides definitive diagnosis; variable yields; slow growth; blood culture instruments speed recovery; hazardous
Serology	Most commonly relied upon in diagnosis
Slide agglutination	Simple; rapid (10 min); relatively good in acute cases; high rate of false negatives in complicated and chronic cases; liable to cross-reaction (i.e., a false-positive result)
Tube (SAT) or microplate agglutination	Widely used; relatively good in acute cases; high rate of false negatives in complicated and chronic cases; liable to cross-reaction (i.e., a false-positive result); takes time to set up and 24 h to read
Indirect Coombs' test	Extension of SAT; takes an additional 24 h to read; detects nonagglutinating or incomplete antibodies; good for complicated and chronic cases; misses around 7% of cases compared to ELISA
Brucellacapt	Based on an immunocapture technique to detect, in a single step, the nonagglutinating IgG and IgA antibodies, as well as the agglutinating antibodies; performance is similar to that of Coombs' test but it is more rapid and easier to carry out
ELISA	Test of choice for complicated and chronic cases; when other tests are negative, reveals total and individual specific immunoglobulins (IgG, IgM, IgA); rapid (4–6 h); objective; highly sensitive and specific
IFA	Generally like the ELISA, but it is subjective and may fail to detect IgA; rapid (2–3 h)
Molecular PCR, reverse transcription-PCR	Promising test, theoretically very highly sensitive and specific; additional documentation is needed vs culture and serology prior to making it routine in clinical laboratories

tests, IFA, and ELISA for detection of immunoglobulin classes and subclasses (68, 87). Generally, agglutination-based tests cannot differentiate the types of antibodies involved, while the enzyme immunoassay (EIA) and IFA can. Commercial EIAs detecting *Brucella* IgG and IgM with a high degree of sensitivity and specificity have been available for a number of years and are considered an excellent method for screening sera for *Brucella* antibodies (68, 87).

Brucellacapt (Vircell, Granada, Spain) has been introduced as a rapid (18 to 24 h) and easy serologic test to carry out. The test is based on immunocapture agglutination methodology that can detect, in a single step, the nonagglutinating IgG and IgA antibodies and seems to be useful in the follow-up of disease activity. The sensitivity and specificity are similar to those of Coombs' test. However, since the latter test is cumbersome because of multiple washing steps, centrifugation, and a long turnaround time (48 h), Brucellacapt can offer a valuable alternative and can be of help in the follow-up of patients (88). Immunochromatographic lateral-flow dipstick tests have been advocated for screening/surveillance of patients with brucellosis in areas of endemicity and as outbreak and field tests. They are simple, rapid, and easy to perform and read with high (>90%) sensitivity and specificity (89).

Recently, numerous protein antigens have been evaluated in microarrays aiming to reveal differential antibody responses among sera from patients with brucellosis at different infection stages for future use as reliable antigens in serologic assays (25, 90).

Interpretation of serologic test results in relation to exposure, diagnosis, and prognosis of the disease necessitates an accurate assessment of the clinical histories and current status of patients and an understanding of the usefulness and pitfalls of the laboratory tests (27, 29, 68). Positive cutoff titers in the *Brucella* agglutination test for diagnosis have generally been considered to be ≥160 in symptomatic patients. However, much lower titers with the SAT have been reported for patients with active disease (27). Moreover, one has to be careful when negative serology is encountered when brucellosis is suspected, since this may be due, for example, to infection with *B. canis*, which can be missed by serologic assays using *B. abortus* or *B. melitensis* antigen. In addition, this may be due to very early disease presentation or due to using slide tests or SAT alone, and thus repeat testing after 1 to 2 weeks is warranted (68, 83). A combination of agglutinating (SAT) and nonagglutinating (e.g., Brucellacapt or indirect Coombs) tests are recommended to definitively exclude *Brucella* species infection.

In acute brucellosis, elevations in *Brucella*-specific IgG, IgM, IgA, IgE, IgG1, and IgG3 are shown, while in those patients with chronic brucellosis, elevations in IgG, IgA, IgE, IgG1, and IgG4 are usually seen (27, 68, 87). Monitoring the treatment response requires a sequential follow-up for patients with serologic titers. A decline indicates good prognosis, persistently high titers necessitate continuous monitoring, and a resurgence in antibody titers most likely indicates relapse or reinfection. Slide and tube agglutination (TA) titers fall faster than with Coombs' test, Brucellacapt, and EIA, as the latter titers can stay positive for years, e.g., 2 to 3 years. Persistence of residual positive titers in cured patients has doubtful meaning (68, 83, 85, 91, 92). Relapse has also been diagnosed by a detection of a resurgence in *Brucella*-specific IgG and IgA antibodies, not IgM (83–85). Markers for differentiating active from inactive disease are being sought. For example, anti-*Brucella* cytoplasmic or periplasmic protein antibodies, as determined by ELISA, increased only in patients with active brucellosis and were a better predictor of cure than antilipopolysaccharide antibodies (91, 93, 94). Also, some interleukins show a decrease posttherapy (95).

Though serologic tests are currently of high importance for the investigation of patients with brucellosis, several limitations can be encountered, mainly lack of standardized antigen preparations and assay methodologies, as well as the detection of sustained high antibody titers in some patients, despite treatment and cure (68). False-positive serologic results are rare. However, two cases were recently reported in the United States, a finding which led to initiating not only unnecessary treatment but also a wide range of public health investigations (96). False-negative results are ascribed mainly to direct agglutination, e.g., SAT in cases of focal complications or chronic brucellosis (27, 68). Although very rare, positive cultures have been described in the absence of any positive serologic test.

ANTIMICROBIAL SUSCEPTIBILITIES

Routine susceptibility testing of *Brucella* species is not indicated for many reasons. These include the rare development of antibiotic resistance against the tetracyclines, rifampin, and aminoglycosides; the lack of plasmids; concerns for laboratory safety; a poor correlation between high levels of *in vitro* activity and clinical efficacy for many agents, including β-lactams, quinolones, tigecycline, and ertapenem; and a general lack of well-established testing conditions and interpretive standards (97–101). Recently, the CLSI presented guidelines to determine the MICs of tetracycline and doxycycline against *Brucella* spp. by a Mueller-Hinton broth dilution method (98). The Etest on Mueller-Hinton agar supplemented with sheep blood or horse serum for drug synergy testing has also been described (102).

ANTI-*BRUCELLA* THERAPY

Appropriate antimicrobial therapy for treatment of human brucellosis reduces morbidity, prevents complications, and minimizes relapses. Several anti-*Brucella* agents have been used (e.g., doxycycline, rifampin, co-trimoxazole, streptomycin-gentamicin, some quinolones, and cephalosporins) with various rates of success. Currently, the most effective treatment regimen and the optimal duration of treatment remain unclear (103, 104).

Fewer relapses with combined regimens than with monotherapy have been reported. For adults with uncomplicated infection, the WHO recommends oral doxycycline and rifampin for 6 to 8 weeks, but a regimen of doxycycline and streptomycin or gentamicin showed higher cure rates. Recommendations for children older than 8 years include doxycycline (45 days or 8 weeks) and gentamicin (5 to 7 days) or streptomycin (14 days), doxycycline plus rifampin, or co-trimoxazole (6 weeks). For children younger than 8 years, gentamicin (5 to 7 days) plus co-trimoxazole (6 weeks) or co-trimoxazole plus rifampin (6 weeks) are recommended (33, 49). Triple regimens using doxycycline, rifampin, aminoglycoside, or ceftriaxone for 2 to 3 months are recommended for patients with endocarditis and neurobrucellosis (56, 97, 105, 106).

Treatment regimens with fluoroquinolones and broad-spectrum cephalosporins have been reported. Although these agents have good MICs *in vitro* against *Brucella* spp., patients treated with these regimens have higher rates of relapses than patients on the standard regimen. The use of fluoroquinolones in combination with rifampin for the treatment of bacteremia and complicated brucellosis has

TABLE 2 Interpretation of commonly used serologic tests based on the immunoglobulin type detected and usefulness in the diagnosis of different disease categories/stages of human brucellosis

Test	Detection of indicated Ig			Usefulness of diagnosis in indicated category of disease			
	IgG	IgM	IgA	Acute	Chronic	Focal	Relapse
Rose Bengal/slide test	+	+	+	+	−	−	−
Tube (SAT) or microtiter plate agglutination	+	+	+	+	−	−	−
Indirect Coombs' test	+	−	−	+	+	+	+
Brucellacapt	+	−	+	+	+	+	+
EIA	+	+	+	+	+	+	+

yielded varied results (104, 106). A systematic review and meta-analysis study covering 30 trials and 77 treatment arms showed that among patients with bacteremia and complicated brucellosis, higher failure and relapse rates and shorter treatment durations (less than 6 weeks) were observed with monotherapy than with multidrug therapy. The preferred treatment should be with dual or triple regimens, including an aminoglycoside (104). A recent study showed that treatment delay beyond 3 months of disease onset resulted in a significantly lower cure rate (107).

The question about postexposure prophylaxis (doxycycline and rifampin therapy for 3 to 6 weeks) after a high-risk exposure in the lab remains debatable. Guidelines for postexposure management are empirical (17, 108). Upon possible exposure, however, recommendations were made to take a baseline blood sample, monitor for symptoms weekly for 6 months, and perform serological surveillance at 0, 2, 4, 6, and 24 weeks (17).

PREVENTION

Vaccines have been successful in the control of livestock infections, which can subsequently reduce infections in humans. Most veterinary vaccines focus on live, attenuated *B. abortus* (strain S19) and a more stable rough mutant of *B. abortus* (strain RB51) for cows, *B. melitensis* (strain Rev-1) for sheep and goats, and *B. suis* 2 for swine. However, the *Brucella* vaccine developed for humans still suffers from limited efficacy and serious medical reactions (109, 110). Heating of dairy products and related foods has also been effective in preventing disease transmission. The most cost-effective approach to control and prevent brucellosis relies on raising public awareness about the disease and greater cooperation between human and animal health sectors (111).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Brucella is a "stealth" and highly infectious pathogen that can result in a wide range of disease spectra, including focal or complicated presentations. Recognition of *Brucella* as a plausible diagnosis can expedite appropriate laboratory testing and prompt treatment to avoid serious complications. With proper interpretation and appropriate use of tests, proper clinical decisions can be reached, and serology is the most relied upon for accurate diagnosis. Laboratories should use a combination of two agglutination tests, namely, the SAT and indirect Coombs' test, the SAT and Brucellacapt, or ELISAs for IgG and IgM. In doing so, one would be able to detect antibodies in different stages of the disease, since in the acute stage, any test can be positive, while in chronic, complicated, or focal disease cases, the SAT can be negative while Coombs' test, Brucellacapt, and ELISA using IgG can be positive (Table 2). Again, one should keep in mind that any serologic test findings need to be interpreted in the context of the patient's clinical history (29, 68). Culture is also mandatory in suspected cases, and when positive, it confirms the diagnosis and offers an opportunity for identifying the organism to the species level to help in epidemiologic tracing and for antimicrobial susceptibility testing. In the United States, laboratories that recover a potential *Brucella* sp. should promptly notify public health authorities and refer the isolate to the nearest LRN reference laboratory for confirmation. Though not yet standardized and still largely research tools, molecular methods may be used in laboratories where optimized assays are available.

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Bartonella[*]

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TAXONOMY

Taxonomy and Description of Genus

Bartonella (including some species formerly known as *Rochalimaea* and *Grahamella*) is a genus of short, facultative, intracellular, pleomorphic, Gram-negative, coccobacillary or bacillary rods that measure 0.2 to 0.6 μm by 0.5 to 1.0 μm (Fig. 1). Members of this genus can induce persistent bacteremia in healthy people and animals and vascular proliferative infections in immunocompromised people. *Bartonella bacilliformis* is the type species and was formerly classified in the family *Bartonellaceae* in the order *Rickettsiales*. The current taxonomic classification incorporates former members of the *Rochalimaea* (*R. quintana*, *R. vinsonii*, and *R. henselae*) and *Grahamella* genera into the *Bartonella* genus, which is now separate from the order *Rickettsiales*, as members of the alpha-2 subgroup of the class alphaproteobacteria within the *Rhizobiales* order. To honor Henrique da Rocha-Lima, who was no longer recognized after the merge of the genera *Rochalimaea* into *Bartonella*, a new species isolated from a patient with bacteremia was named "*Bartonella rochalimae*" (1). There are now 32 recognized species or subspecies of *Bartonella* (http://www.bacterio.net/bartonella.html), many of which have been associated with human disease (2). Because of the slow growth of these bacteria on agar plates (dividing time, approximately 24 h), standard biochemical methods for identification are not useful for clinical diagnosis of infection. *Bartonella* are oxidase and catalase negative and do not produce acid from carbohydrates. All *Bartonella* species are highly hemin dependent. For primary agar culture of enriched blood-containing media, all members of the genus typically require 8 to 45 days in the presence of 5% CO_2 to form visible colonies (Fig. 1). Optimal growth temperatures for various *Bartonella* species vary from 25 to 30°C for *B. bacilliformis* to 35 to 37°C for *B. henselae*, *B. quintana*, *B. koehlerae*, or *B. elizabethae*. On primary isolation, some *Bartonella* species, such as *B. henselae*, *B. clarridgeiae*, *B. vinsonii*, and *B. elizabethae*, form colonies with a white, rough, dry, raised appearance that create a depression in the agar medium. Colonies are therefore hard to extract or transfer to subsequent plates. Other *Bartonella* species, such as *B. quintana*, form colonies that are usually smaller, gray, translucent, and sometimes viscous or mucoid. A few members of the genus, including *B. bacilliformis*, *B. clarridgeiae*, *B. capreoli*, *B. rochalimae*, and *B. schoenbuchensis*, are motile by means of unipolar flagella.

B. bacilliformis, the type species of the genus, is the etiologic agent of Carrion's disease, which is characterized by either an acute hemolytic bacteremia, known as Oroya fever, or a chronic vasoproliferative form with cutaneous nodular vascular eruptions known as verruga peruana. *Bartonella quintana*, the agent of trench fever, causes a febrile bacteremic illness with symptoms that include myalgia, chills, headache, and severe leg pain. *B. quintana* is also a cause of the disease bacillary angiomatosis (BA), which is characterized by vascular proliferative lesions in immunocompromised individuals, particularly those with AIDS (3). *B. quintana* is a common cause of culture-negative endocarditis and bacteremia in homeless individuals (4). BA can also be caused by *B. henselae*. Although *B. henselae* is historically associated with cat scratch disease (CSD), a self-limiting infection in immunocompetent individuals, CSD can be complicated by neuroretinitis, granulomatous hepatitis, and fever of unknown origin. *B. henselae* is also associated with endocarditis in humans (although much less frequently than *B. quintana*) and in dogs. A fourth human pathogen, *B. elizabethae*, caused a single case of endocarditis in an immunocompetent individual (5). Since the description of infections associated with these 4 initial *Bartonella* species, *Bartonella vinsonii* subsp. *arupensis*, *B. koehlerae*, and *Bartonella alsatica* have been associated with a small number of endocarditis infections in humans (6), and "*Bartonella washoensis*" was described as the cause of human myocarditis in a single patient (7). *Bartonella grahamii* has been associated with a case of human neuroretinitis and bilateral retinal artery branch occlusions (8). *B. vinsonii* subsp. *arupensis* has similarly been isolated from a bacteremic rancher who had fever and mild neurological manifestations (9). More-recent and less well-studied *Bartonella* species isolated from or detected in humans include *B. rochalimae*, "*B. tamiae*," *B. tribocorum*, and "*B. melophagi*" (1, 2, 10, 11).

EPIDEMIOLOGY AND TRANSMISSION

Bartonella species are transmitted by insect vectors, such as fleas, sand flies, body lice, and, potentially, ticks, biting flies, and wingless flies. Transmission also occurs by animal scratches or possibly bites. Some species are geographically very limited; an example is *B. bacilliformis*, which lives at specific altitudes in the Andes Mountains of South America

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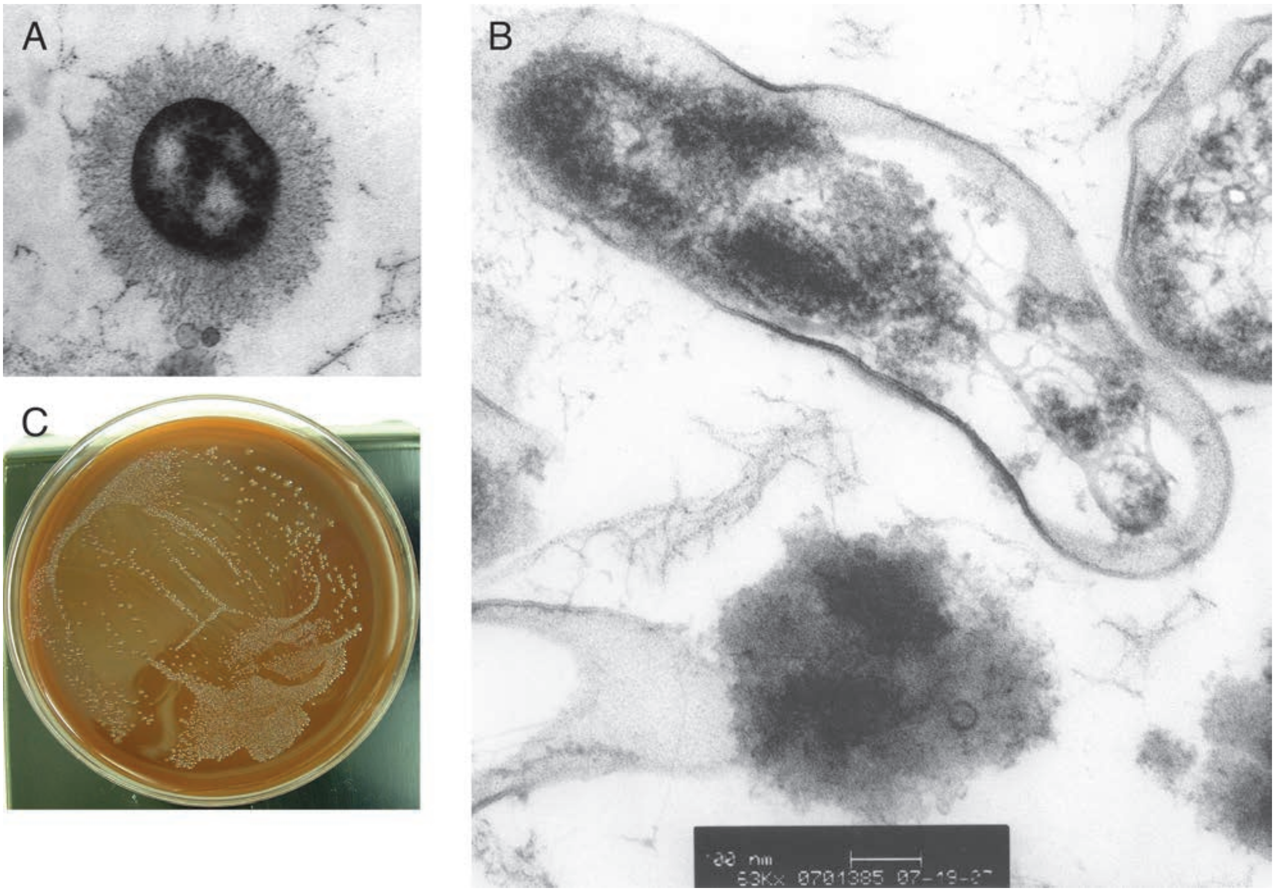

FIGURE 1 (A and B) Electron micrographs of *B. henselae* Marseille (A) and *B. vinsonii* subsp. *berkhoffii* genotype III (B); (C) *B. henselae* strain Houston I isolated on a blood agar plate after 10 days in culture at 36°C and 5% CO_2.

due to the limited distribution of its sand fly vector, *Lutzomyia verrucarum*. Others, such as *B. quintana*, *B. henselae*, *B. elizabethae*, and *B. vinsonii* subsp. *berkhoffii*, appear to have a worldwide distribution (2). *B. quintana* outbreaks are associated with populations of homeless individuals living under conditions of poor sanitation and personal hygiene. Infestation by the human body louse, *Pediculus humanus*, results in inoculation of *B. quintana* in arthropod excreta through broken skin (12, 13).

Cats are the main reservoir for *B. henselae*, *B. clarridgeiae*, and *B. koehlerae*. CSD, caused by *B. henselae* infection, is transmitted from cat to cat mainly by the cat flea, *Ctenocephalides felis*; however, direct cat flea transmission as a cause of human CSD has not been confirmed. More likely, human infection results from the inoculation of infective flea feces at the time of the scratch (6). Stray cats, cats living outdoors, and young cats are more likely to be bacteremic. The prevalence of *B. henselae* infection is usually highest in warm and humid climates where cat fleas are abundant. *B. henselae* antibody prevalence in domestic cat populations can range from 14 to 81% (14–16) Genotype I (Houston) is more common in cats in the Far East (Japan, the Philippines), whereas type II (Marseille, France) is predominant in Western Europe, North America, and Australia (6). Coinfection of cats with different *Bartonella* species or genotypes is reported, as is coinfection in humans and dogs (17–19). Additionally, *B. quintana*, *B. koehlerae*, and *B. clarridgeiae* DNA can be detected in cat fleas, suggesting their possible role as vectors for these organisms. *Bartonella* species DNA has been amplified from species of *Ixodes* ticks from numerous sites throughout the world, but vector competency for transmission of *Bartonella* from ticks to humans has never been confirmed (20, 21).

Twelve *Bartonella* species and subspecies, including *B. bacilliformis*, *B. tamiae*, *B. grahamii*, *B. koehlerae*, *B. alsatica*, *B. quintana*, *B. elizabethae*, *B. henselae*, *B. vinsonii* subspecies *arupensis* and *berkhoffii*, "*B. washoensis*," and *B. rochalimae* (22), can infect humans and can elicit a wide spectrum of clinical manifestations, including cutaneous vasculitis, endocarditis, myocarditis, bacillary angiomatosis, peliosis hepatis, and granulomatous inflammatory disease (Table 1). Other *Bartonella* species, such as *B. vinsonii* subsp. *vinsonii*, *B. doshiae*, *B. taylorii*, *B. peromysci*, *B. birtlesii*, *B. tribocorum*, *B. talpae*, *B. bovis*, *B. chomelii*, *B. schoenbuchensis*, and *B. capreoli*, have been isolated only from blood from animals, including wild rodents, squirrels, felids, canids, and ruminants, such as cattle, deer, and elk (2, 23, 24). Domestic and wild canids represent the main reservoir of *B. vinsonii* subsp. *berkhoffii*, with high antibody prevalence in dogs from tropical countries (6) and high prevalence of bacteremia in coyotes (*Canis latrans*) in California (25).

In addition, a wide range of rodent hosts worldwide are infected with rodent-adapted *Bartonella* species (2). Finally, several *Bartonella* species can be isolated from domestic and wild ruminants, for which biting flies may be important vectors for transmission among these animal species (2).

TABLE 1 *Bartonella* species or subspecies presently described, their main reservoirs, confirmed or possible vector, and reported accidental hosts

Bartonella sp.	Nomenclatural status	Main reservoir	Vector or potential vector	Accidental host(s)
B. acomydis sp. nov.	Approved	Rodentia	?	?
B. alsatica	Approved	Rabbits (*Oryctolagus cuniculus*)	Fleas?, ticks?	Humans
B. bacilliformis	Approved	Humans	Sand flies	?
B. birtlesii	Approved	Wood mice (*Apodemus* species)	Fleas?	?
B. bovis ("*B. weissii*")	Approved	Domestic cattle (*Bos taurus*)	Biting flies?, ticks?	Cats, dogs
B. callosciuri sp. nov.	Approved	Rodentia	?	?
B. capreoli	Approved	Roe deer (*Capreolus capreolus*)	Biting flies?, ticks?	?
B. chomelii	Approved	French domestic cattle (*Bos taurus*)	Biting flies?, ticks?	?
B. clarridgeiae	Approved	Cats (*Felis catus*)	Fleas?, ticks?	Humans?, dogs
B. coopersplainsensis	Approved	Australian rats	?	?
B. doshiae	Approved	Meadow voles (*Microtus agrestis*), Rats (*Rattus* species)	Fleas?	?
B. elizabethae	Approved	Rats (*Rattus norvegicus*), gerbils (*Meriones crassus*)?	Fleas	Humans, dogs
B. grahamii	Approved	Voles (*Clethrionomys* species), mice (*Apodemus* species)	Fleas?	Humans
B. henselae	Approved	Cats (*Felis catus*)	Fleas, ticks?	Humans, dogs
B. jaculi sp. nov.	Approved	Rodentia	?	?
B. japonica	Approved	Field mice (*Apodemus argenteus*)	?	?
B. koehlerae	Approved	Cats (*Felis catus*)	Fleas?	Humans
B. melophagi	Without standing[a]	Sheep (*Ovis* species)	Keds (*Melophagus ovinus*)	Humans
B. pachyuromydis sp. nov.	Approved	Rodentia	?	?
B. peromysci	Approved	Field mice (*Peromyscus* species)	Fleas?	?
B. queenslandensis	Approved	Australian rats	?	?
B. quintana	Approved	Humans	Human body lice	Cats, dogs
B. rattaustraliani	Approved	Australian rats	?	?
"*B. rattimassiliensis*"	Without standing	Rats (*Rattus* species)	Fleas?	?
B. rochalimae	Approved	Gray foxes (*Urocyon cinereoargenteus*), dogs (*Canis familiaris*)	Fleas?	Humans
B. schoenbuchensis	Approved	Roe deer (*Capreolus capreolus*)	Deer keds?, biting flies?, ticks?	?
B. silvatica	Approved	Japanese field mouse (*Apodemus speciosus*)	?	?
B. talpae	Approved	Moles (*Talpa europaea*)	Fleas?	
"*B. tamiae*"	Without standing	Rat (*Rattus* species)	Fleas?	Humans
B. taylorii	Approved	Mice (*Apodemus* species), voles (*Clethrionomys* species)	Fleas?	?
B. tribocorum	Approved	Rats (*Rattus* species), mice (*Apodemus* species)	Fleas?	?
B. vinsonii subsp. *arupensis*	Approved	White-footed mice (*Peromyscus leucopus*)	Fleas?, ticks?	Humans
B. vinsonii subsp. *berkhoffii*	Approved	Coyotes (*Canis latrans*), dogs (*Canis familiaris*), foxes (*Urocyon* species)	Ticks?	Humans
B. vinsonii subsp. *vinsonii*	Approved	Meadow voles (*Microtus pennsylvanicus*)	Ear mites? (*Trombicula microti*)	?
"*B. volans*"	Without standing	Southern flying squirrels (*Glaucomys volans*)	Fleas?	?
"*B. washoensis*"	Without standing	California ground squirrel (*Spermophilus beecheyi*), rabbits (*Oryctolagus cuniculus*)	Fleas?, ticks?	Humans, dogs

[a] Approved as *Wolbachia melophagi*.

CLINICAL SIGNIFICANCE

Human Pathogens

Oroya Fever and Verruga Peruana (Carrion's Disease): *B. bacilliformis*

The disease caused by *B. bacilliformis*, especially its chronic form known as verruga peruana, has been recognized since pre-Columbian times in populations inhabiting the Andes Mountains. However, the suspected link between the acute form (Oroya fever) and the chronic form was finally confirmed in 1885, when Daniel Carrion, a medical student, died of Oroya fever after inoculating himself with material from a verruga (wart-like lesion). The acute form of disease, usually seen in nonnative populations in the zone of endemicity, is a progressive, severe, febrile anemia with intravascular hemolysis associated with *B. bacilliformis*-infected erythrocytes. Mortality was reported to range from 40 to 90% of cases prior to the discovery and use of antibiotics (26). Chronic bacteremia occurs in people from regions of endemicity, although evidence for persistent infection is reported from zones where the disease is not endemic (26, 27). The second stage of infection occurs weeks to months following acute infection and is characterized by cutaneous nodular angioproliferative lesions known as verruga peruana; mucosal and internal lesions are also known to occur (28). These lesions can persist for several months, though prognosis for full recovery is favorable at this stage. A newly emerging pathogen, "*Candidatus* Bartonella ancashi," is now also considered an additional etiologic agent of verruga peruana in the Ancash region of Peru (29).

Trench Fever, Bacillary Angiomatosis, Endocarditis, and Prolonged Fever: *B. quintana*

Trench or quintan fever is a recurrent fever characterized by 3 to 5 (or more) febrile episodes lasting 4 to 5 days, with fever occurring after an incubation period of 15 to 25 days. Severe headaches and shin pain, in addition to malaise, anorexia, abdominal pain, restlessness, and insomnia, are common symptoms. Mild forms and asymptomatic carriage are also reported (4, 30). *B. quintana* endocarditis is one of the most common causes of culture-negative endocarditis (31–33). In HIV-infected individuals, bacteremia caused by *B. quintana* develops insidiously and is characterized by recurrent fever, headaches, and myalgias. *B. quintana* and *B. henselae* are the two *Bartonella* species that cause bacillary angiomatosis (BA). BA, also called epithelioid angiomatosis, is a vasculoproliferative disease of the skin characterized by violaceous, pink, or colorless papular to nodular skin lesions that can clinically suggest Kaposi's sarcoma (KS) but histologically appear quite distinct from the lesions of KS. Fever, weight loss, and malaise can develop in people with disseminated BA. Subcutaneous nodules and lytic bone lesions are also associated with *B. quintana* infection but not with BA caused by *B. henselae* (34).

Zoonotic Bartonellae

Cat Scratch Disease

B. henselae is the sole or predominant etiologic agent of cat scratch disease (CSD) in humans (35, 36), whereas *B. clarridgeiae* (37, 38) and *B. grahamii*, based on multilocus sequence typing (39), are suspected in a few cases as well. There are now strong arguments against *Afipia felis* being an etiological agent of CSD (31). It is estimated that at least 22,000 human cases of CSD occur yearly in the United States. Between 55% and 80% of CSD patients are <20 years old. There is a seasonal pattern, with most cases occurring in autumn and winter (40).

In classical CSD, 1 to 3 weeks elapse between the cat scratch or bite and onset of clinical signs. In 50% of cases, a small skin lesion, often resembling an insect bite, appears at the inoculation site, usually the hand or forearm. The lesion evolves from a papule to a vesicle and in some instances becomes a partially healed ulcer. These lesions generally resolve within a few days to a few weeks. Lymphadenitis develops approximately 3 weeks after exposure and is generally unilateral. Epitrochlear, axillary, or cervical lymph nodes are most frequently involved. Lymph nodes are usually swollen and painful, and lymphadenopathy persists for several weeks to several months. In 25% of CSD cases, suppuration occurs. The large majority of cases present with signs of systemic infection: fever, chills, malaise, anorexia, and headache. In general, the disease is benign, and lymphadenopathy resolves spontaneously without sequelae or antibiotic treatment. Atypical manifestations of CSD occur in 5% to 10% of the cases. The most common of these is Parinaud's oculoglandular syndrome (periauricular lymphadenopathy and palpebral conjunctivitis); however, retinitis, meningitis, encephalitis, osteolytic lesions, and thrombocytopenic purpura can occur (41, 42). Encephalopathy is one of the most serious complications of CSD; it usually occurs 2 to 6 weeks after the onset of lymphadenopathy but typically resolves in a complete recovery and few or no sequelae (43).

B. henselae is also a frequent cause of prolonged fever and fever of unknown origin in children. Rheumatic manifestations of *B. henselae* infection in children, including cases of myositis and arthritis with skin nodules, have been described. However, arthritis is described in a very limited number of cases. Other rheumatic manifestations include erythema nodosum, leukocytoclastic vasculitis, and fever of unknown origin with myalgia, osteolytic lesions, and arthralgia (6).

Fever, weight loss, malaise, and enlargement of internal organs can develop in people with disseminated BA due to *B. henselae*. When visceral parenchymal organs are involved with *B. henselae*-induced vascular proliferation, the condition is referred to as bacillary peliosis hepatis (liver) or splenic peliosis. These lesions are comprised of multiple, blood-filled, partially endothelial-cell-lined cystic structures in the liver or spleen (44). Bacillary peliosis hepatis does not occur with *B. quintana* infection. BA patients with *B. henselae* infection are epidemiologically linked to cat and cat flea exposure (34). *B. henselae* and *B. quintana* are implicated rarely in cases of HIV-associated brain lesions, meningoencephalitis, encephalopathy, dementia, or neuropsychological decline (45). *B. henselae* infection is also described in adult and pediatric solid organ transplant patients (46, 47).

Zoonotic *Bartonella* Species Associated with Endocarditis, Myocarditis, Neuroretinitis, Fever, and Neurological Symptoms

Several zoonotic *Bartonella* species are recognized as causative agents of blood culture-negative endocarditis or myocarditis in humans, including *B. henselae* (48), *B. koehlerae*, *B. elizabethae*, *B. alsatica* (48), *B. vinsonii* subsp. *berkhoffii* (48), *B. vinsonii* subsp. *arupensis* (49), and *B. washoensis* (6). *Bartonella* species account for 3 to 4% of all human cases of endocarditis in France, a percentage similar to that of endocarditis cases caused by *Coxiella burnetii*, the agent of Q fever (48). Some rodent-borne *Bartonella* species are also associated with cases of neuroretinitis (*B. elizabethae*, *B. grahamii*) or fever with bacteremia and neurological symptoms (*B. vinsonii* subsp. *arupensis*) (9).

Other *Bartonella* Species or Subspecies

The clinical impact on animals or humans of many *Bartonella* species is still unknown. *Bartonella* species are routinely detected in asymptomatic, bacteremic human and animal patients, many of which are detected via blood donor programs (50, 51). Although "*Candidatus* B. melophagi" was isolated from the blood of two women (11) and *B. bovis* DNA was amplified from heart valves of cows with endocarditis, no other specific pathology has been associated with *Bartonella* species infecting domestic and wild ruminants or for many of the rodent-borne *Bartonella* species.

Canine and Feline *Bartonella* Species

Several of the 32 identified *Bartonella* species or subspecies have been detected in or isolated from pet dogs and cats, thereby highlighting the zoonotic potential of these bacteria for persons with extensive domestic animal contact (19, 23, 52–59). *B. henselae*, *B. clarridgeiae*, *B. koehlerae*, *B. quintana*, *B. bovis*, *B. elizabethae*, and *B. vinsonii* subsp. *berkhoffii* are pathogenic for dogs (60), and *B. henselae*, *B. clarridgeiae*, *B. washoensis*, *B. quintana*, *B. rochalimae*, *B. elizabethae*, and *B. vinsonii* subsp. *berkhoffii* are also known to infect felines (61, 62). A number of manifestations, including endocarditis (63), myocarditis, epistaxis, proliferative vascular disease, and lethargy, can develop in dogs with *Bartonella* infection (60, 64–66). *Bartonella*-infected cats are more likely to have kidney disease and urinary tract infections, stomatitis, and lymphadenopathy than noninfected cats (23), but most cats infected with *B. henselae* appear to be asymptomatic. In experimentally infected cats, fever, lymphadenopathy, mild neurologic signs, and reproductive disorders have been reported (23). The clinical spectra of *Bartonella* infection in dogs and cats can be highly varied, ranging from no apparent abnormalities to subclinical infections accompanied by lethargy and weight loss.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Most specimens used for *Bartonella* isolation are either blood or tissue. *Bartonella* species are easier to isolate from blood than tissue. For patients with *B. henselae* infection, this species is rarely isolated from immunocompetent individuals unless they have endocarditis. CSD patients rarely have bacteremia or cultivable bacilli in CSD lymph nodes. Blood samples can be collected either in Isolator blood lysis tubes (Wampole, Cranbury, NJ), sodium citrate tubes, or plastic EDTA tubes. If storage of specimens prior to culture is necessary, samples should be kept frozen (at least –20°C). Blood collected from *B. henselae*-infected cats into EDTA and Isolator blood lysis tubes yields good recovery and no loss of sensitivity for EDTA tubes kept at –65°C for 26 days (15). Specimens should be collected prior to antimicrobial therapy.

Tissue from enlarged lymph nodes, cutaneous lesions, or various organs can be cultured after homogenization or processed for DNA extraction and PCR. Fresh tissues are preferred for PCR amplification, but paraffin-embedded tissue can be used (67). Fine-needle aspiration has also been successful for detection of *Bartonella* and is less invasive than biopsy (68). *Bartonella* species have successfully been cultured from aqueous humor (69, 70).

DIRECT EXAMINATION

Microscopy

With the exception of *B. bacilliformis*, *Bartonella* species cannot be visualized in erythrocytes on stained blood smears from animal or human patients (18). In rats experimentally infected with *B. birtlesii*, there can be up to 8 bacteria per erythrocyte (71). Warthin-Starry silver stain is recommended for microscopic detection of *Bartonella* organisms in fixed tissue sections but is not highly specific and is relatively insensitive, even when lymph node biopsy specimens from CSD patients are examined. In patients with BA, a larger number of bacilli are usually identifiable by Warthin-Starry silver staining of tissues.

Antigen Detection

Immunocytochemical staining is a specific technique but is not widely available (3). Direct immunofluorescence of blood smears allowed rapid diagnosis of *B. quintana* in a patient with acute trench fever (52) and in bacteremic homeless patients (31).

Nucleic Acid Detection

Although PCR and agar plate culture are useful tests to document infection with *Bartonella* species in cats, these traditional techniques are not generally sensitive enough to detect active infection with a *Bartonella* species in human or dog blood samples. Therefore, PCR amplification directly from blood or other diagnostic samples is of limited value in patients with low levels of bacteremia.

Advances in PCR methodologies and equipment have facilitated an impressive increase in the sensitivity of molecular detection of *Bartonella* DNA in patient samples. The 16S rRNA gene (rrs) was first used by Relman et al. in 1990 for the identification of the microbial etiology of bacillary angiomatosis (72). However, this gene does not discriminate among all *Bartonella* species. The most widely targeted genes are those coding for citrate synthase (*gltA*), heat shock protein (*groEL*), riboflavin synthase (*ribC*), a cell division protein (*ftsZ*), and the 17-kDa antigen (73) (Table 2). Sequences of *gltA* and *rpoB* (RNA polymerase beta-subunit) are congruent with DNA-DNA hybridization for *Bartonella* speciation (73). The intergenic spacer (ITS) region located between the 16S and 23S rRNA genes is a useful diagnostic target for *Bartonella* detection, speciation, and genotyping (17, 19, 54, 74–78). Inclusion of positive, negative, and processing controls is critical, and confirmatory tests (e.g., PCR followed by sequencing or the use of a second target gene PCR assay) must be performed to avoid potential nonspecific amplification and false-positive results (77).

ISOLATION PROCEDURES

Blood

In immunocompromised patients, including transplant recipients, the level of bacteremia is often higher than in immunocompetent individuals, except in the presence of endocarditis, when blood cultures can be equally sensitive for isolating *Bartonella* species from immunocompetent and immunocompromised patients (3). However, after inoculation of *Bartonella*-infected blood into the broth of CO_2 detection-based blood culture systems, *Bartonella* species do not usually produce turbidity or convert enough oxidizable substrate to CO_2. Thus, these routinely used, automated blood culture systems rarely detect *Bartonella* species from

TABLE 2 List of sequences for PCR primers or probes used to amplify or identify *Bartonella* spp.[a]

Bartonella species	Gene	Direction of primer	Primer	Sequence	Reference(s)
All (except *B. bacilliformis*)	ITS	F	16SF	AGAGGCAGGCAACCACGGTA	132, 133
		R	23S1	GCCAAGGCATCCACC	132, 133
All *Bartonella* spp.	35-kDa antigen	F	35KD1fa	GTCGCTAAAGGCTGATGA	134
		R	35KD2ra	GACTGATATCGTGCGTGTG	134
		F	35KDs1f	GGTACGACGACAGTAATTGTT	134
		R	35KDs2r	GATTTAAGAGATACCAACCA	134
	ftsZ	F	FTS2p	CAGCCTCTTCACGATGTG	135
		F	BaftsZF	GCTAATCGTATTCGCGAAGAA	135
		R	BaftsZR	GCTGGTATTTCCAAYTGATCT	135
		F	FTS1p	GCCTTCTCATCCTCAACTT	135
	gltA	F	BhCS.781p	GGGGACCAGCTCATGGTGG	136
		R	BhCS.1137n	AATGCAAAAAGAACAGTAAACA	136
		F	CS.877p	GGGGGCCTGCTCACCGCGG	137
		R	CS.1258n	ATTGCAAAAAGTACAGTGAACA	137
		F	1240F	GATYCTTTCCGCCTTATG	138
		R	1497R	GAAATCCTAGAGCTTTTAATG	138
	groEL	F	HSPps4	GCTGGNGGTGTTGCNGTTA	139
		F	HSPps3	GCTGTNGAAGANGGNATTGT	139
		F	BbHs233.p	CGTGAAGTTGCCTCAAAAACC	139
		R	BbHs1630.n	AATCCATTCCGCCCATTC	139
		F	HSPps1	CAGAAGTTGAAGTGAAAGAAAA	139
		R	HSPps2	GCNGCTTCTTCACCNGCATT	139
	ITS	F	321s	AGATGATGATCCCAAGCCTTCTGG	15, 54, 71, 78
		R	983as	TGTTCTYACAACAATGATGAT	15, 54, 71, 78
		F	QHVE1	TTCAGATGATGATCCCAA	132, 133
		R	QHVE2	TTGGGATCATCATCTGAA	132, 133
		F	QHVE3	GATATATTCAGACATGTT	132, 133
		R	QHVE4	AACATGTCTGAATATATC	132, 133
		F	16s1	C/TCTTCGTTTCTCTTTCTTCA	76
		R	16s2	GGATAAACCGGAAAACCTTC	76
		F	16s3	C/TCTTCGTTTCTCTTTCTTCA	76
		R	16s4	AACCAACTGAGCTACAAGCC	76
		F	16s5	CTCTTTCTTCAGATGATGATCC	76
		R	16s6	AACCAACTGAGCTACAAGCCCT	76
	pap	F	PAP1fa	CTTTAATGACGACTTCTGTT	134
		R	PAP4ra	CCGAAATCTGAGTAACGGTA	134
		R	PAP2r	CCCTAAATGTTTCAAGTTCA	134
		F	PAP3f	GCTGACAGAGAAGACGCAA	134
	pap31	F	PAPn1	TTCTAGGAGTTGAAACCGAT	140
		R	PAPn2	GAAACACCACCAGCAACATA	140
		R	PAPns2	GCACCAGACCATTTTTCCTT	140
		F	PAPns1	CAGAGAAGACGCAAAAACCT	140
		F	Pap31 1s	GACTTCTGTTATCGCTTTGATTT	78
		R	Pap31 688as	CACCACCAGCAAMATAAGGCA	78
	ribC	F	PBH3	CCAAGTGCTACATAACCATC	141
		R	PBH4	CGGGTTGTTATTGCTCTTAC	141
	rpoB	F	1400F	CGCATTGGCTTACTTCGTATG	142
		R	2300R	GTAGACTGAATTAGAACGCTG	142
		F	QVE1	TTCAGATGATGATCCCAAGC	142
		R	QVE3	AACATGTCTGAATATATCTTC	142
	rrs	S	probe	ATTTGGTTGGGCACTCTAGGGG	8
		R	Rp2	ACGGCTACCTTGTTAGGACTT	134
		F	357f	TACGGGAGGCAGCAG	134
		R	357ra	CTGCTGCCTCCCGTA	134
		F	536F	CAGCAGCCGCGGTAATAC	134
		R	536R	GTATTACCGCGGCTGCTG	134

(Continued on next page)

TABLE 2 *(Continued)*

Bartonella species	Gene	Direction of primer	Primer	Sequence	Reference(s)
		F	800F	ATTAGATACCCTGGTAG	134
		R	800R	CTACCAGGGTATCTAAT	134
		F	1050F	TGTCGTCAGCTCGTG	134
		R	1050R	CACGAGCTGACGACA	134
		F	Bart	CACTCTTTTAGAGTGAGCGGCAA	143
		R	rBh	CCCCCTAGAGTGCCCAACCA	143
B. bacilliformis	*ftsZ*	F	Bb ftsZseqF	GCGCATGTTCTTAGTGAAAT	135
		R	Bb ftsZseqR	CCTGTATACGTGATGCATTT	135
	ITS	F	BABF	CTGGATCACCTCCTTTCTAA	132, 133
		R	BABR	ATGCCCTTAAGACACTTGAT	132, 133
	ribC	F	PBH-L1	GATATCGGTTGTGTTGAAGA	141
		R	PBB-R1	AAAGGCGCTAACTGTTC	141
B. clarridgeiae	*ribC*	F	PBC5	TACATAACGAGCCAATT	141
		R	PBC15	TAGCTTTAGAACAATATGGT	141
B. henselae	*ribC*	F	PBH-L1	GATATCGGTTGTGTTGAAGA	141
		R	PBH-R1	AATAAAAGGTATAAAACGCT	141
B. henselae, *B. clarridgeiae*	*ftsZ*	R	Bh ftsZ 1393.n	GCGAACTACGGCTTACTTGC	135
		F	Bh ftsZ 1247.p	CGGTTGGAGAGCAGTTTCGTC	135
B. quintana	*ftsZ*	F	Bq ftsZseqF	GCACATATTCTTGATGAGAT	135
		R	Bq ftsZseqR	CCCCTATCATCTCATCAAG	135
	ITS	F	BQF	CTCCACCATTTTAGGTCATC	132, 133
		R	BQR	GGTTTTGAGAATTCCCTTGC	132, 133
	ribC	F	PBH-L1	GATATCGGTTGTGTTGAAGA	141
		R	PBQ-R1	AAAGGGCGTGAATTTTG	141

[a] Abbreviations: F, forward primer; R, reverse primer; S, sense probe; ITS, 16S rRNA-23S rRNA gene intergenic spacer region (*rrs-rrl* intergenic spacer); *rrs*, 16S rRNA gene.

patients' blood. Instead, *Bartonella* species can be recovered from the blood of bacteremic patients if specific blood collection tubes are used in conjunction with centrifugation and plating directly onto special agar. *Bartonella* species can be isolated directly from blood using the lysis centrifugation system (Isolator) or from tubes containing EDTA anticoagulant, followed by plating onto fresh chocolate and heart infusion agars containing 5% fresh rabbit blood in the absence of antibiotics. There is preferential growth of *B. henselae* on heart infusion agar, and *B. quintana* grows preferentially on chocolate agar (62). *B. koehlerae* will not grow well on heart infusion agar and requires fresh chocolate agar plates (62). Commercial sheep or horse blood agar plates have also been used but are less sensitive for primary isolation of *Bartonella* species. Plates inoculated with blood should be incubated at 35°C for at least 4 weeks in 5% CO_2 and at high humidity. Incubation in candle jars or sealed, gas-permeable plastic bags aids in preserving moisture and limiting contamination, both of which are critical when incubating these rich, nonselective agars for long periods of time. Colonies usually appear after 5 to 15 days (79). Due to prolonged incubation times, other slow-growing pathogens, including Mycobacterium tuberculosis and *Histoplasma capsulatum*, can grow on the plates; therefore, appropriate safety precautions should be taken with all positive cultures. Broth-based or biphasic culture system vials used for blood culture, such as the BACTEC Peds Plus vials (Becton, Dickinson, Sparks, MD) can be used for *Bartonella* isolation (80). DNA staining (e.g., acridine orange staining) and blind subculture from negative bottles before they are discarded at 7 days can increase the likelihood of identifying *Bartonella* (81). In a different assay, samples of heparinized blood are sedimented and plasma is then collected for inoculation into shell vials. Culture is then performed by the centrifugation-shell vial technique using the T84 bladder carcinoma cell line (inaccurately designated ECV304 human endothelial cells) (3).

Tissue

Recovery of *Bartonella* species from cutaneous lesions, liver, spleen, or lymph nodes is possible after homogenization in supplemented M199 medium and plating directly onto solid agar (3). Cocultivation with an endothelial cell line (3) or use of the shell vial method is laborious but can occasionally yield bacterial growth (3), as with isolation of *Bartonella* from heart valve tissues (31). Various liquid media have been developed for primary isolation of *B. henselae* from blood, serum, and other tissues (10, 11, 17–19, 63, 71, 74, 75, 82–89), but isolation in liquid media is of low sensitivity.

IDENTIFICATION

Colonies of *B. henselae* have two morphological types that can grow simultaneously from primary culture: (i) irregular, raised, off-white, dry, rough (cauliflower-like) colonies that pit and adhere to the agar and (ii) small, circular, tan, and moist colonies that are more easily scraped from the agar surface. The predominant colony morphologies of primary isolates of *B. henselae* are those described for type i above. *B. quintana* colonies are usually smooth, flat, and shiny, they are more tan than *B. henselae*, and they do not pit the agar (3). Highly passaged and confluent *B. quintana* colonies can appear somewhat mucoid. *B. clarridgeiae* produces small, white, raised, indurated, and cohesive colonies which can also appear to spread during primary isolation. Most *Bartonella* species appear uniformly smooth after repeated subcultures. The bacilli are small (2 µm by 0.5 µm) and stain best with Gimenez stain (3). With Gram staining, *Bartonella* species are weakly counterstained with safranin or basic fuchsin. *Bartonella* species appear in Gram stains as small, Gram-negative, slightly curved rods somewhat resembling *Campylobacter*, *Helicobacter*, or *Haemophilus*. Colony morphology, in conjunction with slow growth requiring more than 7 days of incubation, along with negative catalase and oxidase reactions, is often sufficient for a presumptive identification. DNA amplification and sequencing is optimal, however, for confirmation of *Bartonella* genus, species, and strain.

B. bacilliformis, *B. clarridgeiae*, *B. capreoli*, *B. rochalimae*, and *B. schoenbuchensis* are the only members of the genus that are motile by means of unipolar flagella. *B. henselae*, as well as several other *Bartonella* species, has a twitching motility on wet mounts, associated with the expression of trimeric autotransporter adhesins (TAAs) (as shown for *B. henselae* [90]). These TAAs are responsible for host cell adherence and may mediate specific interactions with host erythrocytes and endothelial cells (91).

Most species are biochemically inert except for the production of peptidases. None of the various commercially available identification systems contain *Bartonella* species in their databases. However, the MicroScan rapid anaerobe panel (Baxter Diagnostics, Deerfield, IL), the RapID ANA II, and Rapid ID 32 A have been used for identification. The MicroScan rapid anaerobe panel is reported to provide species identification (code 10077640 for *B. henselae*, code 10073640 for *B. quintana*, and code 10077240 for *B. bacilliformis*). Overall, these identification kits are of limited use for accurate diagnosis of *Bartonella* infections. Measurements of preformed enzymes and standard testing reveal minor differences between species.

Identification of *Bartonella* isolates is largely based on nucleic acid techniques, some of which allow for species determination, strain determination within a given species, or even genotyping. Methods include Southern blotting, gel and capillary electrophoresis, PCR, DNA hybridization, restriction fragment length polymorphism (RFLP) analysis, and gene sequence analysis (81). As described above, PCR and sequencing of target genes, such as *gltA*, *rpoB*, the internal transcribed spacer (ITS) region, *rrs* (16S rRNA), *groEL*, or *ribC*, are the most widely used (Table 2). Matrix-assisted laser desorption–ionization time of flight (MALDI-TOF) mass spectrometry has been applied to the identification of *Bartonella* species as isolated colonies and was able to differentiate 17 distinct species (92). The current Bruker MALDI Biotyper CA system library database lacks *Bartonella* species on the "Organisms Claimed List."

TYPING SYSTEMS

Several molecular methods, such as pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MSLT), multispacer typing (MST), and multiple-locus variable-number tandem-repeat analysis (MLVA) (93), can be used to genotype *B. henselae* (and in some cases *B. quintana*) isolates. Also, a genomic fingerprinting technique using infrequent-restriction-site PCR (IRS-PCR) can be used to identify pathogenic *Bartonella* species (94). Even when isolation of infecting species is not possible, PCR amplification of ITS DNA directly from diagnostic samples, followed by nucleic acid sequencing, is an invaluable tool for the primary identification at the species, subspecies, and genotype levels (11, 15, 17–19, 54, 71, 74, 75, 77, 78, 84, 87, 95, 96). *B. vinsonii* subsp. *berkhoffii* can be separated into 4 distinct genotypes based upon 16S-23S ITS sequences (78).

SEROLOGIC TESTS

Due to difficulties with traditional culture methods for isolation, serologic testing for *Bartonella* infection, including immunofluorescent-antibody assay (IFA), enzyme-linked immunosorbent assay (ELISA), and Western blotting (WB), is the cornerstone for clinical diagnosis. ELISA is the simplest to perform and can easily be automated but has a low sensitivity (17% to 35%) (14). IFAs, using commercial antigen slides for *B. henselae* and *B. quintana*, have become the most frequently used serological tests worldwide. Human infection with *B. henselae* or *B. quintana* is evaluated by detecting the presence of IgM and/or IgG antibodies directed against the *Bartonella* bacteria.

The first IFA for CSD used *B. henselae* bacilli that were cocultivated with Vero cells to inhibit auto-agglutination (35). This test was found to have good sensitivity (84% to 95%) and specificity (94% to 98%) using sera from patients with CSD (35, 36, 97). For this test, titers ranging from 64 to 256 or higher are considered positive and usually indicative of recent or ongoing infection. In cases of endocarditis caused by either *B. henselae* or *B. quintana*, high IgG antibody titers (≥800) are usually detected (98). A titer of IgG antibody to either *B. henselae* or *B. quintana* of ≥800 has positive predictive values of 0.810 for detection of *Bartonella* infection in the general population and 0.955 for detection of *Bartonella* infections among patients with endocarditis (98).

Nevertheless, IFA is time-consuming, requires appropriate equipment and expertise, and is subject to interobserver variation due to difficulty in reading the immunofluorescence (81). In humans, it has been postulated that the sensitivities of different IFAs may range from 14 to 100%, depending on antigen source and cutoff values used by different laboratories (14, 99–104). Antigenic variability among *Bartonella* test strains can result in false-negative serological results for some patients (100–103, 105–108). Cross-reactivity can occur among different *Bartonella* species. Antigen adsorption can be used to reduce cross-reactivity (81). Cross-reactivity to different *Bartonella* species can be present in up to 95% of samples (109). In addition, cross-reactive antibodies to other pathogens, e.g., *Chlamydophila pneumoniae*, *Coxiella burnetii*, and spotted fever group *Rickettsia*, have been reported (74, 75, 104, 110–113). In humans with late-stage AIDS and *Bartonella* coinfection, antibodies were detected in only 75% of culture-positive or tissue PCR-positive patients (114). In addition, several reports describe detection and/or isolation of *Bartonella* species from seronegative patients (4, 74, 75, 99, 105, 106, 115–117). This discrepancy between little or no serological detection of *Bartonella* antibodies despite detection of *Bartonella* DNA and/or bacterial isolation suggests that seronegative infection is more common in humans than currently recognized.

Dihydrolipoamide succinyltransferase, the trimeric autotransporter adhesins (BadA, Vomps), and other proteins of *B. henselae* and *B. quintana* are immunodominant target proteins potentially useful for diagnostic immunoblots (118–120). However, immunoblot-based serological tests do not show clear immunoreactive profiles, which diminishes their utility for routine serological diagnosis of *Bartonella* infections.

ANTIMICROBIAL SUSCEPTIBILITIES AND SUSCEPTIBILITY TESTING

Antimicrobial susceptibility testing can be performed by agar dilution methods using either blood or chocolate agar or by microdilution techniques using various media supplemented with blood (121). There are no current recommendations or guidelines for the interpretation of antimicrobial susceptibility of *Bartonella* species offered by the Clinical and Laboratory Standards Institute (CLSI) or EUCAST. The Etest (AB Biodisk, Solna, Sweden) can also be used to determine antibiotic susceptibility (122). Results of susceptibility testing against *Bartonella* species are summarized in Table 3. Of paramount importance, however, is the observation that there is often not a correlation between demonstrated susceptibility *in vitro* and clinical response *in vivo*. Penicillin G provides an excellent example. All five *Bartonella* species tested show good susceptibility *in vitro* in Table 3, yet patients treated with penicillin G have had progressive *Bartonella* infection and development of new BA lesions (122). In addition, *Bartonella henselae* isolates have various *in vitro* susceptibilities to trimethoprim-sulfamethoxazole, and *Bartonella henselae* has been isolated from a patient taking this antibiotic (122).

Evaluation of susceptibility to antibiotics has been performed either with cell cultures or with axenic media. Determination of antibiotic susceptibility in axenic medium has been carried out both on solid media enriched with 5 to 10% sheep or horse blood and in liquid media (123, 124). It should be noted that the conditions required to grow *Bartonella* during susceptibility testing do not meet standardized criteria established by the CLSI. Bacteria of the genus *Bartonella* when grown axenically are susceptible to many antibiotics, including β-lactams, aminoglycosides, chloramphenicol, tetracyclines, and macrolide compounds, including telithromycin, rifampin, the fluoroquinolones, and co-trimoxazole–trimethoprim–sulfamethoxazole (121, 123, 125, 126), which may not accurately represent *in vivo* activity.

TABLE 3 MICs for *Bartonella* species determined by the agar dilution technique with Columbia agar supplemented with 5% horse blood[a]

Antibiotic group and drug name	MIC range (μg/ml) for:				
	B. henselae	*B. quintana*	*B. bacilliformis*	*B. vinsonii*	*B. elizabethae*
Aminoglycosides					
Amikacin	2–4	4–8	2–8	4	1
Gentamicin	0.12–0.25	0.12–2	1–2	0.5	0.12
Streptomycin	ND	ND	4	ND	ND
Tobramycin	0.5–1	0.5–4	2–4	2	0.25
Cephalosporins					
Cefotaxime	0.12–0.25	0.12–0.25	0.03–0.12	0.12	0.06
Cefotetan	0.25–0.5	0.12–0.5	2	1	1
Ceftazidime	0.25–0.5	0.25–0.5	0.12–0.25	0.25	0.5
Ceftriaxone	0.12–0.25	0.06–0.25	0.003–0.006	0.06	0.12
Cephalothin	8–16	8–16	4–8	16	8
Macrolides					
Azithromycin	0.006–0.015	0.006–0.03	0.015	0.015	0.006
Clarithromycin	0.006–0.03	0.006–0.03	0.015–0.03	0.03	0.015
Erythromycin	0.06–0.25	0.06–0.12	0.06	0.25	0.12
Roxithromycin	0.015–0.03	0.015–0.06	0.03	0.12	0.06
Telithromycin	0.003	0.006	0.015	ND	ND
Penicillins					
Amoxicillin	0.6–0.12	0.03–0.06	0.03–0.06	0.06	0.03
Oxacillin	1–2	1–4	0.25–0.5	1	4
Penicillin G	0.03–0.06	0.03	0.015–0.03	0.03	0.015
Ticarcillin	0.25	0.06–0.25	0.06–0.12	0.25	0.12
Quinolones					
Ciprofloxacin	0.25–1	0.5–2	0.25–0.5	1	0.5
Pefloxacin	4–8	2–8	1–2	4	2
Sparfloxacin	0.06	0.06–0.12	0.25	0.06	0.06
Tetracycline					
Doxycycline	0.12	0.06–0.25	0.03–0.06	0.25	0.06
Miscellaneous					
Clindamycin	2–4	4–16	32–64	8	8
Colistin	4–16	4–16	16	8	4
Fosfomycin	16–32	32–64	8–16	16	16
Imipenem	0.5	0.25–1	0.5–1	2	0.25
Rifampin	0.03–0.06	0.06–0.25	0.003	0.12	0.03
TMP-SXT	1, 5	0.25, 1.25–1, 5	0.4, 2–0.8, 4	1, 5	0.5, 2.5
Vancomycin	2–8	8–16	4–8	8	8

[a] Adapted from reference 123. Abbreviations: TMP-SXT, trimethoprim-sulfamethoxazole; ND, not done.

In vitro antibiotic susceptibilities can also be examined for *Bartonella* species cocultivated with eukaryotic cells. As with agar-based susceptibilities, these *in vitro* studies demonstrate that *Bartonella* species are susceptible to many antibiotics (127). However, many of the tested antibiotics show solely bacteriostatic activity (127, 128). Only aminoglycosides are bactericidal *in vitro* when *Bartonella* species are grown either in liquid medium (121), endothelial cells, or erythrocyte cocultures.

Choice and Use of Antibiotics *In Vivo*

CSD typically does not respond to antibiotic therapy. There is generally only minimal benefit with antibiotic treatment. A randomized, open trial of gentamicin plus doxycycline versus no treatment for immunocompetent patients with *B. quintana* bacteremia (without endocarditis) established the efficacy of doxycycline plus gentamicin (129). However, addition of gentamicin to doxycycline for treatment of *Bartonella* endocarditis patients has often been complicated by renal failure. *Bartonella* endocarditis is a chronic, indolent infection that is often present for months before diagnosis; increasing reports of glomerulonephritis at or shortly after the time of diagnosis prompt caution in the use of gentamicin to treat *Bartonella* endocarditis patients (130). It may be prudent to treat *Bartonella* endocarditis patients with doxycycline 100 mg *per os* (p.o.) or intravenously (i.v.) twice daily plus rifampin (300 mg po) twice daily for 6 weeks, instead of doxycycline plus gentamicin.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Diagnosis of *Bartonella* infection in humans, especially for typical forms of CSD, is based mainly on serologic tests, which is the most cost-effective approach. However, the sensitivities of IFA range from 14 to 100%, depending on selected test antigens, laboratory cutoff, and test procedures used (14). For endocarditis, the best approach is serological testing and performance of nucleic acid amplification methods on cardiac valves. A single-step serological assay against *Coxiella burnetii* and *Bartonella* species found a sensitivity of 100% and a positive predictive value of 98% for the diagnosis of *Bartonella* infection in patients with blood culture-negative endocarditis (131), but this test is not readily available.

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Legionella

PAUL H. EDELSTEIN AND CHRISTIAN LÜCK

49

TAXONOMY

The *Legionellaceae* are composed of a single genus, *Legionella*, and 58 validly named species and three subspecies (http://www.bacterio.cict.fr/l/legionella.html) (Table 1). *Legionella pneumophila*, *Legionella micdadei*, *Legionella longbeachae*, and *Legionella dumoffii* are the most important from a clinical standpoint, with *L. pneumophila* causing more than 90% of cases of Legionnaires' disease (LD). The *Legionellaceae* are most closely related to the *Coxiellaceae*, and these two families comprise the proposed order "*Legionellales*," within the class *Gammaproteobacteria* and phylum *Proteobacteria* phy. nov. *Coxiella burnetii*, the agent of Q fever, shares many characteristics with *L. pneumophila*, including intracellular parasitism and close homologies with several virulence genes (1). Some investigators proposed the use of *Tatlockia* and *Fluoribacter* as additional genera within the *Legionellaceae* (2), but a subsequent study of 16S rRNA demonstrated that the *Legionellaceae* are monophyletic (3); use of these genus names was never widely accepted and is of historical interest only. A number of *Legionella*-like bacteria have been described to grow only within free-living amoebae and have been designated "LLAPs," for "*Legionella*-like amoebal pathogens." One of the LLAPs, *Legionella lytica*, has been shown to cause human disease, and four have been assigned novel *Legionella* spp.; three of these four have been grown axenically at low temperature (4).

Eight different *L. pneumophila* serogroup 1 strains have been completely sequenced (5). Analysis of these sequences shows that *L. pneumophila* contains a number of eukaryote-like genes, some of which have been shown to allow the bacteria to create bacterium-friendly intracellular environments by subverting the normal cellular machinery (6, 7). Analysis of the *L. pneumophila* genome also shows that it is a genetically diverse species and that some virulent strains are disseminated worldwide (7). Genes encoding the lipopolysaccharide core region and an O side chain predominate in clinical isolates of *L. pneumophila* serogroup 1 and may be the reason why this serogroup predominates as a cause of Legionnaires' disease (7). Genomic sequencing of several other *Legionella* species, including that of *L. longbeachae*, a common pathogen in Australia and New Zealand, has been completed or is in progress (8–10). Many more novel *Legionella* spp. have been identified but are not yet validly published (11).

DESCRIPTION OF THE AGENT

The *Legionellaceae* are a diverse group of mesophilic, motile, asaccharolytic, obligately aerobic, nutritionally fastidious Gram-negative rods that share growth dependence for L-cysteine, growth enhancement by iron, and cellular branched-chain fatty acids and ubiquinones, which are unusual for Gram-negative bacteria (12). *L. pneumophila* is the most extensively studied of the *Legionella* spp., with relatively little known about most of the other *Legionella* spp. Almost all of the *Legionella* spp. have been isolated from aqueous environmental sources, and about a third of the 58 validly named species have been isolated from both humans and the environment. It is assumed that the natural reservoir of all the *Legionellaceae* is our aqueous or soil environment and that humans are an accidental host of the bacterium. While almost all *Legionella* spp. have been isolated from environmental water sources, *L. longbeachae* appears to be mainly a soil organism equipped with genes that may aid its residence/pathogenesis in soil insects or worms (13). Environmental *L. pneumophila* is a facultative intracellular parasite of several different free-living amoebae, such as *Acanthamoeba* and *Naegleria*, existing in microbial consortia in biofilms and free-flowing water.

L. pneumophila grows optimally at 35 to 37°C and at a temperature range from 20 to 42°C. Other *Legionella* spp. infecting humans may have more restricted growth temperatures, including one new species that does not grow at temperatures greater than 35°C (14). Growth on solid media is enhanced by increased humidity. Incubation in 2 to 5% CO_2 can enhance the growth of some *Legionella* spp. Bacterial phenotype, including immunogenicity, cell size, and virulence, can be altered by growth at different temperatures (12).

Amino acids, rather than carbohydrates, are used as energy sources by the *Legionellaceae* growing *in vitro*. Primary isolation of all known *Legionella* spp. requires medium supplementation with L-cysteine, as does successful propagation of all but a few species. Iron supplementation of growth media is required for optimal growth, although many *Legionella* spp. can grow, albeit poorly, in the absence of the mineral. Growth of *L. pneumophila* is enhanced by the addition of α-ketoglutarate (0.1%) to media via an unknown, nonnutritive mechanism.

Growth of *L. pneumophila* in artificial media can be inhibited by a number of factors. These include the presence of high (100 mM, or 0.6%) NaCl concentrations, toxic

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TABLE 1 Selected characteristics of the *Legionella* spp.

Legionella species[a]	Clinical?[b]	No. of SG[c]	Color under long-wave UV light[d]	Comment(s)[e]
L. adelaidensis	N	1	NC	
L. anisa	Y	1	BW/YG	
L. beliardensis	N	1	YG	
L. birminghamensis	Y	1	YG	
L. bozemanae	Y	2	BW	AN, *Fluoribacter bozemanae*
L. brunensis	N	1	NC	
L. busanensis	N	1	NC	
L. cardica	Y	1	NC	
L. cherrii	N	1	BW	
L. cincinnatiensis	Y	1	YG	
L. drancourtii	N	NK	NK	Amoebic pathogen; no axenic growth
L. dresdenensis	N	1	wR	
L. drozanskii	N	1	NC	Grows at 30°C but not 37°C
L. dumoffii	Y	1	BW	AN, *Fluoribacter dumoffii*
L. erythra	N	2	R	
L. fairfieldensis	N	1	NC	
L. fallonii	N	1	NC	Grows at 30°C but not 37°C
L. feeleii	Y	2	NC	
L. geestiana	N	1	NC	
L. gormanii	Y	1	BW	AN, *Fluoribacter gormanii*
L. gratiana	N	1	NC	
L. gresiliensis	N	1	YG	
L. hackeliae	Y	2	YG	
L. impletisoli	N	1	NC	
L. israelensis	N	1	NC	
L. jamestownensis	N	1	YG	
L. jordanis	Y	1	YG	Partial L-cysteine independence with serial passage
L. lansingensis	Y	1	NC	
L. londiniensis	Y	1	NC	
L. longbeachae	Y	2	YG	
L. lytica	Y	NK	BW	Grows at 30°C but not 37°C
L. maceachernii	Y	1	YG	AN, *Tatlockia maceachernii*
L. massiliensis	N	1	NC	AN, *Tatlockia micdadei*, *Legionella pittsburghensis*
L. micdadei	Y	1	YG	
L. moravica	N	1	NC	
L. nagasakiensis	Y	1	NC	Partial L-cysteine independence with serial passage
L. nautarum	N	1	NC	
L. oakridgensis	Y	1	YG	Partial L-cysteine independence with serial passage
L. parisiensis	Y	1	BW	
L. pneumophila	Y	16	YG	3 subspecies: *fraseri*, *pascullei*, *pneumophila*
L. quateirensis	N	1	NC	
L. quinlivanii	N	2	YG	
L. rowbothamii	N	1	BW	
L. rubrilucens	Y	1	R	
L. sainthelensi	Y	2	YG	
L. santicrucis	N	1	YG	
L. shakespearei	N	1	NC	
L. spiritensis	N	2	YG	
L. steelei	Y	1	Y/BW	Probable human colonizer without disease, no growth at >35°C
L. steigerwaltii	N	1	BW	
L. taurinensis	N	1	R/YG	
L. tucsonensis	Y	1	BW	
L. tunisiensis	N	1	NC	
L. wadsworthii	Y	1	YG	
L. waltersii	N	1	NC	
L. worsleiensis	N	1	NC	
L. yabuuchiae	N	1	NC	

[a] Several other yet-unnamed species probably exist.

[b] Isolated from humans, yes (Y) or no (N). Severely immunosuppressed patients may acquire infections with *Legionella* spp. not previously isolated from humans.

[c] Number of recognized serogroups (SG). NK, not known.

[d] NC, no color; BW, bright blue-white; YG, pale yellow-green; BW/YG, some strains are BW and some YG; NK, not known; Y/BW, bright-yellow diffusible, with some strains having in addition colony-bound bright blue-white cells; R, dark red; R/YG, the majority of strains are red, with the remainder being yellow-green; wR, weakly red.

[e] AN, alternative name. The valid genus names *Fluoribacter* and *Tatlockia* are not in widespread use.

peroxides, products of other bacteria and fungi, and some lipids (12). In addition, optimal growth occurs over a very narrow pH range from 6.7 to 6.9. Solid growth media contain activated charcoal to inactivate toxic lipids and peroxides, and an organic buffer [morpholinepropanesulfonic acid (MOPS) or *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)] to reduce sodium content and provide the required pH. Preparation of growth media for *Legionella* spp. can be complex and is usually best left to competent commercial sources or to specialized laboratories.

EPIDEMIOLOGY, TRANSMISSION, AND PATHOGENESIS

LD was first recognized as a distinct entity when epidemic pneumonia with a 15% fatality rate developed during and after a convention of Pennsylvania State Legionnaires in Philadelphia, PA, in July 1976 (15, 16). Joseph McDade and colleagues at the CDC determined that a novel Gram-negative bacterium was the cause of the outbreak (17). Neither the disease nor the bacterium was found to be novel, with the first known epidemic of LD occurring in 1957 (18) and the bacterium being isolated multiple times from the 1940s on.

Environmental studies found that the bacterium was widespread in natural bodies of water and occasionally at high concentrations in warm waters found in plumbing systems, water heaters, warm water spas, and cooling towers. Many different *Legionella* spp. exist in nature within free-living amoebae, and as a result, these otherwise fastidious bacteria can multiply within the amoebae and be protected from biocides (19). *Legionella*-infected amoebae are often found in complex consortia of microorganisms within biofilms. The bacteria are present in very low concentrations in freely flowing cold water and biocide-treated waters but can multiply in warm, and especially stagnant, water. Devices that aerosolize these contaminated waters serve to disseminate the bacteria. In contrast to this, *L. longbeachae* infections appear to be acquired mainly through exposure to soil, especially some types of potting soil (20).

Legionella pneumophila serogroup 1 causes 95 to 98% of cases of community-acquired LD. The Pontiac/monoclonal antibody (MAb) 3-1 subgroup of *L. pneumophila* serogroup 1 constitutes about 80 to 90% of clinical isolates of this serogroup. Just a few clonal types of *L. pneumophila* serogroup 1, Pontiac subgroup, are responsible for about 50% of sporadic community-acquired infection (7, 21, 22). Several *L. pneumophila* serogroup 1 strains that predominate as clinical isolates are uncommon in the environment, whereas most *L. pneumophila* strains that are commonly found in the environment are unusual causes of LD (22–24). Infection caused by the *L. pneumophila* serogroup 1 Pontiac subtype is less common in nosocomial LD, especially that involving immunocompromised patients. Up to 60% of nosocomial LD may be caused by other *L. pneumophila* serogroup 1 subtypes, other *L. pneumophila* serogroups, and other *Legionella* species (25, 26).

Infection is acquired by aerosol inhalation of contaminated water, although microaspiration may also be a mechanism for acquiring the disease (15). The majority of community epidemics of LD are from *Legionella*-contaminated cooling towers or other aerosol-generating devices. Contaminated potable water systems, such as water heaters and warm water in pipes, can also be a major source of disease, although these sources are not usually the cause of explosive outbreaks of the disease but rather of more prolonged and slower-paced outbreaks. Either source type may cause sporadic cases of LD.

Despite the ubiquity of *Legionella* spp. in our environment, LD is an unusual cause of pneumonia. About 0.5 to 5% of adults requiring hospitalization for pneumonia have LD. Passive reporting indicates that the disease incidence is from 4 to 20 cases per million people per year, and a prospective study estimated that the disease incidence is about 80 cases per million people per year or between 8,000 and 18,000 LD cases annually in the United States (27). This rate may be an underestimate, as a recent German study found that the annual rate was 180 to 360 cases/million population (28), which was roughly equivalent to 4% of all cases of community-acquired pneumonia, not just patients hospitalized for pneumonia. Underdiagnosis of LD is common in the United States and elsewhere, with only about 3,400 cases being reported to the CDC in 2010; underdiagnosis is due to both the failure to order laboratory tests for the disease and the insensitivity of laboratory diagnostic methods (29). Sporadic community-acquired LD is much more common than epidemic-associated disease, with only about 4% of U.S. cases being associated with known clusters (30). About 20% of LD cases are travel associated, with overnight travel being a risk factor for the disease. LD occurs year-round, with seasonal peaks in the warmer months (from the late spring until early fall); about 60% of reported U.S. and European cases occur between June and October (30, 31).

The incubation period of LD is estimated to be between 2 and 14 days, with a median value of about 4 days. A study of a large outbreak extended the incubation period to as long as 19 days, with a median value of 7 days (32). Epidemiologic case definitions for LD used by public health authorities can be found online (www.cdc.gov/legionella/health-depts/CSTE-position-statement.html, www.ecdc.europa.eu/en/activities/surveillance/ELDSNet/Pages/EU%20case%20definition.aspx).

L. pneumophila causes disease by infecting human mononuclear cells, primarily alveolar macrophages. After the bacterium is inhaled into the lungs, it invades lung macrophages and multiplies in them. The molecular and cellular pathogenesis of *L. pneumophila* has been studied in detail, but relatively little is known about the pathogenic mechanism of the other *Legionella* spp., except that of *L. longbeachae*, which has been the subject of a few studies. *L. pneumophila* injects hundreds of pathogenicity effectors, via a type IV secretion system, into host macrophages almost as soon as the bacterium makes contact with the phagocytic cell. Bacterial strains lacking this type IV secretion system, termed Dot or Icm, are avirulent. The injected effectors hijack normal cellular host defenses that would ordinarily destroy the bacterium within a fused phagolysosome. Instead, the virulent bacteria reside in a nonfused phagosome that is associated with host ribosomes, and rather than being killed, it derives nutrients from the cell and multiplies within the specialized phagosome. After multiplying many thousandfold within the phagocyte, the bacteria effect cell death. At the same time, the bacteria change their phenotype to become very motile, small, and primed for invading adjacent uninfected phagocytes. The bacteria possess other secretion systems, by which they secrete a number of proteases and other potentially toxic substances, many of which are either proinflammatory or tissue destructive. Intracellular iron acquisition is important for bacterial survival and growth within the cell; a number of bacterial effectors aid in iron acquisition. The host response to the bacterium, as well as secreted toxic substances, produces intense inflammation that destroys host tissues in the lung. The bacteria are controlled primarily by the innate immune system and by acquired cellular immunity. Detailed descriptions of pathogenesis can be found elsewhere (15, 33).

CLINICAL SIGNIFICANCE

LD is a type of bacterial pneumonia caused by *L. pneumophila* and other *Legionella* spp. The pneumonia ranges in severity from mild to fatal, with an average fatality rate of 12% (34). Major risk factors for the disease include endogenous or exogenous suppression of the cellular immune system, cigarette smoking, overnight travel outside the home, use of well water, chronic heart or lung disease, and chronic renal failure. Patients at particularly high risk of LD are those receiving high-dosage glucocorticosteroids, those with solid organ transplants, and those receiving anti-tumor necrosis factor therapy for a variety of autoimmune diseases (15).

LD cannot be readily distinguished from other forms of community-acquired pneumonia by clinical, roentgenographic, or nonspecific laboratory studies (15). Several attempts at developing a clinical scoring system to distinguish LD from other pneumonias have failed. The severity of pneumonia at presentation, underlying diseases, and promptness of specific antibiotic therapy are important prognostic factors. Promptly treated LD can be cured in 95 to 99% of cases in an otherwise healthy person. Less than half of patients may respond if there is a delay in therapy, immunosuppression, or respiratory failure (15). Untreated disease causes death in about 15% of previously healthy patients and up to 75% of severely immunocompromised ones (15).

Prospective, randomized controlled studies of adequate size have not been performed to determine the optimal therapy for LD, so great reliance is placed on experimental tissue culture and animal model studies, as well as results of nonrandomized studies (15, 35). Erythromycin, clarithromycin, azithromycin, a tetracycline, levofloxacin, ciprofloxacin, and moxifloxacin all appear to have roughly equivalent efficacies for nonimmunocompromised outpatients with mild LD (15). The quinolone antimicrobials, especially levofloxacin, and azithromycin are the drugs of choice for severe disease and for immunocompromised patients (15, 36). Antimicrobial therapy with more than one agent is sometimes used but is of questionable benefit and may be harmful (37).

Pontiac fever is an acute influenza-like illness that has been associated with exposure to *Legionella* species-containing environmental aerosols (38, 39). The etiology and pathogenesis of this disease are unknown, but it appears that the disease is caused by inhalation of bacterial toxins, such as endotoxin, or perhaps by an acute allergic reaction to a bacterium. Since multiple microorganisms and endotoxin have been found in aerosols causing Pontiac fever, it is unclear if *Legionella* spp. play any role at all in disease causation. Pontiac fever is self-limited, with no reported deaths, little to no need for hospitalization, and no need for antibiotic therapy.

COLLECTION, STORAGE, AND TRANSPORT

Expectorated sputum and other lower respiratory tract specimens are the most common sources of *Legionella* spp. Other less common sources include pleural fluid and blood. Rare sources have included pericardial fluid and specimens from kidney, liver, spleen, myocardium, respiratory sinuses, skin and soft tissues, infected wounds, peritoneal fluid, prosthetic and native heart valves, joint fluids, bone marrow, and intestine. Culture of available sputum, bronchoscopy specimens, lung biopsy specimens, and pleural fluid should be routine for laboratory diagnosis of LD. Lung biopsy specimens have the highest yield but may be negative. Culture of expectorated sputum or other lower respiratory tract secretions, second in yield to lung biopsy specimens, should always be performed for optimal detection of legionella infection. Pleural fluid has low yield but should be cultured if it is available. Culture of other specimen types for *Legionella* spp. is not indicated unless there is a high clinical suspicion of the disease affecting these sites or specific laboratory evidence of the disease elsewhere.

Sputum microscopic scoring criteria cannot be used to determine which sputum specimens should be cultured for legionella bacteria because of limited purulence and scanty secretions in patients with LD. Up to 80% of specimens culture-positive for *Legionella* spp. may be rejected on the basis of the criterion of the presence of sputum purulence for processing specimens (40, 41).

Urine for antigen detection should be collected in a sterile container (42). Boric acid preserves the antigen, but use of commercial urine transport systems containing boric acid have not been studied for antigen preservation and freedom from interactions. Urine can be transported to the laboratory at room temperature if no more than a several-hour delay is anticipated. Longer transport times require specimen refrigeration; antigen in frozen (−20 to −70°C) urine samples is stable for several months (43).

Blood for serum antibody testing is collected in standard tubes and transported at room temperature (44). Test performance is not adversely affected by storage of the clotted unseparated blood at room temperature for several days. Long-term storage of aliquots to allow parallel testing without freeze-thawing, which can lower antibody levels, is at –20°C. *Legionella* spp. are hardy and generally survive for up to a week in clinical specimens. Sputum and other respiratory tract specimens, including lung biopsy specimens, should be collected in sterile containers and transported to the laboratory promptly at room temperature. Transportation and storage should be at 2 to 5°C if more than a several-hour delay is anticipated before the specimen can be plated. Very long term storage is best at –70°C, although this can reduce bacterial concentration to below the level of detection when the starting concentration is low or the specimen is primarily aqueous. Repeated freeze-thawing is harmful to the bacteria. Some tissues, especially spleen, contain growth-preventing substances and must be plated promptly; even overnight storage at 5°C dramatically reduces culture yield (note that this is not true of lung specimens).

Valid data on the stability of *Legionella* species DNA in clinical samples have not been published. Regardless, special conditions for the transportation of respiratory materials or serum samples used solely for *Legionella* species PCR do not appear to be required. General guidelines for the transport and preanalytical storage of these specimen types are found in chapter 18 of this *Manual*.

DIRECT EXAMINATION

Microscopy

The morphology of *L. pneumophila* found in lung and sputum is a small coccobacillus to short rod 3 to 5 µm in length (Fig. 1). This is much different from that observed for the bacterium taken from a culture plate, which is usually a long filamentous bacillus 10 to 25 µm in length. *L. pneumophila* is

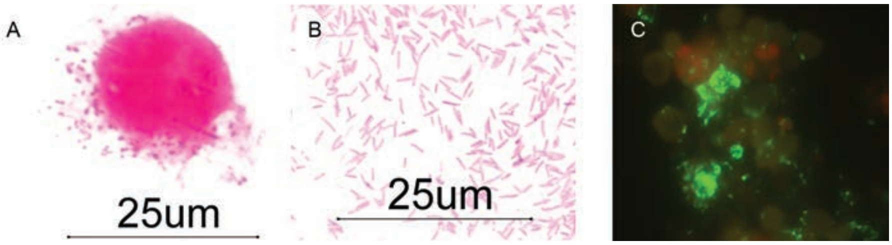

FIGURE 1 Photomicrographs of *L. pneumophila*. (A) Gimenez stain of intracellular bacteria from a patient with lung infection. (B) Gram stain using a basic fuchsin counterstain of a colony taken from a BCYEα plate. Note the dramatic size and shape differences between the intracellular and extracellular bacteria. (C) Direct immunofluorescent staining of *L. pneumophila* in lung. doi:10.1128/9781555817381.ch49.f1

very difficult to detect by Gram staining sputum or lung biopsy specimens. Use of 0.1% basic fuchsin, rather than safranin, greatly enhances the staining of the bacterium from culture plates, but even with use of this stain, it is very difficult to visualize the bacterium in sputum and tissues. Less than 0.1% of *L. pneumophila* cells present in lung tissue or sputum can be visualized by Gram staining using basic fuchsin. The small size of intracellular *L. pneumophila*, the form present in human tissues, makes visualization difficult with Gram stain, as does stain uptake by the surrounding proteinaceous material found in sputum and tissue.

Enhancement of bacterial staining by silver precipitate stains, such as the Warthin-Starry stain and its modifications and the Dieterle stains (45, 46), was an early approach to the detection of *L. pneumophila* in embedded tissues as well as sputum. These silver stains are useful for detection of the bacterium in embedded tissues but have no present role in the staining of the bacterium in sputum or other nonembedded specimens. Silver stains are not highly sensitive, can produce artifacts, and require expert use and interpretation for optimal sensitivity and specificity.

Some *Legionella* spp., in particular *L. micdadei*, may stain with acid-fast stains, both in fresh specimens and from Formalin-fixed tissues (47, 48). The small coccobacillary morphology of *Legionella* spp. should be a clue that the acid-fast organism is not a mycobacterium.

Immunofluorescence microscopy is the most sensitive and specific microscopic method for the detection of *L. pneumophila* in tissues and sputum (Fig. 1) (49, 50). Optimal sensitivity and specificity require exacting staining methods and great expertise by the microscopist. Even when the test is well performed, its sensitivity has been low compared to those of other diagnostic methods (51). For these reasons, this test is now rarely used for direct examination, except perhaps in the examination of autopsy lung specimens. Detailed discussions of this test can be found elsewhere (50).

Antigen Detection

LD due to *L. pneumophila* serogroup 1 can often be diagnosed by detection of bacterial antigenuria (42). Several immunoassays are commercially available for this purpose, the most convenient of which is a rapid single-test immunochromatographic card assay. Immunochromatographic card assays are made by at least six companies, and three are FDA cleared (Alere, BinaxNOW; SA Scientific, SAS Legionella; and Meridian, TRU Legionella); of these, only the BinaxNOW assay has been extensively evaluated. Several other assays utilize a microtube-based enzyme immunoassay. The strength of all these assays is their detection of infections with *L. pneumophila* serogroup 1 and, in particular, its Pontiac/MAb 2/MAb 3-1 monoclonal subtype (25, 26, 52, 53). The immunochromatographic card assays can be up to 40% less sensitive than the microtube-based immunoassays (54), although prolonged incubation and urine concentration can enhance card test sensitivities (55); neither of these enhancements is in the manufacturer's directions. The ability to quickly test a single specimen, combined with ease of use and robustness, makes up for a decrease in sensitivity of the immunochromatographic card assays.

Clinical test performance for all assays is dependent on the pretest probability of *L. pneumophila* serogroup 1 and on the probability of Pontiac monoclonal subtype *L. pneumophila* serogroup 1 infection (25, 52). The assays detect about 60 to 70% of *L. pneumophila* serogroup 1 Pontiac monoclonal subtype epidemic infections and up to 90% of sporadic cases of pneumonia caused by this subtype. The differences in test sensitivity for the same bacterial subtype are probably due to differences in disease severity, the other major factor determining test sensitivity. Patients with severe *L. pneumophila* serogroup 1 LD, for example, those requiring intensive-care nursing and ventilator assistance, are the most likely to have positive urine antigen tests; test sensitivity in this population is probably in the range of 90 to 95% of those infected with the Pontiac monoclonal subtype. On the other hand, urine testing may detect only 50% of outpatients with mild epidemic disease caused by the same monoclonal subtype, perhaps 40% of hospitalized patients with other *L. pneumophila* serogroup 1 subtypes, and fewer than 5 to 40% of those with infections caused by other serogroups and species (26, 56). The test may be negative during the first day of illness, but those with severe disease are likely to be positive on presentation to the hospital. Repeat testing 2 to 3 days after the onset of illness may lead to detection of a small number of patients who had negative tests initially.

Test sensitivity can be enhanced by concentrating the specimen using ultrafiltration devices such as Amicon concentrators (Millipore, Billerica, MA). In some studies, this has increased test sensitivity by about 30%, without affecting specificity (57, 58). Prolonging the incubation time for the BinaxNOW assay to 60 min also increases sensitivity without decreasing specificity (26, 59). Test sensitivity may decrease if specimens are frozen for months before being tested (43).

The urine antigen assays are very specific, i.e., in the range of 99 to 99.9%. False-positive tests can be due to rheumatoid arthritis-like factors in urine, freeze-thawing of urine, and excessive urinary sediment. All together, these causes of false-positive tests account for no more than a few percent of all positive tests. Regardless, all positive tests should be confirmed after boiling urine clarified by centrifugation; these steps increase test specificity but are not mentioned in the test product inserts. Patients who previously had LD may continue to excrete detectable urinary antigen for days to months after successful treatment, with those having very extensive pneumonia being most likely to have positive tests for weeks to months afterwards. Such prolonged urinary excretion is not evidence of treatment failure.

Molecular Diagnosis of LD

Nucleic acid-based detection of *Legionella* spp. in sputum, urine, and blood has been successfully used in reference and research laboratories, with detection of *L. pneumophila* being the most extensively studied. The best results show that molecular diagnosis is a more sensitive method of diagnosis than culture (60, 61), although some studies showed rough equivalence (62). Test sensitivities have been estimated to be 80 to 100% and 30 to 50% for lower respiratory tract secretions and serum, respectively; test specificities are estimated to be >90% (60–62). Sputum digestion may be important to increase yield (63). Prior studies indicated that urine is a suitable PCR analyte, but more-recent work shows that urine is not a suitable specimen type for the detection of *Legionella* species DNA (60).

Both conventional and real-time assays have been utilized (62, 64). Most laboratories use the macrophage infectivity protein (*mip*) gene target to detect *L. pneumophila*. *Legionella* spp. are most commonly detected using an rRNA gene target (usually the 16S rRNA gene, although 23S rRNA is claimed to have advantages) (65). Recent work shows that the *wzm* target can be used to specifically detect *L. pneumophila* serogroup 1 (66). A multiplex assay that detects *L. pneumophila* serogroup 1 (*wzm*), *L. pneumophila* (*mip*), and *Legionella* spp. (*ssrA*) has been described to be a sensitive and specific assay for use with clinical specimens (67). Multiplex assays are most commonly used because they have no disadvantage over uniplex methods, although one study showed the superiority of a nucleic acid sequence-based amplification (NASBA) uniplex assay over a multiplex format (68, 69).

No FDA-cleared commercial PCR assay is marketed in the United States. Several commercial PCR assays are marketed elsewhere for use in testing respiratory specimens. Tests based on reverse hybridization include Gen ID CAP Bac (AID Diagnostika, Germany) and Chlamylege (bioMérieux, France). Examples of non-FDA-cleared commercial real-time PCR assays that detect *L. pneumophila*, *Legionella* spp., or both targets include Duplica real-time assays (EuroClone, Italy), *Legionella pneumophila* plus species (Diagenode Diagnostics, Belgium), Unyvero (Curetis, Germany), and *Legionella pneumophila* PCR (GeneProof, Czech Republic). Sufficient evaluations of the performances of these commercial assays have not been published, requiring extensive internal and external validation before use. European external quality assessment schemes are available for PCR assays (www.qcmd.org, www.instandev.de).

The added benefit of nucleic acid amplification-based detection of *Legionella* spp. in sputum over that obtained by urine antigen testing appears to be slight, with an 11% greater yield than from urine testing alone. This is likely because of the predominance of *L. pneumophila* serogroup 1, Pontiac subgroup, in community-acquired disease (70). The performance of nucleic acid testing for the detection of nosocomial LD and LD in immunocompromised patients is probably significantly better than urine antigen testing.

A problem with molecular detection of *Legionella* spp. in clinical specimens has been the inability to type detected bacteria for epidemiologic purposes. This is now possible for the majority of PCR-positive specimens using a nested PCR method (60, 71, 72). Descriptions of primers and detailed protocols are available online (http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php).

Because *Legionella* spp. are commonly found in water, contamination of almost any molecular reagent with *Legionella* species nucleic acid is a concern. False-positive PCR tests for *Legionella* spp. have been attributed to contaminated commercially produced “pure” water and nucleic acid extraction columns (73–75). Since sequencing of false-positive products yielded *Legionella* species sequences, the possibility of contamination cannot be excluded by the ability to sequence the product and assign it to a particular *Legionella* spp. (74). In the case of extraction column contamination, only a few columns of the same lot may be contaminated and not the entire lot. This means that multiple negative controls are required for optimal specificity, including extraction controls as well as no-template controls. Finally, contamination of specimens with tap water containing *Legionella* spp. is a concern and has been reported to result in pseudo-outbreaks of LD related to bronchoscopy, for both culture and DNA detection methods (76, 77).

ISOLATION PROCEDURES

Specimen Plating

Optimal yield of *Legionella* spp. from clinical specimens usually requires (i) that specimens be diluted to reduce inhibition by tissue and serum factors, as well as antibiotics, (ii) that the specimen be pretreated to reduce contaminating microbiota, and (iii) that a variety of selective and nonselective media be used (Table 2). Practically, this entails the inoculation of a nonselective medium and two different selective media, with the specimen diluted in broth or water, and the same three plates should be inoculated with the specimen diluted in an acid buffer, for a total of six inoculated plates. Culture of *Legionella* spp. from normally sterile fluids and tissues, such as pleural fluid, aseptically obtained lung tissue, or blood, is often successful without the use of multiple selective media and specimen decontamination.

Dilution (1:10) in tryptic soy broth or distilled water increases the culture yield of most specimen types, including sputum and other liquid respiratory tract specimens, lung tissue, lymph nodes, spleen, and probably other organs, such as liver and kidney. Sputum and other respiratory tract specimens should first be examined in a petri dish for purulent-appearing material, and this material should be selected for culture. Tissues (about 1 g) are ground in a tissue grinder with a small amount (1 ml) of broth, which adequately dilutes most tissues except for spleen; this tissue requires an additional 1:10 dilution for the best recovery of bacteria. Liquid specimens are roughly diluted by adding about 0.1 ml of vortex-mixed liquid specimens to 0.9 ml of the dilution broth. Pleural fluid, joint fluid, and blood subcultured from blood culture bottles do not require dilution before being plated; in fact, pleural fluid yield may be enhanced by concentration by centrifugation.

Decontamination is required to reduce contaminating microbiota in most sputum and other respiratory tract secretions. This is done by diluting (1:10) the specimen in a low-pH KCl-HCl buffer (pH 2.2) and incubating it at room temperature (4.0 min) before plating the suspension onto culture media. Timing is critical here, with resultant low yield if the timing is off by as little as a minute. The culture

TABLE 2 Composition and selectivity of media used to grow *Legionella* spp. from clinical and environmental specimens

Medium	Synonym	Selective agents[a]	Main use	Selectivity[a,b]
BCYEα	CYE	None	Clinical, culture maintenance	None
BMPA	PAC	Cefamandole, poly B, antifung	Clinical, environmental	Norm. resp. microbiota, 3+; enterics, 3+; yeasts, 3+; molds, 1+; *Legionella* spp., 1+ to 4+
PAV	VAP	Vanco, poly B, antifung	Clinical, environmental	Norm. resp. microbiota, 2+; enterics, 2+; yeasts, 3+; molds, 1+; *Legionella* spp., 1+
MWY	VGP	Vanco, poly B, antifung, glycine	Environmental	Norm. resp. microbiota, 2+; enterics, 2+; yeasts, 3+; molds, 1+; environmental bacteria, 2+; *Legionella* spp., 1+
CCVC		Cephalothin, poly E, Vanco, cycloheximide	Environmental	Norm. resp. microbiota, +; enterics, 3+; yeasts, 2+; molds, 2+; *Legionella* spp., 1+ to 4+
BCYEα–L		None (made without L-cysteine)	Organism identification	*Legionella* spp., 4+ (no growth of *Legionella* spp. on this medium)

[a] Poly B or E, polymyxin B or E, respectively; antifung, either anisomycin or natamycin antifungal compounds; Vanco, vancomycin; norm. resp. microbiota, normal upper respiratory tract bacteria.

[b] The selectivity scale is from 0 to 4+, where 0 means that the agents do not inhibit these organisms, 1+ means that the agents cause slight inhibition (they allow about 75% growth), 2+ means that about 25 to 50% growth is allowed, 3+ means that about 10% growth is allowed, and 4+ means that less than 1% growth is allowed.

medium is sufficiently buffered so that the acidified specimen is neutralized upon being plated. An alternative to specimen acidification is heating at 50°C for 30 min. Most aseptically collected tissue specimens do not require decontamination, although occasionally lung tissues contain multiple contaminating bacteria and fungi. In this case, heat or acid treatment of tissue ground in sterile distilled water may help; sometimes dilutions of the ground tissues are also required for optimal yield, with or without pretreatment. For specimens critical for epidemiologic purposes, culture yield can sometimes be enhanced by plating multiple 10-fold dilutions of specimens onto a wide variety of media, by using a variety of specimen treatments, such as heat and acid, and by performing coculture with amoebae (78, 79).

Inoculation of Plates

Approximately 0.1 ml is inoculated onto each plate, with the bulk of the inoculum applied to the first quadrant. Comparative studies to show whether it is better to streak plates for isolation or to uniformly distribute the inoculum over the entire plate are lacking. The plates must be thoroughly dry before being inoculated to aid in absorption of the relatively large-volume inoculum and to retard spreading of contaminants throughout the plate.

Culture Media

Buffered charcoal yeast extract medium supplemented with 0.1% α-ketoglutaric acid (BCYEα) is used for the isolation and growth of *Legionella* spp. Use of BCYE without α-ketoglutarate supplementation cannot be recommended for clinical use, as this amino acid greatly enhances the growth of the bacterium (80).

BCYEα can be made selective by the addition of antimicrobial agents (Table 2). A variety of different antifungal agents are used in the media. Cycloheximide is a poor choice for media used for clinical specimens, as it fails to inhibit *Candida albicans*. Both anisomycin and natamycin inhibit more yeasts than does cycloheximide. An array of media exists because no one selective medium is best for all purposes. Optimal yield of *Legionella* spp. from clinical specimens requires the use of three different media, one plate with nonselective medium (BCYEα) and two with selective media (BCYEα medium with antimicrobial agents [BMPA] and BCYE with polymyxin B, anisomycin, and vancomycin [PAV]). BMPA is an excellent selective medium for the vast majority of *L. pneumophila* strains, but the cefamandole present in the medium inhibits the growth of some other *Legionella* spp. and, rarely, *L. pneumophila* strains. Use of the less selective medium, PAV, is required for optimal growth of some *Legionella* spp. other than *L. pneumophila*. No selective medium inhibits multiresistant Gram-negative bacteria, reducing culture yield in nosocomial disease.

Selective and nonselective media are optimized for the isolation of *L. pneumophila*, and their performance for the isolation of other *Legionella* spp. is not accurately known. One study showed that *L. micdadei* in guinea pig spleen had enhanced recovery on BCYEα medium prepared with 1% bovine serum albumin; the growth was enhanced because of less growth inhibition by spleen tissue (81). Whether addition of bovine serum albumin to BCYEα medium enhances *L. micdadei* recovery from human lung or sputum is unknown and probably unlikely. A BCYEα-based selective medium containing natamycin, aztreonam, and vancomycin has been reported to be useful for the isolation of *L. longbeachae* from soil (82).

The medium shelf life is around 1 year for nonselective plates and slants (83). This long shelf life requires thick plates (25-ml pour), complete drying of plates before storage at 2 to 4°C in sealed plastic bags, and protection from light. Selective media lose selectivity after about 3 months' storage time, but depending on the incorporated antibiotic, the media may last considerably longer.

Quality control testing of media is required before they are put into use. Current CLSI standards are inadequate for proper quality control (QC) testing of these media. About 1% of commercial media fail laboratory QC testing (personal observations). The CLSI QC testing protocol utilizes a heavy inoculum of medium-adapted *Legionella* species strains and a growth/no-growth test. Minor variations in the ways media are manufactured, such as the addition of excess salt, overlong autoclaving, and degradation of buffers, can all seriously affect the ability of the medium to support wild strain growth but not necessarily that of medium-adapted strains. The optimal method for QC medium testing is the inoculation of the test media with several hundred non-artificial-medium-passaged *L. pneumophila* bacteria (obtained from infected guinea pig lung) and quantification of the bacterial colonies after 3 to 4 days of incubation (84).

In the absence of the availability of lung-passaged *L. pneumophila* bacteria, low-passage-number clinical strains should be used, with care taken to plate only several hundred bacteria per plate. QC testing of selective media for the ability to suppress non-*Legionella* bacteria can be done by inoculation of the plate with relatively antibiotic-susceptible *Escherichia coli* and *Staphylococcus aureus* strains, such as ATCC 25922 and 25923; the growth should be markedly suppressed.

Medium Incubation

Inoculated media are incubated at 35 to 37°C in humidified air. Regardless of the humidification method, care must be taken to keep the incubators or jars very clean and to regularly sterilize the containers or incubators. A small amount of CO_2 supplementation (2 to 5%) may enhance the growth of some of the more fastidious *Legionella* spp., such as *L. sainthelensi* and *L. oakridgensis*. This low level of CO_2 supplementation will not harm the growth of *L. pneumophila*, but CO_2 levels higher than 5% may inhibit growth. Since the more capnophilic species are very rare human isolates, many laboratories do not use CO_2 incubation of media for *Legionella* spp.

Plate Inspection

Legionella species colonies begin to appear on culture plates on day 3 of incubation. It is very unusual for the bacterial colonies to appear on plates after 5 incubation days. Some very rarely isolated *Legionella* spp. may require up to 14 days of incubation before growth appears; this is an extremely rare event. Regardless, it is reasonable to inspect culture plates on days 1 to 5 and then again at day 14.

The late appearance of *Legionella* spp. on culture plates can be used to great advantage if a careful record is kept of the colonies present on days 1 and 2 postincubation. New colonies appearing after day 2 should be suspected of being *Legionella* spp. Very rarely, *Legionella* spp. may grow from heavily infected lung (usually after autopsy of a fatal untreated case) on day 2, so some latitude in growth rate assumptions needs to be applied in the case of autopsy lung cultures. *Legionella* spp. never grow from clinical specimens on day 1 postincubation, a critical point in the distinction of *Pseudomonas aeruginosa* colonies from those of *Legionella* spp., as very early colonies of the latter superficially resemble those of the former.

Proper observation of culture plates requires the use of a dissecting microscope illuminated with direct light aimed at the plate surface at approximately a 30° angle. Failure to use a dissecting microscope, or use of improper lighting, will result in missed positive cultures, especially when there is mixed bacterial growth on the plates. In addition, very young *Legionella* species colonies are very small and difficult to see with the naked eye. Therefore, use of a dissecting microscope can speed up the time to colony detection by as much as a day. *Legionella* species growth occurs almost exclusively in the first streak quadrant and sometimes at the edge of the plate.

The size and morphology of *Legionella* species colonies change with time. Very young colonies (day 3) are flat, entire, and 0.5 to 1 mm in diameter, and when observed using a dissecting microscope and incident visible light, they usually have a speckled blue, blue-green, or red color. Within 6 to 24 h of additional incubation, these colonies become smooth, convex, iridescent, entire, and about 1 to 3 mm in diameter, and they look opal-like when observed with a dissecting microscope (Fig. 2). A thick string may form when a loop is inserted in the colony and then removed from the colony. Unlike with several mimics, the edges of the colonies are of the same consistency as the central portion and are not watery or clear. In another 1 to 2 days, the colonies may increase in size to up to 5 to 7 mm, become umbonate, sometimes with a tuberculated or inhomogeneous texture, and develop spreading edges; their iridescent nature may be lost at this stage. It is these late-stage colonies that are most difficult to distinguish from non-*Legionella* spp., making daily plate observation crucial for accurate detection. Very rarely, some *Legionella* species colonies do not change morphology with prolonged incubation.

Biosafety level 2 precautions should be used for the manipulation of *Legionella* species cultures. It is safe to inspect culture plates, pick typical colonies, and subculture them on an open bench in a properly ventilated laboratory. Making an organism emulsion on microscope slides for the purposes of Gram staining can also be safely carried out on an open bench. However, vortexing suspensions, sonication, tissue grinding, primary plating, and manipulations that may result in generation of a high-concentration aerosol should be performed in a biological safety cabinet. No well-documented case of laboratory-acquired LD has been reported.

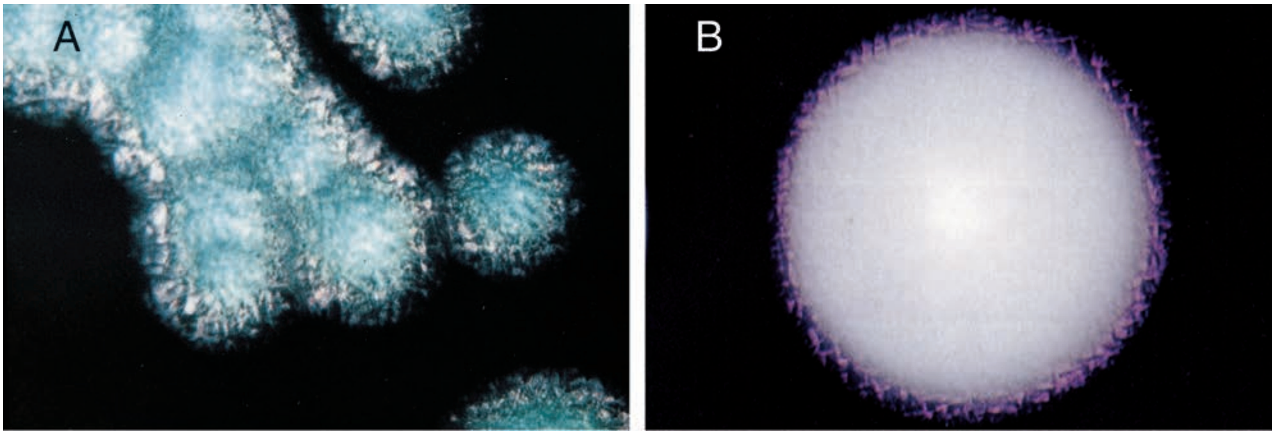

FIGURE 2 Photographs of *L. pneumophila* colonies growing on BCYEα agar. Note the internal speckling and different colors that may be seen, sometimes in the same culture.
doi:10.1128/9781555817381.ch49.f2

Initial Workup of Suspect Colonies and Look-Alike Bacteria

Colonies suspected of being *Legionella* spp. should first be stained with Gram stain to ascertain that the bacteria are small and sometimes filamentous Gram-negative rods. A small amount of the colony should be emulsified in sterile water or saline on a glass slide. It is important to completely suspend the bacteria in the liquid, as nondispersed clumps may stain as Gram-positive rods. It is also crucial to use 0.1% basic fuchsin counterstain because safranin stains these bacteria very poorly. Depending on the colony age and on the strain and species, *Legionella* spp. taken from plates vary in size from short rods that are 0.5 by 5 μm to very long filamentous bacteria that are 1 by 25 μm.

Gram-negative bacteria should then be plated to two different media, BYCEα and either tryptic soy blood agar (TSBA) or BCYEα made without L-cysteine (BCYEα–L), in approximately equal amounts in a small (1-cm^2) area; eight or more isolates can be plated to each plate if needed (Fig. 3). Rather large amounts of the picked colony should be inoculated into these media to enable growth after 16 to 18 h of incubation, as the small inocula normally used for other bacteria may otherwise take several days to produce visible colonies on plates. If only a single small colony is available, then it can be emulsified in a small amount (~0.5 ml) of sterile distilled water (not saline) and used for staining, plate inoculation, and sero-identification. *Legionella* spp. should grow in 16 to 36 h on BCYEα medium but not on TSBA or BCYEα–L medium; this takes advantage of the L-cysteine growth dependence of *Legionella* spp. Sometimes *Legionella* spp. will grow poorly on TSBA or BCYEα–L medium; at most, growth is only about 10% of the growth on BCYEα. Nutrient carryover from the primary-isolation plate is the explanation for this light growth; this can be proven by making a subculture of growth on BCYEα–L or TSBA on a second plate of the same medium. Rare *Legionella* spp. partially lose growth dependence for L-cysteine upon serial passage but, even so, grow more poorly on BCYEα–L than on BCYEα; these species include *L. nagasakiensis*, *L. oakridgensis*, and *L. jordanis*, none of which have been reported to cause more than two cases of LD each. TSBA performs almost as well as does BCYEα–L for determining L-cysteine dependence for clinical isolates and may be less expensive, depending on the number of isolates tested per plate. If a blood-containing agar medium, rather than BCYEα–L, is used as the screening plate for L-cysteine dependence, great care must be taken that the medium base is not too rich. For example, *Brucella* blood agar will support *L. pneumophila* growth almost as well as BCYEα medium, and other blood-containing media have been described to do the same (85). The plates are incubated overnight at 35 to 37°C or until there is visible growth on the BCYEα plate. Compare the relative amounts of growth on the plates to determine if there is L-cysteine dependence. Most *Legionella* spp. produce a characteristic dank odor that is very specific to the trained nose when they exhibit pure growth.

Common mimics of *Legionella* species colonies on BCYEα plates include *Eikenella corrodens*, *P. aeruginosa*, *Flavobacterium* spp., *Sphingomonas* spp., and some *Bacillus* spp. All of these bacteria grow equally well on BCYEα and BCYEα–L media, but when young, they often grow as speckled colonies on BCYEα plates. *Francisella tularensis* can grow well on BYCEα agar but has no resemblance to *Legionella* species colonies. However, *F. tularensis* is the only Gram-negative bacterium other than *Legionella* spp. that exhibits L-cysteine growth dependence. The colony morphology of *F. tularensis* is not speckled but rather is opaque and homogeneous. Adding to the confusion is that some serotyping reagents for *Legionella* spp. may cross-react with *F. tularensis*. There is one case report of the misidentification of *F. tularensis* as *L. pneumophila* (86). When tularemia is suspected, more-stringent safety precautions are needed. Of note, some *Bacillus* species mimics can stain as Gram-negative rods, have inapparent sporulation, and do not grow on TSBA (but will grow on BCYEα–L) (87). With prolonged incubation, colonies of *E. corrodens*, *Sphingomonas* spp., and *Flavobacterium* spp. change color and no longer resemble *Legionella* spp.; *E. corrodens* colonies become light to dark green, *Flavobacterium* spp. bright yellow, and *Sphingomonas* spp. a grayish yellow. Very young *P. aeruginosa* and *Bacillus* species colonies resemble the speckled flat to slightly convex young *Legionella* species colonies but with prolonged incubation change their morphology, making them easily recognizable as non-*Legionella* species colonies. *Bordetella pertussis* colonies may appear late on BCYEα plates, although this bacterium neither is cysteine dependent nor possesses colony morphology similar to that of *Legionella* spp. *B. pertussis* has been reported to be misidentified as *Legionella* spp., abetted by serological cross-reactivity (88). Because many different bacteria may cross-react with serological reagents used for typing and identifying *Legionella* spp., it is crucial to become familiar with the morphology and growth characteristics of this genus; relying exclusively on serotyping to identify *Legionella* spp. could result in mistaken identification.

Microbiologists should know that some pathogenic fungi and higher bacteria grow well on BCYEα medium, presenting both potential biohazards and the opportunity to diagnose unsuspected infections. *Coccidioides* spp. often grow within a day or two on this medium and can rapidly form arthroconidia; as such, they present a biohazard. *Blastomyces dermatitidis* also grows well and converts to the mold phase within a few days. It is likely that other pathogenic fungi grow equally well on this very rich medium. *Nocardia* spp. and rapidly growing mycobacteria often grow quite well on this medium, making BCYEα medium and its selective variants plating media of choice for the laboratory diagnosis of nocardiosis (89, 90). Bacteremia from *Nocardia* spp. can sometimes be diagnosed by subculture of blood culture bottles to BCYEα.

IDENTIFICATION FROM BACTERIAL COLONIES

Basic Identification

Once L-cysteine dependence has been confirmed, further identification of *Legionella* spp. in a clinical laboratory relies almost exclusively on serotyping the bacteria, using either immunofluorescence or agglutination methods (50, 91). Prior to attempting identification by serotyping, the plate should be illuminated with long-wave UV light in a darkroom; some *Legionella* spp. other than *L. pneumophila* fluoresce a brilliant bluish white and some a brilliant red; such bacteria are best identified by a reference laboratory and are not *L. pneumophila*. *L. pneumophila* fluoresces a very pale yellow-green, usually with diffusion of the fluorescent pigment into the culture medium; this is not specific for this species, and sometimes young cultures are completely nonfluorescent. An excellent and specific FDA-cleared fluorescein isothiocyanate (FITC)-labeled monoclonal antibody to all serogroups of *L. pneumophila* is available (Monofluo; Bio-Rad). Also an excellent non-FDA-cleared

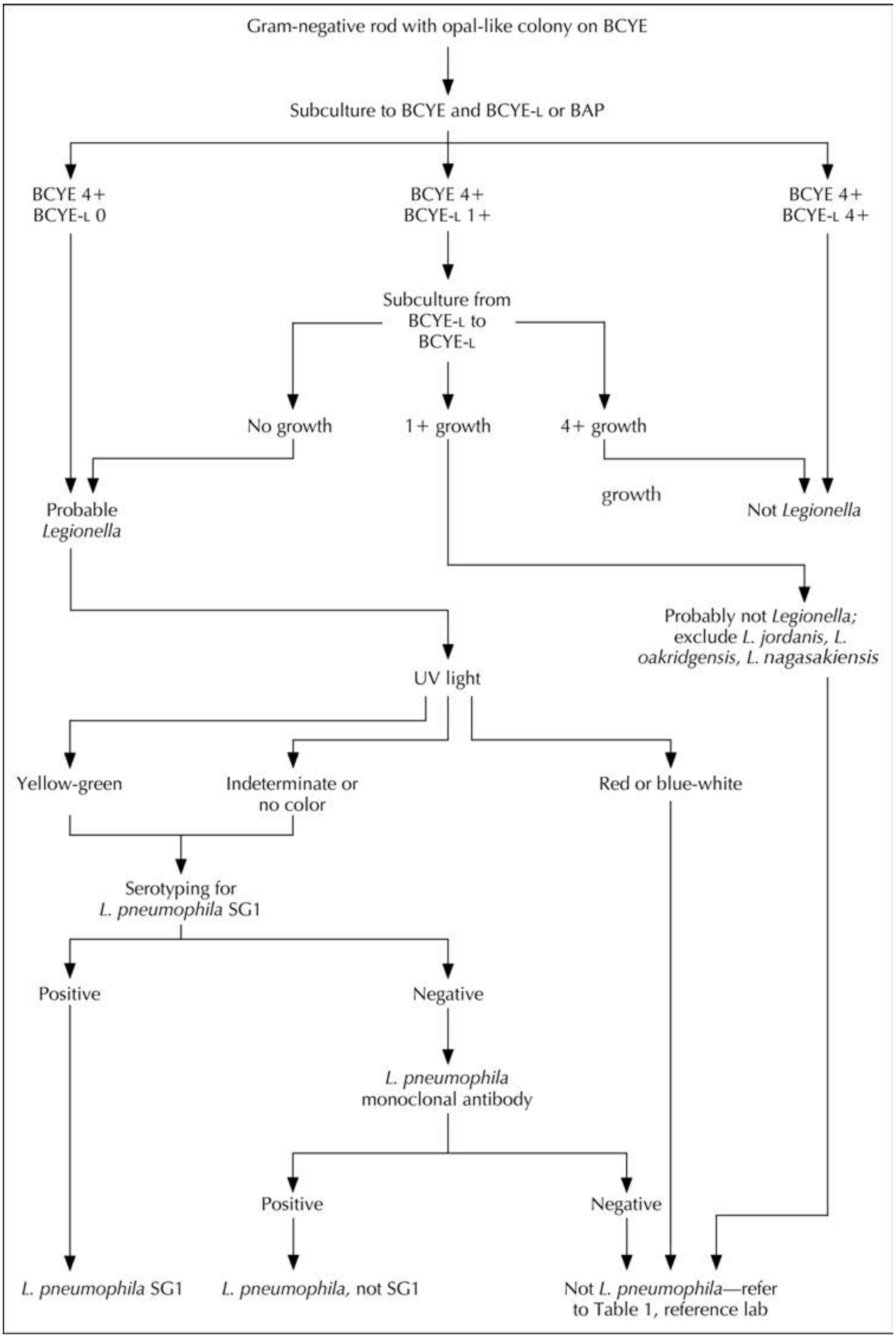
Gram-negative rod with opal-like colony on BCYE
Subculture to BCYE and BCYE-L or BAP
BCYE 4+
BCYE-L 0
BCYE 4+
BCYE-L 1+
BCYE 4+
BCYE-L 4+
Subculture from BCYE-L to BCYE-L
No growth
1+ growth
4+ growth
Probable *Legionella*
growth
Not *Legionella*
Probably not *Legionella*; exclude *L. jordanis*, *L. oakridgensis*, *L. nagasakiensis*
UV light
Yellow-green
Indeterminate or no color
Red or blue-white
Serotyping for *L. pneumophila* SG1
Positive
Negative
L. pneumophila monoclonal antibody
Positive
Negative
L. pneumophila SG1
L. pneumophila, not SG1
Not *L. pneumophila*—refer to Table 1, reference lab

FIGURE 3 Identification flow scheme for basic identification of *Legionella* spp. grown from a BCYEα plate. Abbreviations: BCYE, BCYEα; BCYE-L, BCYEα made without LL-cysteine; BAP, tryptic soy blood agar; UV light, colony fluorescence and color when illuminated with long-wave (360-nm) UV light. doi:10.1128/9781555817381.ch49.f3

FITC-labeled *L. pneumophila* serogroup 1 (Philadelphia 1 strain)-specific polyclonal antiserum is available (m-TECH, http://www.4m-tech.com/). Serotyping can also be performed using non-FDA-cleared latex agglutination antisera, available in a variety of formats (Oxoid, United Kingdom; Denka Seiken, Japan; bioMérieux, France); few data on the performance of these reagents are available.

Since more than 90% of *Legionella* species isolates are *L. pneumophila* serogroup 1, a reagent and time-saving technique is to first test with *L. pneumophila* serogroup 1 antibody; strains negative with this antibody can then be tested with the species-specific monoclonal antibody. Extraordinarily rarely, some *L. pneumophila* serogroup 1 strains do not react with Philadelphia 1 strain antibodies, making it possible that a serogroup 1 strain could be missed with this reagent; additional serogroup 1 antibodies are available (m-TECH). Testing has to be conducted properly for valid results, with the commonest errors being false-negative results because of a prozone phenomenon and false-positive results from contaminated buffers, contaminated wash solutions, or cross-contamination from controls (50). Strains that fail to react with *L. pneumophila* antibodies are best identified by reference or public health laboratories that have other typing sera and molecular identification methods in use.

Gram-negative rods isolated from clinical specimens that are morphologically consistent with *Legionella* spp. and are L-cysteine dependent for growth can be reported as presumptively *Legionella* spp. in the absence of reactivity with typing sera. Gram-negative rods characteristic of *Legionella* spp. that react with *L. pneumophila* monoclonal antibody or *L. pneumophila* serogroup 1 polyvalent antibody can be presumptively identified as *L. pneumophila* or *L. pneumophila* serogroup 1, and the identification can be finalized the next day once L-cysteine growth dependence is confirmed. If the isolate is nonreactive with *L. pneumophila* species-specific antibody or if it is *L. pneumophila* but not serogroup 1, then the isolate should be further typed by a reference laboratory. All clinical isolates should be frozen at −70°C in tryptic soy broth in 10% glycerol and subcultured on a BCYEα slant for possible analysis by public health authorities.

Infections caused by more than one *Legionella* spp. or more than one *L. pneumophila* serogroup have been reported (92–94). This can sometimes be of importance in determination of the source of an epidemic. Colonies with different morphologies usually represent variants of the same strain but sometimes represent different species and serogroups. Identifying and typing multiple *Legionella* species colonies is the only way to detect such cases.

Advanced Identification

Accurate identification of *Legionella* spp. other than *L. pneumophila* and *L. pneumophila* serogroup 1 can be quite difficult because of serologic cross-reactivities between species and serogroups, biochemical inertness, and the phenotypic identity of different species. Identification to the species or serogroup level for these bacteria is usually not of major clinical significance but may have significant public health and scientific importance. Investigation of LD outbreaks and sometimes single cases of the disease requires knowledge of *Legionella* species identity and subtype; this identification if needed can be performed by a public health or reference laboratory as long as isolates are frozen. In addition, strain typing can be performed directly from the original specimen using sequencing of PCR products (60). The identification techniques used by reference laboratories include serotyping using collections of antisera and molecular methods.

Serotyping of *Legionella* spp. is carried out using polyclonal antisera produced by the CDC, other public health agencies, and commercial laboratories (m-TECH, Denka Seiken, Oxoid, and others). Either immunofluorescence or agglutination reactions are used, with no clear evidence of superiority of one technique over the other (95). Some producers make polyvalent serum pools that react with a large number of species or serogroups, which can be useful to reduce the number of monovalent antisera used. The specificity of the polyvalent antisera has not been studied in great detail, although at least one product reacted with a number of non-*Legionella* spp. (96). This makes it important that monovalent typing be performed and that the bacteria meet minimal phenotypic criteria for *Legionella* spp. Unfortunately, cross-reactions between different species and serogroups occur even when monovalent antisera are used (4, 97–101). Use of cross-adsorbed polyvalent antisera has been described as a research tool to avoid this problem of cross-reactions, but these reagents are not available outside some research laboratories (102–104). In addition to intragenus cross-reactions, a large number of cross-reactions of monovalent polyclonal antibodies to non-*Legionella* spp., including *P. aeruginosa*, *Flavobacterium* spp., *Bacteroides fragilis*, *Capnocytophaga ochracea*, *B. pertussis*, *Bordetella bronchiseptica*, and possibly *Burkholderia pseudomallei*, have been reported (105–111). Antibody to *L. pneumophila* serogroup 1 is quite specific, but otherwise enough cross-reactions exist to make serological identification only presumptive. Antisera to newer *Legionella* spp. are often unavailable.

Monoclonal typing antibodies reduce the number of cross-reactions with Gram-negative bacteria but are commercially available only for the identification of *L. pneumophila* (112–115). However, great care must be used by experienced microbiologists, as cross-reactions with some non-*Legionella* spp. have been reported with the *L. pneumophila*-specific monoclonal antibody, including *S. aureus*, yeasts, and *Bacillus* spp.

Legionella spp. are relatively inert biochemically and will not be identified using conventional tube or commercial panel biochemical tests. The few biochemical characteristics described for *Legionella* spp. that can be determined in most laboratories, such as oxidase, catalase, and β-lactamase tests, are unhelpful for identification of these bacteria.

The gold standard for the identification of new *Legionella* spp. has been DNA-DNA hybridization analysis (116). This method is tedious, expensive, and labor-intensive and requires special expertise as well as a large collection of reference DNA standards. As such, DNA-DNA hybridization analysis is not used to identify already-known species.

Molecular identification of *Legionella* spp. has replaced other identification techniques in research and specialty laboratories for several reasons. These include the labor cost and time required to serotype a strain, serological cross-reactions, the limited availability of antibodies to newer strains, the lack of specific and easy biochemical characterization methods, and the increasing availability of inexpensive DNA sequencing and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) instrument systems.

MALDI-TOF MS is a rapid and cost-effective method for the species-level identification of *Legionella* spp. (117).This technique is currently unable to perform serogroup-level identification. Major disadvantages are a lack of comprehensive databases and the fact that MALDI-TOF can identify only known species. About 80 to 90% of isolates tested by reference laboratories are correctly identified to the species level by this method, with some portion of this low sensitivity being due to incomplete databases. Almost all isolates not identified by this method are given low reliability scores but correct identifications (117). Once the databases are more complete, MALDI-TOF MS should be able to identify almost all known *Legionella* spp. present in the databases. If an isolate cannot be identified by MALDI-TOF MS, DNA sequence analysis is used to identify the bacterium.

DNA sequence-based identification methods take advantage of the specific 16S rRNA or *mip* gene sequences of the different *Legionella* spp. (3, 118–120). A *mip* database, procedure instructions, including primer sequences, *mip* sequence alignment software, and other genomic software tools are all available online (http://webarchive.nationalarchives.gov.uk/20140714084352; http://www.hpa.org.uk/web/HPAweb&HPAwebStandard/HPAweb_C/1195733805138). Sequence analysis of 16S rRNA has been used to identify several new *Legionella* spp. (121–124). Partial sequencing of 16S rRNA (500 bp) is able to accurately identify all *Legionella* spp. to the genus level, all *L. pneumophila* strains, and about 90% of *Legionella* spp. other than *L. pneumophila* to the species level; incorrect species-level identification is a problem for the more unusual *Legionella*. spp. (14, 125). The DNA sequence of the *rpoB* gene has been shown to distinguish between species as well as or better than 16S rRNA or *mip* gene sequencing (126). Intergenic 16S-23S ribosomal spacer PCR analysis has been successful for species identification and does not require DNA sequencing (127); the lack of available databases and the paucity of strain data for many species limit the usefulness of these techniques. The relative performances of *mip* sequencing and full-length 16S rRNA sequencing are not known, but partial 16S rRNA sequencing should not solely be relied on when an unusual *Legionella*. sp. is identified. Limited numbers of reference strains, especially of some unusual species, have been tested by these methods, leaving open the possibility of incorrect classifications.

PCR-based methods can be used to distinguish between *L. pneumophila* serogroup 1 and other serogroups (66, 128). PCR can also be used to distinguish Pontiac/MAb 1/MAb 3-1 strains of *L. pneumophila* serogroup 1 from non-Pontiac strains (24, 128).

There are several reports of genotypic discordances for identical serogroups and vice versa (129–131). Sequencing of the *L. pneumophila dnaJ* gene appeared to be able to distinguish between some, but not all, *L. pneumophila* serogroups (118); different serogroups that had very similar genotypes had been shown by other methods to have discordant genotypes and serogroups. More-extensive testing of this method needs to be performed before it is put into routine use.

TYPING SYSTEMS

Typing of *Legionella* spp. is important for public health investigations to help link culture-positive environmental sites with clinical isolates during an epidemic of the disease. Typing cannot be used by itself to determine the environmental source of an outbreak and must be accompanied by an epidemiologic investigation. Otherwise, incorrect conclusions may be made about epidemic sources (132, 133). This problem is due to clonal distributions of environmental and clinical *Legionella* spp. (134–137) and to the poor specificity of some typing techniques (136, 138).

Monoclonal antibody typing plays a major role in subtyping *L. pneumophila* serogroup 1 isolates and, when used with molecular methods, can increase typing specificity (136, 139, 140). Used by itself, monoclonal antibody typing may not be specific enough to distinguish between closely related strains, but this method can be very helpful in epidemic situations as a quick screening test of environmental isolates to determine which isolates to genotype.

Sequence-based typing appears to be the most specific and precise molecular subtyping system for both *L. pneumophila* and *L. pneumophila* serogroup 1 (141). A standardized pulsed-field gel electrophoresis method yields reproducible results and is used as a reference typing method by one national laboratory (137). As whole-genome sequencing and sequence analysis become less expensive, more automated, and less labor-intensive, it is possible that this method will be used in the future for molecular typing of isolates (142).

ANTIBODY DETERMINATION

LD can be diagnosed by demonstration of an increase in antibodies to killed bacterial cells (44). The immunofluorescent-antibody assay (IFA) is considered the gold standard method. While most patients develop both IgG and IgM responses, some develop IgM-only, IgG-only, or IgA-only responses, making it necessary to test for total immunoglobulin response and not just IgG. In addition, IgM antibodies may persist for as long as a year after infection, making the presence of IgM a poor marker of acute disease (143). About 75% of patients with culture-proven nosocomial *L. pneumophila* serogroup 1 LD develop seroconversion to the bacterium, whereas the test seems to have higher sensitivity in LD epidemics. Seroconversion requires weeks to months after infection, with only about a 50% seroconversion rate after 2 weeks; for optimal test sensitivity, acute-phase serum should be frozen while convalescent-phase sera are collected at 2, 4, 6, 9, and 12 weeks postinfection. Parallel testing of sera is required for the best specificity. The most specific testing is for seroconversion to *L. pneumophila* serogroup 1 only, and the least specific is the use of polyvalent antigen preparations, with approximate test specificities of 99 and 90 to 95%, respectively. Serologic diagnosis is best used for epidemiologic studies because of the retrospective nature of serologic diagnosis and limitations of test specificity and sensitivity.

ANTIMICROBIAL SUSCEPTIBILITIES AND SUSCEPTIBILITY TESTING

The antimicrobial susceptibility of *L. pneumophila* grown in broth or on agar can give results having no clinical correlate. This is because of the intracellular location of the bacterium in human infection, to which not all antimicrobial agents gain access and retain activity (35). In addition, the complex broth and agar media used to grow *L. pneumophila* inactivate many drugs. There is no indication for performing antimicrobial susceptibility testing against *Legionella* spp. except in a research setting, where correlative studies of intracellular and experimental animal infection models can be performed. The microbiologist must not assume that a particular drug will be effective for the treatment of LD simply because the drug is active against *L. pneumophila in vitro*, nor should there be an assumption that drugs having low MICs for the bacterium *in vitro* will be more clinically effective than will drugs with higher MICs for the organism. Antimicrobial resistance to drugs used for LD treatment has never been documented to be responsible for clinical treatment failures. Low-level ciprofloxacin resistance of a clinical isolate of *L. pneumophila* was recently described;

whether this had any effect on clinical outcome, or whether the resistance developed during ciprofloxacin treatment, is unknown (144). When antimicrobial susceptibility testing is performed for research purposes, it is best done using buffered yeast extract broth in a microtube dilution format (145). Use of the Etest method has no useful role in the clinical or research testing of *Legionella* spp.

Antimicrobial agents having good intracellular activity against *L. pneumophila* include most macrolide, tetracycline, ketolide, and quinolone antimicrobial agents (15, 35). No β-lactam agent or aminoglycoside has acceptable intracellular activity against the bacterium. It is unknown if the intracellular activities of antimicrobial agents against *L. pneumophila* can be extrapolated to all other *Legionella* spp. and to treatment of infections caused by *Legionella* spp. other than *L. pneumophila*. Some of these other *Legionella* spp. may reside in subcellular compartments different from those of *L. pneumophila* and thus may respond differently to antimicrobial agents (146). However, macrolide and quinolone antimicrobials appear to be effective for the treatment of LD caused by *L. micdadei*, *L. longbeachae*, *L. bozemanae*, and *L. dumoffii* (147).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Multiple laboratory methods have to be used for optimal laboratory diagnosis of LD. Culture of *Legionella* bacteria from sputum, lung, or other respiratory specimens is the most specific (100%) method for diagnosis of the disease; it is very sensitive (~80 to 90%) in cases of severe untreated disease and insensitive (~20%) in those with mild disease. Culture may be the only positive diagnostic test, especially when *Legionella* bacteria other than *L. pneumophila* are causing the infection. Because of the technical difficulty of culture diagnosis, its expense, and its low sensitivity for nonsevere disease, several alternative diagnostic methods have been developed. The antigenuria assay is more sensitive than culture for the detection of community-acquired disease and especially epidemic disease. Even so, the antigenuria test is only about 60% sensitive under the best of circumstances, performs poorly for detection of nosocomial infection, and detects almost exclusively *L. pneumophila* serogroup 1. Antibody detection complements other laboratory diagnostic methods but is retrospective, as it requires seroconversion for greater test specificity and sensitivity. In addition, antibody results obtained using only polyvalent antigens must be viewed with circumspection. Molecular amplification tests are highly sensitive, but in the absence of FDA-cleared and marketed tests in the United States, extensive in-lab validation of homebrew assays is required before implementation. The performance of all laboratory diagnostic tests for non-*L. pneumophila* serogroup 1 LD is unknown but presumably is not as good as it is for the diagnosis of *L. pneumophila* serogroup 1 disease.

A major hindrance to the evaluation of all laboratory diagnostic methods is the lack of a good gold standard of LD. Culture diagnosis, while very specific, is known to be imperfect and of limited sensitivity, especially for epidemic LD. The performances of the diagnostic tests are often compared to that of culture diagnosis, which tends to overestimate test sensitivity and underestimate specificity.

Positive cultures for all *Legionella* spp. are virtually diagnostic of LD, providing there are supportive clinical findings, such as pneumonia. In contrast, single serum specimens showing elevated antibodies to *Legionella* spp. or to *L. pneumophila* are often not the result of LD. Only increases in titers of antibody to *L. pneumophila* serogroup 1, a test not usually commercially available, are specific enough for diagnosis, but even then, the use of appropriate techniques is required for optimal specificity. Detection of *L. pneumophila* serogroup 1 antigenuria is almost as specific as a positive culture, once heat-labile factors capable of causing false-positive tests are excluded. Positive test results must be reported promptly to the patient's clinician as well as to infection control and public health authorities.

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Approaches to Identification of Anaerobic Bacteria

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Anaerobic bacteria must be considered etiological agents in a number of clinical syndromes, including aspiration pneumonia, brain abscess, and intra-abdominal infection (1). Infections of prosthetic shoulder joints and corneal implants often involve *Propionibacterium acnes* (2, 3). Thus, complete diagnostic microbiology laboratories must have protocols in place to detect anaerobes, identify them, and determine their antimicrobial susceptibilities. New technologies such as pyrosequencing and other metagenomic tools are showing that anaerobes are involved in more types of infectious processes than were previously suspected based on culture methods alone, so future laboratory processing must adapt to include more molecular methods (4). Even autism has been associated with anaerobes in the bowel (5). *Clostridium difficile* has continued to be one of the most important agents of diarrhea for both outpatients and those in health care settings (6, 7). Lemierre's disease and its agent, *Fusobacterium necrophorum*, are still an often overlooked but important cause of chronic sore throat (8). New anaerobic species continue to be identified at an increasing rate due to new molecular tools (see the following chapters). This chapter presents an approach to general diagnostic (not research-oriented) anaerobic bacteriology based on the resources and capabilities of laboratories.

Specimen choice, collection, transport, and handling are important activities leading to clinically relevant results involving anaerobes. Because of the presence of anaerobes in all mucous membranes of mammals, specimen collection requires extreme caution to avoid contamination by the resident microbiota. A recently published guideline document contains syndrome-based specimen collection and handling procedures (9). Thus, aspirates, curetting specimens from deep wound tissue, and tissue biopsy specimens are recommended (also see chapter 18).

Respiratory tract samples must be collected to avoid oral and nasal secretions. For example, chronic sinusitis diagnosis requires an aspirate obtained by needle and syringe through the palate or aspirated endoscopically through a protected collector (10). Because transtracheal aspiration is rarely performed today, protected specimen brush samples obtained during endoscopy are the most common acceptable sample for anaerobic culture of lung abscess or other lung infection (11). They must be cultured quantitatively to determine the clinical relevance of isolates. The surgeon should be provided with a freshly boiled tube containing 1 ml chopped-meat broth or anaerobic broth (thioglycolate), into which the brush is dropped after it is cut off the shaft using sterile scissors. This protocol requires notifying the laboratory in advance so that the broth can be supplied to the operating attendants. Once received in the laboratory, the broth can be vortexed and a 0.01-ml calibrated loop can be used to inoculate anaerobic and aerobic media. Potential pathogens are usually present in numbers >1,000 CFU/ml (>10 colonies per plate). Physicians often obtain a percutaneous needle aspiration (also called fine-needle aspiration) through intact skin overlying an area of infiltration in the lung, using radiological guidance (12). These samples contain only the tiniest volume of material and must be ground up (if solid tissue) and/or diluted in anaerobic broth to stretch the sample for inoculation of all necessary media. This process naturally results in lower recovery of pathogens present in small numbers. Throat swabs for detection of *F. necrophorum* require special consideration (13).

Because of the relative intolerance of anaerobes to atmospheric oxygen, specimens for anaerobic culture must be transported in containers that exclude air. Some types of flocked swabs have been shown to maintain the viability of anaerobes during transport (<24 h), but due to the predilection of swabs to pick up contaminating biota and the very small volume that they absorb, they should be reserved for special cases where no other specimen type can be obtained, such as in cases of brain abscess (14). Incubation of blood in at least one anaerobic blood culture bottle should be part of the standard blood culture protocol (15). Chapter 18 of this *Manual*, the *Wadsworth-KTL Anaerobic Bacteriology Manual* (1), and Clinical and Laboratory Standards Institute (CLSI) Document M56-A, *Principles and Procedures for Detection of Anaerobes in Clinical Specimens* (16), expand on those sites from which anaerobic bacteriology cultures should be performed. Transport devices include small vials with special media and glass beads to break up clumps designed for dental samples (only laboratories with specific expertise should attempt culturing periodontal and other dental infection samples), larger tubes with anaerobic atmosphere and oxygen-absorbing gel to maintain organism viability (Anaerobe Systems, Morgan Hill, CA), sterile tubes for fluids, citrated or EDTA tubes for serosanguinous fluids in danger of clotting, and plain sterile containers for larger tissue samples (17).

For some anaerobic infections, a Gram stain is a critical step in the diagnosis and allows timely clinical management.

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Urinary tract infections caused by anaerobic bacteria can be detected first by Gram staining (18). When urinary tract infections persist and routine aerobic cultures are negative, prolonged incubation in 5% CO_2 or anaerobic-medium inoculation should be considered. Molecular analysis of urine has shown previously unsuspected anaerobes in large numbers; these methods may be standard in the future (19). A sputum Gram stain displaying numerous polymorphonuclear leukocytes (PMNs) or degenerating PMNs, rare or no squamous epithelial cells, and mixed morphologies, including small, Gram-positive cocci in chains; fusiform, Gram-negative rods; and Gram-negative coccobacilli, may be the only laboratory test suggesting aspiration pneumonia, as the culture will yield only normal respiratory biota. For serious necrotizing fasciitis, myonecrosis, or clostridial gas gangrene, Gram stains showing rare degenerative PMNs and anaerobic morphotypes (boxcar-shaped, Gram-positive or variable rods, for example) should be supportive evidence that a patient requires emergent surgery. The special syndrome of bacterial vaginosis, indicated by lack of *Lactobacillus* morphotypes and numerous Gram-variable coccobacilli (see chapter 18 for more information), is best diagnosed by Gram staining. In fact, culture is not recommended. Newer studies using molecular methods have shown that many organisms implicated in bacterial vaginosis cannot be recovered in culture (20, 21).

Finally, *C. difficile* infection is best diagnosed by toxigenic culture, but given that this method takes too long for clinical impact, molecular detection of the toxin B gene yields the next-best results (22). Popular enzyme immunoassays for toxins A and B are now known to be unacceptably insensitive and even nonspecific (23, 24). If culture is being done, such as for epidemiological studies or method comparisons, stool should be inoculated both to selective media and to enrichment broth. Taurocholate-containing agar enhances vegetation of spores and yields the best recovery (25). Stool can be pretreated by heating at 80°C for 10 min (to select for spores) or, alternatively, by mixing it 1:1 in 95 to 100% ethanol for 1 h (to kill vegetative cells) and then incubated in broth and plated to selective media (1).

Initial anaerobic culture processing should always include Gram staining and, for most specimens, plating to anaerobic blood agar (containing horse or sheep blood, additional hemin, and vitamin K), *Bacteroides* bile esculin agar, and kanamycin-vancomycin agar with laked sheep blood and an anaerobic broth. Commercial media prepared totally without oxygen exposure has been shown to enhance recovery of some anaerobes (26). If the plates recover organisms, the broth need not be evaluated. However, the broth should be held for up to 14 days in some circumstances, such as for detection of joint infection. As soon as possible after inoculation, media should be placed into an anaerobic atmosphere. Methods such as nitrogen flushing holding jars have been used. Today, smaller incubation containers, such as plastic envelopes, boxes, and shorter jars, and automated gas flushing instruments, e.g., Anoxomat (Mart Microbiology, Drachten, The Netherlands) and Whitley Jar Gassing System (Microbiology International, Frederick, MD), are used to shorten the exposure of plated samples to air (toxic oxygen). Use of an anaerobic chamber for all sample manipulations and incubation is the best method to ensure viability of fastidious anaerobes. If rapid creation of an anaerobic atmosphere is not possible for inoculated plates, it would be better to wait until enough anaerobic samples have been received to fill up one jar and then plate them all at once, closing and gassing the jar as quickly as possible. Jars and boxes should not be opened until after 48 h of incubation to prevent premature death of some slower-growing microbes by exposure to air during their logarithmic growth phase. *Clostridium perfringens*, the agent of gas gangrene, however, grows very quickly and can be identified after overnight incubation. If the clinical situation or initial Gram stain suggests this microbe, it may be prudent to incubate plates individually in plastic anaerobic envelopes (GasPak EZ Anaerobe Pouch System [Becton Dickinson Microbiology Systems, Cockeysville, MD]; AnaeroPack [Mitsubishi Gas Chemical America, Inc., New York, NY]) so that the plates may be examined early (27).

The initial Gram stain will yield preliminary information about the culture. Clinically important information should be telephoned to the physician or caregiver. For some laboratories, this may be the only anaerobic procedure possible. It is better to interpret Gram stains well and report relevant results quickly than to perform inadequate cultures, which will lead to misleading results. Poor specimen handling or transport, exposure to air, lack of good anaerobic media or atmosphere, early opening of incubation chambers, and other factors will result in growth of only the hardiest anaerobes, generating incomplete results. As more information becomes available, the use of newer molecular tools may be necessary for complete anaerobic microbiology.

Initial examination of colonies should be performed using a stereomicroscope or at least a strong magnifying glass. Colony morphologies that appear similar when observed at a distance can be differentiated when magnified, and the presence of tiny colonies near larger ones can be discerned. For culture methods, use the pointed end of a broken sterile wooden stick, touch the tip of a colony, and then touch the colony paste to an anaerobic blood plate, a chocolate agar plate, and a spot on a glass slide. This ensures that the same colony goes onto both plates and the slide. The blood plate should be streaked in quadrants, and special potency disks of 1,000 µg of kanamycin, 5 µg of vancomycin, and 10 µg of colistin can be arranged on the first quadrant. Susceptibility (≥10-mm zone diameter) of the different antibiotics is used to help with further identification (28, 29). The chocolate agar plate is incubated in 5% CO_2 to test a large number of anaerobes in a pie plate format for aerotolerance. Those that grow are not strict anaerobes and can be identified using routine methods. Some organisms can be identified quickly based on colony and Gram stain morphology and a few spot tests; others will require more-extensive methods. The multiparameter anaerobic (and corynebacteria) identification card for the VITEK system (ANC ID card; bioMérieux, Durham, NC) has acceptable performance for common anaerobic genera and would be a good choice for those laboratories without molecular assessment capability (30).

If more-involved phenotypic methods are necessary, available, and warranted by clinical considerations, and when more advanced methods such as matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry and sequencing are not possible, prereduced anaerobically sterilized biochemical agents, gas chromatography, and some newer technologies may be employed. MALDI-TOF has been used to identify anaerobes quickly and inexpensively (after initial purchase of the instrument) (31–33). Commercial MALDI-TOF instruments (MALDI Biotyper, Bruker Daltonics, Billerica, MA; VITEK MS, bioMérieux) are gaining favor in diagnostic laboratories (33). Sequencing of genetic markers, such as portions of the 16S rRNA gene and other useful genetic elements, is another method used for anaerobic identification today in clinical laboratories with molecular-assessment capability (34). If the filter “bacteria [ORGN] not uncultured [TITL]” is entered in the BLAST field named “Entry Query,” the BLAST database search will not be cluttered with uncultured clones.

Modifications such as pyrosequencing are also expected to be useful for anaerobic identifications (35). Chapters 4 and 6 also discuss general principles and the utility of these and other methods. The following chapters of this book (chapters 51 through 54) contain up-to-date taxonomic information, including changes from the last edition.

Susceptibility testing should be performed for clinically important anaerobes. As outlined by several authors, clinically important isolates include those isolated from blood cultures, brain abscess, heart valve or vascular graft tissue, bone biopsy from patients with osteomyelitis, joint aspirates, and isolates from well-collected prosthetic device infection sources. Others to test include likely pathogens from sterile body sites and those from patients who failed initial therapy. An excellent overview of current antimicrobials for anaerobes and testing methods was recently published (36).

The Etest (bioMérieux) has been a method to test individual organisms for many years, but its correlation to reference broth methods is still imperfect; which result best correlates to patient response is not clear (37). Another similar gradient strip method, the M.I.C. Evaluator Device (Thermo Fisher Scientific, Lenexa, KS), has also been evaluated with inconclusive results (38). Other systems include a spiral gradient endpoint system (36). Broth dilution performed in an anaerobic chamber is also acceptable, but interpretation of results may be difficult and current guidelines recommend its use only for members of the *Bacteroides fragilis* group (39). The CLSI standard method (agar dilution) is generally not utilized in clinical laboratories, of which only 21% did anaerobic susceptibilities at all in a 2008 survey (39, 40). A European group (EUCAST Subcommittee on Antimicrobial Susceptibility Testing of Anaerobe Bacteria) is also working on protocols.

It is clear that anaerobic bacterial protocols occupy a separate and distinct place in the clinical microbiology laboratory. Laboratories must determine the extent of effort they can devote to anaerobes and then develop their processes to perform only those protocols that they can guarantee will yield reliable, timely, and accurate results. Organisms of importance can always be sent to a reference laboratory for further studies in anaerobic chopped-meat broth or anaerobic transport vials.

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Peptostreptococcus, *Finegoldia*, *Anaerococcus*, *Peptoniphilus*, *Veillonella*, and Other Anaerobic Cocci

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TAXONOMY

Gram-positive anaerobic cocci (GPAC) are better known to most bacteriologists as peptococci or peptostreptococci. *Peptococcus* is only remotely related to other species of GPAC and is rarely cultured from human clinical specimens. *Peptococcus niger* is now the sole remaining representative of this genus. Until recently, most clinical isolates of GPAC were identified as species in the genus *Peptostreptococcus*; however, data obtained by molecular methods have led to extensive taxonomic changes during the last decades and also to the identification of new genera and species. Since 1998, the genus *Peptostreptococcus* has been divided into six novel genera (1–3). The type species, *Peptostreptococcus anaerobius*, and the recently described species *Peptostreptococcus stomatis* (4) are the only two species in the genus *Peptostreptococcus* that have been isolated from human specimens. *Peptostreptococcus magnus* and *Peptostreptococcus micros* were assigned to two new genera, *Finegoldia* and *Parvimonas*, respectively (3, 5). For the remaining peptostreptococci, three genera were proposed: *Anaerococcus*, *Peptoniphilus*, and *Gallicola* (1). *Anaerococcus* has eight species (*Anaerococcus hydrogenalis*, *A. lactolyticus*, *A. murdochii*, *A. octavius*, *A. prevotii*, *A. tetradius*, *A. vaginalis*, and a newly described species, *A. senegalensis* [6]); *Peptoniphilus* has 14 species (*P. asaccharolyticus*, *P. gorbachii*, *P. harei*, *P. indolicus*, *P. ivorii*, *P. lacrimalis*, *P. olsenii*, and, more recently, *P. methioninivorax* [7]; *P. tyrrelliae* and *P. coxii* [8]; and *P. duerdenii* and *P. koenoeneniae* [9], with *P. grossensis* [10] and *P. timonensis* [11] being effectively but not validly described to date). *Gallicola* contains only one species, *Gallicola barnesae*, which has not been reported from human specimens. Most recently, two new GPAC genera were described: *Murdochiella* (12) and *Anaerosphaera* (13).

Taxonomy of the other GPAC from human clinical specimens has also been under revision. *Streptococcus parvulus* has been transferred to the genus *Atopobium* as *Atopobium parvulum*. *Peptostreptococcus productus* was reclassified in a newly proposed genus, *Blautia*, as *Blautia producta* (14); *Peptostreptococcus saccharolyticus* has been transferred to the genus *Staphylococcus*. Table 1 shows the changes in classification of GPAC species.

The Gram-negative anaerobic cocci (GNAC), including the genera *Veillonella*, *Acidaminococcus*, *Megasphaera*, and *Anaeroglobus* plus the recently described *Negativicoccus*, have been classified in a single family, the *Acidaminococcaceae* (15). The genus *Veillonella* has 13 species, of which only *Veillonella alcalescens*, *V. atypica*, *V. denticariosi*, *V. dispar*, *V. parvula*, *V. rogosae*, and the newly described species *V. tobetsuensis* have been isolated from human oral cavities (16–19). Besides *Veillonella* spp., *Acidaminococcus fermentans* and *A. intestini* of the genus *Acidaminococcus*, *M. elsdenii* and *M. micronuciformis* of the genus *Megasphaera*, and *A. geminatus* of the genus *Anaeroglobus* have also been isolated from human clinical samples.

DESCRIPTION OF THE GROUP

The organisms included in this chapter are obligately anaerobic non-spore-forming, sometimes elongated cocci. The genera *Anaerococcus*, *Anaerosphaera*, *Finegoldia*, *Gallicola*, *Murdochiella*, *Parvimonas*, *Peptococcus*, *Peptoniphilus*, and *Peptostreptococcus* are Gram-positive, coccobacillary or occasionally coccoid cells. In Gram-stained preparations of pure cultures, cells vary in size from 0.3 mm to 2.0 mm and can be arranged in pairs, short chains, tetrads, small clusters, or irregular masses; most species are present as either chains or clumps. The ability to utilize carbohydrates varies greatly; some genera are asaccharolytic, but a few are strongly saccharolytic. For most species, the products of protein digestion appear to be the principal energy source. The genus *Staphylococcus* contains two species, *Staphylococcus saccharolyticus* and *Staphylococcus aureus* subsp. *anaerobius*, which initially grow under anaerobic conditions and become aerotolerant on subcultures. Strictly anaerobic *Staphylococcus epidermidis* is reported to be occasionally isolated from clinical specimens (20). The genera *Veillonella*, *Acidaminococcus*, *Megasphaera*, *Anaeroglobus*, and *Negativicoccus* are Gram-negative cocci. Cells vary in size from 0.3 μm to 2.5 μm. They characteristically occur in pairs, but single cells, masses, or chains may also occur. Carbohydrates are weakly fermented or not fermented. Gas is produced. The metabolic end products are the principal characteristics by which the genera can be differentiated.

EPIDEMIOLOGY

GPAC are part of the commensal microbiota of human mouth, upper respiratory and gastrointestinal tracts, female genitourinary system, and skin (21). *Parvimonas micra* is

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TABLE 1 Changes in classification since the last edition of this *Manual* of GPAC species isolated from human clinical specimens

Current classification	Previous classification(s)
Peptococcus niger	*Peptococcus niger*
Peptostreptococcus stomatis	*Peptostreptococcus anaerobius*
Peptostreptococcus anaerobius	*Peptostreptococcus anaerobius*
Parvimonas micra	*Peptostreptococcus micros*, *Micromonas micros*
Peptoniphilus asaccharolyticus	*Peptostreptococcus asaccharolyticus*
Peptoniphilus coxii	New species
Peptoniphilus duerdenii	New species
Peptoniphilus gorbachii	New species
Peptoniphilus indolicus	*Peptostreptococcus indolicus*
Peptoniphilus harei	*Peptostreptococcus harei*
Peptoniphilus ivorii	*Peptostreptococcus ivorii*
Peptoniphilus koenoeneniae	New species
Peptoniphilus lacrimalis	*Peptostreptococcus lacrimalis*
Peptoniphilus olsenii	New species
Peptoniphilus tyrrelliae	New species
Anaerococcus murdochii	New species
Anaerococcus prevotii	*Peptostreptococcus prevotii*
Anaerococcus tetradius	*Peptostreptococcus tetradius*
Anaerococcus octavius	*Peptostreptococcus octavius*
Anaerococcus hydrogenalis	*Peptostreptococcus hydrogenalis*
Anaerococcus lactolyticus	*Peptostreptococcus lactolyticus*
Anaerococcus vaginalis	*Peptostreptococcus vaginalis*
Finegoldia magna	*Peptostreptococcus magnus*
Murdochiella asaccharolytica	New species
Anaerosphaera aminiphila	New species
Slackia heliotrinireducens corrig.	*Peptostreptococcus heliotrinreducens*
Atopobium parvulum	*Streptococcus parvulus*
Blautia producta	*Peptostreptococcus productus*, *Ruminococcus productus*
Blautia coccoides	*Clostridium coccoides*
Blautia wexlerae	New species
Ruminococcus gauvreauii	New species
Staphylococcus saccharolyticus	*Peptostreptococcus saccharolyticus*

usually considered to be the predominant species of GPAC in the oral flora. The skin flora contains GPAC, with *Finegoldia magna* as the species identified most frequently, followed by *P. asaccharolyticus*. GPAC are opportunistic pathogens and account for approximately one-third of isolated anaerobic bacteria from clinical specimens (21, 22). They can be isolated from a wide variety of sites, of which the dominating are abscesses and infections of the skin and soft tissue, the mouth, bones, and joints, and the upper respiratory and female genital tracts. With increasing studies of GPAC as pathogens, certain species are being associated with specific types of infection. *F. magna* is most commonly associated with infections of the skin and soft tissue and with bone and joint infections. *P. anaerobius* and *A. prevotii* are most common GPAC associated with infections of the abdominal cavity and female urogenitary tract. *Parvimonas micra* is mainly recognized as an oral pathogen.

GNAC form part of the oral, genitourinary, respiratory, and intestinal floras of humans (21). *Veillonella* species are part of the normal mouth and urogenital floras. They are found in the greatest concentrations in saliva and on the surface of the tongue (23, 24). *Veillonella* can also be found in the upper respiratory tract and intestine. Occasionally, *Veillonella* species have been isolated from sites of infection, where they are typically part of a mixed culture. *Acidaminococcus* and *Megasphaera* are part of the intestinal flora but may also be recovered from certain infections.

CLINICAL SIGNIFICANCE

GPAC are opportunistic pathogens; they can cause various infections involving all areas of the human body, ranging in severity from mild skin abscesses to more serious and life-threatening infections such as brain abscess, epidural abscess, bacteremia, endocarditis, necrotizing pneumonia, and septic abortion. Of all isolated anaerobic bacteria from clinical specimens, GPAC account for approximately 25 to 30% (21, 25). The incidence of anaerobic cocci in pleuropulmonary infections such as lung abscess, necrotizing pneumonia, aspiration pneumonia, and empyema is about 40% (26, 27). Anaerobic cocci are also often isolated from skin and soft tissue infections and a range of chronic and acute wound infections such as anaerobic streptococcal myonecrosis, progressive bacterial synergistic gangrene, necrotizing fasciitis, crepitant cellulitis, chronic burrowing ulcer, and synergistic necrotizing cellulitis (21, 28, 29). These are severe infections, and the mortality rates may be as high as 75%. Other infections in which anaerobic cocci have been recognized as significant pathogens are oral and dental infections, female genital tract infections, and intra-abdominal infections (30–32). GPAC are also involved as pathogens in ocular infections, ear, nose, and throat infections, head and neck infections (including serious neck space infections), meningitis, pericarditis, bone and joint infections (including prosthetic joints), breast abscess, urinary tract infections, and anorectal sepsis with and without anal fistula (33, 34).

Estimation of the clinical significance of anaerobic cocci isolated from clinical specimens is often difficult, partly due to their fastidiousness and thus difficulty to isolate. The polymicrobial nature of GPAC infections, in addition to inadequate classification, has most likely contributed to the neglect of the clinical significance of individual species of GPAC. Molecular methods have led to improved identification of GPAC within various infections and have led to better acknowledgment of the clinical importance of GPAC. For example, La Scola et al. (35) recently identified GPAC in blood cultures and osteoarticular samples using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and 16S rRNA gene sequencing. Furthermore, a case of bacteremia caused by *Finegoldia* was also recently reported by Rosenthal et al. (36). Using pyrosequencing, several studies have demonstrated that besides aerobic species, anaerobes including *Peptoniphilus*, *Finegoldia*, and *Anaerococcus* are prominent colonizers of wound infections (37–41). Another interesting study indicated that when the microbial flora was identified with pyrosequencing for patients with chronic rhinosinusitis, anaerobic genera like *Peptoniphilus* predominated, in contrast to *Staphylococcus* species, which were detected using traditional methods (42).

Mostly, GPAC are isolated from polymicrobial infections, but in many cases, the organisms are isolated in pure culture; this is the case mainly for *F. magna*, although other species, like *P. anaerobius*, *P. asaccharolyticus*, *P. indolicus*, *P. micra*, *A. vaginalis*, *A. prevotii*, and *P. harei*, also occur (21, 22, 43) in pure culture.

F. magna is the most pathogenic and one of the most frequently isolated GPAC species found in human clinical specimens. It has been isolated in pure culture from a wide variety of infections at various body sites. These include cases of endocarditis, meningitis, and pneumonia, some of which have been fatal (44). *F. magna* is most commonly associated with infections of the skin and soft tissue, with bone and joint infections, and with chronic wounds, including diabetic ulcers and pressure ulcers, but it has also been isolated from cases of septic arthritis, prosthetic implant infections, breast abscess, diabetic foot infections, and upper respiratory tract infections, such as sinusitis and otitis media. Using culture-independent methods, *F. magna* was detected from patients with culture-negative endocarditis and prosthetic joint infections (45, 46). More recently, *F. magna* has been implicated in a case of toxic shock syndrome (36). These reports suggest that the overall significance of *F. magna* as a pathogen is underestimated and that more accurate detection and identification of this bacterium might lead to a changed view of the frequency of *F. magna* in relation to certain clinical conditions. Several virulence factors have been described for *F. magna*, such as protein L, peptostreptococcal albumin binding protein (PAB), SufA, and *F. magna* adhesion factor (FAF), as reviewed by Murphy and Frick (22). Recently, whole-genome sequencing furthered the understanding of the pathogenicity of this organism by elucidating both chromosomally encoded and mobile plasmid-mediated virulence factors (47). In a recent study by Donelli et al. (48), *F. magna* was described to have the ability to form biofilm, which could protect it from host immune defense as well as targeted antibiotic therapies.

P. anaerobius is involved in polymicrobial infections, including abscess of the brain, ear, jaw, pleural cavity, pelvis, urogenital tract, external genitalia, abdominal regions, nasal septum, and a range of acute and chronic wound infections (21, 22). It is one of the most common GPAC associated with infections of the abdominal cavity and female urogenital tract (21). The isolation of *P. anaerobius* from endocarditis patients has also been reported (49, 50). It is mostly associated with mixed-infection sites, but there have been some reports of isolation in pure culture (51–53). *P. anaerobius* has also been associated with various oral infections such as gingivitis and periodontitis and is one of the species found most frequently in the root canals of teeth with periapical abscess. *P. stomatis*, which was misidentified as *P. anaerobius* previously, is associated with infections of the human oral cavity, such as dentoalveolar abscesses and endodontic infections (4). This organism was detected in one-quarter of the cases from a large number of necrotic root canal samples (54).

Parvimonas micra is well recognized as an important oral pathogen. Although it is considered a natural commensal of the oral cavity, elevated counts of this organism are associated with periodontal destruction. It is also commonly isolated from other oral infections such as endodontic disease and peritonsillar infections. Recent advances in bacterial identification using molecular methods confirmed the association of *P. micra* with oral infections (55). *P. micra* is not restricted to the oral cavity; it is often isolated in mixed anaerobic infections from different body sites, including a range of skin infections, wound infections, otitis media, sinus infections, pleural empyema, intra-abdominal infections, anorectal abscess, septicemia, gynecological infections, vertebral osteomyelitis, and prosthetic joint infections.

The clinical importance of the genera *Peptoniphilus* and *Anaerococcus* has been supported by using molecular methods to analyze clinical samples. *Peptoniphilus* species such as *P. harei*, *P. ivorii*, and *P. indolicus* have been frequently found in chronic wound infection samples (37). Other studies using pyrosequencing reported high prevalences of *Peptoniphilus* species in diabetic ulcers (38, 39) and in chronic rhinosinusitis (42). *A. prevotii* is frequently recovered from clinical specimens derived from vaginal discharges and ovarian, peritoneal, sacral, and lung abscesses (56). In a recent survey of the bacterial diversity in biofilms of various wound types, *A. lactolyticus* and *A. vaginalis* were identified among the predominant species in diabetic foot ulcers and pressure ulcers (37). *A. vaginalis* and *A. prevotii* were also identified after recovery from blood cultures by mass spectrometry and 16S rRNA gene sequencing (35).

GNAC account for a very small percentage of the anaerobic cocci isolated from human specimens. Occasionally, *Veillonella* species have been isolated from sites of infection, where they are typically part of a mixed culture. The risk factors for *Veillonella* infection include periodontal disease, immunodeficiency, intravenous drug use, and premature birth (57, 58). Very rarely, *Veillonella* species have been the only etiologic agents identified in serious infections such as meningitis, osteomyelitis, prosthetic joint infection, pleuropulmonary infection, bacteremia, and endocarditis. The specific role of *Veillonella* as a pathogen is unclear, and very little is known about the virulence mechanisms of this organism.

The variety of infections in which anaerobic cocci are involved has been thoroughly reviewed and summarized (21, 22). Although the spectrum of infections has remained relatively unchanged since the extensive review by Murdoch in 1998 (21), the prevalence of these organisms as pathogens is clearly increasing.

COLLECTION, TRANSPORT, AND STORAGE OF CLINICAL SPECIMENS

Most GPAC isolated from human clinical material are not extremely oxygen sensitive. Specimens suspected of harboring anaerobic cocci should be collected, transported, and stored by methods outlined elsewhere (see chapters 18 and 50).

DIRECT EXAMINATION

Direct examination of specimens frequently provides the most rapid indication of microbial infection. A variety of microscopic, immunologic, and molecular techniques have been developed for direct examination of GPAC.

Microscopic morphology of the anaerobic cocci is varied. *P. anaerobius* and *B. producta* are large coccobacilli that form chains, especially in broth cultures. Other anaerobic cocci, such as *A. tetradius*, *A. prevotii*, and *F. magnus*, have cells greater than 0.6 µm in diameter in pairs and clusters and may resemble staphylococcal cells. *P. micra* cells are less than 0.7 µm in diameter and occur in packets and short chains; difference in cell size has been used as one characteristic to distinguish *P. micra* and *F. magnus*. Serologic studies have described an indirect fluorescent-antibody test for *P. anaerobius*, *P. micra*, and *B. producta* (59), but such tests have not been developed or commercialized further. Molecular methods, such as nucleic acid probe hybridization and PCR amplification, are not yet standardized or available commercially for the direct demonstration of medically important GPAC from clinical specimens, but the PCR technique has been applied for detection of anaerobic cocci (60–64) for research purposes. Several studies reported using the checkerboard DNA-DNA hybridization method to directly detect microbes from oral clinical samples, including *P. micra* (65–68). Most recently, high-throughput pyrosequencing analysis of 16S rRNA genes has significantly increased our understanding of the microbiological etiology of an infection. For example, pyrosequencing was used to detect GPAC in various wound types, including diabetic foot ulcers, pressure ulcers, infected root canals, and brain abscesses (37–39, 69).

ISOLATION PROCEDURES

Routinely used anaerobic plate media, such as brucella, Columbia, or Schaedler agar base supplemented with 5% sheep blood, vitamin K_1, and hemin, support the growth of these microorganisms. The usual procedures for anaerobes should be followed. Many of these organisms require high moisture content for optimal growth, so fresh media should be used. Laboratories unable to prepare their own media may wish to consider the use of commercially prepared, prereduced, anaerobically sterilized blood agar (Anaerobe Systems, USA; bioMérieux, France; HiMedia, India). These media have an extended shelf life, up to 6 months, and yield results comparable to or better than to those obtained with fresh media.

GPAC are heterogeneous; a single medium is unlikely to support the growth of all representatives and be reasonably selective. Wren (70) showed that nalidixic acid-Tween (NAT) blood agar gave better isolation than neomycin blood agar, possibly due to the particularly inhibitory nature of neomycin against GPAC, but recommended that a combination of different media (the best combinations being NAT and neomycin-vancomycin agar or NAT and neomycin blood agar) be used to maximize recovery rates. Turng et al. (71) described *Parvimonas micra* medium, a selective and differential medium for *P. micra* which contains colistin-nalidixic acid agar (Difco, Detroit, MI), a selective base for Gram-positive cocci supplemented with glutathione and lead acetate. Strains of *P. micra* can use the reduced form of glutathione to form hydrogen sulfide, which reacts with lead acetate to form a black precipitate under the colony. Tween 80 supplementation (0.02%) of media may improve growth of some GPAC.

Gutierrez de Ferro et al. (23) tested different media for recovery of *Veillonella* spp. from saliva samples and concluded that a selective medium for *Veillonella* with vancomycin and laked blood gave the greatest recovery of *Veillonella*.

IDENTIFICATION

Some GPAC, particularly strains of *P. asaccharolyticus*, decolorize readily with Gram stain and can be confused with Gram-negative anaerobes such as veillonellae. GPAC can be distinguished from GNAC by special potency disks (vancomycin, 5 g; kanamycin, 1,000 g; and colistin, 10 g). The cell morphology of older cultures of GPAC can be very irregular, with many coccobacillary and rod-like forms. It is also important to distinguish GPAC from microaerophilic organisms, such as strains of *Streptococcus* species. A simple and reliable test is to apply a 5-g metronidazole disk to the edge of an inoculum; GPAC show a zone of inhibition of 15 mm or greater, whereas microaerophilic strains show no zones after incubation for 48 h (72).

P. anaerobius is the only GPAC that gives a zone of inhibition of ≥12 mm around a 5% sodium polyanethol sulfonate (SPS) disk. *Parvimonas micra* also exhibits a zone of inhibition with SPS; however, the zone is usually <12 mm. Most *P. anaerobius* strains form distinctive colonies on enriched blood agar; they are 1 mm in diameter after 24 h and gray with slightly raised off-white centers and a distinctive sweet odor (72). *Parvimonas micra* and *F. magna* can be readily distinguished by a combination of colonial morphology and proteolytic enzyme profiles, supported by Gram-stained cell morphology to assess the cell size. An anaerobic coccus with a milky halo around the colonies and small cells (<0.6 µm) can be presumptively identified as *Parvimonas micra* (72). *F. magna* cells are larger than those of most peptostreptococci. Published data (5) also indicate that they can be differentiated by enzymatic tests for proteolytic activity such as proline arylamidase, phenylalanine arylamidase, and tyrosine arylamidase.

P. asaccharolyticus is another GPAC species that was reported to be frequently isolated from human clinical specimens, but studies indicated that most of the clinical strains identified as *P. asaccharolyticus* in the past are *P. harei* (73), which has the same biochemical features as *P. asaccharolyticus* and can be differentiated from *P. asaccharolyticus* only by its irregular colony and cell morphologies. Therefore, the incidence of *P. asaccharolyticus* in clinical material is potentially highly overestimated. Cells of *P. harei* vary considerably in size (diameter, 0.5 to 1.5 µm) and shape (circular, oval, or elliptical), whereas cells of *P. asaccharolyticus* are more uniform. Colonies of 5-day cultures on enriched blood agar are approximately 1 mm in diameter, entire, flat, and translucent.

Similar observations were made for *A. prevotii* and *A. tetradius*, which were reported as common species of GPAC in human clinical material in early surveys. However, nucleic acid studies indicate that they are very heterogeneous. Again, our study based on 16S rRNA gene sequencing indicated that a large percentage of organisms identified as *A. prevotii* or *A. tetradius* are strains of *A. vaginalis* (74). It is likely that strictly defined strains of *A. prevotii* and *A. tetradius* are only occasionally recovered from most clinical specimens. The activity of pyroglutamic acid arylamidase might be useful for differentiation of *A. prevotii* and *A.*

tetradius; however, distinctions cannot be generalized because insufficient numbers of strains of each species have been reliably identified. *A. prevotii* and *A. tetradius* can be distinguished from other recognized species of GPAC by production of α-glucosidase and β-glucuronidase. Strains of other saccharolytic GPAC, such as *A. vaginalis* and *A. lactolyticus*, can be differentiated by their enzyme profiles.

Table 2 summarizes the differential characteristics of GPAC. Based on published data from our group and others (75), we developed a flowchart for rapid identification of GPAC (Fig. 1). The identification is based on phenotypic tests that can be performed in any diagnostic laboratory. Most of the information presented here relates to the phenotypic characteristics of strains isolated from humans.

Several identification systems, such as the Rapid ID 32A (bioMérieux, Marcy L'Etoile, France) and RapID ANA II (Remel, Inc., Lenexa, KS) systems, are available commercially for the rapid identification of anaerobes. Our most recent evaluation of the Rapid ID 32A kit for identification of GPAC by comparison with 16S rRNA gene sequencing identification showed that the system is good for accurate identification of *P. micra*, *P. anaerobius*, *F. magna*, and *P. asaccharolyticus* but not the others.

Veillonella, *Acidaminococcus*, and *Megasphaera* comprise the principal genera of GNAC. The identification of *Veillonella* at the species level remains uncertain and inconvenient owing to the lack of conventional phenotypic and biochemical discriminating tests. Moreover, serologic tests are no longer available. Table 3 contains a key for differentiating the genera of GNAC (15).

IDENTIFICATION

Molecular Methods

The introduction of molecular-based methods for bacterial identification enabled a real paradigm shift to take place in the early 1990s. 16S rRNA gene-based methods have been used for identifying clinically important GPAC. Monoplex or multiplex PCR, 16S rRNA gene-based methods were developed for rapid identification of clinically significant GPAC isolates from clinical samples (43, 55, 76–79). Lin et al. (80) developed an oligonucleotide array for clinically important anaerobes, including *A. prevotii*, *A. tetradius*, *F. magna*, *P. asaccharolyticus*, *P. anaerobius*, and *P. micra*. Beighton et al. (18) reported using *rpoB* gene sequencing for identifying *Veillonella* strains isolated from tongue samples, and subsequently, a simple two-step PCR procedure was developed for the identification of the recognized oral *Veillonella* species (77).

MALDI-TOF MS

More recently, a second revolution occurred. MALDI-TOF MS was introduced and broadly accepted as a new diagnostic gold standard for microbial species identification. MALDI-TOF MS is utilized for identification of GPAC isolated from clinical specimens (35). By constructing a database using commonly isolated strains of GPAC as a reference, Veloo et al. (81) could identify clinical isolates by MALDI-TOF MS, and results were then compared with those of other identification methods. This method is a rapid and inexpensive alternative to molecular identification that offers equivalent accuracy. Additionally, complete bacterial identification by MALDI-TOF MS is more cost-effective than by conventional methods.

ANTIMICROBIAL SUSCEPTIBILITIES

New information regarding the antimicrobial susceptibilities of individual GNAC is increasing due to the improvement in classification and identification. For example, in a recent study, the susceptibilities of 14 species of 115 GPAC, which were identified using genetic approaches, were determined for 14 antibiotics (82). However, the new information is sparse compared with the information available for other anaerobic species. And it is difficult to compare the new data to other published data because the previous studies failed to give results for specific species, opting instead to combine data for the group.

Antibiotics such as penicillins, clindamycin, and metronidazole are generally considered to be effective against GPAC. However, studies indicate that the susceptibilities are variable between various species (83–87) and should continue to be studied, to monitor a potential increasing resistance of GPAC to antibiotics. Most evidence suggests that *P. asaccharolyticus*, *F. magna*, and *P. micra* are usually susceptible to penicillins, although Wren (70) reported 16% and 8% resistance among isolates of *F. magna* and *P. micra*, respectively. In other studies, *F. magna* showed lower rates of resistance (10 to 20%) to clindamycin, metronidazole, and penicillin and higher rates of resistance (>20%) to erythromycin and tetracycline (70, 84, 85, 88–91), but two other studies reported a higher prevalence of resistance to clindamycin (86, 92). Brazier et al. (84) suggests that *A. prevotii* is resistant to tetracycline, erythromycin, and clindamycin; the study also found that the highest percentage of overall resistance detected among GPAC was 41.6% resistance to tetracycline, followed by 27.4% resistance to erythromycin. Among GPAC as a group, 7.1% of isolates were resistant to penicillin and clindamycin and 3.5% of isolates were resistant to amoxicillin-clavulanate. Other studies have shown resistance of *A. prevotii*, isolated from diabetic foot infections, to clindamycin, levofloxacin, and ceftazidine (93, 94). Clinical isolates of *A. murdochii* (six strains) were reported to be resistant to colistin sulfate, two strains to kanamycin, one to clindamycin, and three showing intermediate resistance to penicillin; in the same study, *P. gorbacchi* showed resistance to clindamycin (95). A recent study reported that *P. coxii* strains were resistant to doxycycline and 29% were resistant to moxifloxacin and clindamycin, whereas all strains of *P. tyrrelliae* were susceptible to doxycycline but resistant to moxifloxacin and 25% were resistant to clindamycin (8). Clindamycin resistance of strains of *P. asaccharolyticus* and *P. micra* has also been reported (85, 94, 96).

In general, GPAC are more susceptible to β-lactams and β-lactamase inhibitors, cephalosporins, carbapenems, and chloramphenicol (97). Cephalosporins are usually, but not always, effective. Carbapenems are extremely active. Several studies (70, 98, 99) indicated that first-generation quinolones, such as ciprofloxacin, have only moderate activity, but more recently developed agents, such as trovafloxacin, clinafloxacin, and Bay y3118, are extremely active (98). Useful references are available regarding the activity against anaerobes for ceftobiprole (93, 100), oritavancin (96), daptomycin (101), dalbavancin (94), tigecycline (102), and linezolid (103). *P. anaerobius* has also been shown to be extremely susceptible to a new antibiotic, oritavancin, which is being developed for infections caused by vancomycin-susceptible and -resistant organisms (96).

It is worth noting that the resistance rates for *P. stomatis* and *P. anaerobius* are different (86). *P. anaerobius sensu lato* exhibits some resistance to several drugs: amoxicillin,

TABLE 2 Differential characteristics of *Peptostreptococcus*, *Peptococcus*, *Peptoniphilus*, *Finegoldia*, and *Anaerococcus*[a]

Species	GLC	Inhibition by SPS	Production of:				Glucose fermentation	Production of saccharolytic and proteolytic enzymes								
			Indole	Urease	ALP	ADH		α-GAL	β-GAL	α-GLU	β-GUR	ArgA	ProA	PheA	LeuA	PyrA
P. asaccharolyticus	A, b	−	d	−	−	−	−	−	−	−	−	+	−	−	d	−
P. indolicus	A, b	−	+	−	+	−	−	−	−	−	−	+	−	+	+	−
P. harei	A, b	−	d	−	−	−	−	−	−	−	−	+	−	−	−/w	−
P. coxii	ND	ND	−	−	−	−	−	−	−	−	−	−	+	−	−	−
P. tyrrelliae	ND	ND	−	−	−	−	−	−	−	−	−	+	−	−	+/w	−
P. lacrimalis	A, b	−	−	−	−	−	−	−	−	−	−	+	−	+	+	−
P. duerdenii	A, b	ND	−	−	−	w	−	−	−	−	−	−	−	−	+	+
P. koenoeneniae	A, b	ND	−	−	−	+	−	−	−	−	−	−	w	+	+	+
P. gorbachii	A, b	−	d	−	−	−	−	−	−	−	−	+	−	+	+	−
P. olsenii	A, b	−	d	−	+	−	−	−	−	−	−	+	−	+	+	−
"*trisimilis*" group	A, b	−	+	−	d	−	+	−	d	−	−	−	−	−	−	+
A. hydrogenalis	B, a	−	+	d	−/w	−	+	−	−	d	−	−	−	−	−	−
A. prevotii	B, a	−	−	+	−	−	−	+	−	+	+	+	−	−	−	+
A. tetradius	B, a	−	−	+	−	−	+	−	−	+	+	+	−	w	+	w
A. lactolyticus	B, a	−	−	+	−	−	+	−	+	−	−	+	−	−	−	−
A. murdochii	B, A	−	−	−	+	+	+	−	+	−	−	+	−	−	+	+
A. vaginalis	B, a	−	d	−	−/w	+	+	−	−	−	−	+	−	−	+	−
"β-GAL" group	B, a	−	−	−	w	d	+	−	w/+	−/w	−	+	−	−	+	w
A. octavius	B, a, c	−	−	−	−	−	+	−	−	−	−	−	+	−	−	w
P. ivorii	IV	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
P. anaerobius	A, IC	+	−	−	−	−	+	−	−	+	−	−	+	−	−	−
P. stomatis	A, IC	+	−	−	−	−	w	−	−	+	−	−	−	−	−	−
P. russellii																
F. magna	A	−	−	−	d	d	−/w	−	−	−	−	+	−	−	+	+
P. micra	A	−	−	−	+	−	−	−	−	−	−	+	+	+	+	+
P. productus	A	−	−	−	−	−	+	+	−	+	−	−	−	−	−	−

[a] Data mainly from the work of Murdoch (21) and Song et al. (75); undescribed strains that cluster in whole-cell composition as assessed by pyrolysis mass spectrometry. Abbreviations and symbols: GLC, gas-liquid chromatography; A/a, acetate; B/b, butyrate; IV, isovalerate; IC, isocaproate; C/c, *n*-caproate; SPS, sodium polyanethol sulfonate; ALP, alkaline phosphatase; ADH, arginine dihydrolase; α-GAL, α-galactosidase; β-GAL, β-galactosidase; α-GLU, α-glucosidase; β-GUR, β-glucuronidase; ArgA, arginine arylamidase (AMD); ProA, proline AMD; PheA, phenylalanine AMD; Leu, leucine AMD; PyrA, pyroglutamyl AMD; ND, not determined; −, >90% negative; w, weakly positive; +, >90% positive; d, different reactions; w, weak positive.

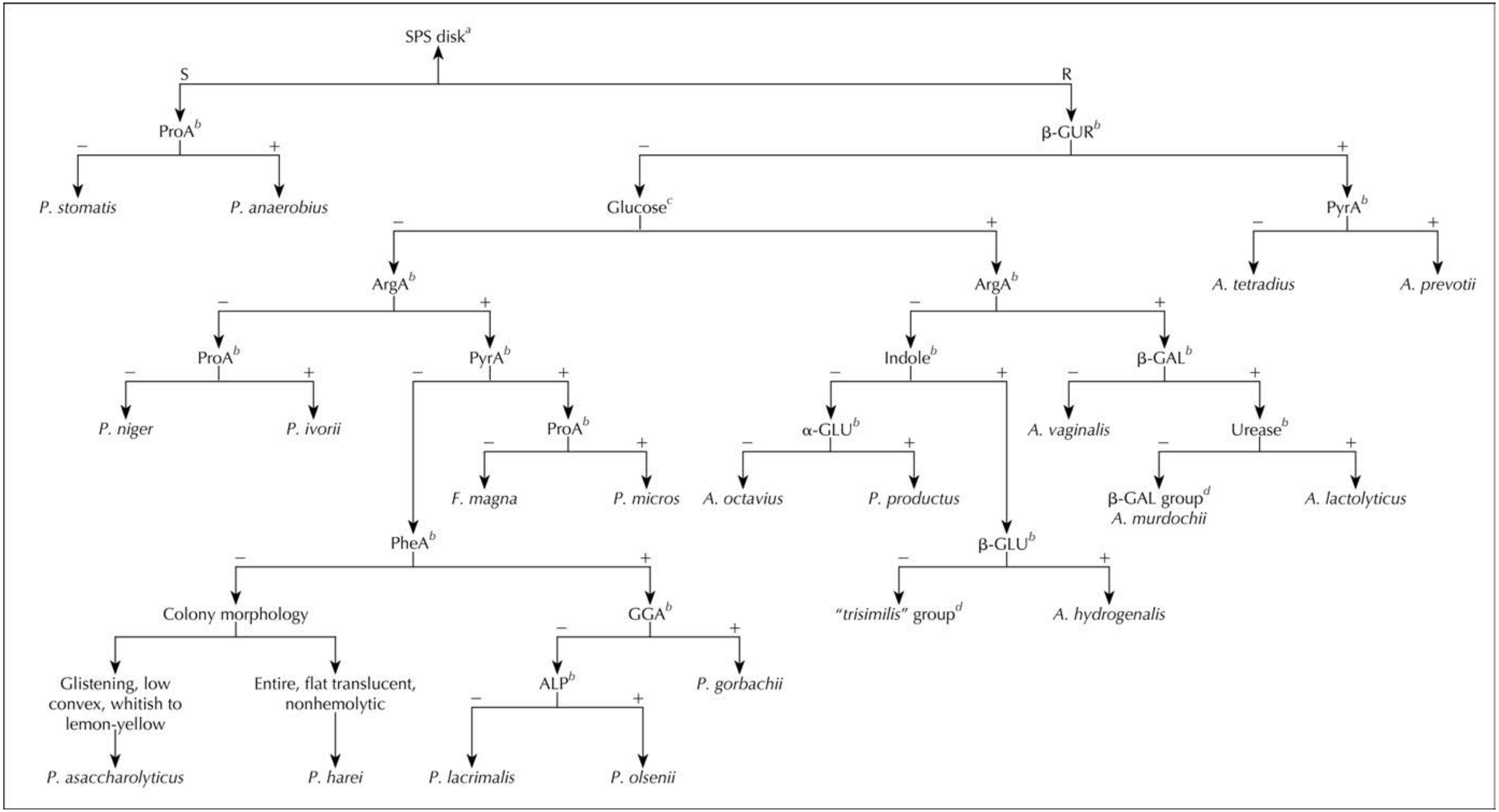
SPS disk[a]
S
R
ProA[b]
−
+
P. stomatis
P. anaerobius
β-GUR[b]
−
+
Glucose[c]
PyrA[b]
A. tetradius
A. prevotii
ArgA[b]
ArgA[b]
ProA[b]
PyrA[b]
P. niger
P. ivorii
ProA[b]
F. magna
P. micros
PheA[b]
Colony morphology
Glistening, low convex, whitish to lemon-yellow
P. asaccharolyticus
Entire, flat translucent, nonhemolytic
P. harei
GGA[b]
ALP[b]
P. lacrimalis
P. olsenii
P. gorbachii
Indole[b]
α-GLU[b]
A. octavius
P. productus
β-GLU[b]
"trisimilis" group[d]
A. hydrogenalis
β-GAL[b]
A. vaginalis
Urease[b]
β-GAL group[d]
A. murdochii
A. lactolyticus

FIGURE 1 Flowchart with key characteristics for identification and differentiation of GPAC. *a*, SPS test was done using an SPS disk (5% SPS; Anaerobe Systems, Morgan Hill, CA). All GPAC are sensitive to SPS except for *P. anaerobius*, which gives a zone of inhibition of ≥ 12 mm around an SPS disk. *P. micra* also exhibits a zone of inhibition with SPS; however, the zone is usually <12 mm. R, no zone or zone of inhibition is <12 mm; S, zone of inhibition is ≥ 12 mm. *b*, All the enzymatic tests were done using Rapid ID 32A systems (API bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. β-GAL, β-galactosidase; α-GLU, α-glucosidase; β-GUR, β-glucuronidase; ArgA, arginine arylamidase (AMD); ProA, proline AMD; PheA, phenylalanine AMD; PyrA, pyroglutamyl AMD; GGA, glutamyl glutamic acid AMD; ALP, alkaline phosphatase. *c*, glucose fermentation tests were performed using prereduced, anaerobically sterilized peptone-yeast-glucose (PYG) broth (Anaerobe Systems). A pH of ≤ 5.5 in the PYG tubes was interpreted as positive and ≥ 5.9 as negative fermentation. *d*, described by D. A. Murdoch (21). doi:10.1128/9781555817381.ch51.f1

TABLE 3 Characteristics of genera of Gram-negative cocci[a]

Characteristic	*Negativicoccus*	*Megasphaera*	*Veillonella*	*Acidaminococcus*	*Anaeroglobus*
Cell size (µm)	0.4	1.7–2.6	0.3–0.5	0.5–1	0.5–1.1
Growth under microaerophilic conditions	+	−	−	−	−
Nitrate reduction	−	−	+	−	−
Ability to ferment carbohydrates[b]	−	+	−	−	+
Fermentation of lactate	−	+	+	−	−
Decarboxylation of succinate	+	−	+	−	−
Amino acids as the main source of energy[c]	−	−	−	+	−
Gas production	−	+	+	+	−
Metabolic end products	A, P (L)[d]	A, P, B, V, C	A, P	A, B, (P), (L)	A, P, iB, B, iV

[a] Modified from the work of Marchandin et al. (15).

[b] Glucose fermentation tests were performed using prereduced, anaerobically sterilized (PRAS) PYG broth tube (Anaerobe Systems, Morgan Hill, CA). The PRAS tubes were inoculated from an actively growing broth culture (without carbohydrate). A pH of ≤5.5 in the PRAS tubes was interpreted as positive, 5.6 to 5.8 as weakly positive, and ≥5.9 as negative fermentation.

[c] Carbohydrates are not fermented for most of the species; amino acids are used as energy sources. Abbreviations and symbols: A, acetic acid; P, propionic acid; L, lactic acid; B, butyric acid; V, valeric acid; C, caproic acid; iB, isobutyric acid; iV, isovaleric acid.

[d] Parentheses indicate that these compounds are produced in variable amounts.

amoxicillin-clavulanate (3/30 strains resistant), cefoxitin (2/30 strains resistant), azithromycin, and moxifloxacin (1/30 strains resistant). There was no resistance found in 31 strains of *P. stomatis*.

Veillonellae show resistance to tetracycline, erythromycin, gentamicin, and kanamycin, and they are susceptible to penicillin G, cephalothin, and clindamycin. Their resistance is intermediate for chloramphenicol and lincomycin (104).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Because anaerobic bacteriology is time-consuming, several interim reports are desirable. The initial report should give Gram stain results and bacterial and human cell morphologies. The relative quantities of different organisms seen in the smear give a good overall impression of the specimen quality, the nature of the polymicrobial infection, and the relative importance of each organism. In general, bacterial isolates that are predominant, virulent, and resistant to antimicrobial agents should be given the greatest attention. Bacteria present in pure culture or in large numbers are probably of major importance, as are organisms recovered in multiple cultures and isolated from normally sterile sites. Furthermore, Gram stain results can guide the laboratory in choosing media for optimal recovery of the predicted organisms.

The significance of finding anaerobic Gram-positive and Gram-negative cocci in clinical specimens depends on the specimen and the likelihood that it was contaminated by the microbiota of the skin or mucous membranes. Hence, interpretation of culture results is dependent on the nature and quality of the specimen submitted to the laboratory.

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Propionibacterium, *Lactobacillus*, *Actinomyces*, and Other Non-Spore-Forming Anaerobic Gram-Positive Rods*

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TAXONOMY AND DESCRIPTION OF THE AGENTS

The anaerobic, Gram-positive, non-spore-forming rods are widely distributed in two Gram-positive phyla: *Actinobacteria* and *Firmicutes* (Table 1).

Phylum *Actinobacteria*

The genus *Actinomyces* and the related clinically relevant genera *Actinobaculum*, *Mobiluncus*, and *Varibaculum* contain anaerobic and aerotolerant, non-acid-fast, Gram-positive organisms with variable morphology, ranging from characteristic branching rods to coccobacilli. The genus *Actinomyces* currently comprises 41 species, of which 24 have been isolated from human sources. The species epithet *A. naeslundii* now applies only to isolates previously termed "*A. naeslundii* genospecies 1," while the novel species *A. oris* and *A. johnsonii* accommodate the former "*A. naeslundii* genospecies 2" and "genospecies WVA 963," respectively (1). The majority of species produce succinic acid from glucose. The genus *Actinobaculum* includes the human-associated species *A. massiliense*, *A. schaalii*, and *A. urinale* (2–4). The genus *Mobiluncus* contains two species, *M. curtisii* and *M. mulieris*, which are strictly anaerobic, curved bacilli with variable Gram reactions and corkscrew motility. They resemble *Actinomyces* in that succinic acid is the major metabolic end product from glucose. *Varibaculum*, currently comprising only *V. cambriense* (5), is related to *Mobiluncus* on the basis of 16S rRNA gene sequence analysis, as is *Actinomyces neuii*, which is distantly related to *Actinomyces sensu stricto* and would appear to be worthy of proposal as a novel genus (6).

Propionibacterium species are anaerobic and aerotolerant, pleomorphic, Gram-positive rods that produce propionic acid from glucose. Five *Propionibacterium* species have been isolated from human clinical infections: *P. acnes*, *P. avidum*, *P. granulosum*, *P. propionicum*, and *P. acidifaciens* (7). A related species, *Propionimicrobium lymphophilum*, formerly a member of *Propionibacterium*, has also been isolated from clinical material, while *Propioniferax innocua* is a member of the normal skin microbiota (8, 9).

Members of the genus *Bifidobacterium* and the closely related genera *Alloscardovia* (10), *Parascardovia*, and *Scardovia* (11) are strictly anaerobic or occasionally microaerobic, Gram-positive, pleomorphic rods, appearing as uniform to branched or club shaped. Typically, bifidobacteria produce fructose-6-phosphate phosphoketolase as well as acetic and lactic acids as major metabolic end products. Bifidobacteria are aciduric and are nutritionally fastidious. There are currently 41 *Bifidobacterium* species. Of these, 11 species have been isolated from the human gut and oral cavity. *B. longum* now includes the former species *B. infantis* and *B. suis* as subspecies (12, 13).

Numerous taxa have been misassigned to *Lactobacillus* in the past, including the so-called "anaerobic lactobacilli," which are now recognized to constitute two genera, *Atopobium* and *Olsenella*, within the family *Coriobacteriaceae* (14, 15). The *Atopobium* species *A. minutum* and *A. rimae* (both formerly *Lactobacillus* species), *A. parvulum* (formerly *Streptococcus*), and *A. fossor* (formerly *Eubacterium*) produce lactic acid as the major glucose metabolic end product (14, 16). The genus *Olsenella* is closely related to *Atopobium* and currently includes two species isolated from the human oral cavity: *O. uli* (formerly *Lactobacillus uli*) and *O. profusa* (15, 17).

The genus *Eggerthella* (18) includes the human pathogens *E. lenta* and *E. sinensis*, while the former *E. hongkongensis* has been moved to the new genus *Paraeggerthella* (19). Other members of the *Coriobacteriaceae* found in human infections include the genera *Collinsella* (20), *Slackia* (18), and *Cryptobacterium* (21), while the genera *Adlercreutzia* and *Gordonibacter* have been isolated recently from feces and the colon, respectively (19, 22, 23).

Phylum *Firmicutes*

Lactobacillus, a large and heterogeneous genus, contains microaerobic, catalase-negative, non-spore-forming, Gram-positive rods, which produce lactic acid as their single or major metabolic end product from glucose fermentation. The majority of *Lactobacillus* species are found within the family *Lactobacillaceae* and order *Lactobacillales*. However, *Catenibacterium mitsuokai* (24), isolated from human feces, forms a cluster with *Kandleria vitulina* and *Eggerthia catenaformis* (formerly *Lactobacillus vitulinus* and *Lactobacillus*

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TABLE 1 Some features of non-spore-forming, anaerobic, Gram-positive genera[a]

Phylum and genus	G+C mol%	Cell characteristics	Aerotolerance[b]	Gram reaction[c]	Major end product(s)[d]	Other features
Actinobacteria						
Actinobaculum	50–57	Straight or slightly curved, branching; singly or in clusters	+/–	(+)	A	Saccharolytic
Actinomyces	55–68	Variable, often branching; singly or in pairs	+/–	+	S, L	Saccharolytic
Adlercreutzia	64–67	Coccobacilli	–	+	None	Asaccharolytic
Alloscardovia	48	Short, irregularly shaped	+	+	ND	Saccharolytic
Atopobium	35–46	Short, elliptical; singly or in pairs or short chains	–/+	+	L	Saccharolytic
Bifidobacterium	57–64	Variable	–/+	+	A, L	Aciduric
Collinsella	60–61	Short; in chains	–	+	A, F, L	Saccharolytic, H_2 production
Cryptobacterium	50–51	Short	–	(+)	None	Asaccharolytic
Eggerthella	62	Coccobacilli or short rods; in pairs or short chains	–	+	(A, L, S)	Asaccharolytic
Gordonibacter	66	Coccobacilli, motile	–	+	ND	Asaccharolytic
Mobiluncus	49–52	Curved with tapered ends; singly or in pairs; motile	–	v	S, L, A	Saccharolytic
Olsenella	63–64	Short, elliptical; singly/in pairs or short chains	–	+	L, A	Saccharolytic
Paraeggerthella	61	Coccobacilli, in chains	–	+	ND	Asaccharolytic
Parascardovia	54–56	Small, slender, variable	–	+	A, L	Saccharolytic
Propionibacterium	59–67	Variable	–/+	+	P	Saccharolytic
Propioniferax	59–63	Variable, in clusters	+	+	P	Saccharolytic
Propionimicrobium	53–54	Variable; often diphtheroid or club-shaped	–	+	P, A, S	Saccharolytic
Scardovia	44–46	Small, coccoid, variable	–	+	A, L	Saccharolytic
Slackia	60–64	Cocci, coccobacilli, or short rods; singly or in clumps	–	(+)	(A)	Asaccharolytic
Varibaculum	52	Short, straight or curved, diphtheroid	–/+	+	L, S	Saccharolytic
Firmicutes						
Anaerofustis	70	Thin rods	–	+	A, B	Saccharolytic
Anaerostipes	46	Thin rods; in short chains	–	(+)	A, B, L	Saccharolytic
Anaerotruncus	54	Thin rods	–	+	A, B	Saccharolytic
Bulleidia	38	Short, straight or slightly curved; singly or in pairs	–	+	A, L	Saccharolytic
"Catabacter"	40	Coccobacilli or short rods, motile	–	+	ND	Saccharolytic
Catenibacterium	36–38	Short; in long tangled chains	–	+	A, B, L	Saccharolytic
Dorea	40–46	Short or long; in pairs or chains	–	+	A, F	Saccharolytic, H_2 production
Eubacterium	30–57	Variable	–	v	B, A, L (F)	Saccharolytic
Faecalibacterium	47–57	Pleomorphic rods	–	–	B, F, L	Saccharolytic
Filifactor	34	Short, regular	–	–	B	Asaccharolytic
Flavonifractor	58–62	Straight or slightly curved rods	–	(+)	A, B	Asaccharolytic
Holdemania	38	Short; in pairs or short chains	–	(+)	A, L	Saccharolytic
Lactobacillus	35–53	Short or long, slender; in chains	–/+	+	L	Aciduric
Marvinbryantia	50	Short; in pairs or short chains	–	+	A (S, L)	Formate required
Mogibacterium	41–50	Short; singly or in clumps	–	(+)	PAA	Asaccharolytic
Oribacterium	42	Elongated, ovoid; singly or in pairs; highly motile	–	–	A, L	Saccharolytic
Pseudoramibacter	61	Pleomorphic; in pairs	–	+	A, B, C, F	Saccharolytic
Robinsoniella	49	Ovoid or short rods singly or in pairs	–	+	S	Saccharolytic
Roseburia	29–42	Thin, pleomorphic rods	–	v	B, L	Saccharolytic, H_2 production
Shuttleworthia	50–51	Short or slightly curved; singly or in pairs or short chains	–	+	B, A (L)	Saccharolytic
Solobacterium	37–39	Short, straight or slightly curved; singly or in pairs	–	+	A, L	Saccharolytic
Turicibacter	37	Irregular, long; in long chains	–	+	L	Saccharolytic

[a] Data compiled from references 2, 15, 17, 24, 27, 28, 37, 38, 41–43, 46, 47, 55, and 147.

[b] +/– or –/+, variable aerotolerance within the genus; –, the genus contains only strictly anaerobic species.

[c] +, positive; –, negative; v, variable; (+), decolorization in old culture.

[d] A, acetic acid; B, butyric acid; C, caproic acid; F, formic acid; L, lactic acid; ND, not determined; P, propionic acid; PAA, phenylacetic acid; S, succinic acid; abbreviations in parentheses indicate strain variation.

catenaformis, respectively) within the family *Erysipelotrichaceae* (25, 26).

Organisms assigned to *Eubacterium* are defined by default; they do not produce propionic acid as a major acid product, lactic acid as the sole major acid product, succinic and lactic acids with small amounts of acetic or formic acids, or acetic and lactic (acetic > lactic) acids, with or without formic acid, as the sole major acid products (27). Due to the number of phylogenetically distinct species within this genus, reclassification of a large number of species is ongoing either to novel genera or to existing ones based on 16S rRNA phylogeny and phenotypic characteristics. It has been proposed that *Eubacterium sensu stricto* should be restricted to *E. limosum*, *E. callanderi*, and *E. barkeri* (28). Using this definition, the family *Eubacteriaceae* then includes the genera *Eubacterium sensu stricto*; *Anaerofustis* (with one species, *A. stercorihominis*, isolated from human feces) (29); and *Pseudoramibacter alactolyticus*, a saccharolytic species found in the oral cavity of humans (28). The *Eubacterium* species *E. biforme*, *E. cylindroides*, and *E. dolichum* fall within the family *Erysipelotrichaceae* mentioned above, together with the saccharolytic species *Bulleidia extructa*, isolated from the human mouth (30), and *Holdemania filiformis* (31) and *Solobacterium moorei* (16), isolated from human feces. *Turicibacter sanguinis*, isolated from a blood culture (32), also belongs to this family (25).

The *Eubacterium* species *E. infirmum*, *E. minutum*, *E. nodatum*, *E. saphenum*, and *E. sulci* (formerly *Fusobacterium sulci*), together with *Mogibacterium*, a genus of five species that are difficult to differentiate by phenotypic tests (33, 34), are a group of asaccharolytic taxa isolated from the human mouth. Many *Eubacterium* species are closely related to clostridia. Phylogenetic analysis indicates that the taxonomic importance of spore formation as a criterion for assignation to the genus *Clostridium* may have been overemphasized. Numerous phylogenetic clusters contain both sporing and nonsporing representatives. The *Eubacterium* species *E. budayi*, *E. moniliforme*, and *E. nitritogenes* are found in clostridial cluster I described by Collins et al. (35), while *E. siraeum* belongs to a group that includes *Clostridium leptum* and *Anaerotruncus colihominis* (36). *E. tenue* and *E. yurii* belong to cluster XI. *E. tenue* is related to *Clostridium ghonii* and *Clostridium sordellii*; further work is required to determine whether these species constitute a novel genus. Strains of *Eubacterium plautii* and *Clostridium orbiscindens* have been shown to belong to the same taxon and renamed *Flavonifractor plautii* (37). *E. yurii* is related to the genus *Filifactor*, which includes *Filifactor alocis* (formerly *Fusobacterium alocis*), isolated from oral infections in humans (38). *E. contortum*, *E. eligens*, *E. hallii*, *E. ramulus*, *E. rectale*, *E. ventriosum*, and *Lachnoanaerobaculum saburreum* (formerly *E. saburreum*) (39) belong to the family *Lachnospiraceae*. *E. rectale* and *E. ramulus* are related to *Roseburia intestinalis* (40). This family also includes the recently described formate-requiring species *Marvinbryantia* (formerly *Bryantella*) *formatexigens* (41, 42), isolated from human feces without any disease association so far, and *Oribacterium sinus* (43), a highly motile species isolated from pus of a human sinus. *E. eligens* and *Lachnospira pectinoschiza* are close phylogenetic neighbors and are both motile rods whose growth in broth culture is stimulated by the presence of fermentable carbohydrate. *E. eligens* should therefore be transferred to the genus *Lachnospira*. *Anaerostipes caccae* (44) and *Anaerostipes hadrus* (formerly *Eubacterium hadrum*) (45) form a loose group with *E. hallii* and *Coprococcus eutactus*, all common species in human feces. "*Catabacter hongkongensis*" is a deep-branching member of the order *Clostridiales*, isolated from blood cultures and associated with high mortality (46, 47), but as yet has not been validly published.

EPIDEMIOLOGY AND TRANSMISSION

The majority of the organisms described in this chapter are part of the commensal microbiota associated with the mucocutaneous surfaces of the human and animal digestive tract, being found in the mouth, small and large intestines, urogenital tract, and skin (48–52). Microbial colonization of an individual occurs in a successive manner during the first weeks and months of life. *Actinomyces* species are among the initial colonizers of the mouth (53), whereas bifidobacteria and lactobacilli play an important role in the development of the healthy gut and its associated immune defenses (54, 55). Where members of this group cause infections, the host itself is the most likely source, although the commensal microbiota of other humans can be responsible, for example, in the case of infections resulting from human bites or clenched fist injuries from striking the face and mouth (56).

CLINICAL SIGNIFICANCE

Non-spore-forming, anaerobic, Gram-positive rods seldom cause infections alone but are typically found in polymicrobial infections associated with mucosal surfaces (Table 2). Many anaerobes involved in infections of the head and neck originate from the oral cavity, whereas most vaginal and bladder pathogens are of fecal origin. In intra-abdominal infections due to organ perforation, the predominant recoveries reflect the microbiota at the site of the leakage (57). For surgical patients, anaerobes are a significant cause of morbidity and mortality (58). Anaerobic bacteria can occasionally spread to adjacent tissues and the bloodstream, with serious consequences. For anaerobic bacteremias, the gastrointestinal tract is the most common source, followed by abscesses, gynecologic infections, and wound infections (59). The incidence and range of anaerobic, Gram-positive rods found in blood cultures may be underestimated because many of them are slow growing and have fastidious nutritional requirements. Anaerobic blood culture methods tend to be targeted at *Clostridium* species and *Bacteroides fragilis*, which grow readily and rapidly in commonly used broth media.

Actinomyces and Related Bacteria

Actinomyces and related bacteria are associated with a wide range of infections, normally as part of a polymicrobial consortium (Table 2) (60). Actinomycosis is a chronic, granulomatous infection affecting the cervicofacial, pulmonary, and abdominopelvic regions and is caused primarily by *Actinomyces* species, particularly *A. israelii*, *A. gerencseriae*, and *A. graevenitzii*, and *P. propionicum* (61, 62). *Actinomyces* organisms make up a significant proportion of the microbiota in dental plaque in healthy individuals but are also associated with a wide range of dental and oral infections, including dental caries, endodontic infections, odontogenic abscesses, and dental implant-associated infections (63–70).

Cervicofacial lesions normally arise as a consequence of untreated dental caries or are associated with dental extractions or trauma. These allow the causative organisms, which are part of the oral commensal biota, to enter the tissues (71). Although actinomyces are regarded as the primary cause and form the characteristic aggregates of branching rods seen macroscopically as sulfur granules, there are always multiple species present, with *Aggregatibacter actino-*

TABLE 2 Genera of non-spore-forming, anaerobic, Gram-positive rods detected in human infections[a]

Site or disease association	Genera isolated
Brain and/or central nervous system	*Actinomyces*, *Eubacterium*, *Propionibacterium*, *Pseudoramibacter*, *Varibaculum*
Eye infections	*Actinomyces*, *Propionibacterium*, *Varibaculum*
Mouth	
Abscesses	*Actinomyces*, *Atopobium*, *Eubacterium*, *Filifactor*, *Mogibacterium*, *Olsenella*, *Pseudoramibacter*, *Slackia*, *Solobacterium*, *Varibaculum*
Dental caries	*Actinomyces*, *Bifidobacterium*, *Lactobacillus*, *Olsenella*, *Parascardovia*, *Propionibacterium*, *Scardovia*
Endodontic infection	*Actinomyces*, *Atopobium*, *Bifidobacterium*, *Eubacterium*, *Filifactor*, *Lactobacillus*, *Mogibacterium*, *Olsenella*, *Propionibacterium*, *Pseudoramibacter*, *Shuttleworthia*, *Slackia*
Periodontal diseases	*Cryptobacterium*, *Eubacterium*, *Filifactor*, *Mogibacterium*, *Olsenella*, *Pseudoramibacter*, *Slackia*, *Solobacterium*
Respiratory tract infections	*Actinomyces*, *Eubacterium*, *Lactobacillus*, *Oribacterium*, *Propionibacterium*
Abdomen, intestine	
Abscesses	*Actinomyces*, *Eggerthella*, *Eubacterium*, *Lactobacillus*, *Solobacterium*
Appendicitis	*Actinomyces*, *Eggerthella*
Cholecystitis	*Actinomyces*, *Lactobacillus*
Peritonitis	*Eggerthella*, *Lactobacillus*
Ulcerative colitis	*Faecalibacterium*, *Roseburia*
Genital tract	
Abscesses	*Actinomyces*, *Atopobium*, *Eubacterium*
Bacterial vaginosis	*Atopobium*, *Eubacterium*, *Mobiluncus*
Intrauterine device infections	*Actinomyces*, *Eubacterium*, *Varibaculum*
Pelvic inflammatory disease	*Actinomyces*, *Atopobium*, *Eubacterium*, *Lactobacillus*
Preterm labor/delivery	*Mobiluncus*
Urinary tract infections	*Actinobaculum*, *Actinomyces*, *Alloscardovia*
Skin and/or soft tissue	
Abscesses	*Actinobaculum*, *Actinomyces*, *Solobacterium*
Infected atheroma	*Actinomyces*
Acne vulgaris	*Propionibacterium*
Cellulitis	*Actinomyces*, *Pseudoramibacter*
Necrotizing soft tissue infections	*Actinomyces*, *Eubacterium*, *Mogibacterium*
Lymphadenitis	*Propionibacterium*, *Propionimicrobium*
Bone and joint infections	*Actinobaculum*, *Actinomyces*, *Bulleidia*, *Propionibacterium*
Wounds	
Bite wound infections (human)	*Actinomyces*, *Collinsella*, *Eggerthella*, *Eubacterium*, *Filifactor*, *Lactobacillus*, *Mogibacterium*, *Propionibacterium*
Postoperative wound infections	*Bifidobacterium*, *Propionibacterium*, *Pseudoramibacter*, *Robinsoniella*
Diabetic foot infections	*Actinomyces*, *Propionibacterium*
Cardiovascular sites	
Bacteremia	*Actinobaculum*, *Actinomyces*, *Alloscardovia*, *Atopobium*, "*Catabacter*," *Eggerthella*, *Eubacterium*, *Lactobacillus*, *Olsenella*, *Paraeggerthella*, *Propionibacterium*, *Robinsoniella*, *Solobacterium*
Endocarditis	*Actinomyces*, *Lactobacillus*, *Propionibacterium*, *Shuttleworthia*
Foreign body infections	*Actinomyces*, *Mogibacterium*, *Propionibacterium*

[a] Data compiled from references 2–4, 7, 10, 15, 19, 21, 27, 29, 20, 30, 39, 47, 54, 56, 57, 62, 70, 75, 77, 91, 99, 101–103, 106–114, 117–120, 123–126, 128–131, 134–138, and 144–151.

mycetemcomitans the most typical, together with a variety of oral organisms, including viridans group streptococci; anaerobic cocci; and Gram-negative, anaerobic rods (72). Oral *Actinomyces* species can be detected in the bloodstream following dental procedures (73), while *A. funkei*, *A. massiliensis*, *A. naeslundii*, *A. odontolyticus*, *A. turicensis*, and *Actinobaculum schaalii* and *Actinobaculum urinale* have been demonstrated to cause infections of the blood, particularly in individuals with predisposing conditions (74–79). *Actinomyces* species have also been implicated in infected osteoradionecrosis lesions, based on detection of the organisms within lesions and evidence of sulfur granule formation (80, 81). Pulmonary actinomycosis most commonly affects the lungs following aspiration of oral bacteria in saliva, resulting in a pulmonary infiltrate or mass. It is commonly misdiagnosed as tuberculosis or malignancy on initial examination. *Actinomyces meyeri* has been shown to have a predilection for causing pulmonary actinomycosis, and dissemination to other organs has also been observed (82, 83). In a study in which anaerobic culture methods were used on sputum samples from adult cystic fibrosis patients, *Actinomyces* species were frequently isolated (84).

The majority of pelvic infections are found in women using intrauterine contraceptive devices (85), and abdominal infection normally arises following perforation of the bowel as a result of disease or surgery (86). A number of *Actinomyces* species, especially *A. israelii* and *A. turicensis* but also *A. cardiffensis*, *A. gerencseriae*, *A. naeslundii*, *A. odontolyticus*, and *A. urogenitalis*, as well as *V. cambriense*, have been isolated from intrauterine device-associated infections (5, 87–90). The most frequently isolated *Actinomyces* species from clinical infections are *A. turicensis*, *A. radingae*, and

A. neuii and are found in a range of soft tissue infections including perianal, groin, axillary, breast, and periaural abscesses (62, 91). *A. europaeus*, *A. funkei*, *A. georgiae*, and *A. timonensis* are also isolated from similar soft tissue infections. In addition, *A. naeslundii* and *A. israelii* have been isolated from infected hip prostheses (92, 93). Pericarditis cases caused by *A. israelii* and *A. meyeri* have been reported (94). Due to the small numbers of human clinical isolates reported to date, the natural habitats and clinical associations of *A. dentalis*, *A. hominis*, *A. hongkongensis*, *A. nasicola*, *A. oricola*, and *A. radicidentis* have yet to be determined.

Actinobaculum spp. are strongly associated with complex urinary tract infections. Increasing evidence suggests that *A. schaalii* is an underestimated emerging uropathogen, which has also been implicated in invasive infections such as bacteremia, endocarditis, cellulitis, and spondylodiscitis (95–98). *A. massiliense* is also associated with soft tissue abscesses (99).

Propionibacterium

Propionibacteria can be found in various systemic or disseminated opportunistic infections (Table 2), such as endocarditis, central nervous system infections, osteomyelitis, osteitis, and arthritis (100–102), and in about 20% of infected dog and cat bite wounds (103).

Because *P. acnes* is a member of the normal skin microbiota, it is frequently discarded as a contaminant or as clinically insignificant, particularly when isolated from the blood (104). However, the pathogenic potential of *P. acnes* should not be underestimated when there are predisposing factors present, such as a foreign body, surgery or trauma, diabetes, or immunosuppression (102, 105–109). Recently, mounting evidence has implicated *P. acnes* as an important pathogen in not only acne vulgaris, but also endocarditis and prosthetic joint infections, and it also has potential roles in prostate cancer and sarcoidosis (110, 111).

One-third of cases of *P. acnes* endocarditis are complicated by intracardiac abscess formation, potentially as a result of delayed treatment due to the initial isolation of *P. acnes* being deemed insignificant (112). Prosthetic joints are particularly susceptible to *P. acnes* infection (113, 114), and biofilm formation appears to be a specific virulence factor associated with invasive strains (115). Controversy remains regarding the optimum incubation time required to recover *P. acnes* from joint infections with suitable sensitivity and specificity for a confident diagnosis. Extended incubation of aspirates and tissue has been suggested; however, this may also increase the potential for false-positive results (116). *P. acnes* has also been isolated as part of the mixed bacterial community found in the sputum of adult cystic fibrosis patients (84).

P. propionicum is part of the normal oral microbiota and causes oral and eye infections (70, 117, 118) as well as actinomycosis, in which it displays a spectrum of pathogenicity similar to those of *A. israelii* and *A. gerencseriae* (61). *P. acidifaciens* is particularly associated with dental caries (7).

Lactobacillus

Despite the reputation of lactobacilli as beneficial organisms, they can be involved in serious infections (Table 2), especially in immunocompromised individuals (100, 119–123). The *Lactobacillus* species most frequently isolated from various human infections are *L. rhamnosus*, *L. casei*, *L. fermentum*, *L. gasseri*, *L. plantarum*, *L. acidophilus*, and *L. ultunensis* (120, 123, 124). Lactobacilli are particularly associated with advanced dental caries (68, 118, 124), where they are considered a secondary colonizer because of their preference for low-pH habitats, but probably play a role in exacerbating existing lesions (125). The clinical infections most commonly caused by lactobacilli are bacteremia and endocarditis, with an associated relatively high mortality rate (120), with the mouth the primary route of entry to the bloodstream, either as a result of normal chewing and brushing or following dental procedures (120). Detection of lactobacilli, alone or with other microorganisms, in blood cultures of patients with underlying diseases may be clinically significant. *L. rhamnosus* was the most frequent species detected in *Lactobacillus* bacteremia and endocarditis (104, 120, 121, 123, 126). Concern has been expressed that probiotic strains consumed in foodstuffs may cause disease in some individuals. Although such reports are rare (127), there have been reports of sepsis and endocarditis attributed to probiotic *Lactobacillus* strains (128, 129). In some cases, these have been attributed to inappropriate dosages and routes of administration, and it should be remembered that organisms used as probiotics are defined at the strain level and that, although infections may be caused by other strains within the same species, this does not imply that probiotic strains are unsafe. Vancomycin-resistant lactobacilli have been implicated in dialysis-related peritonitis after extended use of glycopeptides (122, 130).

Pyrosequencing has demonstrated that a limited number of *Lactobacillus* spp. (*L. crispatus*, *L. gasseri*, *L. jensenii*, and *L. iners*) are responsible for sustaining the normal vaginal bionetwork (131). The depletion of these *Lactobacillus* spp. from the vaginal microbiota is considered one of the characteristic features of bacterial vaginosis. In contrast to other *Lactobacillus* species, *L. iners* has been associated with an intermediate state of bacterial vaginosis (131, 132).

Eubacterium and Related Bacteria

The genus *Eubacterium* remains poorly defined, but species belonging to this genus and its relatives in the phylum *Firmicutes* are commonly isolated from oral infections (Table 2), particularly when nutrient-rich media and extended incubation times are used. For example, careful isolation of tiny-colony-forming anaerobes from periodontal pockets in adult patients with advanced periodontitis showed that "*Eubacterium*" species (mainly asaccharolytic) dominated (133), suggesting a role in the etiology of chronic periodontitis. When molecular identification methods were applied to a collection of *Eubacterium*-like strains from oral infections, *Mogibacterium timidum* was one of the most frequently detected species (134). *Mogibacterium vescum*, *B. extructa*, *F. alocis*, and *P. alactolyticus* were also found among the isolates from severe (some of them requiring treatment in intensive care units) odontogenic infections. Less frequently isolated were *E. sulci*, *E. yurii*, and *L. saburreum* (134). Many species found in odontogenic infections are also common in endodontic infections (70, 135–137). *F. alocis*, *E. nodatum*, *E. saphenum*, and *M. timidum* have been associated with periodontal diseases (138–142), and "*Eubacterium*" species in general with failing dental implants (143). Due to their presence in the oral cavity, various *Eubacterium* and related species are among the anaerobic findings in human bite wound infections (103). *Filifactor villosus*, a species of animal origin, has been isolated from infected cat bite wounds in humans (144).

Recently, a number of species have been identified as the etiological agents in nondental infections: *Shuttleworthia satelles* in a case of endocarditis (145) and *B. extructa* from a periprosthetic hip infection (146). *S. moorei* and *Robin-*

soniella peoriensis have been isolated from cases of bacteremia and wound infections (147–150).

Changes in abundance of *Faecalibacterium prausnitzii* may act as biomarkers of intestinal well-being, as reduced levels of this organism have been implicated in a number of disorders, in particular ulcerative colitis (151). Also, *E. nodatum* has been found in infections of the female genital tract (152). *E. tenue* and *E. callanderi*, an environmental anaerobe, have been detected in clinically significant bacteremias (27, 104, 153).

Eggerthella and Related Bacteria

Species of the genera *Eggerthella* and *Paraeggerthella* are recovered from a wide range of human infections (Table 2). *E. lenta* (formerly *Eubacterium lentum*) is a well-recognized pathogen particularly of intra- and periabdominal sites (100, 104, 119, 154). *E. lenta*, *E. sinensis*, and *P. hongkongensis* have been found in blood in association with clinically significant infections of relatively high mortality (104, 155). *Cryptobacterium curtum* and *Slackia exigua* have been associated with chronic periodontitis (138, 140). The latter has also been associated with endodontic infections (136) and polymicrobial wound and abscess infections thought to be of intestinal origin (156). An as yet unnamed, *Eggerthella*-like taxon is positively associated with bacterial vaginosis (131, 157).

Atopobium

Several species of the genus *Atopobium* are isolated from various infections (Table 2). Although *A. vaginae* is a prominent member of the commensal microbiota of the healthy vagina (158, 159), it has been increasingly reported to be involved in infections of the genital tract. A strong association between *A. vaginae* and bacterial vaginosis has been reported in several studies. The presence of this organism or *Gardnerella vaginalis* combined with the depletion of *Lactobacillus* spp. is being proposed as an accurate predictor of bacterial vaginosis (131, 132, 160–164). *A. minutum* has been isolated from various infections of the lower part of the body, and *A. parvulum* has been isolated from respiratory specimens (165). Although *A. parvulum* and *A. rimae* have been detected in the pockets formed as a result of periodontitis (142, 165), in a comprehensive study of the microbiota of the subgingival region, these species were found to be associated with oral health rather than disease (140). Among *Eubacterium*-like isolates from severe odontogenic infections, *A. rimae* was the most frequently isolated (134).

Olsenella

Olsenella species show disease associations similar to those observed for lactobacilli in the oral cavity and have been found in root caries (166), with *O. profusa* specifically detected in dental caries lesions (68), and *O. uli*, in particular, in endodontic infections (118, 167–169) and acute dental abscesses (169). Both species can also be found in subgingival sites of periodontitis patients (15, 165). In addition, *O. uli* has been reported as one of the causative organisms in clinically significant bacteremia (104).

Bifidobacterium and Related Bacteria

Culture-independent analyses have shown that although members of the phyla *Bacteroidetes* and *Firmicutes* dominate the gut microbiota numerically, bifidobacteria appear to be functionally of great importance to intestinal health (170). Because of this, they are generally considered to be nonpathogenic but nevertheless are isolated from infections of polymicrobial etiology (Table 2). Dental caries is the most common clinical entity in which *Bifidobacterium*, mainly *B. dentium*, and the related species *Parascardovia denticolens*, *Scardovia inopinata*, and *Scardovia wiggsiae* (formerly known as *Scardovia* species C1) may have a pathogenic role (118, 171–174). *B. adolescentis*, *B. dentium*, *B. breve*, and *B. longum* are occasionally isolated from other infections, mainly in immunocompromised individuals (94, 100, 119, 175). Although *B. scardovii* has been isolated from human clinical samples, including blood, urine, and hip (176), its clinical relevance is not known. In addition, a novel species related to *Bifidobacterium*, *Alloscardovia omnicolens*, has been detected in infections at various body sites, including urine and the genitourinary tract, in particular, and the oral cavity, tonsils, lung and aortic abscesses, abdominal wounds, and blood (175).

Mobiluncus

The presence of vibrio-like *Mobiluncus* species in smears of vaginal fluid has been widely used as one of the indicators of bacterial vaginosis (177). Indeed, *M. curtisii* is seldom present in the vaginas of healthy women but, instead, is highly associated with bacterial vaginosis and its treatment failure due to persistence of the organism (178, 179). The altered vaginal microbial ecology seen in bacterial vaginosis can be a risk for adverse pregnancy outcome when ascending to the upper genital tract (180). In addition to bacterial vaginosis, *M. curtisii* has been isolated occasionally from endometrial smears and pus specimens of the female genital tract (181) and from blood (182, 183).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Many of the organisms described in this chapter are part of the human commensal microbiota and cause disease as opportunistic pathogens. This makes specimen collection difficult because at most sites of infection, the local commensal microbiota is close by. Thus, appropriate and careful specimen collection is critical to avoid contamination of the specimen with the commensal microbiota. Anaerobic transport techniques are also essential for the successful recovery of clinically significant anaerobic bacteria (see chapters 18 and 50). Specimens suitable for the isolation of non-spore-forming, Gram-positive, anaerobic rods include aseptically collected peripheral blood, tissue biopsy specimens, aspirates (e.g., cerebrospinal fluid, joint fluids, and pus), root canal exudates, and subgingival plaque. Mucosal or cutaneous swabs are not recommended for the reasons mentioned above. Instead of collecting periprosthetic tissue, sonication of the removed implant followed by sonicate fluid culture has proven to be useful for microbiologic diagnosis of prosthetic joint infection (114, 184).

DIRECT EXAMINATION

Direct examination is of unequivocal value in the confirmation of a diagnosis of actinomycosis. The macroscopic presence of "sulfur granules" in pus, which when crushed, Gram stained, and viewed under the microscope reveal a mass of Gram-positive, branching filaments, is characteristic of this disease. Similarly, in cervical smears of women with an intrauterine contraceptive device, the presence of branching, Gram-positive organisms suggests an infection with *Actinomyces* (91, 185). Gram stains of vaginal smears have been considered more useful than culture for laboratory confirmation of bacterial vaginosis, and the diagnostic criteria for this common infection have been based on the standardized Nugent scoring system (177).

In cases in which there is no typical microbiota associated with a particular infection, care should be taken in determining appropriate empiric antimicrobial treatment on the basis of the Gram stain. For example, branching/pleomorphic rods can be tentatively identified as facultatively anaerobic *Actinomyces* or strictly anaerobic *E. nodatum* (152), and the coccoid cells of *A. radicidentis* are atypical for the genus *Actinomyces* (65), while easily decolorizing species (e.g., *Eubacterium*-like species) can yield a false Gram-negative reaction (134). The misinterpretation can lead to antimicrobial coverage targeted against facultative organisms instead of anaerobes and/or Gram-negative bacteria.

ISOLATION PROCEDURES

Specimens should be processed without delay using appropriate culture media, including standard anaerobic blood agar enriched with hemin and vitamin K_1 and a variety of selective media based on the expected microbiota at the collection site or, in the case of bite wounds, on the oral microbiota of the attacker (human or animal). Fresh or prereduced culture media, including phenylethyl alcohol blood agar and/or colistin-nalidixic acid blood agar, can be useful for enhanced recovery rates of Gram-positive organisms (186). The growth of many asaccharolytic species on solid media is enhanced by the addition of 0.5% arginine (187). In general, members of the aciduric genera *Bifidobacterium* and *Lactobacillus* can be selectively cultured using agar media with an acidic pH, such as Rogosa or deMan, Rogosa, Sharpe agar. However, some nutritionally fastidious *Lactobacillus* strains fail to grow on these agar media. Lactobacilli isolated from dental caries were recovered equally well on nonselective blood-containing media and on Rogosa agar, and it was concluded that acidic-pH medium is not required for their detection (167). Notably, *L. iners*, one of the predominant lactobacilli in the vagina, can grow only on blood agar and not on typical solid media used for *Lactobacillus* (188).

Although some members of this group, particularly *Actinomyces* species, are facultative anaerobes and can grow well on aerobically incubated culture media, anaerobic incubation is recommended for optimal recovery. If anaerobic jars are used for incubation, anaerobic growth should not be exposed to oxygen by opening the jar before 48 h of incubation, in order to facilitate the detection of slow-growing, oxygen-sensitive organisms (186). The availability of an anaerobic chamber may enable examination of the culture whenever necessary. For reliable detection of slow-growing organisms, the incubation time should be sufficient; for instance, an extended incubation period may be needed for some clinically relevant *Actinomyces* spp. and *Eubacterium*-like species (21, 33, 133, 134, 153). In heart tissue specimens from endocarditis patients, grinding the tissue can improve the detection of anaerobic bacteria (77, 99). A lytic anaerobic medium can increase the recovery rate of anaerobes and facultative bacteria in automated blood culture systems (189).

Growth of *S. wiggsiae* on Fastidious Anaerobe Agar (Oxoid Ltd., Basingstoke, United Kingdom) may be enhanced by close proximity to other organisms such as *Staphylococcus aureus* (V. Hall, unpublished data). The viability of *T. sanguinis* is extremely difficult to maintain *in vitro* in solid or liquid media and under usually adequate freezing regimens (32; Hall, unpublished).

IDENTIFICATION

Traditionally, the identification of bacterial isolates in clinical microbiology laboratories is performed by phenotypic tests. For organisms inert in most conventional biochemical tests or with unusual biochemical profiles, as is the case for many *Eubacterium* and related species, or in cases where fastidious organisms require specific nutrients or temperatures, identification strategies based on phenotypic characteristics can be challenging. Some *Actinobacteria* are poorly differentiated in phenotypic tests available to clinical laboratories. In these cases, additional testing is required to achieve identification to the species level. This may include gas-liquid chromatography (GLC), a technique that has now been largely superseded by DNA sequence analysis and/or matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS).

Presumptive Identification

The initial differentiation is based on aerotolerance (growth in air or in air plus 5% CO_2), colonial morphology, pigmentation, fluorescence under long-wave UV illumination (365-nm wavelength), and presence of hemolysis. Colony morphology may provide clues regarding the organism involved; for instance, a "molar tooth" appearance is typical for *A. israelii*, *A. gerencseriae*, *A. nasicola*, *A. oricola*, *P. propionicum*, *S. wiggsiae*, and *E. nodatum*. *A. graevenitzii* may produce both molar-toothed and smooth, entire colonies (186). However, the majority of species discussed in this chapter produce small, relatively nondescript colonies. Other rapidly recognizable features are fluorescence and/or pigment production: *A. graevenitzii*, *P. propionicum*, and *E. lenta* show orange or red fluorescence under UV light (117, 190; Hall, unpublished), and pink/red pigmentation of colonies is typical for *A. odontolyticus*, but some other *Actinomyces* spp. can also produce pigment, especially on rabbit laked blood agar, with *A. graevenitzii* appearing as nearly black, *A. radicidentis* as brown, and *A. urogenitalis* as reddish colonies (53, 191).

Gram stain morphology can contribute to a presumptive identification; it can show whether organisms are Gram-positive, anaerobic rods, which can be very short (e.g., *C. curtum* and *E. lenta*), long (e.g., many *Lactobacillus* spp.), pleomorphic (e.g., *Bifidobacterium*), branching (e.g., many *Actinomyces* spp.), or curved and motile (e.g., *Mobiluncus* spp.); sometimes a specific cell morphology can be seen, such as "flying birds" (e.g., *P. alactolyticus*) and the almost ring-shaped *V. cambriense* (5). The morphology may vary when cells are grown on different culture media. The two *Mobiluncus* species can be tentatively separated based on the length of the curved, motile cells: in contrast to the short, Gram-variable cells of *M. curtisii*, *M. mulieris* reveals clearly longer cells, which often appear as Gram negative (192). Although *Actinomyces* organisms have been traditionally described as branching rods, many new species within the genus are nonbranching, and some have very short or even coccoid cells (65, 193). Staining of cells can vary with different culture conditions. Certain Gram-positive anaerobes, e.g., *F. alocis* and *M. mulieris*, routinely stain Gram negative, whereas older cultures (>3 days) of *Actinobaculum* and some *Eubacterium* species and species of related genera are Gram variable (4, 21, 34). Decolorization of Gram-positive organisms may be due to exposure to oxygen or to damage from fixatives and reagents causing a breakdown of the physical integrity of the cell wall; therefore, anaerobic working conditions or, if not available, limited exposure time to oxygen between incubation and staining improves the reliability of the Gram stain for anaerobic bacteria (194). For rapid confirmation of the Gram reaction, a simple test based on dissolution of the Gram-negative cell wall and cytoplasmic membrane with a solution of 3% potassium hydroxide (195) can be used: when suspended in the solution, Gram-negative cells display increased viscosity and stringing within 30 s, whereas the absence of stringing, i.e., a negative reaction, suggests that the isolate is Gram positive. Routine screening of special-potency antibiotic susceptibility disk patterns is valuable in confirming the accuracy of the Gram stain reaction (196): Gram-positive species are generally resistant to colistin (10 µg) and susceptible to vancomycin (5 µg) and often to kanamycin (1 mg). However, the intrinsic resistance of some *Lactobacillus* species/strains, e.g., *L. rhamnosus*, to glycopeptides should be considered (121, 122, 130), in addition to the intrinsic resistance of *Bifidobacterium* to aminoglycosides (197). *H. filiformis* has been reported to be resistant to vancomycin (31).

Additional rapid tests for initial grouping of non-spore-forming, Gram-positive anaerobes include testing for production of catalase (H_2O_2 at a concentration of 15%) and indole, nitrate reduction, and motility (186). If presumptive identification to the genus level has been made correctly, this may give valuable information to clinicians in deciding the initial treatment. However, differentiating members of the "normal flora" or normal microbiota of human skin and mucous membranes from pathogenic, non-spore-forming, Gram-positive rods can be difficult. Identification of non-sporing, Gram-positive rods to the species level should be performed whenever they are present in pure cultures in clinical specimens or as the predominant organism from normally sterile sites; otherwise, the potential pathogenicity of these less often suspected species may remain undetected.

Biochemical Testing

For a more advanced phenotypic classification of anaerobic organisms and distinguishing of individual species, sugar fermentation reactions, preferably using prereduced, anaerobically sterilized carbohydrates, and enzyme profiles with individual diagnostic tablets, fluorogenic substrate tests, or preformed enzyme kits must be determined. Insufficient growth or poor reproducibility of reactions can cause difficulties in interpretation of results obtained with biochemical tests; therefore, young cultures and heavy inoculum should be used (198). A well-designed selection of key tests provides a tentative identification of various isolates to the species level prior to confirming their identifications by more definitive methods. Table 3 presents some biochemical characteristics of *Actinomyces* and related organisms. Since the description of many novel species, such as *A. dentalis*, *A. hongkongensis*, *A. massiliensis*, *A. nasicola*, *A. oricola*, *A. urinale*, and "*Actinobaculum massiliae*," is based on a single strain (2, 3, 66, 67, 79, 199, 200), discrepancies in test reactions may appear. In addition, the clarification of the taxonomy of the *A. naeslundii*/*Actinomyces viscosus* group, while taxonomically valuable and consistent with their ecology, has resulted in a group of species that cannot be differentiated by phenotypic tests alone. Housekeeping gene sequence analysis is required but may be beyond the scope of routine laboratories. Table 4 presents simple enzymatic reactions useful in distinguishing propionibacteria encountered in human infections, and Table 5 shows tests for *Atopobium* and *Olsenella* species. Although the cultivation and identification of *Eubacterium*-like species can be very laborious, not only because of their oxygen sensitivity and slow growth but also due to their nonreactivity in conventional biochemical testing, some simple reactions are helpful for grouping these organisms (Table 6).

In culture-based identification of anaerobic, non-spore-forming, Gram-positive rods, the determination of major volatile fatty acid end products of glucose metabolism, as detected by GLC, is useful for assigning isolates to the genus level (Table 1). Typically, *Actinomyces* strains produce succinic and lactic acids as their major metabolic end products, but *A. dentalis* is reported not to produce succinic acid (67). The *Actinomyces*-like *Propionibacterium* species, *P. propionicum*, is easily separated from *Actinomyces* based on its production of propionic acid (201). For *Lactobacillus* spp., defining characteristics are their ability to grow in acid media and ferment carbohydrates to produce lactic acid as the major end product with acetate production, whereas *Bifidobacterium* spp. produce acetic acid as a major product.

Preformed enzyme and carbohydrate fermentation profiles can be obtained using commercially available identification test kits, such as the API Rapid ID 32A (bioMérieux, Marcy-l'Etoile, France), RapID (Remel, Lenexa, KS), and BBL Crystal (Becton Dickinson Diagnostic Systems, Sparks, MD) systems, according to manufacturers' instructions. Although this approach is often hindered by similarities in fermentation profiles of separate species within a genus, kits serve as a widely used adjunct to anaerobe diagnostics in most hospital laboratories (202), since they are easy to use and much faster than conventional anaerobic procedures. The main problem with these kits is their incomplete or inaccurate databases (203, 204). Recently, the semiautomated VITEK 2 ANC card (bioMérieux) has been evaluated as a rapid, less subjective method that utilizes colorimetric enzymatic tests. However, this too appears limited by its database, particularly with identification of *Actinomyces* spp. (205). In addition, the same test performed by different methodologies may give conflicting results; this is particularly true for the commercial identification kits in which the tests are "poised," to give a definitive positive or negative reaction to aid interpretation. This can have the effect of making the test insufficiently sensitive, giving false negatives compared to conventional tests, or oversensitive, giving rise to false positives (198, 204). Isolates of *A. vaginae* have been misidentified as *Gemella morbillorum* by the API Rapid ID 32A and RapID ANA II (Remel) test kits (161, 162). In contrast, a clinically relevant *Bifidobacterium* species, *B. scardovii*, was readily separated from other bifidobacteria by using the Rapid ID 32A kit (176). The carbohydrate fermentation test kit API 50 CH (bioMérieux), which is specifically designed for lactobacilli, can be valuable in identification to the genus level but fail at the species level (203). Despite the potential issues, commercial test kits can be useful for the detection of positive reactions and identification of many organisms from clinical sources to the genus level.

A combination of phenotypic tests, specifically the determination of metabolic end products by GLC together with sugar fermentation of carbohydrates and enzyme profiles generated by commercial kits, have successfully been used to identify a number of oral *Eubacterium*-like isolates to the genus and species level (134). For example, the lack of enzyme activity and formation of caproic acid or phenylacetic acid distinguish *P. alactolyticus* or *Mogibacterium* spp., respectively, from other related taxa. However, as already mentioned, phenotypic criteria are particularly unreliable for identification of many *Actinomyces* species (192) and members of the *L. acidophilus* complex and related species (206). In addition, gas chromatographic analysis of cellular fatty acids (196) and examination of protein patterns by polyacrylamide gel electrophoresis have been used taxonom-

TABLE 3 Biochemical characteristics of *Actinomyces* and related bacteria encountered in human infections[a]

Genus and species	Aerotolerance	Pigment[b]	Production of:		Nitrate reduction	Esculin hydrolysis	Production of[c]:			CAMP test	Fermentation of[d]:				
			Catalase	Urease			α-Glu	β-NAG	β-Gal		Mal	Man	Raf	Suc	Tre
Actinobaculum															
A. *massiliense*	+	−	−	−	−	−	+	−	−	−	+	−	+	ND	+
A. *schaalii*	+	−	−	−	−	−	+	−	−	Weak	+	−	−	v	v
A. *urinale*	(+)	−	−	+	−	−	−	−	−	−	v	−	−	v	−
Actinomyces															
A. *cardiffensis*	(+)	−	−	−	v	−	+	−	−	−	v	−	v	v	−
A. *dentalis*	−	−	−	−	−	+	+	−	+	−	+	−	+	+	+
A. *europaeus*	+	−	−	−	v	v	+	−	+	−	+	−	−	v	v
A. *funkei*	+	−	−	−	+	−	+	v	v	v	v	−	−	+	−
A. *georgiae*	+	−	−	−	v	+	+	−	+	−	+	−	−	+	+
A. *gerencseriae*	+	−	−	−	v	+	+	−	+	−	+	v	v	+	v
A. *graevenitzii*	+	+	−	−	v	−	v	+	+	−	+	−	v	+	v
A. *hominis*	+	−	+	−	+	−	+	+	+	+	+	+	+	+	ND
A. *hongkongensis*	−	−	−	−	−	−	−	−	−	−	ND	ND	−	ND	−
A. *israelii*	(+)	−	−	−	+	+	+	−	+	−	+	−	+	+	+
A. *johnsonii*	+	−	v	v	+	−	+	ND	+	ND	ND	ND	ND	ND	v
A. *massiliensis*	+	−	−	−	+	ND	+	−	+	ND	+	−	−	+	+
A. *meyeri*	−	−	−	−	v	−	+	+	+	v	+	−	−	+	−
A. *naeslundii*	+	−	−	+	+	v	v	−	+	−	+	−	+	+	+
A. *nasicola*	(+)	−	−	−	−	−	+	+	+	−	−	−	−	−	−
A. *neuii*															
subsp. *anitratus*	+	−	+	−	−	−	v	−	+	+	+	+	v	+	v
subsp. *neuii*	+	−	+	−	+	−	+	−	+	+	+	+	v	+	v
A. *odontolyticus*	+	+	−	−	+	v	−	−	+	−	+	−	−	+	−
A. *oricola*	+	−	−	−	−	+	+	−	v	−	−	−	+	+	+
A. *oris*	+	−	v	−	+	v	v	ND	v	ND	ND	ND	ND	ND	v
A. *radicidentis*	+	+	+	v	v	+	+	−	+	−	+	+	+	+	+
A. *radingae*	+	−	−	−	v	+	+	+	+	v	+	−	v	+	v
A. *timonensis*	+	−	−	−	−	+	−	−	−	ND	+	+	+	+	+
A. *turicensis*	+	−	−	−	−	−	+	−	−	−	+	−	v	+	v
A. *urogenitalis*	+	+	−	−	+	+	+	+	+	−	+	−	−	+	+
A. *viscosus*	+	−	+	v	+	v	v	−	v	−	+	v	+	+	v
Varibaculum															
V. *cambriense*	(+)	−	−	−	+	−	+	−	v	−	+	v	−	+	−

[a] Biochemical data from references 1, 2, 66, 67, 79, 193, 196, 198–201, and 242. Abbreviations and symbols: +, positive; (+), better growth in anaerobic conditions; −, negative; v, variable; ND, no data.

[b] A. *graevenitzii* colonies are nonpigmented on brucella agar but almost black on rabbit laked blood, whereas other pigment-producing *Actinomyces* spp. appear as pinkish/brownish colonies on brucella agar and as darker colonies on rabbit laked blood.

[c] Abbreviations: α-Glu, α-glucosidase; β-NAG, β-*N*-acetyl-glucosaminidase; β-Gal, β-galactosidase.

[d] Abbreviations: Mal, maltose; Man, mannitol; Raf, raffinose; Suc, sucrose; Tre, trehalose.

TABLE 4 Biochemical characteristics of propionibacteria encountered in human infections[a]

Genus and species	Aerotolerance	Catalase	Indole	Nitrate reduction	Esculin hydrolysis
Propionibacterium					
P. acidifaciens	−	−	−	−	−
P. acnes	+	+	+	+	−
P. avidum	+	+	−	−	+
P. granulosum	+	+	−	−	−
P. propionicum	−	−	−	+	−
Propioniferax					
P. innocua	+	+	−	v	−
Propionimicrobium					
P. lymphophilum	−	v	−	v	ND

[a] Biochemical data compiled from references 7–9 and 196. Abbreviations and symbols: +, positive; −, negative; v, variable; ND, no data.

ically to distinguish among strains within a species and among organisms within a genus or family.

Clinical microbiologists should be aware of the possibility of erroneous identification and adjust the interpretation of their results accordingly, in conjunction with cellular and colonial morphology and other information available. Indeed, practical, discriminatory, and cost-effective methods are needed for identification of fastidious Gram-positive bacteria.

Identification by DNA Sequence Analysis

As discussed above, the use of conventional and biochemical tests for the identification of this group carries a significant risk of misidentification. Far-more-precise identifications can be obtained by 16S rRNA gene sequence analysis (207). DNA can be rapidly and reliably purified from members of this group by using commercially available kits, such as GenElute (Sigma-Aldrich, St. Louis, MO), and the 16S rRNA gene can be amplified using "universal" primers that amplify all members of the domain *Bacteria* (208, 209). Amplicons can be sequenced in-house or submitted to commercial sequencing facilities. The 5′ region of the gene is the most informative for identification purposes; the use of primer 519R for sequencing is recommended (209). Sequences are identified by comparison with those held in the DNA sequence databases, such as GenBank. BLAST interrogation (210) is useful, but care needs to be taken when using the nr/nt database, as a small number of sequences are mislabeled and some nomenclature is inconsistent with current taxonomy. Alternatively, the RefSeq RNA database, which includes only type strains, can be utilized; however, this database is less comprehensive. Also, pairs or even groups of species may have virtually identical 16S rRNA gene sequences and so may require additional tests for identification to the species level. Sequencing of an alternative housekeeping gene (*met*G or *atp*A) or a modified technique such as multilocus sequence typing is necessary to differentiate *A. naeslundii*, *A. johnsonii*, and *A. oris* to the species level (1, 211).

Identification to the genus level is ensured, but at the species level, some investigation of the phylogenetic status of the genus should be made. This technique, while extremely powerful, should not be regarded as an infallible "black box" method. A major advantage is that methods do not need to be adapted for different groups of organisms; thus, there are no special considerations needed when analyzing Gram-positive, non-spore-forming anaerobes, except that the DNA extraction method needs to be suitable for lysing the rigid, Gram-positive cell wall.

A major impetus for the study of as yet uncultured bacteria has been the development of 16S rRNA/PCR amplification/cloning/sequencing methodology (212). The phylum *Firmicutes*, in particular, has been found to harbor a number of lineages without culturable representatives. For example, branches within the families *Eubacteriaceae* and *Lachnospiraceae* have been found in advanced carious lesions, endodontic infections, and subgingival plaque in periodontitis (142, 167, 213). Similarly, the distal esophagus, stomach, and colonic microbiotas include novel branches within the *Clostridiaceae*, *Erysipelotrichaceae*, and *Lachnospiraceae* (49, 51, 214). Culture-independent analysis of the microbiota in bacterial vaginosis identified a novel taxon related to *A. vaginae* (164), and novel taxa belonging to both the *Actinobacteria* and *Firmicutes* were found in a corneal ulcer (215).

Identification by MALDI-TOF MS

Recently, MALDI-TOF MS has been applied to a wide range of organisms; however, relatively few studies have

TABLE 5 Enzyme reactions useful in distinguishing species within the genera *Atopobium* and *Olsenella*[a]

Genus and species	Aerotolerance	Production of:		Nitrate reduction	Hydrolysis of:		Acid phosphatase	β-Gal
		Catalase	Indole		Esculin	Arginine		
Atopobium								
A. minutum	−	−	−	−	ND	+	−	−
A. parvulum	−	−	−	−	v	−	+	+
A. rimae	−	−	−	−	v	v	+	−
A. vaginae	(−)[b]	−	−	−	−	+	+	−
Olsenella								
O. profusa	−	−	−	−	+	−	+	+
O. uli	−	−	−	−	+	+	+	−

[a] Biochemical data compiled from references 14, 15, 17, 152, and 159. Abbreviations and symbols: +, positive; −, negative; v, variable; ND, no data; β-Gal, β-galactosidase.

[b] In the original description, *A. vaginae* is facultatively anaerobic.

TABLE 6 Biochemical characteristics of some human *Eubacterium*-like organisms[a]

Phylum and species	Glucose fermentation	Production of:		Nitrate reduction	Hydrolysis of:	
		Catalase	Indole		Esculin	Arginine
Actinobacteria						
Collinsella aerofaciens	+	−	−	−	v	v
Collinsella intestinalis	+	ND	ND	ND	ND	ND
Collinsella stercoris	+	ND	ND	ND	ND	ND
Cryptobacterium curtum	−	−	−	−	−	+
Eggerthella lenta	−	+	−	+	−	+
Eggerthella sinensis	−	+	−	−	ND	+
Paraeggerthella hongkongensis	−	+	−	−	ND	+
Slackia exigua	−	−	−	−	−	+
Firmicutes						
Bulleidia extructa	+	−	−	−	−	+
Catenibacterium mitsuokai	+	−	ND	−	−	ND
Eubacterium brachy	−	−	−	−	−	−
Eubacterium limosum	+	−	−	−	+	v
Eubacterium minutum	−	−	−	−	−	−
Eubacterium nodatum	−	−	−	−	−	+
Eubacterium rectale	+	−	−	−	+	−
Eubacterium saphenum	−	−	−	−	−	−
Eubacterium sulci	−	−	−	−	−	−
Eubacterium tenue	Weak	−	+	−	−	ND
Eubacterium yurii	Weak	−	+	−	−	−
Filifactor alocis	−	−	−	−	−	+
Flavonifractor plautii	Weak	ND	−	−	−	ND
Holdemania filiformis	+	−	−	−	+	−
Lachnoanaerobaculum saburreum	+	−	+	−	+	+
Mogibacterium spp.	−	−	−	−	−	−
Pseudoramibacter alactolyticus	+	−	−	−	−	−
Robinsoniella peoriensis	+	ND	−	−	ND	ND
Shuttleworthia satelles	+	−	+	−	+	−
Solobacterium moorei	+	−	−	−	+	+

[a] Biochemical data compiled from references 15, 16, 20, 24, 27, 28, 30, 31, 33, 37–39, 128, 130, 149, and 224. Abbreviations and symbols: +, positive reaction; −, negative reaction; v, variable reaction; ND, no data.

focused specifically on the organisms included in this chapter. The majority of published studies have utilized the Microflex MALDI-TOF MS analyzer (Bruker Daltonik GmbH, Bremen, Germany) or the VITEK MS MALDI-TOF (bioMérieux). Direct inoculation of organism onto a MALDI-TOF MS plate has been found to generate good identification of common anaerobic organisms. However, some authors found identification rates of only 61.5 to 67.2% using this method (216, 217). Fedorko et al. found that pretreatment was beneficial in the identification of Gram-positive organisms (218). The off-plate method involving a 70% ethanol extraction may be used, but in other studies, simpler and more rapid on-plate extraction methods have been found to give equivalent results (216, 219). Identification is then performed by comparison of spectra generated against a database of reference spectra. Several databases have been developed, including the Biotyper (Bruker) and the Saramis (bioMérieux), both of which utilize spectra from extracted samples, and the Andromas database (Paris, France), comprising data from the direct colony method (220).

In a comprehensive study employing partial (~500 bp) 16S rRNA sequencing as the gold standard for identification, MALDI-TOF MS of 290 isolates representing 24 *Actinomyces* species yielded correct identification rates to the genus and species level of 60 and 50%, respectively, using the Microflex MALDI-TOF MS and the Flex Control version 4613 database (221). Identification rates for *Actinobaculum* and *Varibaculum* species were 47 and 0%, respectively. In the same study, some genera yielded higher identification rates: *Propionibacterium* species, 71% ($n = 51$); *Eggerthella*, 92% ($n = 12$); *Atopobium*, 71% ($n = 17$); and *Bifidobacterium*, 81% ($n = 16$). However, genera such as *Lactobacillus*, *Eubacterium*, and *Scardovia* were not identified well to the species level, with rates of 0 to 36% (221). Other studies have reported good identification of *Actinomyces* spp. to the genus level, although identification of some species such as *A. neuii* remains poor (222).

A number of clinically significant genera and species, e.g., *Mogibacterium* species and *Actinobaculum massiliense*, are not included in the current database, which potentially results in either no reliable identification or incorrect identification: *M. timidum* was identified as *Clostridium* species in a study by Schmitt et al. (219). Furthermore, for some species, e.g., *V. cambriense* and *P. propionicum*, the database comprises few or poorly representative strains, and poor scores may result from intraspecies variation within clinical isolates. This includes *P. acnes*, within which several phylogroups have been identified. Recently MALDI-TOF MS has been utilized to discriminate between these phylotypes, which may aid in differentiating between clinically significant *P. acnes* isolates and possible contaminants (223). Changes in nomenclature may also result in distorted identification rates, as *E. catenaformis* is listed as *L. catenaformis* in the current database.

In summary, MALDI-TOF MS shows great promise for the future provided that the databases are further developed and regularly updated.

SEROLOGIC TESTS

Serologic tests are of little diagnostic value for this group of organisms.

ANTIMICROBIAL SUSCEPTIBILITIES

In the clinical setting, empirical information is used for the initial diagnosis of infection and choice of antimicrobial therapy, while awaiting culture and susceptibility test results. This is particularly important for this group of organisms because many of them are slow growing, and if they are isolated as part of a mixed infection, it may take some time to obtain pure cultures for testing.

Published data regarding antimicrobial susceptibilities of nonsporing, Gram-positive anaerobes can be difficult to interpret. Changes in bacterial taxonomy, e.g., among the species of the former *Eubacterium* genus, and more precise classification of tested isolates may result in antimicrobial resistance patterns different from those given in previously published surveys (224). In general, penicillin and other β-lactams are active against Gram-positive bacteria, together with parenteral carbapenems, including meropenem (197, 224–228). Metronidazole has been considered a drug of choice for treatment of anaerobic infections; however, the facultative anaerobes among the genera *Propionibacterium*, *Actinobaculum*, *Actinomyces*, *Bifidobacterium*, and *Lactobacillus* are intrinsically resistant, and resistant strains can also be found among the strictly anaerobic genera *Atopobium*, *Eggerthella*, *Eubacterium*, and *Mobiluncus* (161, 162, 227, 229, 230). Failures or relapses are common in the treatment of bacterial vaginosis, but whether metronidazole-resistant *A. vaginae* or *M. curtisii* (161, 162, 181) plays a role is not known. Occasional strains among various genera of non-spore-forming, Gram-positive, anaerobic rods show resistance to clindamycin (224, 225, 229, 231–234). Although vancomycin and teicoplanin are considered active against most Gram-positive bacteria, species-related resistance to glycopeptides is frequent among species of the genus *Lactobacillus*. Less than one-quarter of the isolates from 80 cases of *Lactobacillus* infections were reported as susceptible to vancomycin (120). Notably, the vancomycin-resistant *L. rhamnosus* is the most common *Lactobacillus* species in clinical specimens (121, 123). In contrast to vancomycin and teicoplanin, ramoplanin showed good activity against lactobacilli (229, 235). A novel glycopeptide, telavancin, has proved to be more active than vancomycin against lactobacilli, except for *L. casei*, and demonstrated very good activity against the tested strains of *Propionibacterium*, *Actinomyces*, *Eggerthella*, and *Eubacterium* (226). Tigecycline has been shown to be active against lactobacilli, including *L. casei*, and *Actinomyces* species (236). Oxazolidinones have relatively good *in vitro* activities against Gram-positive cocci but also against anaerobes, and ranbezolid may have lower MICs than linezolid (225, 237). Also, streptogramin antimicrobial agents, such as pristinamycin and quinupristin-dalfopristin, have considerable activities against non-spore-forming, Gram-positive rods (197, 225, 232). Fluoroquinolones have a broad spectrum of antibacterial activity and good absorption from the gastrointestinal tract. Novel quinolones, such as garenoxacin, gatifloxacin (topical application), and moxifloxacin, exhibit better antianaerobic activity than the older quinolone compounds levofloxacin and ciprofloxacin (225, 230, 231, 233), suggesting their potential in treating mixed-organism infections.

The testing of anaerobic isolates for susceptibility to antimicrobials by clinical laboratories remains problematic (see chapter 75). The Clinical and Laboratory Standards Institute (CLSI) defines the agar dilution method as the gold standard but recommends it only for reference laboratories (238). Specific guidance is available for lactobacilli (239). Broth microdilution is recommended for clinical laboratories but is currently limited to fragilis group *Bacteroides*. There are a number of reasons for the current lack of susceptibility testing for this group of organisms. Firstly, anaerobic, Gram-positive rods are frequently isolated from polymicrobial infections from which 10 or more species may be cultivated. The relevance of individual susceptibility testing and its interpretation in this scenario are unclear. Secondly, as has been described in this chapter, there are a large number of species that may be found in clinical material, but each laboratory may encounter them relatively rarely. There are therefore insufficient reference data on the susceptibility profile of each species, but even if appropriate data were available, quality control procedures and strains would be required for each species. The most commonly used method for testing anaerobes is the gradient strip (202), which has been found to be useful and reliable (240, 241). Tests should be performed on brucella blood agar and are optimally read after 48 h, to allow sufficient bacterial growth. Some slow-growing species may require longer incubation.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The primary considerations when interpreting laboratory data are the site from which the sample was collected, the collection method, and the likelihood of contamination by the commensal biota. Culture plates from samples from mucosal and cutaneous sites should be interpreted with reference to the normal commensal biota expected for that site and any recent or current antimicrobial therapy. It is important that incubation times are sufficiently long to allow growth of the slow-growing members of this group. Premature reporting of only the fastest-growing species can be misleading since growth rates *in vivo* and *in vitro* may be very different. Incubation should be continued for at least 7 days before the final report is issued. The finding of a culture from a specimen dominated by a restricted number of organisms is normally suggestive of infection, particularly if suspected clinically, although recent administration of broad-spectrum antimicrobials may also reduce the diversity of the commensal microbiota.

All isolations of members of this group from normally sterile sites are significant, and the organism should be identified. Members of the group are generally of low-grade pathogenicity and do not produce classical virulence factors, such as protein toxins. All isolates should be regarded as equally important and reported with susceptibility to antimicrobials appropriate to the clinical diagnosis and the site of the infection. Obtaining pure cultures of all of the organisms present in a polymicrobial infection can be difficult and time-consuming but should be attempted. Collation of data regarding the identity and antimicrobial susceptibility profiles of isolates causing confirmed infections will be invaluable in formulating recommendations for empirical treatment, which are lacking at present, and in allowing associations between particular species and diseases to be made.

One specific disease caused by Gram-positive, nonsporing anaerobes is actinomycosis. Pus collected from suspected lesions should be examined macro- and microscopically for the presence of sulfur granules; if present, this should be reported as confirmation of a clinical history consistent with actinomycosis. Culture of *Actinomyces* or related genera alone from a site where the specimen is likely to have been contaminated with the commensal microbiota should be interpreted with caution. This is particularly the case for the head and neck regions since *Actinomyces* is one of the predominant genera among the normal oral microbiota.

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Clostridium[*]

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TAXONOMY

The genus *Clostridium* comprises obligately anaerobic (or occasionally aerotolerant), Gram-positive rods. Currently, >200 clostridial species and subspecies are validly published (http://www.dsmz.de); however, the number of clinically significant clostridia from human infections is limited (Table 1).

Phylogenetically, the genus *Clostridium* is heterogeneous, with many species intermixed with other spore-forming and non-spore-forming genera. Traditionally, the different species have been defined based on morphological, ultrastructural, and physiological features. During the past 2 decades, analyses of 16S rRNA gene sequences indicated that the "clostridia" could be divided into 19 clusters (1). Cluster I forms the basis of the genus *Clostridium* and is analogous to group I proposed by Johnson and Francis almost 40 years ago (2). The type species, *C. butyricum*, and most of the clinically relevant *Clostridium* species cluster within rRNA homology group I (reviewed in reference 3). The heterogeneous non-group I clostridia require reclassification; however, 16S rRNA gene sequences may not be adequate alone in distinguishing genera, and it is necessary to find genetic and phenotypic characteristics that enable rapid discrimination among genera within this group.

Two new species clustering within the *C. coccoides* rRNA group, *C. hathewayi* (4) and *C. bolteae* (5), were described from human feces. Phenotypically, *C. clostridioforme* is a relatively heterogeneous anaerobic species. Sequencing analyses of 16S rRNA genes from 107 strains that were previously identified phenotypically as *C. clostridioforme* in various clinical laboratories revealed that "*C. clostridioforme*" in fact represents three distinct species: *C. bolteae*, *C. clostridioforme*, and *C. hathewayi* (6). *C. bartlettii* is another new *Clostridium* species described from human feces (7); the clinical significance of this organism remains unknown. "*C. neonatale*" was proposed as a novel species recovered from bacteremia in patients with necrotizing enterocolitis (NEC) (8). *Anaerotruncus colihominis* is a new genus and species within the *C. leptum* rRNA cluster of organisms originally described from human feces (9) and subsequently found in patients with bacteremia (10). Though it was originally described as a non-spore-forming organism, further studies have revealed that sporulation occurs under some conditions (10) and should therefore be considered in *Clostridium* identification schemes. On the basis of biochemical properties, phylogenetic position, DNA G+C content, and DNA-DNA hybridization, the unification of *Clostridium orbiscindens* and *Eubacterium plautii* into the new species *Flavonifractor plautii* has been proposed (11).

DESCRIPTION OF THE GENUS

Clostridia belong to the phylum *Firmicutes* and comprise a heterogeneous (paraphyletic) group consisting of at least 12 lineages. Clostridia have a wide range of G+C contents, from 22 to 55 mol%, while the toxigenic species have a much narrower range of G+C contents, 24 to 29 mol% (3). Morphological and phenotypic properties that have traditionally been used to define the genus include (i) the formation of endospores, (ii) anaerobic energy metabolism, (iii) an inability to reduce sulfate to sulfide, and (iv) a Gram-positive cell wall structure.

Vegetative cells of *Clostridium* species are pleomorphic, rod shaped, and arranged in pairs or short chains; the cells have rounded or sometimes pointed ends (12, 13). Rods may join to form tight coils or spiral configurations in species such as *C. cocleatum* and *C. spiroforme*. Clostridia stain Gram positive in early stages of growth, although some species, such as *C. clostridioforme*, *C. hathewayi*, *C. innocuum*, and *C. ramosum*, may appear Gram negative. Several species (e.g., *C. tetani*) appear Gram negative by the time that spores have formed. Endospores are often wider than the vegetative organisms, imparting characteristic spindle shapes to clostridia. Most strains are motile by means of peritrichous flagella. Nonmotile species include *C. perfringens*, *C. ramosum*, and *C. innocuum* (12).

Clostridium species are metabolically diverse. As currently designated (1), most species are chemoorganotrophic; some species may be chemoautotrophic and chemolithotrophic. They can be saccharolytic, proteolytic, neither, or both; they do not carry out dissimilatory sulfate reduction. They usually produce mixtures of organic acids and alcohols from carbohydrates, proteins and peptides, or purines and pyrimidines.

Most species are obligately anaerobic, although the tolerance to oxygen varies widely; some species (e.g., *C. tertium*) grow but do not sporulate in the presence of air, and a few aerotolerant species, such as *C. carnis*, *C. histolyticum*, and occasional strains of *C. perfringens*, give scant growth on

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[*] This chapter contains information presented by Eric A. Johnson, Paula Summanen, and Sydney M. Finegold in chapter 57 of the 9th edition of this *Manual* and by Dennis L. Stevens, Amy E. Bryant, Anja Berger, and Christoph von Eichel-Streiber in chapter 50 of the 10th edition of this *Manual*.

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solid media incubated under 5 to 10% CO_2. Aerotolerant clostridia and certain *Bacillus* species may be distinguished by several means: (i) clostridia usually form spores only under anaerobic conditions, (ii) they grow better anaerobically than in air, (iii) they usually do not produce catalase, and (iv) they have straight-chain, saturated, and monounsaturated cellular fatty acid (CFA) compositions, whereas *Bacillus* species have branched-chained CFAs. Although *Clostridium* species are usually catalase and superoxide dismutase negative, trace amounts of these enzyme activities may be detected in some strains, such as *C. perfringens*. In addition, clostridia lack a cytochrome system and are thus oxidase negative. Clostridia often occur in nature and in infections as consortia of mixed species, wherein aerobic and facultative organisms utilize oxygen, provide nutrients or other factors, and create an environment favorable for clostridial growth.

Clostridia produce more kinds of protein toxins than any other bacterial genus, and >25 toxins lethal to mice have been identified (reviewed in reference 14). At least 15 species of cluster I *Clostridium* produce protein toxins, and new toxins and virulence proteins have been discovered through traditional isolation techniques and genomic analyses (15, 16). These proteins include neurotoxins, enterotoxins, cytotoxins, collagenases, permeases, necrotizing toxins, lipases, lecithinases, hemolysins, proteinases, hyaluronidases, DNases, ADP-ribosyltransferases, neuraminidases, and some others that are simply known as lethal toxins. Botulinum neurotoxin and tetanus neurotoxin (BoNT and TeNT) are the most potent toxins known, with lethal doses of 0.2 to 10 ng per kg of body weight for various animals, including humans (17). Epsilon toxin is a 33-kDa protein produced by *C. perfringens* type B and D strains, and in animals it causes edema and hemorrhage in the brain, heart, spinal cord, and kidneys. It is among the most lethal of clostridial toxins and is considered a potential bioterrorism agent (17, 18).

Many genomic sequences of pathogenic clostridia are now available and should facilitate a comprehensive approach for understanding virulence factors involved in clostridial pathogenesis:

C. perfringens (http://www.ncbi.nlm.nih.gov/genome/158)
C. difficile (http://www.ncbi.nlm.nih.gov/genome/535)
C. botulinum (http://www.ncbi.nlm.nih.gov/genome/726)
C. tetani (http://www.ncbi.nlm.nih.gov/genome/1098)

EPIDEMIOLOGY AND TRANSMISSION

Clostridium species are widespread in nature due to their ability to form resistant endospores. They are commonly found in soil, feces, sewage, and marine sediments. The ecology of *C. perfringens* in soil is greatly influenced by the degree and duration of animal husbandry (reviewed in reference 19), and this has relevance to the incidence of gas gangrene caused by contamination of war wounds with soil. For example, the incidence of clostridial gas gangrene was higher in agricultural lands in Europe than in the Sahara Desert of Africa (19). Similarly, the incidences of tetanus and foodborne botulism are also clearly related to the presence of clostridial spores in soil, water, and many foods (19). Outbreaks of hospital-acquired enteric *C. difficile* infections (CDIs) are often traceable to environmental sources and other typical background factors for nosocomial infection (20). Clostridia are present in large numbers in the indigenous microbiota of the intestinal tracts of humans and animals, in the female genital tract, and in the oral mucosa as well.

CLINICAL SIGNIFICANCE

Although exogenous clostridial infections or intoxications, such as tetanus, foodborne botulism, and gas gangrene, have been feared for centuries, severe cases of hospital-acquired and community-acquired *C. difficile* colitis have recently emerged. Endogenous clostridia, in association with non-spore-forming anaerobes and facultative or aerobic organisms, also cause severe infections in diabetic patients and in patients in whom the mucosal integrity of the bowel or respiratory system has been compromised. Head and neck infections, brain abscesses, sinusitis, otitis, aspiration pneumonia, lung abscesses, pleural empyemas, cholecystitis, intra-abdominal infections, gynecologic and obstetric infections, soft tissue infections, myonecrosis, and septic arthritis and bone infections all may involve clostridia. Common predisposing factors are surgical procedures, trauma, vascular stasis, bowel obstruction, malignancy, immunosuppressive agents, diabetes mellitus, prior aerobic infection, and use of antimicrobial agents with poor activity against clostridia (see the section on *C. difficile* below).

Clostridial Bacteremia

Clostridium species are important causes of bloodstream infections (21–23). *C. septicum* is isolated only rarely from the feces of healthy individuals but may be found in the appendixes of normal individuals. More than 50% of patients whose blood cultures are positive for this organism have some gastrointestinal anomaly, such as diverticular disease, or an underlying malignancy, such as carcinoma of the colon. Another clinically important association has been observed between *C. septicum* bacteremia and neutropenia of any origin (24) and, more specifically, neutropenic enterocolitis involving the terminal ileum or cecum (13, 25, 26). Patients with diabetes mellitus, severe atherosclerotic cardiovascular disease, or anaerobic myonecrosis (gas gangrene) may also develop *C. septicum* bacteremia (27). The clinical importance of recognizing *C. septicum* bacteremia and starting appropriate treatment immediately cannot be overemphasized. Patients with this condition are usually gravely ill and may have metastatic spread to distant anatomic sites, resulting in spontaneous myonecrosis (13). Mortality rates are very high. *C. septicum* has also been recovered from cirrhotic patients with bacteremia, as have *C. perfringens*, *C. bifermentans*, and other clostridia (28). Some of these patients have demonstrated septic shock (26).

Another clostridial species of importance in patients with serious underlying disease, such as malignancy and acute pancreatitis, is *C. tertium*. This organism, as well as *C. septicum* and *C. perfringens*, may be seen among the bacteria in the blood of such patients, with or without neutropenic enterocolitis (29). *C. tertium* may present special problems in terms of both identification and treatment. This organism may appear to be Gram negative, and it is aerotolerant and resistant to metronidazole, clindamycin, and cephalosporins. *C. sordellii* and *C. perfringens* have been associated with toxic shock syndrome and abortion (30, 31).

Studies of anaerobic bacteremia by Woo et al. (32) and Simmon et al. (22) identified clostridia based on sequencing of genes encoding 16S rRNA. *C. perfringens* and *C. tertium*

TABLE 1 Characteristics of *Clostridium* species of clinical significance[a]

Species	**Gelatin hydrolysis**	**Lecithinase**	**Lipase**	**Indole**	**Esculin hydrolysis**	**Nitrate**	**Milk digestion**	Fermentation of:		
								Glucose	**Arabinose**	Cellobiose
Saccharolytic, proteolytic										
C. *bifermentans*[b]	+	+	–	+	$+^{-}$	–	+	+	–	–
C. *botulinum*[c]										
Types A, B, and F	+	–	+	–	+	–	+	$+^{-}$	–	–
Types B, E, and F[d]	+	–	+	–	–	–	–	+	w^{-}	–
Types C and D	+	$-^{+}$	+	$-^{+}$	–	–	+	+	–	–
C. *cadaveris*	+	–	–	+	–	–	+	+	–	–
C. *difficile*[e]	+	–	–	–	+	–	–	$+^{w}$	–	v
C. *novyi* A	+	+	+	–	–	–	–	+	–	–
C. *perfringens*	+	+	–	–	v	v	+	+	–	$-^{+}$
C. *putrificum*	+	–	–	–	$-^{+}$	–	+	$+^{w}$	–	–
C. *septicum*[f]	+	–	–	–	+	v	+	+	–	$+^{w}$
C. *sordellii*[b]	+	+	–	+	$-^{+}$	–	+	+	–	–
C. *sporogenes*[f]	+	–	+	–	+	–	+	+	–	$-^{w}$
Saccharolytic, nonproteolytic										
C. *baratii*	–	+	–	–	+	$+^{-}$	–	+	–	+
C. *bolteae*[g]	–	–	–	–	$-^{+}$	–	–	+	+	$-^{+}$
C. *butyricum*	–	–	–	–	+	–	–	+	$+^{-}$	+
C. *carnis*[h]	–	–	–	–	+	–	–	+	–	w^{+}
C. *clostridioforme*[g]	–	–	–	–	+	–	–	+	+	+
C. *glycolicum*	–	–	–	–	$-^{+}$	–	–	+	–	–
C. *hathewayi*[g]	–	–	–	–	$+^{w}$	–	–	+	v	+
C. *indolis*	–	–	–	+	+	$+^{-}$	–	+	w^{-}	$+^{w}$
C. *innocuum*[e]	–	–	–	–	+	–	–	+	$-^{+}$	+
C. *paraputrificum*	$-^{w}$	–	–	–	+	$-^{+}$	–	+	–	+
C. *ramosum*	–	–	–	–	+	–	–	+	–	+
C. *sphenoides*	–	–	–	+	+	$+^{-}$	–	+	$-^{w}$	$+^{w}$
C. *symbiosum*	$-^{w}$	–	–	–	–	–	–	$+^{w}$	v	–
C. *tertium*[h]	–	–	–	–	+	$+^{-}$	–	+	–	$+^{w}$
Asaccharolytic										
C. *argentinense*	+	–	–	–	–	–	+	–	–	–
C. *hastiforme*	+	–	–	–	–	$-^{+}$	$-^{+}$	–	–	–
C. *histolyticum*[h]	+	–	–	–	–	–	+	–	–	–
C. *limosum*	+	+	–	–	–	–	+	–	–	–
C. *subterminale*	+	$-^{+}$	–	–	$-^{+}$	–	+	–	–	–
C. *tetani*[f]	+	–	$-^{w}$	v	–	–	+	–	–	–

[a] Abbreviations and symbols: +, positive reaction; –, negative reaction; v, variable reaction; w, weakly positive reaction; ST, subterminal; T, terminal. A superscript indicates rare variability. Boldface type indicates key reaction results. Capital letters indicate major metabolic products from peptone-yeast-glucose (PYG), lowercase letters indicate minor products, and parentheses indicate a variable reaction for fatty acids as follows: A, acetic; B, butyric; IB, isobutyric; IC, isocaproic; IV, isovaleric; L, lactic; P, propionic; PA, phenylacetic; S, succinic; and V, valeric.

[b] C. *bifermentans* is urease negative, and C. *sordellii* is urease positive. C. *bifermentans* usually forms chalk-white colonies on egg yolk agar.

[c] A toxin neutralization test is required for identification. Send suspected isolates or C. *botulinum*-containing material to the appropriate local or state public health agency.

were the two most frequently identified species, causing up to 79% and 5%, respectively, of clostridial bacteremias. The mortality rate of clinically relevant clostridial bacteremia ranged from 29 to 35%, and risk factors for mortality (33) were liver disease and older age. The C. *clostridioforme* group (including C. *clostridioforme*, C. *hathewayi*, and C. *bolteae*) has also caused bacteremia (6, 34).

Enteric Infections

Food Poisoning

C. *perfringens* is one of the most common bacterial causes of foodborne illness in the United States and Canada (35–37), and virtually all cases have been due to type A strains (18, 35, 38). In C. *perfringens* type A foodborne disease, the food vehicle is typically improperly cooked meat or a meat product, such as gravy, that has cooled slowly after being cooked or may have been inadequately reheated. Spores surviving the initial cooking germinate, and vegetative cells proliferate during slow cooling or insufficient reheating. Illness results from the ingestion of food containing about 10^8 or more viable vegetative cells, which sporulate in the alkaline environment of the small intestine, producing an enterotoxin (C. *perfringens* enterotoxin [CPE]) in the process. Diarrhea develops within 7 to 30 h of ingestion of such food and is generally mild and self-limiting (18); however, in the very young, the elderly, and the immunocompromised, symptoms are more severe, occasionally resulting in death (39). Enterotoxin-producing C. *perfringens* has been implicated as an etiologic agent of persistent diar-

TABLE 1 Characteristics of *Clostridium* species of clinical significance[a]

Fermentation of:										Spore location	Metabolic end products from PYG
Fructose	Lactose	Maltose	Mannitol	Mannose	Melibiose	Ribose	Salicin	Sucrose	Xylose		
$-^{w}$	–	w^{-}	–	$-^{w}$	–	–	–	–	–	ST	A (iv, ic, p, ib, b, l, s)
$-^{w}$	–	$-^{w}$	–	–	–	–	–	–	–	ST	A, B, IV, ib (ic, v, p)
$+^{w}$	–	$+^{-}$	$-^{w}$	$+^{w}$	$-^{w}$	v	–	$+^{w}$	–	ST	B, A (1)
v	–	v	–	v	$-^{w}$	v	–	–	–	T	B, P, A (v, l, s)
v	–	–	–	$-^{w}$	–	–	–	–	–	T	B, A
$+^{w}$	–	–	w^{+}	v	–	$-^{w}$	$-^{w}$	–	$-^{w}$	ST^{t}	B, A, ic, iv, ib (v, l)
$-^{w}$	–	v	–	–	$-^{w}$	v	$-^{w}$	–	–	ST	A, B, P
+	+	+	–	+	v	v	$-^{+}$	+	–	ST	A, B, L (p, s)
$-^{w}$	–	$-^{w}$	–	–	–	–	–	–	–	T^{ST}	A, B, ib, iv (p, ic, v, l, s)
+	+	+	–	+	–	v	v	–	–	ST	B, A (p, l)
v	–	w	–	$-^{w}$	–	$-^{w}$	–	–	–	ST	A (IC, p, ib, iv, l)
–	–	$-^{w}$	–	–	–	–	–	–	–	ST	A, B, iv, ib (p, ic, v, l, s)
+	w^{+}	w^{+}	–	+	$-^{w}$	w^{-}	$+^{-}$	+	–	ST	B, A, L (p, s)
+	–	+	–	+		–	$-^{+}$	+	+	ST	A (l)
+	+	+	$-^{w}$	+	$+^{w}$	$+^{w}$	$+^{w}$	+	+	ST	B, A (l, s)
v	v	w^{+}	–	w^{+}	–	–	w	w^{+}	–	ST	B, A, L (s)
$+^{w}$	+	+	–	+	v	v	+	$+^{w}$	$+^{w}$	ST	A (l)
$+^{w}$	–	v	–	–	–	–	–	–	$+^{-}$	ST	A, IV, IB (p, l, s)
+	+	+	–	+	+	+	+	+	+	ST	A (l)
w^{+}	w^{+}	w^{+}	$-^{w}$	$-^{w}$	$-^{w}$	$-^{w}$	w^{-}	v	v	T	A
+	$-^{w}$	–	$+^{w}$	+	–	v	$+^{w}$	$+^{w}$	$-^{w}$	T	B, L, a (s)
$+^{w}$	+	+	–	+	–	w^{-}	+	+	–	T^{ST}	B, A, L (s)
+	+	+	$+^{-}$	+	$+^{-}$	v	+	+	$-^{w}$	T	A, l (s)
$+^{w}$	w^{+}	$+^{w}$	w^{+}	$+^{w}$	v	$-^{w}$	v	w^{-}	v	ST^{t}	A (l, s)
+	$-^{+}$	–	–	v	–	–	–	–	–	ST	A, B, L
+	+	+	w^{+}	$+^{w}$	$+^{w}$	$+^{w}$	$+^{w}$	+	v	T	A, B, L
–	–	–	–	–	–	–	–	–	–	ST	A, b, ib, iv (l)
–	–	–	–	–	–	–	–	–	–	T	A, B, iv, ib (p, ic)
–	–	–	–	–	–	–	–	–	–	ST	A (l, s)
–	–	–	–	–	–	–	–	–	–	ST	A (l, s)
–	–	–	–	–	–	–	–	–	–	ST	A, B, IV, ib (p, ic, l, s)
–	–	–	–	–	–	–	–	–	–	T	A, B, p (l, s)

[d] Nonproteolytic.

[e] L-Proline aminopeptidase differentiates C. *difficile* and C. *innocuum*. C. *difficile* is positive, and C. *innocuum* is negative.

[f] Swarming.

[g] Cigar shaped. C. *bolteae* is lactose and β-NAG negative, C. *clostridioforme* is lactose positive and β-NAG negative, and C. *hathewayi* is lactose and β-NAG positive.

[h] C. *tertium*, C. *carnis*, and most C. *histolyticum* isolates grow aerobically.

rhea in elderly patients in nursing homes and tertiary-care institutions and has been considered to play a role in antibiotic-associated diarrhea (AAD) without pseudomembranous colitis.

C. *perfringens* strains associated with food poisoning produce the CPE, which generally acts by forming pores in membranes of host cells (18). C. *perfringens* strains isolated from nonfoodborne diseases, such as AAD and sporadic diarrhea, carry *cpe* on a plasmid (40, 41), which may be transmitted to other strains.

Enteritis Necroticans (Pigbel and Darmbrand), Necrotizing Enteritis, and NEC

Enteritis necroticans is caused by alpha-toxin- and beta-toxin-producing strains of C. *perfringens* type C. Beta toxin is located on a plasmid (41) and is mainly responsible for pathogenesis (18, 42, 43). Enteritis necroticans is a life-threatening infection causing ischemic necrosis of the jejunum. In Papua New Guinea during the 1960s, it was found to be the most frequent cause of death in children; it has been associated with pig feasts and occurs both sporadically and in outbreaks. Immunization against the beta toxin decreased the incidence of the disease in New Guinea (44). Enteritis necroticans has also been recognized in the United States, the United Kingdom, Germany, and other developed nations, especially involving adults who are malnourished or who have diabetes, alcoholic liver disease (45, 46), or neutropenia (47). It should be noted that NEC, a disease resembling enteritis necroticans but associated with C. *perfringens* type A, has been found in North America in previously healthy adults (48).

NEC is a serious gastrointestinal disease affecting low-birth-weight (premature) infants hospitalized in neonatal intensive care units. The etiology and pathogenesis of this disease have remained an enigma for more than 4 decades (49). Pathological similarities between NEC and enteritis necroticans include their patterns of bowel necrosis and degrees of inflammation (50). Both diseases may manifest intestinal gas cysts (50). The sources of the gas, which contains hydrogen, methane, and carbon dioxide, are probably the fermentative activities of intestinal bacteria, including clostridia. Epidemiological data support an important role for *C. perfringens* or other gas-producing microorganisms (e.g., "*C. neonatale*," certain other clostridia, or *Klebsiella* spp.) in the pathogenesis of NEC.

***C. difficile* Infection**

Prevalence of CDIs

C. difficile, the major cause of antibiotic-associated pseudomembranous colitis, is also the most frequently identified cause of hospital-acquired diarrhea and is responsible for >250,000 cases of diarrheal disease per year in the United States, with a cost exceeding $1 billion annually (51). *C. difficile* has been isolated from feces of 3 to 5% of the healthy population, 30% of healthy neonates, and 20 to 30% of sedentary patients (52). McFarland et al. (53) reported that 21% of 399 patients with negative cultures on admission to a hospital with a high prevalence of *C. difficile*-associated disease (CDAD) acquired *C. difficile* during hospitalization. Of these patients, 63% remained asymptomatic, while 37% developed diarrhea.

Role of the PaLoc in CDI

Only strains that carry the pathogenicity locus (PaLoc) (54) possess the genetic information for the *C. difficile* enterotoxin, TcdA, and the cytotoxin, TcdB (*tcdA* and *tcdB*, respectively). Only strains producing TcdA and/or TcdB cause CDI. A limited number of cases of CDI or pseudomembranous colitis are caused by TcdA$^-$ TcdB$^+$ strains (55–58). Results with a hamster model indicate that TcdB may be more important for disease induction than TcdA (59). Strains that carry only the genes for the binary toxin CdtA/B do not cause CDI or pseudomembranous colitis. The role of toxins in the pathogenesis of CDI is reviewed in reference 60.

TcdA and TcdB, together with toxins from *C. sordellii*, *C. perfringens*, and *C. novyi* (61), belong to the family of large clostridial cytotoxins (LCCs). The molecular masses of TcdA and TcdB are 308 kDa and 270 kDa, respectively. Such LCC toxins glycosylate small GTP-binding signal proteins of the Ras family, leading to a breakdown of the cell's cytoskeleton and thus causing apoptosis (62). Both TcdA and TcdB are autoactivated once inside the cell. However, in contrast to A and B toxins of the diphtheria type, they are single chained.

Two accessory proteins, TcdR and TcdC, of the PaLoc (54) are regulatory elements that control toxin expression (56, 63). The *tcdC* gene has gained diagnostic attention since it is shortened in endemic hypervirulent ribotype 027/NAP1 (North American pulsotype 1) isolates (herein called ribotype 027 isolates) (64). Some studies suggest that such strains overproduce toxins and may lead to more severe cases of CDI (65, 66), although this is controversial (60).

Risk Factors and Course of CDI

Acquisition of *C. difficile* alone does not induce CDI. Several other risk factors, like age, hospitalization, severe bowel surgery, treatment with proton pump inhibitors plus a change in colonization resistance due to such treatments plus colonization with a TcdA/TcdB-producing *C. difficile* strain, are necessary for development of CDI.

The spectrum of symptoms ranges from mild self-limiting diarrhea to bloody-slimy diarrhea (called *C. difficile*-associated diarrhea) to the development of full-scale pseudomembranous colitis (67). The onset of CDI may begin immediately following antibiotic treatment or as long as 4 to 6 weeks after the course of antibiotics has been finished. Antibiotics most commonly associated with CDI are clindamycin, expanded- and broad-spectrum cephalosporins, and fluoroquinolones (68).

Bloody, mucus-filled stools generally indicate greater destruction of the colonic mucosa and hence are associated with more severe disease. Clinical diagnosis may be established by rectoscopy and the identification of pseudomembranes on the colonic mucosa. Severe cases are typically observed among the elderly, in nursing home residents, and in immunocompromised patients (65–67).

Epidemic Outbreaks

Hypervirulent strains (such as those of ribotype 027) have caused outbreaks in Canada, the United States, Europe, and even worldwide (65, 66, 69). These outbreaks have occurred among younger age groups, in patients with no underlying diseases, and even among outpatients. These cases are associated with megacolon and rupture of the large bowel and are often lethal. There is evidence that use of fluoroquinolones may be an essential trigger in the onset of such outbreaks (70).

Particularly vexing complications of CDIs are relapses after antibiotic treatment caused by the initial causative strain or by reinfection with a second *C. difficile* strain (71). Published data report relapse rates of 20 to 50%. Even the first relapse should be treated with a vancomycin step therapy (see below). Fecal transplantation has been shown to be highly effective for treatment of recurrent CDI (reviewed in reference 72), and two newer drugs, fidaxomicin and rifaximin, have been approved for recurrent CDI (73). The use of the probiotic *Saccharomyces boulardii* (74) has also been suggested, but results are not yet definitive. Eradication of *C. difficile* from the hospital environment is a worthy objective but a difficult task for infection control practitioners. Commonly used disinfectants are not sporicidal. Rather, isolation of symptomatic patients has been the main control mechanism. However, recent studies suggest that asymptomatic carriers are an important reservoir for *C. difficile* in both the community and hospital environments (75, 76).

Other Etiologies of AAD

C. difficile is responsible for ≤20% of cases of AAD (77, 78). Enterotoxin-producing *C. perfringens* type A has been isolated from AAD patients who are negative for *C. difficile* and who have no other apparent cause of the disease. Coinfection with *C. difficile* and enterotoxigenic *C. perfringens* type A has also been reported for AAD patients (79). Though the incidence of *C. perfringens*-associated AAD has been estimated to be 5 to 20% (80), additional epidemiologic studies are needed to accurately determine the role of this organism in AAD.

Histotoxic Clostridial Skin and Soft Tissue Infections

Histotoxic clostridial species such as *C. perfringens*, *C. histolyticum*, *C. septicum*, *C. novyi*, and *C. sordellii* cause aggressive necrotizing infections of the skin and soft tissues attributable, in part, to the elaboration of bacterial proteases, phospholipases, and cytotoxins (26). Necrotizing clostridial soft tissue infections (gas gangrene) are rapidly progressive and characterized by marked tissue destruction, gas in the tissues, shock, and frequently death (81).

Clostridial Myonecrosis

Traumatic Gas Gangrene due to C. *perfringens*

C. *perfringens* myonecrosis (gas gangrene) is one of the most fulminant Gram-positive infections of humans. Predisposing conditions include crush-type injury, laceration of large- or medium-sized arteries, and open fractures of long bones that are contaminated with soil containing the bacterial spores. Gas gangrene of the abdominal wall and flanks occurs after penetrating injuries, such as knife or gunshot wounds, sufficient to compromise intestinal integrity, with resultant leakage of bowel contents into the soft tissues. Cutaneous gas gangrene caused by C. *perfringens*, C. *novyi* type A, and C. *sordellii* have been described in the United States and northern Europe among drug abusers injecting "black-tar heroin" subcutaneously (26, 82–86).

Clostridial gas gangrene is characterized by the sudden onset of excruciating pain at the infection site (87) and rapid development of a foul-smelling wound containing a thin serosanguinous discharge and gas bubbles. Brawny edema and induration develop and give way to cutaneous blisters containing bluish-to-maroon fluid. Later, such tissue may become liquefied and slough. The margin between healthy and necrotic tissue often advances several inches per hour despite appropriate antibiotic therapy (87), and radical amputation remains the single best life-saving treatment. Shock and organ failure frequently accompany gas gangrene, and when patients become bacteremic, the mortality exceeds 50%.

Diagnosis is not difficult because the infection (i) always begins at the site of significant trauma, (ii) is associated with gas in the tissue, and (iii) is rapidly progressive. A Gram stain of drainage or a tissue biopsy specimen is usually definitive, demonstrating large, Gram-positive rods and an absence of inflammatory cells. Using experimental models, Bryant and colleagues have demonstrated that the severe pain, rapid progression, marked tissue destruction, and absence of neutrophils in C. *perfringens* gas gangrene is caused by alpha-toxin-induced occlusion of blood vessels by platelets and neutrophils (26, 88, 89).

Spontaneous, Nontraumatic Gas Gangrene due to C. *septicum*

The first symptom of spontaneous C. *septicum* gas gangrene may be confusion, followed by the abrupt onset of excruciating pain and rapid progression of tissue destruction, with demonstrable gas in the tissue (13, 19, 87, 90). Swelling increases, and bullae appear filled with clear, cloudy, hemorrhagic, or purplish fluid. The surrounding skin has a purple hue, perhaps reflecting vascular compromise resulting from bacterial toxins diffusing into surrounding tissues (13). The mortality of patients with spontaneous gangrene ranges from 67 to 100%, with the majority of deaths occurring within 24 h of onset. Predisposing host factors include colonic carcinoma, diverticulitis, gastrointestinal surgery, leukemia, lymphoproliferative disorders, cancer chemotherapy, radiation therapy, and AIDS (13, 26, 90). Cyclic, congenital, or acquired neutropenia is also strongly associated with an increased incidence of spontaneous gas gangrene due to C. *septicum*, and in such cases, NEC, cecitis, or distal ileitis is commonly found. These gastrointestinal pathologies permit bacterial access to the bloodstream; consequently, the aerotolerant C. *septicum* can proliferate in normal tissues (19). Patients surviving bacteremia or spontaneous gangrene due to C. *septicum* should have aggressive diagnostic studies to rule out gastrointestinal pathology.

Gynecologic Infections due to C. *sordellii*

Gas gangrene of the uterus, especially that due to C. *sordellii*, has historically occurred as a consequence of illegal or self-induced abortions but in modern times also follows spontaneous abortion, normal vaginal delivery, and cesarean section (reviewed in reference 31). C. *sordellii* has also been implicated in medically induced abortions (31). Young, previously healthy women with fatal postpartum C. *sordellii* infections present with a unique clinical picture of little or no fever, a lack of a purulent discharge, refractory hypotension, extensive peripheral edema and effusions, hemoconcentration, and a markedly elevated white blood cell count (31). Death in these cases ensues rapidly, and the infection is almost uniformly fatal (31).

Other Clostridial Skin and Soft Tissue Infections

Crepitant cellulitis, also called anaerobic cellulitis, is seen principally in diabetic patients and characteristically involves subcutaneous tissues or retroperitoneal tissues and can progress to fulminant systemic disease; the muscle and fascia are not involved.

Cases of C. *histolyticum* infection with cellulitis, abscess formation, or endocarditis have also been documented in injecting drug users (91). C. *sordellii* was responsible for endophthalmitis after suture removal after a corneal transplant (92). C. *perfringens* endophthalmitis due to penetrating injuries is a fulminant infection (93).

Exotoxins of the Histotoxic Clostridia

Our current understanding of the potent toxins produced by these clostridia is based on studies done between World Wars I and II, when gas gangrene was a major complication of battlefield injuries. Investigators of this period designated the major lethal toxins of these bacteria with Greek letters, with the letter "α" always used to designate the most potent or most significant lethal factor. The original monograph by Smith and Williams (19) remains an important compilation of the known exotoxins of the histotoxic clostridia and their roles in pathogenesis. Over the ensuing decades, modern technology has provided a greater understanding of the mechanisms of action of some of these factors (reviewed in references 94 and 95), as described in individual sections that follow.

Major Extracellular Toxins of C. *perfringens*

The major C. *perfringens* extracellular toxins implicated in gas gangrene are alpha toxin and theta toxin. Alpha toxin is a lethal lecithinase that has both phospholipase C and sphingomyelinase activities and has been implicated as the major virulence factor based on the observation that immunization of mice with purified recombinant protein consisting of the C-terminal alpha-toxin domain (amino acids 247 to 370) provided protection against lethal challenge with C. *perfringens* (96). In addition, intravascular activation of platelets by alpha toxin leads to platelet aggregation (88, 97) and formation of occlusive thrombi that completely and irreversibly occlude capillaries, venules, and arterioles (88, 89). Without adequate tissue perfusion, the anaerobic niche is extended and rapid destruction of viable tissue, so characteristic of clostridial gas gangrene, ensues.

Theta toxin from C. *perfringens* (also known as perfringolysin) is a member of the thiol-activated cytolysin family, now termed cholesterol-dependent cytolysins, that includes streptolysin O from group A streptococci, pneumolysin from *Streptococcus pneumoniae*, and several others. Upon contact with cholesterol in the host's cell membranes, theta-toxin monomers oligomerize and insert into the membrane, forming a pore and resulting in cell lysis (98). The signature undecapeptide sequence, ECTGLAWEWWR, is highly conserved among all cholesterol-dependent cytolysins and mediates this activity (99). Theta toxin contributes to the pathogenesis of gas gangrene, likely by its ability to modulate the inflammatory response to infection (100, 101).

Major Extracellular Toxins of C. *septicum*

C. *septicum* produces four main toxins, alpha toxin (α, lethal, hemolytic, necrotizing activity), beta toxin (β, DNase), gamma toxin (γ, hyaluronidase), and delta toxin (δ, septicolysin, an oxygen-labile hemolysin), as well as a protease and a neuraminidase (19). Unlike the alpha toxin from C. *perfringens*, the C. *septicum* alpha toxin does not possess phospholipase activity. Active immunization against alpha toxin significantly protects against challenge with viable C. *septicum* (102).

Major Extracellular Toxins of C. *sordellii*

Pathogenic strains of C. *sordellii* produce up to seven identified exotoxins. Of these, lethal toxin (LT) and hemorrhagic toxin (HT) are regarded as the major virulence factors. LT and HT are members of the LCC family, all having molecular masses between 250 and 308 kDa. Other members include the C. *difficile* toxins A and B and C. *novyi* alpha toxin. All LCCs possess remarkable amino acid similarity, with identities ranging between 26 and 76%. LT and C. *difficile* toxin B have the highest homology, with amino acid sequences being 76% identical and 90% homologous to one another. All LCCs possess glycosyltransferase activity and modify signaling molecules that control the cell cycle, apoptosis, gene transcription, and the structural functions of actin, such as cell morphology, migration, and polarity. Once modified, these proteins become inoperative. Modification of actin cytoskeletal assembly and organization presumably leads to the massive capillary leakage characteristic of C. *sordellii* infection. The C. *sordellii* neuraminidase has been shown to contribute to the leukemoid reaction, in part, by enhancing the proliferation of granulocyte progenitor cells (103). Other exotoxins include an oxygen-labile hemolysin, DNase, collagenase, and lysolecithinase; however, their roles in pathogenesis have not been extensively investigated.

Botulism

The Organism and Its Toxin

C. *botulinum* is the cause of the rare but frequently fatal illness known as botulism, which is characterized by sudden flaccid paralysis. Spores of C. *botulinum* are widely distributed in soil and aquatic habitats. C. *botulinum*, along with unique strains of C. *butyricum*, C. *baratii*, and C. *argentinense*, can produce BoNT, the most lethal poison known. The intravenous lethal dose for BoNT has been estimated as 0.1 to 0.5 ng per kg of body weight, and BoNT is among the most potent protein toxins by oral ingestion, with an estimated oral lethal dose of 0.2 to 1 μg per kg (104). There are seven antigenic serotypes of BoNT (A through G) (105), which serve as useful clinical and epidemiological markers (106). Recently a strain of C. *botulinum* was identified that produced both type B and H toxins (107). Toxin serotypes A, B, and E of C. *botulinum* are the principal causes of botulism in humans (108). Neurotoxigenic strains of C. *butyricum* (109) and C. *baratii* (110–112) that produce type E and F neurotoxins, respectively, have been implicated mainly in infant botulism. Type E botulinal-toxin-producing C. *butyricum* strains were confirmed by sequencing of the 16S rRNA gene (113), leading to the conclusion that neurotoxigenic C. *butyricum* must be regarded as an emergent foodborne pathogen. C. *argentinense*, which produces type G neurotoxin (108), has been isolated from soil in Argentina. Its reported isolation from autopsy materials from five individuals who died suddenly has not been substantiated, and C. *argentinense* has not been clearly implicated in botulism. C. *botulinum* types C and D are associated primarily with botulism in birds and mammals (114, 115). Strains of C. *botulinum* that produce more than one serotype of BoNTs, generally with one serotype being formed in much higher levels, have been isolated from the environment and human and animal botulism cases (108, 116). The BoNTs are coexpressed with nontoxic proteins of toxin gene clusters (117), and evidence suggests that the complexes are much more stable than the labile BoNTs in the gastrointestinal tract. The genes for BoNT complex formation are associated with unstable genetic elements in certain serotypes, enabling toxin gene transfer to nontoxigenic clostridial species that are closely related to C. *botulinum*, such as C. *sporogenes* and C. *subterminale* (118).

There are four naturally occurring types of botulism: (i) classical foodborne botulism, an intoxication caused by the ingestion of preformed botulinal toxin in contaminated food; (ii) wound botulism, which results from elaboration of botulinal toxin *in vivo* after the growth of C. *botulinum* in an infected wound; (iii) infant botulism, in which botulinal toxin is elaborated *in vivo* in the gastrointestinal tract of an infant colonized with C. *botulinum*; and (iv) botulism due to intestinal colonization in children and adults (108, 119). Intestinal colonization in adults has been associated with surgery and administration of antibiotics (108). C. *botulinum* has been isolated from patients colonized with C. *difficile* (109), with viral infections (120), or with Crohn's disease (121). In 2008, an international outbreak of botulism caused by commercial carrot juice was reported by Sheth et al. (122).

Regardless of the category of botulism, the toxin enters the bloodstream at a peripheral site (e.g., gut, wound, or lung) and is transferred to the neuromuscular junctions of motor neurons, where it binds irreversibly to the presynaptic membranes. The site of action of all serotypes of BoNT is the presynaptic terminal of motor neurons (123–126). Elucidation of the three-dimensional structures of botulinum and tetanus toxins and their constituent domains has provided considerable insights into their mechanisms of action (125–128). BoNT penetrates the plasma membrane by receptor-mediated endocytosis, and the light chain of 50 kDa (the catalytic domain) is internalized into the nerve cell through a protein channel (125, 126). Once internalized, BoNT specifically cleaves proteins involved in vesicle trafficking of neurotransmitters to the membrane (126). Exocytosis of acetylcholine is prevented at the nerve terminal to the neuromuscular junction, with consequent blockage of innervation of muscle activity (126). The clinical hallmark of botulism is an acute flaccid paralysis, which begins with bilateral cranial nerve impairment involving muscles of the eyes, face, head, and pharynx and then descends symmetrically to involve muscles of the thorax and extremities. Botulinum toxin, unlike TeNT, probably does not enter the central nervous system (CNS). In naturally occurring foodborne botulism, gastrointestinal symptoms (e.g., abdominal cramps, nausea, vomiting, or diarrhea [more often constipation or obstipation]) may precede the neurologic signs of descending flaccid paralysis. Death results from respiratory failure caused by paralysis of the tongue or muscles of the pharynx, leading to occlusion of the upper airway, or from paralysis of the diaphragm and intercostal muscles (104, 124). Generally, the patient's hearing remains normal, consciousness is not lost, and the victim is cognizant of the progression of the disease, which of course can be a terrifying experience.

Wound Botulism in Intravenous Drug Users

An association between botulism and subcutaneous injection of Mexican black-tar heroin into muscle or skin (skin popping) has been reported in the United States and in the United Kingdom (129, 130). A study found 33 clinically diagnosed cases of wound botulism in the United Kingdom and Ireland between 2000 and 2002 (129). The clinical diagnosis was confirmed by laboratory tests in 20 of these cases; 18 cases were caused by type A toxin and 2 by type B toxin. Wound botulism has also occurred after snorting of cocaine (131), cosmetic injection of an unlicensed Botox preparation (132), and a tooth extraction (133).

Infant Botulism

Infant botulism is the most frequently recognized form of botulism in the United States (45% of cases in California) and has been reported in at least 15 other countries (119, 134–138). The geographic distribution of toxin types in infant botulism cases has paralleled the spore distribution of C. *botulinum* toxin types in soils sampled from different locations (119). Type A has been the most frequent BoNT type in cases of infant botulism in states west of the Mississippi River, whereas type B cases have predominated east of the Mississippi River (119, 139). Three cases have been caused by a strain(s) of C. *botulinum* that produced toxins requiring both type B and F antitoxins for neutralization (108). Type E infant botulism, caused by neurotoxigenic strains of C. *butyricum*, was initially confirmed in two infants from Italy (138), and later in additional patients. Type F infant botulism has been caused by neurotoxigenic C. *baratii* (138).

Most infants that contract botulism are 3 weeks to 6 months old (119), and the only clearly defined risk factors have been exposure to soil, dust, and honey (119, 137). Since C. *botulinum* spores have not been detected in any food or liquid ingested by these infants other than honey (119), it is recommended that honey not be fed to infants <1 year of age. Whatever the sources, the ingested spores of C. *botulinum* germinate within the intestinal tract, and the vegetative cells multiply and produce the neurotoxin, which is then absorbed into the bloodstream (108, 119). The first sign of illness is usually constipation, which is often overlooked. Infants develop lethargy and mild weakness, with feeding difficulties, pooled oral secretions, and an altered cry (119). They eventually lose head control and may go on to develop ophthalmoplegia, ptosis, flaccid facial expression, dysphagia, other signs of cranial nerve deficits, generalized muscular weakness, and finally respiratory insufficiency and the inability to swallow. There is likely a spectrum of clinical features in infant botulism, ranging from mild illness not requiring hospitalization to severe botulism requiring intensive care. Human immune globulin that neutralizes BoNT (BabyBIG; intravenous BIG-IV) has been licensed to the California Department of Public Health Infant Botulism Treatment and Prevention Program [www.infantbotulism.org; 24-h/7-day phone, (510) 231-7600] since 2003 (137, 140). Since 2007, it has been made available to physicians outside the United States on a case-by-case basis. Early treatment has shortened hospital stays and significantly reduced the associated costs of hospitalization (141). A recent Cochrane review substantiated that administration of BIG is more effective than any other medical intervention, though the findings were of moderate quality (141).

Botulinum Toxin as a Bioterrorism Agent

Inhalational botulism, which results from aerosolization and inhalation of botulinum toxin, has been considered a fifth category of botulism (104, 142, 143). Botulism could also result from covert contamination of foods (104, 144). Inhalational botulism has been demonstrated experimentally in monkeys (104, 143), accidentally in three veterinary personnel in Germany who were exposed to reaerosolized BoNT from rabbits and guinea pigs with aerosolized BoNT on their fur (104), and in three researchers who were exposed to an aerosol during BoNT manipulations (145). Terrorists have attempted to use aerosolized botulinum toxin as a bioweapon in Japan but were not successful. Although inhalational botulism is possible, the toxin is unstable in aerosols, and the more likely route of intentional intoxication is by food contamination and oral ingestion.

Tetanus

Tetanus, caused by C. *tetani*, is often associated with puncture wounds that do not appear to be infected. The organism and its spores can be isolated from a variety of sources, including soil and the intestinal contents of numerous animal species. A potent neurotoxin (TeNT), often referred to as tetanospasmin, is elaborated at the site of trauma and rapidly binds to neural tissue, provoking a characteristic paralysis and tonic spasms (146). Tetanus is a totally preventable infection with immunization with tetanus toxoid. However, a 2009 study from the United Kingdom demonstrated that, overall, only 83% of the population had protective levels of anti-tetanus toxin antibody (147) and that the age group with the greatest susceptibility was those >70 years of age (32%), followed by those <1 year of age (29%) and, remarkably, those between 45 and 69 years of age (>20%). Thus, immunization of women during pregnancy is recommended, as is routine immunization of all age groups at roughly 5-year intervals.

Tetanus is an intoxication analogous to botulism except that it occurs solely through wound infection and production of tetanospasmin (TeNT). TeNT is synthesized as a single, inactive polypeptide chain (150 kDa), which is cleaved by an intrinsic protease to produce an active form, consisting of a heavy chain (100 kDa) and a light chain (50 kDa) linked by a disulfide bond (126). The heavy chain binds to neuronal cells, and the three-dimensional structure of this region has been elucidated (126). The light chain, a zinc endopeptidase, enters the cell cytoplasm and traverses from the nerve terminal to the nerve cell body by retrograde axonal transport (126, 146), eventually reaching neurons in the spinal cord and brain stem, where it affects glycinergic and GABA (γ-amino-*n*-butyric acid)-ergic neurotransmission (126, 146). Inhibitory impulses to CNS neurons are blocked, while uninhibited firing of motor nerve transmission continues, resulting in prolonged muscle spasms of both flexor and extensor muscles that can persist for weeks. The mechanism by which exocytosis of neurotransmitter release is inhibited is analogous to that of BoNT; in fact, TeNT cleaves the vesicle-associated membrane protein at the same peptide bond as BoNT B (125). Unlike with the pathophysiology of botulism, TeNT is retrogradely transported in neurons to the CNS and its site of action (126, 146).

The worldwide incidence of tetanus has been estimated to be as many as 500,000 cases per year (146). Neonatal tetanus is endemic in developing countries due to a lack of vaccine programs for infants or adult women. In developed countries, injection of drugs (i.e., skin popping) has become an important risk factor (148, 149). Neonatal mortality is highest in low-birth-weight newborns (150).

Additional Clostridial Species of Interest

C. *innocuum* is associated with bacteremia in immunocompromised hosts and has also been recovered from patients with recurrent CDAD (151). It is often resistant to multiple drugs used to treat anaerobic infections (151). C. *ramosum* was the second-most-common *Clostridium* species (after C. *perfringens*) identified from clinical specimens from children, including those with abscesses, peritonitis, bacteremia, and chronic otitis media (152), and the third-most-common *Clostridium* species in adult cases of bacteremia (21). This species may be resistant to clindamycin and multiple cephalosporins. As noted earlier, C. *tertium* is often isolated from blood cultures from immunocompromised patients and has been reported as a cause of neutropenic enterocolitis and meningitis (29, 153–155). C. *hathewayi* and C. *bolteae* have been isolated from a variety of human infections (6, 34), including a fatal case of sepsis (156). Phenotypically similar C. *clostridioforme* is one of the clostridia most commonly isolated from human infections and appears to be associated with human infections that are more serious or invasive than infections with C. *hathewayi* or C. *bolteae*.

The emergence of 16S rRNA gene sequencing technology has provided a means of identification of strains that may previously have been misidentified or classified as *Clostridium* without species identification. Examples are from cases of bacteremia caused by C. *hathewayi* (34), C. *intestinale* (157), and C. *symbiosum* (158); fatal sepsis due to C. *fallax* in a previously healthy 16-year-old (159); and abscesses yielding C. *celerecrescens* (160). Microarray analysis of DNA from fecal samples has also been useful in the determination of predominant species in the large bowel (161). It is likely that in this era of molecular identification techniques a more accurate picture of clostridial infections will emerge.

CLINICAL MICROBIOLOGY OF CLOSTRIDIAL DISEASES

General Methods for Collection, Transport, and Storage of Clinical Specimens

The proper selection, collection, and transport of clinical specimens are extremely important for the laboratory diagnosis of clostridial infections. For recommended collection and transport procedures in general, refer to chapter 18.

Specific Methods for Collection and Direct Examination of Clinical Specimens

In addition to requiring aspirates and tissues, selected clostridial illnesses require special specimens. The methods for collection and direct examination of these specimens are described below.

Suspected Gas Gangrene or Necrotizing Fasciitis

Gas gangrene and necrotizing fasciitis represent extremely urgent situations requiring rapid clinical diagnoses. Multiple tissue specimens should be sampled from the active sites of infection when gas gangrene is suspected, because clostridia are often not distributed uniformly in pathologic lesions. The direct examination of a Gram-stained smear of the wound is of major importance for the early presumptive diagnosis of gas gangrene (81). Characteristic findings in C. *perfringens* infections include the absence of leukocytic infiltration and the presence of clostridia in smears prepared from central areas of the lesion. Special note should be made of Gram-positive rods, with or without spores, because sporulation in tissue is not common for the two species most frequently encountered in wound and abscess materials, C. *perfringens* and C. *ramosum*. C. *perfringens* usually appears as large, relatively short, fat, Gram-positive rods with blunt ends and often in short chains in tissue smears; the cells of C. *ramosum* are more slender and longer (Fig. 1). C. *perfringens* may or may not be encapsulated in smears from wounds; capsules usually are present in smears of endometrial specimens from postabortion C. *perfringens* infections. Spore stains offer no advantage over Gram stains for demonstration of spores, but examination with a phase-contrast or dark-field microscope may be helpful if the spores are close to maturity. If spores are present, shapes (spherical or oval) and positions (terminal, subterminal, or central) in the cells should be noted.

Suspected *C. perfringens* Foodborne Illness

A freshly passed fecal specimen and the suspected food are the preferred specimens for C. *perfringens* culture and toxin assays. These specimens should be placed into sterile containers, stored at 4°C, and shipped on cold packs as soon as possible. For optimal recovery, stool specimens should be processed within 24 h of collection. Swab specimens are inadequate for the toxin assay because the sample volume is insufficient.

Several methods have been described for the detection of CPE in feces, including cell culture assays, enzyme-linked immunosorbent assay (ELISA), and reversed passive latex agglutination (RPLA) (162, 163). The cell culture assay using Vero cells is not as sensitive or as reproducible as other methods (80, 164). The results of the RPLA kit (PET-RPLA; Oxoid Ltd., Basingstoke, United Kingdom, and Remel Inc., Lenexa, KS) are reproducible, and the test is reasonably sensitive; however, nonspecific interference by fecal matter has been reported (163). Similarly, the background bacterial DNA in stool has been reported to interfere with PCR amplification of the enterotoxin gene (163). While an in-house ELISA system developed by the Food Safety Microbiology Laboratory of the Central Public Health Laboratory, London, United Kingdom, has been reported to be the most sensitive assay and is considered the gold standard, the TechLab (Blacksburg, VA) CPE ELISA system has also provided a specific, reliable, and practical tool for detecting CPE in fecal samples (80, 164).

Suspected Enteritis Necroticans (*C. perfringens* Type C)

If enteritis necroticans is suspected, the appropriate specimens include three blood cultures from three different venipuncture sites, stool (at least 25 g, or 25 ml if liquid), and bowel luminal contents or tissue from the involved bowel (e.g., surgical specimen or autopsy material). Specimens should be transported in tightly sealed, leakproof containers for the following: direct Gram staining, culture, isolation, identification, and typing of C. *perfringens*. PCR assays for genotyping C. *perfringens* are being used in certain research

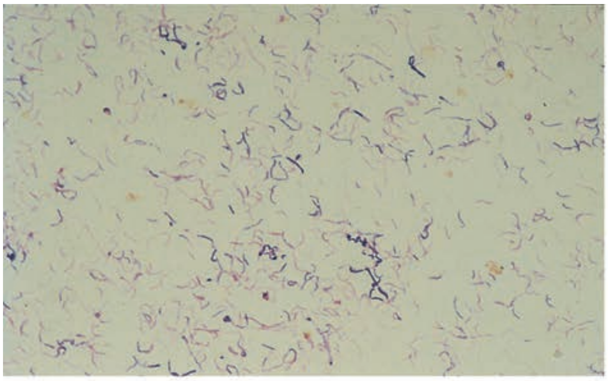
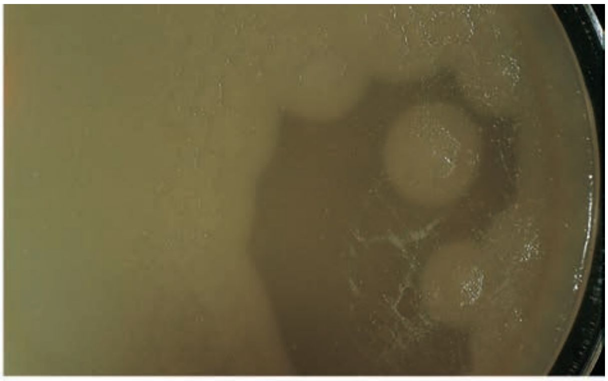
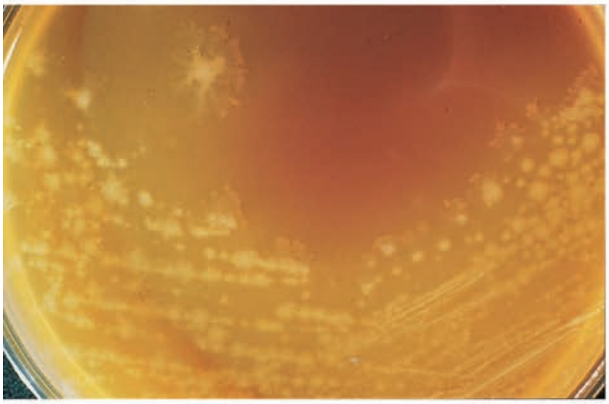
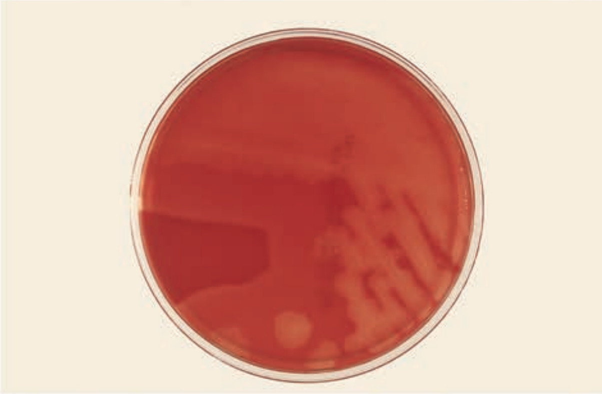
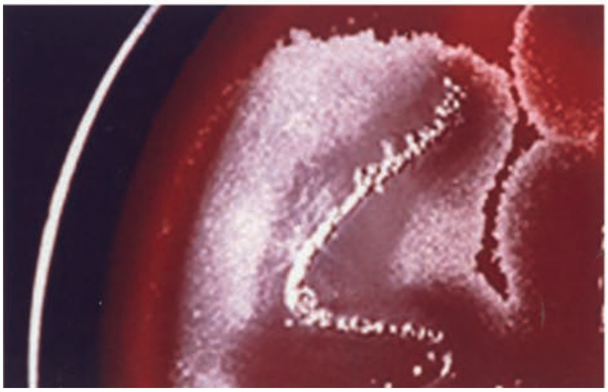
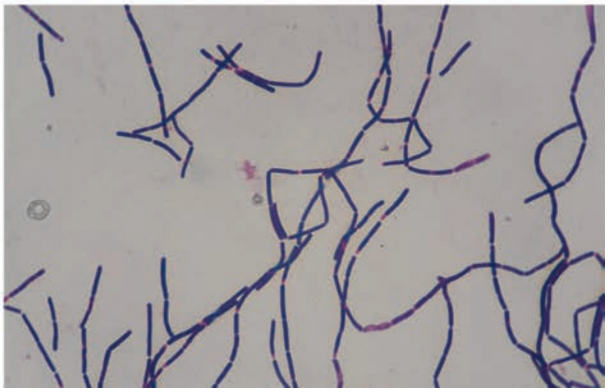

FIGURE 1 (top row, left) Gram stain of C. *ramosum*. Note the thin, Gram stain-variable bacilli with distinct spores. Reprinted with permission from reference 184. doi:10.1128/9781555817381.ch53.f1

FIGURE 2 (top row, right) Lecithinase reaction on egg yolk agar. Note the opacity of the agar surrounding colonies due to precipitation of complex fats. Reprinted with permission from reference 184. doi:10.1128/9781555817381.ch53.f2

FIGURE 3 (middle row, left) C. *difficile* on CCFA. Note the yellow, ground-glass appearance of colonies. Reprinted with permission from reference 184. doi:10.1128/9781555817381.ch53.f3

FIGURE 4 (middle row, right) C. *perfringens* on blood agar showing a double zone of beta-hemolysis. Reprinted with permission from reference 184. doi:10.1128/9781555817381.ch53.f4

FIGURE 5 (bottom row, left) C. *septicum* on blood agar after 12 h of incubation. Note the spreading colonies. Courtesy of E. J. Baron. doi:10.1128/9781555817381.ch53.f5

FIGURE 6 (bottom row, right) Gram stain of C. *septicum*. Note the long, filamentous bacilli with rare spores. Courtesy of E. J. Baron. doi:10.1128/9781555817381.ch53.f6

or referral laboratories to aid in diagnosis (42). Accordingly, DNA can be extracted for this purpose from formalin-fixed intestinal tissue or culture and amplified by PCR using primers specific for the *cpa* and *cpb* genes of C. *perfringens* type C.

Suspected CDI

Among the risk factors that warrant initiating a detailed microbiologic diagnosis of CDI in patients with diarrhea are (i) antibiotic exposure; (ii) host factors, such as advanced age (>65 years), immunosuppression, or severe underlying gastrointestinal disease; (iii) exposure to acute or chronic health care facilities; and (iv) use of proton pump inhibitors (165–167).

CDI is a clinical disease that is confirmed by various laboratory methods. The diagnosis of CDI has gained more attention since the appearance of hypervirulent strains, such as ribotype 027, which have contributed to the increased incidence and severity of infection. The optimum diagnostic assay or algorithm remains a matter of debate, but methods can be stratified into two major categories—those that detect toxin A, toxin B, or both in fecal samples (enzyme immunoassays [EIAs] and cell culture cytotoxicity neutralization assays [CCCNAs]) and those that detect the organism (anaerobic culture, glutamate dehydrogenase [GDH], and nucleic acid amplification tests [NAATs]). Since nontoxigenic strains can colonize individuals, assays that detect the organism alone should be combined with a test that can also assess toxin status.

Submission of Specimens

A single, freshly passed fecal specimen (ideally 10 to 20 ml of watery stool; minimum of 5.0 ml or 5 g) is the preferred specimen for C. *difficile* culture and toxin assays. To lessen the chance of obtaining positive culture results from patients merely colonized with the organism, only liquid or unformed stool specimens should be processed. Swab specimens of stool are inadequate for toxin tests because the sample volume is insufficient. However, swabs can be processed for culture or NAATs obtained for epidemiological reasons. Other appropriate specimens include bowel luminal contents and surgical or autopsy samples of the large bowel.

Specimens should be transported in tightly sealed, leakproof plastic or glass containers. For optimal recovery, stool specimens should be cultured within 2 h of collection. Although spores survive in refrigerated stool for several days, there will probably be a large decrease in the number of viable vegetative cells of C. *difficile* in refrigerated specimens. Stools should be placed in an anaerobic environment (anaerobic transport vial or bag) if culture must be performed after storage. Adequate recovery of C. *difficile* organisms may be expected from stools stored at 4°C for up to 2 days. Specimens for toxin assay may be stored at 4°C for up to 3 days or should be frozen at –70°C if performance of the assay is delayed. Freezing at –20°C results in a dramatic loss of cytotoxin activity, so detection limits may no longer be reached.

Methods for the Detection of C. *difficile* in Clinical Samples

Anaerobic toxigenic culture. Cultivation of C. *difficile* is encouraged for subsequent molecular strain typing and epidemiologic studies and is considered one of two diagnostic gold standards. Anaerobic culture and C. *difficile* identification methods are described in "Isolation Procedures" below. As recovery of C. *difficile* from fecal samples using culture does not assess toxicity, culture is usually combined with a method to assess toxin production.

Antigen detection (of GDH) for diagnosis of CDI. GDH is a metabolic enzyme expressed at high levels by all strains of C. *difficile*, both toxigenic and nontoxigenic strains, so a positive test must be combined with an assay that detects toxin. GDH assays exist in two forms, as a solid-phase microtiter plate format and as a lateral flow immunochromatographic membrane version of the test combined with a toxin A and B EIA (reviewed in references 165 and 166). Before the widespread availability of NAATs, many laboratories turned to GDH testing in two-step algorithms and performed toxin testing on specimens that were GDH positive, while GDH-negative samples were deemed negative for C. *difficile*. Patients whose samples were positive by GDH assay but negative by a toxin test were considered colonized with nontoxigenic isolates. Such algorithms predicated on GDH as the initial step were supported by the reportedly high negative predictive value (NPV) of GDH tests, 98.4 to 100%, compared to both toxigenic culture and CCCNAs (165, 166), an observation that has recently been reconfirmed (NPV of 99.5 and 99.8% compared to toxigenic culture and a cytotoxin assay, respectively) in the largest diagnostic study to date of CDI (168).

Nucleic acid amplification methods. The first nucleic acid amplification assay was approved by the U.S. Food and Drug Administration (FDA) in 2009. At the time of this writing, close to a dozen platforms are approved and are available for testing using a variety of techniques that include PCR, loop-mediated isothermal amplification, and microarray technologies (166, 169). Some platforms are designed for low-volume laboratories, and others are more amenable to high-throughput testing. These assays detect a variety of gene targets, including *tcdA*, *tcdB*, *cdt*, and the Δ117 deletion in *tcdC*, the latter two as surrogates for ribotype 027. Although NAATs are rapidly replacing other methods in clinical microbiology laboratories, these new technologies have yet to be endorsed by professional society guidelines. Like GDH assays, NAATs have a very high NPV and analytical and clinical sensitivity (166, 169), but there are concerns regarding specificity and positive predictive values (PPVs). In the study by Dubberke et al. (170), when clinical parameters were used to assess assay performance, NAATs had the lowest specificity among diagnostic assays (170). This was in part due to inappropriate test ordering, as 36% of patients tested did not have diarrhea and 20% of tested patients had received a laxative (170). Likewise, in a very large prospective multicenter study in the United Kingdom, the authors found that tests that were positive by a NAAT but negative for toxin by CCCNA correlated with neither mortality nor prolonged length of stay (168). The authors concluded that the use of NAATs leads to overdiagnosis of CDI (168). They suggested that NAATs could be used as first-stage tests to exclude the presence of C. *difficile*, followed by a more specific toxin test to identify those patients most likely to have C. *difficile* disease (168). The major limitation to algorithms that begin with either a GDH assay or a NAAT is that a sensitive assay for detection of toxin does not exist. Some recommend a three-tiered approach, with a GDH assay followed by a toxin EIA and progression to a NAAT for confirmation of GDH-positive, toxin EIA-negative samples. Such three-step algorithms complicate and delay testing, may not be reimbursed, and may be less cost-effective than NAAT testing alone (166, 171, 172). Regardless of how a laboratory chooses to utilize a NAAT (i.e., alone or as part of a multistep algorithm), to optimize interpretation of results, testing should be limited to patients who clearly have diarrhea.

Methods to detect C. *difficile* toxins. CCCNAs have long been considered diagnostic gold standards of C. *difficile* toxin testing in spite of the fact that there is no agreed-upon standard method. CCCNAs involve inoculating a filtrate of stool onto a monolayer of an appropriate cell line and observing for cytopathic effect after 24 to 48 h of incubation at 37°C. A number of different cell lines, such as human foreskin fibroblasts, Vero cells, and MRC-5 lung fibroblasts, among others, have been used. Once cytopathic effect is observed, neutralization is required to assess the specificity of cytopathic effect, and can be performed using *C. sordellii* or *C. difficile* antisera. The need for neutralization of the cytopathic effect marks a limitation of the test. Performance characteristics of CCCNAs vary considerably, likely related to the comparative method and also to technical factors that can affect accurate performance (reviewed in reference 166). However, in a large prospective study in the United Kingdom, a CCCNA performed using Vero cells showed the best correlation with clinical outcome (168) compared to assays that detected the presence of the organism. As laboratories embrace molecular techniques for viral diagnostics, many have abandoned cell culture facilities, making CCCNAs less attractive assays for *C. difficile* diagnosis. In addition, the length of time required to complete testing has also limited their utility (Table 2).

Toxin immunoassays for diagnosis of CDI. A number of commercial EIAs are available for detection of *C. difficile* toxins. These assays detect toxin A alone or both toxins A and B using monoclonal or polyclonal antibodies embedded in rapid immunochromatographic/lateral flow membranes or in microwell and solid-phase devices. The result of toxin testing is the declaration of the sample as being toxin positive or negative without any differentiation of TcdA and TcdB. Testing using assays that detect TcdA alone is not recommended, since some epidemic strains produce only TcdB (78). Immunoassays generally show lower sensitivities and specificities (31 to 99% and 84 to 100%, respectively) than CCCNAs (165, 166). Such variability in performance led to the common practice of submitting multiple specimens to the laboratory ("stools for *C. difficile* times three"), adding expense and reducing the PPV of such testing (173). Due to the poor sensitivity and specificity of these tests, EIAs are considered suboptimal for the diagnosis of CDIs, and many laboratories have replaced them with GDH- or NAAT-based testing algorithms.

In summary, a variety of diagnostic test methods are available for the diagnosis of *C. difficile* disease (Table 2). The optimum method is still a matter of debate, but multistep algorithms predicated on the initial results for GDH followed by a sensitive toxin test and/ or a NAAT test provide the best options at present. EIAs for toxin detection lack sensitivity and specificity. Positive CCCNA results seem to correlate the best with poor patient outcomes. Diagnostic accuracy will be enhanced by restricting testing to patients with appropriate clinical symptoms regardless of the method. Toxigenic culture remains useful for epidemiologic studies.

Suspected Neutropenic Enterocolitis Involving *C. septicum*

The specimens of choice for suspected neutropenic enterocolitis involving *C. septicum* are (i) three blood cultures collected from three different venipuncture sites, (ii) stool (at least 25 g, or 25 ml if liquid), and (iii) luminal contents or tissue from the involved ileocecal area collected at surgery or autopsy and transported in tightly sealed, leakproof containers. In addition, a biopsy sample of muscle (or an aspirate of fluid from the involved area, taken with a needle and syringe) should be collected if the patient is also suspected of having myonecrosis or another form of progressive infection.

Suspected *C. botulinum* or *C. tetani* Infection or Intoxication

Most hospital laboratories are not properly equipped to process specimens from patients suspected of having botulism. In the United States, before collecting any specimens, medical care providers who suspect a diagnosis of botulism in a patient should immediately call their state health department's emergency 24-h telephone number or the Centers for Disease Control and Prevention (CDC) in Atlanta, GA [(770) 488-7100, 24-h/7-day emergency service] so that appropriate action can be taken to establish the diagnosis, initiate treatment, and investigate the case.

TABLE 2 Commonly used tests for the diagnosis of *C. difficile* disease

Entity detected	Method	Principal advantage(s)	Principal limitation(s)
Organisms	Anaerobic toxigenic culture	Sensitive, specific Must add method for toxin detection	No agreed-upon standard for performance Useful for epidemiology Takes 2 to 5 days to perform Can be expensive
GDH	Solid-phase microtiter EIA	Rapid High NPV	Does not distinguish between toxigenic and nontoxigenic *C. difficile*; must be combined with a toxin test
	Membrane EIA combined with toxin EIA	Rapid High NPV	EIA toxin test component lacks sensitivity GDH-positive, toxin EIA-negative results must be confirmed with another method such as a NAAT
Toxin B	Cell culture neutralization assays	Sensitive, specific	Requires 24 to 48 h to complete Special expertise required for optimum performance
Toxins A and B	Solid-phase microtiter EIA Membrane EIA	Rapid Inexpensive	Sensitivity is poor with all products (<90%) Specificity may also vary
Genes encoding toxins A and B, binary toxin, and Δ117 deletion in *tcdC*	NAATs	Rapid Easy to perform Sensitive, high NPV	Poor PPV if not used appropriately Cost may be an issue

In Canada, suspected botulism cases should be reported according to the following website (http://www.hc-sc.gc.ca/fn-an/legislation/guide-ld/botulism-botulisme-prof-eng.php). Acceptable specimens include feces, enema fluid, gastric aspirates or vomitus, tissue or exudates, and postmortem specimens. These specimens should be placed into sterile, unbreakable containers. Serum specimens (preferably >10 ml) should be collected as soon as possible after the onset of symptoms. Clinical swabs should be collected in an anaerobic transport medium; environmental swabs (from which spores may be isolated) may be sent in plastic containers without any medium. Food specimens should be left in their original containers, if possible, or placed in sterile, unbreakable containers. All specimens should be stored at 4°C and shipped on cold packs as soon as possible. Further information can be found at the CDC botulism website (http://www.bt.cdc.gov/agent/botulism). Laboratories should have all of the pertinent information and contact numbers handy. During investigations of possible bioterrorism, sera, gastric aspirates, feces, and environmental or nasal swabs could be useful for detecting aerosolized botulinum toxin that may have been inhaled (174, 175). All specimens should be refrigerated until they can be transported to the laboratory for testing.

Certain clostridial toxins, particularly BoNT, TeNT, and iota toxin, are extremely toxic molecules and are considered very potent poisons. The CDC recommends biosafety level 3 primary containment and personnel precautions for facilities producing BoNTs for study. Ideally, personnel who work in laboratories should be immunized with a pentavalent (A to E) toxoid; however, the vaccine is no longer available from the CDC. A biosafety manual should be posted in the laboratory and should contain the proper emergency phone numbers and procedures for emergency response. Current U.S. regulations governing personnel safety for research with select agents are maintained in the electronic Code of Federal Regulations (http://www.ecfr.gov) and in the CDC's manual *Biosafety in Microbiological and Biomedical Laboratories* (http://www.cdc.gov/biosafety/publications/bmbl5). For Canada, biosafety standards can be found at http://canadianbiosafetystandards.collaboration.gc.ca/ and toxin manipulations must be performed according to the Canadian Human Pathogens and Toxins Act (http://www.phac-aspc.gc.ca/lab-bio/regul/hpta-lapht-eng.php).

Direct Toxin Detection

Bioassays for BoNT and TeNT are currently the most important laboratory tests for the diagnosis of botulism and tetanus (108, 176, 177). The definitive diagnosis of botulism is the detection of BoNT (not the organism) (108). Currently, the only reliable assay for BoNT is the mouse bioassay, together with neutralization of mouse toxicity with type-specific antitoxins (108, 178). Detection of neurotoxins is usually performed on fecal specimens, blood (serum), suspect foods in cases of foodborne botulism, and culture fluid following enrichment by growth of the organism (108, 176). ELISAs, cell culture systems (179), and biosensor platforms (180, 181) have also been used to detect BoNT (177, 181). Real-time PCR assays for detection of *C. botulinum* BoNT gene fragments specific to BoNT A, B, and E have been developed as alternatives to the mouse bioassay; this approach was found to demonstrate a sensitivity and specificity similar to those of conventional approaches (182, 183). Potential problems with PCR detection are strains that have the gene but do not produce toxin. Thus, the bioassay remains the study of choice.

ISOLATION PROCEDURES

Isolation and Appearance on Plated Media

A summary of useful procedures for culture and isolation of clostridia is provided below. Clostridia usually produce good growth on commercially available CDC anaerobe blood agar and phenylethyl alcohol blood agar (PEA) after 1 to 2 days of incubation. Brucella agar with 5% sheep blood, Columbia agar, or brain heart infusion agar supplemented with yeast extract, vitamin K, and hemin may also be used as the nonselective blood agar medium. Colony characteristics vary on different media. A few species, such as *C. perfringens*, form colonies after overnight incubation or in as little as 6 h. When clostridia are suspected in wound or abscess specimens (e.g., gas gangrene), egg yolk agar (modified; see chapter 19) should also be inoculated.

After incubation, the blood agar and PEA cultures should be examined under a dissecting microscope, with attention being paid to the hemolysis pattern, colony structure, and evidence of swarming or motile colonies. Egg yolk agar should be examined for evidence of lecithinase (Fig. 2) or lipase production. Lecithinase activity is indicated by the development of an insoluble, opaque, whitish precipitate within the agar. An iridescent sheen or oil-on-water appearance (pearly layer) indicates lipase activity. Proteolysis, the third reaction that can be seen on egg yolk agar, is indicated by a zone of translucent clearing in the medium around the colonies. The same reactions can be visualized on the hemin-supplemented egg yolk agar formulation recommended by Jousimies-Somer et al. (184) or on Lombard-Dowell egg yolk agar (185), in addition to on the modified McClung-Toabe egg yolk agar formulation (186).

Isolation of additional strains in the presence of swarming *Proteus* species or *C. septicum* may require short incubation times (18 to 24 h), subculture onto PEA, or use of anaerobe blood agar with 4% agar ("stiff blood agar"). When isolated colonies can be picked, they should be subcultured to chopped meat medium and incubated overnight for the inoculation of differential media. Prereduced, anaerobically sterilized (PRAS) peptone-yeast-glucose media may be inoculated for detection of metabolic products by gas-liquid chromatography (GLC) if the laboratory has that capability.

Spore Selection Techniques

Heat or ethanol treatment procedures can aid in detecting spores (184, 187). Ethanol may be more effective than heat if the specimen contains relatively heat-sensitive clostridia (e.g., *C. botulinum* type E and some strains of *C. perfringens* involved in foodborne outbreaks). Heat treatment may be more effective than alcohol if homogenization is incomplete and the specimen contains particulate matter that is not penetrated adequately by the alcohol. For any spore selection technique, an untreated control subculture should be prepared.

For alcohol treatment, an equal volume of absolute (or 95%) ethanol is added to a 1-ml sample of a fecal suspension or homogenate of a wound or exudate in a sterile screw-cap tube. The specimen is gently mixed at room temperature (22 to 25°C for 1 h). An Ames aliquot mixer (Miles Laboratories, Inc., Elkhart, IN) is a convenient way to provide continuous mixing. The treated material is used to inoculate chopped meat-glucose or thioglycolate medium, anaerobe blood agar, or egg yolk agar. The culture is incubated and inspected for growth.

For heat treatment, a tube of chopped meat-glucose or thioglycolate medium (5 ml) is preheated in an 80°C water bath for 5 min, and 1 ml of sample suspension is added.

The culture is incubated for 10 min at 80°C, and the tube is removed and cooled in cold water. The treated sample suspension is subcultured into an unheated tube of chopped meat-glucose or thioglycolate medium, anaerobe blood agar, or egg yolk agar. The cultures are incubated anaerobically and examined for growth.

Isolation of *C. difficile*

Since *C. difficile* can be isolated from stool in asymptomatic patients, culture alone is not sufficient to diagnose CDI and may misdiagnose AAD caused by other agents unless stool samples are also assayed for the presence of *C. difficile* toxins. However, the recent emergence of epidemic, hypervirulent strains has reinforced the need for cultivation of *C. difficile* for subsequent typing, molecular studies, and determination of antimicrobial susceptibility.

A variety of media exist for recovery of *C. difficile* from fecal samples or rectal swabs, but there are very few studies that compare media and culture conditions. The original cycloserine-cefoxitin-fructose agar (CCFA), as described by George et al. (188), was based on the ability of *C. difficile* to ferment fructose. Subsequently, additives such as horse blood, taurocholate, and lysozyme (all of which stimulate vegetation) have been shown to enhance recovery (165, 166, 189). Routine cultivation is done at 35 to 37°C, and prereduction of the medium is required for better growth. Growth depends on strict anaerobic conditions. Typically, the culturing time ranges from 2 to 7 days depending on the methods used. Chromogenic agars have also been developed for *C. difficile*. Studies have shown that they are as sensitive as other selective agars and more rapid, yielding identifiable colonies within 24 h of incubation (166, 190). The major drawback is the high cost of some of these products.

Some reports indicate that the best results are achieved if specimens are inoculated into an enrichment broth containing taurocholate, such as cycloserine-cefoxitin-mannitol broth with taurocholate-lysozyme-cysteine broth (CCMB-TAL; Anaerobe Systems, Morgan Hill, CA) (166, 191, 192). If an enrichment broth is not used, a spore enrichment step using either alcohol or heat shock will improve *C. difficile* isolation on selective media (165, 192). In the recent study by Hink et al. (192) that compared a variety of specimen processing and culture methods, the most cost-effective and sensitive procedure for recovery of *C. difficile* from rectal swabs and stool was heat shock followed by inoculation of CCMB-TAL and subsequent plating of growth onto prereduced blood agar.

Following incubation, plates should be examined using a dissecting microscope. Colonies of *C. difficile* are yellowish to white, circular to irregular, and flat, with a rhizoid or erose edge and a ground-glass appearance (Fig. 3). The colonies have a distinctive odor like *para*-cresol (or horse manure). In addition, *C. difficile* colonies on CCFA fluoresce chartreuse under UV light (184).

Gram staining of *C. difficile* reveals rods that are Gram positive to Gram variable, thin, with parallel sides, and 0.5 µm wide by 3- to 5-µm long. Isolation may be difficult due to the presence of both vegetative and spore-forming bacteria. Presumptive identification of *C. difficile* can be made by demonstrating typical colonies, Gram stain morphology, and characteristic odor. Biochemical differentiation is easiest with detection of proline aminopeptidase. Definitive identification depends on demonstration of the unique pattern of short-chain fatty acid metabolic products by GLC, by biochemical characterization of isolates, by 16S rRNA gene sequencing (12, 193–195), or by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) (Table 1). It is likely that the latter method will replace the other more time-consuming and expensive methods (see "Identification" below) in laboratories that have this technology available.

IDENTIFICATION

Preliminary Identification

Identification of *Clostridia* in specimens from sites of infection due to mixed organisms can be time-consuming and expensive. Use of selective and differential media for initial processing can provide rapid and relevant information to the clinician. When isolated from normally sterile sites and sites of serious infection, bacteria should always be completely identified. Some of the organisms that warrant identification include *C. septicum* (associated with gastrointestinal malignancy), *C. ramosum*, *C. innocuum*, and *C. clostridioforme* (which are frequently resistant to antibiotics), and *C. perfringens* (196).

Clostridia are typically Gram-positive rods by microscopic morphology. Some clostridia appear to be Gram negative, especially *C. ramosum*, *C. innocuum*, and the *C. clostridioforme* group, but the special-potency antibiotic disk pattern (see below) verifies the presence of Gram-positive organisms. Second, it may be difficult to detect spores, so an ethanol treatment, heat spore treatment, or malachite green stain may be necessary, and phase-contrast or dark-field microscopy may be helpful. Third, the colonial morphology of pure cultures may be variable, so the culture may appear to be mixed. Subcultures of single, well-isolated colonies yield the same variable morphologies. Examination of colonies by stereomicroscopy is helpful for noting colonial characteristics. Fourth, the aerotolerant clostridia may be confused with *Bacillus* or *Lactobacillus* spp. *Clostridium* species sporulate anaerobically only, grow much better anaerobically (larger colonies), and are almost always catalase negative. *Bacillus* spp. sporulate aerobically only, usually grow better aerobically, and are usually catalase positive. Aerobically grown *C. tertium* has colonial and cellular morphologies similar to those of *Lactobacillus* spp. Certain clostridia can be identified with relative ease by Gram staining, colony morphology determination, a positive indole reaction, hemolysis on blood agar, and the tests described below (Table 3).

Special-Potency Disks

The isolate should be subcultured on blood agar with special-potency disks containing vancomycin (5 µg), kanamycin (1 mg), or colistin (10 µg) and incubated anaerobically for 48 to 72 h at 35 to 37°C. Clostridia are colistin resistant and are usually susceptible to kanamycin and vancomycin (Table 3), except for occasional *C. innocuum* isolates, which may be only moderately susceptible to vancomycin (184, 197).

Lecithinase and Lipase

The isolate should be subcultured on egg yolk agar and incubated anaerobically for 48 to 72 h at 35 to 37°C. Lecithinase activity is demonstrated by a white, opaque, diffuse zone around the colonies that extends into the medium (Fig. 2). Lipase activity is indicated by an iridescent sheen on the surface of bacterial growth and on the agar surface around the colonies.

Spore Test

Media for the demonstration of spores include chopped meat agar or broth and thioglycolate medium. The culture

TABLE 3 Rapid identification of some lecithinase-positive and/or swarming *Clostridium* spp. of clinical significance[a]

Species	Lecithinase	Lipase	Indole	Swarming	Urease	Spore location	Other characteristic(s)
C. bifermentans	+	−	+	−	−	ST	Chalk-white colonies on egg yolk agar
C. novyi A	+	+	−	+	−	ST	Robust beta-hemolysis
C. perfringens	+	−	−	−	−	ST	Double zone of beta-hemolysis, reverse CAMP test positive
C. sordellii	+	−	+	−	+	ST	Large, Gram-positive bacilli
C. septicum	−	−	−	+	−	ST	Rare spores; spreading, adherent colonies
C. sporogenes	−	+	−	+	$-^{+}$	ST	Abundant oval spores
C. tetani	−	$-^{w}$	v	+	−	T	Drumstick shaped

[a] Some information presented here is also found in Table 1. Abbreviations and symbols: +, positive reaction; −, negative reaction; v, variable reaction; w, weakly positive reaction; ST, subterminal; T, terminal.

should be incubated anaerobically at 5 to 7°C below the optimum temperature (30°C) for the growth and sporulation of clostridia, except with *C. perfringens* (should be induced at 37°C). Actively growing cultures may stand at room temperature for several days to 1 week, and ethanol or heat spore treatments can be performed as described above.

Definitive Identification of *Clostridium* Species

The traditional method for the phenotypic characterization and identification of clostridia is the use of PRAS media for the determination of fermentation profiles and other characteristics, combined with GLC analysis of metabolic end products (198, 199). However, only a few laboratories have PRAS media or GLC available. Table 1 lists characteristics that are useful for definitive identification of clinically relevant clostridia. The key reactions (bold in Table 1) require minimal PRAS medium and can be used in conjunction with commercial identification kits or individual preformed-enzyme tests, such as Wee tabs (Key Scientific, Round Rock, TX) or Rosco diagnostic tablets (Rosco, Taastrup, Denmark). Gelatin and esculin hydrolysis, carbohydrate fermentation reactions, and metabolic end product analysis are based on results obtained with PRAS media (Anaerobe Systems).

PRAS Biochemical Inoculation

Actively growing broth cultures (without carbohydrate) or cell pastes suspended in broth medium (e.g., peptone-yeast or thioglycolate) may be used to inoculate PRAS media. Cultures are incubated for 48 to 72 h at 35 to 37°C, but overnight incubation is sufficient for many clostridia.

Gelatin Hydrolysis

A PRAS gelatin tube with an actively growing culture is refrigerated along with an uninoculated tube for at least 1 h. The tubes are removed to room temperature, inverted immediately, and observed for liquefaction every 5 min. In a positive reaction, the gelatin is hydrolyzed and thus fails to solidify, dropping to the top of the inverted tube immediately (184). In a negative reaction, the medium fails to liquefy when it reaches room temperature (>30 min). A weakly positive reaction yields liquid medium at the time that it reaches room temperature (<30 min).

Esculin Hydrolysis

Five drops of 1% ferric ammonium citrate are added to a tube of actively growing bacteria in a PRAS esculin tube, and the tube is observed for a color change and fluorescence under UV (366-nm) light. In a positive reaction, a black or dark-brown color develops, and there is no fluorescence under UV light. In a negative reaction, no color develops, and the tube fluoresces white-blue under UV light. Since many clostridia produce hydrogen sulfide (H_2S), which also reacts with the reagent to form a black complex, all tubes that darken after the addition of reagent should be confirmed under UV light (184).

Carbohydrate Fermentation

The pH of actively growing organisms (>2+ turbidity) should be measured in a PRAS carbohydrate tube. A positive reaction ("acid") yields a pH ≤5.5, and a negative reaction results in a pH ≥5.9. "Weak acid" is indicated by a pH of 5.6 to 5.8. Details of GLC procedures used for the analyses of metabolic end products listed in Table 1 are outlined elsewhere (184).

Commercial kits, based on the detection of preformed enzymes with chromogenic or fluorogenic substrates, have been marketed for the rapid identification of anaerobes. These panels include RapID ANA II (Remel), API 20A and Rapid ID 32A (bioMérieux, Durham, NC), VITEK ANI card and VITEK 2 ANC card (bioMérieux), and the BBL Crystal anaerobe identification system (Becton Dickinson, Franklin Lakes, NJ). The overall performances of these panels vary, and the panels are not always satisfactory as the sole identification method for clostridia (197, 200–205). In general, Gram stain reaction, cellular morphologies, colonial characteristics, and aerotolerance of isolates (characteristics noted above and in Tables 1 and 3) should be determined in conjunction with the use of commercial microsystems. Supplementation of tests in these kits with individual tablets (e.g., Wee tabs or Rosco tablets) can be helpful. Other useful supplemental tests for clostridia include the tests outlined above, such as lipase and lecithinase production; the reduction of nitrate; gelatin and esculin hydrolysis; carbohydrate fermentation; and metabolic end product analysis using GLC.

Clostridial biochemical activity is quite variable, being saccharolytic/proteolytic and saccharolytic/nonproteolytic to asaccharolytic. The identification of asaccharolytic species is most sophisticated. Liquid chromatography-mass spectroscopy (206) and molecular biological methods such as 16S rRNA gene sequencing (22, 32) can be useful in these cases. 16S rRNA gene sequencing is becoming more popular, though interpretation of results must be done by those with special training. Other promising methods for the identification of *Clostridium* species are fluorescent *in situ* hybridization (207, 208) and MALDI-TOF MS (209).

Numerous reports have been published on the utility of MALDI-TOF MS for identification of anaerobic bacteria, including *Clostridium* species. This method, described in detail in chapter 4, identifies a broad range of organisms based on the analysis of ribosomal proteins ranging in size from 2,000 to 20,000 Da. Two commercial MALDI-TOF MS systems, the Bruker Microflex (Bruker Daltonik GmbH, Bremen, Germany) and the VITEK MS (bioMérieux) (previously Shimadzu MS; Shimadzu Corp., Kyoto, Japan), are available. The VITEK MS has FDA clearance for identification of both Gram-positive and Gram-negative bacteria as well as yeasts, while the Bruker system is FDA cleared for only Gram-negative bacterial identification. Neither system is FDA cleared for identification of anaerobic bacteria.

One of the first papers specifically evaluating the utility of MALDI-TOF MS for anaerobic bacterial identification focused on optimizing the system for characterization of clostridial strains (209). The authors carefully controlled the temperature (37°C), anaerobic atmosphere, and incubation time prior to testing. A subanalysis evaluated the effect of sporulation on the spectral patterns. In addition, the authors evaluated the impact of various types of agars to establish whether the medium had any impact on the mass spectra (209). All isolates underwent an inactivation step using 80% trifluoroacetic acid followed by addition of water and acetonitrile; the suspension was centrifuged for 2 min at 6,500 × *g*. One microliter of the supernatant was then transferred to the MALDI target in five separate spots. The instrument used in this study was the Bruker Microflex LT, and the spectra generated were analyzed using the Bruker Biotyper 1.1 software (209). Sixty-four reference strains representing 31 different species, including various *C. botulinum* subtypes and strains otherwise difficult to identify such as *C. chauvoei* and *C. septicum*, were evaluated and used to construct a library for identification of 25 "field strains" representing 6 species, most of which were *C. perfringens* (209). Several important observations were made. The culture medium used had minimal effects on the mass spectra. However, allowing the isolates to sporulate did result in significant changes to the spectra, especially as the cultures aged, so the authors suggested that when testing isolates, fresh cultures should be used. That said, the authors noted that spore spectra could also be used to differentiate among the clostridia (209). All 31 species in this study displayed characteristic mass spectrometric patterns that provided unequivocal identification to the species level and could be used to generate a dendrogram similar to what is generated after 16S rRNA gene sequencing. Strains of the same species clustered into distinguishable groups, including the *C. botulinum* metabolic groups (209). The authors concluded that MALDI-TOF MS can replace cumbersome and often inaccurate biochemical methods for the identification of *Clostridium* species (209).

After the landmark study by Grosse-Herrenthey et al. (209), most of the studies evaluating MALDI-TOF MS for identification of *Clostridium* species have been in papers that have evaluated large numbers of different anaerobes recovered from clinical specimens and have, in general, included fewer *Clostridium* species (210–214).

In the study by Nagy et al. (210), the authors evaluated the Bruker cutoff values for species-level identification of a variety of Gram-positive and Gram-negative, anaerobic bacteria. The manufacturer uses a log score between 0 and 3.0, which is calculated from comparison of the spectra of the unknown organism to those spectra in the reference database. The somewhat arbitrary cutoff that has been established for interpretation is as follows: a log score of ≥1.7 is indicative of genus-level identification, while a log score of ≥2.0 is indicative of a species-level match. In this 2-year study, 283 anaerobes, including 68 isolates of 11 species of *Clostridium*, were evaluated after a formic acid extraction procedure prior to target inoculation in duplicate and testing on the Microflex LT (Bruker Daltonik), using the Biotyper 3.0 software for spectra interpretation (210). Compared to conventional phenotypic methods and 16S rRNA gene sequencing, MALDI-TOF accurately identified 67 of the 68 isolates using the manufacturer's suggested cutoff. An isolate identified as *C. baratii* (log score, 1.727) was identified as *C. fallax* by phenotypic methods and could not be resolved by 16S rRNA gene sequencing (210).

Three groups have directly compared the Shimadzu/bioMérieux VITEK system to the Bruker system (215–217) for the identification of a variety of anaerobes. Both systems performed equally well for identification of *Clostridium* species that were contained in the respective databases at the time of the evaluation. In one study that compared extraction methods, *C. ramosum* was the only species that required pretreatment with formic acid for accurate identification (215).

Important caveats to consider as one evaluates the literature are the instrument manufacturer, the method used (direct application to the target versus pretarget or on-target extraction), the software version of the database, and finally the version of the library used for spectra interpretation. Investigators have shown that on-plate formic acid preparation and direct smear without extraction yield acceptable results for many genera and species of anaerobes (211, 214, 215). Both groups noted that the major limitation of MALDI-TOF MS is the number of anaerobic species in the libraries (211, 214). As databases are frequently updated, readers are encouraged to seek out the latest information provided by the manufacturers.

Characteristics of Commonly Encountered Clostridia

Key characteristics that aid in the presumptive identification of the most common species are listed below. See also Tables 1 and 3.

C. bifermentans: colonies chalk-white on egg yolk agar; irregular, scalloped edge; many free spores, often in chains; urease negative; indole and lecithinase positive. *C. sordellii* is similar but is usually urease positive.

C. bolteae: colonies usually have a slightly irregular edge; greening of agar around colonies; Gram negative; tapered ends; spores are rare; lactose negative and β-*N*-acetylglucosaminidase (β-NAG) negative.

C. butyricum: very large, irregular colonies with mottled-to-mosaic internal structure; subterminal spores; ferments many carbohydrates.

C. cadaveris: white-gray; entire or slightly irregular; raised to slightly convex; oval terminal spores; spot indole positive.

C. clostridioforme: same as for *C. bolteae* but lactose positive and β-NAG negative.

C. difficile: colonies creamy yellow to gray-white (Fig. 3); irregular, coarse, mottled-to-mosaic internal structure; matte or dull surface; horse stable odor (*para*-cresol); subterminal and free spores or spores infrequent; gelatin hydrolysis can be slow; mannitol and proline positive; colonies fluoresce chartreuse on selective CCFA.

C. glycolicum: colonies are gray-white with an entire or scalloped edge and convex; subterminal and free spores.

C. hathewayi: same as for *C. bolteae* but lactose and β-NAG positive.

- C. *innocuum*: gray-white–to–brilliant-greenish colonies; coarsely mottled-to-mosaic internal structure, entire edge usually; terminal spores may be difficult to find; nonmotile; mannitol positive; lactose, maltose, and proline negative.
- C. *novyi* type A: lecithinase and lipase positive; may swarm; strong beta-hemolysis.
- C. *perfringens*: double zone of beta-hemolysis around colonies (Fig. 4); boxcar-shaped rods; spores rare; lecithinase positive (Fig. 2).
- C. *ramosum*: colonies resemble *Bacteroides fragilis* but usually have a slightly irregular edge; Gram stain variable; palisading, slender rods; small round or oval terminal spores (Fig. 1); nonmotile; mannitol positive.
- C. *septicum*: swarms (Fig. 5); large, filamentous bacilli (Fig. 6); subterminal spores often in "lemon" forms; DNase positive and sucrose negative.
- C. *sporogenes*: Medusa-head colonies; possible swarming; colonies adhere firmly to agar; subterminal and many free spores; lipase positive.
- C. *symbiosum*: rods with tapered ends, football shaped; may form chains; often has spores.
- C. *tertium*: aerotolerant; terminal spores when anaerobically incubated.
- C. *tetani*: may form a thin film of growth over entire agar plate, especially on moist media; drumstick spores.

Toxin tests are necessary for the identification of a few species. C. *sporogenes* cannot be differentiated with certainty from the proteolytic group I strains of C. *botulinum* unless toxin tests are used. A few strains of C. *botulinum* produce lecithinase as well as lipase and are difficult to distinguish from C. *novyi* type A except by toxin tests. As a supplement to the methods described, the various types of C. *botulinum* and other clostridia can be presumptively identified on the basis of differences in their CFA profiles and by typing methods such as pulsed-field gel electrophoresis (PFGE) or other molecular analyses. Finegold et al. (6) described a multiplex PCR procedure for rapid distinction of the three species of the C. *clostridioforme* group.

TYPING SYSTEMS

Typing of C. *difficile* fosters understanding of the epidemiology of infections and has become increasingly important with the emergence of outbreaks caused by hypervirulent strains. In the event of a pattern of an increase in the number of severe cases of disease in communities or increased transmission in hospitals, typing of strains may provide useful information on a dominant, more transmissible clone. Physicians should be aware of their local C. *difficile* situation and should know whether hypervirulent strains are circulating in the community, as this allows for risk assessment for patients and perhaps heightened infection control practices. It is obvious from investigations of C. *difficile* strains that every region may have its particular pattern of strains, with ribotypes differing between different countries in Europe but also between different regions of a single state (218).

To resolve endemic-disease situations, to monitor the spread of infection, and to assess the genetic relatedness of the associated strains, the successful cultivation of C. *difficile* is required. Starting with the pure culture, several approaches have been used for such analyses. Restriction endonuclease analysis (REA), PFGE, PCR ribotyping, multilocus sequence typing (MLST), and whole-genome sequencing represent the most common methods used in epidemiologic studies (166, 219). For example, the initial epidemic North American hypervirulent isolates were typed as 027 by PCR ribotyping, designated toxinotype III by typing the toxin A and B genes of the PaLoc, and designated NAP1 by PFGE and type BI by REA (66). These methods are briefly discussed here, as each has distinct advantages and disadvantages. Various tools (Simpson's index of diversity, Rand coefficients, and Wallace coefficients) have been used to assess the congruence between results of various typing methods (166, 220). In general, the more cumbersome and time-consuming restriction digest methods are being replaced by PCR and sequencing-based methods. Restriction endonuclease DNA analysis of C. *difficile* using HindIII restriction digest of total genomic DNA was one of the earlier molecular methods to be adopted for strain characterization (221). Several studies over a decade describe it as a sensitive, discriminating, and reproducible method (220, 222). In the study by Tenover et al. (220) that compared REA to PCR ribotyping and PFGE, REA typing appeared to define a broader group of isolates than the other two methods, consistent with the sampling of total genomic DNA. This study also demonstrated that extrapolation of the results of one typing method to another must be done carefully and that there may be advantages to combining methods for outbreak investigations (220). Currently, few laboratories employ this method of strain typing C. *difficile*, likely because of the complexity involved in its performance and interpretation (221).

PFGE has been the major method used for strain characterization of C. *difficile* in North America (221). SmaI has been the most-often-used infrequently cutting restriction enzyme for this purpose. Early technical issues have been resolved by using younger cultures and adding thiourea to the electrophoresis buffer (223). While this method has excellent discriminatory power, it is laborious, expensive to perform, and may yield patterns that cannot be assigned to existing NAP types (220, 221).

PCR ribotyping has been used globally to define the epidemiology of C. *difficile* and has been more readily adopted by clinical laboratories. PCR ribotyping is done with two specific primers that amplify the spacer region in between the 16S and the 23S rRNAs. The spacer region is known for its heterogeneous nature, as opposed to the highly conserved rRNA genes themselves. C. *difficile* contains 10 rRNA copies, and variations in the spacer regions are seen between different strains but also between different rRNA copies of a single strain. PCR ribotyping is less complex than PFGE and REA and has shown good interlaboratory agreement (219). More than 200 ribotypes exist. The Health Protection Agency in the United Kingdom created the *Clostridium difficile* Ribotyping Network as part of an enhanced national surveillance program (224). Prospective C. *difficile* typing results provided an understanding of regional and national prevalence of C. *difficile* ribotypes and their associated complication rates, particularly with respect to ribotype 027 (224). It was believed that access to these data would contribute to heightened infection control and clinical interventions (224). Variations of PCR ribotyping methods are discussed by Huber et al. (221).

MLST involves partial amplification and sequencing of several housekeeping genes. Data generated with this and other sequence-based methods are unambiguous compared to fragment-based methods and thus may be more easily compared between laboratories (166, 219, 221). The costs with MLST are high compared with PCR methods.

Whole-genome sequencing is being used by several investigators to elucidate the evolutionary history of virulent clones such as ribotype 027 (225). Availability of next-generation sequencing platforms will likely allow laboratories to employ rapid whole-genome data in routine epidemiologic investigations. Next-generation sequencing has the potential to replace existing methods, as the extra resolution provided clarifies differences that may not be detected by more traditional typing methods, and costs are decreasing. One group detected a probable outbreak of community transmission of *C. difficile* using a next-generation sequencing platform (226). The availability of adequate bioinformatics is the major impediment at this time to more widespread implementation.

MLST has been successfully used as a reproducible and discriminating system for strain typing of *C. botulinum* type A using clinical and food isolates (227), *C. perfringens* isolates from necrotic enteritis outbreaks in broiler chicken populations (228), and *C. septicum* isolates recovered from poultry flocks experiencing episodes of gangrenous dermatitis (229). Leclair et al. (230) described a modified PFGE protocol to be the most useful method for typing epidemiologically related *C. botulinum* type E strains, in comparison with randomly amplified polymorphic DNA analysis and automated ribotyping using clinical and food isolates associated with four botulism outbreaks. Furthermore, analysis of the variable numbers of tandem repeats (VNTRs) within the genome, called multiple-locus VNTR analysis, has been described for *C. perfringens* (231, 232). Epidemiologically related isolates previously typed by PFGE were also examined by multiple-locus VNTR analysis, and the congruency of the two methods was found to be very high. Macdonald et al. (106) described VNTR regions in *C. botulinum* strains, providing a rapid and highly discriminatory tool to distinguish among *C. botulinum* BoNT/A1 strains for investigations of botulism outbreaks.

SEROLOGIC TESTS

Serologic procedures are not practical for secure strain identification from colonies. Furthermore, no standardized tests are available for the detection of antibodies against *Clostridium* species in clinical specimens to confirm diagnoses of clostridial infections. To evaluate the vaccination status, determination of immunoglobulin G antibodies against tetanus toxin may be useful, but in cases of an unclear immunization status, preventive vaccination should be done.

ANTIMICROBIAL SUSCEPTIBILITIES

Antimicrobial susceptibility studies with strains of a number of clostridial species are summarized in Table 4. Basic methodology for such testing of anaerobes can be found in two new references (233, 234). Now that more laboratories are identifying anaerobes by 16S rRNA gene sequencing, more accurate species identification will be available and more-reliable susceptibility data can be generated. In addition, because of the inherent difficulty in performing susceptibility studies in anaerobes, new methods using phenotypic and molecular techniques are being developed (235). Drugs lacking antimicrobial activities against various clostridia include trimethoprim-sulfamethoxazole, ampicillin, and clindamycin. No resistance of clostridia to ampicillin-sulbactam or piperacillin-tazobactam has been noted, and antimicrobial resistance is uncommon among clostridia with respect to imipenem, metronidazole, and vancomycin. Five species (all with small numbers of strains) and *C. perfringens* show little or no resistance to the antimicrobial agents under consideration (Table 4). Organisms with some resistance to three drugs include *C. ramosum*, *C. innocuum*, and *C. clostridioforme*.

Multiple studies have described resistance to various antibiotics among clostridia. Resistance to penicillin is especially common in *C. ramosum*, *C. clostridioforme*, and *C. butyricum*; these species produce β-lactamases that are induced by β-lactam antibiotics. *C. tertium* has resistance features unusual among clostridia, including resistance to β-lactam antibiotics, metronidazole, and clindamycin. When treating CDIs with metronidazole, a considerable time lag in the onset of its antibiotic activity has to be considered due to its prodrug nature (195). Resistance to clindamycin has been documented for strains of *C. perfringens* (as noted above), *C. ramosum*, *C. difficile*, *C. tertium*, *C. subterminale*, *C. butyricum*, *C. sporogenes*, and *C. innocuum* (185).

Chloramphenicol, piperacillin, metronidazole, imipenem, and combinations of β-lactams with β-lactam inhibitors (e.g., ampicillin-sulbactam) were active against most clostridia (185), and this has not changed significantly over the last 10 years (236). In a 2009 study by Finegold et al. (237), ampicillin-sulbactam and nitazoxanide had the best activities in comparison with neomycin, rifaximin, teicoplanin, and vancomycin. The clostridia are variably resistant to cephalosporins and tetracyclines, and they are usually resistant to the aminoglycosides. Many clostridia other than *C. perfringens* (particularly *C. ramosum*, *C. clostridioforme*, and *C. innocuum*) are resistant to cefoxitin, cefotaxime, ceftazidime, ceftizoxime, cefoperazone, and other broad-spectrum β-lactams (185, 197). Most strains of *C. innocuum* were only moderately susceptible to vancomycin (197, 237, 238).

Earlier quinolones, such as ciprofloxacin, levofloxacin, and lomefloxacin, have demonstrated low or intermediate activities against anaerobes. More newer quinolones, like moxifloxacin, gatifloxacin, and garenoxacin, initially had good activities *in vitro* against most anaerobes, including clostridia (239, 240), though this is changing rapidly in Europe (234). A high frequency of moxifloxacin resistance has also been described among *C. difficile* isolates (241).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The isolation of a *Clostridium* species from a clinical specimen, even a blood culture, may or may not be significant clinically, and culture results should be interpreted in relation to the patient's clinical findings. Clostridia of the patient's own intestinal microbiota may be present on the skin and may contaminate blood samples or other specimens. Bacteremia may be transient or clinically insignificant. In addition, most clostridia currently encountered in wounds, exudates, blood, and other normally sterile body fluids are opportunistic and may not cause serious or progressive disease unless conditions are suitable in the host. As discussed earlier in this chapter, one exception to this generalization is *C. septicum*, which is rarely encountered in blood cultures except from patients who have an underlying malignancy or neutropenic sepsis. *C. septicum* sepsis is an infectious disease emergency that requires prompt and clear communication between the laboratory and the clinician in order to institute early surgical measures and treatment with antimicrobial agents to improve outcomes. *C. tertium*, *C. perfringens*, and other clostridia, to a lesser extent, may be involved in serious infections that require emergency measures. The best approach for preventing tragic consequences

TABLE 4 Activities of various drugs against *Clostridium* spp. (Wadsworth agar dilution procedure)[a]

Antimicrobial agent	CLSI MIC breakpoint (μg/ml)		% Susceptible																	
	Susceptible	Intermediate	*C. bifermentans*	*C. bolteae*	*C. butyricum*	*C. cadaveris*	*C. clostridioforme*	*C. difficile*	*C. disporicum*	*C. glycolicum*	*C. hathewayi*	*C. innocuum*	*C. paraputrificum*	*C. perfringens*	*C. ramosum*	*C. septicum*	*C. sordellii*	*C. sporogenes*	*C. subterminale*	*C. tertium*
Penicillin/ampicillin[b]	≤0.5	1	100	32	86	100	24	0	100	100	7	100	94	100	100	100	100	100	100	0
Ampicillin-sulbactam	≤8/4	16/8	100	10	100	ND[c]	ND	100	100	100	100	100	100	100	100	100	100	100	100	100
Piperacillin-tazobactam	≤32/4	64/4	100	100	ND	ND	ND	100	ND	ND	100	100	ND	100	100	100	100	100	ND	100
Cefoxitin	≤16	32	ND	90	100	ND	81	0	ND	ND	98	ND	ND	100	ND	ND	ND	ND	ND	83
Clindamycin	≤2	4	100	90	100	100	91	5	100	92	88	83	87	96	82	100	94	8	85	0
Imipenem	≤4	8	100	100	100	100	100	20	ND	ND	100	100	100	100	100	ND	100	100	ND	100
Metronidazole	≤8	16	100	100	100	100	100	100	100	100	100	98	100	100	98	100	95	100	100	100
Moxifloxacin	≤2	4	ND	ND	ND	ND	0	78	ND	ND	9	ND	ND	99	ND	ND	ND	ND	ND	ND

[a] Clinical and Laboratory Standards Institute (CLSI) approved method M11-A8 (242); data compiled from references 6, 236, and 243–245.
[b] Strains producing β-lactamase should be considered resistant.
[c] ND, no data.

that may be avoidable is good communication between microbiologists and clinicians.

The accurate and timely reporting of preliminary results (e.g., findings from direct microscopic examinations of clinical specimens), as well as early culture results (after 24 and 48 h of incubation), can be extremely valuable to the physician. For smaller laboratories without anaerobic chambers, incubation of the appropriate media in anaerobic jars provides acceptable recovery for most clinically significant anaerobes, assuming that optimal collection and transport of specimens are performed. The colony characteristics and microscopic features of some clostridia (e.g., *C. perfringens*, *C. sordellii*, and *C. sporogenes*) may be distinctive, so preliminary or presumptive reports may be released before aerotolerance studies are completed. Accurate, definitive identification is needed to better define the role of clostridia in disease, to aid the clinician in selecting optimal treatment, and for public health purposes (e.g., hospital-acquired *C. difficile* disease).

Potentially life-threatening diseases due to *Clostridium* species or their toxins, such as botulism, tetanus, or severe cases of CDI, should be carefully examined by the physician and the microbiologist together to ensure optimal sample collection and transport, immediate processing, and initiation of specific therapy. Furthermore, health care institutions require accurate and rapid diagnosis for early detection of possible outbreaks and to implement effective control measures.

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Bacteroides, Porphyromonas, Prevotella, Fusobacterium, and Other Anaerobic Gram-Negative Rods*

EIJA KÖNÖNEN, GEORG CONRADS, AND ELISABETH NAGY

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TAXONOMY AND DESCRIPTION OF THE GROUP

Most of the obligately anaerobic, Gram-negative, non-spore-forming rods of clinical relevance belong to the phylum *Bacteroidetes*, the order *Bacteroidales*, including the families *Bacteroidaceae*, *Porphyromonadaceae*, *Prevotellaceae*, and *Rikenellaceae*, and to the phylum *Fusobacteria*, the order *Fusobacteriales*, including the families *Fusobacteriaceae* and *Leptotrichiaceae*. In addition, clinically important taxa representing anaerobic, Gram-negative, non-spore-forming rods exist in some other phyla, such as the *Firmicutes*, *Proteobacteria*, and *Synergistetes*. There have been a considerable number of changes in the taxonomy of the taxa that are covered in this chapter; some new genera have been named, and several novel species have been described in the past few years. The changes made since 2010 are listed in Table 1.

Within the family *Bacteroidaceae*, the genus *Bacteroides* consists of saccharolytic, bile-resistant, and nonpigmented species, mainly isolated from the gut (1). Currently, the genus is limited to species within the *Bacteroides fragilis* group, which includes more than 40 species, of which about 25 have been recovered from humans (2) (for an update, see http://www.bacterio.net). Of these, especially *B. fragilis*, *B. thetaiotaomicron*, and *B. ovatus* are highly relevant in human infections. Only a few taxonomic changes have occurred within the genus since 2010 (Table 1); some isolates from feces of healthy subjects have been described as novel *Bacteroides* species (3, 4), while *B. capillosus* has been reassigned to a new genus, *Pseudoflavonifractor*, which represents a distinct lineage in clostridial cluster IV of the phylum *Firmicutes* (5), and *B. ureolyticus* has been reclassified as *Campylobacter ureolyticus* (6).

The genus *Alistipes*, which belongs to the family *Rikenellaceae*, currently includes *A. finegoldii*, *A. indistinctus*, *A. onderdonkii*, *A. putredinis*, and *A. shahii* (7–9). Recently, three new species, "*A. obesi*," isolated from feces of a young woman with morbid obesity (10), and "*A. senegalensis*" and "*A. timonensis*," both isolated from feces of the same, healthy subject, were proposed (11, 12). Of those, *A. senegalensis* was described as having weak growth under microaerobic conditions and as being resistant to metronidazole (12), while *A. obesi* was described as being motile (10), thus differing from other members of the genus. In general, *Alistipes* species are anaerobic, nonmotile, and straight or slightly curved rods, except for *A. indistinctus* cells, which are coccus shaped. Most of the species are pigmented, weakly saccharolytic, and bile resistant.

The family *Porphyromonadaceae* includes five genera with species detected in humans: *Barnesiella*, *Odoribacter*, *Parabacteroides*, *Porphyromonas*, and *Tannerella*. Of these genera, *Porphyromonas* is clinically the most relevant. There are currently 17 validly published *Porphyromonas* species, many of which are of animal origin. *P. asaccharolytica*, *P. bennonis*, *P. catoniae*, *P. endodontalis*, *P. gingivalis*, *P. somerae*, and *P. uenonis* are frequently detected in humans (13–17). Most species are asaccharolytic and, except for *P. catoniae*, pigmented and are generally considered pathogens. Human *Parabacteroides* species consist of three former *Bacteroides* species, *P. distasonis*, *P. goldsteinii*, and *P. merdae*, and the novel species *P. gordonii* and *P. johnsonii* (18–20), and the genus is phylogenetically closely related to the genera *Tannerella* (21) and *Barnesiella* (22). *Parabacteroides* organisms are saccharolytic and resistant to 20% bile. The genus *Tannerella* contains only one species, *T. forsythia* (formerly *T. forsythensis*) (21), which is an important oral pathogen, and the genus *Barnesiella* contains one human species, *B. intestinihominis*, isolated from feces (23). Both of these species are anaerobic, nonpigmented, nonmotile, pleomorphic rods. The recently described genus *Coprobacter* with *C. fastidiosus*, a nonmotile Gram-negative rod isolated from infant feces, is most closely related to *Barnesiella* (24). The new genus *Odoribacter* includes two human-derived species, *O. splanchnicus* (formerly *Bacteroides*) and *O. laneus*, and one canine-derived species, *O. denticanis* (7, 225). *O. splanchnicus* is saccharolytic and bile resistant, while *O. laneus* is asaccharolytic and susceptible to 20% bile.

The family *Prevotellaceae* includes saccharolytic or moderately saccharolytic short rods that produce acetic and succinic acids as their major end products of fermentation (25). This family has been confronted by a tremendous number of novel species in the past years. Most novel *Prevotella* species have been isolated from the oral cavity (26–30) but also from feces (31) and from other sites of the body, where they have been associated with various clinical conditions (32–37). *P. heparinolytica* and *P. zoogleoformans*, isolated mainly from infected cat and dog bite wounds (38), are only loosely connected to other *Prevotella* species and, instead, cluster phylogenetically with *Bacteroides* species (39). A new genus phylogenetically close to *Prevotella*,

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TABLE 1 Recently classified or reclassified genera and species (from 2010 onwards) of non-spore-forming anaerobic Gram-negative rods isolated from humans

Phylum and genus	New genus	Species	Previous nomenclature	Reference
Bacteroidetes				
Alistipes		*A. indistinctus*	New species	7
		A. obesi	New species	10
		A. senegalensis	New species	12
		A. timonensis	New species	11
Alloprevotella	Yes	*A. rava*	New species	40
		A. tannerae	*Prevotella tannerae*	40
Bacteroides		*B. clarus*	New species	4
		B. faecis	New species	3
		B. fluxus	New species	4
		B. oleiciplenus	New species	4
Coprobacter	Yes	*C. fastidiosus*	New species	24
Odoribacter	Yes	*O. laneus*	New species	7
		O. splanchnicus	*Bacteroides splanchnicus*	7
Prevotella		*P. aurantiaca*	New species	30
		P. fusca	New species	26
		P. jejuni	New species	35
		P. saccharolytica	New species	29
		P. scopos	New species	26
Fusobacteria				
Leptotrichia		*L. hongkongensis*	New species	46
Sneathia		*S. amnii*	*Leptotrichia amnionii*	48
Synergistetes				
Fretibacterium	Yes	*F. fastidiosum*	New species	49
Firmicutes				
Christensenella	Yes	*C. minuta*	New species	60
Phascolarctobacterium	Yes	*P. succinatutens*	New species	59
Pseudoflavonifractor	Yes	*P. capillosus*	*Bacteroides capillosus*	5
Proteobacteria				
Parasutterella		*P. secunda*	New species	61
Succinatimonas	Yes	*S. hippei*	New species	66

Alloprevotella, was recently described (40); it contains the former *Prevotella tannerae* (reassigned to *A. tannerae*) and a novel species, *A. rava*. Another genus distantly related to *Prevotella*, *Paraprevotella*, consists of *P. clara* and *P. xylaniphila*, which were isolated from human feces (41).

In the family *Fusobacteriaceae* of the phylum *Fusobacteria*, the genera of clinical interest are *Fusobacterium*, *Leptotrichia*, and *Sneathia*. These organisms are nonmotile, pleomorphic rods, isolated mainly from the oral cavity and female genital tract. In a study using sequencing of the 16S-23S rRNA gene internal transcribed spacer regions of *Fusobacterium* species (42), three phylogenetic clusters were formed. The first cluster included *F. mortiferum*, *F. varium*, and *F. ulcerans*. The second cluster contained *F. nucleatum* subspecies, *F. simiae*, *F. periodonticum*, and *F. naviforme* (note that there are considerable inconsistencies in the phenotypic and genotypic characteristics between *F. naviforme* strains obtained by different laboratories; the strain used in the study by Conrads et al. [42] fits with the original description of the species). The third cluster included *F. necrophorum* subspecies and *F. gonidiaformans*. *F. russii* and *F. perfoetens* formed separate branches. The somewhat fuzzy phylogeny of fusobacteria and wide heterogeneity of *F. nucleatum*, in particular, have been explained by potential horizontal gene transfer that occurred in the close interaction of oral bacteria within dental biofilms (43).

The genus *Leptotrichia* consists of nonmotile, highly saccharolytic, long rods that typically produce lactic acid. Currently, there are six validly described *Leptotrichia* species: *L. buccalis*, *L. goodfellowii*, *L. hofstadii*, *L. shahii*, *L. trevisanii*, and *L. wadei* (44, 45). In addition, a blood isolate from a patient with disseminated breast carcinoma was characterized, and a novel species, *L. hongkongensis*, was proposed (46) and validated. By using a molecular method, this species was also detected in an oral specimen of a healthy subject. "*L. amnionii*," originally isolated from amniotic fluid (47), is not validly published, and in fact, it phylogenetically clusters closer to *Sneathia sanguinegens* (formerly *Leptotrichia sanguinegens*) than to other *Leptotrichia* species (44). A strain phenotypically and phylogenetically similar to *L. amnionii* from a vaginal specimen was recently characterized, and a novel species, *Sneathia amnii*, was proposed (48).

Among the phylum *Synergistetes*, only a few cultivable species, namely, *Fretibacterium fastidiosum* (49), *Jonquetella anthropi* (50), and *Pyramidobacter piscolens* (51), have been isolated from humans so far. They are obligately anaerobic, nonmotile, Gram-negative organisms. Fluorescent *in situ* hybridization analysis has revealed that the oral cavity harbors a diverse population of unculturable *Synergistetes* spp., which are large, curved bacilli (52). Also, *Synergistes* organisms are widely distributed in various human ecosystems in settings of both health and disease (53).

In the Gram-positive phylum *Firmicutes*, the novel class *Negativicutes* harbors organisms with a traditional Gram-negative cell wall structure (54), including some clinically important genera, such as *Dialister*, *Megamonas*, *Selenomonas*, and *Centipeda*. The genus *Dialister* includes five species of human origin: *D. invisus* (55) and *D. pneumosintes* (formerly *Bacteroides pneumosintes*) isolated from the oral cavity, *D. micraerophilus* and *D. propionicifaciens* (56) from clinical specimens, and *D. succinatiphilus* from feces (23). These anaerobic or microaerobic, Gram-negative coccobacilli are asaccharolytic and largely unreactive in biochemical tests (56). The two human species of the genus *Megamonas*, *M. hypermegale* and *M. funiformis* (57), are anaerobic, Gram-negative, very large rods. In the genus *Selenomonas*, *S. sputigena*, *S. artemidis*, *S. dianae*, *S. flueggei*, *S. infelix*, and *S. noxia* and also the closely related *Centipeda periodontii* have been isolated from the human oral cavity (58). They are anaerobic, Gram-negative, curved motile rods. Recently, two strictly anaerobic, Gram-negative, nonmotile, long-rod-shaped strains from human feces were characterized and classified as a novel species, *Phascolarctobacterium succinatutens*, in the family *Acidaminococcaceae* (59). The novel *Christensenella minuta* from human feces is an anaerobic short rod with a Gram-negative cell wall (60). Phylogenetically, it belongs to a novel family, *Christensenellaceae*, within the phylum *Firmicutes*.

Various families in the phylum *Proteobacteria* include genera and species of clinical importance in humans. The genera *Sutterella* and *Parasutterella* were recently placed in a novel family, *Sutterellaceae* (61). They consist of asaccharolytic, bile-resistant, Gram-negative, short rods or coccobacilli, of which *S. wadsworthensis*, *S. parvirubra*, *P. excrementihominis*, and the novel *P. secunda* (61) have been isolated from human specimens. The family *Desulfovibrionaceae* contains two genera of clinical interest, *Bilophila* and *Desulfovibrio*. *Bilophila wadsworthia* is an anaerobic, asaccharolytic, bile-resistant, Gram-negative rod and is a significant pathogen in humans (62). Of the more than 60 *Desulfovibrio* species and subspecies, only a few infrequently cause a variety of human infections (63). In the family *Desulfomicrobiaceae*, the genus *Desulfomicrobium* includes one human species, *D. orale*, which is associated with periodontal diseases (64). In the family *Succinivibrionaceae*, the genus *Anaerobiospirillum* includes two species, *A. succiniciproducens* and *A. thomasii*, isolated from feces of humans, cats, and dogs (65), and the novel genus *Succinatimonas* has one species, *S. hippei*, isolated from human feces (66). While the last species is nonmotile and has nonspiral cells without flagella, *Anaerobiospirillum*, *Desulfovibrio* (except *D. piger*), and *Desulfomicrobium* are motile, spiral-shaped bacteria that reduce sulfate.

Since 2002, genome projects have produced a tremendous number of complete-genome sequences for a variety of Gram-negative anaerobic species, currently including members of the genera *Bacteroides*, *Alistipes*, *Porphyromonas*, *Parabacteroides*, *Tannerella*, *Odoribacter*, *Prevotella*, *Alloprevotella*, *Paraprevotella*, *Fusobacterium*, *Leptotrichia*, *Jonquetella*, *Pyramidobacter*, *Dialister*, *Megamonas*, *Selenomonas*, *Centipeda*, *Sutterella*, *Bilophila*, and *Desulfovibrio*. Readers are directed to the NCBI home page (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) for detailed information. Interestingly, the genome sequence for *F. nucleatum* revealed that although this species has a Gram-negative cell wall, including an outer membrane, a significant proportion of its genes are related to homologues from Gram-positive species in the phylum *Firmicutes*, suggesting that *Fusobacterium* has a Gram-positive evolutionary history (67).

Current methods used for taxonomic studies are based mainly on nucleic acid analyses, in particular, sequencing of the 16S rRNA gene and the comparison of these sequences, in order to reveal the phylogenetic relatedness of taxa. This approach does not necessarily correlate with phenotypic characteristics, such as cell and colony morphologies, atmospheric growth requirements, and various biochemical test results, which are still widely used in clinical microbiology laboratories. However, appropriate atmospheric requirements should be determined for all isolates, because the true anaerobes can be differentiated from facultatively anaerobic bacteria by their inability to grow in the presence of oxygen and by their susceptibility to metronidazole (68). Table 2 presents differential characteristics of clinically relevant genera within the Gram-negative anaerobic rods.

EPIDEMIOLOGY AND TRANSMISSION

Gram-negative anaerobic rods inhabit the mucosal surfaces of the oral cavities and gastrointestinal tracts of animals and humans. Infants acquire oral and intestinal anaerobic commensals mainly from their mother and become simultaneously colonized by multiple strains of commensal species, e.g., *Prevotella melaninogenica*, *Fusobacterium nucleatum*, or *Bacteroides fragilis* (69–71). In fact, some of these organisms, such as *Fusobacterium* and *Prevotella*, are ubiquitous members of the mouth from the early months of life and, when teeth erupt, form an integral part of dental biofilms (69, 72). Furthermore, *Prevotella* has been found among the dominant genera in other habitats, such as the esophagus, stomach, and lungs (73–75), which have previously been considered to have a very limited microbial diversity. In the gut, *Bacteroides* organisms become part of the microbiota in early infancy (71), although as a result of cesarean section, colonization by the *B. fragilis* group of organisms can be delayed in infants and their levels are greatly reduced (76). Moreover, the composition of *Bacteroides* populations varies depending on the type of milk feeding (77). Two bacterial phyla, *Firmicutes* and *Bacteroidetes*, dominate in the gut. Changes in microbial composition affect the gut barrier function, with consequences for host physiology, energy balance (obesity risk), or mucosal immune system. In this context, Gram-negative anaerobes play a key role (2, 78). In the female genital tract, when the vaginal hydrogen peroxide-producing lactobacilli decrease in numbers, some *Prevotella* species increase and become an important part with other commensal vaginosis-associated microorganisms (79, 80). Notably, the microbiotas at different body sites are still poorly defined due to considerable proportions of not-yet-cultivated bacteria (53, 81–83).

Also, an important point to be mentioned is the considerable variation observed in the microbiotas due to geographical and ethnic differences. This fact clearly influences the rates of recovery of disease-associated organisms in different populations (80, 84, 85).

CLINICAL SIGNIFICANCE

Anaerobes, originating mainly from the indigenous microbiota, are detected typically in polymicrobial infections associated with mucosal surfaces close to the site where they reside. Most infections are acquired when the integrity of the colonized mucosa or lumen is breached by trauma, by underlying disease, or during surgery. Biofilm-associated infections, such as periodontitis and bacterial vaginosis, occur when certain indigenous organisms with virulent properties are enriched within subgingival or vaginal biofilms, respectively, thus causing dysbiosis of the local microbiota (80, 86). Exceptions to endogenous acquisition include acquisition from clenched-fist wounds and animal and human bite

TABLE 2 Characteristics of genera representing Gram-negative anaerobic rods frequently isolated from clinical specimens[a]

Genus	Growth in a microaerobic atmosphere	Cell morphology	Motility	Pigment production[h]	Growth in 20% bile	Susceptibility to[i]:	
						Vancomycin (5 µg)	Kanamycin (1,000 µg)
Bacteroides[b]	–	Short	–	–	+	R	R
Alistipes[c]	–	Short	–	V	V	R	R
Odoribacter[d]	–	Pleomorphic	–	–	V	R	R
Porphyromonas[e]	–	V	–	+	–	S	R
Parabacteroides	–	Short	–	–	+	R	R
Tannerella	–	Pleomorphic	–	–	–	R	S
Prevotella	–	Coccobacillary	–	V	–	R	V
Fusobacterium	–	V	–	–	V	R	S
Leptotrichia/*Sneathia*[f]	–	V	–	–	–	R	S
Dialister	V	Coccoid	–	–	–	R	S
Selenomonas	–	Curved	+	–	–	R	S
Sutterella	V	Straight	–	–	V	R	S
Bilophila	–	Straight	–	–	+	R	S
Desulfovibrio[g]	–	Curved	+	+	V	R	S
Anaerobiospirillum	–	Spiral, long	+	–	V	R	S

[a] Symbols: +, positive; −, negative; V, variable reaction.

[b] *Bacteroides sensu stricto*.

[c] *A. senegalensis* can grow microaerobically, *A. obesi* is motile, and *A. putredinis* is asaccharolytic.

[d] The susceptibility pattern of *O. laneus* is not reported.

[e] *P. catoniae* is nonpigmented, is resistant to vancomycin, and does not produce butyric acid. *P. bennonis* produces succinic acid but not butyric or propionic acid.

wounds. Gram-negative anaerobes, such as *B. fragilis*, are involved in a variety of infections, with considerable morbidity and mortality (2). Table 3 summarizes the infectious sites where Gram-negative anaerobic organisms have been frequently isolated from clinical specimens. Anaerobic bacteria can occasionally spread to adjacent tissues and the bloodstream, with serious consequences. Localized dentoalveolar infections can result in life-threatening spread of oral anaerobes along tissue spaces of the head and neck down to the mediastinum (87). In cases in which Gram-negative anaerobes gain entrance to the bloodstream and trigger a systemic inflammatory response, the result may be sepsis or infective endocarditis, with a fatal outcome. The gastrointestinal tract and the oropharynx are the most common sources for anaerobic bacteremias, with gastrointestinal surgery and underlying malignancies being the major predisposing factors (88–90). In the oral cavity, inflamed periodontal tissues offer an open portal for a myriad of oral anaerobes to the circulation via daily practices of oral hygiene and chewing food (91, 92). This calls attention to the importance of prevention of oral infections, especially in patients at increased risk for infective endocarditis.

Bacteroides and Related Genera

Among the anaerobes in clinical specimens, members of the bile-resistant *B. fragilis* group are the most commonly encountered and are more virulent and resistant to antimicrobial agents than most other anaerobes. Although other intestinal *Bacteroides* species outnumber *B. fragilis* 10- to 100-fold, *B. fragilis* has been proved to be the most frequent *Bacteroides* species found in specimens from blood, ulcers, abscesses, bronchial secretions, bone, intra-abdominal infections, inflamed appendixes, and the head (2). In the Wadsworth Anaerobe Collection database, consisting of more than 3,000 clinical specimens, *B. thetaiotaomicron* and *B. ovatus*, as well as *B. capillosus* (currently known as *Pseudoflavonifractor capillosus*), in descending order, were also detected in these specimens but less frequently. A large U.S. study series that included over 6,500 isolates of the *B. fragilis* group from clinical specimens, collected yearly between 1997 and 2007, confirmed this; *B. fragilis* was the most common (51%), followed by *B. thetaiotaomicron* (19%) and *B. ovatus* (10%) (93). Most isolates came from blood and intra-abdominal infections. In children, *B. fragilis* is the main anaerobic organism recovered from intra-abdominal infections (2, 94); for instance, it is isolated from nearly all tissue specimens of acute appendicitis (95). Around 10 to 20% of the *B. fragilis* strains are able to produce enterotoxin; these strains have been associated with diarrhea in children as well as in adults, and in addition, their proportion seems to be higher than that of nontoxigenic strains among blood culture isolates (96). The *B. fragilis* group organisms (here also including *Parabacteroides distasonis*) and other *Bacteroides* species are the most frequently isolated pathogens from bloodstream infections with involvement of anaerobes (2, 88–90, 97). In a population-based 9-year retrospective analysis on anaerobic bloodstream infections conducted in the province of Alberta, Canada, the *B. fragilis* group was found in 277 (31%) of the 904 cases (90). Comorbidities, especially colorectal cancer, formed a common risk factor for anaerobic bacteremia. Also, recoveries of novel *Bacteroides* species, *B. dorei*, *B. finegoldii*, and *B. nordii*, have been reported from blood (97, 98). In cases of infective endocarditis in which *Bacteroides* species are involved (*B. fragilis* of gastrointestinal origin is the most frequent finding in such infections), the endocarditis tends to be more serious than that caused by other anaerobes and sometimes has a fatal outcome (99, 100). The *B. fragilis* group has been recovered from samples from patients with pericarditis (101) and, often as a single isolate, from patients with septic arthritis as well as osteomyelitis (102). Although spondylodiscitis caused by anaerobes is not common, the involvement of *B. fragilis* needs to be taken into account in cases of potential bacteremia of intestinal origin (103). In peritoneal-dialysis patients, anaerobic peritonitis is a rare event; however, *B. fragilis* and *B. thetaiotaomicron* can then be isolated (104). By hematogeneous spread, the *B. fragilis* group and other *Bacteroides* organisms can reach the brain, causing abscesses

Susceptibility to[i]:	Catalase production	Indole production	Nitrate reduction	Carbohydrate fermentation	Major metabolic end product(s)[j]	Type species
Colistin (10 µg)						
R	–	V	–	+	A, SA	*B. fragilis*
R	V	V	–	+	SA	*A. putredinis*
R	V	+	–	V	A, P, SA	*O. splanchnicus*
R	V	V	–	V	A, B, P	*P. asaccharolytica*
R	V	–	–	+	A, SA	*P. distasonis*
S	V	–	–	–	A, B, IV, P, PA	*T. forsythia*
V	–	V	–	+	A, SA	*P. melaninogenica*
S	–	V	–	V	B	*F. nucleatum*
S	V	–	–	+	L	*L. buccalis*
V	–	–	–	–	A, P	*D. pneumosintes*
V	–	–	+	+	A, P	*S. sputigena*
S	–	–	V	–	SA	*S. wadsworthensis*
S	+	–	+	–	A	*B. wadsworthia*
R	V	–	V	–	A	*D. desulfuricans*
V	–	–	–	+	A, SA	*A. succiniproducens*

[f] Some strains can grow in a microaerobic atmosphere in subsequent cultures.

[g] *D. piger* is nonmotile.

[h] Pigmentation on blood agar varies from tan to black. In some cases, it may take up to 2 weeks. A rabbit laked blood (RLB) agar enhances pigment production.

[i] Special-potency antimicrobial identification disks. R, resistant; S, susceptible; V, variable reaction. A zone size of >10 mm in diameter is considered to indicate susceptibility.

[j] A, acetic acid; B, butyric acid; IV, isovaleric acid; P, propionic acid; PA, phenylacetic acid; SA, succinic acid.

(105–107). In addition, members of this group are among the predominant isolates from burn wound infections, with potential involvement in sepsis in this context (108). *Bacteroides* species can be involved in part in polymicrobial necrotizing soft tissue infections (2), and the *B. fragilis* group is, after Gram-positive anaerobic cocci, one of the most common findings of anaerobes in infected moderate-to-severe foot wounds in diabetics (109, 110). Many novel *Bacteroides* species, such as *B. massiliensis*, *B. nordii*, and *B. salyersae*, have been detected in various clinical materials (98). In cat and dog bite infections, *B. pyogenes*/*B. tectum* can be found (38, 111, 112).

Pigmented, bile-resistant *Alistipes* species, *A. finegoldii*, *A. onderdonkii*, and *A. shahii*, have been strongly connected to appendicitis, both in children and in adults (9, 95). In addition, all three species, but especially *A. onderdonkii*, have been isolated from intra-abdominal fluid, and *A. onderdonkii* has been isolated from intra-abdominal abscesses and urine (9, 97, 113). There is one report of two cases of *A. finegoldii* in blood (114); by phylogenetic reanalysis, one isolate was later identified as *A. onderdonkii* (113).

Porphyromonas and Related Genera

Pathogenic potential varies between different *Porphyromonas* species. Of the three oral *Porphyromonas* species, *P. endodontalis* and *P. gingivalis* are known significant pathogens. The detection rate of *P. gingivalis*, one of the major periodontal pathogens, increases with age (115, 116). In addition to being detected in cases of periodontitis, it has frequently been detected in oral specimens from necrotizing ulcerative gingivitis, infected root canals, peri-implant lesions, and acute apical abscesses (85, 117–119). It has been detected, besides in the oral cavity, in clinical specimens from various body sites, e.g., intra-abdominal sites (120), the vaginas of women with bacterial vaginosis (121), amniotic fluid (122), and synovial fluid of patients with rheumatoid arthritis and psoriatic arthritis (123); together with some other periodontal organisms, it has also been found in occluded arteries of the lower extremities of patients with Buerger's disease (124). *P. endodontalis* is one of the dominant organisms in infected root canals and in acute dental abscesses (85, 118, 125) but may also be involved in chronic periodontitis (126). *P. uenonis*, which is phenotypically similar to *P. endodontalis* and *P. asaccharolytica*, has been detected in polymicrobial infections below the waistline: appendicitis, peritonitis, pilonidal abscess, an infected sacral decubitus ulcer, and bacterial vaginosis (13, 127). *P. asaccharolytica* and *P. somerae* were among the anaerobic isolates from moderate-to-severe foot infections of diabetics (109). At the VA Wadsworth Medical Center laboratory, the 58 *P. somerae* isolates originated from a variety of specimens, including lower extremity skin and soft tissue or bone, in particular, inguinal or sacral area abscess, intra-abdominal abscess, transtracheal aspirate, axillary abscess, mastoiditis, blood culture, brain tissue, and infected scalp (presented in order of their frequency) (16). *P. bennonis* has been detected in human wound infections and abscesses, especially in patients with chronic skin and soft tissue lesions in the perirectal, buttock, and groin regions (17). Although *P. catoniae* inhabits the oral cavity without any disease association described to date, it has been isolated from an abdominal abscess (14). *P. gingivalis* and *Porphyromonas* species of animal origin, e.g., *P. cangingivalis*, *P. canoris*, *P. cansulci*, and *P. macacae*, have been encountered in humans with animal bite infections (112).

Parabacteroides species are common inhabitants of the human gut, and *P. distasonis* is one of the anaerobes of clinical importance in specimens from intra-abdominal infections and inflamed appendixes, where *P. goldsteinii* and *P. merdae* can also be found (2, 128). In addition, *P. distasonis*, *P. merdae*, *P. goldsteinii*, and *P. gordonii* have been isolated from human blood (2, 20, 97, 129, 130).

Tannerella forsythia is considered one of the major periodontal pathogens (115, 131). In addition, it is one of the predominant organisms in root canal infections (85, 125) and has been found in infected sites around dental implants (119). Moreover, *T. forsythia* can be recovered in substantial amounts from tonsillar crypts of adults (82). In laryngecto-

TABLE 3 Common genera of anaerobic Gram-negative rods detected in human infections

Site(s) of isolation and/or type of infection or condition[a]	Genera isolated[b]
Brain and/or CNS	*Bacteroides*, *Dialister*, *Fusobacterium*, *Porphyromonas*
Mouth	
Abscesses	*Dialister*, *Fusobacterium*, *Prevotella*, *Porphyromonas*
Endodontic infections	*Centipeda*, *Dialister*, *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Tannerella*
Periodontal diseases	*Desulfomicrobium*, *Dialister*, *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Selenomonas*, *Tannerella*
Peri-implant diseases	*Fusobacterium*, *Porphyromonas*, *Prevotella*, *Tannerella*
Spreading odontogenic infections	*Fusobacterium*, *Prevotella*
Ear, nose, and throat	*Fusobacterium*, *Prevotella*, *Porphyromonas*, *Tannerella*
Lower respiratory tract	*Bacteroides*, *Dialister*, *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Selenomonas*
Abdomen/intestine	
Abscesses	*Alistipes*, *Bacteroides*, *Bilophila*, *Fusobacterium*, *Parabacteroides*, *Porphyromonas*, *Sutterella*
Appendicitis	*Alistipes*, *Bacteroides*, *Bilophila*, *Fusobacterium*, *Parabacteroides*, *Porphyromonas*, *Sutterella*
Peritonitis	*Alistipes*, *Bacteroides*, *Desulfovibrio*, *Parabacteroides*, *Porphyromonas*, *Sutterella*
Diarrhea	*Anaerobiospirillum*, *Bacteroides*
Urogenital tract	
Abscesses	*Bacteroides*, *Prevotella*
Bacterial vaginosis	*Leptotrichia*, *Porphyromonas*, *Prevotella*, *Sneathia*, *Tannerella*
Intra-amniotic infection	*Dialister*, *Fusobacterium*, *Leptotrichia*, *Porphyromonas*, *Sneathia*
Preterm delivery	*Fusobacterium*, *Leptotrichia*, *Sneathia*
Urinary tract infection	*Bacteroides*, *Dialister*, *Leptotrichia*, *Prevotella*
Skin and/or soft tissue	
Abscesses	*Bacteroides*, *Dialister*, *Fusobacterium*, *Jonquetella*, *Porphyromonas*, *Prevotella*
Ulcer/chronic wound	*Bacteroides*, *Dialister*, *Porphyromonas*, *Prevotella*
Bite wound infection (animal)	*Bacteroides*, *Fusobacterium*, *Porphyromonas*, *Prevotella*
Bite wound infection (human)	*Dialister*, *Fusobacterium*, *Prevotella*
Bone and joints	
Arthritis	*Bacteroides*, *Porphyromonas*, *Tannerella*
Osteomyelitis	*Bacteroides*, *Fusobacterium*, *Porphyromonas*
Cardiovascular sites	
Bacteremia	*Alistipes*, *Anaerobiospirillum*, *Bacteroides*, *Bilophila*, *Desulfovibrio*, *Dialister*, *Fusobacterium*, *Leptotrichia*, *Parabacteroides*, *Porphyromonas*, *Prevotella*, *Sneathia*
Endocarditis	*Bacteroides*, *Fusobacterium*, *Leptotrichia*
Pericarditis	*Bacteroides*, *Fusobacterium*

[a] CNS, central nervous system.

[b] Presented in alphabetical order; order is not related to the frequency of isolation.

mized patients with voice rehabilitation, *T. forsythia* proved to be one of the main biofilm-forming anaerobes on voice prostheses that needed replacement (132). This oral bacterium has also been recovered from vaginal samples in women with bacterial vaginosis (121) as well as from synovial specimens of patients with rheumatoid arthritis and psoriatic arthritis (123).

Prevotella and Related Genera

Prevotella species are among the dominating microorganisms of the oral cavity, where they, despite their commensalism, can be involved in nearly all types of oral infections. Interestingly, *Prevotella melaninogenica*, which is a common anaerobic organism in saliva (69, 133), is also one of the most prevalent anaerobes, together with *F. nucleatum*, *Prevotella intermedia*, and *Prevotella buccae*, in infected human bite lesions (134). Although the cariogenic microbiota consists mainly of Gram-positive species, some proteolytic Gram-negative taxa, including *P. denticola* and *P. tannerae* (currently *Alloprevotella tannerae*), can frequently be encountered in advanced carious lesions (135). *P. intermedia* (*sensu stricto*) has been strongly linked to chronic and aggressive periodontitis (84, 116, 136), and *P. intermedia* and/or the phenotypically identical *P. nigrescens* has been detected in samples from pregnant patients and patients with gingivitis, necrotizing ulcerative gingivitis, pericoronitis, periimplantitis, root canal infections, and dentoalveolar abscesses (85, 117–120, 137, 138). Also, *P. baroniae* proved to be common in root canal infections and acute dental abscess aspirates (85, 125). In noma (*cancrum oris*), *P. intermedia* is considered a key organism (139, 140). In spreading odontogenic infections, members of the genus *Prevotella* seem to play an important role, with *P. buccae* and *P. oris* being the most prominent findings in this context (87, 141). Recently, *P. melaninogenica* and *P. histicola* were found to be significantly associated with recurrent tonsillitis in adults

(82). A new concept of the polymicrobial bacteriology of patients with cystic fibrosis has been presented, and indeed, not only aerobic *Pseudomonas aeruginosa* or *Staphylococcus aureus* but also some anaerobes, especially *Prevotella*, such as *P. melaninogenica*, *P. denticola*, *P. oris*, and *P. salivae*, have been detected as one of the persistently dominating organisms in the sputa of these patients (142). Also, *Prevotella* taxa were recovered from bronchoalveolar lavage fluid of patients with ventilator-associated pneumonia (143). Furthermore, various *Prevotella* species are found in extraoral infections and abscesses at a wide range of body sites; for instance, *P. amnii*, *P. bivia*, *P. corporis*, *P. disiens*, *P. intermedia*, *P. nigrescens*, and *P. timonensis* in the female genital tract (36, 80, 121, 136, 144); *P. buccalis* in urine (145); *P. bergensis*, *P. bivia*, and *P. melaninogenica* in infectious lesions of the skin and soft tissues, including foot lesions in diabetics (33, 109); *P. intermedia* and *P. nigrescens* from intra-abdominal and soft tissue abscesses (120); *P. intermedia* (*sensu lato*) and *P. melaninogenica* from peritonsillar and retropharyngeal abscesses (144); *P. timonensis* from breast abscesses (34); *P. intermedia* in the synovial fluid of arthritis patients (123); and *P. intermedia* and *P. nigrescens* (with some other periodontal bacteria) in occluded arteries of lower extremities of Buerger's disease patients (124). Several *Prevotella* species, such as *P. bivia*, *P. buccae*, *P. denticola*, *P. disiens*, and *P. nigrescens*, have been found among anaerobic organisms in patients with bloodstream infections (97, 120). *P. heparinolytica* is a relatively common anaerobic isolate from animal bite wounds (38, 112).

Fusobacterium and Related Genera

Clinically, the most important *Fusobacterium* species are *F. nucleatum* and *F. necrophorum* (146–149). Over the past few years, there has been an increased interest in the role of fusobacteria in human infections, especially recurrent tonsillitis, Lemierre's syndrome, and bacteremia (82, 150–154), but also in diseases not usually regarded as typical infections, such as acute appendicitis, inflammatory bowel disease, and colon cancer (155–157). Moreover, in children, fusobacterial infections of otogenic origin deserve attention due to potential invasive complications (158, 159). *F. nucleatum* is an oral species which has been divided into several subspecies with variable pathogenic potentials (146). It is a key organism in the maturation of pathogenic biofilms in periodontal pockets (160) and is considered an important pathogen in peri-implantitis, root canal infections, dentoalveolar abscesses, and spreading odontogenic infections (85, 119, 125, 141). *F. nucleatum* was found as a dominant species in tonsillar crypts (82), which may explain its dominance in biofilms on voice prostheses that needed replacement (132). It is also an important etiologic agent in extraoral infections and abscesses at a wide range of body sites, being detected from blood, brain, chest, heart, lung, liver, appendix, joint, long bone, abdomen, gut, genitourinary tract, and fetal membranes as well as infected human bite lesions (95, 97, 99, 101, 102, 105, 134, 145, 148, 150, 153–155, 157, 161–166). Bacteremia cases with the involvement of *F. nucleatum* often have underlying comorbidities (150, 153, 154). Nosocomial *F. nucleatum* bacteremia may be seen as a significant mortality predictor (154). In a tertiary-care hospital where all episodes of documented brain abscess cases between 1991 and 2000 were reviewed, in 40% of cerebral puncture specimens, only anaerobes were found; of these, *F. nucleatum* proved to be the most frequently isolated organism, found in one of three of the patients diagnosed (105). In another study, which used molecular methods for bacterial detection in 51 brain abscess pus specimens, *F. nucleatum* was among the most frequent findings, especially in patients with preceding sinusitis or dental treatment (161). The presence of *Fusobacterium* adhesin A seems to allow separation of oral fusobacteria, i.e., *F. nucleatum*, *F. periodonticum*, and *F. simiae*, from nonoral fusobacteria, including *F. gonidiaformans*, *F. mortiferum*, *F. naviforme*, *F. russii*, and *F. ulcerans* (167). Because the adhesin was present in *F. nucleatum* isolated from intrauterine infections but absent among the vaginal species *F. gonidiaformans* and *F. naviforme*, it was hypothesized that intrauterine *F. nucleatum* originates from the oral cavity rather than the vaginal tract. As a novel observation, an overabundance of *F. nucleatum* organisms was demonstrated in colorectal tumor tissue, and furthermore, this had a positive association with metastases of lymph nodes (156). Of the two *F. necrophorum* subspecies, *F. necrophorum* subsp. *funduliforme* (biovar B) is the dominating isolate in humans, while *F. necrophorum* subsp. *necrophorum* is a common pathogen in animals (147, 149). Important virulence factors, explaining the invasiveness of *F. necrophorum*, are the leukotoxin and plasminogen binding virulence factors (168). *F. necrophorum* is best known for its connection to Lemierre's syndrome (necrobacillosis), which can be considered an invasive *F. necrophorum* disease, often with pleuropulmonar involvement, in previously healthy adolescents and young adults (147, 149, 151, 152, 169). It is notable that invasive disease with *F. necrophorum* may be on the increase (147). In Denmark, the overall mortality of Lemierre's syndrome, originating mainly from oropharyngeal sites, was reported to be 9% in adolescents, and that of disseminated *F. necrophorum* infections, originating from lower parts of the body, was 26% in elderly patients with predisposing diseases (169). In the latter type of cases, underlying cancers should be considered. Interestingly, *F. necrophorum* can be found in adolescents as the causative agent in approximately 10% of the cases of tonsillitis (persistent sore throat) not caused by group A streptococci (147, 149, 170). In a Danish retrospective study including 847 patients with peritonsillar abscesses in the years 2001 to 2006, *F. necrophorum* was detected in 23% of the pus aspirate or swab specimens, in most of which the organism grew as a pure culture (171). Another Danish study of 1,930 throat swabs taken from 15- to 24-year-old patients by general practitioners between 2007 and 2009, reported the detection rate of 22% for *F. necrophorum* (172). A wide variety of infections can be caused by *F. necrophorum*: in the head and neck, tonsillitis, peritonsillar abscess, deep neck space infection, mediastinitis, otogenic infection, mastoiditis, sinusitis, and odontogenic infection; as an intracranial complication, sinus thrombosis, cerebral abscess, and meningitis; and as systemic manifestations, bacteremia, septicemia, pleuropulmonary infections, bone and joint infections, soft tissue infections, intra-abdominal sepsis, endocarditis, and pericarditis (149, 150–153, 158, 159, 171, 173). Other *Fusobacterium* taxa, such as *F. necrogenes*, *F. ulcerans*, and the *F. mortiferum*-*F. varium* group, have only occasionally been isolated from human clinical specimens (94, 109, 148, 158, 174). *F. nucleatum* and an *F. nucleatum*-like species of animal origin, *F. canifelinum*, and *F. russii* are of importance in cat and dog bite wounds (112, 175).

Commensal *Leptotrichia* species are increasingly implicated in anaerobic bacteremias in immunocompromised patients with lesions of the oral or gastrointestinal mucosa but are also occasionally considered etiologic agents of bacteremia and infective endocarditis in immunocompetent individuals (45, 88, 176–179). *L. buccalis*, *L. goodfellowii*, *L. trevisanii*, and *L. wadei* are the main *Leptotrichia* findings in blood specimens. According to a recent 5-year retrospective analysis of *Leptotrichia* organisms in a national reference laboratory in Utah, *L. trevisanii* comprised 75%, *L. goodfellowii*, *L. wadei*, and the newly described *L. hongkongensis* each comprised 9%, and *L. buccalis* comprised 1.5% of the 68 *Leptotrichia*-positive cultures (177). Interestingly, *L. hongkongensis* findings came especially from multiple myeloma patients. In an immunocompetent subject, *L. wadei* was reported as a causative organism of a severe pneumonia (180). In the female genital tract, some infectious cases, including bacterial vaginosis, intrauterine fetal demise, and septic abortion, have been reported in connection with *L. amnionii* (currently *Sneathia amnii*) (47, 80, 127, 162, 181). In fact, this organism significantly associates with all four Amsel's criteria (pH, whiff test, vaginal discharge, clue cells) used in clinical diagnostics of bacterial vaginosis (80). Furthermore, a few bacteremia cases in connection to delivery (182) and one neonatal meningitis case (183) have been reported. However, it has also been isolated from a knee joint specimen of a male patient (184), revealing that the organism is not restricted to the female genital tract. Based on the high number of *L. amnionii* organisms in urine samples from renal transplant recipients, it may also be considered one of the etiologic agents of urinary tract infections (145). One case of septic arthritis with an involvement of a *Sneathia* organism most closely related to *Sneathia sanguinegens* has been reported in an immunocompetent woman (185). Although *S. sanguinegens* was originally isolated from blood (186), it has been reported mainly from various infectious conditions of the female genital tract, such as preterm labor, neonatal sepsis, postpartum bacteremia, and pyosalpinx, and in amniotic fluid associated with various clinical syndromes (127, 162, 166, 182, 186). In a recent study using metagenomic 16S rRNA gene identification for *Sneathia* in midvaginal samples from 736 women in an urban outpatient clinic in Virginia, the target organism was detected in 30 to 43% of the samples, depending on the selected abundance treshold (48). It was also shown that *S. amnii* is able to adhere to the cervical epithelium and, presumably, to invade the uterine cavity. *Leptotrichia*/*Sneathia* species can be considered clinically relevant intra-amniotic pathogens (162, 163), and in fact, their role in preterm labor may be currently underestimated.

Other Gram-Negative Anaerobic Rods

In the novel phylum *Synergistetes*, there are three cultivable species representing three genera, i.e., *Fretibacterium fastidiosum*, *Jonquetella anthropi*, and *Pyramidobacter piscolens*, which have been isolated from human clinical specimens. *J. anthropi* isolates have been found in breast abscess, pelvic abscess, sebaceous cyst, wound, and peritoneal fluid specimens (50), while infectious *F. fastidiosum* and *P. piscolens* have been found in patients with periodontitis, dental root canals, and odontogenic abscesses (49, 51, 85).

Two *Dialister* species, *D. pneumosintes* and *D. invisus*, are inhabitants of the oral cavity, where they have been implicated as pathogens in endodontal and periodontal infections as well as acute dental abscesses (55, 85, 187–189). In addition, they have been recovered from infectious specimens from blood, brain abscesses, urine, human bite wounds, and bronchoalveolar lavage fluid from patients with ventilator-associated pneumonia and cystic fibrosis (91, 134, 142, 143, 145, 190, 191). A considerable part of *D. pneumosintes* isolates originate from various cutaneous/soft tissue infections (190). It may be that the patient's poor dental hygiene is a predisposing factor in certain nonoral cases, e.g., bacteremia and brain abscess (143, 191). *D. micraerophilus*, which is not a strict anaerobe, and *D. propionicifaciens* have been isolated from a variety of clinical specimens, mainly below the waistline (56, 80, 190).

Selenomonas species and *Centipeda periodontii* are motile organisms recovered from the oral cavity, where they are components of dental biofilms (69, 192). *Selenomonas sputigena* has been found in specimens from necrotizing ulcerative gingivitis, generalized aggressive periodontitis, and acute dental abscesses (84, 85, 117), *Selenomonas noxia* has been reported in chronic and aggressive periodontitis lesions (84, 193), and *Centipeda periodontii* has been recovered from endodontic infections (194). Furthermore, there have been a few reports on the involvement of *Selenomonas* in nonoral infections, including cystic fibrosis and bacteremia (142, 195, 196).

Sutterella wadsworthensis, a microaerobic organism phenotypically but not phylogenetically close to fastidious *Campylobacter* species, has been isolated from a variety of infections, such as appendicitis, peritonitis, and rectal or perirectal abscesses (197, 198). Recently, *Sutterella* was suggested to play a dominant role in gastrointestinal disturbances in children with autism after these organisms were detected in ileal mucosal biopsy specimens but not in those from control children with gastrointestinal symptoms (199).

Bilophila wadsworthia is a significant human pathogen that is being isolated from polymicrobial intra-abdominal infections, especially appendicitis, at an increasing frequency, with deteriorating disease status (62). *B. wadsworthia* is even more common in gangrenous appendixes of children than in those of adults (95). Moreover, *B. wadsworthia* has been isolated from abscesses at various body sites as well as from blood (62, 200).

Some *Anaerobiospirillum* and *Desulfovibrio* organisms reside in the gastrointestinal tracts of humans but can also be infrequently encountered in clinical specimens; typical cases are bacteremia and abdominal infections in immunocompromised patients (63, 196, 201–203). Of the two *Anaerobiospirillum* species, *A. succiniciproducens* has been connected to bacteremia and diarrheal illness, whereas *A. thomasii* is considered a potential cause of diarrhea but not of bacteremia (65, 201). In most cases, *A. succiniciproducens* bloodstream infections have an underlying condition, and patients often suffer from gastrointestinal symptoms (201). In the case of diarrhea, the possibility of zoonotic transmission exists (202). The main *Desulfovibrio* species isolated from human infections include *D. desulfuricans*, "*D. fairfieldensis*," *D. piger*, and *D. vulgaris* (63, 196, 204–206). *D. desulfuricans* is also common in the environment, whereas *D. fairfieldensis* and *D. piger* have been detected only in humans so far. The first human findings of *D. intestinalis* came from vaginal specimens of four Japanese women (207). *D. fairfieldensis*, a taxon never validly described, has been isolated from blood, in particular, but also from various intra-abdominal sites, urine, and periodontal pockets (205). *D. desulfuricans* bacteremia cases have also been reported (208, 209). In addition to *D. fairfieldensis*, an oral *Desulfomicrobium* species, *D. orale*, has been connected to periodontitis (64). On the basis of differences in pyrosequencing results of stools collected from autistic children with gastrointestinal symptoms and their sibling and nonsibling controls, *Desulfovibrio* was recently suggested as being linked to regressive autism (210).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

General guidelines for the collection, transport, and storage of specimens are discussed in chapters 11 and 18 of this *Manual*. Specimens suitable for the isolation of anaerobes include aseptically taken blood, tissue biopsy specimens, aspirates (e.g., cerebrospinal fluid, joint fluids, and pus), dental root canal exudates, and subgingival plaque. Appropriate respiratory tract specimens include bronchoscopic, protected bronchoalveolar lavage fluid, and expectorated sputum samples may be collected from patients with cystic fibrosis (211). Also, wound and ulcer specimens should preferably be taken by tissue biopsy, wound curettage, or aspiration. For example, for diabetic foot ulcers, the lesion is cleaned and carefully debrided, and tissue samples are collected from the base or progressive edge, where bacteria actively multiply (109). Infected tissue, obtained by excision or biopsy, is always preferable to pus as a clinical specimen. However, when pus is collected, it is best aspirated into a syringe through a needle and injected into an anaerobic transport vial containing an oxidation-reduction indicator (e.g., products from Anaerobe Systems, Morgan Hill, CA, and Becton, Dickinson, Sparks, MD). It is notable that syringes used for aspiration should not be used as transporters because of the potential danger of needle stick injuries or accidental expulsion and because oxygen diffuses through plastic syringes (68). Mucosal or cutaneous swabs are not recommended. In cases where bacteremia is suspected, a 20-ml volume of blood (at least two separate samples) is recommended for cultures. Anaerobic culture is important, especially in patients with complex underlying diseases and when the source of bacteremia is unknown (89, 130). Also, blood collected from patients with abdominal or gynecological processes, peritoneal abscess, dirty wounds, decubitus ulcers, osteomyelitis, or spreading oropharyngeal disease should be examined for anaerobes.

Specimens must be transported to the laboratory under anaerobic conditions (e.g., in Anaerobe Systems PRAS anaerobic transport medium [ATM] AS-911, in Becton, Dickinson BBL Port-A-Cul transport medium or a BBL Vacutainer anaerobic specimen collector [the last is medium free in order to minimize sample dilution], or in AnaeroGRO [Hardy Diagnostics, Santa Maria, CA]) without delay for further processing. An optimal transport system is able to maintain the viability of anaerobic organisms without allowing the overgrowth of aerobic bacteria. However, if clinical specimens contain fastidious organisms, the transport to the clinical laboratory should occur within 24 h (212). Tissue samples are best transported in specific anaerobic transport vials or in loosely capped containers sealed in gas-impermeable bags in which an anaerobic atmosphere has been generated. For small tissue and biopsy specimens and for subgingival and dental root canal samples, a semisolid anaerobic transport medium (e.g., those of Anaerobe Systems, Becton, Dickinson, and Hardy Diagnostics), in which the specimen can be submerged, can be used.

Further guidance for the collection of specimens from different body sites and by various methods as well as transport systems and anaerobic techniques can be found in more detail elsewhere (68).

For long-term storage, 2- to 3-day-old cultures can be transferred into vials containing sterilized 20% skim milk and kept frozen at −70°C.

DIRECT EXAMINATION

The gross appearance, fluorescence under long-wave UV light, and odor of the specimen can give the laboratory valuable clues as to the presence of anaerobes. A fetid or putrid odor due to volatile short-chain fatty acids and amines is always associated with the presence of anaerobes in the sample. Black necrotic tissue and/or red fluorescence of the sample may be indicative of the presence of pigmented Gram-negative rods (68).

The Gram stain is still the fastest, simplest, and most likely to yield significant information and should be prepared from all specimens accepted for anaerobic culture. Many Gram-negative anaerobic rods, e.g., different species within the genus *Fusobacterium*, have unique cell morphology (see Table 7). For instance, a Gram-stained smear with highly pleomorphic Gram-negative rods in a positive blood culture bottle from a septic patient following a sore throat may indicate invasive *F. necrophorum* infection (147). The morphotypes and relative quantities of the bacteria and host inflammatory cells present in the preparation should be reported. Gram staining using the Nugent criteria for interpretation of vaginal discharge is still considered the best method for diagnosis of bacterial vaginosis (213).

Molecular Detection

Molecular methods are increasingly used for direct detection of bacteria from clinical specimens or for confirmation of identity. As yet, they are used mainly in specialized oral and research microbiology laboratories, but their use also for routine diagnostics is expanding.

For the detection of fastidious organisms and potential pathogens, almost-complete or partial sequencing (the V3-V6 regions are most informative) of the 16S rRNA gene has been successfully used not only for bacterial identification in typical polymicrobial lesions, such as periodontitis, endodontic infections, and spreading odontogenic infections (52, 83, 126, 141, 188), but also in infections where the involvement of anaerobic bacteria has not traditionally been taken into account, e.g., cystic fibrosis (142). It is notable that a culture-based approach can underestimate the presence of etiologic but fastidious or uncultivable organisms in clinical specimens. For example, *Prevotella* species are highly prevalent in the lungs of cystic fibrosis patients (142, 211), but routine culture of sputum does not include anaerobes and potential anaerobic pathogens have not been reported. Furthermore, in women who experienced preterm labor and whose amniotic fluid tested positive by culture or PCR, seven species, including *Sneathia sanguinegens* and *Leptotrichia amnionii* (currently *Sneathia amnii*), were detected by PCR only (162). In addition, in a study of urinary tract specimens from renal transplant recipients (145), the 16S rRNA PCR method detected a wide range of bacteria, including Gram-negative anaerobes, such as *Bacteroides vulgatus*, *Dialister invisus*, *Fusobacterium nucleatum*, *L. amnionii*, *Prevotella buccalis*, and *Prevotella ruminicola*, in culture-negative samples. A recent study that subjected pus specimens from 51 brain abscesses to 16S rRNA gene amplification, cloning, and sequencing revealed 19 cases of polymicrobial infections with 76 different species, including 22 uncultured taxa, among them 10 *Bacteroides* phylotypes (161). Pyrosequencing studies by the same research group have demonstrated that the species diversity in such polymicrobial infections is much richer than previously assumed. With decreasing prices and new methods, e.g., bar code labeling and V6 tagging, next-generation sequencing will expand the power to investigate health- and disease-associated mixed anaerobic microbial communities or outbreak histories (214–216). Challenges associated with molecular techniques include differences in lysis efficiency depending on the bacterial cell wall composition, the presence of human competitor DNA in clinical samples, inaccurate primer discrimination because of mismatches or limited accessibility to the template site, the persistence of bacterial DNA after successful antimicrobial killing, an inability to test for antimicrobial susceptibility, and different interpretations of complex results and clinical consequences (217, 218).

ISOLATION PROCEDURES

Except for blood and joint fluid cultures, the use of liquid medium as the only anaerobic culture technique is not acceptable (68). The use of solid nonselective medium together with selective medium increases the yield and saves time in terms of recognition and isolation of colonies. The selective media are chosen based on the expected microbiota at the collection site or, in the case of bite wounds, on the oral microbiota of the biter (human or animal). Freshly prepared or prereduced and anaerobically sterilized medium should be used (68). Different basal media differ in their abilities to support the growth of anaerobes; brucella base and fastidious anaerobe agar (Lab M, Bury, United Kingdom) may be the best basal media for the isolation of Gram-negative anaerobic rods. In particular, fastidious-anaerobe agar enhances the growth of fusobacteria (147). In academic centers performing large-scale anaerobic bacteriology, it would be ideal to use two different basal media to maximize isolation efficiency.

Culture methods are found in chapters 18 and 19 of this *Manual*. The minimum medium setup for isolating Gram-negative anaerobic rods includes (i) a nonselective, enriched, brucella base sheep blood agar plate supplemented with vitamin K_1 and hemin (BA); (ii) a kanamycin-vancomycin laked sheep blood agar plate for the selection of *Bacteroides* and *Prevotella* species; and (iii) a *Bacteroides* bile-esculin (BBE) agar plate for specimens from areas below the diaphragm for the selection and presumptive identification of the *B. fragilis* group and *Bilophila* species. BBE and kanamycin-vancomycin laked sheep blood are also available as biplates. A phenylethyl alcohol sheep blood agar plate used to prevent overgrowth by aerobic Gram-negative rods and swarming of some clostridia is indicated for purulent specimens and in the case of mixed infections. Use of a metronidazole disk (5 μg) on nonselective agar is useful for the detection of Gram-negative obligate anaerobes; however, it may mask the presence of infrequently encountered metronidazole-resistant organisms.

After inoculation, the anaerobic plates are immediately incubated at 36 to 37°C in an anaerobic environment, such as an anaerobic bag, jar, or chamber. Alternatively, setup and incubation may all be done in an anaerobic chamber. Plates should not be exposed to air during the first 48 h to avoid loss of the more oxygen-sensitive species. The availability of an anaerobic chamber enables the examination of the culture whenever necessary. An incubation period of 48 h will reveal the presence of rapidly growing strains, such as *Bacteroides* or clostridia, but reincubation for 5 to 7 days for primary plates is recommended, since some species, such as *Bilophila*, *Desulfovibrio*, and *Porphyromonas*, may not be detected with shorter incubation times and require at least 4 to 5 days for growth (62, 63, 68). For successful culture and susceptibility testing of anaerobes, a strictly anaerobic atmosphere needs to be maintained inside the chamber, jar, or bag. Chambers especially may fail to keep a proper gaseous condition when kept in busy use, and therefore, repeated quality controls should be performed; e.g., with a simple method of growing an aerotolerant *Clostridium perfringens* strain with a metronidazole (5 μg) disk, an inhibition zone of >27 mm indicates acceptable anaerobiosis (219).

Increased awareness of the importance of anaerobic organisms as a cause of systemic infections may contribute to the increase of their isolation and detection in blood samples in clinical microbiology laboratories. For instance, to reliably detect anaerobic organisms, the LYTIC 10 anaerobic/F BACTEC medium (Becton, Dickinson) has been shown to be a rapid and reliable method, improving the detection of low levels of anaerobic bacteria, such as *Prevotella* and *Fusobacterium*, in the sample (91).

IDENTIFICATION

After anaerobic incubation, the relative quantities of distinct colony types are recorded. Plates should be examined with a dissecting microscope to facilitate detection. Even after incubation for 7 days, certain species, such as *Desulfovibrio* and *Dialister*, grow as transparent colonies that are pinpoint in size and are easily overlooked in mixed cultures (56, 63, 204, 205). The isolates should then be subcultured onto BA, and at this point, a rabbit laked blood agar plate for the rapid demonstration of pigment production and an egg yolk agar plate for the demonstration of lipase, lecithinase, and proteolytic activities may also be inoculated. The primary plates are reincubated along with the purity and test plates.

Presumptive Identification

Colony morphology of an isolate in pure culture can be useful for presumptive identification. For example, *F. nucleatum* can appear on the plate as speckled, iridescent, or bread crumb-like colonies, while *B. wadsworthia* typically has black-centered colonies on BBE (68). Colony morphology, together with the capability of erythrocyte agglutination, is among the features that can be used to separate the two *F. necrophorum* subspecies by phenotypic testing (220). The agglutination procedure, using human and chicken erythrocytes, is performed by a glass slide method in which agglutination is observed by mixing a drop of bacterial suspension and a drop of erythrocyte suspension on a microscope slide. *F. necrophorum* subsp. *funduliforme* (agglutination-negative) colonies are pulvinate, creamy, and glistening, with entire margins, whereas *F. necrophorum* subsp. *necrophorum* (agglutination-positive) colonies are convex or umbonate, waxy, and dull, with erose margins. Also, observation of hemolysis may be of diagnostic value; for instance, both *F. necrophorum* subspecies exhibit beta-hemolysis when grown on horse blood agar (220). Hemolytic properties on human blood may aid in the separation of different *Leptotrichia* species (44). Production of pigment is another visible characteristic valuable in presumptive identification; pigmented Gram-negative anaerobic rods are saccharolytic and asaccharolytic species of the genera *Prevotella* and *Porphyromonas* and the pigment-producing *Alistipes* species *A. finegoldii*, *A. onderdonkii*, *A. shahii*, and *A. timonensis*. In this context, it is notable that the statement "after 4 days incubation on laked rabbit blood agar, colonies appear black" in the original description of *A. onderdonkii* and *A. shahii* (9) is incorrect. Instead, the grade of pigmenting is light or moderately brown on rabbit laked blood agar and, under long-wavelength (365-nm) UV light, colonies appear black. Appearance of pigment requires fresh medium and expanded incubation (113). As a novel observation, a diffusible brown pigment on BBE can be useful in the presumptive identification of *Alistipes* species (113). The pigmented *Prevotella* and *Porphyromonas* species vary greatly in degree and rapidity of pigment production (2 to 21 days), which ranges from buff to tan to black, depending primarily on the type of blood and the composition of the base medium used in the agar (68). Fluorescence under long-wavelength UV light can be helpful in presumptive identification; pigmented *Prevotella* and *Porphyromonas* colonies typically fluoresce red, *F. nucleatum* and *F. necrophorum* fluoresce yellow-green, and *Desulfovibrio* and *Bilophila* species, when tested with a drop of 2 N NaOH on a swab of cell paste, fluoresce red due to the presence of desulfoviridin pigment (68, 206, 220).

Microscopic determination of the morphology of Gram-stained bacterial cells can aid in the presumptive identification of the organisms present. Among fusiforms, *F. nucleatum* usually exhibits long, spindle-shaped cells with tapered ends, while *F. necrophorum* and *F. mortiferum* have highly pleomorphic cells, with or without swollen areas and large round bodies (8, 147, 220). *Leptotrichia* cells, which often stain Gram positive in fresh cultures, have usually been considered long rods; however, this description fits only *L. buccalis*, *L. hofstadii*, *L. shahii*, and *L. trevisanii* (44, 179). *Dialister* species are small coccobacilli, making their separation from Gram-negative cocci difficult (56). *Desulfovibrio piger* typically stains in a bipolar manner (206). Wet slide preparations for microscopic examination reveal the motility of Gram-negative anaerobes; *Selenomonas* displays a characteristic tumbling motility, often moving laterally across the field, *Anaerobiospirillum* cells are spiral with corkscrew-like motility, and *Desulfovibrio* species, except for *D. piger*, appear as curved rods with rapid, progressive motility (63, 65, 221).

The *B. fragilis* group, in particular, and most *Bacteroides* species are typically bile resistant (2, 68). Pigment-producing *Alistipes* can readily be distinguished from pigmented *Porphyromonas* and *Prevotella* species by their resistance to 20% bile (9, 11). Among fusobacteria, *F. mortiferum*, *F. varium*, and some strains of *F. necrophorum* grow in the presence of bile, whereas *F. nucleatum* does not. The profile of susceptibility to special-potency antimicrobial disks (a zone size of ≥10 mm is considered susceptible) containing vancomycin (5 µg), kanamycin (1,000 µg), and colistin (10 µg) is useful in the presumptive identification of many Gram-negative anaerobic taxa (Table 2). Gram-negative anaerobic rods are typically resistant to vancomycin, with pigmented *Porphyromonas* species and *S. amnii* being the only exceptions (13, 16, 17, 48, 68). Susceptibility to both kanamycin and colistin is characteristic of *Fusobacterium* and *Leptotrichia* species and *S. wadsworthensis*. To differentiate members of the genera *Dialister* and *Veillonella*, special-potency disks can be helpful; *Dialister* species are resistant to colistin, whereas *Veillonella* species are usually susceptible, except for *V. montpellierensis* and *V. ratti* (56). Among motile Gram-negative organisms, *Anaerobiospirillum* is usually susceptible to colistin, unlike most *Desulfovibrio* and *Selenomonas* isolates.

In addition to tests used to determine the characteristics listed above, there are some simple tests that are within the scope of most clinical laboratories. For example, a bile-resistant organism with typical darkening of the center of colonies on BBE can easily be recognized as *B. wadsworthia* by its strong catalase reaction with 10 to 15% H_2O_2 (62), and by combining positive indole and lipase reactions, a *Fusobacterium*-like organism can be tentatively identified as *F. necrophorum* (147). An indole- and lipase-positive short rod that forms black-pigmented colonies and fluoresces red can be identified as *P. intermedia*/*P. nigrescens*, while *Prevotella pallens* resembles these indole-positive species but has a lighter pigment and is lipase negative (68). *Bilophila* and *Sutterella* typically reduce nitrate. A characteristic smell may guide the identification; a foul smell produced by butyric acid and other metabolic products is typical for *Fusobacterium* species, while a strong sulfur smell is typical for the presence of *Desulfovibrio* species (63, 206).

Biochemical Testing

In culture-based biochemical testing, the main techniques for classification of anaerobic organisms and distinction of individual species include sugar fermentation reactions, the use of prereduced, anaerobically sterilized (PRAS) carbohydrates or commercial test kits, and the determination of enzyme profiles with individual diagnostic tablets or preformed enzyme kits. In addition, the determination of major volatile fatty acid end products of glucose metabolism, as detected by gas liquid chromatography (GLC), is a useful adjunct to biochemical and physiological tests (68), but together with analysis of the long-chain fatty acids found in bacterial cell walls, it is beyond the scope of most clinical laboratories.

Commercially available test kits, such as the API 20A, API ZYM, and Rapid ID 32A (bioMérieux, Marcy-l'Etoile, France), RapID ANA II (Remel, Lenexa, KS), BBL crystal identification (Becton, Dickinson), and AN microplate (Biolog Inc., Hayward, CA) systems, are used for testing preformed enzyme and carbohydrate fermentation profiles in clinical microbiology laboratories (222). Diagnostic tablets (e.g., from Rosco, Taastrup, Denmark, and Key Scientific, Stamford, TX) are also useful for determining individual enzyme reactions of anaerobic isolates. A heavy inoculum from 2- to 3-day-old cultures should be used for testing to obtain optimal reactions. When different test systems are used, variation of test results is expected due to differences in the substrate specificities. These rapid, easy-to-use systems are best suited for fast-growing and biochemically reactive anaerobes, such as the *B. fragilis* group of organisms, but even then, it may not be possible to reliably identify the isolate to the species level. The Vitek 2ANC card (bioMérieux) is a new automated system for rapid identification of anaerobic bacteria from clinical specimens (223, 224). According to the results of a clinical trial performed in three large tertiary-care centers (224), the Vitek 2ANC card (bioMérieux) proved to be acceptable for routine use in laboratories; however, the system incorrectly identified a considerable number of clinically relevant species, such as *F. necrophorum*, *P. intermedia*, and *P. melaninogenica*, and did not include clinical isolates that were not in the system's rather-limited database. However, skillful reading of Gram stain preparations considerably improves the percentage of correct identifications (223).

The most commonly encountered bile-resistant organisms in clinical specimens belong to the *B. fragilis* group of organisms. They grow as gray, circular, convex, and entire colonies on BA and, in addition, grow well on BBE, where they (except for *B. vulgatus*) blacken the agar by hydrolyzing esculin. Based on their resistance to special-potency antibiotic disks (vancomycin, kanamycin, and colistin) and a few rapid tests, such as the catalase, indole, esculin, and α-fucosidase tests, they can initially be reported as the *B. fragilis* group or as organisms most closely related to this group (68). The genus *Parabacteroides* includes the former *Bacteroides distasonis* and *Bacteroides merdae* (18), and *Bacterioides splanchnicus* has been moved to the genus *Odoribacter* (225). In addition, clinically relevant species include *B. massiliensis*, *B. nordii*, *B. salyersiae*, *P. goldsteinii*, and *P. gordonii* (20, 128, 226, 227). All *Parabacteroides* species are negative for indole and α-fucosidase, which distin-

TABLE 4 Identification scheme for *Bacteroides fragilis* group, *Parabacteroides*, and *Odoribacter* organisms isolated from humans[a]

Species	Production of[b]:			Fermentation of[c]:		
	Ind	Cat	α-Fuc	Arab	Tre	Xyl
B. caccae	−	−	+	+	+	+
B. cellulosilyticus	ND	−	W	W	−	+
B. clarus[d]	+	−	−	−	W	+
B. coprocola	−	−	+	−	−	+
B. coprophilus	−	ND	+	−	ND	−
B. dorei	−	ND	+	+	−	+
B. eggerthii	+	−	−	+	−	+
B. faecis	+	−	+	+	−	+
B. finegoldii	−	ND	−	+	−	+
B. fluxus[d]	+	−	+	+	+	+
B. fragilis	−	+	+	−	−	+
B. intestinalis	+	ND	+	+	−	+
B. massiliensis	−	−	+	−	−	−
B. nordii	+	−	−	−	−	+
B. oleiciplenus[d]	+	+	−	+	+	+
B. ovatus	+	+	+	+	+	+
B. plebeius	−	−	+	+	−	+
B. salyersiae	+	−	−	+	−	+
B. stercoris	+	V	V	−	−	+
B. thetaiotaomicron	+	+	+	+	+	+
B. uniformis	+	−	+	+	−	+
B. vulgatus	−	−	+	+	−	+
B. xylanisolvens	−	−	+	+	+	+
O. laneus[d]	+	−	+	−	−	−
O. splanchnicus	+	V	+	+	−	−
P. distasonis	−	+	−	−	+	+
P. goldsteinii	−	V	−	−	+	+
P. gordonii	−	V	−	+	−	+
P. johnsonii	−	+	−	+	+	+
P. merdae	−	−	−	−	+	+

[a] +, positive; −, negative; V, variable; W, weak reaction; ND, no data.
[b] Cat, catalase; Ind, indole; α-Fuc, α-fucosidase.
[c] Arab, arabinose; Tre, trehalose; Xyl, xylose.
[d] Reactions are based on testing of a single strain.

guishes them from the *B. fragilis* group organisms. *P. goldsteinii* is phylogenetically and phenotypically very similar to *P. merdae*; however, *P. goldsteinii* is positive for α-glucosidase and β-glucosidase (Rosco), whereas *P. merdae* is not (128). Furthermore, its positive β-glucuronidase reaction separates it from *Parabacteroides distasonis*. Unlike other *Parabacteroides* species, *P. gordonii* does not hydrolyze esculin and does not ferment trehalose (20). Most of the *B. fragilis* group and related organisms are highly fermentative. Table 4 presents the key characteristics for distinguishing the *B. fragilis* group organisms, including *Parabacteroides* species and *O. splanchnicus*.

In general, most *Alistipes* species can be separated from *Bacteroides* by their pigment production; however, culture on fresh rabbit laked blood agar and prolonged incubation are then requested (9, 113). Exceptions are the type species of the genus, *A. putredinis*, and the newly described species *A. senegalensis* and *A. obesi*; not only are *A. senegalensis* and *A. obesi* nonpigmented, but *A. putredinis* is also bile sensitive and asaccharolytic (8) and *A. obesi* is motile (10), while *A. senegalensis* is able to grow microaerobically, being resistant to metronidazole (12). *A. finegoldii*, *A. onderdonkii*, and *A. shahii* strains grow well on solid media, where colonies appear to be beta-hemolytic, but hardly in liquid media, with or without supplements (9, 113). They are variably saccharolytic due to their poor growth in liquid media and are bile resistant and catalase negative. Two enzyme reactions may be able to separate pigmented *Alistipes* species; *A. finegoldii* is positive for α-fucosidase but negative for β-glucosidase, whereas *A. onderdonkii* is negative and *A. shahii* is positive for both enzymes (9).

Tannerella forsythia is a fastidious oral pathogen, and human strains require exogenous *N*-acetylmuramic acid for growth in pure cultures (21, 131). Key characteristics of *T. forsythia* are its sensitivity to bile and positive trypsin and esculin reactions. Notably, strains from animal bite wound infections are positive for catalase and indole (228).

Porphyromonas species, except for *P. catoniae*, produce pigment. The identification of the closely related and phenotypically similar *P. asaccharolytica*, *P. endodontalis*, and *P. uenonis* is difficult due to their slow pigment production and inactivity in biochemical testing. Testing with prereduced, anaerobically sterilized carbohydrates is not helpful in distinguishing these species, but glyoxylic acid and glycerol in the AN microplate system (Biolog Inc.) enable their separation; *P. asaccharolytica* is positive for glyoxylic acid and *P. uenonis* is positive for glycerol (13). *P. asaccharolytica* is also positive for α-fucosidase, while the other two species are negative. In addition, the differences in the degrees of pigmentation may be helpful, with *P. uenonis* being the most and *P. endodontalis* the least pigmenting (13). Positive indole, *N*-acetyl-β-glucosaminidase, and trypsin reactions comprise a typical pattern for *P. gingivalis* (68). Unlike most *Porphyromonas* species, *P. bennonis*, *P. somerae*, and *P. catoniae* are indole negative, and the last two species are also weakly saccharolytic (14, 16, 17), which may lead to their misidentification as *Prevotella* species in clinical laboratories. However, the sensitivity to vancomycin and a satellite-like growth pattern (i.e., larger colonies surrounded by smaller colonies), together with negative indole and α-fucosidase but positive *N*-acetyl-β-glucosaminidase reactions, identifies the isolate as *P. somerae* (16). Also, *P. catoniae* isolates grow as satellite-like colonies but are nonpigmented and resistant to vancomycin but susceptible to sodium polyanethol sulfonate in the special-potency antimicrobial disk test; they also produce major amounts of propionic acid, as shown by GLC (14). *P. bennonis* is a slow-growing organism with very slight pigmentation after extended incubation of at least 10 days and has a variable susceptibility to vancomycin (17). Unlike other *Porphyromonas* species, it produces major amounts of acetic and succinic acids by GLC, and some strains are catalase positive. Most *Porphyromonas* species of animal origin have been differentiated from the human strains by a positive catalase reaction (68). Table 5 presents the key reactions for distinguishing *Porphyromonas* species.

A small number of *Prevotella* species produce indole: *P. aurantiaca*, *P. intermedia*, *P. nigrescens*, *P. pallens*, *P. micans*, and *P. massiliensis* (28, 30, 68, 229). Except for *P. massiliensis*, these species are pigment producers, as are *P. corporis*, *P. denticola*, *P. histicola*, *P. loescheii*, *P. melaninogenica*, *P. shahii*, and the novel *P. fusca*, *P. scopos*, and *P. jejuni* (26, 27, 35, 68). *P. fusca*, *P. scopos*, and some strains of *P. histicola* have a bull's-eye appearance due to pigmentation of the colony center (26, 27). Noteworthy is that pigment production, as well its degree, on BA may take up to 14 days and vary from tan to brown to black depending on the species. Colonies of the novel *P. jejuni*, isolated from a small intestine biopsy specimen, are surrounded by various degrees of hemolysis (35). Detection of lipase on egg yolk agar may be useful; *P. intermedia* and *P. nigrescens* and many *P. loescheii* strains are positive. Notably, the separation of *P. intermedia* from *P. nigrescens* is not possible by phenotypic methods. Besides being able to produce pigment and indole, *Prevotella* organisms are characterized by their ability to ferment a variety of sugars. *P. massiliensis* is an exception, being very unreactive; however, the description of the species is based on one strain (229). *Prevotella* species produce acetic and succinic acids as their major metabolic end products of glucose fermentation by GLC; however, *P. marshii*, unusually, also produces propionic acid (230). Due to the high number of *Prevotella* species which have been described based on 16S rRNA gene sequencing, their precise identification by biochemical tests alone is challenging. Table 6 lists reactions that may be of value in the identification of *Prevotella* species and related genera.

Fusobacteria can be presumptively identified by a limited number of simple laboratory tests, including cell morphology with Gram stain, growth in 20% bile, and indole production, but their definitive identification to the species level may require additional biochemical testing (Table 7). Using GLC for volatile fatty acid determination, the detection of propionate produced from lactate and/or threonine has been used to separate different *Fusobacterium* species (68). *F. nucleatum*, appearing as white, speckled, or bread crumb-like colonies, is known for its wide heterogeneity. It includes five subspecies, *F. nucleatum* subsp. *animalis*, *F. nucleatum* subsp. *fusiforme*, *F. nucleatum* subsp. *nucleatum*, *F. nucleatum* subsp. *polymorphum*, and *F. nucleatum* subsp. *vincentii*, but phenotypic tests are not able to identify the organism to the subspecies level. Also, *F. periodonticum* is indistinguishable from *F. nucleatum* by phenotypic methods. In the case of increasingly clinically important *F. necrophorum*, there are two subspecies, *F. necrophorum* subsp. *funduliforme*, isolated mainly from human infections, and *F. necrophorum* subsp. *necrophorum*, isolated usually from animal infections (147, 149, 220). Both subspecies are indole and lipase positive, but colony morphology and a few phenotypic tests, including erythrocyte agglutination, are able to distinguish them (220). A selective agar for *F. necrophorum*, containing nalidixin and vancomycin as well as defibrinated horse blood without heat treatment, that allows the direct detection of beta-hemolysis was found to be nearly as reliable as real-time PCR in detecting the organism at medium and high concentrations in throat swab samples after a 2-day incubation (231). An interesting point of view economically is the concept of culturing throat swabs from adolescents for *F. necrophorum*, followed by antimicrobial treatment when indicated, to reduce preventable invasive complications (172). An indole-negative pleomorphic organism which grows on BBE may presumptively be identified as *F. mortiferum* (68). *F. ulcerans* closely resembles indole-negative *F. varium* strains but reduces nitrate. All fusobacteria produce major amounts of butyric acid as their metabolic end product (Table 2), and in addition, *F. naviforme*, *F. russii*, and *F. varium* produce lactic acid, as detected by GLC (68).

Although *Leptotrichia* species are anaerobic upon first isolation, in subcultures, some strains can grow aerobically in the presence of CO_2 (45). These organisms have been known as large fusiforms; however, their cell morphologies vary between the species. *L. buccalis*, *L. hofstadii*, *L. shahii*, and *L. trevisanii* are long rods, and *L. goodfellowii* and *L. wadei* are relatively short rods, while *L. amnionii* has been seen as pleomorphic coccobacilli and fusiforms and *S. sanguinegens* as pleomorphic rods with some filaments (44, 47,

TABLE 5 Some phenotypic characteristics of *Porphyromonas* species of human origin[a]

Species	Production of:							Sugar fermentation
	Pigm	Ind	Cat	α-Fuc	β-NAG	Trypsin	Chymotrypsin	
P. asaccharolytica	+	+	−	+	−	−	−	−
P. bennonis	W	−	V	−	+	−	+	−
P. catoniae	−	−	−	+	+	V	+	W
P. endodontalis	+	+	−	−	−	−	−	−
P. gingivalis	+	+	−	−	+	+	+	−
P. somerae	+	−	−	−	+	−	+	W
P. uenonis	+	+	−	−	−	−	−	−

[a] +, positive; −, negative; V, variable; W, weak reaction; Pigm, pigment; Ind, indole; Cat, catalase; α-Fuc, α-fucosidase; β-NAG, *N*-acetyl-β-glucosaminidase.

TABLE 6 Biochemical identification scheme for *Prevotella* species[a]

Species	Production of[b]:					Hydrolysis of[c]:		Fermentation of[d]:			
	Pigm	Ind	Lip	α-Fuc	β-NAG	Gel	Esc	Arab	Cellob	Lact	Salic
P. amnii	−	−	−	−	+	−	+	ND	−	+	−
P. aurantiaca	+	+	+	+	−	+	−	−	−	−	−
P. baroniae	−	−	−	+	+	W	+	−	+	+	+
P. bergensis	−	−	−	−	V	−	+	+	+	+	+
P. bivia	−	−	−	+	+	+	−	−	−	+	−
P. buccae	−	−	−	−	−	+	+	+	+	+	+
P. buccalis	−	−	−	+	+	−	+	+	+	+	+
P. copri	−	−	−	−	−	−	+	+	+	+	+
P. corporis	+	−	−	−	−	+	−	−	−	−	−
P. dentalis	−	−	−	−	+	−	+	+	+	+	−
P. denticola	V	−	−	+	+	+	+	−	−	+	−
P. disiens	−	−	−	−	−	+	−	−	−	−	−
P. enoeca	−	−	−	+	+	+	V	−	−	+	−
P. fusca[e]	+	−	ND	+	+	−	−	−	+	+	W
P. heparinolytica	−	+	−	+	+	−	+	+	+	+	+
P. histicola	V	−	−	+	+	+	−	−	−	+	−
P. jejuni	+	−	ND	+	+	+	−	−	−	+	−
P. intermedia	+	+	+	+	−	+	−	−	−	−	−
P. loescheii	+	−	V	+	+	+	+	−	+	+	−
P. maculosa	−	−	−	−	+	−	+	+	+	+	+
P. marshii	−	−	−	−	−	+	−	−	−	−	−
P. melaninogenica	+	−	−	+	+	+	V	−	−	+	−
P. micans	+	+	−	+	+	+	−	−	+	+	+
P. multiformis	−	−	−	V	+	+	−	−	+	+	−
P. multisaccharivorax	−	−	−	−	+	+	+	V	+	+	V
P. nanceiensis	−	−	−	+	+	−	+	−	V	+	−
P. nigrescens	+	+	+	+	−	+	−	−	−	−	−
P. oralis	−	−	−	+	+	V	+	−	+	+	+
P. oris	−	−	−	+	+	V	+	+	+	+	+
P. oulorum	−	−	−	+	+	−	−	−	−	+	−
P. pallens	+	+	−	+	−	+	−	−	−	−	−
P. pleuritidis[e]	−	−	−	+	+	+	−	−	−	+	−
P. saccharolytica	−	−	ND	−	+	W	+	+	+	+	+
P. salivae	−	−	−	W	+	−	+	+	+	+	+
P. scopos[e]	+	−	ND	+	+	+	−	−	−	+	−
P. shahii	+	−	−	W	+	+	−	−	−	+	−
P. stercorea	−	−	−	+	+	−	−	−	−	+	−
P. tannerae	V	−	−	+	+	+	−	−	−	V	−
P. timonensis[e]	−	−	−	+	+	+	−	−	−	+	−
P. veroralis	−	−	−	+	+	V	+	−	+	+	−
P. zoogleoformans	−	V	−	+	+	V	+	V	+	+	V

[a] +, positive; −, negative; V, variable; W, weak reaction; ND, no data.

[b] Pigm, pigment; Ind, indole; Lip, lipase; α-Fuc, α-fucosidase; β-NAG, *N*-acetyl-β-glucosaminidase.

[c] Gel, gelatin; Esc, esculin.

[d] Arab, arabinose; Cellob, cellobiose; Lact, lactose; Salic, salicin.

[e] Reactions are based on testing of a single strain.

179, 186). Interestingly, the cells of the novel *Sneathia* species *S. amnii* (formerly *L. amnionii*) were described as variously sized rods of over 10 μm; however, tandem electron microscopy revealed that the long rods were actually formed of chains of bacilli with rounded ends (48). Fresh cells may stain Gram positive (45). Lactate production as the major end product of glucose fermentation is characteristic for *Leptotrichia* and *Sneathia* (Table 2). *Sneathia* species are extremely fastidious, and thus, not much is generally known about their biochemical characteristics (47, 48, 182, 186). The genome sequence analysis of *S. amnii* revealed a small genome size that might explain its fastidious nature (48). Positive reactions by Rapid ID 32A (bioMérieux) have been reported for β-glucuronidase, alkaline phosphatase, arginine arylaminidase activity, and raffinose fermentation (182, 186). Table 8 presents some characteristics helpful in the biochemical identification of *Leptotrichia* and *Sneathia* organisms.

Within the phylum *Synergistetes*, *Fretibacterium fastidiosum* (49), *Jonquetella anthropi* (50), and *Pyramidobacter piscolens* (51) are the human-derived species that have been cultured so far. They are extremely fastidious in nature, requiring the incubation time of 7 to 21 days, and thus are characterized with the utmost difficulty. *Synergistes* organisms are asaccharolytic rods, and acetic acid is their major metabolic end product of glucose fermentation. *F. fastidio-*

TABLE 7 Some phenotypic characteristics of *Fusobacterium* species[a]

Species	Cell morphology	Growth in 20% bile	Production of: Indole	Production of: Lipase	Conversion to propionate from: Lactate	Conversion to propionate from: Threonine
F. gonidiaformans	Gonidial	–	+	–	–	+
F. mortiferum	Pleomorphic with large, round bodies	+	–	–	–	+
F. naviforme	Boat shape	–	+	–	–	–
F. necrophorum		V	+	+	+	+
Subsp. *funduliforme*	Coccoid/pleomorphic, curling, tangling					
Subsp. *necrophorum*	Pleomorphic, entire					
F. nucleatum	Slender, long, pointed ends	–	+	–	–	+
F. periodonticum	Slender, long	–	+	–	–	+
F. russii	Large with rounded ends	–	–	–	–	–
F. varium	Large with rounded ends	+	V	V	–	+
F. ulcerans	Large with rounded ends	+	–	–	–	+

[a] +, positive; –, negative; V, variable reaction.

sum forms very small, highly convex colonies on blood agar, but the colony morphology can vary (49). A coculture with *Fusobacterium nucleatum* subsp. *nucleatum* is requested for optimal growth. *Fretibacterium fastidiosum* is motile, unlike *J. anthropi* and *P. piscolens*. *J. anthropi* is susceptible to bile and forms pinpoint colonies on blood agar, while the colonies of *P. piscolens* are somewhat bigger and highly convex to pyramidal, and its culture has a specific fishy odor (50, 51). All three species are unreactive to most biochemical tests. In the Rapid ID 32A system (bioMérieux), *F. fastidiosum* and *J. anthropi* are positive for both glycine arylamidase and leucyl glycine arylamidase, while *P. piscolens* is highly positive for glycine arylamidase (49–51).

Dialister species are coccobacilli that form tiny colonies on blood agar. They are asaccharolytic and grow poorly in liquid media; lack of reactivity in conventional biochemical tests hampers their identification. They are often confused with *Veillonella* because of their tiny cell size. There are a few enzymes in the Rapid ID 32A system (bioMérieux) that may be useful in distinguishing three *Dialister* species. *D. pneumosintes* is positive for glycine arylamidase, *D. micraerophilus* for alanine, phenylalanine, serine, tyrosine arylamidases, and arginine dihydrolase, and *D. succinatiphilus* for alkaline phosphatase (23, 56). However, *D. invisus* and *D. propionicifaciens* are negative for all tests in this kit. The latter species produces propionate, which can be detected by GLC (56). Molecular methods, such as 16S rRNA gene sequencing, are often needed for the accurate detection of *Dialister* species in clinical specimens (56, 191).

B. wadsworthia isolates are asaccharolytic, bile resistant, and strongly positive for catalase, and most strains are urease positive (62). This species also produces hydrogen sulfide, and its growth is stimulated by bile (oxgall) and pyruvate. This pattern, together with its typical colony characteristics on BBE, may result in its reliable identification.

Two human *Sutterella* species, *S. wadsworthensis* and *S. parvirubra*, and two human *Parasutterella* species, *P. excrementihominis* and *P. secunda*, are asaccharolytic and very unreactive organisms, of which only *S. wadsworthensis* has been isolated from infections so far. *P. excrementihominis*, *P. secunda*, and *S. parvirubra* are strictly anaerobic, have coccoid cells, and are negative for nitrate reduction (57, 61, 232), while *S. wadsworthensis* is a straight rod that is able to grow under microaerobic conditions and to reduce nitrate (198).

Of the motile Gram-negative anaerobic genera isolated from human specimens, *Selenomonas* and *Anaerobiospirillum* are saccharolytic, while *Phocaeicola* and *Desulfovibrio* are asaccharolytic. Unlike the others, *Desulfovibrio* species are positive for desulfoviridin, which can be detected by adding 2 N NaOH to cell paste on a swab and observing for red fluorescence under UV light (63, 206). *Desulfomicrobium orale* is a straight rod and is negative for desulfoviridin, but typically, both *D. orale* and *Desulfovibrio* species reduce sulfate (64). *P. abscessus* is a slow-growing coccobacillus with a lophotrichous flagellum arrangement (233). Flagellae of *Selenomonas* are arranged on the concave side of the cell, while those of *Centipeda* are around the cell (58).

TABLE 8 Some phenotypic characteristics of *Leptotrichia* and *Sneathia* species[a]

Species	β-Hemolysis	Production of: Cat	α-Gal	β-Gal	α-Glu	β-Glu	β-NAG	PAL
L. buccalis	–	–	+	–	+	+	–	+
L. goodfellowii	+	+	–	+	–	+	+	+
L. hofstadii	+	+	–	–	+	+	–	+
L. hongkongensis[b]	–	–	–	–	+	+	–	+
L. shahii	–	+	–	–	+	–	–	–
L. trevisanii	ND	+	–	–	+	+	+	+
L. wadei	+	+	–	–	+	+	–	–
S. amnii	ND	–	–	–	–	–	–	+
S. sanguinegens	ND	–	–	–	–	–	–	+

[a] +, positive; –, negative reaction; ND, no data; α/β-Gal, α/β-galactosidase; α/β-Glu, α/β-glucosidase; β-NAG, *N*-acetyl-β-glucosaminidase; PAL, alkaline phosphatase.

[b] Reactions are based on testing of a single strain.

Anaerobiospirillum species have a corkscrew shape and a jerky motility. *A. succiniciproducens* is sensitive to colistin, and it ferments glucose, maltose, lactose, and sucrose. *A. thomasii* can be differentiated from *A. succiniciproducens* by carbohydrate fermentations and by α-glucosidase and β-galactosidase activities, the former being negative and the latter positive for both reactions (65). *Desulfovibrio* species are curved rods with a rapid, progressive motility (except the nonmotile *D. piger*) and are resistant to colistin and to 20% bile (except for bile-sensitive *D. desulfuricans*) (206). A rather simple test scheme, including catalase, indole, nitrate, and urease tests, is able to separate the four *Desulfovibrio* species isolated from human clinical specimens. *D. desulfuricans* is positive for nitrate and urease, *D. fairfieldensis* is positive for catalase and nitrate, and *D. vulgaris* is positive for indole, while *D. piger* is negative for all these reactions (206). Table 9 presents an identification scheme for motile Gram-negative anaerobic genera.

Advanced Techniques for Identification

Whole-cell bacterial identification by matrix-assisted laser desorption–ionization time of flight mass spectrometry (MALDI-TOF MS) has proven to be a promising method for the identification of anaerobic bacteria, including Gram-negative organisms (234–238). Conserved proteins at the taxon level, such as ribosomal proteins, are used as identification targets. These characteristic peptides and small proteins (ranging in *m/z* mainly from 3 to 15 kDa) can be measured after laser desorption and ionization of whole cells, cell lysates, or crude bacterial extracts. Accurate identification depends on the unknown organism being present in the database. At present, two systems are available for the routine identification of aerobic and anaerobic bacteria: the MALDI Biotyper system (Bruker Daltonik GmbH, Bremen, Germany), which uses microflex or autoflex MS with the MALDI Biotyper database (research use only [RUO] and *in vitro* diagnostic [IVD] versions), and the Vitek MS (bioMérieux), which uses the Shimadzu Axima MS with an IVD database and SARAMIS as the RUO database. Currently, more anaerobic species are included in the Biotyper (Bruker Daltonik) system. The percentage of correct identifications of Gram-negative anaerobes at the species or genus level, compared to those of phenotypic identification or 16S rRNA gene sequencing, depends on the active development of the databases of the different systems in the field of anaerobic bacteria (234, 238, 239). Different sample preparation methods may also influence the correct identification; however, no significant differences have been observed in success of species identification among the tested strains of *Bacteroides* and *Fusobacterium* species, whether the direct smear or a full chemical extraction was used (240). *Bacteroides* strains resulted in species-level identification, whereas *Fusobacterium* strains were usually identified at the genus level, but problems encountered within the *Fusobacterium* genus may be resolved by adding well-characterized strains into the databases. Even phylogenetically closely related species within the genera *Bacteroides* and *Prevotella*, such as *B. salyersiae* and *B. nordii* (235) or *P. intermedia* and *P. nigrescens* (236, 237), are correctly identified by MALDI-TOF MS. As with some other aerobic and anaerobic species, peak shifts can be found and used for typing of divisions I and II of *B. fragilis* (240, 241). As only isolates in division II harbor the *cfiA* gene, which is responsible for the production of carbapenemase, typing of *B. fragilis* by MALDI-TOF MS may have a direct influence on the selection of antibiotic treatment. The main problem today with the MS-based identification of newly described Gram-negative anaerobes is that they are not frequently isolated (or are misidentified) in routine microbiology laboratories, and therefore, integrating them into the database does not, unfortunately, have any priority.

Another method which can reliably detect as-yet-unnamed taxa as well as identify known species is 16S rRNA gene sequence analysis (242). For clinical purposes, a 500-bp sequence from the 5′ end of the gene is able to identify most, but not all, members of this group to the species level.

TABLE 9 Biochemical identification of motile Gram-negative anaerobic rods[a]

Genus/species	Nitrate reduction	Esculin hydrolysis	Production of:								Fermentation of:	
			Cat	Ind	Urea	α-Glu	α-Gal	β-Gal	α-Fuc	β-NAG	Man	Raf
Anaerobiospirillum												
A. succiniproducens	–	–	–	–	–	+	–	+	–	+	ND	+
A. thomasii	–	–	–	–	–	–	–	–	–	+	ND	–
Selenomonas												
S. artemidis	V	–	–	–	–	V	–	–	–	–	+	–
S. dianae	+	–	–	–	–	–	–	–	–	–	+	V
S. flueggei	+	–	–	–	–	+	V	–	–	–	+	V
S. infelix	+	+	–	–	–	V	V	–	–	–	+	+
S. noxia	–	–	–	–	–	–	–	–	–	–	–	–
S. sputigena	V	–	–	–	–	W	+	V	–	–	–	+
Desulfovibrio												
D. desulfuricans	+	ND	–	–	+	ND	ND	ND	ND	ND	"–"	"–"
D. fairfieldensis	+	ND	+	–	–	ND	ND	ND	ND	ND	"–"	"–"
D. piger[b]	–	ND	–	–	–	ND	ND	ND	ND	ND	"–"	"–"
D. vulgaris	–	ND	–	+	–	ND	ND	ND	ND	ND	"–"	"–"
Phocaeicola												
P. abscessus	–	–	–	–	ND	W	+	+	+	+	"–"	"–"

[a] +, positive; −, negative; "–," *Desulfovibrio* and *Phocaeicola* are asaccharolytic; V, variable; W, weak reaction; ND, no data; Cat, catalase; Ind, indole; α-Glu, α-glucosidase; α-Gal, α-galactosidase; β-Gal, β-galactosidase; α-Fuc, α-fucosidase; β-NAG, *N*-acetyl-β-glucosaminidase; Man, mannose; Raf, raffinose.

[b] Nonmotile.

Even more information per amplicon length can be derived by using V6-specific primers (215). These methods are being increasingly used for identification of anaerobic bacteria, because (partial) sequencing of the gene is faster and more accurate than biochemical testing and, notably, independent of the growth characteristics (97, 243). Recently, sequencing of the *rpoB* gene has also proven its potential for bacterial identification (244). For instance, *rpoB* gene analysis has been successfully used for distinguishing two closely related *Fusobacterium* species, *F. nucleatum* and *F. periodonticum*, and for distinguishing oral isolates from isolates from intestinal biopsy specimens (245). However, sequencing as a routine method may not be feasible for many clinical laboratories. The development of algorithms to screen for those isolates that can be adequately identified by conventional methods and to refer difficult-to-identify isolates for 16S rRNA gene sequencing has been proposed (97, 242). Also, commercial 16S rRNA gene sequence-based identification kits containing reagents for DNA extraction and amplification are available. However, they require instruments, including a thermal cycler, automated gene sequencer, and software for interpretation, and the data needed to assess their value in identifying Gram-negative anaerobes are not available. Although sequencing of the 16S rRNA gene is a useful method for identification of fastidious anaerobic organisms, providing a much faster turnaround time than conventional methods, molecular analysis should complement but not replace culturing in the clinical setting. Phenotypic characteristics obtained by culture and biochemical testing assist in correlating the sequence-based data, which can sometimes be difficult to interpret, for example, due to incomplete sequences stored in the reference database. More importantly, culture is necessary for antibiotic susceptibility testing of isolates from clinical specimens.

Unculturable Anaerobic Gram-Negative Rods

In many chronic infections, if not in all, several as-yet-uncultivated phylotypes representing Gram-negative anaerobic phyla can be detected. In the human mouth, organisms with unculturable phylotypes in the genera *Prevotella*, *Fusobacterium*, *Dialister*, and *Selenomonas*, as well as members of the phylum *Synergistetes*, have been associated with various oral and nonoral infections (83). Microbiotas in tonsillar crypts include unculturable organisms; for instance, a *Porphyromonas* genomospecies (most closely related to *P. catoniae*) was significantly associated with children, while two *Fusobacterium* genomospecies were related to adults with recurrent tonsillitis (82). A metagenomic analysis of brain abscesses revealed several unculturable taxa belonging to the phyla *Bacteroidetes* and *Firmicutes*, among polymicrobial consortia in pus specimens from patients with preceding sinusitis or dental treatment (161). In the female genital tract, several organisms with *Prevotella*-like phylotypes have been strongly associated with bacterial vaginosis (80). Currently, chronic venous leg ulcers are considered polymicrobial infections in which unknown *Bacteroidales* are among the most ubiquitous organisms (246).

ANTIMICROBIAL SUSCEPTIBILITIES

Trends among Gram-negative anaerobes of increasing resistance to antimicrobial agents with antianaerobic activities still continue (93, 94, 98, 247, 248). Although susceptibility to antibiotics can vary considerably among species within the same genus, most clinical laboratories neither perform the accurate species-level identification of the isolated organism nor test the susceptibilities of anaerobic isolates (222). Without knowledge of the local susceptibility patterns, the choice of proper antimicrobial therapy can be hampered and make the treatment outcome of anaerobic infections less predictable.

According to recent surveys conducted in the United States, Canada, Argentina, Kuwait, Taiwan, and several countries in Europe, members of the *B. fragilis* group are among the anaerobes most resistant to various antimicrobial agents; this situation is independent of geographical location (93, 94, 98, 247, 249–253). Some variation, however, exists in resistance rates of different species between countries and areas. In a large U.S. study series, yearly changes in the *B. fragilis* group (here also including *P. distasonis*) patterns of susceptibility to ertapenem, imipenem, meropenem, ampicillin-sulbactam, piperacillin-tazobactam, cefoxitin, clindamycin, moxifloxacin, tigecycline, chloramphenicol, and metronidazole were followed from 1997 to 2007 (93). The first time that metronidazole-resistant isolates were detected in the United States was in 2002, but the susceptibility to metronidazole has remained stable. The latest 3-year study period included >1,300 isolates for testing, and *B. fragilis* continued to be the most susceptible species. Different species within the tested isolates showed high rates of resistance to several antimicrobials, especially to clindamycin and moxifloxacin. In addition, some isolates that were resistant to carbapenems were detected among strains of *B. fragilis*, *B. ovatus*, *B. thetaiotaomicron*, and *B. uniformis* (93). A European susceptibility survey examining 824 *B. fragilis* group isolates collected from 13 countries between January 2008 and March 2009 revealed increased rates of resistance to amoxicillin-clavulanate, piperacillin-tazobactam, cefoxitin, clindamycin, and moxifloxacin, whereas rates of resistance to imipenem, metronidazole, and tigecycline were low (98). In a Canadian study, the susceptibilities of 387 *B. fragilis* group isolates from nine hospital laboratories obtained between January 2010 and August 2011 were determined for 10 antimicrobial agents (247); *B. fragilis* was encountered in over half of the tested isolates, and *B. ovatus* and *B. thetaiotaomicron* were also common (>10%). The lowest resistance rates observed were to metronidazole, piperacillin-tazobactam, imipenem, and ertapenem, while *B. ovatus* and *B. thetaiotaomicron*, in particular, were often resistant to several antimicrobials (247). This underlines the significance of species-level identification as well as susceptibility testing for individual strains in the treatment of severe infections. In a Taiwanese hospital survey, the proportion of isolates of *Bacteroides*, *Prevotella*, and/or *Fusobacterium* species that were susceptible to many antimicrobials, especially cefmetazole, clindamycin, and the combination of ampicillin-sulbactam, decreased during the period from 2000 to 2007 (94). Noteworthy was the presence of strains that were intermediate or resistant to carbapenems among the tested *B. fragilis*, *Fusobacterium*, and *Prevotella* isolates from blood; one *B. fragilis* isolate was even resistant to all four carbapenems tested (94). Indeed, blood isolates seem to be less susceptible than those from intra-abdominal, obstetric, or other infections (254).

Multidrug-resistant *B. fragilis* as well as a few multidrug-resistant *B. vulgatus* and *P. distasonis* strains have been identified, mainly in blood and pus specimens after surgical procedures, with the strains being resistant to antianaerobic agents, such as carbapenems, β-lactam–β-lactamase inhibitors, clindamycin, and/or metronidazole (247, 255). Interestingly, one of these cases was successfully treated with linezolid (256). The *cfiA* gene regulates the metallo-beta-lactamase behind carbapenem resistance; increased MICs of imipenem and meropenem have been observed for *cfiA*-positive *B. fragilis* isolates, while *cfiA*-negative isolates were susceptible (241). Moreover, the presence of the *cfiA* gene can be associated with *nim* genes, which are responsible for resistance to nitroimidazoles (257).

Despite the extensive use of metronidazole against anaerobes, acquired resistance has generally been considered rare (258). In susceptibility surveys conducted in the United States and Taiwan, metronidazole-resistant *Bacteroides* isolates were hardly ever found (93, 94, 259), whereas in Europe, resistance to metronidazole is more common (98, 252, 253). Prolonged exposure of *nim* gene-carrying *Bacteroides* and *Prevotella* strains to metronidazole can select for therapeutic resistance (260, 261). Among 188 *Prevotella* strains isolated from various infections in two university centers in France, all together 30 strains (most after prolonged incubation) showed reduced susceptibility to metronidazole, with MICs ranging from 8 to 32 μl/ml (260). Interestingly, none of the strains harbored known *nim* genes, whereas a novel *nim* gene was found in seven susceptible strains identified as *P. baroniae*. Rare strains of *Prevotella* species may appear highly resistant to metronidazole, resulting in poor treatment outcome (262). A considerable number of *Dialister* strains isolated from a variety of clinical specimens showed decreased susceptibility to metronidazole but did not harbor *nim* genes (190).

Newer fluoroquinolones have previously been considered to have good antianaerobic effects, but the resistance to moxifloxacin is rapidly increasing (98, 248). The situation is worsening, especially among non-*fragilis Bacteroides* species; for instance, *B. merdae*, *B. stercoris*, *B. uniformis*, *B. vulgatus*, and *P. distasonis* (formerly *Bacteroides*) were listed with the highest rates of moxifloxacin resistance in Argentina (249). *Pseudoflavonifractor capillosus* (formerly *Bacteroides*) has been shown to be prevalent and to exhibit a high rate of resistance to moxifloxacin in Greece, with the caveat that the isolates examined in the study were not identified using sequence-based methods (263). The relatively new antimicrobial drugs tigecycline and linezolid have demonstrated good antianaerobic effects; however, *Bacteroides* and *Parabacteroides* strains with increased rates of resistance to tigecycline have been reported (248, 259). A significant number of intermediate/resistant strains have been found especially among non-*fragilis Bacteroides* species, such as *B. caccae* and *B. thetaiotaomicron* (259). Noteworthy is that *Bacteroides* strains for which MICs are elevated (>4 μg/ml) can harbor the *tetX* and *tetX1* genes, indicating the potential development and spread of resistance to tigecycline (264). Moreover, concern over the efficacy and safety of tigecycline therapy has recently been raised (265).

Although the agar dilution method is recommended as the method of choice for susceptibility testing of anaerobic species, many studies have shown that the Etest (bioMérieux) provides reliable results with regard to the susceptibilities of anaerobic isolates from clinical specimens (260, 261). The Etest is simple to perform and readily available when needed, offering a useful method for susceptibility testing in clinical microbiology laboratories. It has been observed that some slow-growing metronidazole-resistant clones can be overlooked when the standard incubation time of 48 h is used; therefore, it has been recommended that labs reexamine the Etest plates after 72 h to look for small colonies within the metronidazole inhibition zone (260, 262). Another gradient endpoint method available for antimicrobial susceptibility testing of clinical isolates, including anaerobes, is the M.I.C. evaluator device (Thermo Fisher Scientific, Basingstoke, United Kingdom). Its performance is equivalent to that of the Etest; however, discordant results between gradient endpoint methods and reference agar dilution have been obtained when testing penicillin and metronidazole susceptibilities of the *B. fragilis* group (266). For isolates for which MICs of carbapenem agents are increased, carbapenemase production can be tested by Etest MBL strips (bioMérieux). Susceptibility testing methods for anaerobes are described further in chapter 75.

In general, carbapenems, some β-lactam–β-lactamase inhibitor combinations, chloramphenicol, and metronidazole are the most useful antianaerobic agents, whereas most cephalosporins, clindamycin, and most fluoroquinolones are currently considered less active for use against Gram-negative anaerobic bacteria in severe infections (2, 93, 94, 98, 247, 249, 251, 254). Table 10 summarizes the current antimicrobial activity rates of the clinically most relevant Gram-negative anaerobic taxa.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Knowledge about the resident microbiota and awareness of the role of anaerobic bacteria in disease permit clinicians

TABLE 10 Antimicrobial activities of potential agents against common Gram-negative anaerobes[a]

Antimicrobial agent(s)	Resistance rate(s) (%) of tested strains of:				
	Bacteroides fragilis	Non-*fragilis Bacteroides*	*Parabacteroides*	*Prevotella*	*Fusobacterium*
Amoxicillin-clavulanate	0–23	4–33	0–21	6	7
Ampicillin-sulbactam[b]	2–17	0–50	21–22	0	0
Piperacillin-tazobactam[b]	1–3	0–15	0–14	0	0
Cefoxitin[b]	4–14	6–67	11–39	0	0
Doripenem	1–7	0–4	0	0	0
Ertapenem	1–5	0–4	0	0	0
Imipenem	<1–3	0–2	0	6	4
Meropenem[b]	1–6	0–5	0–22	0	0–8
Tigecycline[b]	0–8	0–42	0–9	0	0
Linezolid	6	0–11	11	ND	ND
Moxifloxacin	7–32	0–56	0–27	19	0
Clindamycin[b]	17–37	21–72	14–55	20–70	7–30
Metronidazole[b]	0–<1	0–2	0	0	0
Chloramphenicol	3	0–4	ND	0	4

[a] Data are compiled from references 93, 94, 98, 247, 249, and 259. ND, no data.

[b] Reference 259 presents combined rates for intermediate/resistant strains.

to anticipate the likely infecting species at different body sites. Training for anaerobic techniques, in general, and introduction of advanced methods for the detection and precise identification of anaerobic organisms are urgently needed in clinical microbiology laboratories, considering the current reports of considerable frequencies of anaerobic bacteremia and septicemia (89, 90) and increasing numbers of patients with fusobacterial infections, especially *F. necrophorum*-associated invasive diseases (147, 159), as well as increasing rates of anaerobes with resistance to various antimicrobials (94, 247, 248). In cases of inaccurate microbiology and inappropriate choices of antimicrobial agents in treating an anaerobic infection, mortality rates and other treatment failures increase significantly (267). Collecting of clinical specimens should avoid the mucosal microbiota, and proper transport medium and times should be used for keeping the anaerobes alive. Factors such as foul-smelling discharge, proximity of infection to mucosal surfaces, abscess formation, and necrotic tissue indicate the presence of anaerobes in the specimen. A definitive identification of an anaerobic isolate should be obtained for all isolates from normally sterile body sites, including blood, spinal fluid, and organs or body cavities, when the patient is gravely ill and not responding to treatment, and when prolonged treatment is necessary. It is desirable for reference laboratories to periodically provide information on local susceptibility patterns of anaerobic species within the clinically important taxa. However, not only an accurate antimicrobial therapy but also proper surgery, such as the drainage of abscesses and excision of necrotic tissue, are important in resolving anaerobic infections. It is notable that chronic infectious lesions, in particular, include consortia of bacteria organized in biofilms. This leads to reduced diffusion of antimicrobials and increased MICs.

The most important, and often difficult, task in the reporting of results of the isolation of Gram-negative anaerobes is determination of whether the organism is involved in the infectious process or is merely a bystander originating from the patient's commensal microbiota. All isolations from normally sterile sites should be regarded as significant. A secondary challenge is that these organisms are often found in polymicrobial infections, with a number of different species present, and often as a biofilm. Obtaining pure cultures for susceptibility testing can therefore be time-consuming, and the resulting susceptibility profiles may be conflicting, making the recommendation of appropriate antimicrobial therapy difficult. Fortunately, such infections typically respond well to empirical treatment, as partial disruption of the bacterial consortium responsible is sufficient to allow the body's defenses to deal with the infection.

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Algorithms for Identification of Curved and Spiral-Shaped Gram-Negative Rods

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Curved and spiral-shaped bacteria have a common microscopic morphology but represent diverse bacterial pathogens. These organisms are curved, helical, or spiral-shaped Gram-negative rods. Specific detection of these organisms may require a combination of tests, including microscopy, histologic staining of tissue, biochemical tests, antigen tests, serologic tests, bacteriologic culture, and molecular diagnostics.

Most bacteria in this group of organisms are isolated from patients with gastrointestinal-tract-related infections. *Campylobacter jejuni* subsp. *jejuni* is the most frequently isolated curved Gram-negative rod associated with diarrheal illness, but under proper culture conditions, other *Campylobacter*, *Helicobacter*, *Arcobacter*, and *Vibrio* species may be detected in routine stool cultures (Fig. 1). *Helicobacter cinaedi* and *Helicobacter fennelliae* are two important *Helicobacter* species isolated from fecal specimens (see chapter 57). *Helicobacter pylori* is the most common curved Gram-negative rod isolated from gastric tissue, but other *Helicobacter* species have also been reported at this site.

Other less commonly isolated curved Gram-negative rods include the anaerobes *Desulfovibrio* spp., *Sutterella wadsworthensis*, *Wolinella succinogenes*, and *Anaerobiospirillum succiniciproducens*, which may be isolated from blood, abscess material, or other clinical samples (Table 1). Several oxidase-positive nonfermenters, including *Herbaspirillum* species (see chapter 43), may also have a curved appearance.

The spirochetes *Borrelia* spp. and *Leptospira* spp. cause systemic infections and are infrequently isolated in clinical laboratories, usually only with specialized media. These bacteria are strictly aerobic, and their optimal growth temperatures are from 28 to 30°C (*Leptospira* spp.) and 30 to 33°C (*Borrelia* spp.). *Treponema* spp. of clinical importance are diagnosed based on clinical and epidemiologic findings, as well as microscopic, serologic, and molecular test procedures.

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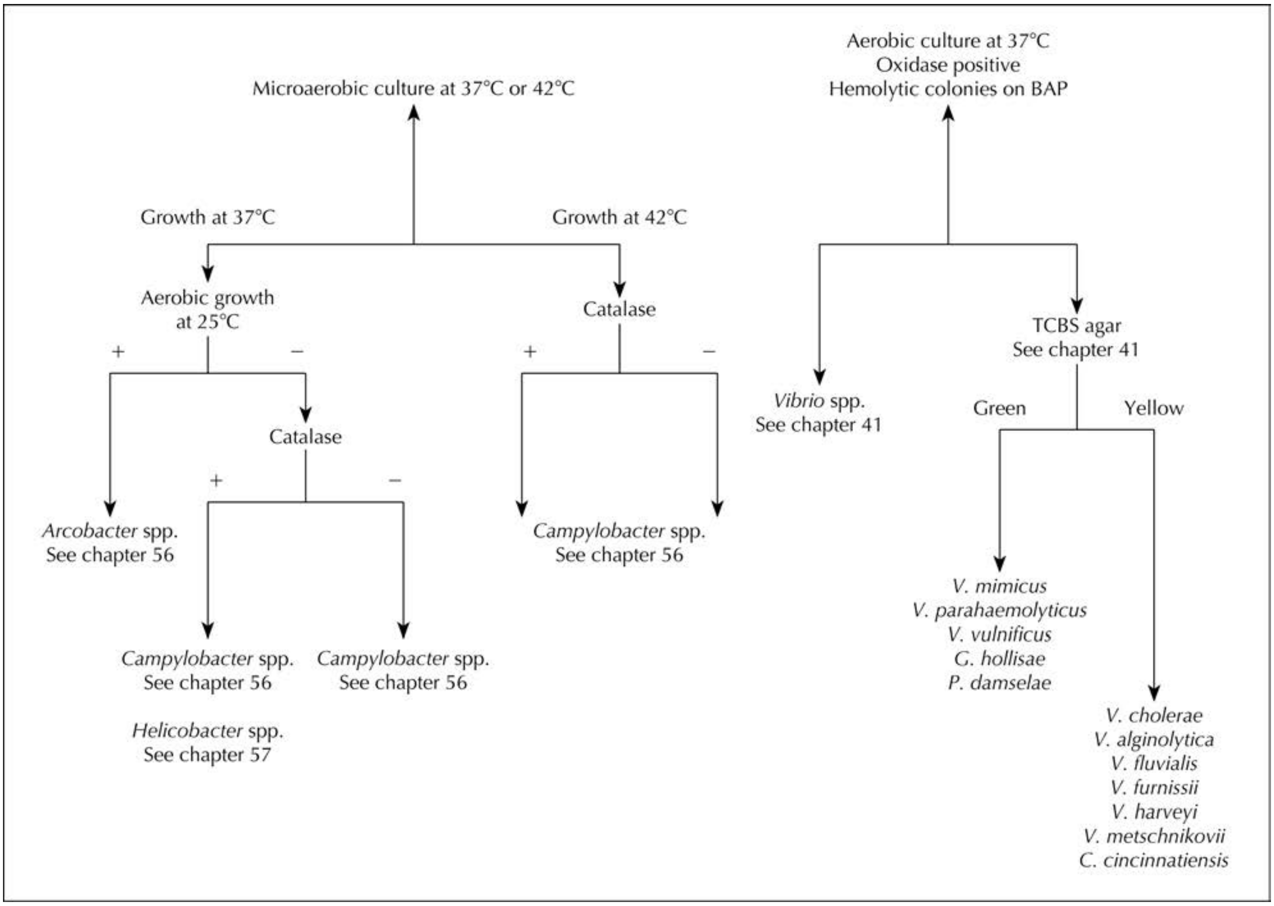
Microaerobic culture at 37°C or 42°C
Aerobic culture at 37°C
Oxidase positive
Hemolytic colonies on BAP
Growth at 37°C
Growth at 42°C
Aerobic growth
at 25°C
+
−
Catalase
+
−
Catalase
+
−
Arcobacter spp.
See chapter 56
Campylobacter spp.
See chapter 56
Campylobacter spp.
See chapter 56
Helicobacter spp.
See chapter 57
Campylobacter spp.
See chapter 56
TCBS agar
See chapter 41
Vibrio spp.
See chapter 41
Green
Yellow
V. mimicus
V. parahaemolyticus
V. vulnificus
G. hollisae
P. damselae
V. cholerae
V. alginolytica
V. fluvialis
V. furnissii
V. harveyi
V. metschnikovii
C. cincinnatiensis

FIGURE 1 Algorithm for identification of curved Gram-negative bacilli from fecal samples. Abbreviations: BAP, sheep blood agar plate; TCBS, thiosulfate-citrate-bile salts-sucrose. doi:10.1128/9781555817381.ch55.f1

TABLE 1 Curved Gram-negative bacilli that may be encountered in clinical specimens[a]

Clinical entity	Specimen type(s)	Curved Gram-negative organism(s) encountered	Species[b]	Microscopic appearance in specimens	Culture conditions and media	Chapter in this *Manual*
Gastroenteritis	Stool, intestinal biopsy	*Arcobacter*	*A. butzleri*, *A. cryaerophilus*, *A. skirrowii*	Slightly curved, curved, S-shaped, or helical	Microaerobic; may grow aerobically or anaerobically; nonhemolytic; grows on nonselective blood agar (with filtration method); may grow on Campy-CVA	56
		Brachyspira	*B. aalborgi*, *B. pilosicoli*	Spirochete	Anaerobic; prolonged incubation (1–2 wk) on anaerobic media; selective media may be required	60
		Campylobacter	*C. jejuni* subsp. *jejuni*, *C. jejuni* subsp. *doylei*, *C. coli*, *C. upsaliensis*, *C. fetus* subsp. *fetus*, *C. lari* subsp. *lari/concheus*, *C. curvus*, *C. concisus*, *C. insulaenigrae*, *C. rectus*, *C. hominis*, *C. lanienae*, *C. hyointestinalis*, *C. peloridis*, *C. sputorum*	Curved, spiral, gull-wing-shaped, S-shaped GNR	Microaerobic; grows at 37 or 42°C; increased H_2 required for some non-*C. jejuni*/*C. coli* species; requires selective media, such as Campy-CVA, and charcoal-based media, such as CCDA; filtration method used for less common species and H_2-requiring species	56
		Helicobacter		Curved, spiral, gull-wing-shaped, S-shaped GNR	Microaerobic; grows optimally at 37°C; increased H_2 required for intestinal species; nonselective blood agar needed for *H. pylori*; selective supplements (Skirrow's, Dent's) may be needed for contaminated gastric samples	57
		Gastric	*H. pylori*, *H. bizzozeronii*, *H. suis*, *H. heilmannii*			
		Intestinal	*H. bilis*, *H. canis*, *H. canadensis*, *H. cinaedi*, *H. fennelliae*, *H. pullorum*			
		Vibrio	*V. cholerae*, *V. parahaemolyticus*, *V. fluvialis*, *V. alginolyticus*, *V. cincinnatiensis*, *V. furnissii*, *V. metschnikovii*, *V. mimicus*, *Grimontia hollisae* (formerly *V. hollisae*), *Photobacterium damselae* (formerly *V. damselae*)	Comma-shaped or straight rods, larger than *Campylobacter* spp.	Aerobic conditions; grows optimally at 37°C; grows on routine laboratory media, blood agar, MacConkey agar; use selective medium for primary isolation from stool samples, such as TCBS	41
Bacteremia	Blood	*Borrelia*	Lyme group, *B. afzelii*, *B. burgdorferi*, *B. garinii*, relapsing fever group, *B. recurrentis*, *B. hermsii*	Not seen in routine BC bottles	Difficult to isolate; special media required for isolation, such as BSK, MKP	59

(Continued on next page)

TABLE 1 *(Continued)*

Clinical entity	Specimen type(s)	Curved Gram-negative organism(s) encountered	Species[b]	Microscopic appearance in specimens	Culture conditions and media	Chapter in this *Manual*
		Campylobacter	*C. jejuni* subsp. *jejuni*, *C. fetus* subsp. *fetus*, *C. upsaliensis*, *C. lari* subsp. *lari*/*concheus*, *C. concisus*	Curved, spiral, gull-wing-shaped, S-shaped GNR	Microaerobic; incubate subcultures at 37°C; increased H_2 required for some non-*C. jejuni*/*C. coli* strains	56
		Herbaspirillum	*Herbaspirillum* species 3	Curved or helical GNR	Growth properties not described	43
		Helicobacter	*H. cinaedi*, *H. fennelliae*	Curved, spiral, gull-wing-shaped, S-shaped GNR	Microaerobic; incubate subcultures at 37°C; increased H_2 required	57
		Leptospira	*L. biflexa*, *L. interrogans*	Not seen in routine BC bottles	Aerobic growth at 28–30°C; specialized media required for isolation, such as EMJH, PLM-5	58
		Vibrio	*V. vulnificus*, *V. metschnikovii*, *V. cincinnatiensis*, *Photobacterium damselae* (formerly *V. damselae*)	Comma-shaped or straight rods	Aerobic growth at 37°C; grows on routine blood agar, MacConkey agar	41
Tissue infection, oral	Tissue biopsy, abscess fluid	*Campylobacter*	*C. concisus*, *C. curvus*, *C. rectus*, *C. gracilis*, *C. showae*, *C. ureolyticus*	Curved, spiral, gull-wing-shaped, S-shaped GNR	Microaerobic; incubate cultures at 37°C; increased H_2 required for oral species; use nonselective blood agar or CCDA with filtration method	56
Tissue infection, skin, wound, other	Skin biopsy, lesion fluid	*Borrelia*	Lyme group	Histologic stains required	Difficult to isolate; special media required for isolation, such as BSK, MKP	59
		Treponema	*T. pallidum* (syphilis), "*T. carateum*" (pinta), "*T. pallidum* subsp. *pertenue*" (yaws), "*T. pallidum* subsp. *endemicum*" (endemic)	Spirochetes (silver staining, dark-field microscopy, DFA)	Has not been isolated *in vitro*	60
		Vibrio	*V. vulnificus* *V. alginolyticus* *V. harveyi Photobacterium damselae* (formerly *V. damselae*)	Comma-shaped or straight rods, larger than *Campylobacter* spp.	Aerobic; grows on routine laboratory media, blood agar, MacConkey agar	41
		Anaerobes	*Desulfovibrio* spp., *Sutterella wadsworthensis*, *Wolinella succinogenes*, *Anaerobiospirillum succiniciproducens*	Curved rods	Grows under anaerobic conditions; use anaerobe media	54
		Herbaspirillum	*Herbaspirillum* species 3	Curved or helical GNR	Growth properties not described	43
Organisms in urine	Urine	*Leptospira*	*L. biflexa*, *L. interrogans*	Spirochete with curved ends (observed by dark-field microscopy)	Aerobic growth at 28–30°C; specialized media required for isolation, such as EMJH, PLM-5	58

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[a] BC, blood culture; GNR, Gram-negative rod; DFA, direct fluorescent-antibody assay; Campy-CVA, Campy-cefoperazone, vancomycin, amphotericin; CCDA, charcoal-cefoperazone-deoxycholate agar; TCBS, thiosulfate-citrate-bile salts-sucrose agar; BSK, Barbour-Stoenner-Kelly medium; MKP, modified Kelley's medium; EMJH, Ellinghausen-McCullough-Johnson-Harris medium; PLM-5, prepared leptospira medium.

[b] Not all species listed in this category have been shown to cause human diseases; they are listed if they have been isolated from human clinical specimens.

Campylobacter and *Arcobacter*

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TAXONOMY

Three closely related genera, *Campylobacter*, *Arcobacter*, and *Sulfurospirillum*, are included in the family *Campylobacteraceae* (1, 2). The family *Campylobacteraceae* includes 24 species within the genus *Campylobacter*, 18 species in the genus *Arcobacter*, and 8 species in the genus *Sulfurospirillum*. Two reviews on comparative genomics of *Campylobacter* have been published (3, 4). Since the last edition of this *Manual*, several new species and subspecies of *Campylobacter* have been published, including *C. subantarcticus* (5), closely related to *C. lari* subsp. *concheus* and isolated from arctic birds (grey-headed and black-browed albatrosses); *C. volucris*, closely related to *C. lari* subsp. *lari* and isolated from black-headed gulls (6); *C. corcagiensis*, isolated from macaques (168); and *C. fetus* subsp. *testudinum*, isolated from blood cultures (169). One *Bacteroides* species, *Bacteroides ureolyticus*, has been reclassified as *Campylobacter ureolyticus* (7). New *Arcobacter* species include *A. anaerophilus*, an obligate anaerobe isolated from estuarine sediment in Gangasagar, India (8), *A. cloacae* and *A. suis* from food and sewage (9), *A. bivalviorum* from mussels and clams (10), *A. defluvii* isolated from sewage samples in Catalonia, Spain (11), *A. ellisii* from mussels (12), *A. marinus* isolated from seawater in the East Sea, Korea (13), *A. molluscorum* from mussels and oysters (10), *A. trophiarum* from pigs (14), *A. venerupis* from mussels and clams (15), *A. aquimarinus* from seawater, and *A. ebronensis* from mussels (170). A detailed review on the taxonomy of *Campylobacteraceae* was previously published (16).

DESCRIPTION OF THE AGENTS

Campylobacter spp. are curved, S-shaped, or spiral rods that are 0.2 to 0.9 µm wide and 0.5 to 5 µm long. Occasional species, such as *C. hominis*, form straight rods. *Campylobacter* species are Gram-negative, non-spore-forming rods that may form spherical or coccoid bodies in old cultures or cultures exposed to air for prolonged periods. Organisms are usually motile by means of a single polar unsheathed flagellum at one or both ends, but some may lack flagella. Species are generally microaerobic with a respiratory type of metabolism, but some strains grow aerobically or anaerobically. An atmosphere containing increased hydrogen (5 to 7%) is required by some species for microaerobic growth (17).

Arcobacter spp. are Gram-negative, slightly curved, curved, S-shaped, or helical non-spore-forming rods that are 0.2 to 0.9 µm wide and 1 to 3 µm long. Organisms are usually motile with a single polar unsheathed flagellum. *A. anaerophilus* is nonmotile (8). *Arcobacter* spp. grow microaerobically at 15, 25, and 30°C but have variable growth at 37 and 42°C. Organisms are microaerobic and do not require increased hydrogen for growth. *Arcobacter* spp. may grow aerobically at 30°C and anaerobically at 35 to 37°C. Most strains are nonhemolytic. *A. skirrowii* may be alpha-hemolytic (18). *A. halophilus* is an obligate halophile and grows poorly on media containing less than 2% NaCl (19).

Originally classified as free-living *Campylobacter* species, *Sulfurospirillum* spp. are slender, curved Gram-negative rods, 0.1 to 0.5 µm wide, and 1 to 3 µm long. All of the species are sulfur reducers and exhibit variable metabolic activity. *S. deleyianum* is the type species of the genus. These species have no known pathogenicity for humans or animals, are environmental organisms isolated from water sediments, and are not further discussed in this chapter (2).

EPIDEMIOLOGY AND TRANSMISSION

Campylobacter species are primarily zoonotic, with a variety of animals implicated as reservoirs for human infection (Table 1). In addition to food animals, such as poultry, cattle, sheep, and pigs, *Campylobacter* species may be present in domestic pets. Humans appear to be the only recognized reservoirs for the periodontal-disease-related species *C. concisus*, *C. rectus*, *C. curvus*, and *C. showae*.

Campylobacter infections are common in both developed and developing countries. The reported incidence of culture-confirmed infections varies considerably from country to country, and as culturing practices and reporting requirements can vary, direct comparison of the reported incidences can be complex. In the United States, where reporting practices vary from state to state, the foodborne disease active surveillance program FoodNet (www.cdc.gov/foodnet) provides uniform reporting from a panel of sentinel sites, giving an accurate incidence of laboratory-confirmed infections. The incidence of culture-confirmed *Campylobacter* infections declined in the United States in the early 2000s (30% decline comparing 2009 to a 1996–1998 baseline) but subsequently showed a 14% increase in 2012 compared with a 2006–2008 baseline, its highest level since 2000 (20). *C. jejuni* subsp. *jejuni* (referred to as *C. jejuni*) continues to be the most common enteric pathogen isolated from patients reported from some states in FoodNet, with 1.3 million cases estimated in the United States annually (21, 22). Because of underdiagnosis and underreporting, the actual incidence in

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TABLE 1 Reservoirs for and diseases associated with *Campylobacter* and *Arcobacter* species[a]

Species	Humans	Cattle	Sheep	Pigs	Wild birds	Marine mammals	Poultry	Shellfish	Reptiles	Pets	Rodents	Type(s) of infection[b] (reference)
C. *avium*							X					NR
C. *canadensis*					X							NR
C. *coli*				X	X		X					GI, B
C. *concisus*	X											D, B, GI
C. *cuniculorum*										X[c]		NR
C. *curvus*	X											D, GI
C *fetus* subsp. *fetus*		X	X						X			B, GI
C. *fetus* subsp. *venerealis*		X										B (160)
C. *gracilis*	X											D
C. *helveticus*										X		NR
C. *hominis*	X											B (161)
C. *hyointestinalis* subsp. *hyointestinalis*		X		X							X	D, B
C. *hyointestinalis* subsp. *lawsonii*				X	X							NR
C. *insulaenigrae*						X						NR
C. *jejuni* subsp. *jejuni*	X	X			X		X			X		GI, B
C. *jejuni* subsp. *doylei*	X											GI, B
C. *lanienae*		X		X								NR
C. *lari* subsp. *concheus*								X				NR
C. *lari* subsp. *lari*					X					X		GI, B
C. *mucosalis*				X								NR
C. *peloridis*	X							X				NR
C. *rectus*	X											D, GI
C. *showae*	X											D
C. *sputorum* bv. paraureolyticus		X										GI
C. *sputorum* bv. sputorum	X	X	X	X								B, T (162)
C. *sputorum* bv. faecalis		X	X									NR
C. *subantarcticus*					X							NR
C. *upsaliensis*							X			X		GI, B
C. *ureolyticus*	X											D
C. *volucris*					X							NR
A. *anaerophilus*												NR
A. *bivalviorum*								X				NR
A. *butzleri*		X		X			X					GI, B, T
A. *cibarius*							X					NR
A. *cloacae*								X				NR
A. *cryaerophilus*		X	X	X			X					GI
A. *defluvii*												NR
A. *ellisii*								X				NR
A. *halophilus*												NR
A. *marinus*												NR
A. *molluscorum*								X				NR
A. *mytili*								X				NR
A. *nitrofigilis*												NR
A. *skirrowii*		X	X	X			X					GI
A. *suis*				X								NR
A. *thereius*				X			X					NR
A. *trophiarum*				X								NR
A. *venerupis*								X				NR

[a] The information in this table is from references 9, 35, and 163–165.
[b] GI, gastrointestinal; B, bloodstream; D, dental/oral; T, soft tissue; NR, not reported to be associated with human infections.
[c] Rabbits.

any country is substantially greater than the reported incidence. The epidemiology of campylobacteriosis in the United States does not appear to have changed over the last 20 years (23). *Campylobacter* infections are usually sporadic; the incidence starts to rise in March, with a peak in the summer, and declines in early fall (23). Infection usually follows ingestion of improperly handled or cooked food, primarily poultry products. Case-control studies in both the United States and Europe continue to find eating poultry to be a significant risk factor for developing campylobacteriosis (23). Outbreaks usually occur in the spring and fall, and in recent years, most outbreaks have been associated with food (poultry or unpasteurized dairy products) or water. Approximately one-half of the outbreaks in the United States from 1998 to 2004 were associated with dairy products or water; the remaining outbreaks were mostly foodborne and 44% were attributed to poultry (23). From 1998 to 2002, there were 64 foodborne *Campylobacter* outbreaks causing 1,628 illnesses, compared to 92 outbreaks and 1,431 illnesses during 2003 to 2007. Outbreaks in other developed countries are also associated with food, water, or dairy contamination (23). In developing countries, *Campylobacter* is often isolated from persons who may or may not have diarrheal disease. Most symptomatic infections occur in infancy and early childhood, and incidence decreases with age. Travelers to developing countries are at risk for *Campylobacter* infection, with isolation rates from 0 to 39% reported in different studies. The incidence of infection follows a bimodal age distribution with the highest incidence in infants and young children, followed by a second peak in adults 20 to 40 years old (22). Secondary transmission of *Campylobacter* from ill persons to other individuals is rare, even though the infectious dose for developing illness is not particularly high, as low as 500 CFU (23).

CLINICAL SIGNIFICANCE

C. jejuni and *C. coli*

C. *jejuni* and C. *coli* have been recognized since the early 1970s as agents of gastrointestinal infection. C. *jejuni* is one of the most common causes of bacterial enteritis in the United States. C. *jejuni* and C. *coli* continue to be the most common *Campylobacter* species associated with diarrheal illness and produce clinically indistinguishable infections. Most laboratories do not routinely distinguish between these organisms. In patients with gastroenteritis caused by C. *jejuni*/C. *coli*, patients' symptoms range from none to severe, including fever, abdominal cramping, and diarrhea (with or without blood/fecal white cells) that lasts several days to more than 1 week (10). The usual incubation period is about 3 days, with a general range of 1 to 7 days. Symptomatic infections are usually self-limited, but relapses may occur in 5 to 10% of untreated patients (10). Hospitalizations are common for *Campylobacter* infections with 15% of laboratory-confirmed infections reported to FoodNet requiring hospitalization in 2012 (20). *Campylobacter* infection may mimic acute appendicitis and result in unnecessary surgery. Extraintestinal infections have been reported following *Campylobacter* enteritis and include bacteremia, hepatitis, cholecystitis, pancreatitis, abortion and neonatal sepsis, nephritis, prostatitis, urinary tract infection, peritonitis, myocarditis, and focal infections including meningitis, septic arthritis, and abscess formation (24, 25). Bacteremia has been reported to occur at a rate of 1.5 per 1,000 intestinal infections, with the highest rate in the elderly (26). Persistent diarrheal illness and bacteremia may occur in immunocompromised hosts, such as patients with human immunodeficiency virus infection, and may be difficult to treat (24). Deaths attributable to C. *jejuni* infection are uncommon (20, 24). The health burden of campylobacteriosis appears to be substantial and may be underrecognized (27).

C. *jejuni* is the most often recognized infection preceding the development of Guillain-Barré syndrome (GBS), an acute paralytic disease of the peripheral nervous system (28). Certain heat-stable (HS) serotypes appear to be overrepresented in some GBS cases, such as HS:19 and HS:41, but other more common serotypes are frequently reported (28). The pathogenesis of *Campylobacter*-induced GBS involves host immune responses to ganglioside-like epitopes present in the core region of the lipooligosaccharide (29), which in the susceptible host mediate damage to the peripheral nerves, where ganglioside targets are highly enriched (30).

Reactive arthritis sometimes follows *Campylobacter* infection, with the onset of pain and joint swelling averaging 2 weeks, with an average range lasting from a few weeks to nearly a year. Reiter's syndrome may also occur in some patients (24). The literature is mixed on the role of HLA B27 as a risk factor for reactive arthritis (24).

The pathogenesis of *Campylobacter* enteric infection is not well understood. The infective dose of *Campylobacter* is not well defined, but as few as 500 organisms may be capable of causing illness (24). The use of proton pump inhibitors increases susceptibility to campylobacter infection (31). The signs and symptoms of infection suggest an invasive mechanism of disease. A variety of determinants may be important in the virulence of C. *jejuni* infection, including adherence to the intestinal mucosa, bacterial effects on the cell, and host inflammatory responses (32).

Campylobacter Species Other than *C. jejuni* and *C. coli*

Campylobacter species other than C. *jejuni* and C. *coli* are increasingly isolated from human infections by improved culture methods that are more optimal for the non-C. *jejuni* and non-C. *coli* species.

C. *fetus* subsp. *fetus* is primarily associated with bacteremia and extraintestinal infections during pregnancy or in the compromised host (33). Although gastroenteritis does occur with this species, the incidence is probably underestimated because the organism may not grow well at 42°C and is usually susceptible to cephalothin (cefalotin), an antimicrobial agent used in some common selective media for stool culture (34). C. *fetus* subsp. *fetus* produces a surface protein microcapsule composed of a high-molecular-weight surface layer protein that is essential for virulence (33). C. *fetus* subsp. *venerealis* causes bovine venereal campylobacteriosis and is a cause of bovine infertility but is rarely the cause of human infection (33).

C. *upsaliensis* is a thermotolerant species that causes diarrhea and bacteremia in humans and is also associated with canine and feline gastroenteritis (35). Over a 10-year period, C. *upsaliensis* was the most common non-C. *jejuni*/non-C. *coli* species isolated from stool samples submitted to the laboratory for culture (36). C. *upsaliensis* is susceptible to many antimicrobial agents present in C. *jejuni* selective media and thus is usually not isolated on routine primary isolation media; it can be recovered using the filtration technique described below.

C. *lari* is a nalidixic acid-resistant, thermophilic species first isolated from gulls of the genus *Larus* and from other avian species, dogs, cats, and chickens. C. *lari* has been infrequently reported from humans with bacteremia and gastrointestinal and urinary tract infections (35). Recent phylogenetic studies have described two subspecies, C. *lari* subsp. *concheus* and C. *lari* subsp. *lari* (37).

Other *Campylobacter* species have been isolated from clinical specimens of patients with a variety of diseases, but their pathogenic role has not been determined (35). C. *jejuni* subsp. *doylei* is a nitrate-negative subspecies of C. *jejuni* rarely recovered from patients with upper gastrointestinal tract infections and gastroenteritis (35). C. *concisus* has been isolated from both fecal and blood specimens. There are reports on the association of this species with gastrointestinal disease, but additional case-control studies are needed to establish the pathogenicity of this organisms (38, 39). C. *avium* is a hippurate hydrolase-positive species that was isolated from broiler chickens and turkeys but has not been reported from human samples (40, 41). C. *canadensis* (41) has been isolated from whooping cranes at the Calgary Zoo and C. *peloridis* (37) from human feces, dialysis fluid, and shellfish. C. *cuniculorum* was isolated from the cecum of rabbits but not reported from humans (42). *C. hyointestinalis* has been occasionally associated with proctitis and diarrhea in human infection. There is some suggestion that *C. curvus* may be an etiologic agent in diarrheal illness (43), but it was rarely isolated from stool samples in another large study (44). *C. curvus* is also isolated from patients with periodontal infections and in patients with a liver abscess and pneumonia (45). *C. gracilis* has been isolated from patients with appendicitis/peritonitis, bacteremia, soft tissue abscesses, and pulmonary infections (46). *C. hominis* has been isolated from fecal samples of healthy individuals and may be a commensal of the oral cavity (47). *C. lanienae* was isolated from two asymptomatic abattoir workers, but its clinical significance is unknown (48). *C. sputorum* has been associated with lung, axillary, scrotal, and groin abscesses (49). *C. sputorum* bv. paraureolyticus, formerly referred to as catalase-negative urease-positive campylobacter, has been isolated from patients with diarrhea, but the significance of this finding is unknown (50). *C. mucosalis* was reported to have been isolated from two children with enteritis, but subsequent testing showed that the isolates were actually *C. concisus* (51). *C. helveticus* (52) has been recovered from domestic cats and dogs and has not been reported from human sources. *C. rectus* is primarily isolated from patients with active periodontal infections but has also been isolated from a patient with pulmonary infection (35, 53) and breast abscess (45). *C. showae* has been isolated from the human gingival crevice (54) and from a blood culture in a patient with cholangitis (55).

Arcobacter

Arcobacter spp. are aerotolerant, *Campylobacter*-like organisms frequently isolated from bovine and porcine products of abortion and feces of animals with enteritis (56). Two of the 18 *Arcobacter* species have been associated with human infection. *A. butzleri* has been isolated from patients with bacteremia, endocarditis, peritonitis, and diarrhea and was recently identified as the causative agent, using molecular methods, of an outbreak of foodborne illness in Wisconsin (34, 35, 57). *A. cryaerophilus* has been previously characterized into two DNA-related groups, 1A and 1B (58). *A. cryaerophilus* group 1B has been isolated from patients with bacteremia and diarrhea (34, 35) and also from healthy individuals (59), suggesting a commensal role for this species. Group 1A has been isolated from animal sources (58). *Arcobacter butzleri* was reported to be the fourth most common *Campylobacter*-like organism isolated from patients with diarrhea by Vandenberg et al. (34) and was also one of the most common non-*C. jejuni*/non-*C. coli* species isolated over a 10-year period from over 73,000 stool samples (36). Thus, *A. butzleri* may be underrecognized if appropriate culture conditions are not used. In a survey of 2,855 *Campylobacter*-like isolates submitted for characterization from laboratories in France, *A. butzleri* was identified in 1%, primarily from fecal samples of patients with a diarrheal illness (60). *A. skirrowii* was reported to be isolated from a human stool culture in a patient with chronic diarrhea, but the role of this species in human disease is unknown (61). *Arcobacter cibarius* has been isolated only from poultry carcasses; the medical significance of this species is unknown (62). *Arcobacter thereius* has been isolated from liver and kidney of spontaneous porcine abortions and from the cloacae of ducks but has not been reported from human samples (63). *Arcobacter mytili* was isolated from shellfish from northeastern Spain and has not been reported from human samples (64). None of the 10 new species described since 2010 have yet been associated with human disease, including *A. nitrofigilis* and *A. halophilus*.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Fecal Samples

Fecal specimens are preferred for isolating *Campylobacter* species from patients with gastrointestinal infections; however, rectal swabs are acceptable for culture. For hospitalized patients, the “3-day” rule (rejection of specimens collected >72 h after admission) should be used as a criterion for acceptability of routine culture requests (65, 66). For routine purposes, a single stool sample has high sensitivity for common enteric pathogens, but two samples may be desirable, depending upon clinical circumstances, such as a >2-h delay in transport of the first sample that could affect recovery (56). A transport medium should be used when a delay of more than 2 h is anticipated and for transporting rectal swabs. Several types of transport media are useful for *Campylobacter*, including alkaline peptone water with thioglycolate and cystine, modified Stuart medium, and Cary-Blair medium (56). Transport media such as commercial Stuart medium and buffered glycerol saline do not appear to perform well. Modified Cary-Blair medium containing reduced agar (1.6 g/liter) appears to be the most suitable single transport medium for *Campylobacter* as well as other enteric pathogens. Specimens received in Cary-Blair medium should be stored at 4°C if processing is not performed immediately. Use of Cary-Blair medium supplemented with laked sheep blood may be useful for prolonged storage of stool samples and recovery of *C. jejuni* (67).

Blood

Campylobacter species, primarily *C. fetus*, *C. jejuni*, and *C. upsaliensis*, have been isolated from blood; however, in only a few studies have optimal conditions for isolating *Campylobacter* from blood culture systems been evaluated. The Bactec (aerobic bottles) and Septi-Chek systems (both from BD, Sparks, MD) appear to support the growth of the common *Campylobacter* species (56). The BacT/Alert system (bioMérieux, Inc.) also supports the growth of *Campylobacter fetus* (68). Other systems such as anaerobic broth or lysis centrifugation may not be as sensitive (56).

DIRECT EXAMINATION

Microscopy

Clinical microbiologists do not normally consider performing Gram stain analysis of stool samples for diagnosis of bacterial gastroenteritis; however, this is a rapid and sensitive method for presumptive diagnosis of *Campylobacter* enteritis. *Campylobacter* spp. are not easily visualized with the safranin counterstain commonly used in the Gram stain procedure and are somewhat thinner than other enteric Gram-negative bacteria; carbol-fuchsin or 0.1% aqueous basic fuchsin should be used as the counterstain for smears of stools or pure cultures (56). Because of their characteristic morphology, *Campylobacter* spp. may be detected by direct Gram stain examination of stools obtained from patients with acute enteritis, with sensitivity ranging from 66 to 94% and specificity above 95%. Phase-contrast and dark-field microscopy have also been used to directly detect motile campylobacters in fresh stool samples; however, the sensitivity of these approaches has not been studied widely, and in our opinion, these methods require significant microscopic expertise (56).

Fecal white cells may be present during *Campylobacter* infection and have been reported in 25 to 80% of culture-proven cases (69). There is no known correlation between the number of cells present and infection. While the likelihood of infection with *Campylobacter* or other enteroinvasive pathogens may be higher in the qualitative presence of fecal leukocytes, the absence of fecal leukocytes does not rule out the diagnosis. Thus, routine examination of stool samples for fecal leukocytes is not recommended as a test for predicting bacterial infection or for selective culturing for *Campylobacter* or other stool pathogens (65, 69).

Antigen Detection

Several commercially available antigen detection systems for *Campylobacter* in stool samples are cleared by the FDA in the United States: the ProSpecT *Campylobacter* assay (Alexon-Trend, Inc., distributed through Remel), the Premier Campy *Campylobacter* assay (Meridian Biosciences), and the ImmunoCard Stat! Campy assay (Meridian Biosciences). When compared with culture, the ProSpecT immunoassay has been shown to vary in sensitivity from 80 to 96% and has a specificity of >97% (70–72). This enzyme immunoassay (EIA) was found to cross-react with *C. upsaliensis* (70). The STAT! Campy antigen assay (Meridian Bioscience) was also found to detect *C. upsaliensis* (73). Antigen may be detected in stored stool samples at 4°C for several days (74). The Premier Campylobacter assay is a microtiter plate-based EIA, while the ImmunoCard STAT! Campy assay is a one-step lateral flow immunoassay; both are reported to be specific for the detection of *Campylobacter jejuni* and *C. coli* but cannot differentiate them. Variable data on their performance characteristics have been reported to date (75–77, 94). Initially shown to have good performance characteristics during an initial verification study, the performance of the Premier Campy EIA following implementation of the test did not reflect initial verification data, with lower sensitivity and poor positive predictive values (78). Other EIAs available outside the United States have variable performance (79). Given that a *Campylobacter* infection is a low-incidence disease, the specificity values described to date for the *Campylobacter* antigen detection assays mentioned above suggest that these tests can lead to poor positive predictive values and thus, we do not recommend that these tests be used as the sole method for diagnosis of campylobacter infection.

Nucleic Acid Detection Techniques

Nucleic acid amplification tests (NAAT) have been used directly to detect *Campylobacter* in stool samples (80, 81). Molecular approaches to detecting *Campylobacter* directly in fecal samples may improve the time to detection, identification to the species level, and identification of the less common *Campylobacter* species often missed by conventional culture. Four NAAT assays are FDA cleared for use in the United States. The xTag Gastrointestinal Pathogen Panel (Luminex Corp.), Prodesse ProGastro SSCS (Hologic Gen-Probe), BioFire Film Array Gastrointestinal Panel (BioFire Diagnostics, Salt Lake City, UT), and Verigene (Nanosphere Inc., Northbrook, IL) are multiplex assays for the detection of numerous gastrointestinal pathogens, including *Campylobacter* spp. While the assays look promising, there is insufficient published information on the U.S. products to assess their performance characteristics or to suggest that they replace culture-based techniques (82–85,171–173).

ISOLATION PROCEDURES

Campylobacter species require a microaerobic atmosphere containing approximately 5% O_2, 10% CO_2, and 85% N_2 for optimal recovery. Several manufacturers produce microaerobic gas generator packs that are suitable for routine use. A tri-gas incubator or evacuation and replacement of an anaerobic jar with the approximate gas mixture may also be used for routine cultures (56). The Anoxomat (Mart Microbiology, distributed through Advanced Instruments, Inc.) is a convenient automated system for the evacuation and gas replacement of jars used for generating different atmospheric conditions, including microaerobic conditions (86). The concentration of oxygen generated in candle jars is suboptimal for the isolation of *Campylobacter* and should not be used for routine laboratory isolation procedures (56).

Some species of *Campylobacter*, such as *C. sputorum*, *C. concisus*, *C. mucosalis*, *C. curvus*, *C. rectus*, and *C. hyointestinalis*, require increased hydrogen for primary isolation and growth. These species will usually not be recovered under the conventional microaerobic conditions, since the amount of hydrogen generated in properly used commercial gas packs is <2% and we recommend that commercial suppliers develop a gas pack with appropriate concentrations of H_2 for isolation of the hydrogen-requiring *Campylobacter* species. The use of anaerobic gas packs without catalyst to generate high H_2 concentrations creates a laboratory safety hazard and should not be used for this purpose. A gas mixture of 10% CO_2, 6% H_2, and the balance N_2 used in an evacuation-replacement jar is sufficient for isolating hydrogen-requiring species. A study by Vandenberg and colleagues reemphasized the requirement of increased hydrogen for isolating certain *Campylobacter* spp. (34).

Media containing selective agents should be used for primary plating of primary stool samples (nonenriched) for reliable isolation of *C. jejuni*/*C. coli*. A number of selective media using blood-containing and blood-free formulations are available from commercial sources. These include two blood-free media, charcoal cefoperazone deoxycholate agar (CCDA) and charcoal-based selective medium (CSM), and two blood-containing media, Campy-CVA (cefoperazone, vancomycin, amphotericin) medium and Skirrow medium (56). Although CVA medium is commonly used in the United States for isolating *Campylobacter* from clinical stool specimens, there are limited data available to assess the ability of CVA to recover *Campylobacter* species from stool specimens, when compared to other *Campylobacter*-selective media; additional evaluation studies are warranted. Charcoal-based media containing cefoperazone, amphotericin, and teicoplanin (CAT media) are selective media for the primary isolation of *C. upsaliensis* (87). Two studies, however, did not isolate *C. upsaliensis* from any stool samples by use of this medium (44, 71). *C. upsaliensis* may occasionally be recovered on some other selective media. *C. upsaliensis* isolates can also be recovered by using the filtration method, and some strains may grow better in a hydrogen-enriched atmosphere (49, 88).

To achieve the highest yield of *Campylobacter* from stool samples, a combination of media that includes either CCDA or CSM appears to be the optimal method (89) and may increase the recovery of *Campylobacter* by as much as 10 to 15% over the use of a single medium. As a single medium, CCDA was found to be the most sensitive for detecting *C. jejuni*/*C. coli* when compared with Skirrow medium, CAT agar, and filtration (44). Recent evaluations of campylobacter-selective media are limited, but based on previous studies, blood-free formulations (e.g., CCDA, CSM) appear to have better performance than blood-containing media, including CampyBAP, Skirrow, and Butzler formulations (44, 90–93). More recently, a multicenter U.S. study found that there was no difference in detection of *C. jejuni*/*C. coli* comparing blood-free media (CCDA and CSM) and blood-containing media (Campy-CVA), although the blood-free media were more selective, making isolation easier (94). If only a single medium is used, we suggest using Campy-CVA, CCDA, or CSM. If *Campylobacter* infection is suspected at the time blood specimens are drawn, broth media should be subcultured after 24 to 48 h to a nonselective blood agar medium and plates incubated under microaerobic conditions at 37°C, preferably with increased hydrogen. This allows for the isolation of thermophilic and nonthermophilic species. While commonly used blood culture systems should support the growth of *Campylobacter* and give appropriate signals if positive, it may be prudent to perform a blind subculture. Similarly, blood drawn in Isolator (Wampole Laboratories, Cranbury, NJ) tubes for bacterial culture should include a nonselective blood agar plate incubated under microaerobic conditions at 37°C if *Campylobacter* infection is suspected. If a curved, Gram-negative rod is observed upon Gram stain examination of a positive blood culture bottle, an aliquot should be cultured on a nonselective blood agar plate and incubated under microaerobic conditions at 37°C. An alternative staining method such as acridine orange may also be useful for detecting campylobacters in blood culture bottles if the Gram stain is negative.

Optimal conditions for recovery of *Arcobacter* from clinical specimens have not been determined. *Arcobacter* spp. were first isolated on semisolid media designed to isolate *Leptospira* spp. (56). *Arcobacter* species are aerotolerant and have been recovered on certain selective media, such as Campy-CVA (95), incubated under microaerobic conditions at 37°C and on nonselective media used in the filtration method (34). Selective media for isolation of *Arcobacter* spp. from human stool samples were evaluated by Houf and Stephan (59). Both selective plates and enrichment broth containing selective supplements with 5-fluorouracil, amphotericin B, cefoperazone, novobiocin, and trimethoprim showed good recovery of *Arcobacter* sp. (59).

Enrichment Cultures

Enrichment broths formulated to enhance the recovery of *Campylobacter* from stool include Preston enrichment, Campy-thio, and *Campylobacter* enrichment broth (56). The clinical advantage and cost-effectiveness of using enrichment cultures as part of the routine stool culture setup have not been studied adequately; therefore, enrichment cultures are not recommended for routine use.

Filtration

Filtration techniques designed to isolate *C. jejuni* and *C. coli* as well as other *Campylobacter* species (34, 44, 56) and *Arcobacter* spp. (34, 36, 58) that are susceptible to antibiotics present in most selective media should be used to complement direct culture to selective plating media. As only stool samples containing ~10^5 CFU/ml of *Campylobacter* will be detected with filtration, it should not be used as a replacement for direct culture, because the filtration method is not as sensitive as primary culture with selective media (88).

The method is based on the principle that campylobacters can pass through membrane filters (0.45-µm to 0.65-µm pore size) with relative ease (because organisms are thin and highly motile) while other elements of the stool microbiota are retained during the short processing time. Cellulose acetate membrane filters with a 0.65-µm pore size are recommended for routine use and available from a number of suppliers (56). Filtration is performed by placing a sterile 0.65-µm-pore-size cellulose acetate filter onto the surface of an agar medium such as antibiotic-free CCDA, CSM, or blood-containing medium. After diluting approximately 1 g of stool in 10 ml of sterile saline, 10 to 15 drops of fecal suspension are placed on the filter, and the plate is incubated at 37°C for 45 to 60 min (96). The filter is then removed, and the plate is incubated at 37°C under microaerobic conditions, preferably with an atmosphere containing increased hydrogen (for the hydrogen-requiring species).

Species within the genera *Campylobacter* and *Arcobacter* have different optimal temperatures for growth. The choice of incubation temperature for routine stool cultures is critical in determining the spectrum of species that will be isolated. By convention, most laboratories use 42°C as the primary incubation temperature for *Campylobacter*. This temperature allows growth of *C. jejuni* and *C. coli* on selective media while inhibiting other fecal microbiota. *C. upsaliensis* also grows well at 42°C but usually is not recovered on selective media. *C. fetus* exhibits variable growth at this temperature and may not be recovered. *Arcobacter* species will generally not be recovered at 42°C.

In contrast, most *Campylobacter* and *Arcobacter* species grow well at 37°C. Selective media, such as Skirrow medium, were devised for use at 42°C and have poor selective properties at 37°C, whereas CCDA and CSM show good selective properties at 37°C (89). Plates should be incubated a minimum of 72 h before being reported as negative. It has been reported that incubation of CCDA medium for 5 to 6 days increased the yield of *C. jejuni* and *C. coli* compared with 2 days of incubation (44).

Because of the expense of including several types of media and the filtration method in the initial workup for *Campylobacter*, a practical approach is to use a single medium for isolation of thermophilic *Campylobacter* spp. in the workup of acute bacterial gastroenteritis, such as Campy-CVA, CCDA, or CSM incubated at 42°C. If the primary culture workup is unrevealing, and for patients with persistent diarrhea, cultures for non-*C. jejuni*/non-*C. coli* species may be appropriate. Additional stool samples should be plated on multiple selective media (e.g., CCDA or CVA), processed by the filtration method as well, and incubated at 37°C under microaerobic conditions with increased hydrogen.

IDENTIFICATION

The identification of *Campylobacter* species is made difficult because of their complex and rapidly evolving taxonomy, fastidious growth requirements, and biochemical inertness (Table 2). These problems have resulted in a proliferation of phenotypic and genotypic methods for identifying members of this group (56).

Campylobacter spp. and *Arcobacter* spp.

Depending on the growth medium used, *Campylobacter* colonies may have different appearances. In general, *Campylobacter* spp. produce gray, flat, irregular, and spreading colonies. Spreading along the streak line is commonly seen, particularly on freshly prepared media. As the moisture content decreases, colonies may form round, convex, and glistening colonies with little spreading observed. Thus, proper storage of media to ensure moisture content is important for optimal isolation and recognition of *Campylobacter* spp. Hemolysis on blood agar is not observed. *Arcobacter*

TABLE 2 Useful phenotypic properties of *Campylobacter* and *Arcobacter* species[a]

Organism	Catalase	H_2 required	Urease	H_2S (TSI)	Hippurate hydrolysis	Indoxyl acetate hydrolysis	Aryl sulfatase	Selenite reduction	Growth in 1% glycine	Growth at 25°C	Aerobic growth
Commonly encountered species of known or presumed clinical importance											
C. *jejuni* subsp. *jejuni*	+	–	–	–	+	+	V	V	+	–	–
C. *jejuni* subsp. *doylei*	V	–	–	–	V	+	–	–	+	–	–
C. *coli*	+	–	–	V	–	+	–	+	+	–	–
C. *fetus* subsp. *fetus*	+	–	–	–	–	–	–	V	+	+	–
C. *lari* subsp. *lari*/C. *lari* subsp. *concheus*[b]	+	–	V	–	–	–	–	V	+	–	–
C. *upsaliensis*	–	–	–	–	–	+	–	+	+	–	–
Infrequently encountered species											
C. *avium*	V	V	–	–	+	+	ND	–	–	–	–
C. *canadensis*	V	–	V	V	–	–	ND	ND	V	–	–
C. *concisus*	–	+	–	V	–	–	+	V	V	–	–
C. *cuniculorum*	+	–	–	–	–	+	ND	–	–	–	–
C. *curvus*	–	+	–	V	V	V	+	–	+	–	–
C. *fetus* subsp. *venerealis*	V	–	–	–	–	–	–	V	V	+	–
C. *gracilis*	V	ND	–	–	–	V	ND	–	+	ND	–
C. *helveticus*	–	–	–	–	–	+	ND	–	V	–	–
C. *hominis*	–	+[c]	ND	–	–	–	ND	–	+	–	–
C. *hyointestinalis* subsp. *hyointestinalis*	+	V	–	+	–	–	–	+	+	V	–
C. *hyointestinalis* subsp. *lawsonii*	+	V	+	+[d]	–	–	–	+	V	–	–
C. *insulaenigrae*	+	–	–	–	–	–	ND	ND	+	–	–
C. *lanienae*	+	–	–	–	–	–	ND	+	V	–	–
C. *mucosalis*	–	+	–	+	–	–	–	V	V	–	–
C. *peloridis*	+	ND	ND	ND	–	ND	ND	ND	+	ND	ND
C. *rectus*	V	+	–	–	–	+	+	–	+	–	–
C. *showae*	+	+	–	–	–	V	+	–	V	–	–
C. *sputorum*[d] bv. sputorum	–	+	–	+	–	–	+	V	+	–	–
C. *sputorum* bv. faecalis	+	+	–	+	–	–	+	V	+	–	–
C. *sputorum* bv. paraureolyticus	–	+	+	+	–	–	+	V	+	–	–
C. *ureolyticus*	V	+[c]	+	–	–	–	ND	–	+	–	–
C. *subantarcticus*	+	–		–	–	–	ND	–	V	–	–
A. *cibarius*	V	–	–	–	–	+	ND	–	–	+	V
A. *cryaerophilus*	V	–	–	–	–	+	–	–	V	+	+
A. *butzleri*	V	–	–	–	–	+	–	–	+	V	+
A. *mytili*	+	–	–	–	–	–	ND	ND	ND	+	+
A. *nitrofigilis*	+	–	+	–	–	+	–	ND	–	ND	ND
A. *skirrowii*	+	–	–	–	–	+	–	ND	V	+	+
A. *thereius*	+	–	–	–	–	+	–	–	+	+	+

[a] Adapted from references 5, 37, 40, 42, 56, 63, and 64. +, positive reaction; −, negative reaction; V, variable reaction; ND, not determined; TSI, triple sugar ion.

[b] C. *lari* subsp. *concheus* can be differentiated from C. *lari* subsp. *lari* by lack of growth on media containing 0.05% safranin (37).

[c] Anaerobic growth only.

[d] Strains of C. *sputorum* and C. *hyointestinalis* subsp. *lawsonii* normally produce large amounts of H_2S in TSI agar (47).

colonies are morphologically similar to those of *Campylobacter* (2, 18).

The Gram stain appearance of *Arcobacter* may differ from that of typical *Campylobacter*. *A. butzleri* is only slightly curved, while *A. cryaerophilus* tends to be much more helical in appearance than *Campylobacter*. Commercial systems for identification of *Campylobacter* species were not found to be more accurate than conventional tests (97). Unfortunately, *Campylobacter* species are difficult to differentiate from *Arcobacter* species based on phenotypic tests. However, an aerotolerant species (i.e., exhibiting growth under aerobic conditions) that grows on MacConkey agar under microaerobic conditions could be presumptively identified as *Arcobacter*. The failure to grow on MacConkey, however, does not rule out *Arcobacter* species.

C. jejuni and *C. coli*

For initial analysis, a Gram stain examination of the colony should be performed along with an oxidase test. Oxidase-positive colonies exhibiting a characteristic Gram stain appearance (e.g., Gram-negative, curved to S-shaped rods) isolated from selective media incubated at 42°C under microaerobic conditions can be reliably reported as *Campylobacter* spp. The most common species, *C. jejuni*, is relatively easy to identify phenotypically; hydrolysis of sodium hippurate is the major test for distinguishing *C. jejuni* (and also *C. jejuni* subsp. *doylei*) from other *Campylobacter* species. Strains isolated on selective media that grow at 42°C, are oxidase positive, show characteristic microscopic morphology, and are positive for hippurate hydrolysis should be reported as *C. jejuni*, and for routine clinical purposes, no other tests need to be performed. Methods for this test are described elsewhere (98). Occasional strains of *C. jejuni* are hippurate hydrolysis negative, making them more difficult to identify. Gas-liquid chromatography for detecting benzoic acid (liberated from hydrolysis of sodium hippurate) or detection of *hipO* by molecular methods can be used for definitive determination.

With the exception of hippuricase activity, which *C. coli* is lacking, *C. coli* and *C. jejuni* are similar biochemically (Table 2). Therefore, molecular methods are needed to accurately identify *C. coli* and differentiate it from hippurate-negative *C. jejuni*; most have proved both accurate and sensitive (99) (Table 3). If molecular testing is not available, strains isolated on selective media that grow at 42°C, are oxidase positive, show characteristic microscopic morphology, and are hippurate negative and indoxyl acetate positive should be reported as hippurate-negative *C. jejuni*/*C. coli*. Additionally, *C. coli* and *Helicobacter pullorum* have similar phenotypic profiles and support the use of molecular identification methods for these species (100). Susceptibility (inhibition) or resistance of *Campylobacter* spp. to nalidixic acid and cephalothin was historically used as an aid for species identification. However, with the increasing prevalence of fluoroquinolone resistance in these species, the use of these disk identification assays can no longer be relied upon. For species other than *C. jejuni*, phenotypic characterization alone will not allow definitive species-level identification; the use of additional molecular identification methods is recommended. If performing phenotypic testing, it is important to use a standardized suspension and inoculum in order to obtain consistent and reproducible results. For growth temperature and oxygen tolerance studies, a suspension of the organism in heart infusion broth or tryptic soy broth with turbidity at a McFarland standard of 1 should be used. A fiber-tipped swab dipped in the broth suspension should be used to make a single streak across the plate (Mueller-Hinton agar with 5% sheep blood is a suitable medium), and the plates should be incubated at the desired temperature and/or atmospheric conditions (101, 102).

Several commercial systems have been developed as an aid to identifying *Campylobacter* spp. to the genus level. Two immunologic reagents are currently available in the United States for culture identification: Campy-JCL (Scimedx Corp., Denville, NJ) and Dryspot Campylobacter test kit (Thermo Scientific-Remel). The Dryspot Campylobacter latex test is reported by the manufacturer to identify but not differentiate *C. jejuni*, *C. coli*, *C. lari*, and *C. upsaliensis* and to yield variable results for *C. fetus* subsp. *fetus* (Oxoid USA; www.oxoid.com).

Because many species of *Campylobacter* and *Arcobacter* are difficult to identify by phenotypic testing alone, tests for detection of species-specific sequences via PCR have been developed. The 16S and 23S rRNA genes are widely used for genus- and species-specific tests; PCR assays based on these targets have been described for different *Campylobacter* (98) and *Arcobacter* (103, 104) species. Broad-range molecular identification schemes involving restriction fragment analysis of PCR-amplified regions of the 16S or 23S rRNA genes have also been described for identification of *Campylobacter* and *Arcobacter* species (105–107).

Many other gene targets have been used in species-specific PCR assays, including *gyrA* (108, 109), *glyA* (110), *ceuE* gene (111), *asp* (112), *lpxA* (113), and a GTPase gene (109). Subspecies identification by PCR within *C. fetus* (114, 115) and *C. jejuni* (116) has also been described. While the use of such PCR tests combines the advantages

TABLE 3 Differentiation of *C. jejuni* and *C. coli* by PCR

Assay	Target gene	Primers (5′–3′ sequence)[a]	Reference
C. jejuni specific	*hipO* (hippuricase)	HIP400F (GAA GAG GGT TTG GGT GGT G) HIP1134R (AGC TAG CTT CGC ATA ATA ACT TG)	112
C. coli specific	*asp* (putative aspartokinase)	CC18F (GGT ATG ATT TCT ACA AAG CGA G) CC519R (ATA AAA GAC TAT CGT CGC GTG)	112
C. jejuni/*C. coli* multiplex	Unknown	JUN3 (CAT CTT CCC TAG TCA AGC CT) JUN4 (AAG ATA TGG CTC TAG CAA GAC) COL1 (AGG CAA GGG AGC CTT TAA TC) COL2 (TAT CCC TAT CTA CAA ATT CGC)	166
C. jejuni/*C. coli* multiplex	*ceu-oxr* [iron(III)-ABC transporter/putative oxidoreductase]	(cc) CeuE-F (ATG AAA AAA TAT TTA GTT TTT GCA) (cc) CeuE-R (ATT TTA TTA TTT GTA GCA GCG) (cj) Oxr-F (CAA ATA AAG TTA GAG GTA GAA TGT) (cj) Oxr-R (GGA TAA GCA CTA GCT AGC TGA T)	167

[a] Abbreviations: cc, *C. coli*; cj, *C. jejuni*.

of being quick and easy to perform with low cost and high-throughput capability, amenable to automation, it is important to validate PCR tests to fully determine their specificity and sensitivity before use. Comparison of 16S rRNA gene sequences is also a useful tool for differentiation of *Campylobacter* spp. from closely related taxa, such as *Arcobacter* and *Helicobacter*. However, it is important to note that species-level identification based on 16S is much more difficult, particularly for the common species of *Campylobacter*. At or above 97% identity, some groups of closely related species such as *C. jejuni*, *C. coli*, and *C. lari*; *C. upsaliensis* and *C. helveticus*; and *C. fetus*, *C. hyointestinalis*, and *C. lanienae* cannot be confidently distinguished from each other based on 16S rRNA gene sequences (117). Conversely, intraspecies 16S rRNA gene diversity is seen in other species, such as *C. hyointestinalis* (118).

Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis may also be used to identify both common and uncommon *Campylobacter* species. In a study of nearly 1,000 isolates, MALDI-TOF (MALDI Biotyper, Bruker Daltronics) had 99.4% accuracy for identifying *C. jejuni* (n = 785), and 100% accuracy for *C. coli* (n = 149), *C. fetus* subsp. *fetus* (n = 40) and *C. lari* (n = 8) as well as *A. butzleri* (n = 14) (119). Other studies also confirm the value of MALDI-TOF identification of *Campylobacter* species (120, 121). Conditions for growth may need to be optimized for successful MALDI analysis (122).

TYPING SYSTEMS

Typing systems for *Campylobacter* epidemiologic studies vary in complexity and ability to discriminate between strains. Common phenotypic methods that have been applied include biotyping, phage typing, and serotyping (123, 124). The heat-labile serotyping scheme, originally described by Lior, can detect over 100 serotypes of *C. jejuni*, *C. coli*, and *C. lari* (124). Uncharacterized bacterial surface antigens and, in some serotypes, flagellar antigens are the serodeterminants for this serotyping system (125). The HS Penner serotyping scheme detects 60 types of *C. jejuni* and *C. coli* (124). Initially thought to detect lipopolysaccharide antigenic determinants, the HS system has been shown to detect a *Campylobacter* capsular polysaccharide (126). Serotyping (HS) is performed in only a few reference laboratories worldwide because of the time and expense needed to maintain quality antisera. A serotyping reagent kit is also commercially available (Denka Seiken USA, Inc., Campbell, CA). Conventional serotyping, while still in limited use, has been instrumental in identifying disease associations with *Campylobacter* and GBS (28). Other systems, such as biotyping and phage typing, do not have any practical applications for modern epidemiologic studies. More recently, multiplex PCR for capsular typing, which correlates quite well with conventional HS serotyping, has been developed (127). A review about the global distribution of HS serotypes was recently published (128).

The limitations of phenotypic subtyping methods and the rapid growth of molecular biology techniques in the 1990s led to the development of a range of molecular subtyping methods such as restriction endonuclease analysis, ribotyping, PCR-based techniques, pulsed-field gel electrophoresis of macrorestricted chromosomal DNA (PFGE), and amplified fragment length polymorphism (123, 124). The development of a rapid 1-day standardized PFGE protocol (129), which is used by participants of the PulseNet national surveillance network for foodborne pathogens (www.cdc.gov/pulsenet), has facilitated the use of this approach for outbreak investigations of campylobacteriosis (130). However, interpretation of data can be difficult since genomic rearrangements can lead to changes in PFGE profiles (131). Advances in DNA sequencing technology have provided a means to investigate strain variation at the nucleotide level and have led to the emergence of DNA-sequencing-based subtyping systems such as multilocus sequence typing (MLST) (132). MLST is useful for studies of the population structure and molecular epidemiology of *C. jejuni* (132), although commonly circulating sequence types can make recognition of outbreaks caused by these strains problematic (133, 134). Rapid advances in next-generation sequencing platforms have made it possible to now apply whole-genome sequence analysis in the context of molecular typing for *C. jejuni*; however, the specialized expertise and information technology infrastructure and tools required for rapid bioinformatic analyses of whole-genome sequence data will likely limit the use of this approach for routine *C. jejuni* subtyping in the short term (135). At present, no method alone is adequate for all applications, and a combination of methods, such as serotyping and molecular methods (PFGE or MLST), should be used for reliable determination of strain relatedness and for studying the epidemiology of *Campylobacter* infections (123). More-detailed reviews of molecular typing methods and their application for use in epidemiological studies of *Campylobacter* species have been published elsewhere (136–138).

A number of typing methods have been applied to the study of *Arcobacter* species (139). An MLST system for *Arcobacter* spp. was reported several years ago (140). Using a set of 374 isolates comprising different species, from different sources and geographic locations, no association of MLST type and location or source was observed.

SEROLOGIC TESTS

Serum immunoglobulin G (IgG), IgM, and IgA levels rise in response to infection, but serum and fecal IgA levels appear during the first few weeks of infection and then fall rapidly (141, 142). Serum antibody assays vary in both sensitivity and specificity for detecting *Campylobacter* infection, and test performance appears to be population dependent. *Campylobacter* antibody assays have been used to study patients with GBS and reactive arthritis (143). Patients with *Campylobacter* infection may give false-positive *Legionella* antibody test results (144). Serologic testing appears to be useful for epidemiologic investigations and is not recommended for routine diagnosis (145).

ANTIMICROBIAL SUSCEPTIBILITIES

C. jejuni and *C. coli* have variable susceptibilities to a variety of antimicrobial agents, including macrolides, fluoroquinolones, aminoglycosides, phenicols, and tetracycline. Azithromycin and erythromycin are drugs of choice for treating *C. jejuni* gastrointestinal infections, and for susceptible organisms, ciprofloxacin or norfloxacin may also be used. Early therapy of susceptible *Campylobacter* infection with erythromycin or ciprofloxacin is effective in eliminating the organism from stool and may also reduce the duration of symptoms associated with infection (24).

C. jejuni is generally susceptible to erythromycin, with resistance rates of less than 10% (24, 146, 147). Nationally, 1.7% of *C. jejuni* and 2.7% of *C. coli* isolates reported by NARMS (National Antimicrobial Resistance Monitoring System for Enteric Bacteria) in 2011 were macrolide resistant (www.cdc.gov/NARMS). Macrolide resistance is generally mediated by mutations in the 23S rRNA gene (148).

Rates of erythromycin resistance in *C. coli* are generally higher than in *C. jejuni* and vary considerably, with up to 25 to 50% of strains showing resistance in some studies (24, 147). Although ciprofloxacin has been effective in treating *Campylobacter* infections, emergence of ciprofloxacin resistance during therapy has been reported (149). Several *in vitro* studies show significant rates of resistance to fluoroquinolones (146, 150, 151). Resistance to fluoroquinolones has ranged from <5% in Australia to approximately 80% reported in Thailand (24, 152). In 2011, 24% of *Campylobacter* strains reported through NARMS at CDC were fluoroquinolone resistant with higher rates of resistance (36%) in *C. coli* (http://www.cdc.gov/NARMS/). Individuals with fluoroquinolone-resistant *C. jejuni* have been shown to have a longer duration of diarrhea, and thus, routine testing of isolates may be indicated (24, 153). *C. jejuni* and *C. coli* are resistant to β-lactam antibiotics, general penicillins, and narrow-spectrum cephalosporins, but imipenem has good anti campylobacter activity.

Parenteral therapy is used to treat systemic *C. fetus* infections; drugs used include ampicillin, aminoglycosides, imipenem, and chloramphenicol, depending upon the type of infection. *C. lari* is resistant to nalidixic acid but may be susceptible to fluoroquinolones, and resistance to macrolides is generally low (152). *C. upsaliensis* is generally susceptible to a variety of antimicrobial agents and shows low rates of resistance to macrolides and fluoroquinolones (152). *Arcobacter butzleri* and *A. cryaerophilus* have variable resistance to macrolides and fluoroquinolones (152).

Agar dilution is the method recognized by the Clinical and Laboratory Standards Institute (CLSI) for testing *Campylobacter* spp.; quality control ranges for several antimicrobial agents have been published (154, 155). A broth microdilution method with published quality control ranges for several antimicrobial agents and a disk diffusion screening method are also approved by CLSI (156, 157). The European committee on antimicrobial susceptibility testing (EUCAST) recently developed a standardized disk diffusion method for ciprofloxacin, erythromycin, and tetracycline with defined breakpoints. Studies testing *Campylobacter* with the Etest have been published (158).

A review of susceptibility testing methods recommended by CLSI and EUCAST was recently published. Methodologies currently vary between different countries and breakpoint committees, and there is a need for harmonization of both methods and interpretive criteria (159).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Campylobacter species, including the common thermophilic species *C. jejuni* and *C. coli*, should be sought in all diarrheic stools submitted to the laboratory for routine culture. Except for epidemiological purposes, cultures of formed stools should not be performed. Isolation of *Campylobacter* from a patient with acute diarrhea is usually significant, since the carrier rate in developed countries is quite low; however, in developing countries, isolation might be more difficult to interpret, especially in the presence of other enteric pathogens. In acute infection, there are usually a high number of organisms in the stool, but the quantity of organisms is not related to the severity of infection or indicative of a carrier state. Gram stain analysis of fecal samples to look for organisms with typical *Campylobacter* morphology is a highly sensitive and specific test that is underused; it should be performed for rapid preliminary diagnosis of *Campylobacter* infection. Other species, such as *C. fetus* subsp. *fetus* and *C. upsaliensis*, may be important causes of diarrhea and are not isolated on routine selective media. Special methods, including alternative incubation techniques, are required as described in this chapter and should be performed by special request. Oxidase-positive, curved, Gram-negative rods that are hippurate hydrolysis positive should be reported as *C. jejuni* without further workup. The importance of identifying other species depends on the clinical circumstance, but identification tests should always be performed with isolates from blood or other sterile sites, since this could influence antimicrobial therapy decisions. Because fluoroquinolone resistance is present in a significant proportion of *C. jejuni* isolates, fluoroquinolone susceptibility testing is suggested for patients who are receiving or being considered for therapy of gastroenteritis. Susceptibility testing should be performed with all isolates from sterile clinical sites.

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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*Helicobacter**

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TAXONOMY

The genus *Helicobacter* is classified in the family *Helicobacteriaceae* of the class *Epsilonproteobacteria*, formerly known as the epsilon subclass of the *Proteobacteria*, with *Helicobacter pylori* as the type species (1). The other genus in the family is *Wolinella*, with the type species *Wolinella succinogenes*, and both genera are phenotypically similar to the genus *Campylobacter*. *Helicobacter* is a genus of expanding diversity. Since the genus name was formally proposed in 1989 (2) with two species (*H. pylori* and *Helicobacter mustelae*) and revised in 1991 to include *Helicobacter cinaedi* and *Helicobacter fennelliae* (3), it has grown to comprise more than 30 species (including a species with "*Candidatus*" status) (Table 1). Species of *Helicobacter* have genomic G+C base contents ranging from 30 (*H. acinonychis*) to 48 (*H. canis*) mol%, which is similar to the G+C content range of *Campylobacter* species. In addition, there are a number of unique *Helicobacter* 16S rRNA gene sequences listed in GenBank that represent sound taxa that have not yet met the criteria for official recognition but could be the basis of future new species.

Interest in gastric spiral bacteria, observed for more than 100 years in animals and humans but rarely cultured, was triggered in 1982 by the discovery of *Campylobacter pyloridis* (later renamed *Helicobacter pylori*) which was cultured from stomach biopsy specimens from human patients with gastritis (4). Phylogenetic analyses based on 16S rRNA gene sequences indicate distinct groups of species within the genus; these fall broadly into groups of gastric (stomach) and enterohepatic (intestine, liver, or biliary tract) origins and include a subgroup of enterohepatic species lacking the characteristic sheathed flagella but possessing an N-linked glycosylation system similar to campylobacter (5) (Fig. 1). Phylogenetic analyses reliant on 16S rRNA gene sequences alone have sensitivity limitations in investigations of closely related helicobacters, which have a high degree of natural transformation and genetic plasticity. Additional phylogenetically informative genes, such as those encoding 23S rRNA and heat shock protein 60, have been used to construct phylogenies of taxa closely related by 16S rRNA gene sequences and to resolve any discordance (6, 7).

The classification of many non-*H. pylori* gastric helicobacters has proven particularly problematic because they are difficult to culture and closely related by 16S rRNA gene sequence analysis (Fig. 1). Large gastric spiral bacteria originally referred to as "*Gastrospirillum hominis*" were subsequently designated "*Helicobacter heilmannii*" (8) and have since been fully characterized as *Helicobacter heilmannii* (9). Further developments have provided clarification that *Helicobacter suis*, recently cultured and formally recognized and named (6), includes samples identified and closely related cultured species (*Helicobacter bizzozeronii*, *Helicobacter salomonis*, and *Helicobacter felis*) typically found in dogs and cats (10). For convenience, *H. heilmannii* and allied species of gastric origin are collectively referred to in this chapter as "*H. heilmannii*"-like organisms (HHLO), as the accuracy of identifications is uncertain in earlier clinical reports (11). In addition, the classification is not fully resolved for organisms with spindle-shaped cells surrounded by periplasmic fibrils and bipolar tufts of sheathed flagella originally referred to as "*Flexispira rappini*" (8). Phylogenetic analysis identified 10 different taxa, of which several grouped with named species of *Helicobacter* (12). Flexispira taxa 2, 3, and 8 are now considered to constitute a single species, *Helicobacter bilis* (13), while taxa 1, 4, and 5 are assigned to *Helicobacter trogontum* (14). Further insights into the taxonomy of the genus are now possible with the availability of complete genome sequences of a number of *Helicobacter* species besides *H. pylori*. Comparative genomics should enable deeper levels of taxonomic analysis as further *Helicobacter* genomes become available.

DESCRIPTION OF THE AGENTS

Members of the genus *Helicobacter* typically are curved, helical or spiral, or fusiform rod-shaped bacteria with or without periplasmic fibers. The cells have sizes ranging from 0.3 to 0.60 μm in width and 1 to 10 μm in length. Cells may become spheroid or form coccoid bodies if they are cultured for a prolonged period or if growth conditions are not optimal. Such forms typically cannot be subcultured. *Helicobacter* species are all Gram negative, cytochrome oxidase producing, and non-spore-forming. Cells are motile and possess either single or multiple flagella. There is considerable diversity among species in flagellum morphology. Flagella are typically sheathed; for example, *H. pylori* has multiple (four to eight per cell) monopolar sheathed flagella with terminal knobs (Table 2). In contrast, *Helicobacter pullorum*, *H. canadensis*, and five other species with unsheathed flagella form a distinct phylogenetic group within

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*This chapter is an updated version of the one that appeared in the 10th edition of this *Manual*; as such, it contains elements contributed by James G. Fox, Francis Megraud, and Robert Owen.

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TABLE 1 *Helicobacter* species, hosts, and disease spectra

Helicobacter species	Primary hosts	Primary site(s)	Disease(s) in humans
Gastric			
H. acinonychis	Large felines (cheetahs)	Stomach	Not reported
H. baculiformis	Cats	Stomach	Not reported
H. bizzozeronii	Dogs, cats	Stomach	Gastritis, ulcer
"*Candidatus* Helicobacter bovis"	Cattle	Stomach	Gastritis
H. cetorum	Dolphins, whales	Stomach	Not reported
H. cynogastricus	Dogs	Stomach	Not reported
H. felis	Cats, dogs	Stomach	Gastritis, ulcer
H. heilmannii	Humans	Stomach	Gastritis, ulcer
H. pylori	Humans	Stomach	Gastritis, ulcer, MALT lymphoma, gastric cancer
H. salomonis	Dogs	Stomach	Gastritis, ulcer
H. suis	Pigs, humans	Stomach	Gastritis, ulcer
Enterohepatic			
H. anseris	Geese	Intestine	Not reported
H. aurati	Rodents (hamsters)	Intestine	Not reported
H. bilis	Rodents, dogs	Intestine, liver	Sepsis
H. brantae	Geese	Intestine	Not reported
H. canis	Dogs	Intestine	Not reported
H. cholecystus	Rodents (hamsters)	Liver	Not reported
H. cinaedi	Rodents, dogs, primates	Intestine	Colitis, sepsis, cellulitis
H. equorum	Horses	Intestine	Not reported
H. fennelliae	Dogs	Intestine	Colitis, sepsis
H. hepaticus	Rodents	Intestine, liver	Not reported
H. macacae	Monkeys	Intestine	Not reported
H. marmotae	Rodents (woodchucks), cats	Intestine, liver	Not reported
H. mastomyrinus	Rodents (*Mastomys*)	Intestine, liver	Not reported
H. muridarum	Rodents	Intestine	Not reported
H. mustelae	Ferrets, minks	Stomach	Not reported
H. pametensis	Birds (terns), pigs	Intestine	Not reported
H. trogontum	Rodents	Intestine	Not reported
H. typhlonius	Rodents	Intestine	Not reported
Unsheathed			
H. canadensis	Chickens, geese	Intestine	Gastroenteritis
H. ganmani	Rodents (mice)	Intestine	Liver disease?
H. mesocricetorum	Rodents (hamsters)	Intestine	Not reported
H. pullorum	Chickens	Intestine	Gastroenteritis
H. rodentium	Rodents	Intestine	Not reported
"*H. winghamensis*"	Rodents	Intestine	Gastroenteritis

the genus (Fig. 1) (note, *H. equorum* clusters with this group by 16S rRNA gene phylogeny but has sheathed flagella and lacks the glycosylation system). Gastric helicobacters found in animals, with the exception of *Helicobacter baculiformis* (a rod-shaped bacterium), have distinctive, tightly spiraled morphologies and can exhibit tufts of up to 20 multiple flagella per cell. The optimum temperature for growth is 37°C. Helicobacters are organotrophs, possess a respiratory type of metabolism, and are microaerobic. The optimal atmosphere for growth varies, as some species, such as *Helicobacter ganmani*, a rodent enteric organism, grow best in an anaerobic cabinet, although strict anaerobiosis is lethal. Successful cultivation of helicobacters typically requires a humid atmosphere maintained at 37°C with reduced levels of oxygen (5 to 10%) and increased levels of carbon dioxide (5 to 12%). The addition of hydrogen (3 to 5%) to the microaerobic atmosphere usually improves growth. *Helicobacter* species grow poorly, if at all, in routine aerobic atmospheres. Key biochemical characteristics, such as urease hydrolysis, nitrate reduction, indoxyl acetate hydrolysis, and alkaline phosphatase activity, vary among species of *Helicobacter* and so are utilized in species identification (Table 2). However, there is no single common feature which reliably distinguishes all species of *Helicobacter* from those of *Campylobacter*. All helicobacters lack the carbohydrate utilization pathways typically exploited in conventional laboratory biochemical tests. Genomic analysis of *H. pylori* shows that it does not appear capable of using complex carbohydrates as energy sources, and comparisons with the *Campylobacter jejuni* genome indicate significant differences in energy metabolism and chemotaxis systems (15).

EPIDEMIOLOGY AND TRANSMISSION

Helicobacter species are isolated from the gastrointestinal and hepatobiliary tracts of a variety of mammalian hosts that include humans, dogs, cats, cattle, sheep, swine, rodents, nonhuman primates, cheetahs, ferrets, rabbits, dolphins, whales, and horses, as well as chickens and wild birds (Table 1).

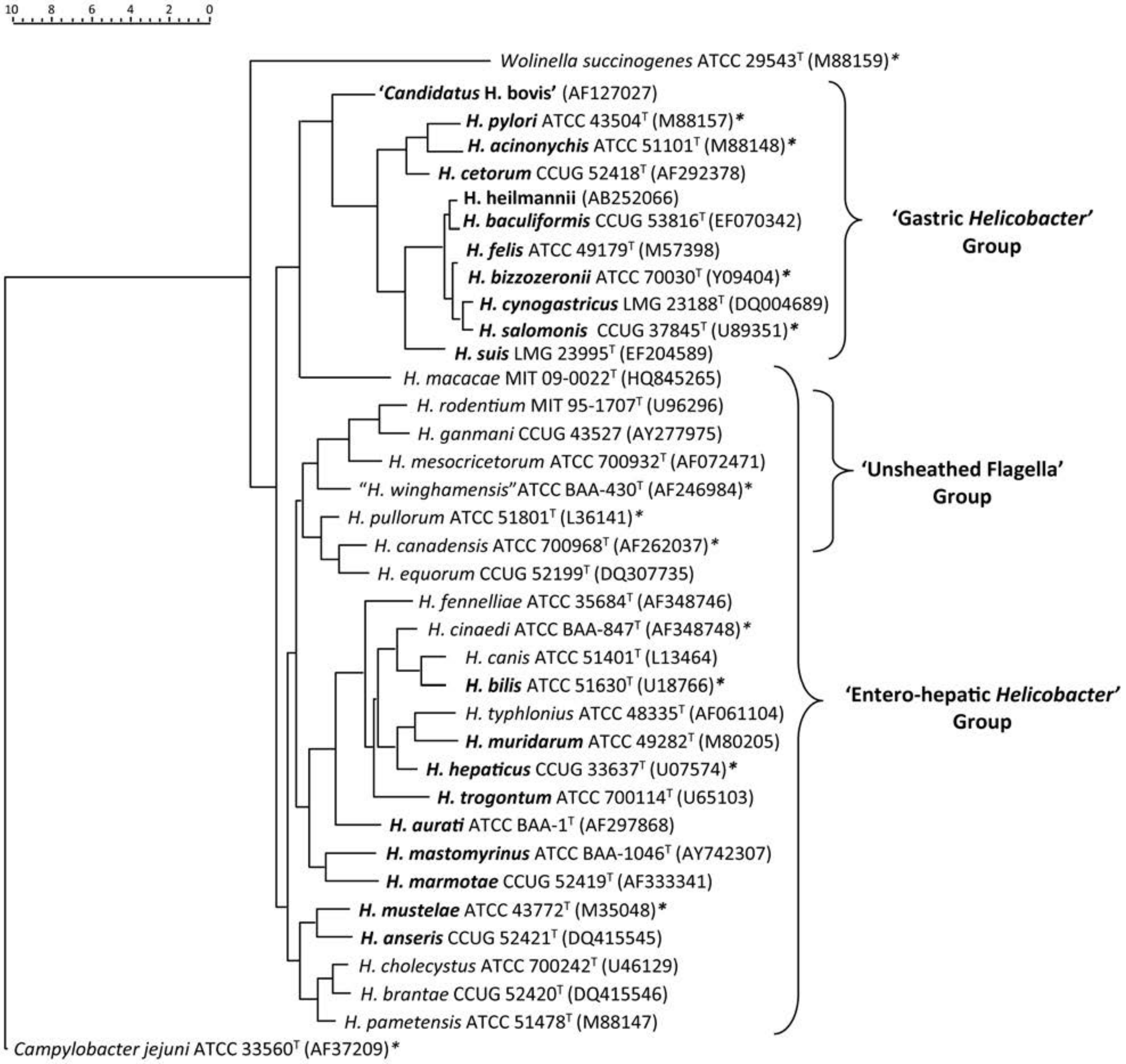
10 8 6 4 2 0
Wolinella succinogenes ATCC 29543^T (M88159)*
'Candidatus H. bovis' (AF127027)
H. pylori ATCC 43504^T (M88157)*
H. acinonychis ATCC 51101^T (M88148)*
H. cetorum CCUG 52418^T (AF292378)
H. heilmannii (AB252066)
H. baculiformis CCUG 53816^T (EF070342)
H. felis ATCC 49179^T (M57398)
H. bizzozeronii ATCC 70030^T (Y09404)*
H. cynogastricus LMG 23188^T (DQ004689)
H. salomonis CCUG 37845^T (U89351)*
H. suis LMG 23995^T (EF204589)
H. macacae MIT 09-0022^T (HQ845265)
H. rodentium MIT 95-1707^T (U96296)
H. ganmani CCUG 43527 (AY277975)
H. mesocricetorum ATCC 700932^T (AF072471)
"H. winghamensis" ATCC BAA-430^T (AF246984)*
H. pullorum ATCC 51801^T (L36141)*
H. canadensis ATCC 700968^T (AF262037)*
H. equorum CCUG 52199^T (DQ307735)
H. fennelliae ATCC 35684^T (AF348746)
H. cinaedi ATCC BAA-847^T (AF348748)*
H. canis ATCC 51401^T (L13464)
H. bilis ATCC 51630^T (U18766)*
H. typhlonius ATCC 48335^T (AF061104)
H. muridarum ATCC 49282^T (M80205)
H. hepaticus CCUG 33637^T (U07574)*
H. trogontum ATCC 700114^T (U65103)
H. aurati ATCC BAA-1^T (AF297868)
H. mastomyrinus ATCC BAA-1046^T (AY742307)
H. marmotae CCUG 52419^T (AF333341)
H. mustelae ATCC 43772^T (M35048)*
H. anseris CCUG 52421^T (DQ415545)
H. cholecystus ATCC 700242^T (U46129)
H. brantae CCUG 52420^T (DQ415546)
H. pametensis ATCC 51478^T (M88147)
Campylobacter jejuni ATCC 33560^T (AF37209)*
'Gastric Helicobacter' Group
'Unsheathed Flagella' Group
'Entero-hepatic Helicobacter' Group

FIGURE 1 Phylogenetic tree of the genus *Helicobacter* based on 16S rRNA gene sequence similarity constructed using the neighbor-joining method. The bar represents the percentage of sequence divergence. Species marked in boldface type are urease positive; those in lightface type are urease negative. An asterisk (*) indicates species for which a genome sequence is either available or currently in progress. Strain collection catalogue and 16S rRNA gene sequence reference numbers (in parentheses) are given where available. A superscript T (^{T}) denotes a type strain. doi:10.1128/9781555817381.ch57.f1

H. pylori

H. pylori, with its characteristic strong urealytic ability, is the gastric helicobacter of humans and is found almost exclusively in the human stomach, which provides the reservoir of infection. Exceptions are isolates from primates previously named *Helicobacter nemestrinae*, which is now considered a synonym of *H. pylori* (16). There is no evidence of animal-to-human transmission. The organism colonizes the cardia, corpus, and antrum of the stomach and may also be found in areas of gastric metaplasia of the proximal duodenum. Studies primarily using serology have been used to study the prevalence of *H. pylori* infection. These studies show a widespread distribution, with estimates that close to half the human global population is colonized, with clinical disease being the exception rather than the rule (17). In the developed world, the prevalence of *H. pylori* seems to be declining; for example, in the United States, an ongoing survey showed that that there had been a significant decline over 2 decades, with an overall decline to 30.7%. The decline is most marked in non-Hispanic whites, but that prevalence was still high amongst Hispanics and African-Americans (18). Likewise, in Europe, the prevalence is higher in eastern countries than in western countries; there is evidence that prevalence rates are declining in developed

TABLE 2 Characteristics of *Helicobacter* species[a]

Helicobacter species	Catalase	Urease	Nitrate	Indoxyl acetate hydrolysis	Alkaline phosphatase	γ-Glutamyl transpeptidase	Growth		Resistance to:		Mol% G+C	Flagellum type (no.)	Flagellum sheath
							At 42°C	With 1% glycine	Nal	Ceph			
Gastric													
H. acinonychis	+	+	−	−	+	+	−	−	R	S	30	B (2–5)	+
H. baculiformis	+	+	+	−	+	+	−	−	I	R	ND	B (11)	+
H. bizzozeronii	+	+	+	+	+	+	+	−	R	S	ND	B (10–20)	+
"*Candidatus* Helicobacter bovis"	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	M/B (1–4)	?
H. cetorum	+	+	−	−	−	+	+	−	I	S	ND	B (2)	+
H. cynogastricus	+	+	+	−	+	+	−	−	ND	ND	ND	B (6–12)	+
H. felis	+	+	+	−	+	+	+	−	R	S	42	B (14–20)	+
H. heilmannii	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	B (10–20)	+
H. pylori	+	+	−	−	+	+	−	−	R	S	35–37	B (4–8)	+
H. salomonis	+	+	+	+	+	+	−	−	R	S	ND	B (10–23)	+
H. suis	+	+	−	−	+	+	−	−	ND	ND	ND	B (4–10)	+
Enterohepatic													
H. anseris	−	+	−	+	−	−	+	W	S	R	ND	B (2)	+
H. aurati	+	+	−	+	+	+	+	−	S	R	ND	B (7–10)	+
H. bilis	+	+	+	−	−	+	−	+	R	R	ND	B (3–14)	+
H. brantae	−	−	−	+	−	−	+	W	S	R	ND	B (2)	+
H. canis	−	−	−	+	+	+	+	−	S	I	48	B (2)	+
H. cholecystus	+	−	+	−	+	−	+	+	I	R	ND	M (1)	+
H. cinaedi	+	−	+	−	−	−	−	+	S	I	37–38	B (1–2)	+
H. equorum	+	−	+	−	+	−	−	−	R	R	ND	M (1)	+
H. fennelliae	+	−	−	+	+	−	−	+	S	S	35	M/B (1–2)	+
H. hepaticus	+	+	+	+	−	−	−	+	R	R	35	B (2)	+
H. marmotae	+	+	−	−	+	−	+	+	R	R	ND	B (2)	+
H. mastomyrinus	+	+	−	−	−	−	+	+	R	R	ND	B (2)	+
H. muridarum	+	+	−	−	+	+	−	−	R	R	34	B (10–14)	+
H. mustelae	+	+	+	+	+	+	+	−	S	R	36	P (4–8)	+
H. pametensis	+	−	+	−	+	−	+	+	S	S	38	B (2)	+
H. trogontum	+	+	+	−	−	+	+	ND	R	R	ND	B (4–7)	+
H. typhlonius	+	−	+	−	−	−	+	+	S	R	ND	B (2)	+
Unsheathed													
H. canadensis	+	−	W	+	−	−	+	+	R	R	33	B (2)	−
H. ganmani	−	−	+	−	−	ND	+	−	R	S	ND	B (2)	−
H. mesocricetorum	+	−	+	ND	+	−	+	−	S	R	ND	B (2)	−
H. pullorum	+	−	+	−	−	ND	+	−	R	S	34–35	M (1)	−
H. rodentium	+	−	+	−	−	−	+	+	R	R	ND	B (2)	−
"*H. winghamensis*"	−	−	−	+	−	ND	−	+	R	R	ND	B (2)	−

[a] +, positive; −, negative; W, weakly positive; Nal, nalidixic acid; Ceph, cephalexin; ND, not determined; S, susceptible; I, intermediate; R, resistant; B, bipolar; M, monopolar; P, peritrichous; ?, unknown.

societies with improvements in sanitation and living standards (19, 20). The prevalence of *H. pylori* infection differs markedly between developing and developed countries (20). In developing countries, infection occurs early in life; most children are infected by the age of 10, and prevalence remains high (up to 90%) for all adult age groups. In contrast, in developed countries, a progressive increase in prevalence is observed, from a low percentage of infection in those born after 1980 (<30 years old) to 40 to 50% infection rates in the older age groups. This is not the consequence of a progressive acquisition of the infection but rather the result of a cohort effect (20, 21). Reported incidences of culture confirm that infections vary considerably from country to country depending on local treatment guidelines and culturing practices.

The modes and routes of transmission of *H. pylori* from person to person remain to be definitely proven. There is epidemiological evidence for both oral-oral and fecal-oral transmission, with the latter being more likely in developing countries, where sanitation and contaminated water supplies may pose a greater risk (20). The role of contaminated public water supplies has never been convincingly proven because of the rarity of culture-positive water samples (22). There is no evidence that viable cells of *H. pylori* can survive the disinfection levels in properly maintained main supplies, although survival may be possible as a viable nonculturable form (23). Biofilms within water distribution systems have been suggested as possible sites of passive accumulation (24).The rationale for oral-oral transmission relies on the presence of *H. pylori* in regurgitated gastric juice, thus allowing *H. pylori* to temporarily colonize the oral cavity. Another possibility is via vomitus, in which *H. pylori* can remain viable for hours (25). Person-to-person transmission appears to be most frequent in intrafamilial settings during childhood, particularly between mothers and siblings, as well as among siblings and between other household contacts (26). Family groups provide the best opportunity to study person-to-person transmission, but interpretation of evidence is complex. Patterns of frequent horizontal spread deduced from DNA sequence types were found both within families and between unrelated individuals in rural South Africa, which may be a situation representative of high-prevalence areas in large parts of the developing world (27). In urban families, in contrast, clonal transmission of *H. pylori* was more frequent between first-degree relatives.

HHLO

Human infections with HHLO are generally considered uncommon, with prevalence rates detected by histological observation ranging from <0.3% in developed countries to about 6% in other regions (27). A higher rate of 2% was indicated for some United Kingdom patients by a direct biopsy PCR assay designed to detect multiple HHLO species (28). Now that individual HHLO taxa are better defined, retrospective reassessment using species-specific assays of past cases attributed to *H. heilmannii* provides evidence of infection with one or more species of zoonotic origin, notably *H. salomonis*, *H. felis*, *H. suis*, and “*Candidatus* Helicobacter bovis” (10). These findings indicate cats, dogs, and swine as possible sources of infection, but modes of transmission are unknown.

Enterohepatic Helicobacters

Enterohepatic helicobacters inhabit the intestinal and hepatobiliary tracts of various mammal and bird hosts, and several species, such as *H. bilis*, *H. canadensis*, *H. canis*, *H. cinaedi*, *H. fennelliae*, *H. pullorum*, and “*H. winghamensis*,” infect humans with clinical symptoms (Table 1). Little is known about prevalence and routes of transmission of these species, but the implications are that they are transmitted to humans from animals. *H. pullorum* is a recognized zoonotic risk, as it has been identified in carcasses of broiler chickens and laying hens (29) and on uncooked retail chicken (30).

CLINICAL SIGNIFICANCE

H. pylori

Marshall and Warren (31) first proposed the association of *H. pylori* with peptic ulcer disease, and since then it has become established as the most clinically important species of *Helicobacter*. It is recognized as the main cause of peptic ulcer disease and a major risk factor for gastric cancer (32). *H. pylori* infection is also an independent risk factor for the development of atrophic gastritis, gastric ulcer disease, gastric adenocarcinomas, and gastric mucosa-associated lymphoid tissue (MALT) lymphomas (21). Individuals infected with *H. pylori* may develop acute gastritis (abdominal pain, nausea, and vomiting) within 2 weeks following infection. The species establishes a chronic infection in the majority of infected people, represented by chronic gastritis. Prominent mucosal inflammation is often evident in the antrum (antrum-predominant gastritis), predisposing patients to hyperacidity and duodenal ulcer disease. Many patients infected with *H. pylori* have recurrent abdominal symptoms (nonulcer dyspepsia) without ulcer disease, and there appears to be a clinical benefit in eradicating *H. pylori* in these patients (33). Duodenitis often occurs with *H. pylori* infection, and duodenal ulcers develop in as many as 16% of infected individuals (34). Gastric MALT lymphoma, a rare stomach cancer, is caused by *H. pylori* infection and is the only cancer which can possibly be cured by antibiotics (35). Eradication of *H. pylori* is also recommended in cases of unexplained iron deficiency anemia and chronic idiopathic thrombocytopenic purpura (36). The clinical significance of *H. pylori* infection remains speculative in a number of other chronic conditions, notably ischemic heart disease, inflammatory bowel disease, and liver and biliary tract diseases (37).

HHLO

HHLO infection has been associated with mild-to-moderate gastritis, peptic ulcer disease, and gastric MALT lymphomas in adults, although it has not unequivocally been established as a causative agent (10, 38). The etiology of HHLO infections is unclear because they are uncommon, and organisms are unculturable in a routine clinical laboratory (10).

Enterohepatic Helicobacters

Isolated cases of infections in adults and children with enterohepatic helicobacters have been reported over the past 25 years, but their clinical significance is often not clearly established. Isolates are mainly from blood and, to a lesser extent, from fecal samples (39, 40). The bacteremia-associated helicobacters, although rare, are the most clinically significant, as they occur more frequently in patients with underlying conditions. It is presumed that these helicobacters are able to invade the bloodstream via colonization of the human lower gastrointestinal tract, possibly from mucosal cells damaged by combined chemo- and radiotherapy.

H. cinaedi infections may present in various clinical manifestations (proctocolitis, gastroenteritis, neonatal meningitis, localized pain and rash, and bacteremia), particularly in individuals with underlying immunosuppressive conditions.

It has also been associated with coronary artery disease (39, 41). *H. fennelliae* has been implicated as a cause of human proctocolitis, gastroenteritis, and bacteremia, particularly in immunocompromised individuals (40, 41). *H. pullorum* has been associated with several cases of human gastroenteritis (42).

Other species of *Helicobacter* isolated occasionally from infected humans but of unclear clinical significance include *H. canadensis* (43) and "*H. winghamensis*" (44) from cases of gastroenteritis. *H. canis* has been associated with inflammatory bowel disease and *H. hepaticus* with hepatobiliary disease (39). Recently, a possible link between *H. suis* and Parkinson's disease has been proposed (45). In this study, *H. suis* DNA was detected by a real-time PCR assay in archived DNA from gastric biopsy specimens with a higher frequency from patients with Parkinson's disease than from a control group.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Gastric Biopsy Specimens

Gastric biopsy specimens for the direct diagnosis of *H. pylori* are routinely obtained from the antrum and corpus by esophagogastroduodenoscopy. While sterile normal saline may be sufficient for short-term (up to approximately 2 h) transport of gastric biopsy specimens, a transport medium should be used if available to maintain the viability of the organisms for culture. *H. pylori* is sensitive to desiccation and to ambient atmosphere and temperature. A semisolid transport medium (e.g., Portagerm pylori [bioMerieux Inc., Durham, NC]) or an in-house transport medium that comprises brain heart infusion broth (3.5%), yeast extract (2.5%), sterile horse serum (5%), and *Helicobacter pylori* selective supplement (Dent's; 10-μg/ml vancomycin, 5-μg/ml trimethoprim, 5-μg/ml cefsulodin, and 5-μg/ml amphotericin B [Oxoid Ltd., Basingstoke, United Kingdom]) may be used. Alternative media include Stuart's transport medium or brucella broth with 20% glycerol. If culture of *H. pylori* is not possible within 24 h, it is recommended that biopsy specimens be stored overnight at 4°C in a transport medium and then transported at ambient temperature. For longer-term storage, biopsy specimens should be frozen at −70°C in a 10% glycerol-containing medium.

Fecal Specimens

H. pylori and other gastric helicobacters cannot ordinarily be isolated from human fecal specimens, so samples are not recommended for routine culture. Fecal samples are used for *H. pylori* stool antigen tests (SAT) and either should be tested immediately or should be stored immediately at −20°C. Repeated thawing and freezing of samples should be avoided. As enterohepatic helicobacters can cause enteric disease, fecal specimens may be required for culture. However, campylobacters are more likely to be tested for in the first instance, and relevant protocols for their collection, transport, and storage also can be used for enterohepatic species of *Helicobacter* (as for *Campylobacter*, see chapter 56).

Blood Specimens

Blood specimens are required for serological diagnosis of an *H. pylori* infection and may be collected, transported, and stored by standard protocols. Also, as the enterohepatic helicobacters may translocate across the intestinal barrier and cause invasive infections, peripheral venous blood from suspected cases may be required for microbiological testing. Blood may be collected in commercially available aerobic and anaerobic blood culture bottles and transported and stored according to the protocols used for campylobacters, which are more likely be tested for in the first instance (for *Campylobacter*, see chapter 56).

Other Clinical Specimens

Laboratory tests requiring the collection of other types of specimen have been developed to assist in the diagnosis of *H. pylori* infection and may be undertaken under some circumstances.

Gastric juice, obtained from the patient either by aspiration after the introduction of a nasogastric tube or by the so-called string test (basically, the patient swallows a string which is then regurgitated), has been used as a possible source of *H. pylori* for culture and PCR. Gastric juice does not offer a satisfactory alternative to a biopsy specimen as a routine specimen because of problems caused in culture by overgrowth of nasopharyngeal microbiota unless preventive steps such as acid pretreatment are taken (46). Specimens, if used, should be transported at 4°C and processed without delay.

Urine

Fresh urine samples required for antigen testing tests should be collected and transported by standard protocols. Urine samples cannot be frozen because any resultant protein precipitation may interfere with the tests.

Saliva

Saliva samples required for serological tests can be collected easily by having the patient spit into a tube. An alternative that may be preferable is use of a special swab device rubbed over the gums that is designed to obtain gingival transudate enriched in immunoglobulin G (IgG) (47). Specimens should then be transported by routine protocols.

DIRECT EXAMINATION

Microscopic Examination of Gastric Biopsy Specimens

Histopathological examination of gastric biopsy specimen sections preserved in a fixative (10% formaldehyde) and embedded in paraffin is widely used for diagnosis of *H. pylori* infection. Standard hematoxylin and eosin tissue staining is not sufficient to detect *H. pylori*, whereas the Warthin-Starry stain allows excellent visualization of bacteria if performed by trained histology personnel. Although the specificity is usually adequate, the presence of bacteria with atypical morphologies may result in misinterpretations. Under optimal conditions, histological diagnosis has a sensitivity and specificity of 95% (20, 48). Immunohistological staining with specific *H. pylori* antibodies can improve specificity. Histological methods and interpretation of histological findings are outside the scope of this chapter, but from the microbiology laboratory perspective, microscopic examinations of a smear prepared directly from a biopsy specimen or from imprint cytology provide rapid bacteriological test results for observation of cells of *H. pylori*. Staining can be performed using Gram stain, rapid Giemsa stain, or the fluorescent acridine orange stain. The less-common gastric HHLO can also be Giemsa stained and, when observed microscopically, can be distinguished from *H. pylori* by their distinct tightly spiral morphology (49).

Microscopic Examination of Feces and Other Pathological Specimens

Direct Gram stain analysis of fecal smears and other clinical samples is not routinely performed for the detection of *H. pylori* or other helicobacters. Direct identification of helicobacters in positive blood cultures may require special stains, particularly if tests are performed by personnel unaccustomed to looking for such organisms. Thin, gull-shaped bacteria such as *H. cinaedi* can be difficult to observe by Gram staining and require acridine orange staining, dark-field microscopy, or Giemsa staining. A modified Gram stain with carbol (0.5%) or basic fuchsin (0.1%) as the counterstain is also recommended for detection (50).

Urease Testing of Gastric Biopsy Specimens for *H. pylori*

H. pylori produces large amounts of extracellular urease, which can rapidly be detected following introduction of gastric biopsy tissue into a urea-containing medium as a rapid urease test (RUT) (20). Urease catalyzes the hydrolysis of urea into ammonia and carbonate. The net effect of ammonia production is to increase local pH. Detection of urease activity forms the basis of several simple, inexpensive, and easy-to-perform tests that are usually performed in an endoscopy unit by clinicians. Biopsy specimens are placed either in an agar gel or on a paper strip containing a pH indicator. If organisms are present in sufficient numbers, a color change will occur as a result of urea breakdown and ammonia production. Commercial RUTs that include agar gel-based tests (e.g., CLOtest [Kimberly-Clark, Neenah, WI]) and paper-based strip tests (e.g., PyloriTek [BARD, Murray Hill, NJ] and *H. pylori* Quick test [Biohit, Helsinki, Finland]) have been critically evaluated, and specificities are usually excellent (20). Detection sensitivity also is high but is dependent on the *H. pylori* density in mucosal biopsy specimens and the number of biopsy specimens sampled. RUTs have their optimal sensitivity after 24 h of incubation, whereas strip tests are optimal within an hour, making them truly rapid tests. Urease broth media commonly available in microbiology laboratories, such as modified Christensen medium and urea-indole medium, can be used but are not optimized to have sensitivities equivalent to those of commercially available kits.

Urea Breath Test

Another important clinically performed test, based on the ability of *H. pylori* to produce urease and developed specifically for detection of an active infection, is the urea breath test (UBT). The UBT has the advantage of being noninvasive, as urea, labeled with a nonradioactive natural isotope (^{13}C), is ingested by the patient. The labeled CO_2 is absorbed by the blood and exhaled in expired air. The testing methodology and factors influencing the result, standardization, and application in different clinical settings have been comprehensively reviewed (20). The use of the UBT has high diagnostic accuracy (>95%) (36) and, where available, is consistently recommended for the diagnosis of *H. pylori* infections in adults in both pre- and posttreatment settings. A recent prospective multicenter study indicated that the ^{13}C UBT was also simple and accurate for diagnosis of *H. pylori* infections in children (51).

H. pylori Fecal Antigen Detection

SAT using an enzyme-linked immunosorbent assay (ELISA) provide another valuable aid in the diagnosis of an active *H. pylori* infection. The test is easy to perform and has the advantage of being noninvasive. Since becoming commercially available, kits consisting of a polyclonal antibody fixed on microwells (e.g., Premier Platinum HpSA [Meridian Bioscience, Inc., Cincinnati, OH]) have been extensively evaluated on samples from adults and children and have proven to be an excellent diagnostic tool. A systematic review of published data up to 2004 confirmed the value of such kits for primary pretreatment as well as for follow-up posttreatment diagnosis (52). The test was further developed by using specific monoclonal antibodies, and reviews and meta-analysis based on evaluations of kits (e.g., IDEIA HpStAR [Oxoid Ltd., United Kingdom] and Premier Platinum HpSA PLUS [Meridian Bioscience, Inc., Cincinnati, OH]) indicated improved sensitivity compared to those of polyclonal tests. For example, high sensitivity (94%) and specificity (100%) were reported for tests on pretreatment adult stools in England (53), and the performance of tests was reported to be excellent for young children in Finland (54). If the UBT is not available, the laboratory-based stool antigen test is recommended for confirmation of eradication between 2 and 4 weeks after treatment (20). The presence of some false positives has been noted in the use of stool antigen tests for posteradication diagnosis, possibly attributable to the presence of antigen in stools from degraded coccoid forms (54). Some stool samples that were transiently positive by ELISA also have been reported for children and were thought to have transient infections with *H. pylori* or other *Helicobacter* species (55). Monoclonal antibodies are used also in immunoenzymatic rapid point-of-care tests for diagnosis of *H. pylori* infection (e.g., the ImmunoCard STAT! HpSA [Meridian Bioscience, Inc., Cincinnati, OH] and *RAPID* Hp StAR [Oxoid Ltd., Basingstoke, United Kingdom]), and their performance in the clinical/near-patient setting has been critically evaluated and in general they are not thought to be as accurate or reliable as the ELISA-based tests (36, 53).

Nucleic Acid Detection

Detection of *H. pylori* in Gastric Biopsy Specimens

Nucleic acid assays based on PCR amplification and on fluorescence *in situ* hybridization with species-specific probes provide useful approaches for the detection of *H. pylori* in gastric biopsy specimens, as they are significantly faster than culture. The commonest targets for amplification are 16S rRNA, *ureA*, *glmM* (formerly named *ureC*), *vacA*, and *cagA* genes (56), and in addition, 23S rRNA genes have been targeted for both detection and antibiotic susceptibility testing (21, 93). There is currently no gold standard method for use in the clinical laboratory setting for PCR of gastric biopsy specimens, and so it is advised that PCR-based assays should not be the sole basis of determining the *H. pylori* status of a patient (56). Nevertheless, PCR assays can provide added value in investigating culture-negative gastric biopsy specimens, particularly those from cases for which other clinical tests indicate an *H. pylori* infection. A systematic study of primers for *H. pylori* detection found that the four best-performing assays each attained a detection limit of <100 CFU/ml from gastric tissue (56). However, no assay had 100% specificity or sensitivity, and all produced false positives. Two of the best all-around assays based on the HP64-f/HP64-r primers for the *ureA* gene and HP1/HP2 primers for the 16S rRNA genes had sensitivities and specificities of >90% with gastric biopsy specimens.

Detection of *H. pylori* in Feces

The PCR assays developed for biopsy specimen testing, in particular those using primers targeting the 16S rRNA and *ureA* genes, have been applied to stools to detect *H. pylori* with various success rates, and their value for routine laboratory use is questionable. Feces is a complex material containing a number of PCR inhibitors (57), and complex DNA purification methods are needed to either eliminate or reduce the levels of such compounds. The performance of the assays is restricted by the low numbers of *H. pylori* cells in feces and by degradation of DNA during transit through the intestinal tract. Another test uses a biprobe 23S rRNA gene real-time PCR assay (58), and a modified version (ClariRes assay [Ingenetix, Vienna, Austria]), as well as clarithromycin susceptibility testing of stool specimens of symptomatic children (59), has been applied to detection (for further details, see "Antimicrobial Susceptibility" below). However, it should be noted this assay was applied to extremely fresh fecal samples.

Detection of HHLO in Gastric Biopsy Specimens

Specialist assays have been developed for direct PCR detection of species of HHLO in gastric biopsy specimens (60), and simultaneous testing for both *H. pylori* and HHLO can be performed using a multiplex PCR assay (28). Fluorescence *in situ* hybridization tests with species-specific probes can also be applied to detect HHLO in human gastric biopsy specimens (61).

Detection of Other Helicobacters in Clinical Samples

With the exception of an unvalidated 16S rRNA gene PCR assay for detection of *H. pullorum* in human fecal extracts (62), there are no assays suitable for fecal detection of enterohepatic *Helicobacter* species of clinical relevance. Genus-level PCR-based assays targeting mainly 16S rRNA genes have been developed and used for direct detection of other helicobacters in a variety of clinical samples that include dental plaque and saliva (63), intestinal tissue biopsy specimens (64), and liver biopsy specimens and associated tissues (bile and gallbladder) (65). These assays are generally undertaken for specific epidemiological and disease association investigations and so are unlikely to be used in the routine laboratory. Results of such PCR-based assays performed in the absence of other evidence therefore should be interpreted with caution (56).

ISOLATION PROCEDURES

Isolation of *H. pylori*

H. pylori is readily isolated by culture from gastric biopsy specimens. Tissue should be streaked over the culture medium with a minimum of delay or first homogenized to facilitate a higher yield of bacteria. Agar-based media such as brain heart infusion agar, brucella agar, Wilkins Chalgren agar, and Trypticase soy agar can be used for primary culture. In our experience, Columbia agar base supplemented with 10% defibrinated horse blood gives excellent results. A selective medium (e.g., *Helicobacter pylori* selective medium [Oxoid Ltd., Basingstoke, United Kingdom]) containing Dent's antibiotic supplement (see "Collection, Transport, and Storage of Specimens" above) also gives adequate isolation results. Plates should be incubated at 35 to 37°C in a humid microaerobic atmosphere (4% O_2, 5% CO_2, 5% H_2, and 86% N_2) achieved using either a gas jar and gas-generating system or an incubator (e.g., the MACS VA500 microaerophilic workstation [Microbiology International, Frederick, MD]). The exact gas mixes used vary between laboratories, but the presence of 5% H_2 in the atmosphere enhances growth. Culture plates should be observed daily for the appearance of small, smooth, circular colonies, which should appear after 48 h of incubation. Plates must be incubated for a minimum of 10 days before a negative result is given. Colonies should be subcultured on nonselective medium for further investigation. Isolates may be stored at −80°C in cryovials (e.g., the Microbank bacterial preservation system [PRO-LAB Diagnostics, Austin, TX]).

Isolation of Other Helicobacter Species

There are no recommended culture methods available currently for use in routine clinical laboratories for isolation of HHLO from human gastric biopsy specimens. The first and only reported successful isolation of "*H. heilmannii*" from a human, achieved after 7 days with a nonselective medium (7% lysed horse blood) in a 5% O_2 and 10% CO_2 atmosphere, was subsequently identified as *Helicobacter bizzozeronii* (66). A novel isolation method using high acidity and modified gaseous conditions has now been developed for isolation of *H. suis* from pig gastric tissue (6) and has recently been applied to human tissue.

Enterohepatic helicobacters such as *H. bilis*, *H. canadensis*, *H. canis*, *H. cinaedi*, *H. fennelliae*, *H. pullorum*, and "*H. winghamensis*" are isolated typically during investigation for campylobacters in feces from humans with gastroenteritis. These organisms grow at 37°C but not uniformly at 42°C, the temperature most often used for isolation of *C. jejuni*. Fresh stool specimens should be examined using a selective medium or the nonselective membrane filter method (67) with incubation for a minimum of 7 days at 37°C in a microaerobic atmosphere (68). Strains of some species may require 5 to 10% H_2 for optimum growth, and recovery may be hindered if they are susceptible to antibiotics present in the selective isolation medium.

Some enterohepatic helicobacters, such as *H. cinaedi*, *H. canis*, and *H. fennelliae*, are isolated occasionally from the blood of patients with suspected bacteremia using commercial blood culture systems (e.g., the Bactec system [BD, Sparks, MD]). Isolates are usually detected in aerobic blood culture bottles only and may be problematic to recover, as they are difficult to see microscopically and will probably grow poorly on subculture if plates are not incubated for an extended period (minimum of 6 days) in a microaerobic atmosphere. The isolation of *Helicobacter* species from other sterile body fluids is rare, but a notable example is the isolation of *H. cinaedi* from joint fluid using a nonselective blood medium (69).

IDENTIFICATION

Identification of *Helicobacter* species is based on a limited range of morphological, physiological, and biochemical characteristics (Table 2). *Helicobacter* species have various colony phenotypes on blood agar, ranging from the discrete, gray, and translucent colonies of *H. pylori* to swarming phenotypes of some gastric helicobacters (e.g., *H. felis*). Most isolates are motile and should be routinely tested for oxidase, catalase, and urease activities according to recommended procedures (70). The advent of whole-cell matrix-assisted laser desorption ionization–time-of-flight spectrometry (MALDI-TOF) promises to make the identification of *Helicobacter* species a great deal easier but will ultimately depend on the quality of the databases used by the laboratories (71).

H. pylori and Other Gastric Helicobacters

In stained gastric biopsy samples, *H. pylori* cells usually have a curved or helical morphology. However, on subculture, this "classical" morphology is often lost, and in Gram-stained preparations, cells may appear curved, U shaped, or even as straight rods. HHLO cells are larger in size and have a more pronounced helical morphology in histological examinations of gastric biopsy specimens (49). *Helicobacter* cells may appear faint on conventional Gram staining and require prolonged counterstaining with carbol fuchsin (0.5%) for enhanced visualization. Urease-negative organisms may be present occasionally in gastric biopsy specimens, as *H. cinaedi*, although not cultured, has been identified by DNA analysis (72). It is important, therefore, to perform other key biochemical tests, such as indoxyl acetate hydrolysis and catalase, to identify isolates of any unexpected species. Ultimately, MALDI-TOF identification will probably be the simplest approach. However, at the time of writing, it is unclear how extensive the Bruker databases for *Helicobacter* species are. However, from personal experience, the most common species are present (71, 73). The MALDI-TOF technique was used to great effect in a recent study of *H. cinaedi* strains (74) where the facility of the technique to subtype strains was explored.

Enterohepatic Helicobacters

Enterohepatic helicobacters may appear as a swarming thin film (e.g., *H. cinaedi* and *H. fennelliae*) or as discrete single colonies (e.g., *H. canadensis* and *H. pullorum*). By light microscopy, they morphologically resemble other Gram-negative spiral or curved bacteria. The enterohepatic species possess several distinguishing characteristics (Table 2), and biochemical and tolerance tests should be carried out according to the recommended procedures (43). Species lacking urease activity isolated from humans, such as *H. canadensis*, *H. canis*, *H. cinaedi*, *H. fennelliae*, and *H. pullorum*, superficially resemble enteric campylobacters, and definitive identification may not be possible from phenotype alone. Useful distinguishing tests are growth at 42°C, as both *H. cinaedi* and *H. fennelliae* are negative, and indoxyl acetate hydrolysis, for which *C. jejuni* and *Campylobacter coli* are positive and *H. pullorum* is negative. A PCR assay is described for identification of *H. pullorum* (42), but the assay does not distinguish *H. pullorum* from *H. canadensis*, which characteristically hydrolyzes indoxyl acetate and is resistant to nalidixic acid (43). *H. canis* is unlike most other helicobacters in being both catalase negative and urease negative, features that may cause confusion with "*H. winghamensis*" and *H. bilis*. Growth at 42°C and the nitrate reduction and indoxyl acetate hydrolysis tests may be useful to distinguish *H. canis* from other catalase-negative campylobacters. It is important to be aware that fecal specimens occasionally can be cocolonized with multiple *Helicobacter* and *Campylobacter* species, so making a complete diagnostic evaluation is challenging. *Helicobacter* genus-specific PCR assays may be useful and sequencing of 16S rRNA genes may be required for a definitive identification. However, 16S rRNA gene phylogeny is not suitable for distinguishing clades of closely related species such as the HHLO (10).

TYPING SYSTEMS

Typing of *H. pylori*

Typing isolates of *H. pylori* has no role in direct patient management (36). Even so, typing data may be useful in monitoring the effects of therapy and to establish whether a persistent infection is due to eradication failure or reinfection, in investigating associations between strain type and disease severity, in epidemiological investigations of routes and modes of transmission, and in investigating the ancestry of strains worldwide that might be relevant in vaccine development. There is no generally agreed-upon system for typing isolates of *H. pylori*; although many different methods have been applied and evaluated, genotypic methods are the most widely used means of characterizing individual isolates of *H. pylori*. A key feature of *H. pylori* is its high genetic diversity, with almost every isolate having a unique genotype arising from within-genome diversification and reassortment by natural homologous recombination (27). This diversification is thought to aid *H. pylori* in persistence during chronic infection and in adapting to new gastric environments.

The highly polymorphic vacuolating cytotoxin (*vacA*) gene provides the basis of a widely adopted PCR-based genotyping scheme with recommended primers (75). The *vacA* allelic type is determined by the presence or absence of short, conserved nucleotide inserts within the signal and middle regions (76). Common *vacA* allelic types identified worldwide are s1/m1 (vacuolating), s1/m2 (selectively vacuolating), and s2/m2 (nonvacuolating). The signal region alleles can be further divided into s1a, s1b, and s1c subfamilies, and likewise, the midregion is subdivided into m2a and m2b subfamilies (77). Genotyping can be performed by using either individual PCR assays or multiplex PCR assays (78, 79). Molecular fingerprinting methods applied to *H. pylori* include electrophoretic protein profiling, ribotyping, restriction fragment length polymorphism analysis, pulsed-field gel electrophoresis, amplified fragment length polymorphism analysis, and plasmid profiling (77). Multilocus sequence typing is probably the most widely used molecular subtyping system for *H. pylori*; it has been used to investigate intrafamilial transmission (73) and to study global patterns of *H. pylori* epidemiology (79). The PubMLST database currently holds somewhere in the region of 2,000 *H. pylori* profiles. It is perhaps a little outside the scope of this chapter, but with the advent of so-called next-generation sequencing, it is possible to sequence whole genomes relatively quickly and inexpensively. The advent of the Bacterial Isolate Genome Sequence Database (BIGSdb) (80) will ultimately allow complex analysis of whole genomes in a scalable fashion.

Typing of Other Helicobacters

The need to type species of *Helicobacter* other than *H. pylori* is unlikely, and the genotyping schemes described are of questionable value for routine use.

SEROLOGIC TESTS

Detection of *H. pylori* Antibody in Blood

H. pylori infection induces a specific systemic immune response to multiple antigens, with only 2% of patients failing to seroconvert (81). The immune response typically shows a transient rise in specific IgM antibodies followed by a rise in IgG and IgA antibodies that persists during infection. Serology is widely used in primary screening for *H. pylori* infection, as it is a simple, noninvasive test. A number of in-house and commercial kits have been developed over the past 20 years for antibody detection, with the essential laboratory technique being the standard ELISA. The performance and diagnostic utility of laboratory ELISA kits (e.g., Cobas Core enzyme immunoassay [Roche, Mannheim, Germany]) and rapid near-patient immunochromatographic tests (e.g., FlexSure HP [Beckman Coulter Inc., Brea, CA]) have been critically evaluated in several reviews and meta-analyses (20). Serology (ELISA) kits that measure IgG antibodies are recommended based on overall performance as an accurate means of diagnosing infection (20, 36). The relevance of IgA in testing is more controversial (82). Some investigators have observed IgA to be equal to IgG in performance, but a recent evaluation concluded that IgA alone yielded poorer overall sensitivity and specificity, although it performed better in samples from children than those from adults (82). IgM has been found to have little diagnostic value, with an unacceptably low sensitivity (82). The Maastricht IV Consensus Report recommended serological test kits with high accuracy (>90%) in validated settings (36).

Serology is not recommended for posteradication follow-up when tests detecting an active infection are preferable (81). Antibody titers decrease very slowly after eradication, so a single serum sample does not differentiate past and ongoing infections. Some 30% of patients have elevated IgG antibodies even after 5 years of successful eradication therapy (83). False positives, therefore, could result in some patients being inappropriately treated for presumed *H. pylori* infection, particularly in low-prevalence populations. Serology is useful in epidemiological studies of *H. pylori* infections, but such analyses likewise need to take into account that some asymptomatic individuals without an active infection may test positive.

Detection of *H. pylori* Antibodies in Urine and Saliva

Specific *H. pylori* IgG antibodies are present in urine at low concentrations. A review of 18 published studies over the period 1998 to 2004, using kits that included commercial ELISAs and rapid immunoenzymatic tests, listed sensitivities and specificities ranging from 82 to 100% and from 68 to 100%, respectively (81). While the accuracy is not affected by the pH or the presence of bacteriuria, it may be influenced by a large amount of total IgG. Detection of *H. pylori* antibody in urine is attractive because it is noninvasive and it could be useful for epidemiological studies. Salivary antibodies are secreted during the immune response to *H. pylori* infections. Several commercial kits and in-house ELISAs have been developed to detect *H. pylori*-specific IgG in saliva, and a review of 15 published studies between 1994 and 2002 listed sensitivities and specificities ranging from 64 to 94% and from 58 to 95%, respectively (81).

Detection of Other *Helicobacter* Antibodies in Clinical Samples

Serology has no application in the routine diagnosis of human infections with gastric HHLO and enterohepatic helicobacters, as there are no validated IgG or IgA assays currently available. Sustained immunoglobulin responses to multiple antigens of *H. cinaedi* and *H. fennelliae* have been documented.

ANTIMICROBIAL SUSCEPTIBILITY

H. pylori Antibiotic Therapy and Relevance of Resistance

The first-choice standard triple therapy to eradicate *H. pylori* comprises a proton pump inhibitor, clarithromycin, and either amoxicillin or metronidazole (36). Therapy should ideally be based on pretreatment antibiotic susceptibility testing, although this is not always practical. The main cause of failure to eradicate *H. pylori* with the standard antimicrobial regimen is clarithromycin resistance (81). In 2004, clarithromycin resistance rates were 13% in the United States (84), while in Europe there are distinct regional variations with resistance rates of 8, 19, and 22% in Northern, Central, and Southern Europe respectively (53). The clinical impact of resistance is marked, with an eradication rate for the standard therapy that decreased by 70% (from 88 to 18%) (81). The key risk factor for clarithromycin resistance is previous consumption of macrolides, and prevalence of resistance after failure of treatment is extremely high. Monitoring local clarithromycin prevalence rates is important, as the recommended threshold at which clarithromycin should not be used or susceptibility testing should be performed is 15 to 20% (53). Resistance to metronidazole, a key component of the triple-therapy regimen, is also widespread and is estimated to decrease treatment success rates by 26% (85). Resistance rates are 25% in the United States (84), with rates in Europe ranging from 29 to 44% (54). In some other countries, resistance rates may be as high as 60 to 90%. *In vitro* resistance to metronidazole may not accurately reflect *in vivo* resistance (85), and for that reason, routine susceptibility testing is not recommended in Maastricht IV guidelines (36). Nevertheless, laboratory testing is important for surveillance of resistance, as a threshold of resistance in the population of 40% provides a guide in deciding on the choice of treatment (36).

Resistance of *H. pylori* to other antibiotics used in therapy, such as amoxicillin and tetracycline (an antibiotic used in second-choice treatment), is rarely found (<1%) in the United States and Europe (48, 84), although higher rates to both antibiotics have been reported in some Asian populations (86). Two other classes of antibiotics have emerged as third choices (rescue therapy) in the treatment of *H. pylori* infection: a fluoroquinolone, levofloxacin, and a rifamycin, rifabutin. Increasing consumption of fluoroquinolones may lead to higher prevalence of resistance in *H. pylori*, as rates of resistance to levofloxacin are currently about 9% in the United States (87) and between 8 and 19% in Europe (48). Resistance to rifabutin is virtually absent in *H. pylori* (48, 88), although its use in eradication therapy has been limited. The efficacy of furazolidone has also been evaluated (89), but no data are available on resistance rates.

Phenotypic Susceptibility Testing of *H. pylori* Cultures

Gastric biopsy isolates of *H. pylori* should be tested against the antibiotics commonly used in eradication therapy, in particular, clarithromycin, as resistance *in vitro* is clinically relevant. Phenotypic methods of susceptibility testing, such as broth microdilution, disk diffusion, the Etest, and agar dilution, can be applied to *H. pylori*. The Clinical and Laboratory Standards Institute (CLSI) and a workgroup of the European Helicobacter Study Group have made a similar recommendation of an agar dilution method and breakpoint for testing susceptibility to clarithromycin (48). In this method, Mueller-Hinton agar base with 5% aged sheep blood is incubated for 72 h at 35°C, with an MIC breakpoint for resistance of 1 μg/ml. The Etest (bioMérieux Inc., Durham, NC) may also be used to determine MIC (90), and its results correlate well with agar dilution results. The disk diffusion method is cost-effective for routine testing, and an inhibitory zone of less than 17 mm around a clarithromycin disk indicates a resistant strain (91).

Metronidazole *in vitro* susceptibility testing is intrinsically less reliable in terms of inter- and intralaboratory reproducibility and is more difficult to standardize, as results appear to be highly dependent on atmospheric conditions (81). Elevated MICs (>8 μg/ml) have been correlated with treatment failures, and 8 μg/ml is the threshold commonly used to define metronidazole resistance (81). The Etest is also used to determine metronidazole MICs for resistant isolates, but comparisons with broth dilution results may not correlate fully (81). The agar diffusion method with disks can be used for testing susceptibility to other antibiotics less commonly used in eradication, such as tetracycline, ciprofloxacin, and rifabutin. For instance, isolates were recorded as resistant if the growth inhibition zone for tetracycline was <30 mm (10-μg disk) and if any inhibition zone was observed for ciprofloxacin (1-μg disk) and rifampin (5-μg disk) (90). The present tentative agar dilution MIC interpretive criteria for resistance to those antibiotics are >1 μg/ml for tetracycline, >0.5 μg/ml for levofloxacin, and >1 μg/ml for rifabutin (81, 90). For resistant isolates, the MICs can be determined using the Etest.

Genotypic Susceptibility Testing of *H. pylori* Cultures and in Biopsy Specimens

Resistance to clarithromycin in *H. pylori* is attributed to point mutations at sites (A2142G and A2143G) in the peptidyl transferase region of domain V of the 23S rRNA gene which inhibit macrolide binding (92). Several methods involving gene amplification, rapid sequencing by pyrosequencing, or fluorescent *in situ* hybridization have been developed for the rapid detection of mutations associated with clarithromycin resistance (81). PCR-restriction fragment length polymorphism analysis was initially used on isolates to detect relevant mutations, but real-time PCR now provides a simpler and more rapid approach. Adaptations allow detection with excellent sensitivity of both *H. pylori* and its resistance to clarithromycin directly from gastric biopsy specimens (28). Real-time PCR assays also are available to ascertain resistance to tetracycline by rapid detection of 16S rRNA gene point mutations (93) and to ciprofloxacin/levofloxacin by rapid detection of point mutations in the quinolone resistance-determining region of the *gyrA* gene (94). Likewise, a real-time PCR test has been developed to ascertain resistance to rifabutin by rapid detection of point mutations in the *rpoB* gene (95). In contrast, development of a DNA-based assay to detect *H. pylori* resistance to metronidazole has proven more problematic, as the mechanisms of *in vitro* resistance have yet to be fully elucidated. Multiple null mutations in the NADPH nitroreductase gene (*rdxA*) and in the NAD(P)H flavin oxidoreductase gene (*frxA*) may contribute to the induction of resistance, but neither provides consistent markers for *in vitro* resistance testing (96, 97).

Genotypic Susceptibility Testing of *H. pylori* in Feces

A biprobe 23S rRNA gene real-time PCR assay has been developed for direct clarithromycin susceptibility testing of *H. pylori* in stool specimens (58), and an evaluation of a modified version, the *Helicobacter pylori* ClariRes assay (Ingenetix, Vienna, Austria), reported that it was at least as sensitive and more specific than the stool antigen test. However, another evaluation of the assay on stool specimens from symptomatic children reported a sensitivity of only 63% (59).

Susceptibility Testing of Gastric HHLO

Optimal treatment remains to be established for HHLO, although there is evidence that eradication by antimicrobial therapy, such as that used in conventional *H. pylori* eradication, results in the resolution of gastritis and peptic ulcer disease (49) as well as *H. heilmannii*-associated, primary, low-grade MALT lymphoma (38). Although susceptibilities *in vitro* were described for multiple isolates (from one patient) of an HHLO subsequently identified as *H. bizzozeronii* (66), usually there are no cultures of HHLO available for testing. Consequently, there is no information for HHLO on their frequency of resistance to clarithromycin and other antibiotics. To ascertain possible treatment options for HHLO, triple therapy was shown to significantly reduce burden in experimentally infected mouse stomachs (98). However, no PCR assays, such as those used to determine *H. pylori* clarithromycin and tetracycline resistance in gastric biopsy tissue, have been developed for direct testing of resistance in HHLO.

Susceptibility Testing of Enterohepatic Helicobacters

No recommended guidelines are available for treatment of a diagnosed infection with the enterohepatic helicobacters *H. cinaedi*, *H. canis*, *H. fennelliae*, and *H. pullorum*. Various antibiotic agents alone or in combination have been successfully used in treating such infections, but there is insufficient information to determine resistance rates for individual species. For the more commonly reported *H. cinaedi*, effective therapy for infection may require prolonged courses for at least 2 to 3 weeks of multiple antibiotics, such as erythromycin, ciprofloxacin, gentamicin, levofloxacin, tetracycline, and beta-lactams (40).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The principal noninvasive tests for diagnosis of an *H. pylori* infection before treatment are the UBT, ELISA SAT, and high-accuracy ELISA-based IgG serology (20). According to the clinical setting, endoscopic investigation may be indicated, and then rapid urease testing, histology, and culture of gastric biopsy specimens can be used. To assess *H. pylori* status for posttreatment follow-up, the UBT and SAT are the recommended noninvasive tests, but not IgG serology, as serum antibody concentrations fall slowly after eradication. In addition to test performance, other factors, such as cost-effectiveness and patient attitudes, need to be considered in test selection (99, 100). To perform antimicrobial susceptibility testing, bacteriological culture of *H. pylori* from gastric biopsy specimens is recommended, especially in cases of repeated treatment failure. Successful culture may be reported if the organism is microaerobic, has a Gram-negative morphology, and is oxidase, catalase, and urease positive. If culture is not positive after 10 days of incubation, it can be reported as negative, but if clinical tests indicate an *H. pylori* infection, it may be informative to perform a species-specific PCR assay directly on the gastric biopsy specimen. Because of the potential unreliability of PCR assays, resulting in false positives, such tests should not be used as the sole basis for diagnosis. Testing for clarithromycin susceptibility should be performed using either the CLSI reference method or a substantially equivalent method. Direct PCR testing of cultures or biopsy specimens provides a rapid alternative to phenotypic testing to detect the presence of discrete mutations conferring macrolide resistance. Eradication therapies are also likely to include other agents, such as amoxicillin, metronidazole, and tetracycline, and in problem cases, possibly rifabutin, levofloxacin, and furazolidone, depending on local clinical practice. Interpretive criteria for these antimicrobials, where available, may be "tentative" but should be used in the absence of recommended guidelines.

Gastric infection with non-*H. pylori Helicobacter* species is less common and should be diagnosed from bacterial morphology in gastric biopsy specimens. In the microbiology laboratory, they may be detected by an HHLO-specific PCR assay. Because of the lack of rapid diagnostic methods for the enterohepatic species, these must be cultured for a definitive identification. Enteric species such as *H. canadensis* and *H. pullorum* may occasionally be isolated by techniques employed for the isolation of *Campylobacter* species, particularly if nonselective media and incubation at 37°C are employed. As *Campylobacter* isolates are typically only cursorily identified routinely, enteric *Helicobacter* species are likely to be missed. Although they may have a limited role in human gastroenteritis, their significance remains unclear. Other species, such as *H. cinaedi*, *H. canis*, and *H. fennelliae*, may be rarely encountered from blood culture and other sites of infection. They are unlikely to grow well aerobically but may be apparent after prolonged incubation in an atmosphere containing additional CO_2 or on plates incubated "anaerobically" (conditions of strict anaerobiosis will not support growth). As these enterohepatic helicobacters are typically urease negative and can be confused with campylobacters, accurate identification is often difficult, and a reference laboratory should be consulted unless MALDI-TOF is available locally. The clinical significance of isolates may be unclear and should be assessed on a case-by-case basis. Determination of antibiotic susceptibilities should be performed if needed to guide antibiotic therapy decisions.

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Leptospira

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58

TAXONOMY

Serologic Classification

The genus *Leptospira* is comprised of spiral-shaped bacteria with hooked ends (1). This genus, along with the genera *Leptonema* and *Turneriella*, makes up the family *Leptospiraceae*, within the order *Spirochaetales* and the class *Spirochaetes* of the phylum *Spirochaetes* (2). The genus was formerly divided into two species: *Leptospira interrogans*, comprising all pathogenic strains, and *L. biflexa*, containing the saprophytic strains isolated from the environment (1). *L. biflexa* and *L. interrogans* were differentiated by a number of biochemical tests (1).

Leptospires are divided into serovars defined by agglutination after cross-absorption with homologous antigens (1, 3, 4). Serovars are considered distinct if more than 10% of the homologous titer remains in at least one of the two antisera on repeated testing (5). Over 60 serovars of *L. biflexa sensu lato* and more than 200 serovars of *L. interrogans sensu lato* are recognized. Serovars that are antigenically related have traditionally been grouped into serogroups (4). Serogroups have no taxonomic standing, but the concept has proved useful for epidemiological understanding, particularly for interpreting serological results from the microscopic agglutination test (MAT). The serogroups of *L. interrogans sensu lato* and some common serovars are shown in Table 1.

Genotypic Classification

The phenotypic classification of leptospires was replaced by a genotypic one, in which 21 genomospecies include all serovars of *Leptospira* (6–15). Table 2 lists all species of *Leptospira*. DNA hybridization studies have also confirmed the taxonomic status of the monospecific genera *Leptonema* (9) and *Turneriella* (16). The genetically defined species of *Leptospira* do not correspond to the previous two species (*L. interrogans sensu lato* and *L. biflexa sensu lato*), and both pathogenic and nonpathogenic serovars occur within some species (17). Neither the serogroup nor the serovar reliably predicts the true species of *Leptospira*. Moreover, genetic heterogeneity resulting from horizontal transfer of genes coding for cell surface antigens occurs within serovars (9, 18), resulting in strains of some serovars being classified as multiple species (Table 3). In addition, the phenotypic characteristics formerly used to differentiate *L. interrogans sensu lato* from *L. biflexa sensu lato* do not differentiate the genetically defined species (6, 9). Both *L. interrogans* and *L. biflexa* are retained as specific names in the genomic classification, and in this chapter, specific names refer to the genetically defined species, including *L. interrogans sensu stricto* and *L. biflexa sensu stricto*.

Classification of leptospires requires the identification of both the species and serovar of each isolate. Identification of *Leptospira* species is readily accomplished by sequencing of *rrs* (19), *rpoB* (20), or *gyrB* (21). Phylogenetic analysis of *rrs* sequences demonstrates three clades of leptospires, representing pathogens, saprophytes, and a group of species of uncertain pathogenicity (Fig. 1). Two hundred strains were recently sequenced through the *Leptospira* Genomics and Human Health Project (http://gsc.jcvi.org/projects/gsc/leptospira/). The analysis of these sequences will further inform the taxonomy of the *Leptospiraceae*. In future, it may be possible to identify serovars by sequence-based analysis.

DESCRIPTION OF THE FAMILY

Leptospires are tightly coiled spirochaetes that are usually 0.1 μm by 6 to 20 μm. The helical conformation is right-handed, with an amplitude of approximately 0.1 to 0.15 μm and a wavelength of approximately 0.5 μm (22). The cells have pointed ends, either or both of which are usually bent into a distinctive hook (Fig. 2). Two axial filaments (periplasmic flagella) with polar insertions are located in the periplasmic space. Leptospires exhibit two distinct forms of movement: translational (rapid back-and-forth movements) and rotational (spinning rapidly about the long axis of the cell) movements (23). Morphologically, all leptospires are indistinguishable.

Leptospires are obligate aerobes with an optimum growth temperature of 28 to 30°C. The optimum pH for growth is 7.2 to 7.6. They produce both catalase and oxidase. They grow in simple media enriched with vitamins (vitamins B_2 and B_{12} are growth factors), long-chain fatty acids, and ammonium salts (1).

EPIDEMIOLOGY AND TRANSMISSION

Leptospires are ubiquitous, either free-living in water or associated with renal infection of animals (24). Leptospirosis is presumed to be the most widespread zoonosis in the world (25). The source of infection in humans is usually either direct or indirect contact with the urine of an infected animal. The incidence is very much higher in countries

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TABLE 1 Serogroups and selected serovars of *Leptospira interrogans sensu lato*

Serogroup	Serovar
Icterohaemorrhagiae	Copenhageni
	Icterohaemorrhagiae
	Lai
Hebdomadis	Hebdomadis
	Jules
Autumnalis	Autumnalis
	Bim
	Fortbragg
Pyrogenes	Pyrogenes
Bataviae	Bataviae
Grippotyphosa	Canalzonae
	Grippotyphosa
	Ratnapura
Canicola	Canicola
Australis	Australis
	Bratislava
	Lora
Pomona	Pomona
Javanica	Javanica
Sejroe	Sejroe
	Hardjo
Panama	Mangus
	Panama
Cynopteri	Cynopteri
Djasiman	Djasiman
Sarmin	Sarmin
Mini	Mini
	Georgia
Tarassovi	Tarassovi
Ballum	Arborea
	Ballum
Celledoni	Celledoni
Louisiana	Lanka
	Louisiana
Ranarum	Ranarum
Manhao	Manhao
Shermani	Shermani
Hurstbridge	Hurstbridge

TABLE 2 Species of *Leptospira*

Phylogenetic cluster[a]	Species
Pathogenic species	*L. alexanderi*
	L. alstonii
	L. borgpetersenii
	L. interrogans
	L. kirschneri
	L. kmetyi
	L. noguchii
	L. santarosai
	L. weilii
Intermediate species	*L. broomii*
	L. fainei
	L. inadai
	L. licerasiae
	L. wolffi
Saprophytic species	*L. biflexa*
	L. idonii
	L. meyeri
	L. terpstrae
	L. vanthielii
	L. wolbachii
	L. yanagawae

[a] Based on 16S rRNA gene sequences (see Fig. 1).

with a warm climate than in temperate regions, due mainly to longer survival of leptospires in the environment under warm, humid conditions. Leptospirosis is seasonal. Peak incidence occurs in summer or fall in temperate regions, where temperature is the limiting factor in survival of leptospires, and during rainy seasons in warm-climate regions, where rapid desiccation would otherwise prevent survival.

Animals, including humans, can be categorized as maintenance hosts or accidental (incidental) hosts. A maintenance host is defined as a species in which infection is endemic, usually transferred from animal to animal by direct contact. Infection is usually acquired at an early age, and the prevalence of chronic excretion in the urine increases with the age of the animal. Other animals (such as humans) may become infected by indirect contact with the maintenance host. Animals may be maintenance hosts of some serovars but incidental hosts of others, infection with which may cause severe or fatal disease. The most important maintenance hosts are small mammals, which may transfer infection to domestic farm animals, dogs, and humans. Different rodent species may be reservoirs of distinct serovars, but rats are generally maintenance hosts for serovars of the serogroup Icterohaemorrhagiae, and mice for serogroup Ballum serovars. Domestic animals are also maintenance hosts; dairy cattle may harbor serovars Hardjo and Pomona, pigs serovars Pomona, Tarassovi, and Bratislava, and dogs serovar Canicola. Distinct variations in maintenance hosts and the serovars they carry occur throughout the world, and these associations may change over time.

Knowledge of the prevalent serovars and their maintenance hosts is essential for understanding the epidemiology of the disease. For example, the incidence of canine leptospirosis has increased in the northeastern regions of North America over the last 20 years. Very few cases have been diagnosed by culture, but serology suggests that they are caused largely by serovar Grippotyphosa (26, 27). Epidemiological evidence implicates raccoons and possibly skunks as the reservoir. Cases are clustered particularly in newly suburbanized areas (27). This change in epidemiology has led to the inclusion of serovars Grippotyphosa and Pomona in canine vaccines.

Human infections may be acquired through occupational, recreational, or avocational exposures. Occupation remains a significant risk factor for humans. Direct contact

TABLE 3 Leptospiral serovars found in multiple species[a]

Serovar	Species
Bataviae	*L. interrogans*, *L. santarosai*
Bulgarica	*L. interrogans*, *L. kirschneri*
Grippotyphosa	*L. interrogans*, *L. kirschneri*
Hardjo	*L. borgpetersenii*, *L. interrogans*, *L. meyeri*
Icterohaemorrhagiae	*L. inadai*, *L. interrogans*
Kremastos	*L. interrogans*, *L. santarosai*
Mwogolo	*L. interrogans*, *L. kirschneri*
Paidjan	*L. interrogans*, *L. kirschneri*
Pomona	*L. interrogans*, *L. noguchii*
Pyrogenes	*L. interrogans*, *L. santarosai*
Szwajizak	*L. interrogans*, *L. santarosai*
Valbuzzi	*L. interrogans*, *L. kirschneri*

[a] Based on data reported by Brenner et al. (9) and by Feresu et al. (18).

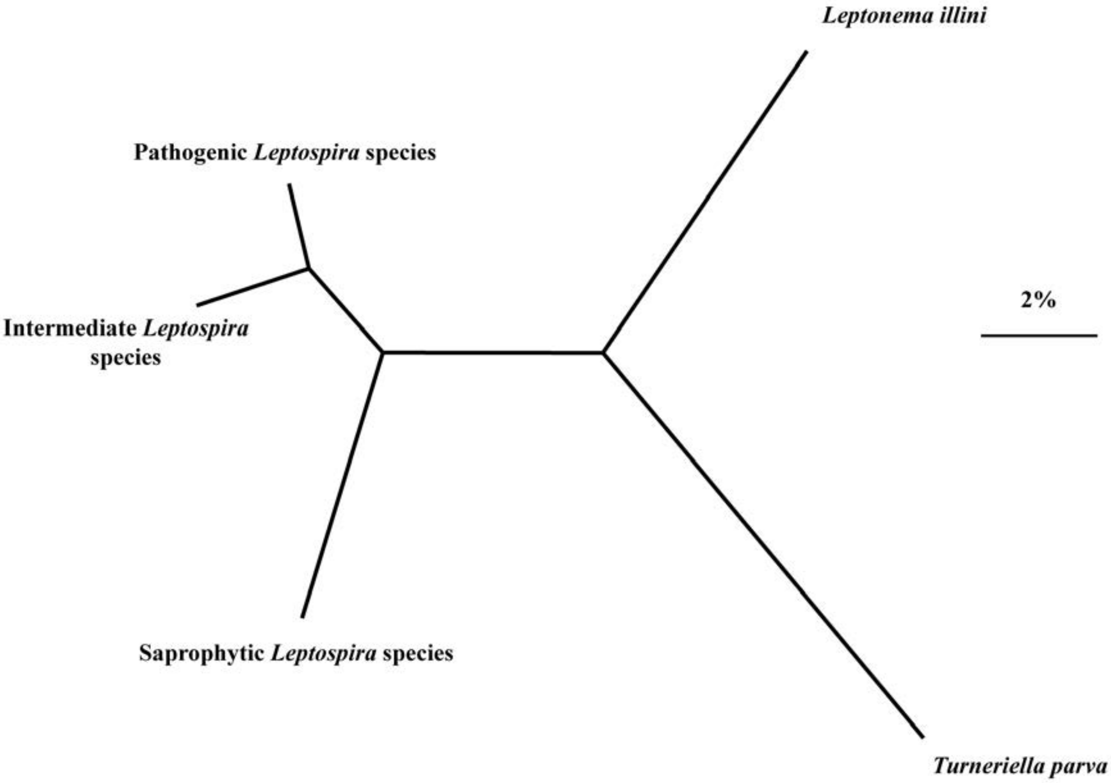
Leptonema illini
Pathogenic Leptospira species
Intermediate Leptospira species
2%
Saprophytic Leptospira species
Turneriella parva

FIGURE 1 Phylogenetic relationships between the genera of the *Leptospiraceae*, based on *rrs* (16S rRNA gene) sequences. The scale bar represents a 2% difference in sequence.
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with infected animals accounts for most infections in farmers, veterinarians, abattoir workers, rodent control workers, and workers in other occupations which require contact with animals, while indirect contact is important for sewer workers, miners, soldiers, septic tank cleaners, fish farmers, rice field workers, and sugar cane cutters. Livestock farming is a major occupational risk factor throughout the world. The highest risk is associated with dairy farming and is associated with serovar Hardjo and, in particular, with milking of dairy cattle. There is a significant risk associated with recreational exposures occurring in water sports (28). The increasing popularity of adventure sports and ecotourism has led to an increase in leptospirosis in travelers (29, 30).

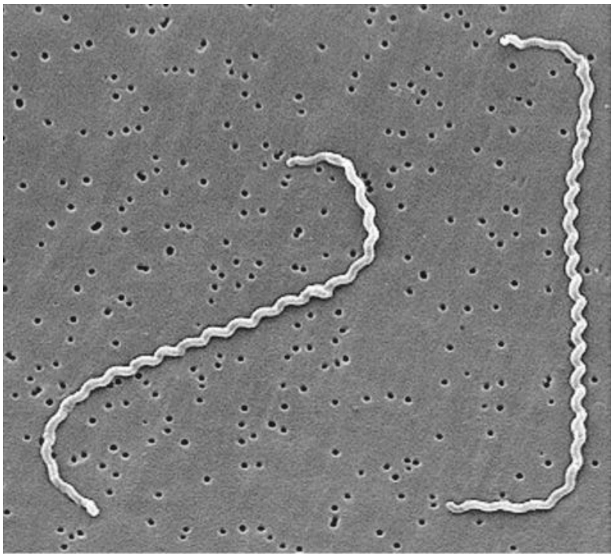

FIGURE 2 Scanning electron micrograph of leptospiral cells bound to a 0.2-µm filter. Magnification, approximately ×3,500. Source: Public Health Image Library.
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CLINICAL SIGNIFICANCE

The usual portal of entry is through abrasions or cuts in the skin or via the conjunctiva. The great majority of infections are either subclinical or of very mild severity, and patients will probably not seek or be brought to medical attention. The clinical presentation of leptospirosis is biphasic, with a septicemic phase lasting about a week, followed by the immune phase, characterized by antibody production and excretion of leptospires in the urine. Most of the complications of leptospirosis are associated with localization of leptospires within the tissues during the immune phase and thus occur during the second week of the illness.

The overwhelming majority of patients with recognized cases will present with a febrile illness of sudden onset, the symptoms of which include chills, headache, myalgia, abdominal pain, and conjunctival suffusion. Aseptic meningitis may be found in ≤25% of all leptospirosis cases. Between

5 and 10% of all patients with leptospirosis have the icteric form of the disease (Weil's disease), in which the clinical course is often very rapidly progressive. In addition to jaundice, patients with icteric leptospirosis may develop acute renal failure, pulmonary hemorrhage, and cardiac arrhythmias. Patients with severe cases often present late in the course of the disease, and this contributes to the high mortality rate, which ranges from 5 to 15%.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Leptospires can be isolated from blood, cerebrospinal fluid (CSF), and peritoneal dialysate fluids during the first 10 days of illness. Specimens should be collected before antibiotic therapy is initiated and while the patient is febrile. Under optimal conditions, one or two drops of venous blood are inoculated directly into culture medium at the bedside. Survival of leptospires in commercial blood culture media for several days has been reported (31). There are no transport media available, but blood can be collected and shipped at ambient temperature in tubes containing heparin, oxalate, or citrate (22). Citrate- or EDTA-containing tubes are optimal for PCR detection, whereas tubes containing heparin, sodium polyanethol sulfonate, or saponin are inhibitory to PCR reactions (32, 33).

Urine can be cultured after the first week of illness. Specimens should be collected aseptically into sterile containers without preservatives and must be processed within a short time of collection; best results are obtained when the delay is less than 1 hour. If culture is not possible, urine specimens intended for PCR testing may be stabilized at ambient temperature by use of one of several commercial products for this purpose, allowing for transport over long distances (33).

DIRECT EXAMINATION

Microscopy

Leptospires may be visualized in clinical material by dark-field microscopy (magnifications of ×100 and ×400) or by immunofluorescence or light microscopy after appropriate staining. Dark-field microscopic examination of body fluids, such as blood, urine, CSF, or dialysate fluid, is insensitive and lacks specificity (34). Approximately 10^4 leptospires/ml are necessary for one cell per field to be visible by dark-field microscopy (35). Direct dark-field microscopy of blood is also subject to misinterpretation of fibrin or protein threads, which may demonstrate Brownian motion (22, 35). Leptospires in tissues were first visualized by silver staining (36), and the Warthin-Starry stain is widely used for histologic examination. More recently, immunohistochemical methods have been applied (37, 38). Immunohistochemical staining can be performed at the Centers for Disease Control and Prevention, Atlanta, GA (contact the Infectious Diseases Pathology Branch prior to shipping specimens; e-mail pathology@cdc.gov).

Antigen Detection

Antigen detection is generally regarded as insensitive. There are no commercial antigen detection assays.

Nucleic Acid Detection

Several primer pairs for detection of leptospiral nucleic acids by conventional PCR have been described (17), and real-time PCR assays have been developed, targeting either *rrs* or pathogen-specific sequences (33, 39–43). Leptospiral DNA has been amplified from serum, urine, aqueous humor, CSF, and tissues obtained at autopsy (44, 45).

Clinical evaluations have shown that PCR detection of leptospiral DNA is comparable to or more sensitive than diagnosis by culture (41, 44, 46, 47), while detection of cases by serologic diagnosis by testing acute- and convalescent-phase sera with the MAT remains more sensitive (46, 47). The principal advantage of PCR is the ability to confirm the diagnosis during the acute illness, while treatment is likely to be most effective (47, 48).

Real-time PCR assays have been applied to quantify the burden of infecting organisms in human cases and animal models (47, 49–51). A limitation of PCR-based diagnosis of leptospirosis is the inability of most PCR assays to identify the infecting serovar. While this is not significant for individual patient management, the identity of the serovar has both epidemiological and public health value.

ISOLATION PROCEDURES

Leptospiremia occurs during the first stage of the disease, beginning before the onset of symptoms, and has usually finished by the end of the first week of the acute illness (52). Therefore, blood cultures should be taken as soon as possible after the patient's presentation. One or two drops of blood (approximately 50 µl) are inoculated into 10 ml semisolid oleic acid-albumin medium (53, 54), such as Ellinghausen-McCullough-Johnson-Harris medium or Fletcher's medium (Difco EMJH or Difco Fletcher's medium; BD Diagnostic Systems, Sparks, MD), containing 0.1% agar and 200 µg/ml 5-fluorouracil, at the patient's bedside. Blood is allowed to drop onto the surface of the medium; mixing is not necessary. Care should be taken to avoid overinoculation of the medium, as blood contains inhibitors of leptospiral growth. For the highest recovery rate, multiple cultures should be performed, but this is rarely possible. Survival of leptospires in commercial blood culture media for up to a week has been reported (31, 55). Thus, if leptospirosis is not initially suspected, blood cultures may be used as a source for PCR testing if other samples are no longer available.

Other samples that can be cultured during the first week of illness include CSF and peritoneal dialysate. Urine cultures can yield growth from the beginning of the second week of symptomatic illness. Survival of leptospires in voided human urine is limited, so urine should be processed immediately, by neutralization of pH with sodium bicarbonate followed by centrifugation. After centrifugation in 15-ml tubes for 30 min at 1,500 × *g*, the sediment is resuspended in 1 ml of phosphate-buffered saline, and one or two drops are inoculated into semisolid EMJH medium containing 5-fluorouracil as described above.

Cultures in EMJH medium are incubated in sealed bottles at 28 to 30°C and examined weekly by dark-field microscopy for up to 13 weeks before being discarded. Growth often develops in a discrete band several millimeters below the surface of the medium, known as Dinger's ring. Cultures that show growth of other bacteria may be passed through a 0.2-µm or 0.45-µm filter before subculture into fresh medium.

IDENTIFICATION

Isolated leptospires are identified either by serological methods or, more recently, by molecular techniques. Traditional methods relied on cross-agglutinin absorption (3). The number of laboratories that can perform these identification methods is very small. The use of panels of monoclonal antibodies allows laboratories which can perform the MAT to identify isolates of frequently encountered serovars with relative rapidity (56). Monoclonal antibodies are available from the WHO/OIE Leptospirosis Reference Laboratory at the Royal Tropical Institute, Amsterdam, the Netherlands.

The current taxonomy of *Leptospira* necessitates the identification of isolates to both the species and serovar levels. Species identification is most practically determined by sequence analysis (57), using the *rrs* (16S rRNA) (19), *gyrB* (21), *rpoB* (20), or *secY* (58) gene. Identification of leptospires by matrix-assisted laser desorption ionization–time of flight mass spectrometry has been reported (59).

TYPING SYSTEMS

Because of the difficulties associated with serological identification of leptospiral isolates, there has been great interest in molecular methods for identification and subtyping (48, 57). Methods employed have included digestion of chromosomal DNA by restriction endonucleases, restriction fragment length polymorphism analysis, ribotyping, and a number of PCR-based approaches (57). The most widely applicable of these for identification of serovars is pulsed-field gel electrophoresis, largely because this technique is widely used for infection control studies and for public health typing of enteric pathogens (60, 61). This approach has been standardized by the PulseNet model, in which standardized profiles can be exchanged electronically (61–63).

The availability of full-genome sequences of several *Leptospira* strains has led to the development of sequence-based methods, such as amplified fragment length polymorphism and variable-number tandem-repeat analyses. Several studies have applied multilocus sequence typing (MLST) to understand the epidemiology of leptospirosis (64–66). The application of these powerful tools will lead to a greater understanding of leptospiral epidemiology at the population level (24, 57).

SEROLOGIC TESTS

Most cases of leptospirosis are diagnosed by serology. Antibodies are detectable in the blood approximately 5 to 7 days after the onset of symptoms. The definitive serologic investigation of leptospirosis remains the MAT, in which patient sera are incubated with live or killed antigen suspensions of leptospires of different serovars. After incubation, the serum-antigen mixtures are examined microscopically for agglutination, and the titers are determined. The MAT is a complex test to control, perform, and interpret (67), and its use is limited to regional or national reference laboratories. Protocols for performing the MAT have been described in detail (22, 68). An international proficiency testing scheme under the auspices of the International Leptospirosis Society has stimulated improvement in the performance of the MAT by participating laboratories (69). The range of antigens used should include those of serovars representative of all serogroups (22, 67). The wide range of antigens is used in order to detect infections with uncommon or previously undetected serovars. The test is read by dark-field microscopy. The endpoint is the highest dilution of serum in which 50% agglutination occurs and is determined by the presence of approximately 50% free, unagglutinated leptospires by comparison with the control suspension (22). Considerable effort is required to reduce the subjective effect of observer variation, even within laboratories.

Interpretation of the MAT is complicated by the high degree of cross-reaction that occurs between different serogroups, especially in acute-phase samples. Paradoxical reactions, in which the highest titers are detected for a serogroup unrelated to the infecting one, are also common (17). The broad cross-reactivity in the acute phase, followed by relative serogroup specificity in convalescent-phase samples, results from the detection in the MAT of both IgM and IgG antibodies and the presence of several common antigens among leptospires (70).

Paired sera are required to confirm a serologic diagnosis with certainty. A 4-fold or greater rise in titer between paired sera confirms the diagnosis, regardless of the interval between samples. The interval between the first and second samples depends very much on the delay between onset of symptoms and presentation of the patient. If symptoms typical of leptospirosis are present, an interval of 3 to 5 days may be adequate to detect rising titers. However, if the patient presents earlier in the course of the disease, or if the date of onset is not known precisely, then an interval of 10 to 14 days between samples is more appropriate. Less often, seroconversion does not occur with such rapidity, and a longer interval between samples (or repeated sampling) is necessary. MAT serology is insensitive, particularly with early-acute-phase specimens (71, 72). Moreover, patients with fulminant leptospirosis may die before seroconversion occurs (72, 73).

A presumptive diagnosis can be made by detection of a single elevated titer in association with an acute febrile illness. The magnitude of such a titer is dependent upon the background level of exposure in the population, and hence the seroprevalence.

Titers following acute infection may be extremely high (≥25,600) and may take months or even years to fall to low levels (74, 75). Thus, in a high-incidence population, a low cutoff titer for presumptive diagnosis is inappropriate and will generate many false-positive diagnoses. In areas where the disease is endemic, a single titer of ≥800 in symptomatic patients is generally indicative of leptospirosis, but titers as high as ≥1,600 have been recommended (68). Rarely, seroconversion may be delayed for many weeks after recovery, and longer serological follow-up will be necessary to confirm the diagnosis.

Formalized antigens have been used in the MAT in order to overcome some of the difficulties associated with the use of live antigens. Titers obtained with these antigens are somewhat lower, and more cross-reactions are detected (22). These antigens are not available commercially but may be obtained from WHO Collaborating Centers.

The MAT is the most appropriate test to employ in epidemiological serosurveys, since it can be applied to sera from any animal species, and because the range of antigens utilized can be expanded or decreased as required. It is usual to use a titer of ≥100 as evidence of past exposure (67). Contrary to a widely held belief, the MAT is a serogroup-specific assay. However, conclusions about infecting serovars cannot be drawn without isolates; at best, the MAT data can give a general impression of which serogroups are present within a population (76, 77).

Because of the complexity of the MAT, rapid screening tests for leptospiral antibodies in acute infection have been developed. Traditional methods based upon agglutination have largely been superseded by IgM detection assays. IgM antibodies become detectable during the first week of illness, allowing the diagnosis to be confirmed and treatment to be initiated while it is likely to be most effective. IgM detection has repeatedly been shown to be more sensitive than MAT when the first specimen is taken early in the acute phase of the illness (72, 78). IgM-based dipstick assays have been shown to be as sensitive as microtiter plate IgM-based enzyme-linked immunosorbent assays (79–81). Other rapid assays include a latex agglutination assay (82) and a lateral flow assay (83).

ANTIBIOTIC SUSCEPTIBILITIES

Leptospires are susceptible to many antimicrobial agents, including β-lactams, macrolides, tetracyclines, fluoroquinolones, and streptomycin. Problems in the determination of susceptibility include the long incubation time required (84), the use of media containing serum (85, 86), and the difficulty in quantifying growth accurately. These constraints limited the development of rapid, standardized methods for susceptibility testing. However, broth microdilution methods were described recently (87), which facilitated the testing of larger numbers of isolates against a wide range of antimicrobial agents (88). Such studies will lead to the identification of potential new agents for inclusion in clinical trials.

Penicillin and doxycycline are both effective for treatment of leptospirosis and remain the drugs of choice (89); clinical studies have shown that third-generation cephalosporins may be equally effective (90, 91). Prophylaxis with once-weekly oral doxycycline can give short-term protection in high-risk environments and is effective at preventing disease but not seroconversion (89).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

A diagnosis of leptospirosis can be made by isolation of the organism or by amplification of leptospiral DNA from blood, urine, or other specimens, by demonstration of leptospires in tissues by immunohistochemical staining, or by detection of a 4-fold or greater rise in titers between acute- and convalescent-phase serum samples tested by the same methodology at the same time. In populations and/or regions where leptospirosis is not endemic, MAT titers of ≥200 in a single specimen obtained after the onset of symptoms are suggestive but not diagnostic of acute or recent leptospirosis. A titer of ≥800 in the presence of compatible symptoms is strong evidence of recent or current leptospirosis. Delayed seroconversions are common. Assays that detect IgM antibodies give presumptive evidence of recent exposure to leptospirosis but require confirmation by another method, such as the MAT, since IgM titers are persistent (75). Negative test results in the presence of compatible symptoms do not rule out the diagnosis of leptospirosis, and further samples should be examined. The isolation of leptospires, the demonstration of leptospiral DNA by molecular methods, or the detection of leptospires in tissues by immunohistochemistry confirms the diagnosis and differentiates between current infection and past exposure, which may not be differentiated clearly by serology.

Despite recent advances in molecular detection and characterization of leptospires and in the development of rapid serologic tests, there are still relatively few laboratories throughout the world with the appropriate capabilities for *Leptospira* diagnostics. In the United States, leptospirosis was reinstated as a nationally notifiable disease in January 2013. Instructions for specimen submission and case reporting are available at the CDC website (http://www.cdc.gov/leptospirosis). Additional information regarding leptospirosis is available at the Leptospira Library website (http://www.kit.nl/biomedical-research/leptospirosis-reference-centre/leptospira-library/), and a current list of international diagnostic centers of expertise (92) is available on the International Leptospirosis Society website (http://www.med.monash.edu.au/microbiology/staff/adler/ils.html).

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Borrelia

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The bacterial genus *Borrelia* is divided into the relapsing fever (RF) and Lyme borreliosis groups based on genetic relatedness among dozens of member spirochete species (Table 1). All species are vectored by blood-feeding arthropods, are environmentally restricted to animal systems, and (with few exceptions) cause zoonotic infections, with humans being rare and dead-end hosts. Human infection is most commonly self-limited, may be asymptomatic, or may cause disease ranging from acute and relapsing fevers to staged illness that may involve the skin and the musculoskeletal, neurological, and cardiovascular systems.

RF borrelia species are vectored by lice (louse-borne RF [LBRF]) or ticks (tick-borne RF [TBRF]). LBRF has been known to humans for thousands of years and has been the cause of massive epidemics as recently as the early 1900s (1). LBRF is one of the few examples of human-specific borrelia disease; it is caused by *Borrelia recurrentis* and is vectored by the human body louse, *Pediculus humanus humanus*. Although improved personal hygiene and use of antibiotics and dichlorodiphenyltrichloroethane (DDT) in the 20th century resulted in global case and morbidity declines, LBRF continues to severely impact impoverished and displaced populations in parts of East Africa (2). In contrast to LBRF, TBRF was first described in the medical literature in the late 1800s, and human disease, which presents in small clusters or as isolated cases, is now known to occur in focal areas throughout most of the world (3–5). In parts of Northwest Africa, however, it is one of the most common bacterial infections, and in Tanzania, it is a leading cause of prenatal and child mortality (2, 6). Over a dozen *Borrelia* species (Table 1) are the agents of TBRF and, with the possible exception of *Borrelia duttonii*, cause zoonotic disease. Most TBRF species are vectored by soft-bodied ticks of the genus *Ornithodoros* (Table 1; Fig. 1).

Lyme borreliosis, first known as erythema chronicum migrans, was reported in late-19th-century medical literature from Europe. Over the next 50 years, numerous reports described its association with bites of hard-bodied ticks of the genus *Ixodes* (Fig. 1), its responsiveness to penicillin, and its likely spirochetal etiology (3, 7). Despite these later-proven correct deductions, the disease garnered little notoriety and outside Europe was largely unknown until the late 1900s. In 1969, a North American case of erythema migrans (EM) in a patient from Wisconsin was described and successfully treated with penicillin (8). However, active investigation of the etiology and natural biology of the disease was not prompted until 1975, when a group of parents in Lyme, CT, expressed concerns to the State Health Department of a seeming epidemic of inflammatory arthritis among local children. Ensuing epidemiological and clinical investigations linked antecedent tick bites with EM and subsequent arthritis among children and adults in this and other regions in the United States (9). The causative agent of Lyme borreliosis, or Lyme disease, as it is known in North America, *Borrelia burgdorferi*, and transmission by the tick *Ixodes scapularis* was described shortly thereafter (10). Lyme borreliosis became a reportable disease in the United States in 1987. Cases reported to the Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/lyme/stats/index.html) have increased steadily over the last 25 years, and it has become and remains the most prevalent vector-borne disease in North America. As with other reportable diseases, Lyme borreliosis is thought to be significantly underreported in the United States, Canada, and much of Europe (11, 12). Recent estimates by the CDC suggest that the true incidence is closer to 300,000 cases/year. Although endemic throughout most of Europe, Lyme borreliosis is a notifiable disease in only a few of the countries in which it occurs; nonetheless, over 85,000 cases/year were reported from 18 countries in 2006, and significant increases in rates of incidence have been documented in parts of this region as well.

TAXONOMY

Borreliae belong to the order *Spirochaetales*, which encompasses the families *Spirochaetaceae* and *Leptospiraceae*. Within the *Spirochaetacea*, two genera, *Borrelia* and *Treponema*, cause human disease. Borreliae are agents of LBRF and both tick-borne Lyme borreliosis and relapsing fever. The type species of the genus *Borrelia* is *Borrelia anserina*, which causes borreliosis in birds. Based on *rrs* (16S rRNA gene) sequence analyses, spirochetes form a distinct entity (division D) within the eubacterial kingdom. They are neither Gram positive nor Gram negative. In the case of the spirochetes, morphological criteria and DNA data produce concordant phylogenies, a rare trait in other bacterial groups.

DESCRIPTION OF THE GENUS

Common Characteristics

Borreliae (Fig. 2 and 3) are similar in length (8 to 30 μm) to, but wider (0.2 to 0.5 μm) than, the two other human-

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TABLE 1 Characteristics of arthropod-borne borreliae[a]

Type of borreliae and *Borrelia* species	Arthropod vector	Animal reservoir	Geographic distribution	Disease
Relapsing fever borreliae				
B. recurrentis	*Pediculus humanus humanus*	Humans	East Africa	Louse-borne (epidemic) relapsing fever
B. duttonii	*Ornithodoros moubata*	Humans	Central, eastern, and southern Africa	Tick-borne (endemic) relapsing fever
B. hispanica	*Ornithodoros erraticus*	Rodents	Mediterranean region	Hispano-African tick-borne relapsing fever
B. crocidurae, "*B. merionesi*," *B. microti*, *B. dipodilli*	*Ornithodoros sonrai*, *Ornithodoros erraticus*	Rodents	Morocco, Libya, Egypt, Turkey, Senegal, Kenya	North African tick-borne relapsing fever
B. persica	*Ornithodoros tholozani*	Rodents	Western China, India, Kashmir, central Asia, Iraq, Iran, Egypt	Asiatic-African tick-borne relapsing fever
B. caucasica	*Ornithodoros verrucosus*	Rodents	Caucasus to Iraq	Caucasian tick-borne relapsing fever
B. hermsii	*Ornithodoros hermsi*	Rodents	Western North America	American tick-borne relapsing fever
B. turicatae	*Ornithodoros turicatae*	Rodents	Southwestern United States	American tick-borne relapsing fever
B. parkeri	*Ornithodoros parkeri*	Rodents	Western United States	American tick-borne relapsing fever
B. mazzottii	*Ornithodoros talajé*	Rodents	Southern United States, Mexico, Central and South America	American tick-borne relapsing fever
B. venezuelensis	*Ornithodoros rudis* (*Ornithodoros venezuelensis*)	Rodents	Central and South America	American tick-borne relapsing fever
Lyme disease borreliae (*B. burgdorferi sensu lato*)				
B. burgdorferi	*Ixodes scapularis*	Rodents	Eastern and north-central United States	Lyme borreliosis
	Ixodes pacificus	Rodents	Western United States	Lyme borreliosis
	Ixodes ricinus	Rodents	Europe	Lyme borreliosis
B. garinii	*Ixodes ricinus*, *Ixodes persulcatus*	Rodents	Europe, Asia	Lyme borreliosis
	Ixodes uriae	Birds	Europe, Asia	?
B. afzelii	*Ixodes ricinus*, *Ixodes persulcatus*	Rodents	Europe, Asia	Lyme borreliosis
B. bavariensis	*Ixodes ricinus*, *Ixodes persulcatus*	Rodents	Europe, Asia	Lyme borreliosis
B. spielmanii	*Ixodes ricinus*	Rodents	Europe	Lyme borreliosis (few cases)
B. japonica	*Ixodes ovatus*	Rodents	Japan	?
B. andersonii	*Ixodes dentatus*	Rabbits	United States	?
B. bissettii	*Ixodes scapularis*, *Ixodes pacificus*	Rodents	United States	Lyme borreliosis (few cases)
B. tanukii	*Ixodes tanukii*, *Ixodes ovatus*	Rodents	Japan	?
B. turdi	*Ixodes turdus*	?	Japan	?
B. sinica	*Ixodes ovatus*		China	?
B. valaisiana	*Ixodes ricinus*	Birds	Europe, Asia	Lyme borreliosis (one case)
B. lusitaniae	*Ixodes ricinus*	Reptiles (birds?)	Europe, North Africa	Lyme borreliosis (few cases)
B. californiensis	?	Rodents	Western United States	?
B. carolinensis	*Ixodes minor*	Rodents	Southeastern United States	?
Other borreliae				
B. lonestari	*Amblyomma americanum*	?	United States	?
B. miyamotoi	*Ixodes persulcatus*, *Ixodes scapularis*, *Ixodes pacificus*, *Ixodes ricinus*, *Ixodes persulcatus*	Rodents	Japan, United States, Europe	? (Japan), Lyme borreliosis-like (United States and Europe)
B. theileri	*Rhipicephalus* sp., *Boophilus* spp.	Cattle, horses, sheep	South Africa, Australia, North America, Europe	Bovine borreliosis
B. coriaceae	*Ornithodoros coriaceus*	Deer?	Western United States	Epizootic bovine abortion?
B. anserina	*Argas* spp.	Fowl	Worldwide	Avian borreliosis

[a] Modified from reference 132 with permission.

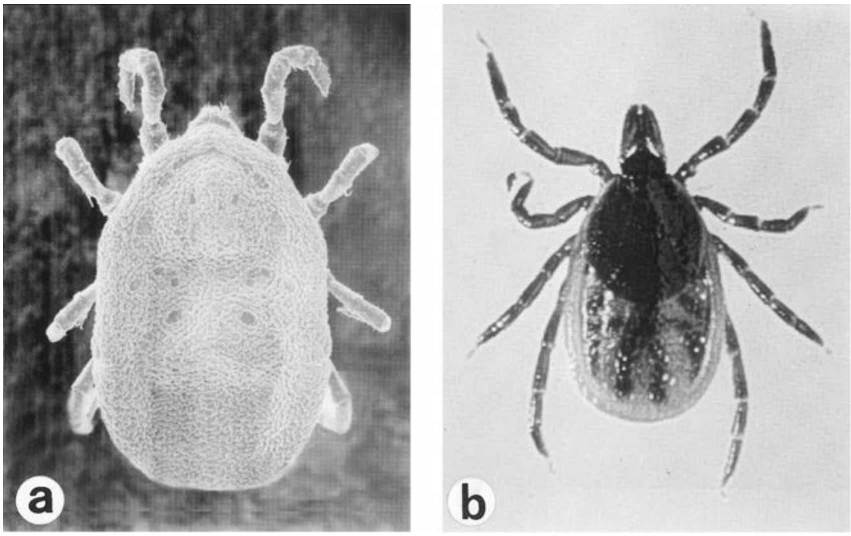

FIGURE 1 Two genera of ticks are vectors for relapsing fever and Lyme borreliosis: *Ornithodoros* (a) and *Ixodes* (b). doi:10.1128/9781555817381.ch59.f1

pathogenic spirochetes, the treponemes and the leptospires (13). They are highly motile organisms, with corkscrew and oscillating motility enabling movement through highly viscous mediums, such as connective tissue. In contrast to the exoflagella of other bacteria, the flagella of spirochetes are endoflagella. The endoflagella (7 to 20 per terminus) are localized beneath the outer membrane and insert subterminally at one end or the other of the protoplasmic cylinder. The protoplasmic cylinder consists of a peptidoglycan layer and an inner membrane which encloses the internal components of the cell (13). If cultivable, borreliae grow slowly under microaerophilic (14) or anaerobic (15) conditions. They require *N*-acetylglucosamine and long-chain saturated and unsaturated fatty acids and produce lactic acid through glucose fermentation (16).

Species Diversity

The causative agent of Lyme borreliosis, *Borrelia burgdorferi*, was first described by Burgdorfer et al. in the early 1980s (10). Studies published since 1992 have divided *B. burgdorferi sensu lato* into 3 prevalent, human-pathogenic species—*B. burgdorferi sensu stricto*, *Borrelia afzelii*, and *Borrelia garinii* (17)—and 12 other species, some of which have been linked to human cases (Table 1) (18). With few exceptions, *B. burgdorferi sensu stricto* is the only well-documented human-pathogenic Lyme borreliosis species found in North America; in contrast, all three species have been isolated from humans in Europe. From central to eastern Asia, *B. garinii* and *B. afzelii* are the agents of almost all human cases of Lyme borreliosis.

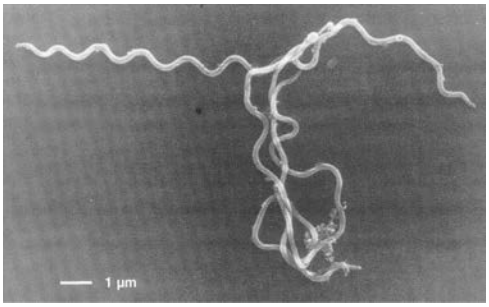

FIGURE 2 Scanning electron micrograph of *B. burgdorferi* (provided by Gerhard Wanner, Munich, Germany). doi:10.1128/9781555817381.ch59.f2

There is a high prevalence of *B. afzelii* among human skin isolates from Europe, whereas isolates from cerebrospinal fluid (CSF) in Europe are most often *B. garinii* (Table 2) (19, 20). All three genospecies cause Lyme arthritis in Europe (Table 2) (21–23).

A few studies have reported the detection of other *Borrelia* species (*B. valaisiana*, *B. spielmanii*, "*B. bissetii*," *B. bavariensis*, and *B. lusitaniae*) in patient samples in Europe (24–28). Similarly, *B. lonestari* and *B. miyamotoi*, two species carried by hard ticks but genetically more closely related to the relapsing fever spirochetes vectored by soft ticks, have been reported in a small number of cases in Russia, the Netherlands, and the United States (29–31).

In North America, the TBRF-causing *Borrelia* species *B. turicatae*, *B. parkeri*, and *B. hermsii* have been isolated from *Ornithodoros turicatae*, *Ornithodoros parkeri*, and *Ornithodoros hermsi* ticks, respectively, although they may be a single species because their DNA-DNA similarity is greater than 70%. The species status of other cultivable borreliae, such

TABLE 2 Distribution of species of *B. burgdorferi sensu lato* in European isolates from CSF, skin, and synovial fluid specimens[a]

Species	% distribution in indicated specimen type[b]		
	CSF (*n* = 78)	Skin (*n* = 560)	Synovial fluid (*n* = 20)
B. burgdorferi sensu stricto	12	1	33
B. afzelii	17	88	29
B. garinii	71	10	38

[a] Data are from references 20, 21, and 23.

[b] *B. burgdorferi sensu lato* species identifications from CSF and skin are based on culture; species identification from synovial fluid samples is based on *ospA* PCR results. Culture isolates from synovial fluid are too few to estimate *Borrelia* species distribution.

as *B. anserina*, *B. crocidurae*, *B. recurrentis*, and *B. coriaceae*, has been supported by greater DNA-DNA dissimilarity findings (32).

Genomes

The genomes of the borreliae are unusual among prokaryotes in that they have a small linear chromosome of approximately 1,000 kb and both linear and circular plasmids. Also unlike most bacteria, borreliae have a low G+C content (approximately 30 mol%). The complete nucleotide sequence of the chromosome and 21 plasmids (9 circular and 12 linear) has been published for the type strain, *B. burgdorferi* B31 (33). A total of 59% of the chromosomal open reading frames (ORFs) have homologs in other bacterial species; in contrast, homologs have been identified for only one-third of the plasmid ORFs. The genome encodes a basic set of proteins for DNA replication, transcription, and energy metabolism but, interestingly, lacks most cellular biosynthetic pathways. Of some surprise is the tremendous number (>150) of genes that encode putative lipoproteins, suggesting an essential role for these molecules in the life cycle of the spirochete. Genome analysis of another Lyme disease spirochete, *B. garinii* (strain PBi), revealed that most of the chromosome is conserved (>90% identity at the DNA and amino acid levels) in the two species. Furthermore, two colinear plasmids (lp54 and cp26) seem to belong to the basic genome inventory of the *Borrelia* species that cause Lyme disease. However, the authors did not find counterparts of the *B. burgdorferi* plasmids lp36 and lp38 or their respective gene repertoires in the *B. garinii* genome (34). The large linear plasmid lp54 encodes two major outer surface proteins, OspA and OspB, which are tandemly arrayed in one operon (35). OspC, another major outer surface protein, is encoded on a circular plasmid (cp26), and sequence analysis of *osp*C from different strains suggests that gene exchange might play a role in diversity and immune evasion of Lyme disease borreliae (36).

Whole-genome microarray analysis revealed that a total of 215 ORFs, 136 of which are plasmid borne, were differentially expressed at 23 and 35°C. These findings highlight the potential importance of plasmid-borne genes in the adaptation of *B. burgdorferi sensu lato* to mammal hosts and tick vectors (37). The linear plasmids of *B. hermsii* and *B. turicatae* contain genes encoding outer membrane lipoproteins, called variable major proteins (Vmps). These genes are silent except when they are translocated to an expression site immediately adjacent to one of the linear plasmid telomeres. Antigenic variation of Vmp-like proteins due to recombination of *vls* (Vmp-like small) gene sequence cassettes has also been described for *B. burgdorferi*. These *vls* genes have highly varied regions, as well as highly conserved sequences which encode immunogenic epitopes important for serodiagnosis (38, 39).

EPIDEMIOLOGY AND TRANSMISSION

The ecological components that maintain *Borrelia* species in nature are quite diverse and are spread throughout the world (Table 1).

Relapsing Fever Borreliae

Rodents serve as reservoirs of most relapsing fever borreliae, which are transmitted by soft-bodied ticks of the genus *Ornithodoros* (Table 1; Fig. 1). One exception, *B. recurrentis*, the agent of LBRF, is found only in humans and transmitted only by the human-specific body louse, *Pediculus humanus humanus*. Although LBRF had a global distribution only 100 years ago, recent reported outbreaks have been limited to parts of East Africa. LBRF is not communicable between its human hosts, but rapid transfer of the infected louse between persons by direct contact and shared clothing and bedding enables efficient disease dissemination among crowded populations, particularly when personal hygiene is compromised.

Over a dozen *Borrelia* species (Table 1) are the agents of TBRF. Endemic cycles of TBRF between rodents and *Ornithodoros* ticks are recognized globally (Table 1). Human infections occur in western Canada and the United States (reportable in 11 western states), portions of Mexico, Central and South America, the Mediterranean, Central Asia, and much of Africa. *Ornithodoros* ticks are rapid (10 to 30 min) and typically nocturnal feeders; human victims most often do not recall tick bites. In parts of northwest Africa, TBRF is one of the most common bacterial infections, and in Tanzania, it is a leading cause of prenatal and child mortality (2, 6).

Lyme Disease Borreliae

The Lyme disease borreliae of *B. burgdorferi sensu lato* are transmitted by hard-bodied ticks (genus *Ixodes*) (Fig. 1). Globally, Lyme borreliosis is limited to temperate regions of the Northern Hemisphere (Table 1). The prevalence of vector-competent ticks and their infection-permissive vertebrate hosts largely defines human risk and case numbers. For example, in the United States during the 15-year period from 1992 to 2006, 93% of the total cases (*n* = 248,074) reported to the CDC by health departments were from 10 of the 50 states (Connecticut, Delaware, Massachusetts, Maryland, Minnesota, New Jersey, New York, Pennsylvania, Rhode Island, and Wisconsin), and these case incidences were mirrored by *B. burgdorferi sensu stricto* infection rates among *I. scapularis* ticks and reservoir vertebrates (40–42).

Ixodes species feed on three different hosts, depending on the developmental stage of the tick. The larvae and nymphs feed primarily on small rodents, whereas adult ticks feed on a variety of mammals (deer, raccoons, domestic and wild carnivores, larger domestic animals, and birds). The feeding period of *Ixodes* ticks is rather long (several days to over a week) and contributes to their geographic dispersal along the course of the movement of the host. Birds, particularly migratory seabirds, can transport the ticks (*Ixodes uriae*) over very long distances and thus distribute borreliae (especially *B. garinii*) worldwide (22).

There appears to be an association between *B. afzelii* and small rodents and *B. garinii* and birds, likely due to different serum sensitivities of the borreliae (43) mediated by complement regulator-acquiring surface proteins (44). In unfed ticks, *B. burgdorferi sensu lato* lives in the midgut. During the blood meal, transcriptional changes are induced in the spirochetes and precede their migration to the salivary glands (45). Migration of spirochetes from the midgut of the feeding *I. scapularis* tick to the skin of the animal host takes >36 h (46). For *Ixodes ricinus*, however, spirochete migration has been observed with ticks feeding for as few as 17 h (47).

CLINICAL SIGNIFICANCE

Relapsing Fever

Relapsing fever is an infectious disease with an acute onset of clinical signs and symptoms, including high fever, shaking chills, severe headache, nausea, myalgias, and severe malaise. Initial physical findings often are conjunctival effusion, petechiae, and diffuse abdominal tenderness. Fever attacks of 3 to 7 days are interspersed with afebrile periods of days to weeks. Detailed descriptions and reviews have been published elsewhere for louse-borne (48) and tick-borne relapsing fever (49, 50).

TBRF is, in general, less severe than LBRF. An exception to this rule is *Borrelia duttonii* TBRF in East Africa. TBRF studies in Tanzania and the Democratic Republic of the Congo have documented severe morbidity among pregnant women, the young, and the elderly; pregnancy loss rates of 47% have been reported in some areas of endemicity (2). In TBRF, up to 13 febrile attacks have been documented, and a rash is more often reported than in LBRF (28% versus 8% of cases). Splenomegaly, hepatomegaly, and jaundice are observed in 41%, 17%, and 7% of TBRF cases, respectively, whereas these signs are reported in 77%, 66%, and 36% of LBRF cases. In TBRF, 16% of the patients have respiratory symptoms and 9% have central nervous system involvement; in patients with LBRF, these figures are 34% and 30%, respectively (51). Complications leading to death (the mortality rate for LBRF patients is up to 40%) are acute heart and hepatic failure and cerebral hemorrhage. Disease severity increases with compromising conditions common to many areas of endemicity.

The initial treatment of relapsing fever cases with appropriate antibiotics may elicit the Jarisch-Herxheimer reaction (JHR) (50). This reaction, associated with the rapid clearance of spirochetes from circulation and an overwhelming release of cytokines, typically occurs within 1 to 4 h of antibiotic treatment. Signs include hypotension, tachycardia, chills, rigors, diaphoresis, and sudden elevation of body temperature. Death caused by JHR associated with LBRF has been reported. While generally not as severe, JHR associated with TBRF in the United States is reported in approximately 50% of cases. Therefore, patients with either LBRF or TBRF should be monitored closely upon initial treatment.

Acute respiratory distress syndrome (ARDS) may occur more frequently in patients with TBRF than previously recognized. In 2004 and 2005, three cases of posttreatment ARDS in severe-TBRF patients were reported from California, Washington, and Nevada. A retrospective investigation of 111 TBRF cases reported from these states during the preceding 10 years revealed two additional ARDS cases, both occurring after 2001. Continued surveillance is needed to determine whether the risk of ARDS in cases of TBRF is increasing. If so, possible correlates might include changed medical practices, use of newer antimicrobials, or the emergence of more-virulent TBRF strains (52).

Lyme Borreliosis

Lyme borreliosis can be defined by early localized, early disseminated, and late-stage manifestations similar to the three stages of syphilis (53). The natural courses of untreated *B. burgdorferi* infections vary considerably, and clinical manifestations can occur alone or in various combinations (53, 54). In the majority of cases, the infection is self-limiting, but in some cases, *B. burgdorferi* will disseminate to other skin sites, the nervous system, joints, heart, or (occasionally) other organs.

EM at the site of the infectious tick bite is the most common manifestation of early (stage I) Lyme borreliosis and occurs in 60 to 90% of patients. The center of the expanding annular lesion often fades to produce a bull's-eye appearance. However, the extents, color intensities, and durations of EM vary considerably. In Europe, the skin lesion often develops more slowly and persists longer, hence, the initial description of chronicum migrans (3). One or more general symptoms, such as fatigue, arthralgia, myalgia, and headache accompany a majority of primary EM cases (55, 56).

In some patients, hematogenous dissemination of spirochetes to other organs and tissues occurs within days to weeks of infection (stage II). Patients often feel quite ill and can present with fatigue, headache, fever, malaise, arthralgia, and myalgia. Multiple (secondary) erythemata are common in the United States but uncommon in Europe. Neurologic structures, including the meninges, brain, spinal cord, peripheral nerves, and nerve roots, are also potential sites of early-dissemination infection. In the United States, 15 to 20% of untreated patients develop neurologic signs, most commonly facial nerve palsy (unilateral or bilateral), meningitis, and radiculoneuropathy. CSF findings in cases of Lyme meningitis almost always include a mononuclear pleocytosis (10 to 1,000 cells/µl) and elevated protein concentration. Meningitis, or even facial palsy without meningismus, is more common among children than adults. Severe encephalitis is occasionally observed in stage II. Bannwarth's syndrome is the most common neurologic manifestation of early, disseminated Lyme borreliosis in Europe. The syndrome is characterized initially by intense, migratory or focal radicular pain, particularly at night, and by cranial nerve palsy. Paresis of the extremities and the trunk are less frequent. Borrelial lymphocytoma, a reddish to livid swelling of the skin that typically occurs in locations such as the earlobe, nipple, or scrotum, is manifested among some patients in Europe (53, 54). Symptomatic infection of the heart is rare in recognized Lyme disease cases and most often presents as atrioventricular conduction blocks, which can fluctuate between first-, second-, and third-degree blocks; these typically occur in conjunction with other manifestations of disease, such as EM, arthritis, or neurological disease. Symptoms, including light headedness, palpitations, shortness of breath, chest pain, and syncope, may occur within days to months of disease onset. Cardiac involvement typically resolves promptly with appropriate antibiotic treatment but can occasionally cause life-threatening cardiac conduction abnormalities. Three cases of sudden cardiac death between 2012 and 2103 in the United States were attributed to previously undiagnosed Lyme carditis (57).

Lyme arthritis and acrodermatitis chronica atrophicans (ACA), occurring months to years after the initial infection, are the most common manifestations of late (stage III) disease. Lyme arthritis can be monoarticular or oligoarticular, typically affecting the knee, and usually takes an intermittent course. Patients with ACA initially develop an infiltrative stage, followed by alterations characteristic of the atrophic stage: creased skin with livid discolorations and plastic protrusion of vessels. ACA is observed almost exclusively in Europe, a finding highly correlated with *B. afzelii* infections. Neuroborreliosis is a very rare manifestation of late (stage III) disease. Paraparesis and tetraparesis are the most common symptoms. Examination of the CSF reveals a marked elevation of protein concentration, with a low to moderate increase of cells in the CSF. The detection of intrathecally produced specific antibodies is currently regarded as the best marker of neuroborreliosis (53, 54, 58).

Early manifestations of Lyme borreliosis are observed most frequently in the spring, summer, and autumn, coinciding with tick activity. Late manifestations do not show a seasonal pattern.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

General Remarks for Collection and Transport

For culture and nucleic acid detection of borrelia, collection and preparation of specimens under sterile conditions are

TABLE 3 Specimen types used for the diagnosis of Lyme borreliosis

Clinical manifestation	Specimen type(s) for:	
	Direct pathogen detection (culture, PCR)	Antibody detection
Stage I (early/localized; days through weeks after tick bite)		
EM	Skin biopsy	Serum
Stage II (early/disseminated; weeks through months after tick bite)		
Multiple erythemata	Skin biopsy	Serum
Borrelial lymphocytoma	Skin biopsy	Serum
Lyme carditis	Endomyocardial biopsy	Serum
Neuroborreliosis	CSF	Paired serum/CSF[a]
Stage III (late/persistent; months through years after tick bite)		
Arthritis	Synovial fluid, synovial biopsy	Serum
ACA	Skin biopsy	Serum
Chronic neuroborreliosis	CSF	Paired serum/CSF[a]

[a] From the same day for antibody index determination.

of utmost importance. Body fluids should be transported without any additives, and biopsy specimens should be placed in a small quantity of sterile saline or suitable culture medium (see "Isolation Procedures," below). Samples should reach the laboratory as quickly as possible (within 2 to 4 h). Before specimens are collected and transported, the laboratory should be contacted so that details of methodology can be agreed upon. If postal transport is unavoidable, overnight delivery is recommended. Specimens for laboratory confirmation of Lyme borreliosis are presented in Table 3.

Blood and Serum

For relapsing fever, blood is the specimen of choice. During febrile attacks, borreliae may be easily detected by dark-field or bright-field microscopy of a wet-mount blood sample or a stained blood smear, respectively (see "Direct Examination" and "Microscopy," below) (Fig. 3). During early febrile periods, the spirochetemia may reach 10^6 to 10^8 cells per ml (16). Blood from acutely ill patients is also the best source for culture confirmation (32). However, the spirochetemia diminishes with each successive relapse, and visualization or culture isolation of borreliae is most often unsuccessful during afebrile periods. In contrast to what occurs in relapsing fever patients, spirochetemia in Lyme borreliosis patients is below the level of microscopic detection, with estimates of 0.1 spirochete/ml of whole blood. The rate of culture recovery from EM patients' blood has generally been 5% or less (59). However, in a series of experiments, it was demonstrated that *B. burgdorferi* culture recovery from untreated adult patients with EM was better from plasma than from serum or from an identical volume of whole blood. Approximately 50% of large-volume plasma cultures from EM patients have yielded *B. burgdorferi* (60). Serum is suitable for indirect (antibody) evidence of *Borrelia* exposure. Specific antibody detection tests are the most widely utilized tests for laboratory confirmation of Lyme borreliosis (see "Serological Tests," below). Serodiagnosis of relapsing fever is performed in only a few specialized laboratories.

Cerebrospinal Fluid

Patients with suspected Lyme neuroborreliosis (LNB) may have evidence of immunoglobulin synthesis against *B. burgdorferi* antigens in their CSF, elevated numbers of CSF inflammatory cells (usually lymphocytes, monocytes, or plasma cells), and elevated protein. CSF along with serum drawn at the same time should be obtained for laboratory demonstration of *Borrelia*-specific, intrathecal (CSF/serum antibody index [AI]) antibody production (see "Serological Tests," below). For culture or PCR detection, rates are only

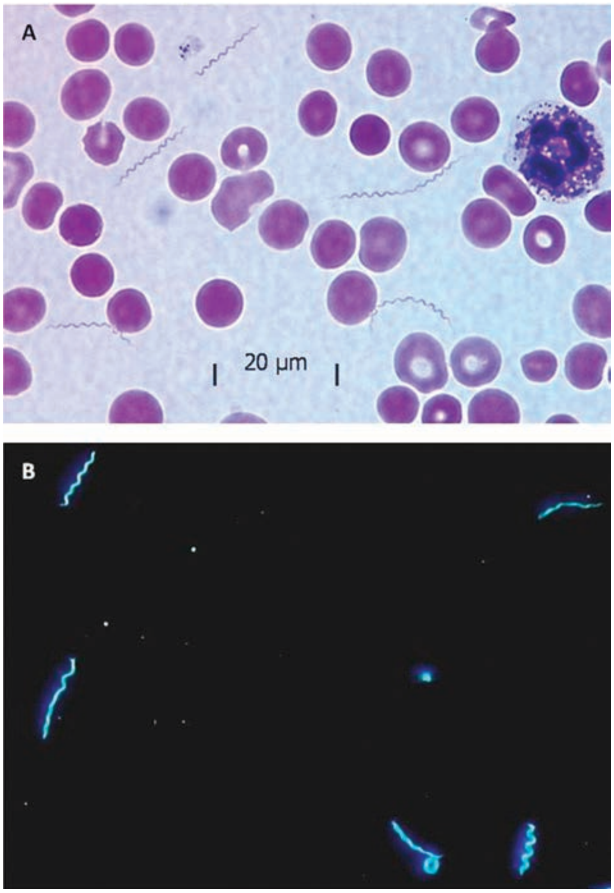

FIGURE 3 Borrelia spirochetes. *B. hermsii* in a thin smear of patient blood (bright-field microscopy, Giemsa stain) (a) and *B. burgdorferi* culture (dark-field microscopy) (b). doi:10.1128/9781555817381.ch59.f3

TABLE 4 Sensitivity of methods for pathogen detection (PCR and culture) in Lyme borreliosis

Specimen	Sensitivity (%)
Skin (EM, acrodermatitis)	50–70 when using culture or PCR
CSF (neuroborreliosis, stage II)	10–30 when using culture or PCR[a]
Synovial fluid[b] (Lyme arthritis)	50–70 when using PCR (culture is rarely positive)
Blood (EM)	15 to 40 when using PCR or culture

[a] Sensitivity can be up to 50% in patients with a disease duration of less than 2 weeks, compared with only 13% in patients for whom the illness duration was greater than 2 weeks.

[b] The sensitivity of direct pathogen detection is higher from synovial biopsy specimens.

about 20% (61). Positive PCR results in CSF also seem to correlate inversely with duration of neurologic disease. Among neuroborreliosis patients, 7 of 14 (50%) with a disease duration of less than 2 weeks had a positive PCR result, compared with only 2 of 16 (13%) patients in whom the illness duration was greater than 2 weeks ($P = 0.045$) (61).

Synovial Fluid or Synovial Biopsy Specimens

Investigation of synovial fluid or a synovial biopsy specimen by PCR can be useful in special circumstances where Lyme arthritis is suspected or the efficacy of antibiotic treatment is questioned (see "Nucleic Acid Detection Techniques," below) (59, 62). Culture is usually negative, with few exceptions. Due to the high protein permeability of the synovium, synovial fluid and serum display roughly equivalent antibody titers. Thus, it is sufficient to monitor antibody in serum.

Skin Biopsy Specimens

Skin biopsy samples are the best sources for isolation of *B. burgdorferi*; spirochetes can be isolated in most untreated cases of EM and acrodermatitis (Table 4). In cases of EM, culture success is highest (up to 86%) with biopsy samples taken close to (4 mm inside) the expanding border of the lesion (63), although this is a technique primarily for research, not for routine diagnosis. There are indications that the number of spirochetes in the skin is rather low or unevenly distributed, since an increase in sensitivity is observed if more than one biopsy sample is investigated (64). Without treatment, *B. burgdorferi sensu lato* can persist for long periods in the skin, as shown by isolation from a 10-year-old acrodermatitis lesion (65). Biopsy samples (taken after thorough disinfection of the skin) should be sent in a small amount of sterile saline or Barbour-Stoenner-Kelly (BSK) medium (with or without rifampin) as soon as possible to a microbiology laboratory capable of culturing *B. burgdorferi*.

Other Materials

Ticks are often tested for borreliae as part of epidemiological studies to assess risk to human populations in a given geographic area. Although specialized laboratories offer diagnostic services for individual ticks, detection of spirochetes within ticks by PCR or other methods has not been shown to provide clinically useful information.

DIRECT EXAMINATION

Microscopy

Direct microscopic visualization of borreliae in clinical samples is applicable only to cases of relapsing fever. During acute phases, spirochetemia often reaches 10^6 to 10^8 borreliae/ml, and motile spirochetes can be visualized by dark-field microscopy from wet preparations made from a drop of blood. This simple confirmatory test is often overlooked because of the increasingly common use of automated differential blood counting. Spirochetes can be visualized by stained (e.g., Giemsa stain) thin or thick films (Fig. 3). Detection of low-level spirochetemias may be assisted by a microhematocrit concentration technique. The hematocrit capillary is filled 75% with anticoagulated (e.g., EDTA- or citrate-treated) blood and centrifuged for 2 min. The buffy coat is then examined directly under the microscope at magnifications of ×400 to ×1,000 (66). Failure to observe spirochetes does not rule out disease, and culture isolation (see "Isolation Procedures," below) can be considered.

Antigen Detection

Enzyme-linked immunosorbent assay (ELISA) and immunoblotting have been used for the detection of borrelial antigen in body fluids, including CSF and urine (67, 68). However, a commercial assay for antigen in urine was shown to lack reproducibility, and its use is not recommended (69).

Nucleic Acid Detection Techniques

Nucleic acid amplification techniques (NAAT) may serve as an adjunct to clinical diagnosis but should be restricted to experienced and specialized laboratories (70, 71). A variety of chromosomal and plasmid targets for NAAT have been developed (for reviews, see references 59 and 72 to 74). For PCR, an analytical sensitivity of approximately 10 to 20 borreliae per test sample has been demonstrated. For Lyme borreliosis, test sensitivities for both NAAT and culture are greater with tissue specimens than with body fluids, except for synovial fluid, with which NAAT is superior. Sensitivities of 96% (62) and 86% (75) were reported for NAAT from synovial fluid from American patients with Lyme arthritis. European authors found NAAT sensitivities ranging between 50 and 70% (21–23, 76). Patients with Lyme arthritis are nearly always seropositive, so PCR of synovial samples is not used as a primary diagnostic technique. A positive PCR result after antibiotic therapy is of uncertain significance, since the presence of *B. burgdorferi* DNA does not necessarily mean that spirochetes are viable (75, 77–79).

From skin biopsy and CSF specimens, NAAT demonstrated diagnostic sensitivities of approximately 60% and 20%, respectively (19, 61, 80). A prospective study of PCR and culture detection of *B. burgdorferi* in EM biopsy samples from Slovenian patients showed comparable sensitivities (36% were culture positive with modified Kelly Preac-Mursic [MKP] medium, 24% were culture positive with BSK II medium, and 25% were PCR positive) (81). PCR targeting *ospA*, a plasmid-borne gene, is more sensitive than flagellin PCR, which uses a chromosomal target (64, 82). Borreliae can shed blebs containing plasmids, leading to greater abundance of plasmid than chromosomal genes.

PCR amplification of *B. burgdorferi* sequences from urine has been described (74, 83) but is not recommended. Although *Borrelia*-specific DNA was demonstrated in over 70% of skin biopsy samples from patients with florid EM, parallel testing of urine samples was uniformly negative (80).

ISOLATION PROCEDURES

Many Lyme and relapsing fever borreliae are successfully cultured in artificial media. However, for diagnostic purposes, culturing is a slow, time-consuming method characterized by low sensitivity, especially from body fluids of patients with Lyme borreliosis (Table 4). For these reasons, culture attempts are most often limited to research applications and performed by reference laboratories (e.g., the National Reference Center for Borreliae in Germany and the CDC in the United States).

Several media (modified Kelly media, e.g., BSK II, BSK-H, or MKP) (14, 15, 84) are capable of supporting the growth of borreliae. It is important to verify the quality of each lot of medium by growing a reference strain from a small inoculum (<10 cells). Optimum growth (the generation time of *B. burgdorferi* is about 7 to 20 h) in these media is obtained at 30 to 33°C under microaerophilic conditions. Positive cultures from EM and synovial biopsy or fluid samples (blood and CSF) may be obtained in as few as 4 days, but most isolates require several weeks of incubation, and negative cultures should be monitored by dark-field microscopy (Fig. 3) for at least 6 weeks.

IDENTIFICATION

Molecular Techniques

Most species of the genus *Borrelia* were initially delineated using DNA-DNA reassociation analysis (16, 32) or diversity at the *rrs* locus. *B. burgdorferi* has an arrangement of its rRNA genes (a single *rrs* and tandemly repeated *rrl* and *rrf* genes) which distinguishes it from the relapsing fever borreliae (which have single copies of each) (85). Sequencing of 5S-23S intergenic spacers and a number of genes, pulsed-field gel electrophoresis of large restriction fragments, PCR, and restriction fragment length polymorphism analysis of multiple targets have all been utilized for species differentiation (18, 19, 86–90). More recently, multilocus sequence analysis has been recommended as a simpler approach to taxonomic species assignment (28, 91). However, in most cases, diagnosis and effective management of individual patients are independent of species determinations beyond the Lyme disease and relapsing fever groupings.

Immunological Techniques

Serotyping methods to identify *Borrelia* species and strains within a species have been described (92, 93). However, as with molecular techniques, diagnosis and effective management of individual patients have yet to utilize characterizations beyond the Lyme disease and relapsing fever groupings.

SEROLOGICAL TESTS

Borrelia Antigens and the Human Humoral Immune Response

Borrelia burgdorferi possesses at least 30 immunogenic proteins, which include the outer surface proteins A to F (OspA to -F), a number of tissue binding proteins, and components of the flagellar apparatus. Proper detection and interpretation of the humoral response against *B. burgdorferi* must consider several variables. All Lyme borreliosis genospecies (or strains within a genospecies) do not produce qualitatively or quantitatively identical sets of antigens. In serological assays that utilize whole-cell culture extracts as the source of reactive antigens, these variables may result in different sizes of a given antigen (e.g., OspC of 21 to 25 kDa) and quantitative antigen differences or even their absence. This is particularly problematic in Europe and Asia, where multiple genospecies are present. Thus, it is imperative, even in North America, that diagnostic laboratories and manufacturers of serologic assays verify that all diagnostic antigens are present in relevant amounts. In the case of Western immunoblotting, diagnostic antigens must also be discernable from each other. For many diagnostic proteins, sequence heterogeneity, even between strains in a given genospecies, may result in amino acid variations and reduced detection of an antibody response in a patient with a heterologous infection. Again, OspC serves as an example: with 21 major OspC types recognized among North American and European isolates (94), patient reactivity in an assay with one selected OspC type may not be sufficiently cross-reactive to enable its detection (95, 96). In addition to having genospecies- and strain-dependent protein profiles as well as antigenic heterogeneity, many antigens are variably expressed in response to environmental cues, both in culture and during infection. OspC and VlsE serve as examples; while both of these potent immunogens are expressed during early infection, they are variably produced in culture, often in very small amounts. Thus, their presence in diagnostic assays must be verified. Similarly, while OspA expression is turned off in early infection (97) and is therefore an insensitive marker of this stage of disease, it is an abundant protein of most cultures. During progression to later stage II and III disease, particularly in North America, expression of OspA is often triggered and patient antibody to this antigen is strongly correlated with arthritic involvement (98).

Among immunogenic *B. burgdorferi* proteins, some proteins have both heterogeneous and conserved antigen epitopes. Despite the overall antigenic heterogeneity of OspC, portions of both the C and N termini harbor highly conserved and immunodominant epitopes (pepC10 and OscC1, respectively), which have been used successfully in peptide-based serodiagnostic enzyme immunoassays (EIAs) (99–101). Finally, at least one protein of great and recent diagnostic interest is capable of switching antigen epitopes during infection. The variable major protein-like-sequence-expressed (VlsE) protein of *B. burgdorferi* is a surface lipoprotein that is expressed early in infection. It contains both variable and invariable regions, and extensive antigenic switching within the variable regions likely contributes to immune evasion (39, 102, 103). Nonetheless, and of some surprise, studies in the late 1990s found that Lyme borreliosis patients developed strong antibody responses to VlsE, particularly to the sixth invariant region of the protein (38), and that this region was highly conserved among the three major Lyme borreliosis genospecies. These findings served as the basis of EIAs in which synthetic peptides representing the sixth invariant (or conserved) region, C6, were developed. Accumulating published studies over the last 10 years have shown that VlsE- and C6-based assays have high sensitivities in most stages of Lyme borreliosis and suggest that they may serve as future, single-tier assays for serodiagnosis (38, 59, 100, 104–108).

The earliest IgM responses to all *B. burgdorferi* infections are directed against OspC (21 to 25 kDa), the flagellar antigens p 41 (FlaB) and p37 (FlaA), and p35 (BBK32, fibronectin binding protein) and are typically detectable within the first few weeks. Detectable IgM against BmpA (39 kDa) is in part strain dependent and most often appears after the response to OspC and FlaB and FlaA (59, 109–111). Although the level of IgM antibody to most spirochetal antigens peaks within the first weeks, it often persists at detectable levels for many months.

The IgG response increases and broadens slowly over the first weeks of disease. Among the reactive antigens to which there is an early IgG response are OspC, p35 (BBK32), p37 (FlaA), VlsE, and p41 (FlaB) (38, 109, 112–114). During early-dissemination (stage II) disease, IgG levels increase and reactivity against Osp17 (DbpA [decorin binding protein A]), p39 (BmpA), and p58 often appears (115). The late-stage immune response (stage III) is characterized by IgG antibodies to a wide variety of antigens (110, 115). Approximately 80% of the sera from European patients with late disease (arthritis and ACA) react with p14, Osp17 (DbpA), p21 (not OspC), p30 (not OspA), p39, p43, p58, and p83/100 (homolog of p93) of *B. afzelii* strain PKo (115). Similarly, among North American patients with chronic neurologic abnormalities or arthritis, close to 100% react with 5 or more of the diagnostic antigens p18, p23 (OspC), p28, p30, p39 (BmpA), p41 (FlaB), p45, p58, p66, and p93 (59, 107, 110).

Notable differences in late-stage disease antibody responses between European and North American patients include those to OspC and OspA. While IgG antibodies against OspC are detected in only 20% of late-stage European patients (115), the frequency of IgG reactivity to OspC in American patients with late disease is 48% (110). Similarly, while only 5 to 7% of late-stage European patients are reactive to OspA (115, 116), over 40% of American patients with late disease are reactive to this antigen (110, 112).

Two-Step Approach in Serodiagnosis

For serodiagnosis of Lyme borreliosis, a two-step approach is recommended by the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD) and the CDC (117, 118). All serum specimens submitted for Lyme disease testing should be evaluated in a two-step process in which the first step is a sensitive serological test, such as an EIA or immunofluorescence assay (IFA). Specimens found to be negative should not be tested further. All specimens found to be positive or equivocal by a sensitive first-tier test should be further tested by a standardized immunoblot procedure (117). This procedure is also recommended in the MiQ Lyme borreliosis standard published by the German expert group on the diagnosis of Lyme borreliosis of the German Society for Hygiene and Microbiology (DGHM) (Fig. 4) (71). The concept of a two-step approach, which aims at increasing the predictive value of a positive result with each step, requires that the tests be performed in succession (71, 119). Omitting the first step, a quantitative assay, and proceeding directly with qualitative immunoblotting reduces the specificity of the procedure (120).

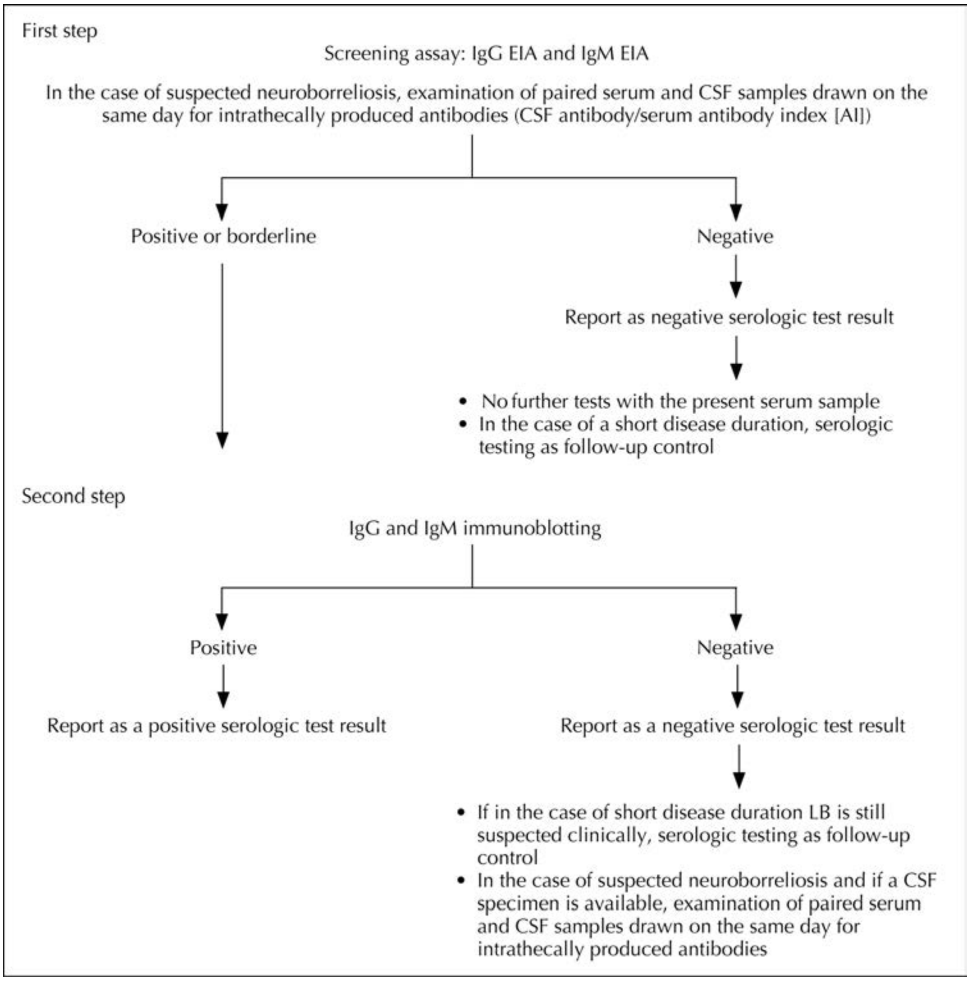
First step
Screening assay: IgG EIA and IgM EIA
In the case of suspected neuroborreliosis, examination of paired serum and CSF samples drawn on the same day for intrathecally produced antibodies (CSF antibody/serum antibody index [AI])
Positive or borderline
Negative
Report as negative serologic test result
• No further tests with the present serum sample
• In the case of a short disease duration, serologic testing as follow-up control
Second step
IgG and IgM immunoblotting
Positive
Negative
Report as a positive serologic test result
Report as a negative serologic test result
• If in the case of short disease duration LB is still suspected clinically, serologic testing as follow-up control
• In the case of suspected neuroborreliosis and if a CSF specimen is available, examination of paired serum and CSF samples drawn on the same day for intrathecally produced antibodies

FIGURE 4 Two-step approach for serodiagnosis. doi:10.1128/9781555817381.ch59.f4

IFA

For the IFA, borreliae fixed on glass slides are used as the antigen. IFA for serodiagnosis of relapsing fever, however, is challenging since expression of the major membrane proteins is variable. The specificity of IFA serodiagnosis for Lyme disease may be improved by adsorption of sera with *Treponema phagedenis* sonicate (IFA with antibodies

[IFA-ABS]) (71). For the IgM test, pretreatment of the sera with anti-IgG-immune serum is recommended to avoid false-positive test results due to rheumatoid factor as well as false-negative results due to high IgG antibody levels. As in all antibody detection assays, it is important to verify expression of OspC within the antigen source cultures. Although IFA is relatively easy to perform, it is not easy to standardize, and evaluation of test results requires expertise not always available in routine laboratories. In general, antibody titers of ≥64 and ≥256 are regarded as positive in the IFA-ABS and unadsorbed IFA, respectively. Sera from patients with syphilis are often positive in the unadsorbed assay and are rarely positive in the IFA-ABS (71).

EIA

Different modifications of the EIA have been used for the diagnosis of Lyme borreliosis. In the indirect EIA, antigen is used to coat the plates, followed by incubation with patient serum, enzyme-labeled anti-IgM or anti-IgG, and the EIA substrate. Capture IgM EIA (μ-capture EIA) has been specially designed to avoid false-positive reactivity due to rheumatoid factor (121). Rheumatoid factor false-positive reactivity can also be overcome by pretreatment of the sera with anti-IgG (116). EIA has the advantage of objective measurement, quantification, and high throughput. Many different antigen preparations have been used, including whole-cell sonicates (122), isolated flagella (123), detergent extracts (116), recombinant protein antigens (116, 124, 125), and synthetic peptides (38). Use of crude antigen preparations, such as whole-cell sonicates, often results in unacceptable specificity. Improved tests which utilize enriched, specific, or recombinant protein antigens are now widely used. Tests using an octyl β-D-glucopyranoside detergent extract and Reiter treponeme absorbent, isolated flagella, recombinant VlsE, or the C6 peptide of VlsE are commercially available (Dade-Behring, Marburg, Germany; Dakopatts, Copenhagen, Denmark; Diasorin, Turin, Italy; and Immunetics, Boston, MA). Since VlsE is not present in relevant amounts in cultivated borreliae, recombinant VlsE has been added to whole-cell extracts to increase sensitivity in some products (Dade-Behring).

Immunoblotting

Western immunoblotting is regarded as a supplementary (United States) or confirmatory (Europe) assay. This implies that it should be employed only when a screening assay is reactive (positive or indeterminate, sometimes called equivocal). Western immunoblotting enables assessment of the humoral immune response to protein antigens as separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Antigen preparations for Western immunoblotting include whole-cell lysates or recombinant protein antigen mixtures that are resolved (largely by molecular weight) by SDS-PAGE and then transferred to blot membranes. Patient antibody against dozens of borrelial antigens can be discerned by an experienced diagnostic laboratorian. However, the procedure is considered technically complex. An alternative test format is the line immunoblot, in which recombinant or native borrelia antigens that have been resolved or purified by means other than SDS-PAGE are directly striped on membranes for immunoprobing. This approach enables discrete spacing or placement of individual antigens in quantified deliveries on the membrane and avoids the overlap of comigrating antigens that often complicate reading in Western immunoblots. By either method, Western immunoblotting or line immunoblotting, it is imperative that antigen identifying and cutoff (minimal band intensity) controls are employed in each diagnostic run. These calibration controls may include antibody preparations provided with commercial kits, monoclonal antibodies (MAbs), or characterized patient samples from commercial or other (e.g., CDC) sources.

Numerous immunoblot tests which use antigens of various strains or genospecies of *B. burgdorferi sensu lato* are commercially available. The ASTPHLD and the CDC, as well as the DGHM, have published recommendations for interpretation of *Borrelia* immunoblots (71, 117). In the United States, immunoblot interpretation rules which refer to detection of antibody against whole-cell antigens of specific *B. burgdorferi sensu stricto* strains have been recommended (110, 111). An IgM immunoblot is interpreted as positive if at least two bands of the following proteins are reactive: p23 (OspC), p39 (BmpA), and p41 (FlaB). An IgG blot is interpreted as positive if five or more bands of the following proteins are reactive: p18, p23 (OspC), p28, p30, p39 (BmpA), p41 (FlaB), p45, p58, p66, and p93. If immunoblotting is used within the first 4 weeks of disease onset (early, stage I, or stage II), both IgM and IgG immunoblotting should be performed. Due to specificity concerns of the IgM immunoblotting criteria potentially yielding false-positive findings in persons with a low pretest likelihood of infection, initial recommendations limited application of IgM Western blotting to the first 4 weeks of infection. Despite these recommendations, errant IgM Western blotting use due to a disease duration of >30 days, lack of an antecedent reactive first-tier test, and misinterpretation of the blot itself continue (120). Beyond this time point, a more specific IgG-reactive Western blot test is expected. Recent studies, however, indicate that some patients do not develop a robust IgG response during the first 4 weeks of infection (107).

Interpretation of the antibody response among European patients is complicated by the risk of infection with different *Borrelia* species. In addition, immunoblot studies have shown that the immune response to European infections, compared with North American infections, is restricted to a narrow spectrum of *Borrelia* proteins (126). Interpretive rules defined in a species- and strain-specific manner have been determined (36, 115) and independently corroborated (43). *B. afzelii* strain PKo is preferred to PBi (*B. garinii*) and PKa2 (*B. burgdorferi sensu stricto*) strains because it permits a two-band criterion for the IgG test; i.e., at least two bands must be positive for p14, p17 (DbpA), p21, OspC, p30, p39 (BmpA), p43, p58, and/or p83/100 (Fig. 5) (71). According to the general Deutsches Institut für Normung (DIN) recommendations on immunoblotting (DIN recommendation 58967, part 40), at least a two-band criterion should be required for the positive interpretation of an IgG immunoblot. In IgM immunoblots, a detectable immune response is restricted to only a few bands. Therefore, the IgM blot is regarded as positive if there is strong reactivity to OspC (71). Specific DIN recommendations for *Borrelia* immunoblotting (DIN 58969, part 44) which include new antigens (i.e., VlsE) and line immunoblotting as a new technique have been published.

Recombinant immunoblotting with Osp17 (DbpA), OspC, p39 (BmpA), truncated p41 (FlaB), p58, and p83/100 has demonstrated sensitivity comparable to that of whole-cell immunoblotting except for patients with isolated EM (127). Recombinant immunoblotting was substantially improved by the addition of several homologs of VlsE and DbpA, which increased the sensitivity of antibody detection in early disease (105, 106). Recombinant blots containing DbpA, OspC, p39 (BmpA), truncated p41 (FlaB), VlsE, and

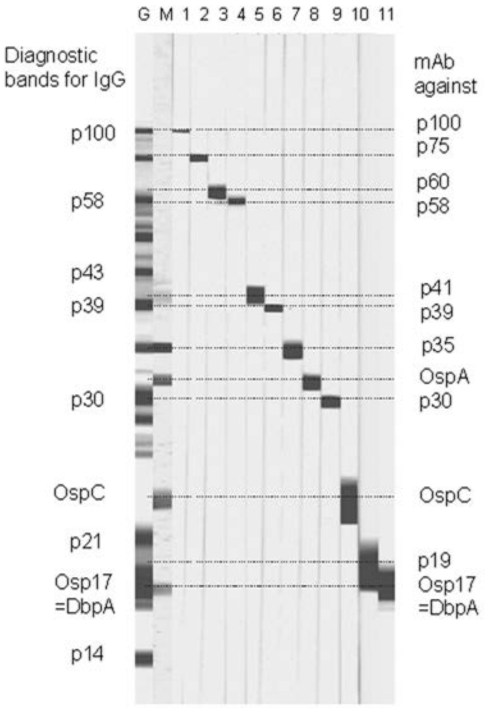

FIGURE 5 Whole-cell immunoblot for identification of diagnostic bands with MAbs. The antigen used is *B. afzelii* strain PKo. Lane G, IgG blot from a patient with late disease; lane M, IgM blot from a patient with early disease; lanes 1 to 11, different MAbs against the respective reactive proteins. (Modified from reference 115.) doi:10.1128/9781555817381.ch59.f5

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p83/100 are commercially available (Mikrogen, Munich, Germany). By the line blot technique, sensitivity among early neuroborreliosis patients was significantly increased compared to that of conventional sonicate immunoblotting (92% versus 69%, respectively) (105). Listings of FDA 510(k)-approved seroassays for commercial distribution can be found at http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMN/pmn.cfm by entering “LSR” as the product code.

Detection of Intrathecally Produced (CSF) Antibodies

Approximately 15% of untreated Lyme borreliosis patients will develop neurologic manifestations. Lyme neuroborreliosis (LNB) has been divided into early-dissemination and late stages. Both the central nervous system (CNS) and the peripheral nervous system (PNS), as well as blood vessels and meningeal coverings, may be involved in either stage. Laboratory testing should be used only to confirm the diagnosis and presence of *B. burgdorferi*-specific antibody in CSF or serum and may be indicative of past or present infection. The pattern of nervous system involvement is largely stage dependent and may affect the correlation between clinical spectrum and serologic test utility. Thus, careful evaluation of a thorough clinical history and presentation are critical to the selection of appropriate diagnostic tests and proper interpretation of their findings (58, 128–130).

In patients in whom the CNS is involved, there should be evidence of CNS inflammation. Rarely, this may be localized to the brain or spinal cord, but in most cases, inflammation involves the CSF and is evidenced by pleocytosis, elevated protein concentrations, and, in cases of protracted infection, antiborrelia-specific-immunoglobulin synthesis. In contrast, for PNS-limited disease, the CSF findings may be normal, as they are in most patients who have toxic metabolic encephalopathy (58).

Although <10% of LNB cases are culture confirmed, subtle differences in clinical presentations between European and North American LNB cases are linked to causative genospecies. North American cases are limited to *B. burgdorferi sensu stricto*, while most European culture-positive LNB cases are *B. afzelii*, and a much smaller percentage are *B. garinii* and *B. burgdorferi sensu stricto*.

The triad of symptoms of early-dissemination LNB, also known as meningoradiculoneuropathy, includes aseptic meningitis, cranial neuropathy, and radiculoneuritis; these may occur singularly or in combination. The single-most-common presentation of early-dissemination LNB in North America is meningitis. Examination of the CSF shows mononuclear pleocytosis and elevated protein. CSF-specific anti-*B. burgdorferi* (IgA or IgG) immunoglobulin is demonstrated in 80 to 90% of patients. Standard two-tier serology of these patients is also most often positive. Cranial neuropathy occurs both in North America and Europe in about 10% of early-dissemination LNB cases. Most frequently, this involves the facial nerve and is manifested by unilateral or bilateral facial palsy. Only about 50% of these cases will demonstrate CSF pleocytosis. European early-dissemination LNB often presents as Bannwarth’s syndrome and is highly associated with *B. garinii* infection. This radiculoneuropathy also occurs in up to 5% of untreated North American Lyme borreliosis patients. Most patients with Lyme radiculoneuritis are reactive in two-tier serologic testing, and CSF findings include pleocytosis and *B. burgdorferi*-specific antibody (58, 128–131).

Late neurological manifestations usually develop months to years after initial infection.

Encephalopathy is more common in North America, while encephalomyelitis is more frequent in Europe. For cases of late encephalopathy, serum immunoreactivity is nearly universal, while CSF pleocytosis, elevated protein, and *B. burgdorferi*-specific antibody are found in only 5%, 20 to 45%, and ~50% of cases, respectively. Most cases of chronic encephalomyelitis are reported from Europe, although North American cases have been described. In these cases, CSF pleocytosis and marked *B. burgdorferi*-specific antibody are almost universal.

Although there are no well-accepted criteria for seroconfirmation of neuroborreliosis in the United States, detection of an intrathecal *Borrelia*-specific immune response is a valuable tool and widely utilized in Europe (132). Methods taking into account potential dysfunction of the blood-CSF barrier, a common finding in neuroborreliosis, are required for accurate assessment of intrathecal antibody production. Long-used procedures for detection of specific intrathecal antibody production in the diagnosis of neurosyphilis have been modified for the diagnosis of neuroborreliosis (133–135). The most frequently used method is the determination of the CSF/serum antibody index (specific antibody index [AI]). CSF and serum must be obtained at the same time. By calculating the AI, CSF and serum are compared with regard to the portion of pathogen-specific IgG antibodies in the total IgG content. An AI of ≥2.0 is considered significantly elevated (71, 136). Lower indices (e.g., ≥1.3) are also considered significant by some investigators. False-positive AI results are likely with neurosyphilis patients when tested with whole-cell or flagellum sonicates by EIA. Here, EIAs with *T. phagedenis* adsorption (Dade-Behring) or recombinant antigens not cross-reacting with *Treponema pallidum* can be helpful for differential diagnosis. Other suitable methods for determination of intrathecal antibody production are the μ- or γ-capture EIA (Dakopatts) (133) and the IgG-matched immunoblot (135). The latter allows comparison of the antibody spectra (against various *Borrelia* proteins) in serum and in CSF and thus permits conclusions as to the specificity of the intrathecal antibody response.

Past and Future Vaccination and Its Impact on Serology

The recombinant OspA vaccine (LYMErix) was withdrawn from the U.S. market in 2002. This vaccine utilized a novel approach to preventing human disease by killing *B. burgdorferi* in the vector tick while it fed on vaccines. Low sales and concerns regarding a putative arthritogenic T-cell epitope of OspA which mimicked human leukocyte function-associated antigen-1 (hLFA-1) led to its withdrawal. Nonetheless, continued efforts to make use of the immunogenic properties of OspA and its mode of action have led to development of a genetically engineered chimera in which the hLFA-1-like epitope has been removed and the variable portions of several predominant serotypes of the antigen are retained (137). While promising, this vaccine has yet to go through phase III trials. Alternate vaccine candidates and strategies remain largely at the research level (138).

Controversial Methods

A variety of diagnostic approaches have been developed as alternatives or adjuncts to the more widely practiced methods described above. These include antigen detection, T-cell proliferation assays, antigen-specific memory/effector T cell responses, and novel culturing methods (139–146). While some of these have been disputed in the scientific literature (147–151), it is often difficult to assess the validity of other tests that have not been validated independently and/or have not been reviewed by the FDA. No test should be utilized for clinical diagnosis and case management without Clinical Laboratory Improvement Amendments (CLIA) certification. In addition, novel assays should be tested in multiple laboratory settings with blind and well-documented (clinically and epidemiologically) patient samples from positive Lyme disease controls, healthy controls, and patients with other diseases (and without a history of Lyme disease), including those that may overlap clinically with Lyme disease. Novel test results should have a positive correlation with clinical, epidemiological, and other well-established test findings of Lyme disease patients. Ideally, tests should be reviewed and cleared by the FDA, which has considerable experience with all facets of Lyme disease diagnostics and is able to address comparative test performances; without FDA clearance, it is often difficult to assess the sensitivity, specificity, and clinical utility of novel assays (41).

ANTIMICROBIAL SUSCEPTIBILITIES

The antimicrobial susceptibility of *Borrelia* species has been studied intensively *in vitro* (16, 152). Standard methods for the determination of the minimal bactericidal concentration have not been established. However, there is general agreement on the *in vitro* susceptibility of borreliae to antimicrobials, as follows. *B. burgdorferi sensu lato* is susceptible to macrolides, tetracyclines, semisynthetic penicillins, and the late second- and third-generation (expanded- and broad-spectrum) cephalosporins; moderately susceptible to penicillin G and chloramphenicol; and resistant to trimethoprim, sulfamethoxazole, rifampin, the aminoglycosides, and the quinolones (16). No significant differences between the Lyme disease borreliae and relapsing fever borreliae (*B. hermsii* and *B. turicatae*) were found with regard to penicillin G, amoxicillin, ceftriaxone, erythromycin, azithromycin, doxycycline, or tetracycline (16). There is no indication for routine antimicrobial susceptibility testing in cases of either Lyme disease or relapsing fever.

Recommendations for Antibiotic Therapy

All clinical manifestations of *B. burgdorferi* infection should be treated with antibiotics. The antibiotic, dosage, duration, and route of application depend on the clinical picture and the stage of the disease (153–156). In cases of solitary EM, oral treatment with doxycycline, amoxicillin, or cefuroxime axetil is recommended. In acrodermatitis, the same antibiotics and daily doses as for EM are recommended. In arthritis, oral treatment with doxycycline may be tried first, but in cases of poor therapeutic response, patients should be treated intravenously with cephalosporins or penicillin G. Intravenous cephalosporins or penicillin G is also recommended for stage III neuroborreliosis.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

General Aspects

Clinical criteria (case history and clinical findings) are decisive factors in the diagnosis and ordering of microbiological laboratory testing. The predictive value of laboratory tests is directly related to the pretest probability. It should be kept in mind that the lower the probability based on the clinical diagnosis, the lower the predictive value of a positive test result. For example, a negative serological result has a high negative predictive value for Lyme arthritis, since nearly all cases are seropositive. Whether or not a positive test corresponds with the patient's presentation is a question that can be answered only by the clinician, e.g., by means of clinical case definitions applied to the various manifestations of Lyme borreliosis. Therefore, the laboratory report should not contain any therapy recommendations.

Serological Report

The serological report should contain the following.

1. Recording of individual test results. Results of the first assay, generally an EIA, are reported as positive, indeterminate, or negative. The immunoblot results are reported as positive or negative. In the case of a positive result, the reactive diagnostic bands may be reported (60). Caution against overinterpretation of minimally reactive blots must be emphasized (e.g., IgG reactivity against p41 is expected in approximately 50% of healthy adults in the United States and Europe and is excluded from the European scoring criteria).
2. Assessment of the final result of the two-step approach with regard to its immunodiagnostic significance (e.g., whether specific antibodies have been detected or not).
3. Assessment of serological findings as to the stage of the immune response, as far as test results allow pertinent statements to this effect (see below).
4. Recommendations for further reasonable diagnostic methods (PCR or culture) or for serological follow-up, if indicated.

TABLE 5 Sensitivity of antibody detection methods in the diagnosis of Lyme disease

Stage	Sensitivity (%)	Remark(s)
I	20–50	IgM predominates
II	70–90	IgM and IgG are present; in cases of long disease duration, IgG predominates
III	Nearly 100[a]	Usually only IgG is present

[a] Tests are negative only for patients with a very short duration of symptoms.

Patterns of Serological Results in Various Stages of Lyme Borreliosis

Antibody tests performed in the early stage of Lyme borreliosis, particularly in cases lacking evidence of spirochetal dissemination, may show a negative or an indeterminate result (Table 5), often due to insufficient time for the full evolution of the immune response. In many cases, seroconversion still occurs after the initiation of treatment. Serological testing of patients with EM alone is not recommended because of the low predictive value of a negative result and the highly characteristic appearance of most rashes. In the presence of a suggestive clinical presentation and inadequate response to therapy, serologic testing is warranted up to 6 weeks after onset of disease. During the first 4 weeks of a positive clinical correlation, detection of IgM is consistent with an active infection. A robust IgG response is expected thereafter. Usually only a few bands (IgM and/or IgG) are detected by immunoblotting during the first weeks of early disease. In late disease, a positive test for IgG antibodies is mandatory for seroconfirmation, and the IgM test is not useful for establishing the diagnosis; the absence of IgG rules out the diagnosis of late Lyme disease, even in the presence of IgM. False-positive IgM results due to a polyclonal B-cell activation immune response in the context of herpesvirus infections or autoimmune diseases and rheumatoid disorders also need to be considered. In many cases, the origin of such IgM responses remains unclear. Both IgM and IgG may persist for many months, and their presence may be compatible with past, asymptomatic, spontaneously resolved, or treated and clinically cured infections. Such patterns are often found among members of high-risk groups with frequent tick exposure (for example, forest workers) who do not show any clinical manifestations.

In cases of CNS neuroborreliosis, detection of pathogen-specific, intrathecal antibodies provides critical laboratory evidence of past or present infection. The presence of anti-borrelia antibodies (IgM, IgG, and IgA) in the absence of CSF pleocytosis suggests previous infection, and since specific antibody and positive AI may be detectable years after treatment and cure, repeat testing is not appropriate for monitoring therapy success.

Influence of Antimicrobial Therapy on Serodiagnosis

Clinicians are often tempted to order repeated posttreatment serologies in an effort to correlate cure and decreasing antibody titers. However, IgG antibodies against *B. burgdorferi* (especially as measured in whole-cell antigen assays) persist for a long time even after successful therapy. Significant titer changes can be expected only several months after the end of therapy; in cases of late manifestations, even years may elapse. Since there is practically no indication for follow-up serological tests, therapeutic success should be based on clinical criteria. A fourfold decline in titer of antibody to VlsE peptide C6 was shown to be an indicator of successful therapy for early Lyme disease (157), but this was not demonstrated for late disease (158) or posttreatment Lyme disease syndrome (159).

Sources of Error in Serodiagnosis

False results, both negative and positive, can occur from the test itself or the nature of the immune response. Seronegative results within the first days of stage 1 illness onset, before a mounting of the humoral immune response, are the norm. In Europe, differences between the test antigen and the species causing infection can also contribute to seronegative findings. Deficiencies in diagnostic antigen expression among cultivated borreliae will compromise the sensitivities of tests; important diagnostic antigens, such as OspC, DbpA, and VlsE, are often not expressed in cultivated borreliae. The high background reactivity of many first-generation whole-cell-based assays often results in lower specificity and therefore frequent false-positive results. Cross-reactivity with treponemes can be largely avoided by use of Reiter treponeme adsorbent, although syphilis serology should be performed in cases where treponeme exposure cannot be ruled out. Second- and third-generation assays with improved sensitivity and specificity are preferable to the first-generation tests. Nonetheless, critical assessment of pretest risk factors, clinical history, and presentation will provide the best guidance for laboratory test use and minimize false test outcomes of current and future diagnostic tests.

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Treponema and *Brachyspira*, Human Host-Associated Spirochetes

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TAXONOMY

The recognition of spirochetes as human host-associated organisms is believed to date from nearly 400 years ago, when Van Leeuwenhoek described spiral, nimble "animalcules" in human oral plaque (1). Determination of taxonomic relationships among spirochetes has been complicated by their fastidious nature and the refractoriness of many to cultivation. Numerous phenotypic traits have been examined in attempts to establish taxonomic hierarchies (2). Paster et al. (3) demonstrated using 16S rRNA sequences that spirochetes can be grouped into a single phylum containing five clusters: *Treponema*, *Spirochaeta*, *Borrelia*, *Serpula* (now *Brachyspira*), and *Leptospira*. The relatedness among the members of individual clusters varied considerably. Interspecies similarities among borreliae were >97%, suggesting recent evolutionary divergence. In contrast, the ~10% sequence differences among treponemes pointed toward divergence over a greater evolutionary time frame, a conclusion consistent with the diversity of vertebrate and invertebrate hosts known to harbor treponemes as symbionts (4).

Many investigators formerly believed that cultivable treponemes were closely related, nonpathogenic forms of *Treponema pallidum* (5). Miao and Fieldsteel (6, 7) dispelled this idea using DNA-DNA hybridization to demonstrate that *T. pallidum* Nichols DNA shared <5% homology with DNAs of cultivable treponemes but was indistinguishable from that of a *Treponema pertenue* (yaws) strain. Their work led to the reclassification of the agents of venereal syphilis, endemic syphilis, and yaws as *T. pallidum* subsp. *pallidum*, *T. pallidum* subsp. *endemicum*, and *T. pallidum* subsp. *pertenue*, respectively, while *Treponema carateum*, the cause of the skin disease pinta, retained its status as a distinct species primarily because no isolates were available for study (8). *Treponema denticola* has long been regarded as the prototype oral treponeme, and its presence strongly correlates with periodontal disease (9, 10); however, this species represents only a small fraction of the resident treponemal phylotypes in patients with gingivitis and periodontitis, most of which are uncultivable (11, 12). In 1997, all *Serpulina* species were reclassified as *Brachyspira* (13). *Brachyspira hyodysenteriae* is the cause of porcine dysentery, a severe mucohemorrhagic colitis of pigs, while *Brachyspira pilosicoli* is recognized as a cause of diarrheal disease in pigs, chickens, and dogs (14, 15). Three *Brachyspira* species have been associated with human intestinal spirochetosis (HIS): *B. aalborgi*, *B. pilosicoli*, and the provisionally named "*B. hominis*" (14, 16, 17).

DESCRIPTION OF THE AGENTS

T. pallidum Subspecies

The four members of the genus *Treponema* that cause venereal syphilis, endemic syphilis, yaws, and pinta are morphologically identical and, despite advances in molecular differentiation based on genomic sequences (18), are distinguished primarily by differences in geographic distribution, epidemiology, clinical manifestations, and host range in experimental animals (Table 1) (19–22). The extraordinarily high degree of similarity (99.8%) between the *T. pallidum* subsp. *pallidum* and *T. pallidum* subsp. *pertenue* genomes (18) implies that these profound differences arise from polymorphisms at a handful of genetic loci. Only *T. pallidum* subsp. *pallidum* is transmitted routinely by sexual contact and vertically from a pregnant woman to her fetus (19, 21, 22). It also is the only subspecies that regularly breaches the blood-brain barrier (19, 22). The type strain of *T. pallidum* subsp. *pallidum* (Nichols) was isolated in 1912 from the cerebrospinal fluid (CSF) of an individual with secondary syphilis (23) and has been propagated since by intratesticular inoculation of rabbits (24). No strain or subspecies of *T. pallidum* can be cultivated continuously *in vitro*, although limited replication has been achieved by cocultivation with mammalian cells (25). Rabbits have long been the animals of choice for studying syphilitic infection, and rabbit inoculation (rabbit infectivity testing, or RIT) is the only means by which strains can be isolated from clinical specimens (26). A murine model for venereal syphilis has been recently developed (27).

T. pallidum is ~0.2 µm in diameter, has tapering ends, and ranges in length from 6 to 20 µm (Fig. 1). Cells typically consist of 6 to 14 flat waves with a wavelength of 1.5 µm and amplitude of ~0.3 µm (Fig. 1). Because of their small diameter, pathogenic treponemes cannot be visualized by bright-field microscopy, nor do they take up Gram stain. Consequently, *T. pallidum* is best visualized by phase-contrast or dark-field (DF) microscopy, the latter being an important diagnostic tool. *T. pallidum* grows slowly (doubling time of 30 to 33 h in rabbits) (28) and poorly tolerates desiccation, elevated temperatures, and high oxygen

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TABLE 1 Characteristics and major clinical features of the treponematoses

- Venereal syphilis (*Treponema pallidum* subsp. *pallidum*)
  - Global distribution
  - Transmitted predominantly sexually, also congenitally and, rarely, by transfusion or organ transplantation
  - Stages/manifestations
    - Primary (local): 10–90 days postinoculation (avg, 21 days)
      - Single or multiple ulcers (chancres) on skin or mucous membranes
      - Regional lymphadenopathy
    - Secondary (disseminated): 6 wk–6 mo postinfection (also seen with early latent relapses)
      - Diffuse lesions, most typically skin, but also visceral
      - Other mucocutaneous lesions: condyloma lata, alopecia
      - Generalized lymphadenopathy
      - Occasionally constitutional symptoms
      - CNS involvement common (usually asymptomatic invasion by *T. pallidum*) but also symptomatic (usually "aseptic" meningitis)
    - Latent (early latency, ≤1 yr duration; late latency, >1 yr duration)
      - Reactive serologic tests for syphilis without manifestations
      - Relapses common during early latency and are infectious
      - Late latency is noninfectious, chronic, "asymptomatic" (may just be clinically inapparent)
    - Tertiary
      - Gummatous (skin, bones, or viscera)
      - Cardiovascular (classically involving proximal thoracic aorta)
      - Neurosyphilis (classically paresis, tabes dorsalis)
    - Congenital
      - Early (perinatal): ranges from asymptomatic to disseminated to fulminant
      - Late: interstitial keratitis, bone and tooth deformities, deafness, neurosyphilis

- Yaws (*Treponema pallidum* subsp. *pertenue*)
  - Tropical areas (Africa, Asia, Latin America, Western Pacific)
  - Transmitted by nonvenereal person-to-person contact
  - Infection usually occurs in childhood
  - Typically does not involve CNS or cause congenital infection
  - Stages/manifestations
    - Early: 10–90 days postinoculation (avg, 21 days)
      - Primary lesion (mother yaw): papular, nontender, often pruritic, crusted, or ulcerated
      - Secondary disseminated lesions (frambesia)
      - Hyperkeratotic plaques, dactylitis
      - Malaise, fever, lymphadenopathy
    - Latent: positive serologic tests but without other signs of infection
    - Late: 10% of untreated individuals
      - Destructive lesions of bone and cartilage
      - Osteitis, periostitis

- Endemic syphilis (*Treponema pallidum* subsp. *endemicum*)
  - Saharan Africa, Middle East
  - Transmitted by nonvenereal person-to-person contact, utensils
  - Infection of children or adults, rarely congenital infection
  - Stages/manifestations
    - Early
      - Primary: mucosal or cutaneous lesions, often undetected
      - Secondary: disseminated oropharyngeal, cutaneous lesions
      - Generalized lymphadenopathy
      - Periostitis
    - Latent: positive serologic tests, no other signs of infection
    - Late: destructive skin, bone, and cartilage lesions

- Pinta (*Treponema carateum*)
  - Semiarid warm areas of Central and South America
  - Transmitted by nonvenereal person-to-person contact, usually children or adolescents
  - Restricted to skin
  - Stages/manifestations
    - Early
      - Primary lesion: hyperkeratotic, pigmented papule or plaque
      - Disseminated skin lesions
      - Regional lymphadenopathy
    - Late: pigmentary changes in skin (hyper- and hypopigmentation)

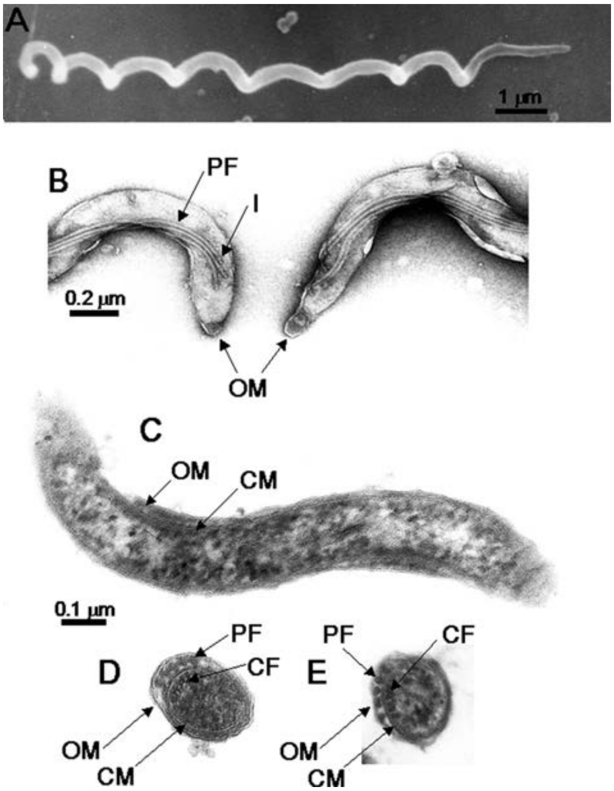

FIGURE 1 Morphology of *T. pallidum*. (A) Scanning electron micrograph showing spiral shape. (B) Negatively stained view of the tips of two organisms. Note the insertion points (I) of periplasmic flagella (PF) near the ends. (C to E) Electron micrographs of ultrathin sections, showing the outer membrane (OM), the cytoplasmic membrane (CM), periplasmic flagella (PF), and the location of the cytoplasmic filaments (CF). Bars, 0.1 μm. Reprinted with permission of Kluwer from reference 187. doi:10.1128/9781555817381.ch60.f1

tensions (29). Once considered to be an anaerobe, it was reclassified as a microaerophile based on the finding that it replicates best *in vitro* in ambient oxygen concentrations of 3 to 5% (29). *T. pallidum* relies entirely on glycolysis for energy production and is unable to synthesize fatty acids, nucleotides, enzyme cofactors, and most amino acids (29, 30). The molecular architecture and cell envelope composition differ markedly from those of Gram-negative bacteria (31). In addition to lacking lipopolysaccharide (30), the outer membrane contains an extraordinarily low density of integral membrane proteins (31, 32). Many of the bacterium's dominant immunogens are lipid-modified, periplasmic proteins involved in transport of nutrients across the cytoplasmic membrane (31, 32).

Host-Associated Spirochetes

Spirochetal phylotypes identified in the human mouth belong exclusively to the genus *Treponema* (11). Oral treponemes are anaerobic, spiral-shaped organisms ranging from 0.15 to 0.30 μm in diameter and from 5 to 16 μm in length (33). They can be differentiated based on genotypic characteristics and biochemical parameters, such as growth requirements, carbohydrate fermentation, and enzymatic activities (8, 34). Ten species of *Treponema* (*T. denticola*, *T. pectinovorum*, *T. socranskii*, *T. vincentii*, *T. lecithinolyticum*, *T. maltophilum*, *T. medium*, *T. parvum*, *T. putidum*, and *T. amylovorum*) have been cultivated from the oral cavity; the large majority of treponemal phylotypes remain uncultivated (10). *T. denticola*, the prototype, binds to host cells and extracellular matrix components and also coaggregates with other bacteria (*Porphyromonas gingivalis* and *Fusobacterium nucleatum*) in periodontal pockets (35). The ability of treponemes to flourish in periodontal pockets also can be attributed to their ability to evade complement-mediated killing in inflamed gingival tissues prone to bleeding (36, 37). Although far less invasive than *T. pallidum*, *T. denticola* displays a limited degree of hematogenous dissemination in a SCID mouse model (38).

Brachyspira spp. are oxygen-tolerant anaerobes differentiated by phenotypic growth characteristics, degree of beta-hemolysis, and biochemical parameters such the ability to produce indole and hydrolyze hippurate (39). *B. aalborgi* was first isolated from rectal biopsy specimens from patients with intestinal spirochetosis in 1982 and has not been detected in or isolated from animals other than nonhuman primates (40, 41). The type strain is comma shaped or helical, 2 to 6 μm long and ~0.2 μm in width, with tapered ends and four flagella at each end (42). Isolation requires weeks of incubation under anaerobic conditions (43). There are no experimental animal models to study disease caused by *B. aalborgi* (14). *B. pilosicoli* colonizes the large intestine of a number of animal species and is thought to have greater pathogenic potential for humans than *B. aalborgi* (14, 44). Compared to *B. aalborgi*, isolates of *B. pilosicoli* are longer (4 to 12 μm), are more coiled, and have more-pointed ends (45). On blood agar plates, *B. pilosicoli* isolates display two morphologically distinct, weakly beta-hemolytic colony types (46). Intestinal spirochetes can be found in all regions of the colon but increase in number from cecum to rectum (47). They reside in the brush border surrounded by microvilli with their proximal tips embedded in invaginations of the host cell membrane (Fig. 2). Because of high density and orientation perpendicular to the mucosal surface, they form a characteristic basophilic fringe often described as a "false brush border" in histological samples stained with hematoxylin and eosin (H&E) (Fig. 2, top) (47). A third *Brachyspira* species, currently named "*B. hominis*" because it has been detected only in humans, has been identified exclusively by molecular means in colon biopsy specimens (17).

EPIDEMIOLOGY AND TRANSMISSION

Venereal Syphilis

In 2008, the World Health Organization (WHO) estimated that there were 36 million prevalent cases of syphilis and 11 million incident cases in adults between the ages of 15 and 49 (48). The majority occur in underdeveloped countries, particularly in sub-Saharan Africa and Asia. Eastern Europe and Russia reported dramatic increases in the incidence of syphilis with the fall of Communism (49). There have been alarming increases in syphilis rates in China during the past 2 decades that have been attributed to the enormous societal and economic changes in that country (50). In 2008, ~1.36 million pregnant women worldwide were estimated to have active syphilis, causing substantial perinatal mortality and morbidity that could have been averted by early testing and treatment (51).

In the United States, the incidence of syphilis fell precipitously in the late 1940s following the introduction of penicillin, reaching a nadir in the 1950s (52). Syphilis rates

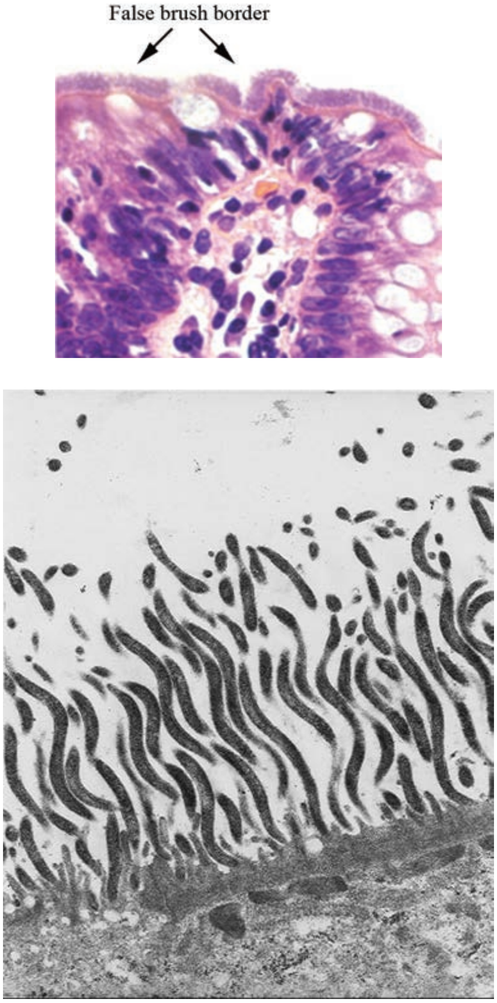

FIGURE 2 Human intestinal spirochetosis. (Top) H&E staining of a colonic biopsy sample showing a false brush border covering the luminal surface of the colon as observed by light microscopy. Reproduced with permission of Blackwell Publishing Asia Pty Ltd. from reference 188. (Bottom) Transmission electron micrograph showing *Brachyspira aalborgi* attached end-on to the colonic mucosa, forming a false brush border. Magnification, ×11,000. Reproduced with permission of the American Society for Microbiology from reference 102.
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then began to rise, with peaks (all well below pre-penicillin era levels) occurring approximately every 10 years. After steady declines following the "sex for drugs" cocaine-related outbreak of the late 1980s and early 1990s (53), the United States experienced a steady increase in rates of primary and secondary (P&S) syphilis from 2001 to 2009 due to a resurgence of risky sexual behaviors among men who have sex with men (MSM) (54, 55). Although rates have started to decline, syphilis continues to be a major health problem among MSM, with an increase in the male-to-female ratio of P&S syphilis from ~2:1 in 2000 to ~8:1 in 2011 (56). In the United States, there has been a decrease in congenital syphilis to 8.5 cases per 100,000 live births in 2011, likely associated with the decline in rates of P&S syphilis among women in the past 5 years (57).

A statistical association between HIV infection and syphilis became evident at the outset of the AIDS epidemic (58) and has been attributed to both behavioral and biological components (59). Similar high-risk behaviors promote transmission of both HIV and *T. pallidum*. Genital ulcers can facilitate HIV acquisition and transmission by breaching protective mucosal barriers, recruiting HIV-susceptible CD4/CCR5-positive immune cells, and increasing HIV shedding in the genital tract (60). A recent review of studies conducted among adults living with HIV/AIDS worldwide reported a median prevalence of 9.5% for syphilis (61). The highest prevalence of coinfection was noted among individuals with newly diagnosed HIV infection.

The primary mode of syphilis transmission is through sexual contact, with exudative lesions of P&S disease being the most infectious (Table 1 and Fig. 3). Estimates of the risk of sexual transmission vary greatly from 10 to 80%; the 30% transmission rate reported by Schroeter et al. (62) is the generally accepted estimate (63). Vertical transmission of syphilis due to transfer across the placenta has been recognized for centuries (21). Neonates also can become infected from exposure to lesional exudate or infected maternal blood within the birth canal (21). Since the 1940s, transmission of syphilis through blood transfusion in the United States has been negligible due to routine testing of donor blood and refrigeration of blood products (64). However, transfusion-associated syphilis remains a concern in underdeveloped countries with inadequate screening programs (65). There have been rare instances of syphilis transmission via solid organ transplantation (66).

Endemic Treponematoses

According to WHO estimates, prior to the advent of penicillin, ~200 million persons were exposed to the endemic treponematoses during their lifetimes. In the early 1950s, the WHO and the United Nations Children's Fund launched an extremely successful campaign that decreased the global prevalence of endemic treponematoses by >95% over an approximately 10-year period (20). Unfortunately, the failure of local health services has led to the resurgence of these diseases in many areas where they were formerly endemic (20). Yaws, the most common of the endemic treponematoses, occurs mainly in poor communities in tropical regions of Africa, Asia, Latin America, and the Western Pacific (67). Pinta is now confined to native populations in remote Central and South America (Table 1) (20). In contrast to yaws and pinta, endemic syphilis (or bejel) is a disease of hot, dry countries; major foci of endemic syphilis still exist in the Sahelian region of Africa (Table 1) (20). The agents of yaws and pinta are transmitted primarily by direct person-to-person nonsexual contact with exudative lesions during childhood or early adolescence (Table 1). Transmission of endemic syphilis also occurs via nonsexual contact with infectious lesions on the skin and mucous membranes or via shared drinking and eating utensils (20).

Oral Treponemes: Gingivitis, Periodontal Disease, and Atherosclerosis

The epidemiologic importance of *T. denticola* and other oral treponemes arises to a great extent from their occurrence as members of the polymicrobial consortium that causes gingivitis and periodontitis (9). Gingivitis is ubiquitous globally in children and adults and is associated with poor oral hygiene; comprehensive oral hygiene programs are effective in preventing or reducing gingival inflammation (68). Chronic periodontitis is most common in adults and

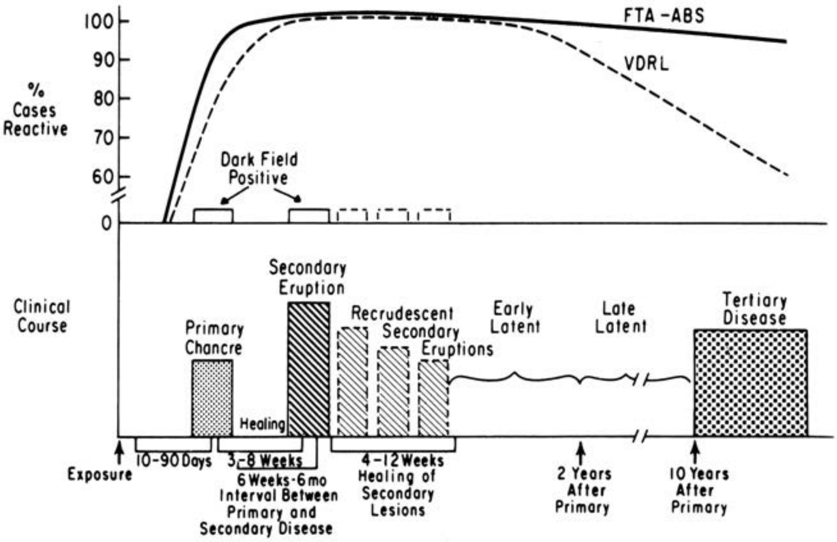

FIGURE 3 The natural course of untreated syphilis. Reprinted with permission of Appleton & Lange from reference 189. doi:10.1128/9781555817381.ch60.f3

seniors and is more common with cigarette smoking, obesity, diabetes, and alcohol consumption (69). The National Survey of Employed Adults and Seniors and the Third National Health and Nutrition Examination Survey found periodontal disease in 24% of employed adults and >60% of seniors (70, 71). Accumulating evidence supports an epidemiologic link between periodontal disease and atherosclerotic vascular disease, although a causal relationship has not been definitively established (72, 73).

Intestinal Spirochetosis

The frequency of spirochetal colonization of the intestinal tract declined dramatically in developed countries during the 20th century but remains high in the developing world (47). *B. aalborgi*, lacking animal reservoirs, is probably transmitted via the fecal-oral route (44). Infection with *B. pilosicoli* likely occurs by ingestion of water contaminated by feces of birds, animals, or infected humans (44). In developed countries, the prevalence of HIS is greatest among MSM with or without HIV infection, with oral-anal contact being the presumed mode of transmission (46, 74).

CLINICAL SIGNIFICANCE

Venereal Syphilis

Figure 3 illustrates the natural history of untreated syphilis, emphasizing the relationship between the infectious and noninfectious stages of the disease and reactivity in serologic tests (see below). Table 2 presents modified case definitions used by the Centers for Disease Control and Prevention (CDC) for syphilis staging and surveillance. Although the clinical consequences of syphilitic infection may be delayed for months to years, venereal syphilis typically commences with the appearance of one or more mucocutaneous lesions days to weeks following inoculation (63). Primary syphilis occurs when spirochetes replicating at the site of inoculation induce a local inflammatory response, giving rise to one or more chancres, the defining lesion(s) of primary syphilis. The clinical consequences of spirochete dissemination, collectively referred to as secondary syphilis, become manifest 4 to 10 weeks after the chancre. Because chancres tend to be painless and often are not visible in females, women tend to present with secondary disease; for the same reasons, a similar trend has been noted in MSM (75, 76). Although mucocutaneous lesions are by far the most common presentation, secondary syphilis can affect any organ (63). Mucocutaneous lesions of secondary syphilis usually resolve in 3 to 12 weeks, leading to the asymptomatic stage referred to as latency. The Oslo Study of Untreated Syphilis showed that 25% of patients experience secondary relapses, mostly within the first year but as late as 5 years after initial infection (77). All forms of tertiary syphilis have decreased markedly in incidence in the post-antibiotic era. Gummas are large, necrotizing, granulomatous lesions that may involve skin, bone, viscera, or the central nervous system (CNS) (78, 79). Cardiovascular syphilis, due to persistent infection of the blood vessels in the wall of the aorta (vasa vasorum), typically presents as aortic valvular insufficiency, coronary stenosis, and/or aortic aneurysm (80). Neurosyphilitic syndromes have been grouped into five major categories with substantial overlap in presentations: asymptomatic, meningeal, meningovascular, parenchymatous, and gummatous (81).

A multicenter, prospective, randomized trial of syphilis therapy found that HIV infection had only a minimal effect on the clinical manifestations of early syphilis (82, 83). However, there have been reports of HIV-infected patients presenting with symptomatic early neurosyphilis occurring within the first 12 months of infection, which is otherwise rare in HIV-negative persons (84). Recent studies also have suggested a higher occurrence of ocular syphilis and syphilitic hepatitis among HIV-infected patients (85, 86).

Most infants with congenital syphilis are asymptomatic at birth (21). Early congenital syphilis is analogous to secondary syphilis and can involve almost any fetal organ, most frequently the liver, kidneys, bone, pancreas, spleen, lungs, heart, and brain (21, 87). Two years of age is used to demarcate early from late congenital syphilis, which corresponds to tertiary syphilis in the adult. The best-known stigmata of late congenital syphilis are Hutchinson's teeth, interstitial

TABLE 2 Clinical and laboratory criteria for diagnosis of syphilis (case definitions)[a]

- Early syphilis
  - Primary
    - Confirmed (requires 1 *and* 2 or 3)
      1. One or more chancres (ulcers)
      2. Identification of *T. pallidum* in lesion exudate by microscopy (DF or DFA-TP)
      3. Detection of *T. pallidum* DNA in lesion exudate by PCR
    - Probable (requires 1 *and* either 2 or 3)
      1. One or more lesions compatible with chancres
      2. Reactive nontreponemal test
      3. Reactive treponemal test
  - Secondary
    - Confirmed (requires 1 *and* either 2, 3, or 4)
      1. Localized or diffuse mucocutaneous lesions consistent with secondary syphilis
         - a. Macular, papular, follicular, papulosquamous, or pustular rash
         - b. Condylomata lata (anogenital region or mouth)
         - c. Mucous patches (oropharynx or cervix)
      2. Identification of *T. pallidum* in lesion exudates by microscopy (DF or DFA-TP)
      3. Identification of treponemes in skin biopsy by silver, immunofluorescence (DFAT-TP), or immunohistochemical staining
      4. Detection of *T. pallidum* DNA in tissue by PCR
    - Probable (requires 1 *and* 2)
      1. Skin or mucous membrane lesions consistent with secondary syphilis
      2. Reactive nontreponemal test titer of ≥4 and a reactive confirmatory treponemal test
  - Early latent
    - Probable (requires 1, 2, 3, *and* either 4, 5, 6, or 7)
      1. Absence of signs and symptoms of syphilis
      2. Reactive nontreponemal and treponemal tests and no evidence of having acquired the disease within the preceding 12 mo
      3. A current nontreponemal test titer demonstrating ≥4-fold increase from the last nontreponemal test titer
      4. Documented seroconversion or ≥4-fold increase in the nontreponemal test titer during the previous 12 mo
      5. A history of symptoms consistent with primary or secondary syphilis during the previous 12 mo
      6. A history of sexual exposure to a partner who had confirmed or probable primary or secondary syphilis or probable early latent syphilis (documented independently as duration of <1 yr)
      7. Reactive nontreponemal and treponemal tests in a person whose only possible exposure occurred within the preceding 12 mo
  - Late latent
    - Probable (requires 1 *and* either 2 or 3)
      1. Absence of signs and symptoms of syphilis
      2. Reactive nontreponemal and treponemal tests and no evidence of having acquired the disease within the preceding 12 mo
      3. A history of syphilis and a current nontreponemal titer demonstrating a ≥4-fold increase from the last titer, with no evidence of having acquired the disease in the preceding 12 mo
- Late syphilis
  - Benign (gummatous) and cardiovascular
    - Confirmed (requires 1 *and* 2 or 3)
      1. Clinically compatible case (e.g., inflammatory lesions of the skin, bones, or cardiovascular system)
      2. Identification of treponemes in tissue sections (usually skin biopsy) by silver, immunofluorescence (DFAT-TP), or immunohistochemical staining
      3. Detection of *T. pallidum* DNA in tissue by PCR
    - Probable (requires 1, 2, *and* 3)
      1. Clinically compatible case
      2. A reactive serum treponemal test
      3. Absence of clinical signs or symptoms consistent with neurosyphilis
  - Neurosyphilis
    - Confirmed (requires 1, 2, *and* either 3, 4, or 5)
      1. Clinical signs consistent with neurosyphilis
      2. A reactive serum treponemal test
      3. A reactive VDRL in CSF

(Continued on next page)

TABLE 2 *(Continued)*

4.Detection of *T. pallidum* DNA in CSF by PCR
5.Identification of treponemes in nervous tissue by silver, immunofluorescence (DFAT-TP), or immunohistochemical staining
Probable (requires 1, 2, *and* 3)
1. Clinical signs consistent with neurosyphilis
2. A reactive serum treponemal test
3. Elevated CSF protein or leukocyte count in the absence of other known causes

Congenital syphilis (neonatal)
Confirmed (requires 1 *and* 2 or 3)
1. Clinically compatible case (e.g., hepatosplenomegaly, rash, condyloma lata, jaundice, or anemia)
2. Demonstration of *T. pallidum* by microscopic examination of specimens from lesions, amniotic fluid (antenatal), placenta, umbilical cord, nasal discharge, or autopsy material
3. Detection of *T. pallidum* DNA in lesions, tissue, blood, and/or CSF by PCR
Probable (requires 1 or 2 *and* 3)
1. Infant born to a mother who had untreated or inadequately treated syphilis at delivery, regardless of findings in the infant
2. An infant or child with a reactive treponemal test result
3. One of the following additional criteria
    a. Clinical signs or symptoms of congenital syphilis on physical examination
    b. Evidence of congenital syphilis on radiographs of long bones
    c. Abnormal CSF cell count or protein without other cause
    d. Reactive VDRL in CSF
    e. Reactive fluorescent treponemal antibody absorbed—19S-IgM antibody test or IgM ELISA
Syphilitic stillbirth
1. A fetal death that occurs after a 20-wk gestation
2. Fetus weighs >500 g and the mother had untreated or inadequately treated syphilis at delivery

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[a] These criteria were modified from the Sexually Transmitted Diseases Surveillance Case Definitions for Nationally Notifiable Infectious Diseases (http://www.cdc.gov/std/stats/casedefinitions-2014.pdf).

keratitis, saddle nose deformity, frontal bossing, and saber shins.

Endemic Treponematoses

Clinical features of the endemic treponematoses are summarized in Table 1. Primary yaws (frambesia) consists of a papule at the site of inoculation that enlarges to a hyperkeratotic papilloma ("mother yaw") before forming a shallow ulcer or pitted scar that can persist for months to years (67). Highly infectious secondary lesions appear weeks to months later, frequently accompanied by early osteoperiostitis of the proximal phalanges or long bones (67). After a several-year period of infectious relapses, patients enter an asymptomatic late latent period; ~10% of untreated patients progress to tertiary disease, generally consisting of solitary destructive lesions of bone or mucocutaneous surfaces.

Endemic syphilis usually begins as a generalized infection in the absence of an obvious primary lesion and is characterized by ulcerative lesions of the oropharyngeal mucosa (mucous patches), angular stomatitis and split papules at the corners of the mouth, intertrigial condylomata (similar to those of venereal syphilis), painful periostitis, and rashes (20). Tertiary gummas, most commonly on the skin, in the nasopharynx, and in bone, may result in severe disfigurement.

Pinta is unique in being limited to skin (20). The initial lesions of primary pinta are small papules, which appear after an incubation period of 1 week to 4 months. Secondary lesions (pintids) usually appear 2 to 6 months later. Tertiary lesions consist of well-defined hyperchromic, hypochromic, achromic, or dyschromic plaques.

Intestinal Spirochetosis

In 1967, Harland and Lee (88) coined the term "intestinal spirochetosis" to describe a noninflammatory condition of the large bowel in which spirochetes attached end-on to the colonic epithelium in a dense, palisade-like arrangement, forming a basophilic "false brush border" (Fig. 2). Although they identified spirochetes in 9 of 100 consecutive biopsy specimens examined, they were unable to relate these findings to symptomatology. The confusion and controversy over the clinical significance of HIS have not abated over the years despite extensive phylogenetic and biochemical characterization of intestinal spirochetes and advances in methodologies for their isolation and/or detection (14, 89, 90). Although generally noninvasive and minimally inflammatory, *Brachyspira* spp. have been observed causing crypt abscesses, ulceration, and necrosis (91). Spirochetemia with multiple-organ failure has also been reported (14, 92).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

T. pallidum: Syphilis

Because *T. pallidum* cannot be cultivated on artificial medium, the diagnosis of syphilis has to rely on the direct detection of treponemes in clinical specimens and/or reactivity in serologic tests. The method used for direct detection of *T. pallidum* depends on the stage of disease, the clinical presentation, and the type of specimen obtained (Table 3). *T. pallidum* can be detected in lesion exudate by DF

TABLE 3 Laboratory tests used for direct detection of *T. pallidum* in clinical samples obtained during the various stages of syphilis

Stage of disease	Test(s) used for specimen type						
	Lesion exudate	Lymph node aspirate	Tissue biopsy	Blood/ plasma/ serum	CSF	Amniotic fluid	Placenta or cord
Primary	DF,[a] DFA-TP,[b] PCR	DF, DFA-TP, PCR	–	–	–	–	–
Secondary	DF,[a] DFA-TP, PCR	DF, DFA-TP, PCR	Silver stain, IHC, DFAT-TP,[b] PCR	PCR	PCR[d]	–	–
Neurosyphilis	–[c]	–	Silver stain, IHC, DFAT-TP,[b] PCR	–	PCR	–	–
Tertiary (not neurosyphilis)	–	–	Silver stain, IHC, DFAT-TP, PCR	–	PCR	–	–
Fetal/congenital[e]	DF, DFA-TP, PCR	–	Silver stain, IHC, DFAT-TP, PCR	PCR	PCR	DF, DFA-TP, PCR	Silver stain, IHC, DFAT-TP, PCR

[a] Applicable to all except oral and anal lesions.

[b] There is no commercial kit for this test, but FITC-conjugated polyclonal or monoclonal antibody can be purchased commercially.

[c] –, not applicable.

[d] CDC recommends against routine lumbar puncture, but it needs to be done for neurologic symptoms.

[e] Nasal discharge or autopsy material can also be examined for *T. pallidum*.

microscopy, direct fluorescent-antibody test for *T. pallidum* (DFA-TP), or PCR. Tissue biopsies are rarely performed on genital ulcers but are used for diagnosing nongenital manifestations of secondary and tertiary disease. PCR can be performed on either fixed or unfixed tissue, although unfixed tissue is preferable. PCR assay of CSF is not recommended under routine circumstances because treponemes are present in a substantial percentage of spinal fluids from early syphilis patients who lack neurologic complaints or findings (93). Other specimens, such as lymph node aspirates and amniotic fluids, although rarely obtained, can be examined by DF microscopy, DFA-TP, and PCR, while placenta or cord tissue can be examined by silver staining, immunohistochemistry (IHC), direct fluorescent-antibody tissue test for *T. pallidum* (DFAT-TP), and PCR.

The order of sample collection from genital ulcers or moist lesions depends on the tests to be performed. Samples should be collected first for DF microscopy (see below), followed by DFA-TP and PCR. Ideally, the specimen for both DF microscopy and DFA-TP should be free of red blood cells, other microorganisms, and tissue debris. A specimen for DFA-TP is collected in the same manner but left to air dry for 15 min. To collect serous exudate for PCR, a sterile Dacron- or cotton-tipped swab should be rolled firmly along the base of the ulcer or lesion. The swab then should be suspended in a cryotube containing 1 to 2 ml of nucleic acid transport medium such as AssayAssure (Sierra Molecular Corporation, Sonora, CA) or universal transport medium (Copan Diagnostics, Murrieta, CA). Tissue or fine-needle aspirates (e.g., lymph nodes) for silver staining, IHC, or DFA-TP should be fixed as soon as possible in 10% buffered formalin at room temperature and sent to the laboratory for paraffin embedding, sectioning, and staining. To test products of conception for congenital syphilis, a 3- to 4-cm section of umbilical cord distal from the placenta should be obtained as soon as possible after delivery and processed as above.

A detailed procedure for collecting a specimen for DF examination has been described by Wheeler et al. (94). A step-by-step instructional video can be obtained without charge from Allan Pillay, Laboratory Reference and Research Branch, Division of STD Prevention, CDC [(404) 639-3446; apillay@cdc.gov]. Briefly, the site is gently cleansed and abraded with sterile gauze moistened with physiological saline until serous fluid appears; the specimen is then collected onto a clean glass slide and a coverslip is applied. Specimens obtained for DF microscopy should be examined within 20 min since diagnosis relies on identification of motile treponemes. Slides containing air-dried lesion exudates and touch preparations for DFA-TP staining can be stored in a slide container at 4 to 29°C for up to 2 weeks; otherwise, specimens should be fixed with acetone and stored at −20°C until testing. Serum, plasma, and CSF should be stored at 4°C if testing will be delayed by more than 4 h and at −20°C or lower if testing will be done more than 5 days from collection. Samples of unfixed tissue, ulcer exudate, mucosal or skin lesions, CSF, and amniotic fluid should be stored at −70°C if PCR testing cannot be performed immediately. Formalin-fixed samples should be stored at room temperature prior to embedding and sectioning or DNA extraction.

If specimens must be sent to a different laboratory for analyses, previously frozen plasma or serum must be shipped on dry ice. Transportation of whole blood for serologic testing can be done at ambient temperature if testing is done on-site; otherwise, samples should be stored at 4°C and transported overnight with cool packs. CSF for serology can be transported at ambient temperature overnight or on cold packs if also being examined by PCR. Samples collected in AssayAssure or universal transport medium for PCR can be shipped overnight at room temperature or with cool packs. Formalin-fixed tissue samples can be shipped at ambient temperature.

Serum is the specimen of choice for conventional nontreponemal and treponemal tests, but plasma can also be used with some of the enzyme immunoassays (EIAs) and newer treponemal assays. Plasma can be used for the rapid plasma reagin (RPR) and toluidine red unheated serum test (TRUST) assays but not for the VDRL test because heat inactivation of plasma enhances fibrin formation, leading to false-positive results. Plasma should be tested within 24 h to avoid false-positive test results. Fingerstick whole blood, serum, or plasma can be used for rapid point-of-care (POC) syphilis tests. Whole blood for PCR testing should be collected in tubes containing EDTA as an anticoagulant. When screening for congenital syphilis, the mother's serum should be used rather than cord blood. For IgM-specific tests, infant's serum is required since cord blood specimens can be contaminated by maternal blood.

T. denticola and Other Oral Treponemes

Specimen collection for detection, isolation, or identification of *T. denticola* and other commensal treponemes is not normally performed in the routine clinical management of gingivitis or periodontitis. Detailed methods for propagating these organisms for research purposes have been published (95).

Brachyspira: Intestinal Spirochetosis

Fecal samples should be collected in sterile containers and transported at ambient temperature to the laboratory or overnight on ice if culture or DF microscopy will be performed off-site. Rectal swabs (96) can be collected and transported in Stuart medium (Becton Dickinson, Sparks, MD). Fecal samples and swabs should be processed for culture within 24 h of collection (97, 98). Fresh colonic or rectal biopsy samples should be used for culture, while samples for histological examination should be processed as described for *T. pallidum*. Biopsy samples placed in physiological saline have been used successfully to culture *B. aalborgi* (97). Fecal samples or rectal swabs can be used for isolation of strains (99) or examination by DF microscopy (100). Biopsy specimens obtained from the colon or rectum can serve as material for culture, PCR, or histological examination by light microscopy or transmission electron microscopy (TEM) (100–102). Cultured spirochetes from solid- or broth-based media can be observed by phase-contrast or DF microscopy (99).

DIRECT EXAMINATION

T. pallidum

DF Microscopy

Samples from exudative primary, secondary, and early congenital syphilis lesions should be examined by DF microscopy whenever possible. Other specimens (e.g., lymph node aspirates and amniotic fluid) can contain enough spirochetes for successful DF examination (Table 3). DF should not be performed on oral lesions, since *T. pallidum* cannot be distinguished easily from commensal oral spirochetes.

DFA-TP

Touch preparations of lesion exudates (103) or tissue impressions (104) can serve as samples for DFA-TP, which utilizes either a fluorescein isothiocyanate (FITC)-conjugated polyclonal or monoclonal antibody for staining. There is no Food and Drug Administration (FDA)-approved DFA-TP test in the United States, although labeled polyclonal antibodies can be obtained commercially (Meridian Life Sciences, Memphis, TN; ViroStat, Portland, ME). Results with polyclonal antibodies should be interpreted with caution, since these reagents are not specific for pathogenic treponemes.

Silver Staining, DFAT-TP, and IHC

Silver staining (either Warthin-Starry or the Steiner modification of the Dieterle method) has long been used to visualize treponemes in paraffin-embedded samples. DFAT-TP is a modification of the DFA-TP test that enables immunofluorescent labeling of treponemes in tissue samples (105). For DFAT-TP, tissue sections are deparaffinized and pretreated prior to immunostaining to enhance epitope accessibility (106). Recently, impressive IHC results have been obtained using commercially available (Biocare Medical, Concord, CA; ViroStat) polyclonal anti-*T. pallidum* antibodies and avidin-biotin immunoperoxidase staining in paraffin-embedded skin biopsy specimens from secondary syphilis patients (107, 108).

PCR

Although PCR is not used routinely for syphilis testing and a commercial test is not available, numerous studies have been published since 1991 using PCR for *T. pallidum* detection based on several gene targets (109, 110). Of these, the *polA* (*tp0105*) (111) and *tpn47* (*tp0574*) (112) are most commonly used. PCR is most useful for genital ulcers and other exudative lesions, which typically contain large numbers of treponemes and can be sampled noninvasively.

Genomic DNA for PCR is usually extracted from 200 µl of nucleic acid transport medium containing a genital ulcer swab sample using a commercial kit (e.g., QIAamp DNA mini kit; Qiagen Inc., Valencia, CA). Laboratories with real-time PCR capability can use the TaqMan-based multiplex PCR assay developed in the Laboratory Reference and Research Branch, Division of STD Prevention at the CDC. This assay simultaneously detects *T. pallidum*, *Haemophilus ducreyi*, and herpes simplex viruses 1 and 2 (HSV-1 and -2), the major causative agents of genital ulcer disease (GUD). Gene targets, primers, and probes for the assay are shown in Table 4. Laboratories that lack real-time PCR capability can use the conventional multiplex PCR assay for simultaneous detection of *T. pallidum*, *H. ducreyi*, and HSV described by Mackay et al. (113). If testing for *H. ducreyi* will not be performed, PCR products from the multiplex reaction can be analyzed by agarose gel electrophoresis. Otherwise, an enzyme-linked hybridization assay (113) must be used because the amplicons for *H. ducreyi* and HSV cannot be distinguished by size on agarose gels. When the primary diagnostic objective is to determine whether a specimen contains just *T. pallidum*, a TaqMan-based real-time PCR targeting the *polA* gene can be used (106). The primers consist of TP-1 (5′-CAGGATCCGGCATATGTCC-3′) and TP-2 (5′-AAGTGTGAGCGTCTCATCATTCC-3′) and a probe, TP-3 (5′-CTGTCATGCACCAGCTTCGACGTCTT-3′), which is labeled with 6-carboxyfluorescein (FAM) at the 5′ end and black hole quencher 1 (BHQ1) at the 3′ end. Laboratories unable to perform real-time PCR can use a conventional assay targeting the *polA* gene of *T. pallidum* with primers F1 (5′-TGCGCGTGTGCGAATGGTGTGGTC-3′) and R1 (5′-CACAGTGCTCAAAAACGCCTGCACG-3′) using PCR conditions described by Liu et al. (114).

Brachyspira

Fresh stool specimens or rectal swabs can be examined by DF microscopy for the presence of spirochetes (100). Colonic and rectal biopsy specimens can be examined by using periodic acid-Schiff, H&E, or silver staining: spirochetes appear as a "fuzzy coat" on the brush border of the epithelium (Fig. 2, top) (97, 115). Using TEM, the organisms appear to attach end-on to the host cell membrane (Fig. 2, bottom)

TABLE 4 Oligonucleotide primers and probes used for real-time multiplex PCR for GUD

Organism or control	Gene target	Sequence of primer/probe (5′–3′)	Nucleotide position	Concn[a] (nM)
HSV-1 and -2	*gD*[b]	CCCCGCTGGAACTACTATGACA	472–493	200
		GCATCAGGAACCCCAGGTTA	535–516	300
		FAM[f]-TTTAGCGCCGTCAGCGAGG-BHQ[g]	496–514	200
H. ducreyi	*hhdA*[c]	AATCGTTAACTGCGGGATTAGG	5153–5174	200
		CAATAGACACATTATCGCCCTTTAAA	5245–5220	300
		JOE[f]-ATGGCCATGGTAGTGAGGTAAATCAGGCTGT-BHQ	5180–5210	200
T. pallidum	*tpn47*[d]	CAACACGGTCCGCTACGACTA	864–884	300
		TGCCATAACTCGCCATCAGA	931–912	300
		ROX[f]-ACGGTGATGACGCGAGCTACACCA-BHQ	887–910	200
Human	*RPP30*[e]	CCAAGTGTGAGGGCTGAAAAG	826–846	80
		TGTTGTGGCTGATGAACTATAAAAGG	905–880	80
		CY5[f]-CCCCAGTCTCTGTCAGCACTCCCTTC-BHQ	851–876	80

[a] Indicates final concentration of primer or probe in multiplex reaction.
[b] Glycoprotein D gene.
[c] Hemolysin A gene.
[d] 47-kDa immunogen gene.
[e] Human ribonuclease P gene target is used as an internal control.
[f] Fluorescent dyes.
[g] Black hole quencher.

(102, 116). IHC also has been performed with polyclonal antibodies to *T. pallidum*, *Mycobacterium bovis*, and an uncharacterized intestinal spirochete (102, 115). Detection of *B. pilosicoli* and *B. aalborgi* DNA can be achieved with the PCR assays described in Table 5 (101, 117) or, alternatively, with a real-time PCR that can simultaneously detect both species plus "*B. hominis*" (17).

ISOLATION PROCEDURES

T. pallidum

RIT is the only means available for isolating *T. pallidum* from clinical specimens. Because of its extraordinary sensitivity (1 to 2 organisms), RIT has long been considered the gold standard for detection of treponemes (26). The method has been described in detail by Lukehart and Marra (24). Because of a number of factors (e.g., cost, the requirement for a specialized animal facility, the need for trained personnel, and the time required for a rabbit to test positive), RIT is not practical for routine diagnostic purposes.

Brachyspira

Fecal samples, rectal swabs, or colon or rectum biopsy specimens can be cultured using brain heart infusion agar (102, 118) or Trypticase soy agar medium (102) with 10% bovine blood, 400 μg/ml of spectinomycin, and 5 μg/ml of polymyxin incubated anaerobically at 37°C. Specimens should be streaked onto agar plates within 1 h of collection. Colonies of *B. aalborgi* appear light gray and weakly beta-hemolytic with a diameter of 1.2 mm on brain heart infusion agar medium after 21 days of incubation (99). *B. pilosicoli* and *B. aalborgi* appear as a thin film or as discrete, pinpoint colonies on Trypticase soy agar medium after 5 to 14 days (102). *B. aalborgi* cultures usually require a longer incubation period. *B. aalborgi* has been successfully subcultured on brain heart infusion agar (99) and propagated in Trypticase soy broth containing 10% fetal calf serum (97). *B. pilosicoli* is less fastidious than *B. aalborgi* and can be subcultured on media used for its isolation.

IDENTIFICATION

Brachyspira

B. pilosicoli and *B. aalborgi* strains can be characterized using API-ZYM (bioMérieux, Inc., Durham, NC) and using biochemical tests such as indole production and hippurate hydrolysis (92, 97, 102). A strong hippurate reaction and weak α-galactosidase activity are often used to identify *B. pilosicoli*,

TABLE 5 Primers and thermocycling conditions for *B. aalborgi*- and *B. pilosicoli*-specific PCR[a]

Organism	Primer sequence (name) (5′–3′)	Predicted product size (bp)	Thermocycling conditions
B. aalborgi 16S rRNA	TAC CGC ATA TAC TCT TGA C (F, Ba 16S) CCT ACA ATA TCC AAG AAC C (R, Ba 16S)	471	94°C for 4.5 min; 33 cycles of 94°C for 30 s, 46°C for 30 s, 72°C for 30 s
B. aalborgi nox	GGT TGA CTC AAG CAC TAC (F, Ba *nox*) AAA CCG TAT TTT GTT CCA GG (R, Ba *nox*)	334	94°C for 4.5 min; 30 cycles of 94°C for 30 s, 46°C for 30 s, 72°C for 30 s
B. pilosicoli 16S rRNA	AGA GGA AAG TTT TTT CGC TTC (Acoli 1) CCC CTA CAA TAT CCA AGA CT	439	94°C for 4.5 min; 33 cycles of 94°C for 30 s, 51°C for 30 s, 72°C for 30 s
B. pilosicoli nox	GTA ACT CCT CCT ATT GAG (F, Sp *nox*) GCA CCA TTA GGT AAA GTC (R, Sp *nox*)	465	94°C for 4.5 min; 30 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 30 s

[a] Adapted from references 101 and 117.

while *B. aalborgi* is negative for α-galactosidase activity and gives a weak hippurate reaction (102).

TYPING SYSTEMS

T. pallidum

Identification of stable polymorphisms within the *T. pallidum* genome made possible the development of a practical molecular typing system for *T. pallidum* (119). Typing is based on PCR amplification and restriction fragment length polymorphism (RFLP) analysis of three members of the *tpr* gene family (*tprE* [*tp0313*], *tprG* [*tp0317*], and *tprJ* [*tp0621*]) (Fig. 4, top) and amplification of a variable number of 60-bp tandem repeats within *arp* (*tp0433*) (Fig. 4, bottom). To type *T. pallidum* strains, an ~1.8-kb region of *tprE*, *tprG*, and *tprJ* is simultaneously amplified using a nested PCR and primer pairs B1 (5′-ACTGGCTCTGCCACACTTGA-3′) and A2 (5′-CTACCAGGAGAGGGTGACGC-3′) and IP6 (5′-CAGGTTTTGCCGTTAAGC-3′) and IP7 (5′-AATCAAGGGAGAATACCGTC-3′), followed by restriction digestion with MseI and RFLP analysis (120). The 60-bp repeat region of *arp* is amplified with PCR primers 1A (5′-CAAGTCAGGACGGACTGTCCCTTGC-3′) and 2A (5′-GGTATCACCTGGGGATGCGCACG-3′); an improved method has been recently developed (121). Strain typing is performed primarily on specimens from genital ulcers and mucosal lesions, but other specimens have also been typed (122, 123). The major strain types identified are 14*a*, 14*d*, and 14*f* (120, 123). Only a few specimens from epidemiologically linked cases have been typed (124). To provide better discrimination among some of the more common subtypes, an enhanced typing method was recently described that utilizes the *arp*/*tpr* method (CDC typing system) described above, along with sequence analysis of an 84-bp variable region of the *tp0548* gene (125).

Brachyspira

B. pilosicoli strains can be characterized by pulsed-field gel electrophoresis (PFGE) and multilocus enzyme electrophoresis, but the PFGE method appears to be more discriminatory. Studies using PFGE show that *B. pilosicoli* strains belong to a genetically diverse group (44, 126). There are no reports on typing of *B. aalborgi* strains.

SEROLOGIC TESTS

General Principles

Syphilis elicits two different types of antibody responses, traditionally designated "nontreponemal" and "treponemal" (Fig. 3) (33). Nontreponemal tests, which detect antibodies directed against lipoidal antigens, were the first to be developed and are still used for screening and evaluation of serologic response following therapy (Table 6). Nontreponemal tests have two inherent problems: (i) they lack sensitivity in primary and late syphilis (Fig. 3); and (ii) they lack specificity because reactive antibodies can be elicited in diseases and conditions unrelated to syphilis, giving rise to biological false positives (BFPs) (127). Conventional treponemal tests, beginning with fluorescent treponemal antibody absorption (FTA-ABS), were developed using *T. pallidum* or *T. pallidum* lysates to address the lack of specificity of the nontreponemal tests (Table 7) (33). The need for high throughput and decreased costs have prompted development of automated EIAs, immunoblots, and chemiluminescence assays, most of which now use recombinant *T. pallidum* antigens (Tables 8 to 13). The challenge of

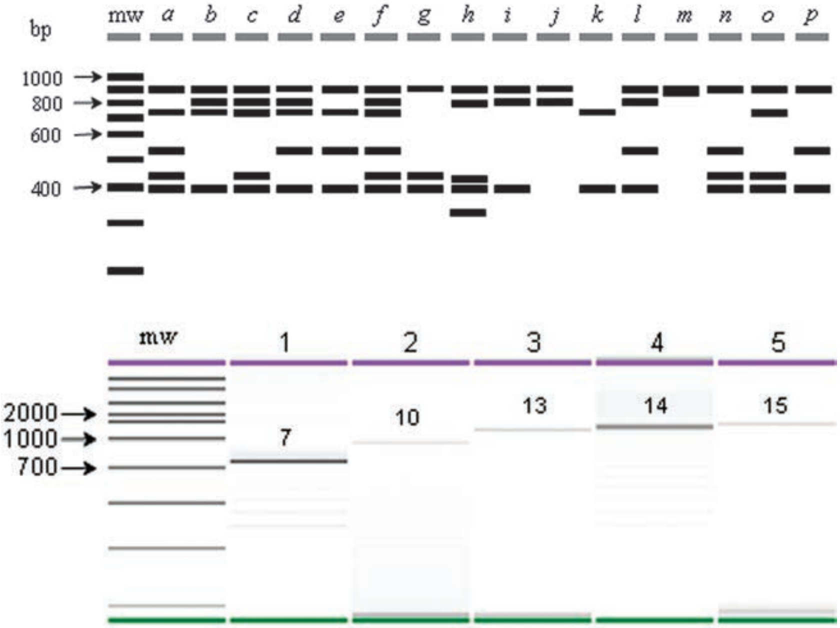

FIGURE 4 PCR-based molecular typing of *T. pallidum* "street strains." (Top) Schematic representation of all *tpr* gene MseI RFLP patterns identified to date. Reproduced with permission of Springer from reference 190. (Bottom) Schematic showing different *arp* repeat sizes obtained by PCR. Molecular weight markers in base pairs are shown to the left in each panel. In panel B, the size of each repeat is indicated above the amplicon. doi:10.1128/9781555817381.ch60.f4

TABLE 6 Nontreponemal tests for syphilis serodiagnosis

Parameter	VDRL	USR	RPR	TRUST
Method	Flocculation	Flocculation	Flocculation	Flocculation
Specimen[a]	S, C	S	S, P	S, P
Sample vol (µl)[b]	50–100	50–100	50–100	50–100
Time to complete (min)[b]	15	15	15	15
FDA approved	Yes	Yes	Yes	Yes
Sensitivity (%)				
Primary	67–78	80	60–86	70–85
Secondary	96–100	100	100	100
Tertiary	85–95	95	98	98
Specificity (%)	96–99	98–99	93–99	98–99
References	33, 151, 176, 191	33, 128	33, 128, 132, 151	33, 128

[a] C, CSF; P, plasma; S, serum.

[b] For technical details, see reference 128.

syphilis diagnosis in developing countries necessitated the development of POC tests that can provide immediate results. The majority of the rapid syphilis tests detect only treponemal antibodies, but a few dual POC tests can also provide qualitative nontreponemal test results.

Nontreponemal Tests

Table 6 presents the predominant features of the FDA-approved nontreponemal tests. These tests use a precisely defined combination of cardiolipin, lecithin, and cholesterol that flocculates upon reaction with IgM and IgG antibodies. Detailed protocols for performing the RPR, VDRL, unheated serum reagin (USR), and TRUST assays can be found in *A Manual of Tests for Syphilis*, published by the American Public Health Association (http://www.apha.org) (128); an online version can be accessed at http://www.cdc.gov/std/syphilis/manual-1998/.

The VDRL test is a quantitative flocculation reaction performed on special glass slides (128). The results are read microscopically at ×100; the highest titer causing flocculation is the endpoint. The USR microflocculation test uses a modified VDRL antigen containing choline chloride, which eliminates the need for heating of the serum sample (129), and is read microscopically like the VDRL. The USR test is rarely used, since the RPR test is essentially equivalent but does not require a microscope for determination of results. The RPR card test, performed on serum or plasma, contains finely divided charcoal particles as a visualizing agent (128). In the RPR card test, serial dilutions of serum or plasma (heated or unheated) are prepared on a plastic-coated card after which the RPR antigen is added. The presence of antibodies causes flocculation, while suspensions without antibodies remain uniformly gray. The TRUST is a macroflocculation assay, very similar to the RPR, in which the charcoal is replaced with toluidine red (128). The sensitivity of the TRUST is similar to that of the RPR (Table 6), while its specificity is slightly higher (33).

The VDRL test can also be performed on CSF to identify cases of neurosyphilis. The VDRL-CSF test is performed identically to the serum VDRL except that the VDRL antigen is diluted 1:1 with 10% saline. RPR testing of CSF is available but has a lower sensitivity of 58%, compared to 67% for the VDRL-CSF, for the diagnosis of symptomatic neurosyphilis (130).

Treponemal Tests

The salient features of the FDA-approved treponemal antibody-based assays, as well as several others that are commercially available worldwide, are summarized in Tables 7 to 13. Detailed protocols for the FTA-ABS, the microhemagglutination assay for antibodies to *T. pallidum* (MHA-TP), the *T. pallidum* particle agglutination assay (TP-PA), and some of the initial treponemal EIAs can be found in *A Manual of Tests for Syphilis* (128).

TABLE 7 Conventional treponemal tests for syphilis

Characteristic	FTA-ABS	MHA-TP	TPHA	TP-PA
Type	Indirect immunofluorescence	Agglutination	Agglutination	Agglutination
Detects	IgG + IgM	IgG + IgM	IgG + IgM	IgG + IgM
Captures antibody with	Whole *T. pallidum*	*T. pallidum* antigen	*T. pallidum* antigen	*T. pallidum* lysate
Conjugate	Anti-human Ig-FITC	None	None	None
Specimen[a]	S, C	S	S	S
Sample vol (µl)	50	100	10	100
Time to complete (h)	1.5	2	1.5	2
FDA approved	Yes	Yes	Yes	Yes
Sensitivity (%)				
Primary	82–90	57–88	86	85–97.5
Secondary	100	96–100	100	100
Latent	96	96–97	100	100
Tertiary	100	98–100	100	96.2–100
Specificity (%)	94.5–98.6	99	100	97.6–100
Reference(s)	33, 179, 192	193, 194	195	132, 151, 179, 196

[a] C, CSF; S, serum.

TABLE 8 Treponemal IgM or IgG EIAs for syphilis[a]

Characteristic	Captia Syphilis-M	Trep-Chek IgM	Mercia Syphilis M	Treponema pallidum μ-capture IgM ELISA	recomWell Treponema IgM	Captia Syphilis-G	Trep-Chek IgG
Manufacturer	Trinity Biotech, Bray, Ireland	Phoenix Bio-Tech, Mississauga, Ontario, Canada	Microgen Bioproducts, Camberley, UK	IBL International, Hamburg, Germany	Mikrogen Diagnostik, Neuried, Germany	Trinity Biotech	Phoenix Bio-Tech
Type	Antibody class capture	Antibody class capture	Antibody class capture	Antibody class capture	Two-step sandwich	Two-step sandwich	Two-step sandwich
Detects	IgM	IgM	IgM	IgM	IgM	IgG	IgG
Captures antibody with	Goat anti-human IgM	Rabbit anti-human IgM	Rabbit anti-human IgM	Rabbit anti-human IgM	*T. pallidum*; 15-, 17-, and 47-kDa forms	Whole *T. pallidum* Ag	r *T. pallidum*; 15-, 17-, 44-, and 47-kDa forms
Conjugate	*T. pallidum* Ag-HRP	*T. pallidum* Ag-HRP	*T. pallidum* Ag-HRP	*T. pallidum* Ag-HRP	r *T. pallidum* Ag-HRP	Anti-human IgG-HRP	Anti-human IgG-HRP
Specimen	S	S	S	S, P	S, P	S, P	S
Sample vol (μl)	20	10	NS	10	10	50	100
Time to complete	3 h	3 h	3 h	3 h	NS	2 h, 20 min	3 h
FDA approved	Yes	Yes	No	No	No	Yes	Yes
Sensitivity (%)							
Primary	94	NS	90	NS	NS	83–95	NS
Secondary	85	NS	100	NS	NS	23	NS
Early latent	82	NS	75	NS	NS	76.5	NS
Sensitivity, stages not determined (%)	NS	97.4	NS	97.4	100	94.7–97.7	95.9–99.5
Specificity (%)	100	100.0	NS	99–100	98–100	94.2–100	98.5–100.0
Reference standard	19S-IgM FTA-ABS	NS	TP-PA	19S-IgM FTA-ABS	WB	TPHA/TP-PA	TPHA/TP-PA, FTA-ABS
Reference(s)	197	Package insert	198	Package insert	Package insert	178, 197, 199	Package insert; 179

[a] Ag, antigen; NS, not specified; P, plasma; r, recombinant; S, serum.

TABLE 9 Combined treponemal IgM/IgG EIAs for syphilis[a]

Characteristic	Bioelisa Syphilis 3.0	Enzygost Syphilis	Enzywell Syphilis Screen	Mercia Syphilis Total	ICE Syphilis	Syphilis EIA II	Pathozyme Syphilis	Syphilis Total Antibody	Trepanostika Tp Recombinant	Trep-Sure
Manufacturer	Biokit, Barcelona, Spain	Dade Behring, Marburg, Germany	Diesse Diagnostica, Siena, Italy	Microgen Bioproducts, Camberley, UK	Murex Biotech, Dartford, UK	Newmarket Laboratories, Newmarket, UK	Omega Diagnostics, Alva, UK	Bio-Rad Laboratories, Hercules, CA	bioMérieux, Marcy l'Etoile, France	Trinity Biotech, Bray, Ireland
Type	Two-step sandwich	Competitive	One-step sandwich	Competitive	Two-step sandwich	One-step sandwich	Competitive	Two-step sandwich	One-step sandwich	Two-step sandwich
Detects	IgG + IgM	IgG + IgM	IgG + IgM	IgG + IgM	IgG + IgM	IgG + IgM	IgG + IgM	IgG + IgM	IgG + IgM	IgG + IgM
Captures antibody with	r *T. pallidum*; 15-, 17-, and 47-kDa forms	*T. pallidum* Ag	r *T. pallidum* Ag	*T. pallidum* Ag	r *T. pallidum*; 15-, 17-, and 47-kDa forms	r *T. pallidum*; 15-, 17-, and 47-kDa forms	*T. pallidum* Ag	r *T. pallidum*; 15-, 17-, and 47-kDa forms	r *T. pallidum*	r *T. pallidum* Ag
Conjugate	r *T. pallidum* (15-, 17-, and 47-kDa forms)-HRP	Anti-*T. pallidum* Ab-HRP	r *T. pallidum* Ag-HRP	Anti-*T. pallidum* Ab-HRP	r *T. pallidum*-HRP	r *T. pallidum* (15-, 17-, and 47-kDa forms)-HRP	Anti-*T. pallidum* Ab-HRP	r *T. pallidum* (15-, 17-, and 47-kDa forms)-HRP	r *T. pallidum* Ag-HRP	r *T. pallidum* Ag-HRP
Specimen	S, P	S	S, P	S, P	S	S, P	S, P	NS	S, P	S, P
Sample vol (μl)	50	25	30	25	50	50	25	50	30	100
Time to complete	2 h, 20 min	2 h, 40 min	1 h, 20 min	2 h, 25 min	2 h, 50 min	1 h, 40 min	2 h, 25 min	2 h, 20 min	2 h, 10 min	2 h
FDA approved	No	No	Yes	No	No	No	No	Yes	No	Yes
Sensitivity (%)										
Primary	98.2	98.2	98.2	NS	75–98.2	99.1	97.4	NS	99.1	NS
Primary, MHA-TP negative	67.3	69.2	NS	NS	75.0	NS	69	NS	76.9	NS
Secondary	100	100	100	NS	100	100	100	NS	100	NS
Latent	100	100	100	NS	100	100	100	NS	100	NS
Tertiary	S	NS	NS	NS	NS	NS	NS	NS	NS	NS
Sensitivity, stages not determined (%)	98.2	98.2	98.2	96.5	98.2	99.1	97.4	97.4	99.1	96.9–98.9
Specificity (%)	100	100	100	100	99.2	100	98.5–100	100	100	94.7–99.9
Reference standard	TPHA/TP-PA	TPHA/TP-PA	TPHA/TP-PA	TPHA/TP-PA	TPHA/TP-PA	TPHA/TP-PA	TPHA/TP-PA	TPHA/TP-PA	TPHA/TP-PA	FTA-ABS
Reference(s)	178, 192	161, 178, 192	178, 192	178	178, 192	178	178, 192	178	178, 192	Package insert; 179

[a] Ab, antibody; Ag, antigen; NS, not specified; P, plasma; r, recombinant; S, serum.

TABLE 10 Rapid treponemal tests for syphilis[a]

Characteristic	Determine Syphilis TP	Espline TP	SD Bioline	Span Crystal Tp	VisiTect Syphilis	Syphicheck-WB	Syphilis Fast	Syphilis Health Check
Manufacturer	Alere Inc., Waltham, MA	Fujirebio Inc., Tokyo, Japan	Standard Diagnostics, Inc., Yongin, Korea	Span Diagnostics, Surat, India	Omega Diagnostics, Alva, UK	Qualpro Diagnostics, Goa, India	Diesse Diagnostica, Siena, Italy	Diagnostics Direct, Stone Harbor, NJ
Type	Immunochromatography	Immunochromatography	Immunochromatography	Immunochromatography	Immunochromatography	Immunochromatography	Latex agglutination	Immunochromatography
Detects	IgG + IgM	IgG + IgM	IgG + IgM+ IgA	IgG + IgM + IgA	IgG + IgM	IgG + IgM	IgG + IgM	IgG + IgM
Captures antibody with	r *T. pallidum*; 47-kDa form	r *T. pallidum*; 15-, 17-, and 47-kDa forms	r *T. pallidum*; 15-, 17-, and 47-kDa forms	r *T. pallidum*; 15-, 17-, and 47-kDa forms	r *T. pallidum*	r *T. pallidum*; 17- and 47-kDa forms	r *T. pallidum*; 15.5-, 17-, and 47-kDa forms	r *T. pallidum*
Conjugate	Anti-human IgG-gold	Anti-human IgG-AlkP	Anti-human IgG-gold	Anti-human IgG-gold	Anti-rabbit IgG-gold	Anti-human IgG-gold	Anti-human IgG-gold	Anti-human IgG-gold
Specimen	S, P, B	S, P	S, P, B	S, P, B	S, P, B	S, P, B	S	S, P, B
Sample vol (μl)	50	25	20	100	25	25–50	20	25–50
Time to complete (min)	15	15	5–20	15	30	15	8	10
FDA approved	No	No	No	No	No	No	No	Yes
Sensitivity (%)	77.1–100	97.7	87.6–95.5	100	72.7–98.2	64–84.5	86–96.8	98.5
Specificity (%)	94.1–100	93.4–100	94.9–99.4	100	98–100	97–99.7	92.8–99.8	97.3
Reference standard	TPHA/TP-PA	TPHA/TP-PA	TPHA/TP-PA	NS	TPHA/TP-PA	TPHA/TP-PA	TPHA/TP-PA	TPHA/TP-PA/ ELISA
Reference(s)	182, 200, 201	Package insert; 182	182, 200	Package insert	182, 200, 202	182, 200	182, 203, 204	Package insert

[a] Ag, antigen; AlkP, alkaline phosphatase; B, blood; NS, not specified; P, plasma; r, recombinant; S, serum.

TABLE 11 Rapid combination nontreponemal and treponemal tests for syphilis[a]

Characteristic	ChemBio Dual Path Platform	Span Signal Spirolipin
Manufacturer	ChemBio Diagnostic Systems, Inc., Medford, NY	Span Diagnostics, Surat, India
Type	Lateral immunochromatography	Flowthrough
Detects	IgG + IgM	IgG + IgM
Captures antibody with	r *T. pallidum* (17-kDa form)-cardiolipin	r *T. pallidum* (17- and 47-kDa forms)-cardiolipin
Conjugate	Anti-human IgG-gold	Anti-human IgG-gold
Specimen	S, P, B	S, P, B
Sample vol (µl)	5–10	100
Time to complete (min)	20	15
FDA approved	No	No
Sensitivity, nontreponemal test (%)	85.5–93.5	94.6
Specificity, nontreponemal test (%)	94.4–100	97.3
Sensitivity, treponemal test (%)	90.7–96.7	98.0
Specificity, treponemal test (%)	99.1–99.6	97.2
Reference standard	RPR/TRUST/TP-PA	RPR/TP-PA
Reference(s)	Package insert; 141	Package insert

[a] B, blood; P, plasma; r, recombinant; S, serum.

FTA-ABS Test

In the FTA-ABS test (Table 7), a serum sample, adsorbed with an extract of *T. phagedenis* Reiter (Sorbent) to remove naturally occurring cross-reactive antibodies, is reacted with treponemes fixed to glass slides; FITC-conjugated anti-human immunoglobulin is used to visualize antibody-labeled organisms. Because of the nature of the conjugate used, the FTA-ABS test cannot distinguish between IgG or IgM antibodies. The serum is subjectively scored based on the fluorescence intensity. Standardized controls, which produce negative, weak, and strong fluorescence readings, must be included in each assay. The test can be performed with unheated CSF for diagnosis of neurosyphilis (see below). Because of the subjectivity involved in reading samples and the need for expensive microscopy equipment, the FTA-ABS is used much less frequently than in the past and is no longer recommended by the CDC (131).

MHA-TP, TPHA, and TP-PA Tests

The MHA-TP test (Table 7) is a passive hemagglutination assay using formalinized, tanned sheep erythrocytes sensitized with *T. pallidum* antigen; the *T. pallidum* hemagglutination assay (TPHA) uses fowl erythrocytes. Both the MHA-TP and the TPHA have been supplanted by the TP-PA. The TP-PA test (Fujirebio Inc., Tokyo, Japan) is a modification of the MHA-TP test that uses gelatin particles sensitized with *T. pallidum* antigens to reduce the number of nonspecific interactions (132). With all three tests, agglutination indicates the presence of IgG and/or IgM antitreponemal antibodies.

EIAs

Since the 1980s, >20 EIAs have been developed for syphilis diagnosis, the majority of which use recombinant *T. pallidum* antigens and detect IgM or IgG (Table 8) or both (Table

TABLE 12 Treponemal immunoblot assays for syphilis[a]

Characteristic	MarDx *T. pallidum* MarBlot	Treponema+VDRL ViraBlot	INNO-LIA Syphilis Score
Manufacturer	Trinity Biotech, Bray, Ireland	ViraMed Biotech, Planegg, Germany	Innogenetics NV, Ghent, Belgium
Type	WB	Pseudo-WB	Pseudo-WB
Detects	IgG or IgM	IgG or IgM	IgG + IgM
Captures antibody with	Whole *T. pallidum*	r *T. pallidum*; 15-, 17-, 44.5-, and 47-kDa forms	r *T. pallidum*; 15-, 17-, and 47-kDa forms
Conjugate	Anti-human Ig-AlkP	Anti-human Ig-AlkP	Anti-human Ig-AlkP
Specimen	S	S	S
Sample volume (µl)	20	20	10
Time to complete	1 h, 40 min	1.5 h	4–6 h
FDA approved	No	No	No
Sensitivity (%)			
Primary	71.8	NS	92.3–94.1
Secondary	90	NS	100
Latent	72.5	NS	94.4
Tertiary	NS	NS	100
Sensitivity, stages not determined (%)	65.2	93.8–95.5	94.1
Specificity (%)	100	97.8–98.5	99.3–100
Reference standard	TP-PA/FTA-ABS	TP-PA/FTA-ABS	TP-PA/FTA-ABS
References	Package insert; 142, 205	Package insert; 179, 206	142, 176, 205, 207.

[a] AlkP, alkaline phosphatase; NS, not specified; r, recombinant; S, serum.

TABLE 13 Treponemal chemiluminescence assays and multiplex flow immunoassays for syphilis[a]

Characteristic	Architect Syphilis TP	Immulite 2000 Syphilis Screen	Liaison Treponema Screen	AtheNA Multi-Lyte	BioPlex 2200 Syphilis
Manufacturer	Abbott Laboratories, Abbott Park, IL	Siemens Healthcare, Diagnostics, Deerfield, IL	DiaSorin S.p.A., Vercelli, Italy	Zeus Scientific, Branchburg, NJ	Bio-Rad Laboratories, Hercules, CA
Type	Bead capture	Bead capture	Bead capture	Bead capture	Bead capture
Detects	IgG + IgM	IgG + IgM	IgG + IgM	IgG	IgG + IgM
Captures antibody with	r *T. pallidum*; 15-, 17-, and 47-kDa forms	r *T. pallidum*; 17-kDa form	r *T. pallidum*; 17-kDa form	r *T. pallidum*; 17-kDa form	r *T. pallidum*; 15-, 17-, and 47-kDa forms
Conjugate	Anti-human Ig-acridinium	Anti-human Ig-AlkP	Isoluminol-antigen conjugate	Anti-human phycoerythrin	Anti-human phycoerythrin
Specimen	S, P	S, P	S	S	S
Sample vol (µl)	30–150	100	70	10	5
Time to complete (min)	30	35	40	75	45
FDA approved	Yes	Yes	Yes	No	Yes
Sensitivity (%)					
Primary	97.5	NS	100.0	90.5	91.40
Secondary	100	NS	100.0	100	89.3
Latent	NS	NS	NS	86.5	100
Tertiary	100	NS	98.7	NS	86.4
Sensitivity, stages not determined (%)	NS	96.4	NS	94.3	96.9–100
Specificity (%)	98.4	100	99.1	98.1	98.2–99.4
Reference standard	FTA-ABS	Liaison	TPHA/WB	TP-PA	RPR/TP-PA/FTA-ABS/EIA
Reference(s)	Package insert; 208	Package insert	Package insert; CDC, unpublished data; 193	Package insert; CDC, unpublished data	Package insert; CDC, unpublished data; 179, 209

[a]AlkP, alkaline phosphatase; C, CSF; NS, not specified; P, plasma; r, recombinant; S, serum.

9). The 15-, 17-, 44.5-, and 47-kDa lipoprotein antigens are the most frequently utilized because they induce strong, persistent antibody responses (133) and are thought to be expressed only by pathogenic treponemes (134). Although EIAs using recombinant antigens might be expected to perform better than assays using *T. pallidum* lysates (135), this has not been borne out (136–138).

EIAs utilize several different formats, including (i) antibody class capture, (ii) sandwich capture, and (iii) competitive assay. Most of the EIAs that specifically detect treponemal IgM (Table 8) are based on antibody class capture using goat or rabbit anti-human antibodies and purified *T. pallidum* antigens labeled covalently to peroxidase (conjugates) to complex with the patient's antitreponemal antibodies. Examples are the Captia Syphilis-M (Trinity Biotech, Bray, Ireland) and the Trep-Chek IgM (Phoenix Bio-Tech Corporation, Mississauga, Ontario, Canada). Two-step sandwich EIAs use either immobilized whole or recombinant *T. pallidum* antigens to coat microplate wells. Serum or plasma patient specimens are incubated in the wells, and antitreponemal antibodies are captured to the antigens on the wells. After washing unbound components, the conjugate consisting of antigens labeled with horseradish peroxidase (HRP) is then added and captured by the antitreponemal antibodies bound to the microplate. Unbound conjugate is then washed away, and bound conjugate is detected by a reaction with a substrate. Examples include the Captia Syphilis-G (Trinity Biotech) and the ICE Syphilis (Murex Biotech Ltd., Dartford, United Kingdom). One-step sandwich EIAs have similar formats but specimen and conjugate are added at the same time; examples include the Enzywell Syphilis Screen (Diesse Diagnostica, Siena, Italy) and Syphilis EIA II (Newmarket Laboratories Ltd., Newmarket, United Kingdom). One disadvantage of EIAs using antigen-antibody conjugates can be high background signals that give rise to false-positive results. An increasing signal indicates a more reactive serum and is proportional to the antitreponemal antibodies in the patient's serum.

The competitive format EIAs, such as the Pathozyme Syphilis (Omega Diagnostics, Alva, United Kingdom) and the Enzygost Syphilis (Dade Behring, Marburg, Germany), use immobilized antigen to capture specific IgG and IgM antibodies from patient serum, which then block binding of an antibody conjugate of identical specificity. With this format, optical density is inversely related to the amount of antibody bound.

Rapid POC Tests

Most of the POC tests for syphilis are immunochromatographic strip assays that detect treponemal antibodies using whole blood from fingerstick specimens, serum, or plasma (Table 10). All of the rapid syphilis tests use recombinant *T. pallidum* antigens to detect IgM and IgG antibodies. These POC tests can provide results within 20 min, but a positive result needs follow-up nontreponemal antibody testing for further evaluation. A recent meta-analysis of treponemal POC tests used for screening in antenatal and sexually transmitted disease clinic populations reported sensitivities and specificities comparable to those of conventional treponemal tests (139).

Two dual POC tests have the ability to detect both nontreponemal and treponemal antibodies (Table 11), features that are highly advantageous in resource-poor settings (140). The ChemBio Dual Path Platform Screen and Confirm (ChemBio Diagnostic Systems, Inc., Medford, NY) is a lateral-flow dual POC test that requires only 5 µl of serum, plasma, or whole blood and can be read visually or with a digital card reader for quantitative results (141). The Span Signal Spirolipin (Span Diagnostics Ltd., Surat, India) is a flowthrough dual POC test in which control, nontreponemal, and 17-kDa antigens are located, respectively, at the 12, 4, and 8 o'clock positions on the test pad.

Immunoblot Assays

Immunoblotting techniques confer specificity because they identify the specific treponemal proteins recognized by serum antibodies; they also can separately detect IgG or IgM antibodies when the appropriate anti-immunoglobulin conjugates are used (Table 12). The Western blot (WB) techniques use *T. pallidum* antigens that are fractionated with polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and reacted with patient serum. The MarDx test (Trinity Biotech), which uses whole *T. pallidum* lysate, is used to define serum reactivity when screening and confirmatory treponemal tests are discrepant (142). The INNO-LIA (Innogenetics NV, Ghent, Belgium) and ViraBlot (ViraMed Biotech, Planegg, Germany) tests are pseudo-WBs that employ several recombinant antigens (15, 17, 44.5, and 47 kDa) applied onto a nitrocellulose membrane. In addition to some of the same recombinant antigens as the INNO-LIA, the ViraBlot strip (IgG or IgM) has five strips with increasing quantities of VDRL antigen, which allows for semiquantitative nontreponemal results.

CLIAs

Automated chemiluminescence immunoassays (CLIAs) can be performed using random access analyzers with a turnaround time of <1 h (Table 13). Recombinant *T. pallidum* antigens are used to coat paramagnetic beads and the conjugate consisting of antigens linked to alkaline phosphatase or isoluminol derivative. After an incubation period with the patient specimen and removal of unbound antibodies-conjugate with a wash cycle, a chemiluminescent substrate is added, which generates a signal relative to the proportion of bound conjugate. Examples of CLIAs include the Architect Syphilis TP (Abbott Laboratories, Abbott Park, IL) and the Liaison Treponema Screen assay (DiaSorin S.p.A., Vercelli, Italy).

Multiplex Flow Immunoassays and Other New Technologies

Multiplex flow immunoassays (Table 13) are based on Luminex fluorescent bead technology. Performed in 96-well microtiter plates, this format enables high throughput. The Luminex assay differs from EIAs in two ways: (i) the capture antibody is attached to a suspension of polystyrene beads instead of the wells; and (ii) the polystyrene beads are dyed with fluorophores of differing intensities that confer on each bead a unique fingerprint, enabling multiplex antibody detection. After the sandwich immunoassay, the bead suspension is analyzed using a dual-laser flow cytometry detection system. Currently, two multiplex flow immunoassays are available: the BioPlex 2200 Syphilis (Bio-Rad Laboratories, Hercules, CA) and the AtheNA Multi-Lyte (Zeus Scientific, Branchburg, NJ).

ANTIMICROBIAL SUSCEPTIBILITIES

T. pallidum

Routine antimicrobial susceptibility testing of *T. pallidum* is not possible due to the lack of an *in vitro* cultivation system. *T. pallidum* strains are highly susceptible to penicillin G, which has been used successfully for the treatment of syphilis over the past 65 years (93). Ceftriaxone is highly active against *T. pallidum in vitro* and is effective in the treatment of early syphilis when a sufficient number of doses is given (93). Because efficacy data for ceftriaxone are limited, its use as an alternative therapy for syphilis is recommended only when better-established regimens are contraindicated (93). Tetracyclines have long been the second-line class of drug recommended for treatment of syphilis in patients allergic to penicillin and have well-established track records; doxycycline, which has a much longer half-life and better CNS penetration, is preferable to tetracycline (93). *In vitro* studies indicate that quinolone compounds have low antimicrobial activity against *T. pallidum* (29).

Erythromycin treatment failures were recognized years ago, and one case was reported to be due to an infection with an erythromycin-resistant *T. pallidum* strain (143). Enthusiasm for azithromycin, administered orally in a single 2-g dose, has been tempered by the discovery of geographically widespread macrolide-resistant strains of *T. pallidum* associated with an A2058G or A2059G mutation in both copies of the bacterium's 23S rRNA genes (144–146). The 23S rRNA gene segment conferring macrolide resistance is detectable by nested PCR and RFLP analysis. A TaqMan-based real-time triplex PCR assay has been recently reported for the rapid detection of both point mutations (144).

Brachyspira

The *in vitro* antimicrobial susceptibilities of *B. pilosicoli* isolates from a number of geographic locations have been tested using an agar dilution method (147). Isolates were found to be susceptible to amoxicillin-clavulanic acid, ceftriaxone, chloramphenicol, meropenem, tetracycline, and metronidazole. Metronidazole remains the drug of choice for treating *B. pilosicoli* infections; resistance has not been reported (148).

EVALUATION, INTERPRETATION AND REPORTING OF RESULTS

Direct Detection of *T. pallidum*

DF Microscopy and DFA-TP

Identification of a single motile *T. pallidum* by DF microscopy is sufficient for diagnosis. The predictive value of DF microscopy is difficult to discern (149), with one study claiming a 97% positivity rate in patients with clinically diagnosed primary syphilis (80% for patients positive on their first clinic visit) (94). The development of PCR provided a much needed standard for assessing the sensitivity and specificity of DF microscopy. In two independent studies in which conventional multiplex PCR was used to evaluate the etiology of genital ulcers (112, 150), the sensitivity and specificity of DF ranged from 39 to 81% and 82 to 100%, respectively. DF microscopy has fallen into disfavor because it requires a specialized microscope and highly trained laboratory personnel. However, the continued need for DF microscopy proficiency is underscored by studies demonstrating that substantial percentages of DF microscopy-positive primary syphilis patients lack detectable antibodies (Fig. 3)

(33, 151). Although DFA-TP is at least as sensitive as DF microscopy and more specific (33), it has never gained wide acceptance and is unlikely to see increased usage in the PCR era.

Visualization of *T. pallidum* in Tissues

Silver impregnation is the traditional method for *T. pallidum* detection in formalin-fixed tissues (152) and should be performed when routine histopathologic findings suggest syphilis. When performed by a credible laboratory and accompanied by reactive serologic tests, visualization of spirochetes by silver staining can be considered definitive evidence for syphilis (Table 2). Of note, rare cases of seronegative secondary syphilis in HIV-infected patients are described in which silver staining was instrumental in establishing a diagnosis (153, 154). Nevertheless, silver staining has some drawbacks: it is prone to staining artifacts, its sensitivity is limited and not well determined, and it is not *T. pallidum* specific. The Steiner modification of the Dieterle stain is said to be more sensitive than the Warthin-Starry stain (155). IHC using commercially available polyclonal antibodies largely circumvents the limitations of silver staining, although it must be borne in mind that these immunological reagents can react with spirochetes other than *T. pallidum* (e.g., *Borrelia burgdorferi*).

PCR Detection of *T. pallidum*

PCR enhances detection of *T. pallidum* in genital ulcer exudates. An additional advantage of PCR for diagnosis of GUD is that multiplex analysis for other causes of GUD, most importantly HSV-1 and HSV-2 (see above), is possible. CSF has also been examined extensively by PCR. In a multicenter study, PCR in conjunction with RIT confirmed the long-held view that neuroinvasion by *T. pallidum* occurs with high frequency in early syphilis patients without neurologic symptoms (82). Unfortunately, very little is known about the utility of CSF PCR for diagnosing symptomatic neurosyphilis. A study from South Africa found that 56% of 50 patients with suspected neurosyphilis had positive CSF PCRs (122), but these findings need corroboration. Cumulative evidence suggests that PCR is also useful for detection of treponemes in fresh and processed tissues. Comparative analysis of paraffin-embedded secondary syphilis skin biopsy specimens revealed that PCR is equivalent to IHC staining and is easier to perform (155). Studies using conventional and real-time PCR indicate that analysis of blood has diagnostic utility, although results have varied (111, 123, 156). Nevertheless, PCR assays of blood should be used cautiously in suspected cases of acquired syphilis given the lack of consensus on the specific clinical scenarios in which it complements conventional diagnostic methods.

Serologic Tests

Serologic tests play an important role in syphilis diagnosis by supplementing direct detection methods for diagnosing early syphilitic lesions, diagnosing latent infection, and confirming suspected tertiary disease (Table 2 and Fig. 3). On the other hand, many problems in syphilis management stem from the fact that the nontreponemal antibody titers used to assist in staging of infection and monitoring response to therapy are poor surrogate markers for syphilitic infection. As noted earlier, nontreponemal and treponemal assays measure two distinctly different kinds of antibody reactivities with different kinetics during untreated and treated infection (Fig. 3). None of the currently available serologic tests can distinguish venereal syphilis from the endemic treponematoses (Table 1).

Nontreponemal Tests

Nontreponemal antibody tests are reported as the highest dilution giving a fully reactive result. Titers for the same serum can differ by 2- to 4-fold when tested using microscopic versus macroscopic nontreponemal tests, underscoring the importance of using the same method for serial serologic tests, preferably in the same laboratory. Sera with extremely high nontreponemal test titers can give weak, atypical, or even negative "rough" reactions at low dilutions when antibody excess prevents agglutination. This prozone phenomenon occurs in 1 to 2% of patients with secondary syphilis (157). Most laboratories circumvent this problem by routinely determining the titers of all samples to at least 16 dilutions.

Traditional algorithms utilize nontreponemal tests as the primary screening tests for suspected syphilis. Nontreponemal tests must be interpreted according to the suspected stage of syphilis as well as the population being tested. Reactive results require confirmation using a treponemal test, since the proportion of false-positive tests increases with decreasing prevalence of syphilis. Approximately 30% of those with early primary syphilis have nonreactive nontreponemal test results on the initial visit (Fig. 3 and Table 6), underscoring the importance of direct detection methods for genital ulcers and the use of treponemal antibody-based tests, which have increased sensitivity. In secondary syphilis, nearly all patients have nontreponemal test titers of ≥1:4. Approximately one-third of patients with tertiary disease have nonreactive nontreponemal tests.

Conditions other than treponemal infection can elicit antilipoidal antibodies that cause BFP reactions, defined as reactivity in a nontreponemal test with a negative treponemal test result. Review articles cite a number of conditions and diseases that cause BFPs (158); however, many of these lack strong scientific evidence. Acute BFP reactions, which last <6 months, are associated with transient diseases or conditions such as malaria (159), brucellosis (160), mononucleosis (161, 162), viral hepatitis (163), lymphogranuloma venereum (164), viral pneumonias (165), tuberculosis (162), and chancroid (164). More recently, smallpox vaccination was shown to cause an increased frequency of BFP reactions (166). Causes of chronic BFP reactions, which last >6 months, include autoimmune diseases, particularly systemic lupus erythematosus (167), HIV infection (163, 168), intravenous drug use (163), and leprosy (169). Patients with hepatitis C infection are noted to be 5 times more likely to have BFPs than hepatitis C-negative controls (170).

Treatment response in syphilis patients is defined by resolution of disease manifestations, at least a 4-fold decline in nontreponemal titers (or a change in 2 dilutions, e.g., from 1:16 to 1:4) using the same assay, or seroreversion to a nonreactive test (93). For serologic monitoring after therapy, repeat nontreponemal titers are recommended at 6 and 12 months for early syphilis and at 6, 12, and 24 months for late syphilis. More frequent serologic monitoring can be conducted for HIV-infected persons and pregnant women after syphilis treatment. A 4-fold increase in nontreponemal titers is suggestive of either reinfection or treatment failure and requires further clinical evaluation for HIV coinfection and CNS involvement from *T. pallidum*.

Early investigators reported that nontreponemal tests serorevert or become nonreactive in the majority of treated patients with primary and secondary syphilis (171). However, a large historical cohort review found that the seroreversion rate ranges from 26 to 74% for patients at 12 to 36 months after treatment for early syphilis, which varies in part based on whether the patient has an initial or repeat episode of infection (172). A more recent analysis of the serologic response to syphilis treatment demonstrated that up to 21% of patients with early syphilis can remain serofast at 6 months after treatment (173). The serofast status is defined as a <4-fold decline in nontreponemal titers from initial titers or persistent low-level nontreponemal titers over time despite adequate treatment. The serofast state is more likely to occur in patients with low baseline titers (i.e., <1:32) or later stages of syphilis infection compared to primary syphilis (173). The clinical relevance of the serofast state is uncertain and requires further investigation to determine its optimal management.

Treponemal Tests

Conventional treponemal tests (i.e., FTA-ABS and TP-PA) and the majority of newer, commercially available treponemal assays measure both IgG and IgM without distinguishing the class responsible for reactivity. This property accounts for the primary strength of treponemal tests, which is their high level of sensitivity for syphilitic infection of all stages, but also their cardinal weakness, the inability to distinguish active from inactive disease. The greatest value of the treponemal tests is in distinguishing between true- and false-positive nontreponemal test results.

In contrast to nontreponemal tests, treponemal tests are performed at a fixed titer or serum dilution and titers are not normally determined. FTA-ABS reactivity is graded on a scale of 1 to 4+; 1+ reactivity, which is highly observer dependent, is considered equivocal evidence for syphilis, while 2+ reactivity also has a significant probability of being false positive due to naturally occurring cross-reacting antibodies (174). Another type of false positivity, beaded fluorescence due to anti-DNA antibodies, is observed in sera from patients with systemic lupus erythematosus and other autoimmune diseases (175). Authorities no longer consider the FTA-ABS to be the gold standard treponemal test (131). In head-to-head comparisons, the TP-PA has been shown to be as sensitive and specific for all stages of syphilis as any treponemal test currently on the market, including EIAs using recombinant antigens (176–179).

Three head-to-head comparisons of treponemal assays, including EIAs, provide some guidance on their use (137, 178, 179). All of the commercially available EIAs have high specificities (at or near 100% in tested panels) and, not surprisingly, nearly 100% sensitivities in diagnosing secondary syphilis. Schmidt et al. (137) evaluated nine EIAs using highly selected sera from patients with primary syphilis, all with nonreactive MHA-TP tests. Higher sensitivity correlated with (i) the volume and dilution of serum used, (ii) assay format (i.e., capture and competitive tests showed higher sensitivities than sandwich-based assays), and (iii) detection of IgM as well as IgG antibodies. The greater sensitivity for primary syphilis of assays that include IgM detection is presumed to be due to the earlier appearance of IgM versus IgG antitreponemal antibodies. IgM-specific EIAs have sensitivities ranging from 88 to 90% in primary syphilis, 76 to 100% in secondary syphilis, and 19 to 69% in early latent syphilis (180). IgM-specific assays can assist in distinguishing between early and late infections but are not currently recommended by the CDC for diagnosis of acquired syphilis (93).

The availability of high-throughput EIAs resulted in a dramatic shift in the way that serologic screening for syphilis is performed in many patient care settings. In recent years, numerous laboratories switched from the traditional (i.e., nontreponemal test followed by treponemal test confirmation) to a reverse algorithm in which screening is performed with a treponemal test, such as an EIA or CLIA, followed by a reflex nontreponemal test when the former is reactive (131). Patients with a positive treponemal assay but negative nontreponemal test could have (i) very early syphilis, (ii) long-standing latent syphilis, (iii) past treated syphilis, or (iv) a false-positive treponemal test. Therefore, a careful clinical history is important to distinguish at-risk individuals in whom a reactive treponemal test is likely to be a true positive from individuals with previously treated syphilis. For persons who do not have a prior history of syphilis treatment, a second treponemal test, preferably the TP-PA, should be performed (131). A reactive second test confirms that the person has or had syphilis while leaving unresolved the issue of disease activity. If the second treponemal test is nonreactive, the clinician could decide that no further evaluation or treatment is indicated or that treatment is indicated for individuals at high risk. The reverse screening algorithm for syphilis testing could lead to increased patient follow-ups, overtreatment, and potentially higher costs (181). Therefore, several issues should be considered prior to its implementation, including disease prevalence, pretest probability of the patient having syphilis, and the laboratory's need for automation.

Rapid POC Tests

Head-to-head comparison of nine rapid syphilis tests at geographically diverse sites has shown good performance relative to TPHA or TP-PA reference standards (182). However, the stability of the test results was affected if the reading was delayed past the recommended period, which resulted in previously negative tests becoming false positives. Since the majority of the rapid syphilis tests are treponemal assays, nontreponemal testing is recommended following a positive result if feasible. The exceptions are the dual POC tests, which can detect both nontreponemal and treponemal antibodies from the same patient specimen.

Tests for Neurosyphilis

CSF abnormalities are common in early syphilis patients without neurologic symptoms, and a high proportion of such individuals have treponemes in their CNS yet do not require enhanced therapy (82). For these reasons, routine lumbar puncture is not recommended in early syphilis (93). Some groups argue that this recommendation should not apply to HIV-infected patients with serum RPR titers of ≥1:32 or CD4 counts of <350 cells/μl (183), although there are no data demonstrating long-term benefits of enhanced therapy in this patient population. The diagnosis of neurosyphilis is based on a combination of clinical and laboratory test criteria (Table 2). CSF examination should include the VDRL-CSF slide test and total protein and leukocyte counts. The VDRL-CSF test is considered to have high specificity but low sensitivity for neurosyphilis (184). A nonreactive VDRL-CSF test result, therefore, does not rule out neurosyphilis. The FTA-ABS CSF test has high sensitivity but low specificity for neurosyphilis because reactivity may be due to the passive transfer of IgG antitreponemal antibodies across the blood-brain barrier rather than intrathecal production of antibodies (185). A negative FTA-ABS CSF test, however, is strong evidence against neurosyphilis (185).

Syphilis Tests in HIV Infection

There is a clear consensus that serologic tests for syphilis perform well for persons coinfected with HIV and can be relied on for accurate diagnosis in such individuals (93). However, some caveats to this statement exist. First, HIV-infected individuals can have a higher incidence of false-positive nontreponemal tests (163, 168). Second, nontreponemal test titers in HIV-infected individuals tend to be higher than those in patients without HIV infection (82), and there is an increased risk of serologic failures among HIV-infected persons with early or late latent syphilis (186). Third, there are a small number of documented cases in which HIV-infected patients with secondary syphilis had nonreactive syphilis serologies (153, 154).

Tests for Congenital Syphilis

The CDC has issued revised criteria for congenital syphilis surveillance (21) (Table 2). A confirmed diagnosis requires demonstration of *T. pallidum* in neonatal tissues, lesion exudates, and/or secretions or in the products of conception (i.e., placenta or umbilical cord) using DF microscopy, fluorescent antibody, or other specific stains. Congenital syphilis is diagnosed presumptively in a symptomatic or asymptomatic infant whose mother had untreated or inadequately treated syphilis at delivery *or* in an infant with a reactive treponemal test and any one of the following: (i) clinical evidence of congenital syphilis (i.e., on physical exam or radiographs); (ii) a reactive VDRL-CSF test; (iii) elevated CSF cell count or protein (without other cause); or (iv) a reactive 19S-IgM FTA-ABS or IgM enzyme-linked immunosorbent assay (ELISA). However, none of the commercially available IgM tests for congenital syphilis is currently recommended by CDC.

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General Approaches to Identification of *Mycoplasma*, *Ureaplasma*, and Obligate Intracellular Bacteria

J. STEPHEN DUMLER

61

The bacteria discussed in chapters 61 to 67 differ from bacteria described in other parts of this *Manual* by several characteristics, including lack of efficient characterization with the Gram stain method and, except for *Mycoplasma* and *Ureaplasma* species and to a limited degree for *Coxiella burnetii* and *Tropheryma whipplei*, the requirement for intracellular growth. Thus, the most frequently used tests in clinical microbiology laboratories, the Gram stain and culture on artificial media, are unable to detect these organisms if present in clinical samples. Diagnosis of infections caused by these bacteria has traditionally been accomplished by Romanowsky staining (Giemsa and Wright stains) of clinical samples, by detection of antibody responses to infection using a variety of serologic tests, or by histopathologic analysis of biopsy samples. Molecular diagnostic tools and better culture methods have significantly improved the ability to detect these agents and to diagnose the diseases that they cause. For some of these infections, molecular tools are standard practice. The following tables summarize the epidemiology of these infections (Table 1) and the diagnostic tests most often used for the detection of the causative bacteria (Table 2).

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TABLE 1 Epidemiology and clinical diseases associated with *Anaplasma*, *Chlamydiaceae*, *Coxiella*, *Ehrlichia*, *Mycoplasma*, *Orientia*, *Rickettsia*, *Tropheryma*, and *Ureaplasma* infections

Organism	Disease	Reservoir(s)	Vector and mode of transmission
Anaplasma phagocytophilum	Human granulocytotropic anaplasmosis (HGA): fever, headache, myalgia, systemic involvement except for central nervous system	White-footed mouse, other small mammals, ruminants, deer	*Ixodes scapularis* (deer or black-legged tick), *I. pacificus* (western black-legged tick), *I. ricinus* (rabbit tick), *I. persulcatus* (taiga tick), *Haemaphysalis* tick bites
Chlamydia abortus	Spontaneous abortion	Ruminants	Exposure to infected sheep or goats during birthing season
Chlamydia pneumoniae	Pneumonia, bronchitis, sinusitis, pharyngitis	Humans	Inhalation of infected aerosols
Chlamydia psittaci	Psittacosis (pneumonia), systemic infections	Birds, domestic animals	Direct contact with or inhalation of infected aerosols
Chlamydia trachomatis	Endemic trachoma, inclusion keratoconjunctivitis, urethritis, epididymitis, endometritis, salpingitis, perihepatitis, pneumonia, lymphogranuloma venereum	Humans	Sexual contact, hand-eye contact, insect fomites, infected birth canal
Coxiella burnetii	Acute Q fever (self-limited febrile illness ± pneumonia, hepatitis); chronic Q fever (endocarditis, endovascular infections); congenital infection	Cattle, sheep, goats, cats, rabbits, dogs, ticks	Inhalation of infected aerosols, ingestion of nonpasteurized dairy products
Ehrlichia chaffeensis	Human monocytotropic ehrlichiosis (HME): fever, headache, myalgia, systemic involvement including central nervous system	White-tailed deer, dogs and other canids, raccoons	*Amblyomma americanum* (Lone Star tick) and potentially *Dermacentor variabilis* (American dog tick) bites
Ehrlichia ewingii	"Ewingii" ehrlichiosis: fever, headache, myalgia, predominantly in immunocompromised individuals	Dogs and other canids	*Amblyomma americanum* (Lone Star tick) bites
Ehrlichia muris-like agent	*E. muris*-like agent (EMLA) ehrlichiosis	Small mammals	*Ixodes scapularis* (deer or black-legged tick)
Mycoplasma genitalium	Urethritis, cervicitis, endometritis, conjunctivitis	Humans	Sexual contact, vertical transmission *in utero* or intrapartum
Mycoplasma hominis	Acute pyelonephritis, cervicitis, endometritis, tubal factor infertility, postabortion bacteremia, bacteremia with immune suppression	Humans	Obstruction or instrumentation of urinary tract, sexual contact, vertical transmission *in utero* or intrapartum
Mycoplasma pneumoniae	Tracheobronchitis, pneumonia, pharyngitis, extrapulmonary complications (meningoencephalitis, arthritis, etc.)	Humans	Contact with infectious aerosols or fomites
Orientia tsutsugamushi	Scrub typhus	Chiggers (larval mites)	*Leptotrombidium* spp. (chigger) bites
Rickettsia africae	African tick-bite fever	Not established	*Amblyomma* spp. tick bites
Rickettsia akari	Rickettsialpox	Mice and other small mammals	*Allodermanyssus sanguineus* (mouse mite) bites
Rickettsia conorii	Boutonneuse fever or Mediterranean spotted fever	Small mammals and ticks	*Rhipicephalus sanguineus* (brown dog tick) bites
Rickettsia felis	Flea-borne spotted fever	Fleas, opossums, cats, dogs	*Ctenocephalides felis* (cat fleas), contamination of infected flea feces into flea bite
Rickettsia honei	Flinders Island spotted fever	Not established (reptiles?)	*Aponomma hydrosauri* (reptile tick)
Rickettsia parkeri	*R. parkeri* spotted fever rickettsiosis	Small mammals	*Amblyomma* spp. ticks
Rickettsia prowazekii	Louse-borne typhus	Humans, lice, flying squirrels	*Pediculus humanus* subsp. *corporis* (body louse), contamination of infected louse feces into louse bite
Rickettsia rickettsii	Rocky Mountain spotted fever	Ticks, small and medium-size mammals	*Dermacentor variabilis* (American dog tick), *Dermacentor andersoni* (wood tick), *Rhipicephalus sanguineus* (North and Central America), *Amblyomma cajennense* (Central and South America) tick bites
Rickettsia typhi	Murine typhus	Rats and other rodents, opossums	*Xenopsylla cheopis* (rat flea) and *Ctenocephalides felis* (cat flea), contamination of flea bite wound with infected flea feces
Tropheryma whipplei	Whipple's disease: chronic diarrhea, malabsorption, migratory arthritis, weight loss, lymphadenopathy, uveitis/retinitis/endophthalmitis, endocarditis, encephalitis, immune reconstitution inflammatory syndrome, acute gastroenteritis, pleural effusion, pulmonary infiltration, mediastinal lymphadenopathy	Humans	Environmental contacts (sewage, human stool, saliva), genetic predispositions, fecal-oral transmission among asymptomatically infected humans
Ureaplasma urealyticum	Urethritis, epididymo-orchitis, urinary calculi, abortion, chorioamnionitis	Humans	Sexual contact, *in utero* or peripartum vertical transmission

TABLE 2 Diagnostic tests for *Anaplasma*, *Chlamydia*, *Coxiella*, *Ehrlichia*, *Mycoplasma*, *Orientia*, *Rickettsia*, *Tropheryma*, and *Ureaplasma*

Organism	Diagnostic test[a]
Anaplasma phagocytophilum	**Microscopy:** Giemsa or Wright stain of peripheral blood or buffy coat smears is positive in ~60% of infected persons. **Antigen tests:** None commercially available; IHC offered at CDC. **Molecular tests:** EDTA-anticoagulated blood collected during the pretreatment acute phase of illness is used for PCR amplification. Species- and genus-specific tests are described. Current test of choice for diagnosis during active infection. **Culture:** EDTA-anticoagulated peripheral blood is inoculated onto HL-60, THP1, or other myelocytic cell lines. Positive cultures may be obtained between 3 and 30 days from many samples if inoculated within 24 h and if obtained before antimicrobial therapy. Lack of timely results mitigates against frequent use. **Serologic tests:** IFA is the most frequently used test. In a patient with typical clinical features of HGA, a ≥4-fold rise in IgG titer confirms infection, and a single peak IgG titer of ≥80 provides supportive evidence. IgG test sensitivity is between 90 and 100%; specificity is ~95%. IgM testing is not recommended. Cross-reactivity with *Ehrlichia chaffeensis* occurs.
Chlamydia pneumoniae	**Microscopy:** Organisms may be detected by Giemsa stain or DFA test directed against LPS, but both tests are insensitive. **Antigen tests:** Available EIAs directed against LPS detect all *Chlamydiaceae* but are licensed only for *C. trachomatis*. **Molecular tests:** FDA-cleared assays are not currently available. **Culture:** Recovered best if inoculated onto HL cells or Hep-2 cells. **Serologic tests:** MIF test is the test of choice. Diagnosis confirmed by ≥4-fold rise in titer or single samples with IgM titer of ≥16 and/or IgG titer of ≥512.
Chlamydia psittacii	**Microscopy:** Organisms may be detected by Giemsa stain or DFA test directed against LPS, but both tests are insensitive. **Antigen tests:** Available EIAs directed against LPS detect all *Chlamydiaceae* but are licensed only for *C. trachomatis*. **Molecular tests:** None commercially available. **Culture:** Recovered in many different cell cultures including McCoy and HeLa cells. **Serologic tests:** MIF test is most sensitive and specific (test of choice).
Chlamydia trachomatis	**Microscopy:** Organisms can be detected by DFA test or Giemsa stain. DFA is more sensitive, but neither test should be used alone. **Antigen tests:** Commercial EIAs for *C. trachomatis* are considered substandard; they are not recommended for use and are not suitable for screening urine or vaginal swabs. Point-of-care tests are only 62–72% sensitive compared to culture and 33–50% sensitive compared to molecular tests. **Molecular tests:** Commercial nucleic acid amplification tests (PCR, transcription-mediated amplification, strand displacement amplification) are available and are tests of choice for confirmation of *C. trachomatis* infections. **Culture:** Recovered in many different cell cultures including McCoy and HeLa 229 cells. Test sensitivity is lower than that of molecular tests and dependent on the quality of the submitted specimen. **Serologic tests:** MIF test is most sensitive and specific (test of choice) for neonatal pneumonia and lymphogranuloma venereum but is not recommended for other *C. trachomatis* infections. Fourfold or greater rise in titers are significant, but rising titers may not be observed with chronic, repeated, or systemic infections. A single IgM titer of ≥32 supports a diagnosis of neonatal pneumonia.
Coxiella burnetii	**Microscopy:** DFA or IHC may be performed, but these tests are not widely available. **Antigen tests:** None commercially available. **Molecular tests:** PCR available through reference laboratories; more sensitive than serology during first few weeks; whole blood or leukocyte fractions are preferred, and serum is less useful. Cardiac valve tissue more sensitive than blood for chronic Q fever endocarditis. **Culture:** Requires biosafety level 3 facility and practices; U.S. category B select agent. Animal (mouse) inoculation is most sensitive. May be cultivated in a variety of cell lines, especially HEL, Vero, RK13, THP1, or A549 cells. Axenic culture has recently been developed but is not yet proven for primary isolation. **Serologic tests:** Most frequently used diagnostic test. Acute Q fever is confirmed by a ≥4-fold rise in IgG titer to phase II antigens. Chronic Q fever is confirmed in a single serum with a ≥1,024 IgG titer to phase I antigen. A decreasing antibody titer suggests successful therapy. The IFA test that detects both phase I and II IgG is recommended. IgM phase II antigen enzyme-linked immunosorbent assay is commercially available but is for acute Q fever diagnosis only.

(Continued on next page)

TABLE 2 *(Continued)*

Organism	Diagnostic test[a]
Ehrlichia chaffeensis	**Microscopy:** Giemsa or Wright stain of peripheral blood or buffy coat smears is positive in up to 29% of infected persons. **Antigen tests:** None commercially available; IHC offered at CDC. **Molecular tests:** EDTA-anticoagulated blood collected during the pretreatment acute phase of illness is used for PCR amplification. Species- and genus-specific tests are described. Current test of choice for diagnosis during active infection. Sensitivity ranges from 56–100%. **Culture:** EDTA-anticoagulated peripheral blood or cerebrospinal fluid is inoculated onto DH82, THP1, HEL-22, Vero, HL-60 or other cell lines. Positive cultures may be obtained between 5 and >30 days from most samples if inoculated within 12 h and if obtained before antimicrobial therapy. Lack of timely results mitigates against frequent use. **Serologic tests:** IFA is the most frequently used test. In a patient with typical clinical features of HME, a ≥4-fold rise in IgG titer confirms infection, and a single peak IgG titer of ≥64 provides supportive evidence. IgG test sensitivity is believed to be high; specificity is ~95%. IgM testing is not recommended.
Ehrlichia ewingii	**Microscopy:** Giemsa or Wright stain of peripheral blood or buffy coat smears occasionally reveals bacterial clusters (morulae) in neutrophils of infected persons. **Antigen tests:** None available. **Molecular tests:** EDTA-anticoagulated blood collected during the pretreatment acute phase of illness is used for PCR amplification. Species- and genus-specific tests are available in some reference and public health laboratories. Current test of choice for diagnosis during active infection. Sensitivity is not currently known but is suspected to be high. **Culture:** No method of *in vitro* culture has been developed. **Serologic tests:** No specific antibody test is available. Antibody tests are based largely on cross-reactivity with *E. chaffeensis* and alone are not diagnostic.
Ehrlichia muris-like agent	**Microscopy:** Giemsa and Wright stains of peripheral blood or buffy coat smears should be examined but have not yet revealed bacterial clusters in human leukocytes. **Antigen tests:** None available. **Molecular tests:** EDTA-anticoagulated blood collected during the pretreatment acute phase of illness is used for PCR amplification. A species-specific test is available in some reference and public health laboratories. Current test of choice for diagnosis during active infection. Sensitivity is not currently known but is suspected to be high. **Culture:** Isolation has rarely been attempted but was successful in ISE6 tick cell and RF/6A endothelial cell cultures. **Serologic tests:** Currently only available at some public health laboratories; cross-reactivity with *E. chaffeensis* occurs.
Mycoplasma genitalium	**Microscopy:** May be detected in genital fluids by use of DNA fluorochrome stains (Hoechst 33258 or acridine orange), but these are not specific. **Antigen tests:** Not recommended for diagnostic purposes. **Molecular tests:** PCR amplification may be the only practical means for detection of the pathogen. Commercial kits are not currently available in the United States but are in Europe. **Culture:** Growth conditions are not well established but can require long periods. Widely considered insensitive for diagnosis confirmation. **Serologic tests:** MIF and Western immunoblot methods have been described, although none are commercially available.
Mycoplasma hominis	**Microscopy:** May be detected in body fluids by use of DNA fluorochrome stains (Hoechst 33258 or acridine orange), but these are not specific. **Antigen tests:** None commercially available. **Molecular tests:** PCR tests have been developed but because of rapid growth are less useful than culture. Commercial assays are available that simultaneously detect *M. hominis*, *M. genitalium*, *Ureaplasma* spp., *Trichomonas vaginalis*, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae*. **Culture:** Organisms are isolated from a variety of clinical samples. Dacron or polyester swabs are preferred; wood-shafted cotton swabs should be avoided. Mycoplasmas are extremely labile, and appropriate transport medium should be used. Can be recovered on SP4 broth and agar supplemented with arginine (best overall), on Shepard's 10B broth, or on A8 agar. Growth occurs within 2–4 days. **Serologic tests:** Not commercially available in the United States and not recommended for routine use.

(Continued on next page)

TABLE 2 Diagnostic tests for *Anaplasma*, *Chlamydia*, *Coxiella*, *Ehrlichia*, *Mycoplasma*, *Orienta*, *Rickettsia*, *Tropheryma*, and *Ureaplasma* (*Continued*)

Organism	Diagnostic test[a]
Mycoplasma pneumoniae	**Microscopy:** May be detected in body fluids by use of DNA fluorochrome stains (Hoechst 33258 or acridine orange), but these are not specific. **Antigen tests:** Not recommended for diagnostic purposes. **Molecular tests:** Real-time PCR amplification is the test of choice and is highly sensitive, but clinical studies have yielded variable results when compared with culture and serology. Commercial kits are available in both the United States and Europe. **Culture:** Organisms are isolated from a variety of clinical samples. Dacron or polyester swabs are preferred; wood-shafted cotton swabs should be avoided. Mycoplasmas are extremely labile, and appropriate transport medium should be used. Can be recovered on SP4 glucose broth or agar. Growth occurs after 21 days or longer. Subculture may improve yield. Widely considered insensitive for diagnosis confirmation. **Serologic tests:** EIAs are more sensitive and specific than complement fixation and IFA; detection of seroconversion by demonstration of a 4-fold increase in antibody titer is preferred, but detection of IgM antibodies in single sera may be useful. The cold agglutinin test is not recommended for diagnosis of M. *pneumoniae* infection.
Orientia tsutsugamushi	**Microscopy:** DFA or IHC on skin or other tissues may be performed, but tests are not widely available. **Antigen tests:** None available. **Molecular tests:** PCR amplification performed on EDTA-anticoagulated blood, buffy coat leukocytes, plasma, or tissue samples obtained during acute phase of illness; available only through reference laboratories. **Culture:** Isolation is performed by intraperitoneal inoculation of mice. Performed only in reference and research laboratories. **Serologic tests:** When IFA test is used on patients in a region of endemicity, a titer of ≥400 is 96% specific and 48% sensitive; lower cutoffs are used for travelers and for populations in which the infection is not endemic. Indirect immunoperoxidase uses light microscopy and is also sensitive and specific with diagnostic cutoffs of 128 for IgG and 32 for IgM. Dot EIA kits have lower sensitivity and specificity than IFA. Weil-Felix (*Proteus*) OX-K febrile agglutinins test is insensitive, nonspecific, and not recommended.
Rickettsia africae, *Rickettsia conorii*, and *Rickettsia parkeri*	**Microscopy:** DFA or IHC on skin biopsy specimen of rash or eschar is sensitive and specific for spotted fever group rickettsiae. Antibodies are not commercially available. **Antigen tests:** Not available. **Molecular tests:** PCR amplification performed on EDTA-anticoagulated blood, buffy coat leukocytes, plasma, skin biopsy specimens, or tissue samples obtained during acute phase of illness; available only through reference laboratories. **Culture:** Heparin-anticoagulated plasma or buffy coat cells or triturated skin biopsy specimens obtained before antirickettsial therapy are inoculated into shell vials seeded with cell lines such as Vero, L-929, HEL, or MRC5. Infected cells are detected by immunofluorescence or PCR after 48–72 h; sensitivity is up to 59%. **Serologic tests:** IFA is sensitive using *R. africae*, *R. conorii*, *R. parkeri*, or other spotted fever group rickettsial antigens (e.g., *R. rickettsii*) but is low during the acute phase of illness. A 4-fold increase in titer is generally considered most specific, but single titers of ≥128 for IgG and ≥32 for IgM are considered diagnostically significant. A Dot EIA that is modestly less sensitive and specific is available for *R. conorii*.
Rickettsia akari	**Microscopy:** DFA or IHC on skin biopsy specimen of rash or eschar is sensitive and specific for spotted fever group rickettsiae. Antibodies are not commercially available. **Antigen tests:** Not available. **Molecular tests:** PCR amplification performed on skin biopsy specimen of eschars obtained during acute phase of illness; available only through reference laboratories. **Culture:** Heparin-anticoagulated blood plasma or buffy coat cells are inoculated into shell vials seeded with cell lines such as Vero, L-929, HEL, or MRC-5. Infected cells are detected by Giemsa, Gimenez, or fluorescent-antibody staining after 48–72 h. Sensitivity is not known. **Serologic tests:** IFA is sensitive using either *R. akari* or other spotted fever group rickettsial antigens (e.g., *R. rickettsii*) but is low during the acute phase of illness. A 4-fold increase in titer is generally considered most specific, but single titers of ≥128 for IgG and ≥32 for IgM are considered diagnostically significant. *R. akari*-specific testing can be obtained in reference or public health laboratories.
Rickettsia felis	**Microscopy:** Not available. **Antigen tests:** Not available. **Molecular tests:** PCR amplification performed on EDTA-anticoagulated blood, buffy coat leukocytes, plasma, or tissue samples obtained during acute phase of illness; available only through reference laboratories. **Culture:** Not available except through research laboratories.

(*Continued on next page*)

TABLE 2 *(Continued)*

Organism	Diagnostic test[a]
	Serologic tests: IFA is sensitive using either *R. felis* or other spotted fever or typhus group rickettsial antigens but is low during the acute phase of illness. A 4-fold increase in titer is generally considered most specific; diagnostic titers have not been established. *R. felis*-specific testing can be obtained in reference or public health laboratories.
Rickettsia prowazekii	**Microscopy:** Not available. **Antigen tests:** Not available. **Molecular tests:** PCR amplification performed on EDTA-anticoagulated blood, buffy coat leukocytes, plasma, or tissue samples obtained during acute phase of illness; available only through reference laboratories. **Culture:** Heparin-anticoagulated blood plasma or buffy coat cells are inoculated into shell vials seeded with cell lines such as Vero, L-929, HEL, or MRC-5. Infected cells are detected by Giemsa, Gimenez, or fluorescent-antibody staining after 48–72 h. Sensitivity is not known. **Serologic tests:** IFA is sensitive using either *R. prowazekii* or *R. typhi* as antigen. A 4-fold increase in titer is generally considered most specific, but single titers of ≥128 for IgG and ≥32 for IgM are considered diagnostically significant.
Rickettsia rickettsii	**Microscopy:** DFA or IHC on skin biopsy specimen of rash is 70% sensitive and 100% specific. Antibodies are not commercially available. **Antigen tests:** Not available. **Molecular tests:** PCR amplification performed on EDTA-anticoagulated blood, buffy coat leukocytes, plasma, or tissue samples obtained during acute phase of illness; available only through reference laboratories. **Culture:** Heparin-anticoagulated blood plasma or buffy coat cells are inoculated into shell vials seeded with cell lines such as Vero, L-929, HEL, or MRC-5. Infected cells are detected by Giemsa, Gimenez, or fluorescent-antibody staining after 48–72 h. Sensitivity is not known. **Serologic tests:** IFA is sensitive using *R. rickettsii* or other spotted fever group rickettsial antigens but is low during the acute phase of illness. A 4-fold increase in titer is generally considered most specific, but single titers of ≥128 for IgG and ≥32 for IgM are considered diagnostically significant.
Rickettsia typhi	**Microscopy:** DFA or IHC on skin biopsy specimen of rash is sensitive and specific. Antibodies are not commercially available. **Antigen tests:** Not available. **Molecular tests:** PCR amplification performed on EDTA-anticoagulated blood, buffy coat leukocytes, plasma, or tissue samples obtained during acute phase of illness; available only through reference laboratories. **Culture:** Heparin-anticoagulated blood plasma or buffy coat cells are inoculated into shell vials seeded with cell lines such as Vero, L-929, HEL, or MRC-5. Infected cells are detected by Giemsa, Gimenez, or fluorescent-antibody staining after 48–72 h. Sensitivity is not known. **Serologic tests:** IFA is sensitive using either *R. prowazekii* or *R. typhi* as antigen. A 4-fold increase in titer is generally considered most specific, but single titers of ≥128 for IgG and ≥32 for IgM are considered diagnostically significant. A Dot EIA that is modestly less sensitive and specific is available for *R. typhi*.
Tropheryma whipplei	**Microscopy:** Tissue biopsy with periodic acid-Schiff stain, IHC, or fluorescence *in situ* hybridization; electron microscopy. **Antigen tests:** None available. **Molecular tests:** PCR tests are currently the preferred method for specific diagnosis; performed on fresh, frozen, or paraffin-embedded small intestinal biopsy specimens, cardiac valve tissues, lymph nodes, liver, synovium, central nervous system samples. Available through reference laboratories. **Culture:** Isolation requires long-term (>30 days) culture methods and is currently available in only a few research and reference/public health laboratories around the world. **Serologic tests:** Not currently useful.
Ureaplasma urealyticum	**Microscopy:** May be detected in body fluids using DNA fluorochrome stains (Hoechst 33258 or acridine orange), but these are not specific. **Antigen tests:** None commercially available. **Molecular tests:** PCR tests have been developed but are less useful than culture. **Culture:** Organisms are isolated from a variety of clinical samples. Dacron or polyester swabs are preferred; wood-shafted cotton swabs should be avoided. Ureaplasmas are extremely labile, and appropriate transport medium should be used. Can be recovered on Shepard's 10B urea broth and A8 agar. Growth occurs within 2–4 days. Subculture increases diagnostic yield. **Serologic tests:** Not recommended for routine use.

[a] Abbreviations: DFA, direct fluorescent antibody; EIA, enzyme immunoassay; IFA, indirect fluorescent antibody; IHC, immunohistochemistry; LPS, lipopolysaccharide; MIF, microimmunofluorescence.

Mycoplasma and *Ureaplasma*

KEN B. WAITES AND DAVID TAYLOR-ROBINSON

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TAXONOMY

Bacteria commonly referred to as mycoplasmas ("fungus-form") are included within the Class *Mollicutes* ("soft skin"), which is comprised of 4 orders, 5 families, 8 genera, and about 200 known species, as shown in Table 1. New species, mainly in animals, are still being identified. Table 2 lists 16 species isolated from humans on multiple occasions, excluding species of animal origin detected occasionally in humans, usually in immunosuppressed hosts, but which are generally considered transient colonizers. *Mycoplasma amphoriforme* is the most recent species to have been recognized, having been recovered from the respiratory tract of several patients with antibody deficiency and chronic bronchitis or bronchiectasis (1). Mollicutes are eubacteria that have evolved from clostridium-like Gram-positive cells by gene deletion. The availability of species-specific PCR technology is ameliorating difficulties of both culture and identification for fastidious mollicutes. Therefore, additional noncultivable, and thus presently unknown, species are likely to be discovered, although this has not happened so far in the human field.

DESCRIPTION OF *MOLLICUTES*

The term "mycoplasma" is often used in a trivial way to refer to any members of the class *Mollicutes*, irrespective of whether they actually belong to the genus *Mycoplasma*. It is more accurate to refer to the class with the trivial term "mollicute(s)" and to organisms within the individual genera as "mycoplasmas," "ureaplasmas," "acholeplasmas," etc. Mollicutes are smaller than conventional bacteria, in cellular dimensions as well as genome size, making them the smallest free-living organisms known. Mycoplasmas associated with humans range from coccoid cells of about 0.2 to 0.3 μm in diameter, as in *Ureaplasma* spp. and *Mycoplasma hominis* (2), to tapered rods 1 to 2 μm in length and 0.1 to 0.2 μm in width in the case of *Mycoplasma pneumoniae* (3). Mollicutes are contained by a trilayered cell membrane and do not possess a cell wall. The permanent lack of a cell wall barrier makes the mollicutes unique among prokaryotes and differentiates them from bacterial L forms, for which the lack of the cell wall is but a temporary reflection of environmental conditions. Lack of a cell wall also renders the mollicutes insensitive to the activity of beta-lactam antimicrobials, prevents them from staining by Gram stain, and is largely responsible for their pleomorphic form. The extremely small genome (<600 kb in the case of *M. genitalium*) and limited biosynthetic capabilities explain the parasitic or saprophytic existence of these organisms, their sensitivity to environmental conditions, and their fastidious growth requirements, which can complicate cultural detection. Mollicutes require enriched growth medium supplemented with nucleic acid precursors. Except for acholeplasmas, asteroleplasmas, and mesoplasmas, mollicutes require sterols in growth media, supplied by the addition of serum. Growth rates in culture medium vary among individual species, with generation times of approximately 1 hour for *Ureaplasma* spp., 6 hours for *M. pneumoniae*, and 16 hours for *M. genitalium* (4).

Typical mycoplasmal colonies vary from 15 to 300 μm in diameter. Colonies of some species, such as *M. hominis*, often exhibit a "fried-egg" appearance owing to the contrast between deeper growth in the center of the colony with more shallow growth at the periphery (Fig. 1), while others, such as *M. pneumoniae*, produce spherical colonies (Fig. 2). Whereas colonies of mycoplasmal species may be observed with the naked eye, those produced by ureaplasmas are typically 15 to 60 μm in diameter and require low-power microscopic magnification for visualization (Fig. 3).

Mycoplasmas and ureaplasmas of human origin can be classified according to whether they utilize glucose, arginine, or urea (Table 2). Except for utilization of urea for generation of metabolic energy, which is unique for ureaplasmas, these biochemical features are not sufficient for species distinction. Anaeroplasmas and asteroleplasmas, which occur in ruminants, are strictly anaerobic and oxygen sensitive, while most other mollicutes are facultative anaerobes.

Attachment of *M. pneumoniae* to host cells in the respiratory tract of humans is a prerequisite for colonization and infection. Cytoadherence, mediated by the P1 adhesin and other accessory proteins, described in detail in other publications (5, 6), is followed by induction of chronic inflammation and cytotoxicity mediated by hydrogen peroxide, which also acts as a hemolysin. *M. pneumoniae* stimulates B and T lymphocytes and induces the formation of autoantibodies, which react with a variety of host tissues and the I antigen on erythrocytes, which is responsible for production of cold agglutinins (6). An ADP-ribosylating toxin with significant sequence homology to the pertussis toxin S1 subunit, now known as the community-acquired respiratory distress syndrome (CARDS) toxin that causes vacuolation and ciliostasis in cultured host cells, has been described in *M. pneumoniae* (7). A murine model has shown that CARDS toxin

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TABLE 1 Classification and some distinguishing features of mycoplasmas (class *Mollicutes*)

Classification of class *Mollicutes*	Distinguishing features			
	Sterol required	Genome size (kbp)	G+C content of DNA (mol%)	Other
Order I: *Mycoplasmatales*				Vertebrate hosts
Family I: *Mycoplasmataceae*				Sterol requirement
Genus I: *Mycoplasma* (118 species)[a]	Yes	580–1,350	23–40	
Genus II: *Ureaplasma* (7 species)[b]	Yes	730–1,170	25–32	Hydrolyze urea
Order II: *Entomoplasmatales*				Plant/insect hosts
Family I: *Entomoplasmataceae*				Nonhelical structure
Genus I: *Entomoplasma* (6 species)	Yes	870–900	27–34	Sterol requirement
Genus II: *Mesoplasma* (11 species)	No	825–930	26–32	Sterol requirement +/−
Family II: *Spiroplasmataceae*				Helical structure
Genus I: *Spiroplasma* (38 species)	Yes	780–2,200	24–31	Sterol requirement +/−
Order III: *Acholeplasmatales*[c]				Sterols not required
Family I: *Acholeplasmataceae*	No	1,500–1,650	26–36	
Genus I: *Acholeplasma* (18 species)				
Order IV: *Anaeroplasmatales*				Obligate anaerobes
Family I: *Anaeroplasmataceae*				
Genus I: *Anaeroplasma* (4 species)	Yes	1,500–1,600	29–33	Sterol requirement
Genus II: *Asteroleplasma* (1 species)	No	1,500	40	Sterols not required
"*Candidatus* Phytoplasma"[c] (35 species)	Unknown	530–1,350	23–29	

[a] The total number includes subspecies. The genus *Mycoplasma* also includes several *Candidatus* species of cell wall-less uncultivated parasitic bacteria. Some were previously classified in the genera *Haemobartonella* and *Eperythrozoon*. Their guanine + cytosine content and sterol requirements are unknown.

[b] *U. urealyticum* and *U. parvum*, formerly considered biovars of *U. urealyticum*, are now classified as two separate species and are the only ureaplasmas of human origin.

[c] Phytoplasmas are *Candidatus* species of mollicutes of plants and insects genetically related to the *Acholeplasmatales*.

concentrations in bronchoalveolar lavage fluid are directly linked to the ability of specific *M. pneumoniae* strains to colonize, replicate, persist, and elicit lung histopathology. This variation among strains may predict the range in severity of pulmonary disease observed among patients (8). The CARDS toxin is also being studied as a gene target for PCR assays and as an antigen for use in serologic assays. *M. genitalium* also possesses a terminal structure, the MgPa adhesin, which facilitates its attachment to epithelial cells (9). Factors involved with *Ureaplasma* and *M. hominis* attachment have not been characterized to the same extent as for *M. pneumoniae*, and these mycoplasmas do not have prominent attachment organelles. Henrich et al. (10) demonstrated the presence of the variable-adherence-associated antigen (Vaa), which is believed to be a major adhesin of *M. hominis* and could also assist in evasion of host immune

TABLE 2 Primary sites of colonization, metabolism, and pathogenicity of mollicutes of human origin[a]

Species	Primary site of colonization		Metabolism of:		Pathogenicity
	Respiratory tract	Genitourinary tract	Glucose	Arginine	
M. salivarium	+	−	−	+	−
M. orale	+	−	−	+	−
M. buccale	+	−	−	+	−
M. faucium	+	−	−	+	−
M. lipophilum	+	−	−	+	−
M. amphoriforme[b]	?+	−	+	−	?
M. pneumoniae	+	−	+	−	+
M. hominis	+	+	−	+	+
M. genitalium[c]	+	+	+	−	+
M. fermentans	+	+	+	+	+
M. primatum	−	+	−	+	−
M. spermatophilum	−	+	−	+	−
M. pirum	?	?	+	+	−
M. penetrans	−	+	+	+	?
Ureaplasma spp.[d]	+	+	−	−	+
Acholeplasma laidlawii	+	−	+	−	−

[a] Symbols: +, positive for trait; −, negative for trait; ?, unknown.

[b] All isolates reported to date have been from the lower respiratory tract, but no other sites have been sampled.

[c] The organism has been found in the oropharynx, but it seems unlikely that this is a common or primary location.

[d] These species metabolize urea.

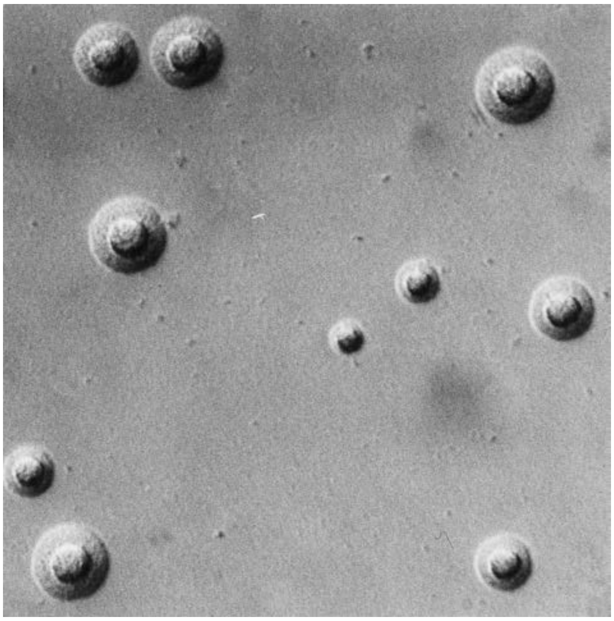

FIGURE 1 Fried-egg-type colonies of *Mycoplasma hominis* up to 110 µm in diameter. Magnification, ×132.
doi:10.1128/9781555817381.ch62.f1

responses through antigenic variation. Ureaplasmas also attach to a variety of cell types, mediated by adhesin proteins expressed on the surface of the bacterial cell. The MB antigen contains serotype-specific and cross-reactive epitopes and is a prominent antigen recognized during human ureaplasmal infections (11). Ureaplasmas are known to produce IgA protease, which may be associated with disease production, and they release ammonia through urealytic activity (11).

EPIDEMIOLOGY AND TRANSMISSION

Mollicutes are common in practically all mammalian species, as well as many other vertebrates in which they have been sought. Although most mollicutes have species-specific host-organism associations, some mycoplasmas and acholeplasmas of animal origin occur in a wide variety of different animal hosts. Mollicutes in the genera *Spiroplasma*, *Mesoplasma*, *Entomoplasma*, and *Acholeplasma* can be isolated from insects and plants.

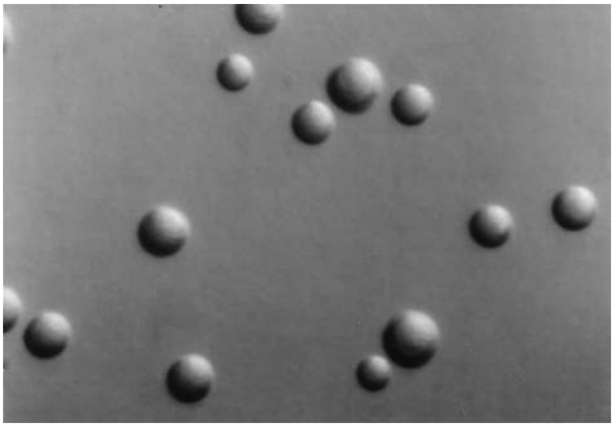

FIGURE 2 Spherical colonies of *Mycoplasma pneumoniae* up to 100 µm in diameter. Magnification, ×126.
doi:10.1128/9781555817381.ch62.f2

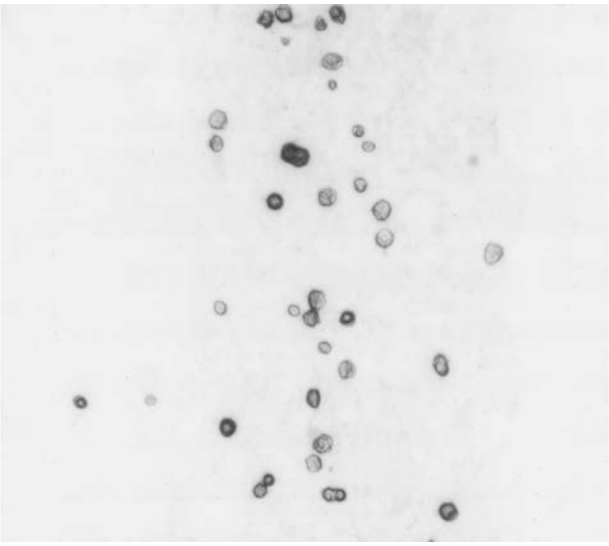

FIGURE 3 Granular brown urease-positive colonies of *Ureaplasma* species 15 to 60 µm in diameter from a vaginal specimen growing on A8 agar. Magnification, ×100.
doi:10.1128/9781555817381.ch62.f3

In humans, mycoplasmas and ureaplasmas are mucosally associated, residing predominantly in the respiratory or urogenital tracts, rarely penetrating the submucosa, except in cases of immunosuppression or instrumentation, when they can invade the bloodstream and disseminate to many different organs and tissues throughout the body. Many mollicutes exist as commensals in the oropharynx or urogenital tract (Table 2) and have been associated with invasive disease only in very rare circumstances. *M. fermentans* has been detected by culture and PCR assays in various body sites, including the urogenital tract, throat, lower respiratory tract, and other body locations, including joints, but its primary site of colonization and true disease potential are incompletely understood (11). Oral commensal mycoplasmas can occasionally spread to the lower respiratory tract and show up in cultures, but they should not cause diagnostic confusion with *M. pneumoniae* if appropriate means of organism identification are employed. The frequent occurrence of pathogenic species such as *M. hominis* and ureaplasmas in the lower urogenital tract in healthy men and women has complicated complete understanding of their disease-producing capabilities. Various investigations have found that *M. genitalium* is much less common in the lower urogenital tract of healthy females than *M. hominis* and *Ureaplasma* spp., with a prevalence of 6% or less (12, 13). Other species, as shown in Table 2, can also occur in the lower urogenital tract but are not known to be associated with disease. PCR assays have demonstrated the frequent occurrence of *M. genitalium* in the urogenital tract in men with urethritis and in lower and upper genital tract sites in women and that of *M. penetrans* in urine of homosexual males with HIV (14, 15). Although mycoplasmas are generally considered to be extracellular organisms, intracellular localization is now appreciated for *M. fermentans*, *M. penetrans*, *M. genitalium*, and *M. pneumoniae* (16). Intracellular localization may be responsible for protecting the organisms from antibodies and antibiotics, as well as contributing to disease chronicity and difficulty in cultivation in some cases. Variation in surface antigens of *M. hominis* and *Ureaplasma* spp. can be related to the persistence of these organisms at invasive sites. In humans, mycoplasmas and ureaplasmas can be transmitted by direct contact between hosts, i.e., venereally through genital-genital or oral-genital contact, vertically from mother to offspring either at birth or in utero, by respiratory aerosols or fomites in the case of *M. pneumoniae*, or even by nosocomial acquisition through transplanted tissues (11).

CLINICAL SIGNIFICANCE

Respiratory Infections

M. pneumoniae was first identified and described in the early 1960s. Prior to this time, it was believed to be a virus. It causes approximately 20% of all community-acquired pneumonias in the general population and up to 50% of pneumonias in certain confined groups (6). Although *M. pneumoniae* has long been associated with pneumonias in school-aged children, adolescents, and young adults, in recent years this organism has also been shown to occur endemically and occasionally epidemically in older persons, as well as children under five years of age (6). The most typical clinical syndrome is tracheobronchitis, often accompanied by upper respiratory tract manifestations, such as acute pharyngitis. Pneumonia develops in about one-third of persons who are infected. The incubation period is generally 2 to 3 weeks, and spread throughout households is common. The organism can persist in the respiratory tract for several months after initial infection, and sometimes for years in hypogammaglobulinemic patients, possibly because it attaches strongly to and invades epithelial cells. Disease tends not to be seasonal, subclinical infections are common, and the disease is ordinarily mild. However, severe infections requiring hospitalization and even death are known to occur (6, 17).

Extrapulmonary complications of *M. pneumoniae* infections can include meningoencephalitis, ascending paralysis, transverse myelitis, Bell's palsy, pericarditis, hemolytic anemia, arthritis, and mucocutaneous lesions (6, 18). An autoimmune response is thought to play a role in some extrapulmonary complications. However, *M. pneumoniae* has been isolated directly from cerebrospinal, pericardial, and synovial fluids, as well as other extrapulmonary sites, and additional evidence of direct invasion by this organism has been documented by the use of the PCR assay (18). Clinical manifestations are not sufficiently unique to allow differentiation from infections caused by other common bacteria, particularly *Chlamydia pneumoniae*. Data from animal models as well as clinical studies have suggested a potential role for *M. pneumoniae* as an etiologic or exacerbating factor in bronchial asthma, and additional clinical studies are linking this organism to stable as well as exacerbating disease (5, 19, 20).

M. fermentans has been recovered from the throats of children with pneumonia, some of whom had no other etiologic agent identified, but the frequency of its occurrence in healthy children is not known. It has been detected in adults with an acute influenzalike illness (21) and in bronchoalveolar lavage fluids, peripheral blood lymphocytes, and bone marrow from patients with AIDS and respiratory disease (22, 23). It is apparent that respiratory infection with *M. fermentans* is not necessarily linked with immunodeficiency, but it can also behave as an opportunistic respiratory pathogen. *M. fermentans* has not been detected by culture or PCR in patients with urethritis or cervicitis but has been detected by PCR in amniotic fluid and in association with histologic chorioamnionitis, suggesting that *M. fermentans* can be transferred transplacentally (24).

Very little is known about *M. amphoriforme* beyond what has been described in the initial reports of its detection in the lower respiratory tract by culture and/or PCR in a series of patients with antibody deficiency and chronic bronchitis or bronchiectasis (1). Its biochemical reactivity, colonial appearance, growth characteristics, and gliding motility are similar to these features in *M. pneumoniae*, but it is genetically distinct. Repeated isolations over time and clinical improvement after antimicrobial therapy, which resulted in the elimination of the mycoplasma, suggest a possible pathogenic role, but more work must be done to determine the extent of disease that may be due to this organism (25). Thus far, there have not been any isolations of *M. amphoriforme* outside Europe and northern Africa.

Genitourinary Infections

Following puberty, *Ureaplasma* spp. and *M. hominis* can be isolated from the lower genital tract in many healthy sexually active adults, but there is evidence that these organisms play etiologic roles in some genital tract diseases. Results of human and animal inoculation studies and observations of immunocompromised persons are supportive of ureaplasmas being a cause of nonchlamydial, nongonococcal urethritis (NGU) in men (26). Since the identification of two distinct biovars of *Ureaplasma urealyticum*, now considered separate species, biovar 2 (*U. urealyticum*) has been implicated in NGU, whereas biovar 1 (*U. parvum)* has not been implicated in this manner in most studies (27–29). There have been attempts to link various disease conditions with individual *Ureaplasma* serovars. However, recent evidence suggests that individual serovars rarely occur alone and horizontal gene transfer among the various serovars occurs to a significant extent, making the individual distinct serovar concept no longer tenable (27). Evidence that *M. hominis* causes NGU is lacking, but *M. genitalium* has been detected by PCR technology significantly more often in urethral specimens from men with acute NGU than from those without urethritis and is now considered to be one of the causes of the disease (14). *M. genitalium*-positive men have been found to have symptomatic urethritis significantly more often than those infected with *Chlamydia trachomatis* (30). Antibody responses have been detected in some men with acute disease, and this mycoplasma has also produced urethritis in nonhuman primates (14). *M. genitalium* also may be a rare cause of conjunctivitis associated with urethritis (31). *M. fermentans*, *M. penetrans*, and *M. pirum* were not detected in the urethras of men with urethritis by PCR assays, suggesting that these organisms are unlikely to have a pathogenic role in this condition (32). In women, there is no evidence that *M. hominis* is a cause of the urethral syndrome, but ureaplasmas may be involved (33).

M. hominis and *Ureaplasma* spp. have not been detected by culture of prostatic biopsy samples from patients with chronic abacterial prostatitis (34), and *M. genitalium* has been found rarely by using a PCR assay (35). In contrast, ureaplasmas have been recovered from an epididymal aspirate from a patient suffering from nonchlamydial, nongonococcal acute epididymo-orchitis accompanied by a specific antibody response (36) and could be an infrequent cause of the disease. *Ureaplasma* spp. produce urease, induce crystallization of struvite and calcium phosphates in urine *in* *vitro* and calculi in animal models, and have been found in urinary calculi of patients with infection-type stones more frequently than those with metabolic-type stones, suggesting a possible causal association (37). *M. hominis* has been isolated from the upper urinary tract only in patients with symptoms of acute pyelonephritis, often with an antibody response, and causes about 5% of cases (38). Obstruction or instrumentation of the urinary tract are predisposing factors. Ureaplasmas have not been associated in the same way.

Mollicutes do not cause vaginitis but are among various microorganisms that proliferate in patients with bacterial vaginosis (BV). Some studies suggest that *M. hominis* contributes to BV (39), but evidence is lacking for an association of ureaplasmas with BV (11, 26). The independent association of *M. genitalium* with BV is controversial, as in some studies an association has been shown (40) while in others a relationship has not been demonstrated (39, 41). BV can lead to pelvic inflammatory disease (PID), and *M. hominis* has been isolated from the endometrium and fallopian tubes of about 10% of women with salpingitis diagnosed by laparoscopy, accompanied by a specific antibody response (42). The significance of this mycoplasma is difficult to assess in an individual case when several microorganisms are present. Nevertheless, serological evidence suggests that *M. hominis* could be an independent factor in tubal factor infertility (43). *Ureaplasma* spp. have been isolated directly from affected fallopian tubes, but not alone. This fact, together with the negative results of serologic tests and of inoculating nonhuman primates as well as fallopian tube organ cultures, does not support a causal relationship for ureaplasmas in PID (26). *M. genitalium*, however, can play a role in PID as indicated by its significant association with cervicitis (41, 44, 45) and endometritis (46). In addition, there is serologic evidence that this mycoplasma causes some cases of tubal infertility (47). That ureaplasmas might cause infertility still remains speculative.

Ureaplasmas have been isolated from internal organs of spontaneously aborted fetuses and from stillborn and premature infants more often than from fetuses in induced abortions or normal full-term infants (11). The results from some serologic and therapeutic studies have also supported a role for these organisms in fetal morbidity (11). BV is a possible confounding factor that must be considered in the association between ureaplasmas in the chorioamnion and low birth weight. Ureaplasmas at this site are directly associated with inflammation and may invade the amniotic sac early in pregnancy in the presence of intact fetal membranes, causing persistent infection and an adverse pregnancy outcome (48).

The notion that *M. hominis* causes fever in some women after abortion, or after normal delivery, is based on the isolation of this organism from the blood of about 10% of such women, but not from afebrile women who have had abortions or from healthy pregnant women (26). In addition, antibody responses have been detected in about one-half of febrile aborting women but in few of those who remain afebrile (26). Similar observations have been made for the isolation of *Ureaplasma* spp., which could be responsible for a few cases of postpartum endometritis (49). The ability of *U. parvum* and *M. hominis* to upregulate amniotic fluid leukocytes, proinflammatory cytokines, prostaglandins, metalloproteinases, and uterine activity to induce chorioamnionitis, a systemic fetal inflammatory response, and contribute to preterm labor and fetal lung injury is supported by experimental studies in rhesus monkeys (50). However, it is difficult to determine whether they have a role independent of all other bacteria occurring in BV, a condition strongly associated with preterm labor and birth. In addition, there are conflicting opinions about the importance of *U. urealyticum* as opposed to *U. parvum* in premature delivery, a situation that needs to be resolved. There is no evidence that *M. genitalium* is a cause of preterm labor or abortion (51, 52).

Neonatal Infections

Colonization of infants by genital mycoplasmas can occur by ascension from the lower genital tract of the mother at the time of delivery or *in utero* earlier in gestation and can be transient and without sequelae. The rate of vertical transmission is 18 to 55% among infants born to colonized mothers (11). *Ureaplasma* spp. and *M. hominis* can be isolated from neonates born to mothers with intact membranes and delivered by cesarean section (11). Congenital pneumonia, bacteremia, progression to chronic lung disease of prematurity with the development of inflammatory cytokines in tracheal aspirates, and even death have occurred in very low birth weight infants due to ureaplasmal infection of the lower respiratory tract (11). A meta-analysis of the literature accumulated since the 1980s supports the association of ureaplasmal infection with development of chronic lung disease, but so far there has been no evidence of a reduction in the incidence of chronic lung disease or death when preterm infants were treated with erythromycin (53). Additional studies using azithromycin are under way in an attempt to answer this important question. Both *M. hominis* and *Ureaplasma* spp. have been isolated from maternal and umbilical cord blood, as well as the blood of neonates. Both *M. hominis* and *Ureaplasma* spp. can also invade the cerebrospinal fluid of neonates (11). Either mild, subclinical meningitis without sequelae or neurological damage with permanent handicaps can ensue. Colonization of healthy full-term infants declines after 3 months of age, and fewer than 10% of older children and sexually inexperienced adults are colonized with genital mycoplasmas (11). Vertical transmission of *M. genitalium* from mother to neonate has been reported, but its significance in neonates is unknown (11).

Routine screening of neonates for genital mycoplasmas is not clinically justified based on the available evidence that many healthy neonates can be colonized without consequence. However, if there is clinical, radiological, or laboratory evidence of pneumonia, meningitis, or overall instability, particularly in preterm neonates in whom there are no obvious alternative etiologies, infection with *M. hominis* or *Ureaplasma* spp. should be considered.

Systemic Infections and Immunosuppressed Hosts

Extrapulmonary and extragenital mycoplasmal infections probably occur more often than currently recognized. *M. hominis* is alone among pathogenic mycoplasmas of human origin that are occasionally detected in routine bacteriologic cultures, so that there have been many instances of accidental discovery when mycoplasmas were not specifically sought. The number of published case reports implicating *Mycoplasma* and *Ureaplasma* spp. in a variety of systemic infections involving persons with and without impaired host defenses has increased in recent years as a result of a more widespread utilization of universal PCR primers and DNA sequencing when infection is suspected and no conventional microbes are detected by culture. Mollicutes can cause invasive disease of the joints as a result of dissemination from the genital or respiratory tracts in immunosuppressed persons, especially individuals with hypogammaglobulinemia, and should always be considered early when attempting to diagnose septic arthritis in the setting of congenital antibody deficiency. M. *hominis* bacteremia has been demonstrated after renal transplantation, trauma, and genitourinary manipulations, and M. *hominis* has also been found in brain abscesses, osteomyelitis lesions, and wound infections (54, 55). Numerous mycoplasmal species, including M. *fermentans*, U. *urealyticum*, and M. *salivarium*, have been detected mainly by a PCR assay in synovial fluid of persons with rheumatoid arthritis and other arthritides, although the precise contribution of these organisms to these disease conditions is still uncertain (56–58). The significance of M. *fermentans* and other mycoplasmas as possible agents of Gulf War Syndrome received a great deal of attention several years ago, but there is no credible evidence supporting such an association or causal role (59). However, in cross-sectional and longitudinal studies, an association between M. *genitalium* and HIV infection has been found. M. *genitalium* can be found more frequently at both urethral and rectal sites of HIV-positive homosexual men than those who are HIV negative (60), and M. *genitalium*-induced cervicitis (61) occurs more often in HIV-positive than in HIV-negative women. Women who have a high burden of M. *genitalium* were more likely to shed HIV-1 than were M. *genitalium*-negative women, an observation in keeping with the ability of mycoplasmas to stimulate HIV replication (62) and possibly enhance viral transmission. The significant positive association between M. *genitalium* and HIV infection is also strongly supported by the results of a meta-analysis (63).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Specimen Type and Collection

Body fluids appropriate for mycoplasmal culture or detection by noncultural methods include blood, synovial fluid, amniotic fluid, cerebrospinal fluid, urine, prostatic secretions, semen, wound aspirates, sputum, pleural fluid, bronchoalveolar lavage fluid, or other tracheobronchial secretions, depending on the clinical condition and organisms of interest. Swabs from the nasopharynx, throat, cervix/vagina, wounds, and urethra are also acceptable. Tissue from biopsy or autopsy, including placenta, endometrium, bone chips, and urinary calculi can also be used. When swabs are used, care must be taken to sample the desired site vigorously to obtain as many cells as possible since mycoplasmas are mainly cell associated. Urine specimens sometimes prove more sensitive than urethral swabs for detection of fastidious mycoplasmas such as M. *genitalium* by PCR (64). If determination of the localization of mycoplasmas in the genitourinary tract is desired, urine specimens can be obtained at various stages during urination or after prostatic massage. Care should be taken to avoid collection of specimens that are contaminated by lubricants or antiseptics commonly used in gynecologic practice. Dacron or polyester swabs with aluminum or plastic shafts are preferred. Wooden shaft cotton swabs should be avoided because of potential inhibitory effects. Swabs should always be removed from specimens before transportation to the laboratory.

Successful isolation of mycoplasmas from blood can be achieved by inoculating blood, free of anticoagulant, into a liquid mycoplasmal growth medium at the bedside in a 1:5 to 1:10 ratio, using as much blood as possible (at least 10 ml is desirable for adults). Mycoplasmas are inhibited by sodium polyanethol sulfonate, the anticoagulant used in most commercial blood culture media, but the inhibitory effect can be overcome by addition of gelatin (1%, wt/vol) (65). Use of commercial blood culture media with or without automated blood culture instruments is not recommended for detection of mycoplasmas. None of the newer continuously monitored, nonradiometric, automated blood culture systems flag bottles containing M. *hominis*, even when additional metabolic substrate and gelatin are added. However, the organisms can survive in these media for several days (66).

Transport and Storage

Mycoplasmas are extremely sensitive to adverse environmental conditions, particularly dryness and heat. Specimens should be inoculated at the bedside whenever possible, using appropriate transport and/or culture media. Liquid growth media such as commercially prepared SP4 or 10B broths (Thermo Fisher, Waltham, MA, or Hardy Diagnostics, Santa Maria, CA) can also serve as transport media; 2 SP (10% [vol/vol] heat-inactivated fetal calf serum with 0.2 M sucrose in 0.02 M phosphate buffer, pH 7.2) and Trypticase soy broth with 0.5% (vol/vol) bovine albumin (Thermo Fisher) are also acceptable transport media. Specialized liquid transport media such as A3B (Thermo Fisher) have been designed by deletion of some of the growth supplements present in other growth media so that an increase in pH caused by the urealytic activity of ureaplasmas can be delayed, resulting in less toxicity to the organisms during transport. Laboratories can choose to stock a single universal transport medium for mycoplasmas, ureaplasmas, chlamydiae, and viruses. Most universal transport media contain inhibitors to prevent bacterial or fungal overgrowth from specimens obtained from nonsterile sites that have their own indigenous microbial flora. M4, M5, and M6 (Thermo Fisher), UTM (Copan Diagnostics, Murrieta, CA), and BD UVT medium (Becton Dickinson, Franklin Lakes, NJ) are examples of universal transport systems suitable for transport of specimens from which mycoplasmas and/or ureaplasmas will be sought by culture.

Liquid specimens do not require special transport media if cultures can be inoculated within 1 h, provided the specimens are protected from evaporation. Tissues can be placed in a sterile container that can be tightly closed and delivered to the laboratory immediately. Otherwise, tissue specimens should be placed in transport media if delay in culture inoculation is anticipated. Specimens should be refrigerated if immediate transportation to the laboratory is not possible. If specimens must be shipped and/or if the storage time is likely to exceed 24 h prior to processing, the specimen in transport medium should be frozen at −80°C to prevent loss of viability and to minimize bacterial overgrowth. Mollicutes can be stored for long periods in appropriate growth or transport media at −80°C or in liquid nitrogen. Frozen specimens can be shipped with dry ice to a reference laboratory if necessary. Storage at −20°C is deleterious to detection, even by nonculture methods. When frozen specimens are to be examined, they should be thawed rapidly in a water bath at 37°C.

DIRECT EXAMINATION

Microscopy

Mycoplasmas, like chlamydiae and rickettsiae, cannot be clearly visualized by routine light microscopy. Lack of a cell wall precludes visualization of mycoplasmas by Gram staining, but this procedure can prove useful to exclude contaminating bacteria. M. *hominis* occasionally appears as pinpoint colonies on bacteriologic media, such as Columbia agar, and the lack of a Gram stain reaction by these colonies gives a clue as to their possible mycoplasmal identity, warranting further specific evaluation and subculture to mycoplasmal media. A DNA fluorochrome or acridine orange stain can be useful to assist in organism visualization when applied to body fluids, but it is not specific for mycoplasmas.

Antigen Detection

Although culture is appropriate for species that can be isolated easily and rapidly from clinical specimens, such as M. *hominis* and *Ureaplasma* spp., it is not ideal for detection of fastidious and/or extremely slow-growing organisms such as M. *genitalium* and, to a considerable degree, M. *pneumoniae*. Therefore, alternate non-culture-based methods of detection should be employed even if culture is attempted for these organisms. Rapid methods for antigenic detection of M. *pneumoniae* were developed in the 1980s, but these techniques were hampered by low sensitivity and cross-reactivity with other commensal mycoplasmas. This approach for rapid diagnosis has now been abandoned in favor of PCR-based assays.

Nucleic Acid Detection

PCR systems have been developed for all of the clinically important mycoplasma species that infect humans, largely because of the advantages that they have over culture and serology, such as the ability to complete the procedure in one day utilizing a single specimen containing organisms that do not have to be viable, as well as the ability to detect nucleic acid in preserved tissues. PCR assays also detect the organisms earlier in the course of illness than serology, since development of measurable antibodies requires up to several days or more. Real-time PCR assays are now the preferred PCR method for detection of mycoplasmas and ureaplasmas. The advantages include a more rapid turnaround time, less handling of PCR product, and improved diagnostic sensitivity over the traditional PCR techniques. Analytical sensitivity is generally high, with some assays capable of detecting a single organism when purified DNA is used. Details of various nucleic acid amplification tests and their applications for detection of mycoplasmas and ureaplasmas have been discussed in depth in a recent review (67).

Gene targets for PCR assays for detection of M. *pneumoniae* in clinical specimens have included 16S rRNA, *P1*, *tuf*, *parE*, *dnak*, *pdhA*, ATPase operon, CARDS toxin gene (*mpn372*), and the noncoding repetitive element *repMp1* (5, 68–72). Both conventional and real-time nucleic acid sequence-based amplification (NASBA) have also been used to detect M. *pneumoniae* RNA (73). NASBA can provide rapid results with sensitivity as good as that of PCR, with a detection threshold as low as 5 to 50 CFU (73, 74). There have been very few side-by-side comparisons to determine whether one assay format or gene target is better than another. Many of the comparisons of PCR using culture or serology as a reference method yielded disparate results. Commercial PCR assays have been available in Europe for several years, and additional products are still in development. Limited evaluations showed that they work in a manner comparable to that of noncommercial assays (75–77).

One multiplex assay, the Biofire Diagnostics (Salt Lake City, UT) FilmArray RP, detects nucleic acids in nasopharyngeal swabs for 20 respiratory tract pathogens, including M. *pneumoniae*. This assay has received FDA clearance for use in the United States and is also available in Europe. Meridian Bioscience (Cincinnati, OH) has also introduced the *illumigene* mycoplasma assay into the United States. This loop-mediated isothermal amplification assay enables detection of M. *pneumoniae* in up to 10 clinical specimens that can be tested simultaneously within 1 h after extracted DNA is set up in the incubator/reader provided by the manufacturer. This instrumentation can easily be incorporated into clinical microbiology laboratories that do not have extensive molecular diagnostic facilities, and it has been reported to produce acceptable results in comparison to culture (78).

Quantitative, rapid, real-time PCR assays for M. *genitalium* have utilized targets such as the MgPa operon, 16S rRNA, the 115-kDa protein-coding gene, and *gap*, encoding glyceraldehyde-3-phosphate dehydrogenase (79–85). Use of the latter target overcomes some of the limitations that have been identified for 16S rRNA and MgPa (67). Strong associations between serology and PCR for M. *genitalium* have been described, but the analytic sensitivity of a single PCR assay for M. *genitalium* was questioned by Baseman et al., who reported that 61% of culture-positive women tested negative by PCR, despite apparently good quality control parameters for their assay (86). In view of the difficulty of culturing M. *genitalium*, culture positivity could have been due to cross-contamination. Assuming that this was not the case, the observation highlights the point that reduced sensitivity of a single PCR assay could be related to the quality of the specimen or the presence of inhibitors. Gen-Probe (San Diego, CA) developed a real-time PCR assay that performs well in comparison to other methods, but it is currently available in the United States only for research purposes (80, 87, 88). Multiplex PCR-based systems for detection of M. *genitalium* along with *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and other urogenital mycoplasmas and ureaplasmas are sold as kits in several European countries by multiple companies using various formats and instrument platforms. PCR assays for M. *hominis* have utilized mainly 16S rRNA as a gene target (89, 90). Since some heterogeneity has been reported in the 16S rRNA gene of M. *hominis* (91), assays that target other genes including *gap*, *fstY*, and *yidC* have been developed (92–94).

PCR assays can be used to detect as well as identify individual *Ureaplasma* species. Gel-based conventional PCR assays targeted sequences of 16S rRNA and 16S rRNA-23S rRNA intergenic spacer regions, the urease gene, and *mba* (95–103), while real-time PCR assays have targeted mainly the urease genes and their subunits or *mba* (84, 104–108). The UAB Diagnostic Mycoplasma Laboratory performs a real-time PCR assay for detection and differentiation of *Ureaplasma* spp. based on *UU063*, which encodes a conserved hypothetical protein that is identical in all 4 *U. parvum* serovars, and a 15,072-bp open reading frame (ORF), *UUR10_0680*, that is conserved (>99.97%) in all 10 *U. urealyticum* serovars (105). This assay detected more positive clinical specimens than a conventional PCR assay based on a urease gene target in intralaboratory method evaluations. Some molecular assays that include detection of *Ureaplasma* spp. are commercially available in various European countries, but not in the United States. Seegene, Inc. (Rockville, MD, USA), markets their products STD6 and STD6B ACE Detection, which simultaneously detect *Trichomonas vaginalis*, M. *hominis*, M. *genitalium*, C. *trachomatis*, N. *gonorr-*

hoeae, and *Ureaplasma* spp. in endocervical/urethral swabs.

Comparison of the PCR technique with culture and/or serology, in the case of *M. pneumoniae*, has yielded varied results that are not always in agreement. Positive PCR results for *M. pneumoniae* in culture-negative persons without evidence of respiratory disease suggests inadequate assay specificity, persistence of the organism after infection, or its existence in asymptomatic carriers. Positive PCR results in serologically negative persons could be due to an inadequate immune response or to the collection of specimens before specific antibody synthesis could occur. Negative PCR results in culture-proven or serologically proven infections raise the possibility of inhibitors or other technical problems with the assay. Use of a second PCR assay with a different gene target can help one to interpret results and resolve such discrepancies. This is particularly important in the setting of a positive PCR assay when culture and/or serology is negative. If mycoplasmacidal antibiotics have been administered, PCR results can be negative, even though serology is positive. There is some evidence suggesting that PCR inhibition occurs more commonly with nasopharyngeal specimens than throat swabs being assayed for *M. pneumoniae* (109). Commercial reagents available for purification of nucleic acid can be helpful in overcoming PCR inhibition.

PCR technology is less valuable for routine diagnostic purposes in the case of the more rapidly growing and relatively easily cultivable organisms, such as *M. hominis* and *Ureaplasma* spp., but this method can be valuable in clinical studies of ureaplasmal infections and can detect a smaller number of organisms. Even though PCR-based techniques permit identification and differentiation of *U. urealyticum* versus *U. parvum*, this is not normally necessary for routine clinical diagnostic purposes.

PCR is a very good tool for identification of an unknown mycoplasma previously obtained by culture. Presently, PCR detection for mycoplasmas is still too complex to be carried out routinely in most hospital-based microbiology laboratories in the United States, although it is becoming the diagnostic method of choice for *M. pneumoniae* in reference laboratories that develop their own assays. Molecular detection of mycoplasmas could be used much more frequently once commercially sold rapid methods become more widely available.

ISOLATION PROCEDURES

Biosafety Considerations

M. pneumoniae, *M. hominis*, and ureaplasmas are considered biosafety level 2 (BSL2) pathogens. Work with these microorganisms and other mycoplasmas of human origin can be undertaken on the laboratory bench and/or in a class 2 biosafety cabinet.

Growth Media and Inoculation

Growth of mycoplasmas pathogenic for humans requires the presence of serum, growth factors such as yeast extract, and a metabolic substrate. No single formulation is ideal for all pertinent species due to different properties, optimum pH, and substrate requirements. SP4 broth and agar (pH 7.5) are the best media overall and can be used for both *M. pneumoniae* and *M. hominis*, provided arginine is added for the latter. Shepard's 10B broth (pH 6.0) can be used for *M. hominis* and *Ureaplasma* spp. with A8 as the corresponding solid medium. Penicillin G or another broad-spectrum beta-lactam should be added to minimize bacterial overgrowth. Addition of a pH indicator, such as phenol red, is important for detection because mycoplasmas usually do not produce turbidity in broth culture owing to their small cell size. The compositions of these media are provided elsewhere (110).

For self-prepared media, quality control is crucial for each of the main components. These controls must consist of the quantitative growth of mycoplasma strain(s) in 2 media that differ only in the component to be tested. New lots or batches of broth are considered satisfactory if the numbers of organisms that grow are within one 10-fold dilution of the reference batch. Agar plates should ideally support growth of at least 90% of the colonies that are supported by the reference media. The sterility of commercially purchased medium components, such as horse serum, must be confirmed prior to their use. If a reference laboratory is to be used for mycoplasma testing, inquiry should be made of whether the medium used is self-prepared or purchased from a manufacturer, and there should also be verification of the type of quality control procedures performed. Quality control test organisms should include type strains and low-passage clinical isolates of the species of interest. When testing ureaplasmas, it is recommended to include at least one serovar representative from each of the two species. Testing inhibitory properties of media against growth of various other organisms likely present in specimens from nonsterile sites may also be worthwhile to prevent loss of mycoplasmas due to overgrowth of contaminating organisms.

Specimens should always be mixed well before inoculating media. Fluids should be centrifuged (600 × *g* for 15 min), and the pellet should be inoculated. Urine can be filtered through a 0.45-μm filter if bacterial contamination is suspected. Furthermore, it is wise to mince, not grind, tissues in broth prior to diluting. Serial dilution of specimens in broth to at least 10^{-3} with subculture of each dilution onto agar is an extremely important step in the cultivation process, since it helps overcome possible interference by antibiotics, antibodies, and other inhibitors, including other bacteria that can be present in clinical specimens. Omission of this critical dilution step can be one reason why some laboratories have difficulty in recovering the organisms. Dilution also helps to overcome the problem of rapid decline in culture viability, which is particularly common with ureaplasmas, and it also provides information about the number of organisms present.

Incubation Conditions and Subcultures

Broths should be incubated at 37°C under atmospheric conditions. Agar plates yield the best growth if they are incubated in an atmosphere of room air supplemented with 5 to 10% CO_2 or in an anaerobic environment of 95% N_2 plus 5% CO_2. A candle jar or anaerobe jar with GasPak catalyst is adequate if dedicated incubators are not available. The relatively rapid growth rates of *M. hominis* and *Ureaplasma* spp. make identification of most positive cultures possible within 2 to 4 days, whereas *M. pneumoniae* usually requires 21 days or more. Several mycoplasmal species can produce similar biochemical reactions, and identification can be accomplished only by specific tests on organisms once isolated. All broths that have changed color should be subcultured into a fresh tube of the corresponding broth (0.1 ml into 0.9 ml) and onto agar (0.02 ml). Subcultures must be performed soon after the color change occurs, particularly if the organism belongs to *Ureaplasma* spp., because the culture can lose viability within a few hours. Subculture also increases the diagnostic yield, since some strains may not grow sufficiently from the original specimen inoculated initially onto solid media. Blind subculture performed periodically during incubation may improve the yield of *M. pneumoniae* and other mycoplasmas, since a color change may not always be evident, even if growth occurs. Cultures should be incubated for at least 7 days before being designated negative for genital mycoplasmas and 6 weeks for *M. pneumoniae*. The growth rate of *M. fermentans* is similar to that of *M. pneumoniae*. However, for *M. fermentans*, *M. genitalium*, and mycoplasmas of human origin other than *M. pneumoniae*, *M. hominis*, or *Ureaplasma* spp., cultivation conditions are not well established. Due to the advent of PCR assays for use in research and reference laboratories, the need to refine culture techniques for these slow-growing and fastidious organisms is less critical, and cultivation methods for them are not discussed here.

Development of Colonies

Broth cultures for *Ureaplasma* spp. should be examined for color change resulting from hydrolysis of urea twice daily for up to 7 days because of the steep death phase of this organism in culture. This is less critical for *Mycoplasma* spp., for which a once-daily inspection of broth cultures is sufficient. Agar plates should be examined, using a stereomicroscope at a magnification of ×20 to ×60, daily for *Ureaplasma* spp., at 1- to 3-day intervals for *M. hominis*, and every 3 to 5 days for *M. pneumoniae* and other slower-growing species. *Ureaplasma* colonies (Fig. 3) can be identified on A8 agar by urease production in the presence of the $CaCl_2$ indicator contained in the medium. The larger *M. hominis* colonies are urease negative and often have the typical fried-egg appearance (Fig. 1). Other species, such as *M. pneumoniae* and *M. genitalium*, produce much smaller spherical colonies, which may or may not demonstrate the fried-egg appearance (Fig. 2). Methylene blue stain applied directly to the agar plate to turn the colonies blue is sometimes useful if there is uncertainty about whether or not mycoplasmal colonies are present. *M. hominis* is the only pathogenic mycoplasma of humans cultivable on bacteriological media such as chocolate agar or blood agar. However, the pinpoint translucent colonies are easily overlooked, and routine bacterial cultures may be discarded sooner than the time needed for *M. hominis* colonies to develop, which may require 4 days or more in some cases. Occurrence of suspicious colonies warrants subculture to appropriate mycoplasma media.

Commercial Media and Culture Kits

In response to the growing desires of many independent or hospital-based clinical laboratories to offer mycoplasmal culturing on-site, numerous companies have developed transport and growth media. A variety of kits for detection, quantitation, identification, and antimicrobial susceptibility testing of *Ureaplasma* spp. and *M. hominis* from urogenital specimens are available in Europe. Two products, Mycoscreen *Plus* and Mycofast US, are now distributed in the United States by Wescor, Inc. (Logan, UT). These kits consist of strips with wells containing specific dried or lyophilized substrates and inhibitors. Specimens are placed in a suspension transport medium that is used to inoculate wells. The detection, identification, and quantitation of organisms are based on the color change of specific wells containing substrates and inhibitors.

Some kits and other commercial products and media sold in various countries have been evaluated by independent investigators (111, 112). Commercial products and kits can be of particular value if the need to detect mycoplasmas arises infrequently in laboratories that do not specialize in mycoplasma detection, but users should be aware of the potential limitations of existing products. If commercially prepared media are to be utilized, it is advisable that laboratories perform internal quality control tests.

IDENTIFICATION

Even though the numerous large-colony mycoplasmal species that can be isolated from humans cannot be identified based on colonial morphology or a particular biochemical profile, the body site of origin and rate of growth, in conjunction with biochemical features, give some clues. Biochemical properties such as glucose, arginine, or urea utilization are determined based on color change in the absence of turbidity in the appropriate broth. Utilization of glucose by a mycoplasma in SP4 broth produces an acidic shift (red to yellow), whereas utilization of arginine produces a red to deeper-red color change in this broth in the presence of the phenol red pH indicator. Urea or arginine utilization in 10B broth causes an alkaline shift from orange to deep red. Thus, a slow-growing glycolytic organism from the respiratory tract that produces spherical colonies on SP4 agar after approximately 5 to 20 days of incubation and exhibits hemolytic activity and hemadsorption with guinea pig erythrocytes is most likely to be *M. pneumoniae*. Oral commensal *Mycoplasma* spp. that can be present alone or simultaneously with *M. pneumoniae* usually result in an alkaline shift (pink to deep red) in SP4 media and produce fried-egg-type colonies on agar. An alkaline color change that occurs after overnight incubation without turbidity in 10B broth containing urea is almost certainly due to *Ureaplasma* spp., whereas a urogenital specimen that produces an alkaline reaction within 24 to 72 hours in broth supplemented with arginine is likely to contain *M. hominis*. Examination of colonial morphology is sufficient to identify *Ureaplasma* spp., and it is important to keep in mind that these organisms often coexist with *M. hominis* in urogenital specimens.

In order to identify a large-colony mycoplasma to the species level, a number of different techniques are available, although they are more appropriately performed by a reference laboratory than by a hospital microbiology laboratory because of their complexity and the lack of commercial availability of the reagents required. PCR is the best overall choice for mycoplasma species identification since it is much simpler to perform than other methods and it does not require immunological reagents that are not readily available. The PCR assay is also less subjective to interpret than some of the older methods such as epi-immunofluorescence. An emerging alternative for bacterial species identification, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), has now been applied by Pereyre and colleagues to the identification and subtyping of human mycoplasmas (113). Following broth culture and protein extraction, MALDI-TOF MS was applied for the identification of 119 human clinical isolates that were previously identified by antigenic or molecular methods. MALDI-TOF MS was shown to accurately identify most of the organisms and to cluster *M. pneumoniae* isolates by their adhesin P1 type.

TYPING SYSTEMS

Several methods for typing mollicutes have been described and used as a means to study epidemiology in the case of *M. pneumoniae* and differential pathogenicity for the 14 serotypes of *Ureaplasma* spp. Results of early studies have been varied and inconsistent due to the inefficient and imprecise methods available, occurrence of multiple cross-reactions, and the fact that many persons can harbor more than one serotype in their urogenital tract in the presence or absence of disease. Development of monoclonal antibodies enabled identification of multiple-band antigens responsible for *Ureaplasma* serotype specificity on the cell surface. PCR-based assays have enabled more-accurate characterization of the two genomic clusters of *Ureaplasma* spp., which led to their designation as two separate species. Pulsed-field gel electrophoresis (PFGE) has been applied to *Ureaplasma* spp. to determine the size of the genome, and this technique can distinguish among most of the 14 *Ureaplasma* serotypes and detect differences within serotypes (114–116).

Restriction fragment length polymorphism, multiple-locus variable-number tandem-repeat analysis (MLVR), Western blotting, two-dimensional gel electrophoresis, and PCR assays have been used to characterize *M. pneumoniae* clinical isolates (117–121). Most evaluations have determined that there are two major genomic groups or subtypes distinguishable by analysis of the P1 adhesin gene, ORF6 gene, P65 gene, and typical DNA restriction fragment pattern. In view of the recent findings of extensive horizontal gene transfer among *Ureaplasma* serotypes and lack of association at the serovar level with any disease condition, serotyping is not recommended for diagnostic purposes (27). Facilities for typing of human mycoplasmas or ureaplasmas for epidemiological purposes are generally unavailable except in specialized research or reference laboratories.

SEROLOGIC TESTS

M. pneumoniae Respiratory Disease

Historically, serology has been the most common laboratory means for diagnosis of *M. pneumoniae* respiratory tract infections. Although culture and PCR are also used to detect the presence of *M. pneumoniae* in respiratory specimens, persistence of the organism for variable lengths of time following acute infection makes it difficult in some cases to assess the significance of a positive culture or PCR assay without additional confirmatory tests such as seroconversion.

M. pneumoniae has both lipid and protein antigens that elicit antibody responses that can be detected after about 1 week of illness, peaking at 3 to 6 weeks, followed by a gradual decline, allowing several different types of serological assays based on different antigens and technologies. Serology is a very useful epidemiologic tool in circumstances where the likelihood of mycoplasmal disease is high, but it is less suited for assessment of individual patients in a timely manner. Its main disadvantage is the need for both acute-phase and convalescent-phase paired sera collected 2 to 3 weeks apart, which are tested simultaneously for IgM and IgG to confirm seroconversion. This is especially important in adults over 40 years of age who may not mount an IgM response, presumably because of reinfection (6). Moreover, IgM antibodies can sometimes persist for several weeks to months, making it risky to base a diagnosis of acute infection on a single assay for IgM (6). Antibody production may also be delayed in some infections or even absent if the patient is immunosuppressed. False-negative tests for IgM can also occur if serum is collected too soon after onset of illness. Since *M. pneumoniae* is a mucosal pathogen, IgA is typically produced early in the course of infection. Measurement of serum IgA may therefore be a better approach for diagnosis of acute infection because of its rapid rise and decline, but very few commercial assays include reagents for its detection. Table 3 provides examples of various serological assays used to detect *M. pneumoniae* infection in the United States.

Complement fixation (CF) was the primary method for serological testing for *M. pneumoniae* in the past. Although CF measures mainly the early IgM response, the test does not differentiate among antibody classes. Cross-reactions with other organisms, most notably *M. genitalium*, are well recognized, and false-positive results due to cross-reactive autoantibodies induced by acute inflammation from other, unrelated causes may occur. In most clinical laboratories, CF has been replaced by alternative techniques.

Immunofluorescent antibody (IFA) assays, direct and indirect hemagglutination using IgM capture, and other particle agglutination antibody assays (PAs) have been developed to detect antibody to *M. pneumoniae* (122–124). IFA assays consist of *M. pneumoniae* antigen affixed to microscope slides and measure IgM and IgG separately. This assay is technically simple to perform, but it is subjective in interpretation, it requires a fluorescent microscope, and the presence of *M. pneumoniae*-specific IgG may interfere with IgM results. Qualitative and semiquantitative PAs using either latex beads or gelatin that detect IgM and IgG simultaneously can be technically easy to perform, but they do not offer any significant advantages over enzyme immunoassays (EIAs). Though commonly used in some countries because of their ease and simplicity of performance, PAs have not been widely used for serological diagnosis of *M. pneumoniae* infections in the United States.

EIAs, developed in the 1970s, have become the most widely used methods for detection of *M. pneumoniae* antibody in the United States. All are classified as having either moderate or high complexity according to the Clinical Laboratory Improvement Amendment (CLIA). They are qualitative or quantitative and may or may not require specialized equipment. EIAs are more sensitive than CF or culture and can be performed with very small volumes of serum. The need for acute- and convalescent-phase sera has limited their utility for prompt point-of-care diagnosis. A membrane-based EIA specific for IgM, the ImmunoCard (Meridian Bioscience), was developed for rapid detection of acute *M. pneumoniae* infection using a single serum specimen. However, in one study this EIA had a sensitivity of only 31.8% when a single serum was analyzed from seropositive children with pneumonia, increasing to 88% when paired sera were analyzed (125). The Remel EIA (Thermo Fisher) is another rapid point-of-care qualitative serologic assay that detects both IgM and IgG simultaneously in an easy-to-read format without the need for instrumentation. This test has shown good sensitivity and specificity compared to other EIAs, IFAs, and CF tests. Several comparison studies have been performed, evaluating each of the EIA kits listed in Table 3 and various others (122–124, 126–131). A comprehensive evaluation of 12 commercial EIAs and PAs was performed in the Netherlands using PCR as a reference standard. The authors found that most assays had problems with sensitivity and specificity, indicating limitations for their use in diagnosis of acute infections, reaffirming the necessity of testing both IgM and IgG in paired sera from adults, and suggested that the PCR assay is a better diagnostic approach (129). In another study (132), sera from a substantial proportion of healthy blood donors had measurable antibody against *M. pneumoniae*, suggesting cross-reactivity of the antigens used in some of the commercial EIAs and the likelihood that their use results in overdiagnosis of mycoplasmal infections. Selection of the best commercial

TABLE 3 Examples of test kits for detection of serum antibodies to M. *pneumoniae* sold in the United States[a]

Product name	Manufacturer	Antibodies measured	Assay format and description	No. of tests/kit	Specimen throughput	Assay time: Start to finish	Assay time: Hands on
M. *pneumoniae* Antibody (MP) Test System	Zeus Scientific Inc., Branchburg, NJ	IgM and IgG separately	Indirect immunofluorescence assay available as a slurry of M. *pneumoniae* antigenic substrate or a CrownTitre of M. *pneumoniae* colonies affixed to microscope slides	100	Each slide contains 10 wells	2.5 h	30 min
Immuno *Card*	Meridian Bioscience, Cincinnati, OH	IgM only	Qualitative, membrane-based, enzyme immunoassay in a single-sample card format consisting of a test port containing M. *pneumoniae* antigen and a control port containing immobilized human IgM	30	1 specimen per card	12 min	10 min
M. *pneumoniae* IgG/IgM Antibody Test System	Thermo Fisher (Remel), Lenexa, KS	IgM and IgG simultaneously	Qualitative, membrane-based, enzyme immunobinding assay consisting of a single sample test containing inactivated M. *pneumoniae* cytoadhesin protein	10 or 40	1 specimen per card	10 min	2–3 min
Mycoplasma IgG and IgM ELISA Test System	Zeus Scientific, Inc., Branchburg, NJ	IgM and IgG separately	Qualitative enzyme immunoassay in multiwell breakaway strips containing partially purified inactivated M. *pneumoniae*	96	Strips of 8 wells	50 min	5–10 min
GenBio ImmunoWELL *Mycoplasma pneumoniae* IgM and IgG	GenBio Diego, CA	IgM and IgG separately	Qualitative enzyme immunoassay in a 96-well microtiter plate format coated with purified glycolipid M. *pneumoniae* antigen	96	Strips of 8 wells	IgG, 2.35 h; IgM, 2.75 h	15–20 min

[a]This table does not include all commercially available test kits for serological diagnosis of M. *pneumoniae* infections sold in the United States. It provides descriptions of tests representing various formats and is limited to those products that have been evaluated in independent studies.

EIA for individual patient diagnosis depends on the age of the patient being tested, the timing of serum collection, the availability of paired sera, the equipment available, and the experience of the laboratory personnel who perform the test. However, maintaining a large variety of different assays within one laboratory is not practical or cost-effective. A combination of IgM or IgA serology and PCR might be an optimum diagnostic approach, but it could be less useful for adults who do not mount an IgM response and would add considerable cost to laboratory testing. Cold agglutinins, detected by agglutination of type O rhesus (Rh)-negative erythrocytes at 4°C, occur in association with M. *pneumoniae* infection, but only in about 50% of cases. Titers of 64 to 128 or a 4-fold or greater rise in titer suggests a recent M. *pneumoniae* infection, but the test is nonspecific. Therefore, detection of cold agglutinins is not recommended for serologic diagnosis of M. *pneumoniae* infection.

Infections Due to Genital Mycoplasmas

Serologic tests for M. *hominis* and *Ureaplasma* spp. using the techniques of microimmunofluorescence, metabolism inhibition, and EIA have been described (133–135). A microimmunofluorescence assay for M. *genitalium* has also been developed (136) and shown to detect antibody responses in men with NGU (137) and women with salpingitis (138). This method is rapid, reproducible, and quite sensitive and specific, there being less cross-reactivity with M. *pneumoniae* than is seen with other methods. A sensitive and specific serological assay for M. *genitalium* using lipid-associated membrane proteins as antigens has also been developed, and this technique has been used in combination with Western immunoblotting to assess the immunoreactivity of women who were regarded as culture positive for M. *genitalium* (86). No serologic tests for genital mycoplasmas have been standardized and made commercially available for diagnostic use in the United States. Therefore, they cannot be recommended for routine diagnostic purposes.

ANTIMICROBIAL SUSCEPTIBILITIES

Methods Used

Several methods of susceptibility testing used for conventional bacteria have been employed for testing mycoplasmas. Agar dilution has been used as a reference method (139). It has the advantages of a relatively stable endpoint over time, the inoculum size does not have a great effect, and it allows detection of mixed cultures readily. However, this technique is not practical for testing small numbers of strains or occasional isolates encountered in diagnostic laboratories. Agar disk diffusion is not useful for testing mycoplasmas, since there has been no correlation between inhibitory zones and MICs, and the relatively slow growth of some of these organisms further limits application of this technology. Studies using the agar gradient diffusion technique (Etest; bioMérieux) for detection of tetracycline and fluoroquinolone susceptibilities in *M. hominis* and *Ureaplasma* spp. yielded results comparable to both broth microdilution and agar dilution, although the procedures for performing the Etest MIC are not standardized (140–142). Staining plates with Dienes' stain helps visualize the ellipse on the agar plate. Etest advantages include the simplicity of agar-based testing, an endpoint that does not shift over time, absence of large inoculum effect, and its easy adaptability for testing single isolates. This technique is adaptable for laboratories not specializing in mycoplasma diagnosis that encounter isolates needing susceptibility tests only on an occasional basis. Etests are commercially available, can be maintained frozen for 3 to 5 years, and work well with commercial SP4 broth and agar. Broth microdilution is the most practical and widely used method to determine MICs. It is economical and allows several antimicrobials to be tested in the same microtiter plate but has some disadvantages in that preparation of antimicrobial dilutions is labor-intensive and the endpoint tends to shift over time (110).

Standardized consensus-based methods, designated quality control reference strains with defined MIC ranges, medium formulations for broth microdilution and agar dilution, as well as MIC breakpoints for selected antimicrobial agents have now been published for human mycoplasmas and ureaplasmas by the Clinical and Laboratory Standards Institute (CLSI) (143). Since standardized methods for MIC determination and quality control are now available, these procedures should be used whenever MIC testing is needed. Bactericidal activity can be tested directly from the wells in broth microdilution MIC assays that have not changed color by removing the mixture of organisms and antibiotic, diluting it to subinhibitory concentrations in fresh medium, and observing for a color change as evidence of growth. This method is described in more detail in another publication (110).

Susceptibility testing kits using the broth microdilution technique, such as Mycoplasma IST2 (bioMérieux, Marcy-l'Etoile, France), SIR Mycoplasma (Bio-Rad, Hercules, CA), MYCOFAST *Evolution2*, *Evolution3*, and *RevolutioN* (ELItech, Puteaux, France), and Myco-Kit-ATB (Eurobio, Courtaboeuf, France), are sold in Europe but not in the United States. Some of these kits combine detection and identification in the same product. They consist of microwells containing dried antimicrobials, generally in one or two concentrations corresponding to the thresholds proposed for conventional bacteria to classify the strains as susceptible, intermediate, or resistant. To date, only the *RevolutioN* kit has adapted the antibiotics tested and their concentrations to the new mycoplasma susceptibility testing guidelines published by the CLSI, which include the designations for susceptibility and resistance.

Susceptibility Profiles and Treatment

A comparison of MICs for several antimicrobial agents is shown in Table 4. Mollicutes are innately resistant to all beta-lactams, sulfonamides, trimethoprim, and rifampin. Resistance to macrolides and lincosamides is variable according

TABLE 4 MIC ranges of various antimicrobials for *M. pneumoniae*, *M. hominis*, *M. fermentans*, *M. genitalium*, and *Ureaplasma* spp.[a]

Antimicrobial	MIC range (μg/ml) for:				
	M. pneumoniae	*M. hominis*	*M. genitalium*	*M. fermentans*	*Ureaplasma* spp.
Tetracycline	0.06–0.25	0.2–2[b]	≤0.01–0.05	0.1–1	0.05–2[b]
Doxycycline	0.02–0.5	0.1–2[b]	≤0.01–0.3	0.05–1	0.02–1[b]
Tigecycline	0.06–0.25	0.125–0.5	ND	ND	1–16
Erythromycin	≤0.004–0.06[c]	32 to >1,000	≤0.01[d]	0.5–64	0.02–4[d]
Clarithromycin	≤0.004–0.125[c]	16 to >256	≤0.01	1–64	≤0.004–2[d]
Azithromycin	≤0.004–0.01[c]	4–64	≤0.01	≤0.003–0.05	0.5–4[d]
Clindamycin	≤0.008–2	≤0.008–2	0.2–1	0.01–0.25	0.2–64
Lincomycin	4–8	0.2–1	1–8	ND	8–256
Telithromycin	≤0.008–0.06[c]	2–32	≤0.015	0.06–0.25	≤0.015–0.25[d]
Solithromycin	<0.000000063–0.5[e]	0.002–0.008	<0.000032	<0.008	0.002–0.063[d]
Chloramphenicol	2	2–25	ND	0.5–10	0.4–8
Gentamicin	4	2–16	ND	0.25 to >500	0.1–13
Ciprofloxacin	0.5–2	0.1–4	2	0.02 to >64	0.1–16
Ofloxacin	0.05–2	0.1–4	1–2	0.02–0.25	0.2–4
Levofloxacin	0.5–1	0.1–2[f]	0.5–1	0.05–1	0.2–2[f]
Moxifloxacin	0.06–0.125	0.06–0.125[f]	0.03–0.06	≤0.015–0.06	0.125–1[f]
Garenoxacin	≤0.008–0.125	≤0.008–0.063[f]	0.06–0.125	≤0.008–0.015	0.016–1[f]
Gemifloxacin	0.05–0.125	0.0025–0.01[f]	0.05–0.125	0.001–0.01	0.03–0.5[f]
Rifampin	>8	>1,000	ND	25 to >50	>1,000
Quinupristin-dalfopristin	0.008–0.06	0.03–8	0.05	0.1–0.5	0.03–0.5
Linezolid	≥64	2–8	ND	ND	>64

[a] Data were compiled from multiple published studies in which different methodologies and often different antimicrobial concentrations were used. ND, no data available.

[b] Tetracycline-susceptible isolates only. Tetracycline MICs for resistant isolates containing *tet*M are 2 to >64 μg/ml.

[c] Macrolide-susceptible isolates only. Erythromycin MICs for resistant isolates containing mutations in domain V of 23S rRNA are ≥32 μg/ml.

[d] Macrolide-susceptible isolates only. Erythromycin MICs for resistant isolates containing 23S rRNA mutations or mutations and/or deletions in L4 ribosomal proteins are usually ≥32 μg/ml.

[e] Data include two macrolide-resistant strains with MICs of 0.5 μg/ml. Corresponding azithromycin MICs were >32 μg/ml.

[f] Fluoroquinolone-susceptible strains only. Levofloxacin MICs for resistant isolates containing *gyr*A and/or *par*C mutations are 4 to 32 μg/ml.

to species, with *M. hominis* being resistant to erythromycin and other 14- and 15-membered macrolides but susceptible to clindamycin. For *Ureaplasma* spp., the reverse is true. Newer macrolides and ketolides have shown *in vitro* activity comparable to or better than that of erythromycin for *M. pneumoniae*.

M. pneumoniae has historically been predictably susceptible to fluoroquinolones, tetracyclines, and macrolides, so that susceptibility testing has not been recommended except for the *in vitro* evaluation of new and previously untested agents. However, studies from Japan, China, Europe, and the United States found that high-level macrolide resistance in *M. pneumoniae* due to mutations in domain V on the 23S rRNA gene is increasing in children and adults with acute respiratory infections (144). This resistance is greatest in China, where the percentage of resistant organisms has exceeded 90%. This high-level resistance can be clinically significant, sometimes necessitating changes to alternative treatments such as fluoroquinolones (145, 146). Molecular methods to detect mutations in rRNA directly in clinical specimens by PCR enable monitoring resistance trends without having to isolate *M. pneumoniae* in culture and can provide rapid diagnostic information (145, 147, 148). Tetracycline resistance has been well documented in both *M. hominis* and *Ureaplasma* spp. since the mid-1980s, mediated by the *tet*M determinant, which codes for a protein that binds to the ribosomes, protecting them from the actions of these drugs. The extent to which tetracycline resistance occurs in *M. hominis* and *Ureaplasma* species varies geographically and according to prior antimicrobial exposure in different populations but may approach 40 to 50% in some locations (11). In recent studies, some isolates of *Ureaplasma* spp. that contain ribosomal mutations conferring high-level macrolide resistance have been found (149, 150). However, such resistance remains uncommon in the United States. *M. genitalium*-positive NGU or women with cervicitis respond better to azithromycin than they do to a tetracycline, possibly because of the lower MICs. However, there has been documentation of clinically significant macrolide-resistant *M. genitalium* due to rRNA mutations (151).

Fluoroquinolones such as levofloxacin and moxifloxacin are usually active against all human mycoplasmal and ureaplasmal species. However, fluoroquinolone-resistant strains of *M. hominis*, *Ureaplasma* spp., and *M. genitalium* with mutations in the DNA gyrase and/or topoisomerase IV genes have been reported (150, 152–156). Other agents such as streptogramins, aminoglycosides, and chloramphenicol can show *in vitro* inhibitory activity against some mollicute species, but these agents are rarely used to treat infections caused by these organisms. Oxazolidinones such as linezolid are inactive *in vitro* against mycoplasmas.

Extragenital infections, often in immunocompromised hosts, can be caused by multidrug-resistant mycoplasmas and ureaplasmas, making guidance of chemotherapy by *in vitro* susceptibility tests important in this clinical setting. Eradication of infection under these circumstances can be extremely difficult, requiring prolonged therapy, even when the organisms are susceptible to the expected agents. This difficulty highlights the facts that mollicutes are inhibited but not killed by most commonly used bacteriostatic antimicrobial agents in concentrations achievable *in vivo* and that a functioning immune system plays an integral part in their eradication. Treatment of mycoplasmal and ureaplasmal infections has been described elsewhere (157).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Tests offered through diagnostic microbiology laboratories should focus on the species known to cause human disease and for which cultivation techniques are best defined. Unusual organisms or those for which cultivation conditions are not established may be detectable by PCR technology offered through a few specialized research or reference laboratories. Such organisms should be sought only after consultation with clinicians and personnel from the reference laboratory. Except for *Ureaplasma* spp., which can be identified by urease production and distinct colonial morphology, until species identification can be confirmed, a preliminary report of "large-colony *Mycoplasma* species" is appropriate.

M. pneumoniae

Detection of *M. pneumoniae* in culture is time-consuming and not overly sensitive. However, isolation of the organism from respiratory tract specimens is clinically significant in most instances and should be correlated with the presence of clinical respiratory disease, since a small proportion of asymptomatic carriers exist. A mycoplasma isolated by culture from the respiratory tract or from a sterile site that is suspected of being *M. pneumoniae* should always have species identification confirmed due to the possibility that one of several commensal species inhabiting the upper respiratory tract could also be present. Detection of *M. pneumoniae* by the PCR assay or other molecular method is becoming more widely available through reference laboratories, but a positive result must still be correlated with clinical events. A 4-fold rise in antibody titer between acute-phase and convalescent-phase sera is considered diagnostic of acute infection. In children, adolescents, and young adults, a single positive IgM result using appropriate immunoglobulin class-specific reagents can be considered diagnostic of acute infection in some, but not necessarily all, cases because of the possibility of prolonged IgM elevation that sometimes occurs. Mild respiratory infections due to *M. pneumoniae* may not merit a costly and time-consuming microbiological workup since empiric treatment is effective in most instances. However, the emergence of clinically significant macrolide resistance may influence choices of empiric antimicrobial agents.

M. hominis

M. hominis can be detected in culture within a few days. It may occasionally be discovered in routine bacteriologic media from appropriate clinical material, but this should not be relied upon. Its isolation in any quantity from normally sterile body fluids or tissues is significantly associated with disease, and complete species identification is necessary. Quantitation of organisms could also be of value. When mycoplasmas are detected in nonsterile sites such as the female lower genital tract in numbers exceeding 10^5 organisms, they are most likely to be associated with BV.

Ureaplasma Species

Ureaplasmas can be detected in culture within 24 to 48 hours. The characteristic colony morphology and urease production are sufficient for identification at the genus level. Isolation in any quantity from normally sterile body fluids or tissues is significantly associated with disease and can result in a serious infection in neonates or other immunosuppressed persons. Detection of fewer than 10^4 organisms in the male urethra is unlikely to be significant. Distinguishing between the 2 species of *Ureaplasma*, *U. urealyticum* and

U. parvum, by the PCR assay could become more important in view of possible differences in pathogenicity in some circumstances, but currently this is not recommended for routine diagnostic purposes.

M. genitalium

Growing evidence for the role of *M. genitalium* as a urogenital pathogen has generated interest in the development of diagnostic methods for its detection, though no molecular assays for direct detection or serology test kits have been sold commercially in the United States thus far. Even though cultivation techniques for *M. genitalium* have been described (4, 86, 158), relatively few clinical isolates have actually been attained since the initial description of this mycoplasma in the early 1980s. The slow growth, requiring 6 weeks or longer even in enriched SP4 media, and poor sensitivity make culture impractical. The potential importance of this organism in sexually transmitted urogenital infections underscores the need for improved and standardized methods for its detection. At present, noncommercial, nonstandardized PCR-based assays are available in the United States through reference laboratories. Results of these assays should be used with caution if they are adapted and employed for diagnostic purposes. When *M. genitalium* is detected in clinical specimens from the urogenital tract such as the male urethra or female cervix in persons with clinical evidence of urethritis or cervicitis, it should be considered medically significant.

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*Chlamydiaceae**

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63

TAXONOMY

Members of the *Chlamydiaceae* are nonmotile, obligate intracellular prokaryotic bacteria characterized by a unique biphasic developmental cycle bearing two chlamydial forms that differ essentially in terms of morphology and function. According to the *Approved List of Bacterial Names*, published in 1980, the *Chlamydiaceae* contained one genus with just two species, *Chlamydia trachomatis* and *Chlamydia psittaci*, which were separated by their capability to accumulate glycogen in inclusions (Fig. 1A) and their susceptibility to sulfadiazine. In the 1990s, the application of DNA-based classification methods contributed to the recognition of the emerging human pathogen *Chlamydia pneumoniae* (1) and of *Chlamydia pecorum* (2), a pathogen of ruminants, as new species of the *Chlamydiaceae*. Phylogenetic analyses of the 16S and 23S rRNA genes were the rationale for the proposal of an emended description of the order *Chlamydiales* and a revised taxonomy of the family *Chlamydiaceae* in 1999 (3). According to this proposal, members of the order *Chlamydiales* are obligately intracellular bacteria that have the unique chlamydialike developmental cycle and more than 80% sequence identity with chlamydial 16S rRNA genes and/or 23S rRNA genes. The emended order now includes four families: *Chlamydiaceae*, *Parachlamydiaceae*, *Simkaniaceae* (4), and *Waddliaceae* (5).

Early divergence of *C. trachomatis*-like strains in the *Chlamydiaceae* was postulated on the basis of sequence data from the rRNA genes and supported by other data such as genome size, glycogen production, and *ompA* sequence analysis. This led to the proposal to divide the family *Chlamydiaceae* into two genera, *Chlamydia* and *Chlamydophila* (3). As a consequence, *Chlamydia pneumoniae*, *Chlamydia psittaci*, and *Chlamydia pecorum* were proposed to be placed into the new genus *Chlamydophila*. However, the newly proposed nomenclature was controversial. The division into two genera was especially objected to by experts in the field, who argued that the new genus designations ignore the unique, highly conserved biology shared by these organisms that was recognized when they were in a single genus (6). Although the new taxonomy was validly published, the scientific chlamydia community has consistently rejected the use of the term *Chlamydophila*, and its use has been abandoned (7).

DESCRIPTION OF THE FAMILY

The *Chlamydiaceae* contain the known human pathogens *C. trachomatis*, *C. pneumoniae*, and *C. psittaci* as well as organisms such as *C. abortus* and *C. felis* that have been rarely associated with human infections. Members of the *Chlamydiaceae* show less than 10% overall 16S rRNA gene diversity and less than 10% overall 23S rRNA gene diversity. The genome sizes of the *Chlamydiaceae* range from 1.0 to 1.24 Mbp with a G+C content of about 40%.

The cell wall harbors a common lipopolysaccharide (LPS) that differs from LPS of other bacteria in its relatively low endotoxic activity. Accounting for about 60% of the protein mass, the 40-kDa chlamydial major outer membrane protein (MOMP), encoded by the *ompA* gene, is an important structural component of the organisms' outer membrane. The variable domains 1 through 4 (VD1 through 4) lead to multiple *C. trachomatis* serovars associated with different clinical manifestations of oculogenital infections. In contrast, *C. pneumoniae* isolates possess a strikingly high MOMP homology and serovars of *C. pneumoniae* have not been described.

Members of the *Chlamydiaceae* share a unique biphasic developmental cycle leading to important consequences in laboratory diagnosis, clinical course, and antibiotic therapy. The elementary body (EB) of chlamydiae infects eukaryotic host cells and can survive for only a limited period of time outside the host cell. Once inside the host cell, EBs differentiate to metabolically active reticulate bodies (RB) that multiply by binary fission (Fig. 1D) within vacuoles that are continuously growing and that develop into large intracytoplasmic inclusions (Fig. 1C). Reticulate bodies reorganize back to EBs at the end of the chlamydial developmental cycle (Fig. 1D). After 48 to 72 h, hundreds of EBs are released from the host cell, using two mutually exclusive pathways to perpetuate the infectious cycle (8). Genomic transcriptional analysis of the chlamydial developmental cycle reveals a small subset of genes that control the differentiation stages of the cycle and have evolutionary origins in eukaryotic lineages (9).

There is accumulating evidence that factors including gamma interferon, antibiotics, and nutrient deprivation may drive chlamydiae into a state of persistence. Persistent chlamydial forms are morphologically characterized by aberrantly enlarged RBs located within small intracellular inclusions that are arrested in a viable but noninfectious state. It was proposed that persistence is an alternative life cycle

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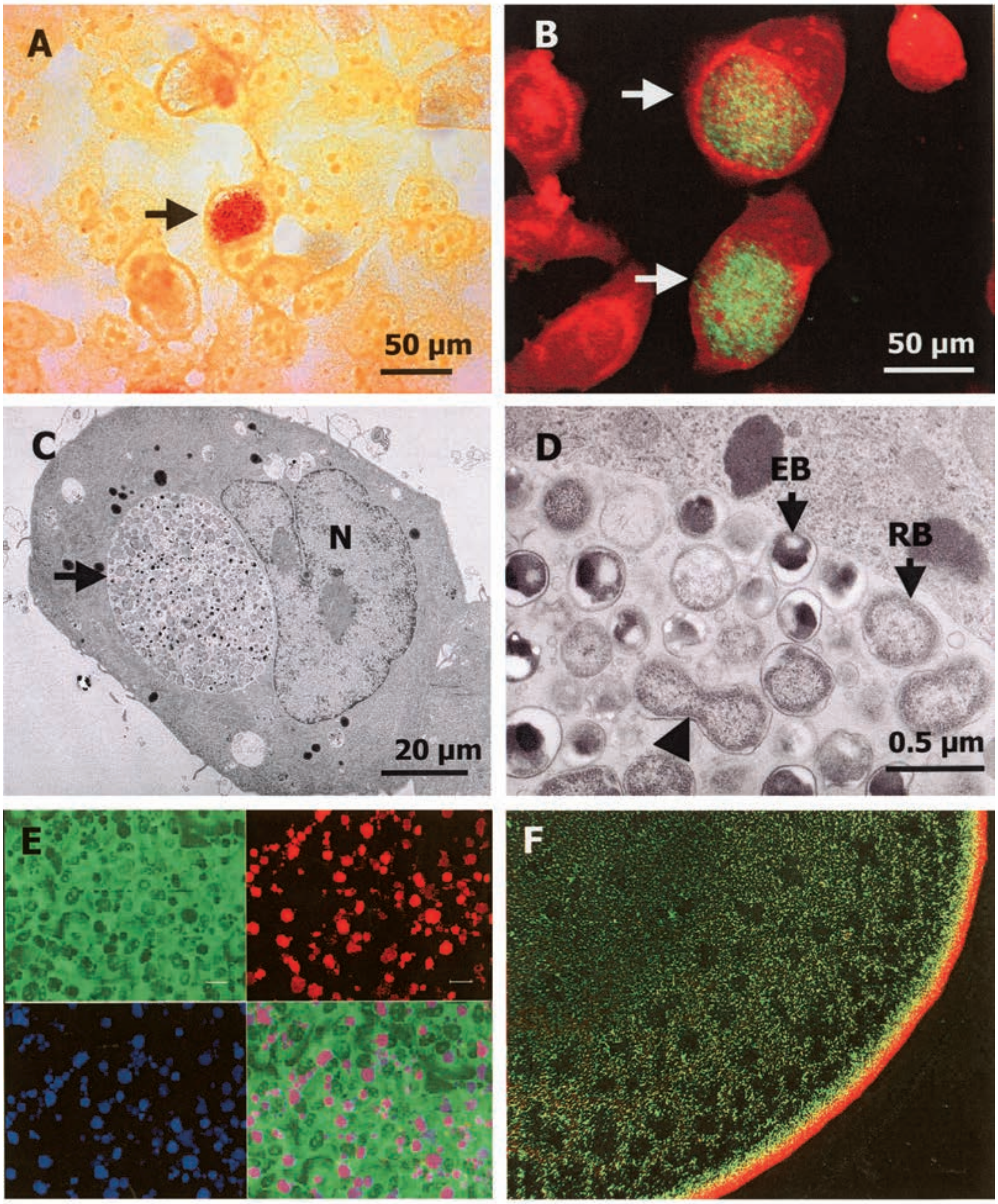

FIGURE 1 Identification of *C. trachomatis* by staining intracytoplasmic inclusions with iodine (A) and FITC-conjugated monoclonal antibody directed at the MOMP (B). Transmission electron microscopy of a *C. pneumoniae*-infected cell shows an intracytoplasmic inclusion impressing the cell nucleus (C) and filled with EBs and RBs (D) at 60 h postinfection; the arrowhead shows a dividing RB. (E) Identification of culture-grown *C. pneumoniae* by fluorescence *in situ* hybridization using rRNA-targeted oligonucleotide probes. Simultaneous use of a Cy5-labeled probe that targets a chlamydial 16S rRNA sequence common to all members of the *Chlamydiaceae* (blue) and a Cy3-labeled probe specific for *C. pneumoniae* (red). Due to the overlap of colors, *C. pneumoniae* appears purple in the composite image; host cells are counterstained by a 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS)-labeled eukaryotic probe (green). (F) IgG-MIF image (magnification, ×400) showing bright homogeneous fluorescence of *C. pneumoniae* EBs at a serum dilution of 1:512. (Photographs courtesy of Sonja Maier, Sven Poppert, and Ulrike Simnacher, Department of Medical Microbiology and Hygiene, University of Ulm; and Matthias Horn, Division of Microbial Ecology, University of Vienna.) doi:10.1128/9781555817381.ch63.f1

used by chlamydiae to avoid the host immune response. As a consequence, chronic infections have been attributed to chlamydial persistence (10). However, the clinical significance of chlamydial persistence is still a matter of debate because diagnostic tools to detect persistence in the human host are lacking.

The chlamydiae can elicit the induction of host cell apoptosis under some circumstances and actively inhibit apoptosis under others (11). This illustrates an important strategy that chlamydiae have evolved to promote their survival through the modulation of programmed cell death pathways in infected host cells.

Sequence information is available for C. *trachomatis* including lymphogranuloma venereum (LGV) isolates (12, 13), C. *psittaci* (14), C. *pneumoniae* (15), C. *caviae* (16), C. *abortus* (17), C. *felis* (18), and C. *muridarum* (19). Comparative analyses of chlamydial genomes have provided a novel understanding of the common biological processes required for infection and survival in mammalian cells and allowed new insights in evolutionary dynamics, host and tissue tropism, and virulence determinants of the chlamydiae (15, 20–23). Genome analysis of a *Chlamydia*-like endosymbiont of *Acanthamoeba* showed that about 700 million years ago the last common ancestor of pathogenic and symbiotic chlamydiae was already adapted to intracellular survival in early eukaryotes and contained many virulence factors found in modern pathogenic chlamydiae, including a type III secretion system (24).

CLINICAL SIGNIFICANCE, EPIDEMIOLOGY, AND TRANSMISSION

C. trachomatis

Based on the antigenic reactivity of the MOMP, C. *trachomatis* is currently divided into 18 serovars. Serovars A, B, Ba, and C can be isolated from patients with clinical trachoma in areas of endemicity in poor countries in Africa, the Middle East, Asia, and South America. Acute manifestations of trachoma primarily include a follicular keratoconjunctivitis, while late-stage manifestations include tarsoconjunctival scarring with trichiasis, entropion, and subsequent loss of vision (25). According to estimates of the World Health Organization (WHO), approximately 1.3 million people in the world suffer from preventable blindness due to trachoma. Trachoma is transmitted under poor hygienic conditions between members of the same family or between families with shared facilities via discharges from the eyes of infected patients. Flies feeding from the mucopurulent eye discharges of infected and weakened humans may carry the organisms on their legs from one person to another across relatively long distances. A WHO Global Initiative aims to eliminate blinding trachoma by 2020.

The C. *trachomatis* serovars D through K, including the serovars Da and Ia and the genovariant Ja, are associated with genital tract disease and are among the most common sexually transmitted bacterial organisms in industrialized countries. According to surveillance data of the Centers for Disease Control and Prevention (CDC), these organisms are responsible for an estimated 2 to 3 million new cases every year in the United States, with 1.4 million cases reported in 2012 in the United States (26). In Europe, more than 300,000 cases were reported in 2011, basically by the United Kingdom and the Scandinavian countries. The C. *trachomatis* serovars D through K typically cause nongonococcal urethritis in men and cervicitis in women. Infection of the urethra and the lower genital tract may cause dysuria, whitish or clear urethral or mucopurulent vaginal discharge, and postcoital bleeding. Urethritis and the rarer manifestations proctitis and conjunctivitis are observed in both men and women. The majority of infections are asymptomatic and therefore remain undetected (27). This can result in ascending infections such as epididymitis in men and endometritis, salpingitis, pelvic inflammatory disease, and perihepatitis (Fitz-Hugh–Curtis syndrome) in women. Manifestations of upper genital infection in women are irregular uterine bleeding, pelvic discomfort, or chronic abdominal pain. Salpingitis may lead to tubal scarring and severe reproductive complications such as tubal factor infertility and ectopic pregnancy. Tubal factor infertility attributable to C. *trachomatis* is the most frequent form of infection-induced infertility. C. *trachomatis*-infected pregnant women may transmit the organisms during delivery to the infants, who are therefore at risk to develop neonatal conjunctivitis and/or pneumonia (28, 29). To prevent chlamydial infection in the infant, pregnant women should be routinely screened for *Chlamydia trachomatis* (30).

Sequelae of C. *trachomatis* infection in both men and women can involve HLA-B27-associated reactive arthritis, presenting most frequently as an acute asymmetric oligoarthritis with or without enthesopathic and extramusculoskeletal symptoms (31). Among sexually active people, young age is strongly associated with infection, with the highest prevalence in those aged 25 years or less. Additionally, sex workers, persons with a new sex partner, or persons who have had several sex partners are at increased risk of infection. In one study, high prevalence rates were found among incarcerated females entering juvenile and adult correctional facilities (32). Screening women who are at risk for C. *trachomatis* infection can prevent serious complications such as pelvic inflammatory disease (27). Consequently, female screening programs have been established in some European countries and the United States to identify and treat infections of asymptomatic individuals and their partners. Screening asymptomatic men has been discussed; however, up to now there is insufficient evidence to recommend routine chlamydia screening in men in the general population (33).

The C. *trachomatis* serovars L1, L2, L2a, and L3 cause lymphogranuloma venereum, a systemic sexually transmitted disease that is endemic in parts of Africa, Asia, South America, and the Caribbean but is rare in industrialized countries. However, ongoing reports about outbreaks with the newly identified variant L2b in Europe, Australia, and the United States show that health care providers should be vigilant for LGV especially among men who have sex with men (MSM) (34–37). The primary lesion, a small, painless papule that tends to ulcerate at the site of inoculation, often escapes attention. Proctitis is more common in people who practice receptive anal intercourse, and abundance of white blood cells in anorectal smear specimens may predict LGV in these patients (38). Ulcer formation favors transmission of HIV and other sexually transmitted and bloodborne diseases. The cardinal feature of LGV is the presence of painful inguinal and/or femoral lymphadenopathy. Complications of LGV include development of coalescing fluctuant lymph nodes (buboes) that result in discharging sinuses and fistula formation. If untreated, fibrosis can lead to lymphatic obstruction, causing elephantiasis of the genitalia.

C. pneumoniae

C. *pneumoniae* causes infections of the upper and lower respiratory tract such as sinusitis, pharyngitis, bronchitis, and pneumonia (39). *C. pneumoniae* was identified as the causative agent in 10 to 15% of cases of community-acquired pneumonia in adults (40) as well as in children (41). However, data from studies yielding prevalence rates under 1% for *C. pneumoniae* pose the question whether its role in community-acquired pneumonia is overestimated (42, 43). Severe and life-threatening *C. pneumoniae* infections have been described in patients with acute leukemia and treatment-induced neutropenia (44). Respiratory outbreaks due to *C. pneumoniae* were observed in military barracks, long-term care facilities, and a federal correctional facility (45). Chronic infection with *C. pneumoniae* was reported among patients with chronic obstructive pulmonary disease and could also play a role in the natural history of asthma, including exacerbations. The clinical symptoms of *C. pneumoniae* infection are nonspecific and do not differ significantly from those caused by respiratory viruses and *Mycoplasma pneumoniae*. Persistent cough does not seem to be strongly associated with *C. pneumoniae* (46). Primary infection occurs mainly in school-age children, while reinfection has been observed in adults. Seroprevalence rates from 40 to 70% show that *C. pneumoniae* is a widely spread organism in industrialized as well as developing countries.

The role of *C. pneumoniae* in the etiology of atherosclerosis, a chronic inflammatory disease of the artery vessel wall, has been discussed since 1988, when Saikku and coworkers presented serological evidence of an association of *C. pneumoniae* with coronary heart disease and acute myocardial infarction (47). In subsequent studies, the organisms were identified in atherosclerotic lesions of patients by culture, PCR, immunohistochemistry, and transmission electron microscopy; however, the discrepancies of study results (48), including those of animal studies, and the failure of large-scale treatment studies (49) have raised skepticism about the organism's causative role in atherosclerosis (50). In addition, a heterogeneous spectrum of extrapulmonary diseases has been linked to *C. pneumoniae*, including multiple sclerosis, Alzheimer's disease, and chronic fatigue syndrome; however, a causal relationship between these diseases and *C. pneumoniae* infection has not been substantiated.

C. psittaci

Psittacine birds and a wide range of other avian species can act as natural reservoirs for *C. psittaci*. In addition, a variety of mammalian species may host *C. psittaci* (3, 51). Based on the *ompA* sequence, *Chlamydia psittaci* harbors 9 genotypes (14). Some strains previously designated as belonging to the *C. psittaci* species have been placed into several animal species such as *C. abortus*, *C. muridarum*, *C. suis*, *C. felis*, and *C. caviae*. All birds are susceptible; however, pet birds (parrots, parakeets, macaws, and cockatiels) and poultry (turkeys and ducks) are most frequently involved in *C. psittaci* transmission to humans. Exposure is greatest in poultry breeders and processing workers, as well as in households with pet birds. Infectious forms of the organisms are shed from symptomatic and from apparently healthy birds and may remain viable for several months. *C. psittaci* can be readily transmitted to humans either by direct contact with infected birds or following inhalation of aerosols from nasal discharges and from infectious fecal or feather dust. Transmission from person to person has been suggested but has never been proven. Symptomatic *C. psittaci* infection in humans may present as a severe chronic pneumonia (52), although mild illness and asymptomatic infections in persons exposed to infected birds have also been observed. Typical symptoms include fever, chills, muscular aches and pains, severe headache, hepato- and/or splenomegaly, and gastrointestinal symptoms. Cardiac complications may involve endocarditis and myocarditis. Fatal cases were common in the preantibiotic era. Due to the quarantine of imported birds and improved veterinary-hygienic measures, outbreaks and sporadic cases of psittacosis are rarely observed nowadays. Since 1996, fewer than 50 confirmed cases have been reported in the United States each year.

C. abortus

Chlamydiae associated with ruminant abortion and formerly contained within the *Chlamydia psittaci* taxon were transferred to a new species: *C. abortus* (53). *C. abortus* has been acknowledged as a cause of abortion and fetal loss in sheep and has also been broadly detected in calves. There are a number of reports of pregnant women who have had spontaneous abortions following exposure to animals infected with *C. abortus* (54, 55). The incidence of this animal-acquired infection is not known, but sheep and goats during the birthing season represent a potential risk to pregnant women. Obstetricians should consider this diagnosis along with early antibiotic treatment and cesarean section delivery in the context of the patient's case history.

Environmental Chlamydiae

The host range of chlamydiae was further broadened with the discovery of *Chlamydia*-related endosymbionts in free-living amoebae. The so-called environmental chlamydiae that have been placed in the family *Parachlamydiaceae* share the chlamydial developmental cycle and represent evolutionary early-diverging sisters of the pathogenic chlamydiae (24). Environmental chlamydiae were discussed as potential emerging pathogens (56); however, clinical evidence for their importance in human infection is still pending. *Simkania negevensis*, currently the only member of the *Simkaniaceae*, is a recently discovered *Chlamydia*-like intracellular agent that has been associated with respiratory infections in infants (4). The natural host of *Simkania* is not known; however, the organism was successfully grown in various cell lines as well as in free-living amoebae and was identified in drinking water and in reclaimed wastewater.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

General Comments

Since chlamydiae are obligate intracellular pathogens, the objective of specimen collection should be to include the host cells that harbor the organisms. Outside their host, chlamydiae survive only briefly, and efforts must be undertaken to maintain the organisms' viability for successful culture. Commercial diagnostic nonculture assays do not require the presence of viable chlamydiae in the specimen; nevertheless, the instructions of the manufacturers given in the package insert should be followed for appropriate collection, transport, and storage of specimens. This includes the use of swabs and transport media specified by the manufacturer.

For successful culture of chlamydiae, the time between collection and processing of the specimens in the laboratory should be minimized and specimens should be kept cold (4 to 8°C). Specimens should be forwarded to the laboratory within 24 h in a special chlamydial transport medium such as 2-sucrose phosphate or sucrose phosphate glutamate supplemented with fetal bovine serum (5 to 10%), gentamicin (10 μg/ml), vancomycin (25 to 100 μg/ml), and amphotericin B (2 μg/ml) or nystatin (25 U/ml). Tetracyclines, macrolides, and penicillins cannot be used in the transport medium since they have activity against chlamydiae. If specimens cannot be processed within 24 h, storage at −70°C in transport medium is acceptable. Specimens for culture should not be stored at −20°C or in frost-free freezers. Swab specimens should be collected on swabs with a Dacron tip and an aluminum or plastic shaft. Swab tips made of calcium alginate and swabs with wooden shafts may inhibit the growth of chlamydiae. It is recommended to check new lots of swabs that are used to collect specimens for culture of chlamydiae for possible inhibition of chlamydial growth (57).

C. trachomatis

The type and anatomical site of specimen collection for laboratory diagnosis of *C. trachomatis* infection depend on both the clinical picture and the laboratory test selection, as comprehensively reviewed elsewhere (25, 58, 59). Table 1 gives an overview of the ranges of sensitivity and specificity for common diagnostic tests for *Chlamydia trachomatis* in urogenital specimens.

Traditional sites for specimen collection in *C. trachomatis* genital tract infection have involved the endocervix in females and the urethra in males, especially for symptomatic patients. The newly recommended specimen additions include so-called noninvasive specimens such as vaginal samples for women and first-void urines (FVU; the first 10 to 30 ml of urine) for men, but only when highly sensitive nucleic acid amplification tests (NAATs) are used (59, 60). Urine or vaginal specimens are specifically not recommended by the CDC for testing by culture and nonamplification tests such as enzyme immunoassay (EIA), direct fluorescence antibody assays (DFA), and nucleic acid hybridization (NAH) because of their very low sensitivity (58).

The noninvasive specimens are ideal for screening asymptomatic patients but can also be used for symptomatic patients. Sensitivity and specificity of NAATs for *C. trachomatis* on noninvasively collected specimens are similar to those obtained with samples collected directly from the cervix or urethra (61, 62). FVU specimens should be obtained at least 2 h after the last micturition. Ambient-

TABLE 1 Ranges of sensitivity and specificity for diagnostic tests for *Chlamydia trachomatis* in urogenital specimens[a]

Diagnostic method	Sensitivity (%)	Specificity (%)
Tissue culture	70–85	100
DFA	80–85	>99
EIA	53–77	95
Direct hybridization	65–83	99
PCR (Roche Cobas 4800 CT/NG PCR) (no claim for male urethra)		
Cervical swabs	89.5–93.0[b]	99.7–100[b]
Vaginal swabs	91.9–93.9[c]	99.7–99.8[c]
Female urine	89.1–94.4[b]	99.7–99.8[b]
Male urine	97.3–98.1[b]	99.5–99.5[b]
PCR (Abbott *m*2000 Real time PCR)		
Cervical swabs	80.9–87.7[b]	99.4–99.7[b]
Vaginal swabs	92.5–94.7[c]	98.8–99.0[c]
Female urine	92.6–95.7[b]	99.2–99.5[b]
Male urine	97.3–97.8[b]	99.6–99.7[b]
Male urethral swabs	88.6–93.3[b]	98.3–99.1[b]
PCR (Cepheid GenXpert CT/NG Realtime PCR) (no claim for male urethra)		
Cervical swabs	95.8–100[b]	99.4–99.8[b]
Vaginal swabs	98.0–100[b,d]	99.4–99.5[b,d]
Female urine	96.1–100[b]	99.8–99.8[b]
Male urine	96.1–100[b]	99.9–100[b]
Strand displacement amplification [ProbeTec *Chlamydia trachomatis* Qx (CTQ) Amplified DNA assay on the BD Viper system with XTR technology]		
Cervical swabs	89.7–93.0[b]	98.0–98.6[b]
Vaginal swabs	94.8–98.2[b]	99.0–99.5[b]
Female urine	92.2–94.7[b]	98.9–99.2[b]
Male urine	96.0–96.2[b]	98.3–98.9[b]
Male urethral swabs	88.6–93.9[b]	97.9–98.9[b]
Transcription-mediated amplification (Gen-Probe/Hologic AptimaCombo2)		
Cervical swabs	92.4–98.4[b]	96.7–98.8[b]
Vaginal swabs	96.6–96.7[c]	97.6–97.1[c]
Female urine	93.8–96.8[b]	98.8–99.0[b]
Male urine	97.0	99.1
Male urethral swabs	95.2	98.2

[a] Sensitivities and specificities are adapted from published clinical trial data (72, 73, 76–80) and are published in package inserts.

[b] Asymptomatic and symptomatic patient results.

[c] Clinician-collected and self-collected vaginal swabs.

[d] All patient-collected vaginal swabs.

temperature storage of fresh unprocessed urine should not exceed 24 h to avoid denaturation of chlamydial DNA. Subsequent processing of the urine specimens for NAAT varies depending on the manufacturers' instructions. Since culture and older, less sensitive tests are falling into disuse, the preferred samples recommended for screening asymptomatic women are vaginal swabs, which can be self-collected for some assays. Self-obtained vaginal swabs are gaining in practical use (63) and have been recommended again in new CDC laboratory guidelines as highly accurate and acceptable samples (64). It was shown that for women, self-collected vaginal swabs had a higher mean chlamydial load than did first-void urines (65).

In cases where culture is performed, although rare today, proficient specimen collection including speculum examination in females is required to obtain appropriate samples that contain sufficient columnar or squamocolumnar epithelial cells. Purulent discharges have to be cleaned before a swab is inserted 1 to 2 cm into the cervical os past the squamocolumnar junction, rotated more than two times, and removed without touching the vaginal mucosa. Urethral specimens from males are collected by placing a dry swab 3 to 4 cm into the urethra and rotating prior to removal. Urination prior to specimen collection may reduce test sensitivity by washing out infected columnar epithelial cells.

In women with salpingitis, samples may be collected by needle aspiration of the involved fallopian tube. Endometrial specimens have also yielded chlamydiae. Further appropriate sites include the conjunctiva in chlamydial eye infection (trachoma, inclusion conjunctivitis, and newborn conjunctivitis) and the nasopharynx and deeper respiratory tract of infants in newborn pneumonia. For men who have sex with men, screening of rectal and pharyngeal specimens is recommended since some reports support the utility of commercial NAATs as a screening test for this population (66, 67). In cases of suspected LGV, ulcer swabs, aspirates of bubo fluid, and rectal or urethral swabs should be collected in transport medium. Buboes of LGV may contain only small amounts of a thin milky fluid, and it may be necessary to inject 2 to 5 ml of sterile saline to obtain any fluid by aspiration.

C. pneumoniae

The optimal sites for specimen collection in *C. pneumoniae* infection are poorly defined. Respiratory specimens from which the organisms were cultured include sputum, bronchoalveolar lavage fluids, nasopharyngeal aspirates, throat washings, and throat swabs (tonsil area). Swab specimens should be collected using a Dacron tip and a plastic shaft (57) and placed immediately in transport medium. Specimens need to be kept at 4 to 8°C in chlamydial transport medium, since the organisms are inactivated rapidly at room temperature. Rapid freezing or freezing and thawing of specimens should be avoided (39). Liquid specimens are collected in transport medium at a specimen-to-medium ratio of 1:2 (57). Testing of vascular tissue specimens and blood samples is of questionable value.

C. psittaci

C. psittaci strains seem to be the most stable organisms among the pathogenic chlamydiae. Nevertheless, specimens should be collected in chlamydial transport medium. Appropriate specimens include sputum, bronchoalveolar lavage fluid, pleural fluid, blood, and tissue biopsy specimens from various anatomical sites. Culture is no longer recommended because of the potential for laboratory-acquired infections.

DIRECT EXAMINATION

Nucleic Acid Amplification Tests

C. trachomatis

Due to their high sensitivity and specificity, NAATs are the tests of choice for the diagnosis of genital *C. trachomatis* infections in routine clinical laboratories. NAATs can be used to detect *C. trachomatis* without a pelvic examination or intraurethral swab specimen by testing self- or clinician-collected vaginal swabs or urine, respectively (60, 68). This facilitates the establishment of screening programs in asymptomatic individuals and may enhance the compliance for testing asymptomatic contact persons of infected individuals. NAATs on urine, with confirmation, were shown to be adequate for use as a new forensic standard for diagnosis of CT and NG in children suspected of being sexually abused (69). Increasing experience is available for the use of NAATs in conjunctival, oropharyngeal, and rectal samples (66, 67) and in LGV (70, 71). All commercial NAATs detect LGV serovars as *C. trachomatis*, but none can differentiate them as LGV. Only research PCR assays have this ability. Thus far, no commercial company has an FDA-cleared test for alternative sample types from extragenital sites, but it is possible for laboratories to use these samples for testing by NAATs if they perform a verification study to indicate their performance. If such verification is performed, Clinical Laboratory Improvement Amendments (CLIA) compliance can be demonstrated (58). Commercial NAATs seem to work in cases of newborn conjunctivitis (28), but no company has an FDA claim. For research studies of trachoma patients, NAATs have been recommended and are now being used by trachoma researchers as the "gold standard" (72). However, the available commercial assays may be too expensive and too complex for use in some national trachoma programs (25). In many evaluations, NAATs detected 20 to 30% more positive specimens than could be detected by earlier technologies.

Licensed NAATs for detection of *C. trachomatis* include (in the order of their introduction) the PCR-based Roche Amplicor (Roche Diagnostics, Basel, Switzerland), the Aptima transcription-mediated amplification (Gen-Probe/Hologic, Inc., San Diego, CA), the BD ProbeTec strand displacement amplification (SDA; Becton Dickinson and Company, Diagnostic Systems, Franklin Lakes, NJ), the *m*2000 real-time PCR (Abbott Molecular, Des Plaines, IL), and the GenXpert CT/NG real-time PCR assay (Cepheid, San Jose, CA). The GeneXpert is a modular cartridge-based platform that allows molecular point-of-care testing (POCT) because it can be performed in on-site laboratories and may provide results at the time of patient visits (73). Licensed assays working on fully automated platforms for use in high-volume laboratories include the Roche Cobas Taqman, Cobas c4800, Abbott *m*2000, BD ProbeTec (Viper), and Aptima (Tigris and Panther) (61, 73–80).

Both the PCR and SDA assays amplify nucleotide sequences of the 7.5-kb cryptic plasmid of *C. trachomatis*, which is present in an average copy number of about four plasmids per chromosome in EBs and up to seven plasmids per chromosome in replicating RBs (81). *C. trachomatis* strains that do not harbor the cryptic plasmid have been sporadically isolated from urethral specimens. A new variant of *C. trachomatis* was discovered in Sweden but did not gain substantial clinical and epidemiological relevance outside Sweden. Due to a 377-bp deletion in the target sequence for nucleic acid amplification, this strain had initially escaped detection by some of the licensed NAATs (82, 83), but manufacturers affected by this discovery moved quickly to modify their primers to enable this variant's detection. The transcription-mediated amplification-based assays target specific sequences of the 23S rRNA, which is also present in multiple copies. Each of the five commercially available NAAT systems offers the option for combination testing of *C. trachomatis* and *Neisseria gonorrhoeae* in the same specimen. The transcription-mediated amplification platform is also offered as individual assays for chlamydia infection or gonorrhea.

Considering the multiplicity of target sites for the amplification procedures being used, NAATs should be able to produce a positive signal from less than one EB; however, the actual sensitivity in clinical specimens is lower because of sampling variability due to Poisson distribution and inefficient nucleic acid isolation. Since inhibitor problems of NAATs can be reduced by dilution of specimens, heating, freeze-thaw cycles, or overnight storage at 4°C, the use of internal inhibition controls of the amplification assays (as supplied by the manufacturers of PCR and SDA) is helpful for identification of clinical specimens containing inhibitory factors. Extraction of nucleic acids by target capture and magnetic bead procedures in second-generation NAATs has almost completely eliminated the presence of inhibitors in processed clinical samples. All of these NAATs are highly specific for chlamydiae if problems with cross-contamination, labeling errors, and mistakes in specimen collection can be avoided (Table 1). Confirmatory testing of positive specimens was once recommended by CDC if a low positive predictive value was expected (≤90%) or if a false-positive result would have serious psychosocial or legal consequences. However, supplemental testing is no longer recommended for chlamydia or for diagnosis of *C. trachomatis* or *N. gonorrhoeae* infection in children suspected of being sexually abused (58, 69, 84).

In settings where resources are limited, including developing countries, the concept of pooling to detect *C. trachomatis* by NAATs has proved to be a simple, accurate, and cost-effective procedure compared to individual testing (85, 86). Specimen pools may consist of aliquots from 4 to 10 processed specimens (FVU or genital swab) combined into one amplification tube. Subsequent testing of individual samples is required only if the pooled sample gives a positive result. Following this strategy, considerable savings of reagent costs can be obtained, especially in low-prevalence populations.

C. pneumoniae

A vast number of PCR-based protocols using different formats and target genes have been developed in research laboratories for detection of *C. pneumoniae* in both respiratory and nonrespiratory samples (87). However, the lack of a reliable gold standard for *C. pneumoniae* infection has made it difficult to evaluate the published protocols thoroughly. Broad application of NAATs for diagnosis of *C. pneumoniae* infection has been hampered because many PCR protocols are not reliable or robust enough to provide reproducible results in routine clinical laboratories. Even in specialized laboratories, there seems to be a substantial interlaboratory variation in the performance of *C. pneumoniae* NAATs, and the need for quality control and standardization of these assays has been recognized (57, 87). Subsequently, specific recommendations for standardizing *C. pneumoniae* PCR assays were made, and it was suggested to compare the performance of newly developed PCR protocols with at least one of four recommended assays that target the *PSTI* fragment (88), the *ompA* gene (89), or the 16S rRNA gene (90, 91). However, all of these assays must be considered research tools (57), because commercial FDA-cleared assays are currently not available. Real-time PCR technology provides promising results that warrant further evaluation of this approach for detection of *C. pneumoniae* infection (92–94). A recent review again stated that standardization and validation particularly of PCR assays are urgently needed because the true role of the organism in respiratory infections as well as in extrapulmonary diseases cannot be ascertained at the moment (87).

C. psittaci

NAATs could be helpful for detection of avian *C. psittaci* strains from clinical samples since culture of these organisms is dangerous, requires biosafety level 3 (BSL-3) facilities, and is not recommended. Some PCR-based assays (52, 89, 90), including a DNA microarray assay (95), have been developed in research laboratories for diagnosis of human ornithosis. Due to the rarity of the disease, the performance characteristics of these assays have been poorly evaluated in clinical specimens.

Nucleic Acid Hybridization Tests

Two NAH tests were previously commercially available for detection of *C. trachomatis*: the Gen-Probe PACE 2 test and the Digene Hybrid Capture II (Digene Corp., Gaithersburg, MD). The PACE 2 test is no longer commercially available. The use of NAH tests has been replaced by NAATs.

Antigen Detection Assays

Direct Fluorescent Antibody Tests

The presence of typical intracytoplasmic inclusions in columnar epithelial cells of the conjunctiva, urethra, or cervix of infected patients can be demonstrated when air-dried smears are fixed on a slide with absolute methanol and stained with Giemsa. Cytological testing was particularly useful in diagnosing acute inclusion conjunctivitis of the newborn, but the more-sensitive immunofluorescence procedures have largely replaced this method. DFAs use fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies directed at a *C. trachomatis*-specific epitope of the MOMP (*Chlamydia* CEL, from Cellabs, Brookvale, Australia; Pathfinder, from Bio-Rad Laboratories, Redmond, WA). DFAs are based on detecting EBs in smears, although staining of inclusions can also succeed if intact infected host cells are collected. Checking for the presence of columnar cells allows assessment of the adequacy of the sample. The procedure offers rapid diagnosis, taking only 30 min to perform, making DFA tests useful especially for laboratories that test only a limited number of specimens. However, this method requires an experienced microscopist who can distinguish between fluorescing chlamydial particles and nonspecific fluorescence. The DFA test has approximately 75 to 85% sensitivity and 98 to 99% specificity compared with culture and a lower sensitivity than NAATs (96, 97). DFA tests can be another alternative for testing endocervical swabs from females or urethral swabs from males when a NAAT is not available or not economical. In addition, DFAs have been recommended for use with conjunctival specimens and for testing of individuals with possible rectal and pharyngeal exposure to *C. trachomatis*, if a *C. trachomatis*

MOMP-specific antibody is used. Nontrachomatis chlamydial conjunctivitis should be considered if DFA testing reveals the presence of chlamydial LPS but not *C. trachomatis*-specific MOMP.

Enzyme Immunoassays

EIA-based tests for detection of *C. trachomatis* use either monoclonal or polyclonal antibodies to detect chlamydial LPS, which is more soluble than MOMP. Although they can theoretically detect all chlamydiae, EIAs have not been well evaluated for the diagnosis of infections with *C. pneumoniae* or *C. psittaci*. Using cultures as reference standards, the sensitivities of EIAs applied to endocervical swabs were in a range from 62 to 72% (97). EIAs are never recommended for testing of noninvasively collected specimens such as urine and vaginal swabs. EIAs are now considered substandard and are not recommended for use as a diagnostic platform by the CDC.

Point-of-Care Tests

Rapid or point-of-care tests designed for office- or clinic-based settings that provide test results in less than 30 min for *C. trachomatis* infection in women have been developed. Similar to EIAs, they also use antibodies against chlamydial LPS with the potential to yield false-positive results due to cross-reaction with other Gram-negative bacteria. Current POCTs are not recommended in laboratory settings because sensitivity and specificity are lower, quality controls are less rigorous, and costs are higher than for tests designed for laboratory use. Although some POCTs are FDA cleared, they were compared to culture as the gold standard and now that the new gold standard is NAATs, the package inserts often overstate performance. When compared to PCR, the Clearview POCT demonstrated a sensitivity of 32.8% for vaginal swabs and 49.7% for cervical swabs (98). New POCTs are highly desirable and are being developed. Technologies such as microfluidics and biosensors appear promising but are not yet FDA cleared (99).

ISOLATION

Biosafety Considerations

C. pneumoniae and *C. trachomatis* are BSL-2 organisms, whereas *C. psittaci* is a BSL-3 organism. Transmission of the organisms from patient specimens or infected cell cultures can occur through aerosols, splashes onto the mucous membranes of the eyes, and hand-to-face actions. In recent years, fewer laboratory-acquired infections have been reported, probably due to the common usage of class II biosafety cabinets in laboratories that work with *Chlamydia*-infected cell cultures. Use of a class II biosafety cabinet protects laboratory staff from exposure to aerosols as well as specimens and cell cultures from contamination. Additional means of preventing laboratory-acquired infection include the use of gloves, alcohol-based hand disinfectants, safety centrifuge caps, and face protection, if appropriate. Laboratory infections with *C. trachomatis* usually manifest as follicular conjunctivitis. The LGV strains are more invasive, and severe cases of laboratory-associated pneumonia and lymphadenitis are reported. *C. psittaci* must be considered a potentially dangerous organism, requiring appropriate BSL-3 facilities. Laboratory-acquired *C. pneumoniae* infections might be underestimated, since the mild clinical course may not prompt infected laboratory workers to seek medical attention.

Specimen Processing

Ocular and Genital Tract Specimens

For culture of chlamydiae from ocular and genital tract sites, only swabs that are rapidly forwarded to the laboratory in a special chlamydial transport medium are acceptable (see above). Specimens to be assayed by commercial EIA, DFA, NAH, or NAATs should be processed as directed by the manufacturer.

Bubo Pus

To prepare bubo pus, the aspirate fluid of fluctuant lymph nodes is ground and then suspended in nutrient broth or cell culture medium to at least 20% by weight. Even when the pus is not viscous, dilution is advisable. The material should be tested for bacterial contaminants and inoculated onto monolayer cultures of McCoy or HeLa 229 cells.

Blood

Blood samples from clotted blood tubes have been used in the past for diagnosis of *C. psittaci* endocarditis. The blood clot was ground, and cell culture medium was added to make a 10% solution. However, culture is no longer recommended for *C. psittaci* due to the possibility of laboratory-acquired infections, so this method is reserved for specialized public health and research laboratories.

Sputum, Throat Washings, and Other Secretions from the Respiratory Tract

Sputum and other respiratory samples are suspended in antibiotic-containing transport medium or cell culture medium at a 1:2 to 1:10 ratio of specimen to medium depending on specimen consistency. Specimens are homogenized by adding sterile glass beads to the sample and vigorously vortexing for 1 to 2 min in a tightly stoppered container. Extracts should be centrifuged for 20 to 30 min at 100 × *g* to remove coarse material before the supernatant fluid is inoculated onto cell monolayers. Serial dilutions may be required if the inoculum is toxic to cells.

Fecal Samples

Human rectal swabs for *C. trachomatis* and avian material for *C. psittaci* are suspended in chlamydial transport medium or antibiotic-containing cell culture medium. The suspension is shaken thoroughly and centrifuged at 300 × *g* for 10 min, and the supernatant is removed. It may be further diluted (1:2 and 1:20) with medium before being inoculated into cell culture. Rectal swabs for commercial NAATs are processed in accordance with the corresponding protocol of the manufacturer.

Tissue Samples

Frozen tissue is thawed in a refrigerator at 4°C. The specimen is weighed, minced with sterile scissors or a scalpel, and ground with a mortar and pestle or homogenizer. A volume of cell culture medium required to make a 10 to 20% suspension is added, and the suspension is thoroughly mixed. For tissue specimens, serial dilutions (1:10 to 1:100) are often required for inoculation to prevent toxicity.

Isolation Procedures

Cell culture was considered the gold standard for diagnosis of genital *C. trachomatis* infection because its sensitivity and specificity were thought to be close to 100%. Problems associated with cell culture isolation of chlamydiae, including technical complexity, long turnaround time, and stringent requirements related to collection, transport, and storage of specimens, have driven the development of commercially available nonculture methods, which have found widespread application in many routine laboratories. With the advent of NAAT detection methods, it became clear that the sensitivity of culture was substantially lower than previously thought, most probably due to the presence of nonviable chlamydiae that died during transport and processing. Culture for detection of chlamydiae in clinical specimens is generally now performed only in specialized laboratories. Culture is recommended in treatment failures (when a viable isolate is needed for susceptibility testing) and in cases related to possible sexual assault for medicolegal reasons, although NAATs on urine have been shown to be adequate for children suspected of being sexually abused (69).

Historically, chlamydiae were cultivated in the yolk sac of embryonated eggs. The yolk sac method (for details, see reference 100) is still used for preparing antigens for the microimmunofluorescence (MIF) test. The ability to propagate chlamydiae in the laboratory has greatly increased the understanding of diagnosis and pathogenesis of chlamydial infections (101). For isolation of chlamydiae from clinical specimens, appropriately collected and transported samples are inoculated onto preformed cell monolayers. A number of susceptible permanent cell lines, including McCoy, HeLa 229, HEp-2, HL, BGMK, Vero, and L cells, have been used. Clinical samples are centrifuged onto monolayers to enhance infection. Strains of *C. psittaci* and LGV biovars are capable of serial growth in cell culture without centrifugation. Cultures are incubated for 48 to 72 h in the presence of the host cell protein synthesis inhibitor cycloheximide. McCoy and HeLa 229 cells are most commonly used for *C. trachomatis*. HL and HEp-2 cells seem to be more sensitive for recovery of the fastidious *C. pneumoniae* from clinical specimens. Visualization of cell culture-grown chlamydiae is achieved by immunostaining of inoculated cell monolayers for intracytoplasmic inclusions. A positive culture shows one or more typical intracellular inclusions (Fig. 1B).

Cell culture methods can vary among laboratories. Host cells are plated either onto 12-mm glass coverslips contained in 15-mm-diameter (1-dram [1 dram = 3.697 ml]) disposable glass vials (shell vial method) or in 6-, 12-, or 24-well tissue culture plates. The cells are seeded in concentrations of 1×10^5 to 2×10^5 cells/ml to give a healthy and confluent monolayer after 24 to 48 h of incubation. For optimal results, cell monolayers should be inoculated with patient specimens within 24 h after reaching confluence. Clinical specimens are thoroughly vortexed with glass beads in tightly closed screw-cap vials to facilitate release of chlamydiae before inoculation. The cell culture medium of the cell monolayers to be inoculated is discarded and replaced by a volume of 0.2 to 2 ml of the vortexed specimen. The inoculated specimen is centrifuged onto the cell monolayers at 900 to 3,000 × *g* for 1 h at 22 to 35°C. Cells are incubated at 35°C for 1 to 2 h to allow uptake of chlamydiae before the medium is replaced with chlamydial isolation medium, consisting of the cell culture medium supplemented with fetal calf serum (10%), L-glutamine (2 mM), cycloheximide (1 to 2 μg/ml), gentamicin (10 μg/ml), vancomycin (25 μg/ml), and amphotericin B (2 μg/ml). Cultures are incubated at 35°C in 5% CO_2 for 48 to 72 h. Then, one coverslip per specimen is removed for immunostaining of inoculated monolayers. Both cell detritus and toxic effects of the inoculum may make it difficult to read slides. Dilution of cell-rich material (bubo pus, sputum, tissue samples, and rectal swabs) and blind performance of subpassages can be helpful for microscopic interpretation of slides.

If a blind subpassage or passage of positive material is to be performed, the corresponding cell monolayers of duplicate wells are scraped and disrupted by vortexing with glass beads. Cell debris of harvested material is removed by low-speed centrifugation (300 × *g*) for 10 min, and the supernatant is passed onto preformed cell monolayers as described above. For *C. pneumoniae*, most laboratories agree that at least two passages are needed to maximize the recovery of the organisms from respiratory specimens. Modifications of the standard procedure, including use of serum-free culture medium, pretreatment of cell monolayers with polyethylene glycol or diethylaminoethyl-dextran, and extension of culture times, have not been sufficiently tested to warrant their routine recommendation (57). Laboratories processing large numbers of specimens may use flat-bottom 48- or 96-well microtiter plates onto which cells are plated directly. Processing and incubation are as described above, but microscopy is modified because cells are stained directly in the well, requiring use of inverted microscopes and long working objectives.

Continuous quality control is important for maintaining a sensitive and specific culture system. Because of its technical complexity, there are multiple opportunities to modify factors in the culture system that may impact the isolation efficiency (25, 101). Therefore, positive controls with a known number of inclusion-forming units should be run routinely to check the sensitivity of the culture system. Negative controls with uninfected human cells may help to evaluate episodes of cross-contamination as a result of handling positive patient specimens or positive controls. Routine testing of cell culture systems for *Mycoplasma* contamination has been recommended because *Mycoplasma* contamination may impair the growth of chlamydiae and may decrease the sensitivity of the culture system (57).

IDENTIFICATION

The basic procedure for detection of isolated chlamydiae involves demonstration of intracytoplasmic inclusions by fluorescent-antibody staining that provides both morphological and immunological identification of chlamydiae. Screening of cultures can be performed with a commercially available FITC-conjugated monoclonal anti-LPS antibody (Pathfinder Bio-Rad), which recognizes all chlamydiae known to cause infections in humans. Confirmation of positive genital cultures can be done by the use of a *C. trachomatis* MOMP-specific monoclonal antibody (Fig. 1B). For respiratory cultures, a *C. pneumoniae*-specific monoclonal antibody may additionally be appropriate. Monoclonal antibodies specific for *C. psittaci* are not commercially available. Using DFA procedures, inclusions of *C. trachomatis*-infected cells are visible at 24 h postinfection. Less expensive but also less sensitive methods that were commonly used before the advent of monoclonal antibodies include Giemsa staining (which needs an experienced and well-trained microscopist for interpretation) and iodine staining for identification of glycogen-containing inclusions, which are produced by *C. trachomatis* but not by *C. psittaci* or *C. pneumoniae* (Fig. 1A).

Identification of replicating chlamydiae can also be done by fluorescence *in situ* hybridization using fluorescently labeled oligonucleotide probes complementary to order-, genus-, and species-specific target sites on the chlamydial 16S rRNA molecules (102). The risk of false-positive signals caused by nonspecific binding of the fluorescent dyes to nontarget organisms or structures of the host cells can be minimized by the simultaneous application of multiple probes with hierarchical specificity labeled with different dyes leading to a characteristic hybridization pattern (Fig. 1E).

TYPING SYSTEMS

Serotyping and genotyping procedures are important tools for epidemiological studies. They are of clinical use if medicolegal issues are involved or if lymphogranuloma venereum is suspected. The most convenient method for serotyping of C. *trachomatis* isolates appears to be the microwell typing system (103), in which inclusions in microtiter plates are stained with pools of monoclonal antibodies (available at Washington Research Foundation, Seattle, WA) that recognize serovar- and subspecies-specific epitopes of the MOMP. Genotyping of C. *trachomatis* isolates usually involves either restriction fragment length polymorphism analysis of the MOMP-encoding *ompA* gene or sequence analysis of the variable domains in the *ompA* gene. These variable regions include the peptides responsible for species, serovar, and serogroup specificities. PCR amplification and sequencing of *ompA* using extracted DNA from patient specimens such as urine or genital samples allow direct genotyping from C. *trachomatis*-positive individuals without isolation of the organisms. In addition, new high-resolution genotyping methods applying a multilocus variable number tandem repeat assay (MLVA) or multilocus sequence typing (MLST) have been introduced (104). *ompA*-based procedures (105), including real-time PCR with high-resolution melt analysis (106) and DNA microarray technology (107), are used to identify all known and additional new genotypes of avian C. *psittaci* strains. Different serotypes or genotypes of C. *pneumoniae* have not been described.

SEROLOGIC TESTS

Serological testing may be helpful in the diagnosis of human ornithosis, lymphogranuloma venereum, neonatal pneumonia caused by C. *trachomatis*, and respiratory C. *pneumoniae* infections. Serological testing for diagnosis of uncomplicated genital infections of the urethra and the lower genital tract as well as for C. *trachomatis* screening in asymptomatic individuals is not recommended. C. *trachomatis* antibody testing has been proposed as the first screening test for tubal factor subfertility (108). Antibodies to C. *trachomatis* were independently associated with reduced rates of pregnancy and elevated rates of recurrent pelvic inflammatory disease (PID) (109).

Since a reference standard has not been defined, the diagnostic value of some serological assays for detection of chronic or persistent chlamydial infections is difficult to estimate. General problems of chlamydial serodiagnosis arise from the difficulty in obtaining paired serum samples, the high seroprevalence of C. *pneumoniae* in adult populations, and the lack of standardized species-specific test methods. The most commonly used serological assay formats include the complement fixation (CF) test, the MIF test, and the EIA to detect immunoglobulin M (IgM), IgA, IgG, or total classes of antibodies with either family, species, or serotype specificity. Some of these assays have been commercialized and are being used by clinical laboratories, although their performance characteristics have been evaluated only in a limited number of studies.

CF Test

The complement fixation test is based on antibody reactivity to the chlamydial LPS antigen common to all members of the *Chlamydiaceae*. The CF test may be useful in diagnosing LGV in patients who present compatible clinical symptoms. A titer of ≥256 strongly supports the clinical diagnosis, while a titer of <32 rules it out except in the very early stages of the disease. In addition, the CF test is useful for diagnosis of psittacosis; however, in the absence of a typical patient history (exposure to birds), C. *pneumoniae* infection might be considered in patients with positive test results. However, due to its potential for cross-reactivity and its low sensitivity for reinfection, CF is not recommended for serodiagnosis of C. *pneumoniae* infections (57). The CF test also lacks sensitivity for the diagnosis of trachoma, inclusion conjunctivitis, and uncomplicated genital infections caused by C. *trachomatis*. The CF test is becoming unavailable in many laboratories, which may limit its usefulness in the near future.

MIF Test

The MIF test developed by Wang and Grayston in the early 1970s is still considered the method of choice for serodiagnosis of chlamydial infections. With this procedure, species- and serovar-specific antibody responses in human chlamydial infection can be detected. The MIF test allows quantitative detection of IgM and IgG antibodies that may be helpful in distinguishing recent from past infections.

The MIF is the diagnostic test of choice for C. *trachomatis* pneumonitis in infants because elevated levels of IgM antibodies are regularly associated with disease. A single IgM titer of ≥32 may support the diagnosis of neonatal pneumonia caused by C. *trachomatis*. IgG antibodies are less useful because infants may present with typical symptoms when they still have a high level of maternal IgG. In LGV-infected individuals, an MIF IgG titer of ≥128 strongly supports the clinical diagnosis, although invasive genital infection with C. *trachomatis* serovars D through K, such as pelvic inflammatory disease, salpingitis, or epididymitis, can also give rise to high titers of antichlamydial antibody in serum. The MIF test may be useful in the diagnosis of psittacosis and is the serological testing method of choice for diagnosis of acute C. *pneumoniae* infection. Criteria for acute infection of C. *pneumoniae* generally include paired sera demonstrating at least a 4-fold rise in titer and single serum samples with IgM titers of ≥16 and/or IgG titers of ≥512. However, single IgG titers of ≥512 should be interpreted with caution because elevated IgG titers may persist for several years in the absence of clinically apparent disease (57). IgG titers in the range of 16 to 256 are suggestive of past infection. The usefulness of IgA as a diagnostic marker in acute or chronic C. *pneumoniae* infections has not been substantiated (87).

The MIF assay is performed using purified formalinized EBs of representative strains or serovars of C. *trachomatis*, C. *psittaci*, and C. *pneumoniae* that are dotted in a specific pattern onto glass slides. MIF antigens are commercially available from the Washington Research Foundation. Serial dilutions of patient sera are placed over the fixed antigen dots and incubated, and bound antibody is detected with fluorescein-conjugated anti-IgG or anti-IgM antibody (Fig. 1F). A more detailed description of the MIF procedure has been summarized elsewhere (110). In addition, recommendations for standardizing the MIF assay in terms of antigen preparation, testing, interpretation of results, and quality assurance should be followed (57).

The MIF assay format is technically demanding, time-consuming, and less useful for higher volume testing. In addition, subjective reading of titers may contribute to intra- and interlaboratory variation in MIF assay results (111). For these reasons, a well-trained and experienced laboratory staff is required. A few standardized kits based on the MIF format have been developed and marketed (Focus Diagnostics, Cypress, CA; Labsystems OY, Helsinki, Finland; Savyon Diagnostics Ltd., Ashdod, Israel). Initial studies suggest that their performance characteristics are similar and seem to correspond well to those of the classical MIF method (112). However, at the time of this writing, none of these assays are cleared by the FDA for use in the United States for the diagnosis of *C. pneumoniae* or *C. trachomatis* infections.

Enzyme Immunoassay

To overcome the problems associated with MIF testing, EIAs that offer a more automated workflow and objective endpoints for serodiagnosis of chlamydial infections have been developed. EIAs based on synthetic peptides from the variable domain 4 (VD4) of the *C. trachomatis* MOMP have been marketed for detection of *C. trachomatis*-specific IgG and IgA antibodies (CT-EIA [Labsystems OY]; SeroCT [Savyon Diagnostics Ltd.]; CT pELISA [Medac, Wedel, Germany]). These assays performed as well as the MIF assay in a few studies (113); however, little is known regarding how long specific antibodies may persist in individuals with resolved infections. For this reason, they cannot reliably differentiate current and past infections. They are not useful in *C. trachomatis* infections of the lower genital tract, for which adequate specimens for direct detection of the organisms can be noninvasively obtained. Further studies are needed to clarify if *C. trachomatis* species-specific antibody tests based on recombinant antigens are convenient tools for diagnosis of upper genital tract infections (114) and tubal factor infertility (115).

The major antigenic determinants of *C. pneumoniae* that are broadly immunodominant among infected individuals are elusive. Commercial assays designed for specific diagnosis of *C. pneumoniae* infection are based on either whole elementary bodies (Savyon Diagnostics Ltd.) or (to obtain more specificity) on LPS-extracted EB preparations (Labsystems OY and Medac). Most kits have been compared only to MIF tests (116), but none has been evaluated adequately with sera from culture- or PCR-positive patients. Thus, their diagnostic value for acute *C. pneumoniae* infections remains to be determined (57).

ANTIMICROBIAL SUSCEPTIBILITIES

Evaluation of antimicrobial resistance and potential clinical treatment failure in chlamydial infection is hampered by the lack of standardized antimicrobial susceptibility tests and the fact that *in vitro* resistance does not correlate with the patient's clinical outcome (117). For these reasons, antimicrobial susceptibility testing of *Chlamydia* organisms has little clinical utility and is currently performed only in some research laboratories. Antimicrobial susceptibility testing in chlamydiae requires growing the organisms in epithelial cells cultured in medium containing increasing concentrations of antibiotics. Cells are stained with an FITC-labeled anti-chlamydial antibody, and the lowest concentration of antibiotic that inhibits inclusion formation after 48 h of incubation is reported as the MIC (117, 118). The minimum chlamydicidal concentration has been defined as the lowest concentration of antibiotic producing no viable bacterial progeny as determined after passage from antimicrobial-containing medium to antimicrobial-free medium. However, variation of antimicrobial susceptibility results is common because they depend on many factors including the cell type used, the inoculum size, and the time between infection and the addition of an antimicrobial.

Tetracyclines, macrolides, fluoroquinolones, and rifampin are commonly used for antibiotic treatment of chlamydial infections. A single 1-gram dose of azithromycin has been shown to be as effective for the treatment of uncomplicated genital *C. trachomatis* infections in adults as a standard 7-day course of doxycycline (33, 119). Alternative regimens include a 7-day course of erythromycin, ofloxacin, or levofloxacin. More data and clinical experience are available to support the efficacy and safety of azithromycin in pregnant women (30, 33). Cotreatment or testing for chlamydiae should be considered among gonorrhea-infected patients because of the frequency of coinfection. Systemic treatment with erythromycin has been recommended for ophthalmia neonatorum as well as for infant pneumonia caused by *C. trachomatis*. In the treatment of adult inclusion conjunctivitis, a single azithromycin dose was as effective as a standard 10-day treatment with doxycycline (120). Doxycycline for 21 days is the antibiotic treatment of choice for both bubonic and anogenital LGV (121). Doxycycline, azithromycin, erythromycin, levofloxacin, and newer macrolides such as clarithromycin and roxithromycin have been recommended for treatment of *C. pneumoniae* infection; however, evidence from clinical trials supporting their use is limited.

Chlamydial resistance to recommended antimicrobial agents appears to be rare and confined to only a few clinical isolates of *C. trachomatis* and has not yet been reported for *C. pneumoniae* or *C. psittaci* infections. Nevertheless, concern has been raised about resistance because recurrent or persistent chlamydial infections were observed in women adequately treated for *C. trachomatis* infection and in a few cases of *C. pneumoniae* infections.

In vitro, chlamydial resistance to fluoroquinolones, macrolides, tetracyclines, and rifampin can be induced with large numbers of organisms cultured in the presence of antimicrobials. In an animal model, persistence of *C. pneumoniae* after antimicrobial therapy has been demonstrated. The emergence of *Chlamydia suis* strains isolated from livestock and displaying a chromosomally stable *tet*(C) resistance gene raises concern about the issue of antibiotic use in animal feeds.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Licensed commercially available NAATs enable the reliable detection of uncomplicated genital *C. trachomatis* infection even from noninvasively obtained specimens such as first-void urine and (self-collected or clinician-collected) vaginal swabs. These specimens are also recommended for screening asymptomatic individuals. Reporting of test results for chlamydiae should include the type of test used and a clinical interpretation if possible. Sexual partners of infected patients should be notified, examined, and treated for *C. trachomatis*. Patients and their partners should be instructed to abstain from sexual intercourse until therapy is completed. Due to the presence of nonviable bacteria, nonculture tests for *C. trachomatis*, especially NAATs, may remain positive when performed ≤3 weeks after completion of therapy (122). Repeat *C. trachomatis* testing of recently infected women and men should be performed approximately 3 months after treatment (30) because recurrent chlamydial infections are common in women and men even after treatment. The use of EIAs, DFAs, and NAH-based assays are increasingly discouraged due to their relatively low sensitivity compared to that of NAATs. In cases of repeated treatment failure, isolation should be attempted and specimens should be forwarded to a specialized reference laboratory.

NAATs could also be helpful for diagnosis of *C. pneumoniae* and *C. psittaci* infections. However, commercial FDA-cleared assays are currently not available. Therefore, respiratory specimens of patients with clinical suspicion of ornithosis or *C. pneumoniae* infection should be directed to a specialized laboratory.

Interpretation of serologic results is particularly challenging in chlamydial infections. Serologic testing may be helpful for screening of chlamydial tubal factor infertility and diagnosis of human ornithosis, lymphogranuloma venereum, neonatal pneumonia, and respiratory *C. pneumoniae* infections. A reliable serologic marker for chronic or persistent chlamydial infection is not available. Especially in *C. pneumoniae*, there is poor agreement between the presence of chlamydial antibody and direct markers of current infection, such as culture or PCR (123). Single-point serology for diagnosis of *C. pneumoniae* infection is discouraged, except when specific IgM antibodies are positive. Paired sera should be tested in the same assay on the same day, and seroconversion or a 4-fold rise or fall in titer is diagnostic for a recent infection. Obviously, there is a general lack of reliable and standardized assays for laboratory diagnosis of *C. pneumoniae*, and this basically hampers the current understanding of the organism's true prevalence and role in respiratory infections as well as in extrapulmonary diseases.

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Rickettsia and *Orientia*

DAVID H. WALKER AND DONALD H. BOUYER

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TAXONOMY

The family *Rickettsiaceae* comprises two genera of small, obligately intracellular bacteria that reside freely within the host cell cytosol, namely, *Rickettsia* and *Orientia*. Although the numbers and diversities of pathogenic strains in these genera are similar, the practices of species designation differ remarkably. The second edition of *Bergey's Manual of Systematic Bacteriology* lists 20 validated names of *Rickettsia* species, and others have been proposed (1). Strains of *Orientia tsutsugamushi* have 0.8% divergence of the *rrs* (16S rRNA) gene. Similarly, other obligately intracellular bacteria have 0.5% *rrs* divergence within a species (e.g., *Ehrlichia chaffeensis*, *Coxiella burnetii*, and *Chlamydia trachomatis*). A proposal of criteria for the limits of divergence of *Rickettsia* species based upon the unacceptable criteria of historical designations would allow different species to be as closely related as having a 0.2% divergence for *rrs*, 0.8% for citrate synthase (*glt*A), 1.2% for outer membrane protein A (*omp*A), 0.8% for outer membrane protein B (*ompB*), and 0.7% for *sca4* (2, 3). There are no common or universal concepts that can be utilized to delineate prokaryotic species as there are with eukaryotes. However, among prokaryotes, 1% divergence of the *rrs* gene is considered to indicate a natural separation between species. Thus, the genus *Rickettsia* has a disproportionate number of designated species relative to the genetic divergence of the relevant bacteria.

The genus is divided by phylogenetic clustering into the typhus group (TG) and the spotted fever group (SFG), defined originally by their distinctive lipopolysaccharide antigens, as well as the transitional group and other basal groups that are widely distributed in arthropods (4). The TG consists of only two members, *Rickettsia prowazekii* and *R. typhi*, whereas the SFG contains bacteria that are generally recognized as human pathogens (*R. rickettsii*, *R. conorii*, *R. africae*, *R. sibirica*, *R. japonica*, *R. honei*, *R. parkeri*, *R. massiliae*, *R. monacensis*, *R. slovaca*, *R. aeschlimannii*, and *R. helvetica*), as well as others that have been identified only in arthropods (1, 4–9). The transitional group, a clade between the TG and SFG, contains the pathogens *R. akari*, *R. australis*, and *R. felis* (10). Most *Rickettsia* spp. of undetermined pathogenicity, including *R. montanensis*, *R. bellii*, *R. peacockii*, and *R. rhipicephali*, are much more prevalent in U.S. ticks than is pathogenic *R. rickettsii*. SFG isolates from Israel and the Astrakhan region of Russia, which have wider geographic distributions, are genetic variants of *R. conorii*. There are reports of SFG rickettsioses in southern Asia for which the agents have not been determined (11, 12).

Orientia tsutsugamushi, which was once considered to be the only species in the *Orientia* genus, diverges from *Rickettsia* by approximately 10% in the *rrs* gene and differs greatly in its cell wall structure, containing completely unrelated proteins and lacking lipopolysaccharide (Table 1). Phylogeny based upon *groEL*, encoding a member of the molecular chaperone family, reveals genetic diversity for the genus *Orientia* similar to that for SFG *Rickettsia*, for which it may be that too many species have been named (Fig. 1) (13, 14). *O. tsutsugamushi*, originally classified serologically, was subsequently analyzed genetically (15). There are several genetic variants in each geographic area. Phylogeny reveals nine genetic clusters that are not identical with serotypes. The genotypes do not have strong evidence of geographic differentiation (15). Genetic variants of *O. tsutsugamushi* appear to correspond to particular arthropod hosts in which divergence most likely occurred. However, a recent change in the *Orientia* genus was the addition of a newly validated species, *Orientia chuto* sp. nov., that was isolated from a patient infected in Dubai (16). This new species has 98.5% homology in *rrs* to *O. tsutsugamushi* strains Ikeda, Kato, and Karp, with only 84.1 to 84.6% identity in the 47-kDa protein gene (16, 17). There is also genetic evidence of a potential third member of the genus in Chile, which was detected in an eschar from a man who was suffering a scrub typhus-like illness (18). Analysis of *rrs* showed 97% sequence similarity to isolates of *O. tsutsugamushi* and a similar difference from *O. chuto* sp. nov. The 47-kDa protein gene could not be amplified, indicating that the genetic sequence for the Chilean agent may be substantially divergent from that of *O. tsutsugamushi*.

DESCRIPTION OF THE GENERA

Rickettsia spp. are small (0.3 to 0.5 μm by 1 to 2 μm), obligately intracellular bacteria of the *Alphaproteobacteria* with a Gram-negative cell wall structure that contains lipopolysaccharide, peptidoglycan, a major 135-kDa S-layer protein (OmpB), a 17-kDa lipoprotein, and, for SFG rickettsiae, a surface-exposed protein (OmpA) containing different numbers of nearly identical tandem repeat units (19, 20). *Rickettsia* organisms have small (1.1 to 1.5 Mb), AT-rich genomes resulting from reductive evolution, with a large proportion (19 to 24%) of noncoding sequence. Among SFG and TG rickettsiae, the genomes have remarkable synteny (21). *Rickettsia* organisms lack genes for enzymes for sugar metabolism, lipid biosynthesis, nucleotide synthe-

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TABLE 1 Characteristics of *Rickettsia* and *Orientia tsutsugamushi*[a]

Organism(s)	LPS	OmpA	OmpB	17-kDa lipoprotein	56-kDa protein
SFG	S	+	+	+	0
TG	T	0	+	+	0
R. canadensis	T	+	+	+	0
R. bellii	B	0	+	+	0
O. tsutsugamushi	0	0	0	0	+

[a] LPS, lipopolysaccharide; S, SFG lipopolysaccharide present; T, TG lipopolysaccharide present; B, *R. bellii*-type lipopolysaccharide present; +, present; 0, absent.

sis, and amino acid synthesis and possess genes encoding enzymes for the complete tricarboxylic acid cycle and several copies of ATP/ADP translocase. These facts suggest both independent synthesis of ATP and acquisition of host ATP, as well as rickettsial utilization of host sources for nutrition and building blocks. Rickettsiae adhere to the host cell receptor Ku70 by OmpB, to an integrin receptor by OmpA, and to unknown receptors for SFG rickettsiae by Sca1 and Sca2. Adhesion triggers signaling pathways that lead to recruitment and activation of induced phagocytosis and escape from the phagosome by membranolytic activities of rickettsial phospholipase D and hemolysin C (TlyC) (22–28). *Orientia tsutsugamushi* (0.3 to 0.5 µm by 0.8 to 1.5 µm) has a 2.1-Mb genome with large proportions of mobile genetic elements, identical repeats, and fragmented genes and a low coding capacity (29). This organisms has a major surface protein of 54 to 58 kDa as well as 110-, 80-, 47-, 42-, 35-, 28-, and 25-kDa surface proteins but lacks muramic acid, glucosamine, 2-keto-3-deoctulonic acid, and hydroxy fatty acids, suggesting the absence of lipopolysaccharide and peptidoglycan. Compared with *Rickettsia*, *Orientia* has a more plastic Gram-negative cell wall, with a thicker outer leaflet and thinner inner leaflet of the outer envelope.

EPIDEMIOLOGY AND TRANSMISSION

Rickettsia spp. reside in an arthropod host (tick, mite, louse, flea, or other insect) for at least a part of their life cycle,

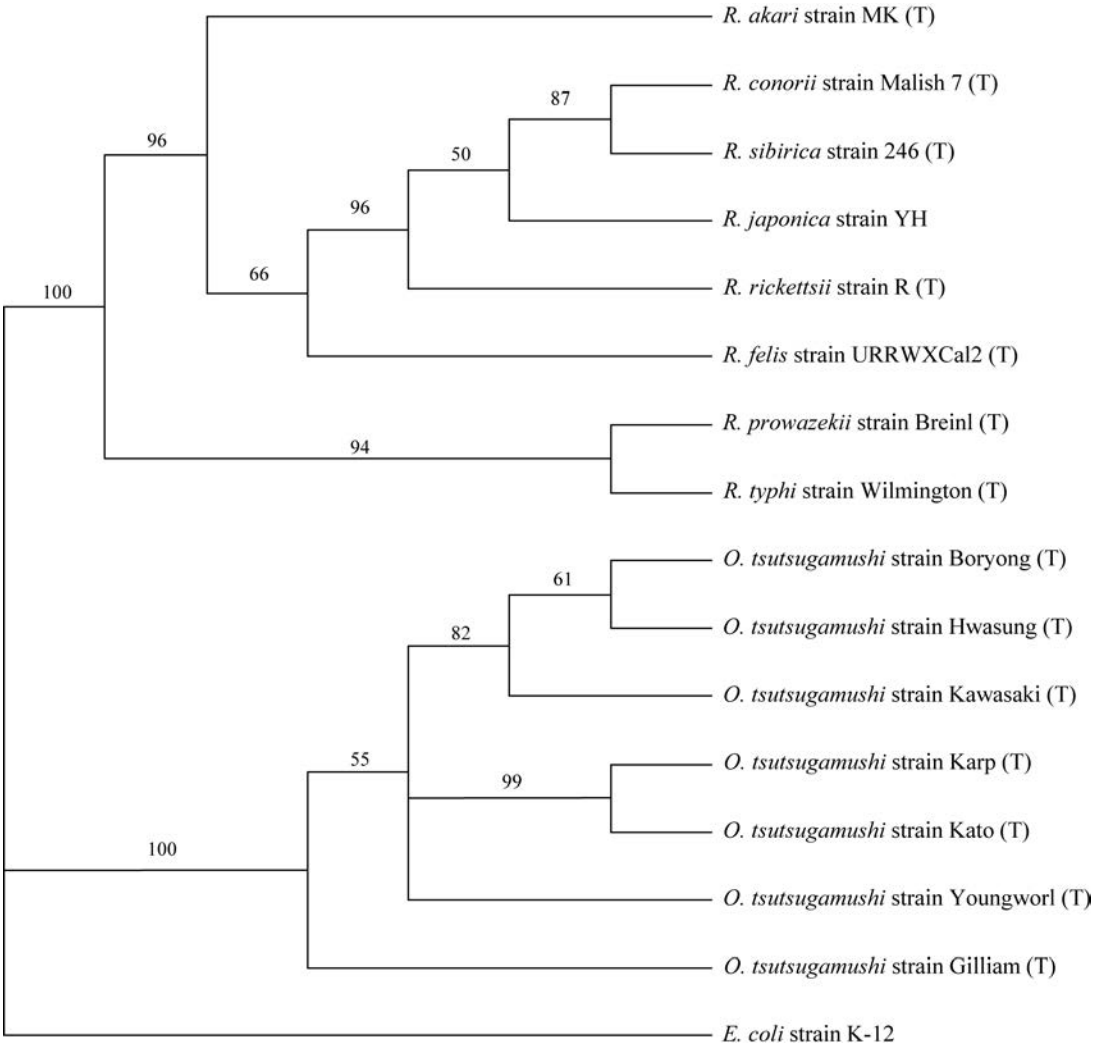
R. akari strain MK (T)
R. conorii strain Malish 7 (T)
R. sibirica strain 246 (T)
R. japonica strain YH
R. rickettsii strain R (T)
R. felis strain URRWXCal2 (T)
R. prowazekii strain Breinl (T)
R. typhi strain Wilmington (T)
O. tsutsugamushi strain Boryong (T)
O. tsutsugamushi strain Hwasung (T)
O. tsutsugamushi strain Kawasaki (T)
O. tsutsugamushi strain Karp (T)
O. tsutsugamushi strain Kato (T)
O. tsutsugamushi strain Youngworl (T)
O. tsutsugamushi strain Gilliam (T)
E. coli strain K-12
87
96
50
96
66
100
94
61
82
55
99
100

FIGURE 1 Phylogeny of *Rickettsia* and *Orientia* as determined by unweighted maximum parsimony analyses of *groEL* gene sequences, with *Escherichia coli* as the outgroup (prepared by PAUP 4.0 software). Numerical values on the branches represent quantities of genetic divergence from the nearest nodes. doi:10.1128/9781555817381.ch64.f1

during which they are maintained by transovarian transmission and/or cycles involving horizontal transmission to vertebrate hosts (30–37) (Table 2). *Orientia tsutsugamushi* resides freely in the cytosol and is maintained in nature by transovarian transmission in trombiculid mites, whose larval stage transmits the infection to humans during feeding (Table 2). Although unconfirmed as vectors of *Orientia* species, in the case of the Chilean agent, leeches have been suggested as a possible vector because the patient recalled being bitten by a leech during a field project (18). There is also the possibility that the patient was bitten by mites that were not detected (18).

CLINICAL SIGNIFICANCE

Rocky Mountain spotted fever (RMSF), rickettsialpox, murine typhus, flying squirrel-associated *R. prowazekii* infection, flea-borne spotted fever, *R. parkeri* infection, and "*Candidatus* Rickettsia philippi" infection are indigenous to the United States. In addition, the potential for imported cases is significant for African tick bite fever, boutonneuse fever, murine typhus, and scrub typhus (Table 2) (38–47). Other rickettsioses, either because of their geographic distribution and the infrequency of travelers' exposure to them or because of their incidence, are unlikely to be imported. RMSF, louse-borne typhus, and scrub typhus are life-threatening illnesses even for young, previously healthy persons. Murine typhus and boutonneuse fever can have a fatal outcome in patients who are elderly or have underlying diseases or other risk factors. A recent study of 140 patients infected with *R. conorii* that were admitted to Portuguese hospitals indicated that alcoholism and infection with the Israeli strain are risk factors for a fatal outcome. Patients with fatal cases more frequently have acute renal failure, hyperbilirubinemia, obtundation, tachypnea, petechial rash, gastrointestinal symptoms, and coagulopathy (48).

An average of 7 days after tick bite inoculation of rickettsiae, patients with RMSF develop fever, severe headache, malaise, and myalgia, frequently accompanied by nausea, vomiting, and abdominal pain, and sometimes cough (40). A rash typically appears only after 3 to 5 days of illness. Rickettsiae infect endothelial cells, frequently leading to increased vascular permeability and focal hemorrhages. In severe cases, noncardiogenic pulmonary edema and rickettsial encephalitis with coma and seizures are grave conditions that often presage death (49, 50).

Rickettsia parkeri causes a milder illness, with tick inoculation site eschar, fever, headache, and myalgia, usually a maculopapular or vesiculopapular rash, occasionally tender regional lymphadenitis, and no reported deaths (51). Rickettsialpox has been recognized mainly as a nonfatal urban disease with disseminated vesicular rash and an eschar at the location of rickettsial inoculation by the feeding mite (41). The complete spectrum of clinical manifestations of *R. felis* infections has yet to be determined. This disease suffers from diagnostic neglect despite its widening recognized geographic distribution and the prevalence of cat flea exposure (44, 52–55).

Murine typhus causes a rash in only slightly more than one-half of patients, cough and chest radiographic infiltrates suggesting pneumonia in many patients, and severe illness in some patients, with seizures, coma, and renal and respiratory failure necessitating intensive care unit admission in 10% of hospitalized patients (39).

Travelers who have returned from Africa and develop fever, one or more eschars, and, in some cases, regional lymphadenopathy and a maculopapular or vesicular rash are very likely infected with *R. africae* (43).

Rickettsia prowazekii, *R. rickettsii*, *R. typhi*, and *R. conorii* are bioterror threats via aerosol exposure to organisms that are infectious at a low dose (56). Only *R. prowazekii* is classified as a select agent, with restrictions on its possession and biosecurity.

Scrub typhus caused by *O. tsutsugamushi* occurs in the geographic area that is bordered by Japan, Korea, and Russia on the north, Australia and Indonesia on the south, Pakistan and Afghanistan on the west, and the Philippines and Micronesia on the east (57). Clinical signs and symptoms of the disease include fever, headache, maculopapular rash, eschar, interstitial pneumonia, temporary deafness, lymphadenopathy, and central nervous system involvement (57–61). In a study of 191 confirmed scrub typhus patients, differences in clinical manifestations were observed between the *O. tsutsugamushi* Boryong and Karp genotypes: weakness (91% versus 61.1%), eschar (97% versus 73.7%), skin rash (94% versus 68.4%), and conjunctival injection (45.1% versus 3%) occurred with increased frequency with the Boryong genotype (62). Without treatment, mortality can reach 30% (58, 61, 63, 64). The index case of *O. chuto* infection was characterized by an eschar, lymphadenopathy, and generalized myalgia followed by fever, lethargy, and rash (16).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Blood should be collected as early as possible in the course of illness. For the isolation of rickettsiae, blood should be obtained in a sterile heparin-containing vial prior to the administration of antimicrobial agents that are active against rickettsiae (40, 65). For isolation and immunocytologic diagnosis, blood can be stored temporarily at 4°C and should be processed as promptly as possible. If inoculation of cell culture or animals must be delayed for more than 24 h, plasma, buffy coat, whole blood, or biopsied tissue should be frozen rapidly and stored at –70°C or in liquid nitrogen. EDTA- or sodium citrate-anticoagulated blood collected in the acute state has been used effectively for the diagnosis of boutonneuse fever, murine typhus, epidemic typhus, Japanese spotted fever, and scrub typhus and, with a lower sensitivity, RMSF and African tick bite fever, by PCR (43, 44, 66–69). PCR provides a higher diagnostic yield when applied to biopsy specimens of rickettsia-infected lesions, particularly eschars (48, 70–73). If whole blood, plasma, buffy coat, or tissue cannot be processed for PCR within several days, it should be stored at –20°C or lower. Serum has a lower sensitivity in PCR but is often diagnostic in fatal cases (74, 75). Overall, PCR has poor sensitivity for diagnosis of rickettsioses owing to the presence of organisms in tissue rather than blood until near death.

For serologic diagnosis, blood is collected as early in the course of disease as possible, a second sample is collected after 1 or 2 weeks, and if a 4-fold rise in antibody titer has not occurred, a third sample is collected 3 or 4 weeks after onset. The serum can be stored for several days at 4°C but should be stored frozen at –20°C or lower for longer periods to avoid degradation of the antibodies. However, blood samples collected by finger stick on appropriate blotting paper in remote areas and sent by ordinary mail can be used for serologic diagnosis (76, 77). Even after transport at ambient temperature, this collection method yields a serologic sensitivity similar to that of testing of fresh serum for immunofluorescence assay (IFA) diagnosis of scrub typhus (77).

TABLE 2 Etiology, epidemiology, and ecology of rickettsial diseases

Organism(s)	Disease	Geographic distribution	Typical mode of transmission to humans	Natural cycle
SFG				
R. rickettsii	RMSF	Western hemisphere	Tick bite	Transovarian in ticks and vertebrate-tick cycles
R. conorii	Boutonneuse fever	Southern Europe, Africa, Middle East	Tick bite	Transovarian in ticks and likely vertebrate-tick cycles
R. africae	African tick bite fever	Sub-Saharan Africa, Caribbean	Tick bite	Transovarian in ticks
R. parkeri	American tick bite fever	North and South America	Tick bite	Transovarian in ticks
R. sibirica	North Asian tick typhus, lymphangitis-associated rickettsiosis	Asia, Europe, Africa	Tick bite	Transovarian in ticks
R. japonica	Japanese spotted fever	Japan, Korea	Tick bite	Ticks
R. heilongjiangensis	Far Eastern tick-borne rickettsiosis	Russia	Tick bite	Ticks
R. honei	Flinders Island spotted fever	Australia, Asia	Tick bite	Transovarian in ticks
R. slovaca	Tick-borne lymphadenopathy	Eurasia	Tick bite	Unknown
TG				
R. prowazekii	Primary louse-borne typhus	Worldwide	Infected louse feces rubbed into broken skin or mucous membranes or inhaled as aerosol	Human-louse cycle, flying squirrel-flea and/or louse cycle
	Brill-Zinsser disease	Worldwide	Recrudescence years after primary attack of louse-borne typhus	
R. typhi	Murine typhus	Worldwide	Infected flea feces rubbed into broken skin or mucous membranes or inhaled as an aerosol	Rat-flea cycle, opossum-flea cycle
Transitional group				
R. akari	Rickettsialpox	USA, Ukraine, Croatia, Korea, Turkey, Mexico	Mite bite	Transovarian in mites and mite-mouse cycle
R. australis	Queensland tick typhus	Australia	Tick bite	Ticks
R. felis	Flea-borne spotted fever	North and South America, Europe, Africa	Not known	Transovarian in cat fleas
Scrub typhus group				
O. tsutsugamushi	Scrub typhus	Japan, eastern Asia, northern Australia, western and southwestern Pacific and Indian Oceans	Chigger bite	Transovarian in mites

A 3-mm-diameter punch biopsy specimen of a skin lesion, preferably a maculopapule containing a petechia or the margin of an eschar, should be collected as soon as possible (78, 79). Although treatment should not be delayed, it is best to perform the biopsy prior to the completion of 24 h of treatment with a tetracycline or chloramphenicol. For immunohistologic detection of rickettsiae, the specimen can be snap-frozen for frozen sectioning or fixed in neutral buffered formalin for the preparation of paraffin-embedded sections (40, 41, 45, 78–81). The former approach yields an answer more rapidly, but freezing artifacts distort the architecture of the tissue, and fixed tissue is more convenient for shipping to a reference laboratory. Aseptically collected autopsy tissues, e.g., spleen and lung, are useful for rickettsial isolation. Ideally, these are inoculated fresh, held for 24 h at 4°C, or stored frozen at –70°C for longer periods if the specimen must be shipped to a public health or reference laboratory. Autopsy tissues can also be examined for rickettsiae by use of immunohistochemistry or PCR.

Body lice (*Pediculus humanus corporis*) removed from the clothing of patients with suspected epidemic typhus can be examined for the presence of rickettsiae. Body lice acquire rickettsiae and remain infected for life, thus providing a useful specimen for PCR diagnosis even after a prolonged period of shipping at ambient temperature and humidity, which do not ensure survival of the lice (82).

DIRECT DETECTION

General Considerations

At this time, to the best of our knowledge, molecular and immunohistochemical diagnostic testing, the most useful methods for establishing a diagnosis during the acute stage of illness, when therapeutic decisions are critical, are available in only a few reference laboratories, including ours. Individual cases for immunohistochemistry can be referred to the following laboratories after contacting the directors for consultation: David H. Walker, Department of Pathology, University of Texas Medical Branch, 301 University Boulevard, Keiller Building, Room 1.104, Galveston, TX 77555-0609 [telephone, (409) 747-3990; fax, (409) 772-1850; e-mail, dwalker@utmb.edu]; Sherif Zaki, Department of Pathology, Centers for Disease Control and Prevention, 1600 Clifton Road, Mail Stop G32, Atlanta, GA 30333 [telephone, (404) 639-3133; e-mail, sxz1@cdc.gov]; and Robert Massung, Rickettsial Zoonosis Branch, Centers for Disease Control and Prevention, 1600 Clifton Road, Mail Stop G-13, Atlanta, GA 30333 [telephone, (404) 639-1082; e-mail, rmassung@cdc.gov].

Immunologic Detection

The diagnoses of RMSF, *R. parkeri* infection, boutonneuse fever, African tick bite fever, murine typhus, louse-borne typhus, and rickettsialpox have been established by immunohistochemical detection of rickettsiae in formalin-fixed, paraffin-embedded sections of biopsy specimens of rash and eschar lesions (40, 45, 49, 72, 79, 81) (Fig. 2, left panel). Monoclonal antibodies that are specific for lipopolysaccharides of either SFG or TG rickettsiae can be used to detect rickettsiae by immunohistochemical staining (Fig. 2, right panel) (45, 80). There is no antibody commercially available. The sensitivity and specificity of immunohistochemical detection of *R. rickettsii* in cutaneous biopsy specimens are 70 and 100%, respectively (40, 79). Eschar biopsies yield sensitive specimens for the diagnosis of SFG rickettsioses that manifest those lesions and should be considered for diagnostic evaluation for patients suspected of having rickettsialpox, *R. parkeri* infection, boutonneuse fever, or Afri-

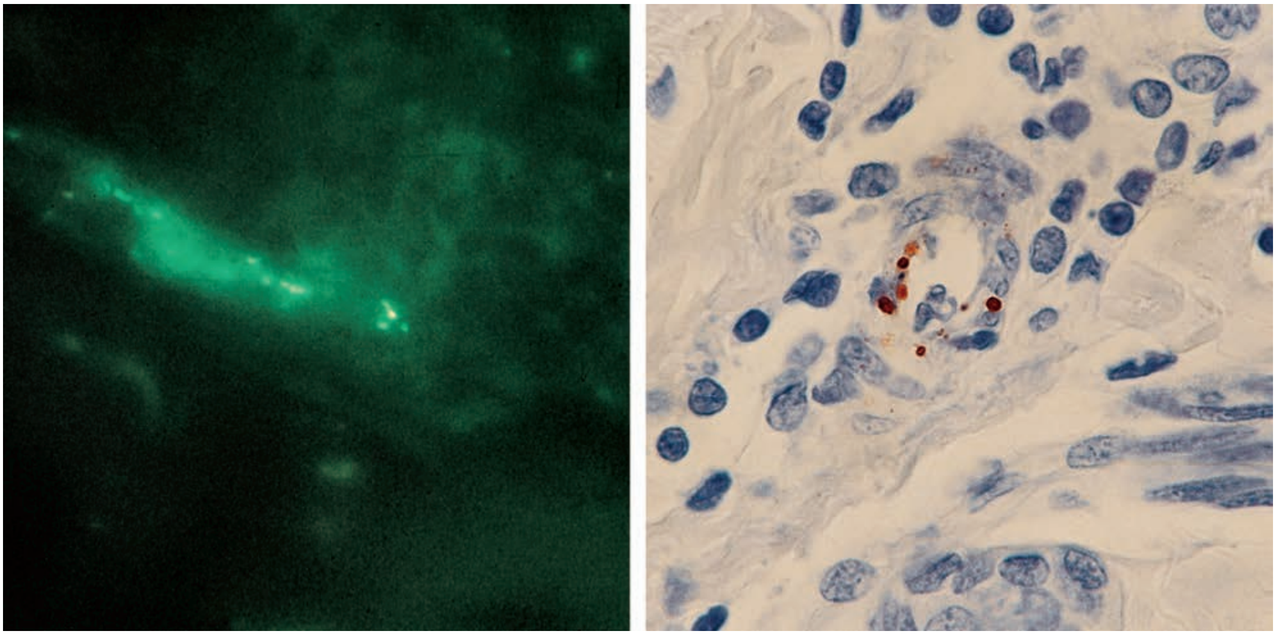

FIGURE 2 (Left) Direct immunofluorescence staining of skin biopsy specimens with anti-SFG *Rickettsia* antibodies facilitates rapid diagnosis. Rickettsiae are present in the vessel wall. (Right) Demonstration of rickettsial organisms in the microvasculature of the dermis in a patient with a history of RMSF. Rickettsiae are seen in the vessel wall. Immunoperoxidase staining was performed using monoclonal antibodies directed against SFG lipopolysaccharide. Skin biopsy magnification, ×800. doi:10.1128/9781555817381.ch64.f2

can tick bite fever. Because histologic processing results in heat denaturation of species-specific antigens, immunohistochemistry distinguishes *Rickettsia* organisms only at the SFG or TG level.

Immunocytochemical detection of *R. conorii* in circulating endothelial cells has been accomplished by capture of endothelial cells from blood samples by use of magnetic beads coated with a monoclonal antibody specific for a human endothelial cell surface antigen followed by immunofluorescence staining of the intracellular rickettsiae (65). This method has a sensitivity of 50% and a specificity of 94%. Rickettsiae are detected in 56% of untreated patients and in 29% of patients receiving antirickettsial treatment. *O. tsutsugamushi* has been identified in human endothelial cells, dendritic cells, macrophages, and cardiac myocytes by use of immunohistochemistry (83). The technique of *in situ* hybridization has been developed but has not been reported for the detection of rickettsiae in clinical samples.

Skin biopsy specimens can also be utilized for the diagnosis of scrub typhus (84). Immunohistochemical staining of maculopapular skin rash samples using a mouse polyclonal anti-*O. tsutsugamushi* Boryong strain antibody has a sensitivity of 65% and a specificity of 100%. Staining of eschar lesions with the same antibody resulted in a sensitivity of 100% and a specificity of 100%. No significant loss of diagnostic effectiveness was noted for patients who had biopsies collected after 3 to 4 days of antibiotic treatment (84).

Molecular Detection

PCR has been applied for the amplification of DNA of *R. rickettsii*, *R. parkeri*, *R. conorii*, *R. japonica*, *R. typhi*, *R. prowazekii*, *R. africae*, *R. sibirica*, *R. felis*, *R. akari*, *R. slovaca*, and *O. tsutsugamushi*, usually from peripheral blood, buffy coat, or plasma but occasionally from fresh, frozen, or paraffin-embedded tissue or arthropod vectors from patients (43, 44, 66, 67, 73, 82, 85–88). Nested PCR applied to skin biopsy specimens, particularly of eschars prior to treatment, has a sensitivity of 78% (89). For all pathogenic *Rickettsia* spp., the 17-kDa lipoprotein gene is a target, amplified by employing the primers 5′-CATTACTTGGTTCTCAATTCGGT-3′ and 5′-GTTTTATTAGTGGTTACGTAACC-3′, which amplify a 231-bp DNA fragment (44). The *gltA*, *rrs*, *groEL*, *ompA*, and *ompB* genes have also been amplified diagnostically, with the *Rickettsia* spp. being identified through either restriction fragment length polymorphism analysis using AluI and XbaI or sequencing of the PCR product. The primer set RP.330(2) Fw (5′-ATGGCTCAAAAACCAAATTTTCTAA-3′) and Rp.330(2) Rv (5′-AGCTCTACCTGCCCATTATCTGTACC-3′), which targets the *ompB* gene, amplifies a 990- to 1,000-bp PCR product (length is species dependent) that, when digested with AluI, discriminates among 11 different rickettsial species (90). The availability of rickettsial genome sequences offers the possible design of an enormous number of primer sets. The approach of using primer sets on a single occasion to reduce the chances of amplicon contamination and false-positive results seems impractical. With batch processing, the delay in laboratory results reduces the clinical value (89). The single use of primers requires that their utility and sensitivity be unknown. The potential for amplicon contamination originating from a positive patient sample remains even if there is no positive control. Recent advances in technology, such as real-time PCR, allow for increased sensitivity in the detection of rickettsiae (91–98). The advantage of real-time PCR is detection of rickettsial organisms during the early or acute stage of disease, before the generation of antibody. The targets for primer design have ranged from housekeeping genes (*gltA*) to antigen genes (*ompA* and *ompB*). The sensitivities for detection vary among primer sets. For example, real-time assays utilizing the primer set RR.190.547F (5′-CCTGCCGATAATTATACAGGTTTA-3′) and RR.190.701R (5′-GTTCCGTTAATGGCAGCAT-3′), generating a product of 154 bp, can detect five copies of rickettsial DNA. The primer set CS-5 and CS-6 detects 1 copy of *R. rickettsii* DNA and 10 copies of *R. bellii* DNA. Perhaps the best potential demonstration of real-time PCR as a diagnostic assay was observed in a recent study that compared real-time PCR evaluation with serology (98). In that study, the primer set PanRick_2_for (5′-ATAGGACAACCGTTTATTT-3′) and PanRick_2_rev (5′-CAAACATCATATGCAGAAA-3′) and the probe PanRick_3_taq (5′-6-carboxyfluoresc n-CCTGATAATTCGTTAGATTTTACCG-TMR-3′), targeting a 70-bp region of the rickettsial *gltA* gene, were utilized for diagnosis of a febrile returned traveler who presented with a macular rash and an eschar on his leg. DNAs were extracted from a small sample of the eschar biopsy and from the leukocyte layer of the EDTA-treated blood samples collected from the patient and analyzed by real-time PCR. The assay was able to detect 1,476 copies of PCR target per ml (1.4 copies per μl) from the initial patient sample. The acute-phase serum of the patient had a positive IFA titer for SFG rickettsial IgM antibodies of 64 and no IgG antibodies. Intracellular bacterial growth was observed in Gimenez-stained Vero cells on day 5 after inoculation. IgG seroconversion was detected using convalescent-phase sera obtained 4 weeks later. This rapid (total processing time is 3 to 4 h) molecular approach is currently being evaluated further for its effectiveness as a timely diagnostic tool (98).

Utilization of group- and species-specific primers in real-time PCR analysis of banked blood and tissue samples by both the CDC and a laboratory in France has shown that real-time PCR analysis is more sensitive than nested PCR assays and can identify the rickettsial agent at the group or species level (99, 100). For patients who have been treated empirically with antibiotics before diagnosis, real-time quantitative PCR is slightly more sensitive (28%) than rickettsial isolation and cultivation (19%) (101).

For *O. tsutsugamushi*, the 56-kDa protein gene is the usual target of diagnostic PCR. The nested 56-kDa protein gene PCR assay is useful for the diagnosis of *O. tsutsugamushi* infections during the acute phase of the disease (67). PCR amplification of DNA from blood samples by using primers p34 (5′-TCAAGCTTATTGCTAGTGCAATGTCTGC-3′) and p55 (5′-AGGGATCCCTGCTGCTGTGCTTGCTGCG-3′), which generate a 1,003-bp product, followed by nested PCR with primers p10 (5′-GATCAAGCTTCCTCAGCCTACTATAATGCC-3′) and p11 (5′-CTAGGGATCCCGACAGATGCACTATTAGGC-3′), amplifies a 483-bp product. This assay detects 10 prototype strains of *O. tsutsugamushi* and does not amplify *R. typhi* or *R. honei*. Real-time PCR assays targeting the *Orientia htrA* gene have been applied to diagnosis with clinical samples (88, 102, 103). The greatest analytical sensitivity level of detection (2 copies per μl of DNA extracted from buffy coat) was observed when *groEL* was the target for analysis in blood collected from 61 Thai patients at the time of clinic admission (94). In this assay, a set of primers (forward primer, 5′-TTGCAACRAATCGTGAAAAG-3′; and reverse primer, 5′-TCTCCGTCTACATCATCAGCA-3′) was used to amplify a 459-bp fragment of the gene. This quantitative PCR method is the preferred molecular method as a diagnostic tool for the acute stage of scrub typhus and is more sensitive than conventional or nested PCR (103). The development of a loop-mediated isothermal PCR assay targeting *O. tsutsugamushi groEL* offers the possibility of a simple method that can be used in locations lacking costly infrastructure (95).

ISOLATION PROCEDURES

Due to high infectivity at a low dose, rickettsial isolation is performed in biosafety level 3 laboratories. Cumbersome historic methods, such as inoculation of adult male guinea pigs, mice, or yolk sacs of embryonated chicken eggs, have been supplanted by cell culture methods, except for isolation of *O. tsutsugamushi*, which is often achieved by intraperitoneal inoculation of mice (40, 65). Vero, L-929, HEL, and MRC5 cells have been used in antibiotic-free media to isolate rickettsiae. The best results reported were achieved with heparin-anticoagulated plasma, buffy coat, or skin lesion biopsy specimens collected prior to administration of antirickettsial therapy.

Samples containing 0.5 ml of triturated clinical material mixed with 0.5 ml of tissue culture medium are inoculated as promptly as possible onto 3.7-ml shell vials with 12-mm-diameter round coverslips having a confluent layer of cells and centrifuged at 700 × *g* for 1 h at room temperature to enhance attachment and entry of rickettsiae into host cells (65, 104). After removal of the inoculum, the shell vials are washed with phosphate-buffered saline and incubated with minimal essential medium containing 10% fetal calf serum in an atmosphere with 5% CO_2 at 34°C. At 48 and 72 h, a coverslip is examined by Giemsa or Gimenez staining or by immunofluorescence with antibodies against SFG and TG rickettsiae. Detection of four or more organisms is interpreted as a positive result. This method yielded a diagnosis for 59% of samples from patients with boutonneuse fever who had neither been treated nor developed antibodies to *R. conorii* prior to collection of the sample (65). Rickettsiae were detected at 48 h of growth in 82% of the positive samples. Universal precautions should be exercised, and work should be performed in a laminar flow biosafety hood with use of gloves, mask, and gown. Although the quantity of rickettsiae in the cell culture is relatively small, avoidance of aerosol, internal, or contact exposure should be taken as for mycobacteria, fungi, and viruses.

IDENTIFICATION OF *RICKETTSIA* AND *ORIENTIA* ISOLATES

Rickettsiae isolated in cell culture can be identified by indirect immunofluorescence with group-, species-, and strain-specific monoclonal antibodies. There is no current commercial source for these reagents. Thus, rickettsial isolates are frequently identified by molecular methods, such as PCR amplification of genes that are genus specific (17-kDa protein, citrate synthase [*gltA*], or *ompB*) or SFG specific (*ompA*) (105). *Orientia tsutsugamushi*, being more distantly related to *Rickettsia* spp., lacks the above cell wall genes but can be identified by PCR amplification of the gene encoding the major immunodominant 56-kDa surface protein, *groEL*, or *rrs* (71, 95).

Identifying the species of rickettsial isolates by microimmunofluorescence serotyping requires intravenous inoculation of mice with large doses of rickettsiae on days 0 and 7 and collection of sera on day 10. The resultant high-titer antibodies react with conformational species-specific epitopes of OmpA and OmpB. Antibodies against group-specific lipopolysaccharide develop later in the murine immune response to high doses of *Rickettsia* spp. This rather cumbersome and expensive method requires propagation of large quantities of the isolate and of the prototype strains for immunofluorescence titration as well as for development of the typing sera. As a result, genetic analysis is currently favored for identification of isolates. An isolate that is known to be a *Rickettsia* spp. should be identified in a biosafety level 3 laboratory, and isolates of *R. prowazekii* must be handled as required by U.S. federal regulations for select agents.

SEROLOGIC TESTS

For most clinical microbiology laboratories, assays for antibodies to rickettsiae are the only tests performed. This situation is unfortunate for patients with life-threatening, acutely incapacitating rickettsial disease because these assays are useful principally for serologic confirmation of the diagnosis in convalescence and usually do not provide information that is helpful in making critical therapeutic decisions during the acute stage of illness. Patients who die of rickettsioses usually have received many antibiotics, none of which have antirickettsial activity, owing in part to the lack of laboratory data providing clinical guidance for a rickettsial diagnosis. The earlier a diagnosis is established, the shorter the course of rickettsial illness will be after an appropriate antirickettsial antibiotic is administered.

Serologic assays for the diagnosis of rickettsial infections focus on the "gold standard," the indirect IFA. Other approaches include indirect immunoperoxidase assay, latex agglutination, enzyme immunoassay (EIA), *Proteus vulgaris* OX-19 and OX-2 and *Proteus mirabilis* OX-K agglutination (Weil-Felix febrile agglutinins), line blotting, Western immunoblotting, and rapid lateral flow assays (39–41, 77, 79, 106–117). Only some of these assays are available as commercial kits or in reference laboratories, and not for all rickettsial diseases. Other serologic tests, such as indirect hemagglutination, microagglutination, and complement fixation, are no longer in general use.

The IFA contains all the rickettsial heat-labile protein antigens and group-shared lipopolysaccharide antigen and thus provides group-reactive serology. IFA reagents for SFG and TG rickettsiae are available commercially from Scimedx Corp., Denville, NJ; Focus Technologies, Cypress, CA; and Fuller Laboratories, Fullerton, CA. They are also available for *O. tsutsugamushi* from Scimedx Corp. and Fuller Laboratories. In cases of RMSF, IFA detects antibodies at titers of ≥64, usually in the second week of illness. Effective antirickettsial treatment of RMSF must be initiated by day 5 of illness to avoid a potentially fatal outcome. For boutonneuse fever, an IFA titer of ≥40 occurs in 46% of patients between days 5 and 9 of illness, in 90% of patients between 20 and 29 days, and in 100% of patients thereafter. For murine typhus, diagnostic IFA titers are present in 50% of cases by the end of the first week of illness and in nearly all cases by 15 days after onset. In areas where particular rickettsial diseases are endemic, a higher diagnostic cutoff titer is required. For example, for the IFA diagnosis of scrub typhus in patients residing in zones of endemicity, an IFA titer to *O. tsutsugamushi* of ≥400 is 96% specific and 48% sensitive, with sensitivity rising from 29% in the first week to 56% in the second week (118). Lowering the diagnostic cutoff titer to 100 raises the sensitivity only to 84% and reduces the specificity to 78%. These considerations are not as important for testing patients who have visited regions of endemicity for only a short period. Each laboratory performing the test should establish its own cutoff titers for the patient population, the microscope and reagents used, and the laboratorian's judgment of the minimal positive signal. Meta-analysis of IFA serology for the diagnosis of scrub typhus led to recommendations that diagnosis be based on a 4-fold or greater rise in titer and that diagnosis not be based on a single antibody titer, unless previous studies had determined a seroprevalence in the local population that justified the cutoff titer (118).

Indirect immunoperoxidase assays for scrub typhus, murine typhus, boutonneuse fever, and presumably other rickettsioses yield results similar to those of IFA when the IgG diagnostic titer is set at 128 and that of IgM is set at 32 (115). Advantages include the use of a light microscope rather than a fluorescence microscope and the production of a permanent slide result. Latex agglutination test reagents are available commercially from Scimedx Corp. only for *R. rickettsii* in the United States. Latex beads coated with an extracted rickettsial protein-carbohydrate complex containing rickettsial lipopolysaccharide are agglutinated mainly by IgM antibodies, with reports of a sensitivity of 71 to 94% and a specificity of 96 to 99% (110). A diagnostic titer of 128 is often detected early in the second week of illness.

EIAs have been developed in various formats, including antigens coating microtiter wells or immobilized on nitrocellulose or other solid phases for use in the commercial reference laboratory setting. Dot-EIA kits from Scimedx Corp. are commercially available in the United States for detecting antibodies against *R. rickettsii*, *R. conorii*, *R. typhi*, and *O. tsutsugamushi* (114, 116). No peer-reviewed publications have evaluated the use of dot-EIA for the diagnosis of RMSF. Compared with an IFA titer of ≥64 for the diagnosis of murine typhus, the dot-EIA showed a sensitivity of 88% and a specificity of 91% (114). The dot-EIA for diagnosis of scrub typhus had sensitivities and specificities of only 80 and 77%, respectively, compared with an IFA cutoff titer of 64 and of 89 and 66%, respectively, at an IFA cutoff titer of 128 (116). These SFG rickettsia and *R. typhi* kits detect cross-reactive antibodies, as demonstrated in an outbreak of African tick bite fever diagnosed by clinical and epidemiologic data (119). The dot-EIA with *R. conorii* antigen provided early diagnostic evidence of an SFG rickettsiosis. Subsequent analysis revealed poor specificity with a high rate of false-positive results. These assays are diagnostic tools that do not require expensive, specialized equipment, but they suffer from an apparently low specificity. Standard EIAs for detecting IgG or IgM antibodies against SFG rickettsiae or *O. tsutsugamushi* are also available from Panbio Diagnostics internationally but are not currently available for purchase in the United States. The utilization of these tests for paired sera from populations with clinical (fever, headache, or rash) and epidemiologic (vector exposure) features consistent with rickettsiosis would most likely yield useful information. A multitest dot-EIA for scrub typhus, murine typhus, and leptospirosis was not useful in Thailand, where nearly all patients had antibodies to more than one agent and others had antibodies against an agent that was not the cause of the illness (120). The diagnosis of scrub typhus by detection of antibodies in clinical samples in an IgM-capture enzyme-linked immunosorbent assay can be utilized for single serum samples from early-stage infections (111). This assay has a sensitivity of 96.3% and a specificity of 99%. A comparison of serologic methods for scrub typhus revealed that an EIA containing recombinant p56 of Karp, Kato, and Gilliam strains was most sensitive (100%), and a rapid lateral flow assay of antibodies against recombinant Karp p56 had a sensitivity of 86% (111). The latter, which is especially useful in situations with limited laboratory facilities and a small number of specimens, is available from Panbio Ltd., Brisbane, Australia.

The assays that were historically most widely used for the diagnosis of rickettsial diseases were the Weil-Felix febrile agglutination tests, using the OX-19 and OX-2 strains of *Proteus vulgaris* for TG and SFG rickettsioses and the OX-K strain of *Proteus mirabilis* for *O. tsutsugamushi* infections. These assays have poor sensitivity and specificity (79, 113), and they should be replaced by more accurate serologic methods, such as IFA or EIA. However, there are situations in developing countries where the choice is between the *Proteus* agglutination tests and none at all for the detection of important public health problems, such as outbreaks of louse-borne typhus (121). In fact, the evidence leading to the discovery of Japanese spotted fever and Flinders Island spotted fever included *Proteus* agglutinating antibodies.

Shared antigens of OmpA, OmpB, and group-specific lipopolysaccharide impede establishment of a species-specific diagnosis by serologic methods. The criterion of a 4-fold or greater difference in IFA titers between the two suspected agents distinguished infections by *R. prowazekii* and *R. typhi* in only 34% of cases and infections by *R. africae* and *R. conorii* in only 26% of cases (43, 122). Western immunoblot detection of antibodies against OmpA or OmpB of only one *Rickettsia* species has also been proposed as a criterion for species-specific diagnosis. However, it was effective in distinguishing *R. prowazekii* and *R. typhi* infections or *R. africae* and *R. conorii* infections in only half of the cases (43, 122, 123). Cumbersome, expensive cross-absorption of sera prior to IFA or Western immunoblotting is more effective in establishing a species-specific diagnosis. However, interpretation of these results requires careful evaluation of the performance of valid controls, the quality and quantity of each antigen preparation, and the potential for the occurrence of infection by an untested or even undiscovered agent. In the past, knowledge of the geographic origin of the case was thought to suffice to designate the specific diagnosis. However, the increasing number and geographic overlap of rickettsioses challenge old assumptions. The report of the reactivity of sera from patients with flea-borne spotted fever but not of those from patients with RMSF, rickettsialpox, or murine typhus with a recombinant fragment of *R. felis* OmpA suggests that species-specific peptide antigens may be identified and incorporated into assays that identify the disease more precisely (124).

ANTIMICROBIAL SUSCEPTIBILITIES

Data supporting the use of doxycycline or another tetracycline antibiotic as the drug of choice for the treatment of infections caused by *Rickettsia* spp. and *O. tsutsugamushi* and the use of chloramphenicol as an alternative drug have been derived principally by empiric experience, retrospective case studies, and a few prospective studies (125–134). In addition to historic studies of the activity of antimicrobial agents against these obligately intracellular bacteria in infected animals and embryonated eggs, studies of the effects of antimicrobial agents in cell culture have supported the consideration of alternative drugs, such as fluoroquinolones, josamycin, azithromycin, and clarithromycin. Indeed, several fluoroquinolones, josamycin, and azithromycin have been used successfully for the treatment of boutonneuse fever under certain circumstances but cannot be recommended for more pathogenic rickettsioses (125, 126, 130, 131). Mediterranean spotted fever has also been treated effectively in clinical trials by use of fluoroquinolones such as ciprofloxacin, azithromycin, and clarithromycin. A retrospective study of patients with murine typhus demonstrated that ciprofloxacin is an effective drug. Evidence for a toxic effect of fluoroquinolones led to concern about its use to treat *R. conorii* infections (135, 136). During pregnancy, chloramphenicol has been used to treat RMSF, and josamycin has been used for boutonneuse fever. Except for cases of scrub typhus in Thailand, which responded poorly to doxycycline or chloramphenicol, but for which azithromycin has been reported to be effective, there is little concern regarding rickettsial development of antimicrobial resistance (129, 134, 137). Thus, antimicrobial susceptibility studies of rickettsiae are not routinely performed clinical laboratory tests. However, it has been reported that fluoroquinolones may exacerbate rickettsial effects (135).

INTERPRETATION AND REPORTING OF RESULTS

In reporting the results of an assay for antibodies in a single serum sample, the laboratorian seldom knows the duration of illness and whether the serum sample is from the acute or convalescent phase of the disease. For sera that are nonreactive by dot-EIA, by IFA at a dilution of 1:64, by indirect immunoperoxidase assay at a dilution of 1:128, by latex agglutination at a dilution of 1:64, or by Weil-Felix *Proteus* agglutination at a titer of 1:160, the laboratory report should state that no antibodies were detected at the particular cutoff dilution. In addition, the report should indicate that this may differ among laboratories and patient populations, that negative results are expected in the acute stage of rickettsial illness, and that a second sample should be submitted to evaluate the possibility of seroconversion if no alternative diagnosis has been established. If paired acute- and convalescent-phase sera separated by an appropriate interval are available, they should be tested simultaneously. It is wise to test for all the rickettsial and ehrlichial agents to which the patient is likely to have been exposed in the United States. SFG rickettsiae, *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, and *R. typhi* are the likely agents, unless travel to an area where scrub typhus is endemic has occurred. If the paired sera contain no antibodies against rickettsiae at the cutoff dilution, the report should state that the results do not support the diagnosis of rickettsial infection but that occasionally antibody synthesis is delayed, particularly in cases with early antirickettsial therapy. If a single serum sample contains an IFA antibody titer of ≥64, an IFA IgM titer of ≥32, an indirect immunoperoxidase antibody titer of ≥128, a latex agglutination titer of ≥64, or a Weil-Felix titer of ≥320, the laboratory report should state (i) that antibodies reactive with the particular rickettsial antigen were detected at the measured titer, (ii) that the result provides supportive evidence for the diagnosis of the rickettsial disease, and (iii) that a convalescent-phase sample should be submitted to assess the possibility of a diagnostic rise in titer. If paired sera measured simultaneously show a 4-fold or greater rise in titer, the interpretation should state that the results strongly support the rickettsial diagnosis indicated by the tested antigen. If a significant titer was detected in the acute-phase sample but no rise or only a single doubling dilution rise was measured, it should be stated that an additional later sample should be tested to evaluate a 4-fold rise or fall in titer. The concept that recrudescent typhus may be distinguished from primary louse-borne typhus by the absence of IgM antibodies to *R. prowazekii* and of *Proteus* OX-19 agglutinating antibodies has been challenged (109). The manufacturers of the dot-EIA have recommended the interpretation that strongly reactive samples (three or four dots) may indicate the presence of a specific antibody response and that weakly reactive samples (one or two dots) are infrequent but possible in normal populations. Retesting 2 to 3 weeks later would establish the diagnosis if three or four dots developed in the convalescent-phase serology and should always be performed.

Isolation of a rickettsia from blood or tissue may be interpreted as indicating an etiologic role. The level of identification of the isolate should be stated, whether it is identified only to the level of a group containing particular organisms or to the species level.

Immunohistologic and immunocytologic diagnostic interpretations should state the method, reactivity of the method (e.g., antibody reactive with SFG rickettsiae), and location of the antigen (e.g., vascular endothelium and, frequently, adjacent vascular smooth muscle for *R. rickettsii*). Detection of three or more rickettsiae in vascular endothelium in biopsy specimens or four or more rickettsiae in captured circulating endothelial cells is diagnostic of rickettsial infection.

Interpretation of PCR results should state the target gene, the organisms that would be detected, and the presence or absence of a DNA product. If a specific oligonucleotide probe or DNA sequence confirmed the specificity of the identification, this result should be stated. For negative immunohistologic, immunocytologic, and PCR results, it should always be stated that the failure to detect the agent does not exclude the diagnosis, along with data regarding the sensitivity and specificity of the assay in the particular laboratory and the effects of antirickettsial treatment on the sensitivity.

Special efforts should be made to establish the diagnosis of fatal cases, including rickettsial isolation, immunohistology, PCR, and serology on samples collected at necropsy.

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Ehrlichia, *Anaplasma*, and Related Intracellular Bacteria

MEGAN E. RELLER AND J. STEPHEN DUMLER

65

TAXONOMY

Members of the genus *Ehrlichia* and *Anaplasma* are now recognized to be important human pathogens. They are obligate intracellular bacteria currently placed in the *Proteobacteria* phylum, section alpha, order *Rickettsiales*, and family *Anaplasmataceae*. Although most closely related to the genera *Rickettsia* and *Orientia*, organisms classically considered ehrlichiae are divided into at least 4 major clades. Taxonomic classification (Fig. 1) is largely based upon sequence analysis of *rrs* (16S rRNA genes) and *groESL* (heat shock operon) (1) and, increasingly, on fully sequenced genomes (2). Serologic cross-reactions, similarities among major immunodominant surface proteins, and the cellular tropisms of these bacteria further support the phylogenetic approach (3, 4). Recent studies also discern a separate clade of related bacteria, the family "*Candidatus* Midichloriaceae," that invade eukaryotic, mostly arthropod, mitochondria. Some serologic and molecular evidence suggests human pathogenicity, but this remains to be critically evaluated. Table 1 delineates current names of selected *Ehrlichia* and *Anaplasma* species that are known human and veterinary pathogens. Minor sequence differences in *rrs* exist that are markers of their biological and ecological differences (5–7). Genetic variants of *Anaplasma phagocytophilum* exist among ticks and mammals in the Northeast United States and Europe, including some that probably do not cause disease in humans (6, 7).

Although *Ehrlichia* species infect predominantly leukocytes of humans and other mammals, individual *Anaplasma* species infect bone marrow-derived cells of each lineage in different animal hosts. *Neorickettsia* species infect predominantly mononuclear phagocytes and occasionally enterocytes in mammalian hosts (8). "*Candidatus* Neoehrlichia mikurensis," a bacterium in a clade equally distant from the *Ehrlichia* and *Anaplasma* branches and present in *Ixodes* ticks, is proposed to be a new genus and species, and infections in humans are now documented (9–13). The genus *Aegyptianella* is currently listed as *incerta sedis*, most closely related to *Anaplasma* (14). *Wolbachia* spp. are "endosymbionts" of insects, helminths, and crustaceans that are transmitted predominantly by transovarial and transstadial (between stages of development) passage (15), although cell-to-cell transmission into mammalian cells has been demonstrated *in vitro*.

DESCRIPTION OF THE GENERA

Ehrlichia and *Anaplasma* spp. are Gram-negative obligate intracellular bacteria that reside and propagate within membrane-lined vacuoles found in the cytoplasm of bone marrow-derived cells, such as granulocytes, monocytes, erythrocytes, and platelets. *Ehrlichia ruminantium* and several other species also infect endothelial cells (16, 17). These intracytoplasmic clusters of bacteria resemble elementary bodies of *Chlamydia* as small dense core forms (0.2 to 0.4 µm); larger forms (0.8 to 1.5 µm) resemble reticulate bodies (18). Both are capable of binary fission. Specific ligands associated with cell adhesion are differentially expressed on the infective dense forms but not on the metabolically active reticulate forms. After a few days, the elementary bodies dividing in the phagosome form an inclusion, also called a morula, which can be seen microscopically. *Neorickettsia sennetsu* organisms grow as bacterial cells that can maintain individual vacuolar membranes with binary fission. Cell lysis leads to the release of bacteria that can infect other competent cells (19). Unlike *Rickettsia*, *Ehrlichia* and *Anaplasma* do not have a thickened outer membrane leaflet. The outer membrane appears more ruffled in *A. phagocytophilum* than in *N. sennetsu* or *E. chaffeensis* (20). As observed by transmission electron microscopy, *Ehrlichia* and *Anaplasma* spp. have a very limited region that corresponds to the peptidoglycan layer, and genes necessary for its synthesis and for lipopolysaccharide synthesis are not present within the genomes of *Ehrlichia*, *Anaplasma*, or *Neorickettsia* spp. (21–23).

The family *Anaplasmataceae* contains five genera. The full genome sequences of more than 25 *Anaplasmataceae* members are established and range in length from 860 kb in *N. sennetsu* to 1,176 kb in *Ehrlichia chaffeensis* and 1,471 kb in *A. phagocytophilum*. Genome sequencing identified a low G+C content common to endosymbionts (except for *Anaplasma marginale*) and a large proportion of noncoding sequences. Many of the genes required for glycolysis are absent, but those that are present suggest the ability of these bacteria to use simple substrates such as glyceraldehyde 3-phosphate and phosphoenolpyruvate for biosynthesis of phospholipids and nucleotides (22). Carbon sources are proline and glutamine. In contrast to the case with *Rickettsiaceae*, all enzymes necessary for the Krebs cycle and for metabolism of purine and pyrimidine are present. All other

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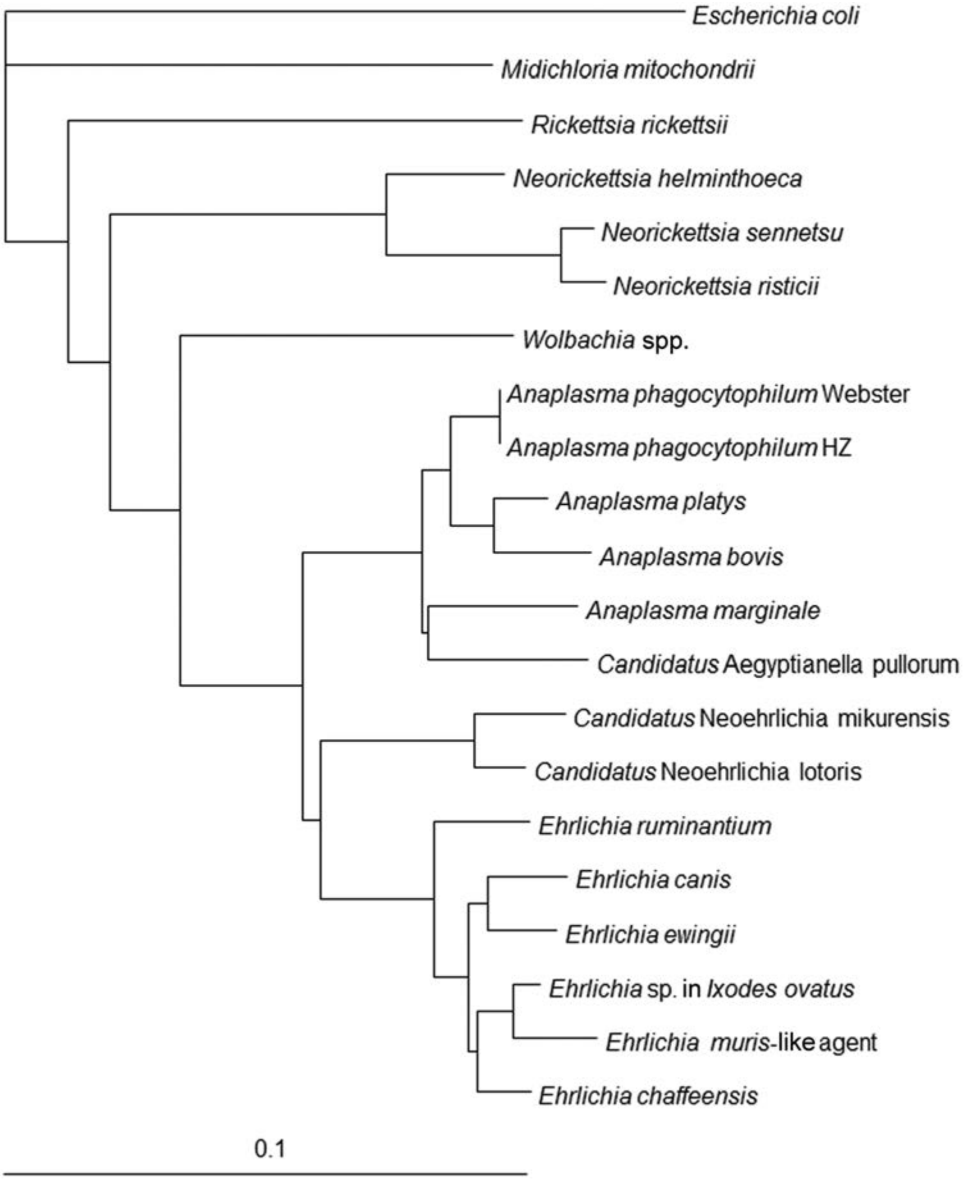
Escherichia coli
Midichloria mitochondrii
Rickettsia rickettsii
Neorickettsia helminthoeca
Neorickettsia sennetsu
Neorickettsia risticii
Wolbachia spp.
Anaplasma phagocytophilum Webster
Anaplasma phagocytophilum HZ
Anaplasma platys
Anaplasma bovis
Anaplasma marginale
Candidatus Aegyptianella pullorum
Candidatus Neoehrlichia mikurensis
Candidatus Neoehrlichia lotoris
Ehrlichia ruminantium
Ehrlichia canis
Ehrlichia ewingii
Ehrlichia sp. in Ixodes ovatus
Ehrlichia muris-like agent
Ehrlichia chaffeensis
0.1

FIGURE 1 Neighbor-joining phylogenetic tree inferred from *rrs* (16S ribosomal RNA gene) sequences of selected *Ehrlichia*, "*Candidatus* Neoehrlichia," *Anaplasma*, *Neorickettsia*, and *Wolbachia* spp. *Escherichia coli* and *Rickettsia rickettsii* are used as outgroups. The bar represents the estimated number of substitutions per site. Modified from Juan P. Olano and Maria E. Aguero-Rosenfeld in chapter 67 of the 9th edition of this *Manual* (156). doi:10.1128/9781555817381.ch65.f1

amino acids must be imported from the host by membrane transporters. ATP synthesis is possible, since these organisms possess the ATP synthase complex and other enzymes required for aerobic respiration. However, unlike in *Rickettsia*, ATP/ADP translocases are absent. Like other *Rickettsiales*, *Anaplasmataceae* possess genes that encode an anomalous type IV secretion system that most likely contributes to virulence, perhaps by transport of effectors into the host cell or by DNA uptake or in conjugation (24). In contrast to the reductive evolution seen in other intracellular bacteria, these organisms have many pseudogenes, which document gene duplication events (21, 22). The active duplication of tandemly repeated sequences likely results in new genes, antigenic variation, immune evasion, and thereby enhanced survival.

Anaplasma marginale infects ruminants, and *Aegyptianella* spp. infect birds, amphibians, and reptiles. These organisms also reside in small membrane-bound inclusions (14, 25) and are not known to cause human disease.

EPIDEMIOLOGY AND TRANSMISSION

Ehrlichia, *Anaplasma*, and "*Candidatus* Neoehrlichia" spp. are zoonotic agents transmitted to animals and humans by ticks. *Neorickettsia sennetsu* rarely causes human disease and is likely acquired by ingestion of fish infested with *Neorickettsia*-in-

TABLE 1 Selected features of *Ehrlichia*, *Anaplasma*, *Neorickettsia*, “*Candidatus* Neoehrlichia mikurensis,” and *Aegyptianella* species of human and veterinary interest[a]

Organism name	Vector	Disease	Hosts developing disease	Infected cells	Reservoir hosts
Anaplasma					
Anaplasma phagocytophilum	*Ixodes persulcatus* group *Haemaphysalis concinna* *H. longicornis*	EGE Tick-borne fever HGA	Horses Ruminants Humans, dogs	Granulocytes	Deer, sheep White-footed mice
Anaplasma marginale, *A. centrale*, *A. ovis*	Several tick species	Ruminant anaplasmosis	Cattle (sheep, goats)	Erythrocytes	Ruminants Wild cervids?
Anaplasma platys	*Rhipicephalus sanguineus?* *Amblyomma* spp.?	Canine cyclic thrombocytopenia	Dogs	Platelets	Ruminants?
Anaplasma bovis	Rhipicephalus appendiculatus *Amblyomma variegatum*	Bovine ehrlichiosis	Cattle	Mononuclear leukocytes	Ruminants? Rabbits?
Ehrlichia					
Ehrlichia chaffeensis	*Amblyomma americanum* *Dermacentor variabilis*	HME	Humans Dogs	Mononuclear leukocytes	White-tailed deer Domestic dogs Others?
Ehrlichia ewingii	*Amblyomma americanum*	CGE *E. ewingii* ehrlichiosis	Dogs Humans	Granulocytes	Canids?
Ehrlichia canis	*Rhipicephalus sanguineus*	CME, VHE	Dogs, humans	Mononuclear leukocytes	Canids
Ehrlichia ruminantium	*Amblyomma* species	Cowdriosis (heartwater)	Domestic and wild ruminants	Granulocytes, endothelial cells	Ruminants
Ehrlichia muris and *Ehrlichia muris*-like agent	*Haemaphysalis flava* *Ixodes scapularis*	*E. muris* ehrlichiosis	Small mammals, Humans	Mononuclear leukocytes	Not known
Ehrlichia species (IOE)	*Ixodes ovatus*	Not named	Laboratory and wild mice and rodents	Mononuclear leukocytes, Kupffer cells, endothelial cells	Not known
Neorickettsia					
Neorickettsia sennetsu	Unknown Possibly acquired by ingestion	Sennetsu fever	Humans	Mononuclear leukocytes	Fluke-infested fish?
Neorickettsia risticii	Ingestion of fluke-infested insects	Potomac horse fever	Horses	Mononuclear leukocytes	*Juga* flukes
Neorickettsia helminthoeca	Ingestion of fluke-infested salmonid fish	Salmon poisoning disease	Dogs, bear	Mononuclear leukocytes	Fluke-infested fish
Genus and species *incertae sedis*					
Aegyptianella spp.	*Amblyomma* spp.	Aegyptianellosis	Birds, amphibians, reptiles	Erythrocytes	Not known
“*Candidatus* Neoehrlichia mikurensis”	*Ixodes ricinus*, *Ixodes ovatus*, *Ixodes persulcatus*	Neoehrlichiosis	Humans, rodents	Endothelial cells, granulocytes	Rodents
“*Candidatus* Neoehrlichia lotoris”	*Ixodes* spp.?	No disease	Raccoons	Not known	Raccoons

[a] Modified from Juan P. Olano and Maria E. Aguero-Rosenfeld in chapter 67 of the 9th edition of this *Manual* (156). EGE, equine granulocytic ehrlichiosis; HGA, human granulocytic anaplasmosis; HME, human monocytic ehrlichiosis; CGE, canine granulocytic ehrlichiosis; CME, canine granulocytic ehrlichiosis; VHE, Venezuelan human ehrlichiosis.

fected flukes (8). *Wolbachia* spp. are symbionts of a broad range of arthropods, helminths, and crustaceans (26). Thus, most species can infect vertebrates and invertebrates (Table 1). Transovarial transmission of these organisms in ticks does not occur, but transstadial, interstadial, and intrastadial transmissions do. The bacteria are acquired when a larval or nymphal tick feeds on an infected animal and are transmitted when the next stage (nymph or adult) feeds on another mammalian host. Ticks and mammals, the latter with bacteremia that can persist, serve as reservoirs (27). In contrast, humans are only inadvertently infected and represent an end-stage host. Therefore, maintenance of tick-borne ehrlichiae in nature depends upon the presence of appropriate tick vectors and mammalian hosts in the local environment (28–31).

Recognized natural reservoirs for *E. chaffeensis* include deer (*Odocoileus virginianus* and *Blastocerus dichotomus*), domestic dogs, and perhaps other animals that host *Amblyomma* ticks (32, 33). Less important reservoirs include opossums, raccoons, voles, coyotes, and goats (34). White-tailed deer (*Odocoileus virginianus*) also are a reservoir of *Ehrlichia ewingii* and of the white-tailed deer agent, an ehrlichia closely related to *Anaplasma platys* that has not yet been associated with human disease (35). *E. muris* was found in *Apodemus* mice and *Haemaphysalis flava* ticks when first detected in Japan (12). In the Perm region of Russia, *E. muris*, or a closely related organism, was also detected in *Ixodes persulcatus* ticks and humans were found to have serologic reactions to this agent (36). In the United States, this organism was isolated from *Ixodes scapularis* ticks from Wisconsin and Minnesota (37). Further study is needed to identify the major animal reservoirs for *E. muris*.

Even for *A. phagocytophilum*, the major reservoirs are incompletely documented. However, small mammals are frequent hosts of the immature stages of *I. scapularis*, *Ixodes ricinus*, *I. persulcatus*, and *Haemaphysalis concinna* (31, 38). These include white-footed mice (*Peromyscus leucopus*), chipmunks (*Tamias striatus*), voles (*Clethrionomys gapperi*), wild rodents, and other small mammals worldwide. Although white-tailed deer can be persistently infected by *A. phagocytophilum*, the *A. phagocytophilum* strains (AP variant 1) that naturally infect deer are not infectious for small mammals, have not been identified in humans, and may represent nonpathogenic variants (39, 40). Persistent or prolonged infection in animal reservoir hosts is essential for maintenance of zoonoses. Mice infected with *A. phagocytophilum* can remain infected for months, which contributes to transmission to different stages of developing *I. scapularis* ticks (41). In Europe, red deer, sheep, cattle, and goats are persistently infected and serve as reservoirs of *A. phagocytophilum*. Cases of "*Candidatus* Neoehrlichia mikurensis" in humans have now been reported among predominantly immunocompromised patients in Europe (Czech Republic, Germany, Sweden, and Switzerland) and immunocompetent patients in China (12, 13, 42–45); the reservoirs for "*Candidatus* Neoehrlichia mikurensis" are also small mammals, including field mice and voles, in both Europe and Asia. Although the reservoir for *N. sennetsu* is also not known, epidemiological data suggest that consumption of raw fish is a risk factor for sennetsu fever (8). Other species of *Neorickettsia* have complex transmission processes involving trematodes. *Neorickettsia risticii*, the agent of Potomac horse fever, is transmitted to horses by accidental ingestion of insects carrying *N. risticii*-infected cercariae (46). Similarly, *Neorickettsia helminthoeca* infects dogs through ingestion of trematode-infested fish.

CLINICAL SIGNIFICANCE

Human Diseases

HME

The causative agent of HME is *E. chaffeensis*, a monocytotropic ehrlichia first identified as a human pathogen in a patient with a severe febrile illness after tick bites in 1986 (47). More than 8,523 cases of HME were reported to the CDC through 2012; however, data from active surveillance efforts suggest that HME occurs much more frequently than is reported (48, 49). The seroprevalence of *E. chaffeensis* ranges from 1.3% to 12.5% in regions of Arkansas and Tennessee where it is endemic. In contrast, active surveillance in Tennessee and Missouri identified 330 to 414 cases/100,000 population (48, 49). Most cases are identified in the south central and southeastern United States, but increasingly infections are identified in the mid-Atlantic region. Prospective evaluation of heavily exposed cohorts shows that approximately 75% of seroconversions are subclinical (50). Reports from Latin America, Africa, Europe, and Asia indicate that *E. chaffeensis*, or closely related microorganisms, could also be found there (51–56).

The median incubation period for HME is 9 days. The median age of HME patients is 51 years, and 60% of patients are males (34, 57). Patients often present with high fever (96%), headache (72%), malaise (77%), myalgia (68%), nausea (57%), and no localizing physical findings (48). Other gastrointestinal (vomiting and diarrhea), respiratory (cough), and osteoarticular (joint pain) symptoms are present in less than 50% of patients. Central nervous system involvement (stiff neck, confusion, and meningitis) is well described (58). Patients with *E. chaffeensis* can develop hemophagocytic lymphohistiocytosis syndrome (59–61). Petechial, macular, and maculopapular rashes occur with varied distribution and onset (34, 62). Rashes are more frequent in children (57% of cases) (63). Abnormal laboratory parameters occur in at least 86% of patients and include thrombocytopenia (68 to 88%), leukopenia (60 to 71%) with lymphopenia and/or neutropenia, and increased serum transaminases (38 to 54%). Severe complications occur in 9 to 17% and include meningoencephalitis and a toxic shock-like syndrome with multiorgan failure, including adult respiratory distress syndrome. Fulminant infections are more common in patients immunocompromised by HIV, high-dose corticosteroids, medications related to organ transplantation, and diabetes (64–70). The case fatality rate is 2 to 3% (71). Male sex, advanced age, immunosuppression, and delayed diagnosis leading to late treatment are independent risk factors for death (57).

HGA

The causative agent of human granulocytic anaplasmosis (HGA) is *Anaplasma phagocytophilum*. HGA was first identified in 1990 in a patient from Wisconsin who reported tick bites. Tick bite is the most frequent route of transmission; however, there are others (72–74), including perinatal transmission (73), accidental inoculation of infected blood, and blood transfusion (72, 74).

Despite a mandate to report all cases of HGA in the United States, the true incidence and prevalence of the infection are unknown. Passive surveillance in northwestern Wisconsin and Connecticut reveals yearly incidence rates of 24 to 58 cases per 100,000 population (49). As of 2012, 13,151 cases were reported nationwide, with the highest annual incidence rates in the Northeast and upper Midwest. Infected patients are identified in other states, as well as in Europe and Asia (56, 72, 75–79). Most infections are subclinical or mild, since between 1% and 15% of persons residing in regions of endemicity in Connecticut and northwestern Wisconsin, respectively, are seropositive (80, 81). The tick vectors for HME and HGA coexist in the mid-Atlantic, the southern New England states, and the southern Midwest. Thus, both diseases occur in these areas (57, 78, 82). Furthermore, human ehrlichioses, including HME, HGA, and infections with *E. ewingii* and an *E. muris*-like agent, are clinically indistinguishable.

HGA has a median incubation period of 5 to 11 days after the bite of an infected tick (*Ixodes* or *Haemaphysalis* spp.). The median age of HGA patients is 53 to 54 years (83 to 85), and the male/female ratio is 1.5:1 (86). Patients most often present with high fever (93%), myalgias (68%), headache (62%), and malaise (93%). Gastrointestinal, respiratory, musculoskeletal, and central nervous system involvement occurs in fewer than half of patients. Rash is observed in 6% of patients, in all cases attributable to erythema migrans with concurrent Lyme disease (87). Leukopenia with lymphopenia, thrombocytopenia, and increased serum aspartate transaminase activity is common early in the disease and may normalize before antimicrobial treatment. Lymphocytosis with atypical lymphocytes can occur after the first week of infection (86, 88). Approximately 36% (89) of infections are severe enough to warrant hospitalization, and 3 to 7% of patients have life-threatening complications or require intensive care unit admission (71, 90). The clinical course is worse for patients with cancer, diabetes, immunosuppression related to treatment of rheumatic conditions, or functional or anatomic asplenia. Severe complications of HGA include a septic shock-like illness with multiorgan failure, adult respiratory distress syndrome, and opportunistic infections (85, 88, 91–93). Meningoencephalitis and cerebrospinal fluid (CSF) pleocytosis are rarely documented (0.2% of cases) (71) in HGA; however, other neurologic sequelae can include facial diplegia, brachial plexopathy, and demyelinating polyneuropathy (89). Meningitis has not been documented. Clinical severity can vary. For example, a subset of 16 patients from one region of China had significantly greater disease severity (including multiorgan dysfunction, gastrointestinal, renal, and hemorrhagic manifestations, and worse laboratory features) and a case fatality rate of 16%, whereas the case fatality rate was 2.6% among 46 other patients with HGA in Shandong Province and is 0.6% in the United States (94).

Ehrlichia ewingii Ehrlichiosis

E. ewingii was recognized to cause human infection when its DNA was found in the blood of patients with an HME-like disease in Missouri (95). As with *E. chaffeensis*, the main vector is the lone star tick (*A. americanum*); therefore, the distribution of the disease is similar to that of HME. White-tailed deer are also reservoirs, and recent epidemiological studies suggest that dogs may be as well (35, 96). *E. ewingii* ehrlichiosis seems to affect mostly immunosuppressed patients, including those with HIV, but is clinically milder than coinfection with HIV and *E. chaffeensis* (68, 95). In dogs, *E. ewingii* is responsible for canine granulocytotropic ehrlichiosis.

Other Human Ehrlichioses

Venezuelan Human Ehrlichiosis

In 1996, *Ehrlichia canis* was isolated from the blood of an asymptomatic man from Venezuela. Since then, 6 additional symptomatic patients have been identified as infected by an *E. canis* strain that differs from canine strains by a single nucleotide polymorphism in *rrs* (97, 98).

Ehrlichia muris-Like Agent Ehrlichiosis

In 2009, infection with an *Ehrlichia muris*-like agent was detected and the organism isolated from patients with suspected ehrlichiosis/anaplasmosis in Wisconsin and Minnesota (37). Some patients had strong serologic reactivity in convalescence to *E. chaffeensis* but not *A. phagocytophilum* antigens. Since the original report, 44 patients, all from Minnesota and Wisconsin, were diagnosed with *Ehrlichia muris*-like agent infection (B. Pritt, personal communication). The mean age of patients is 60 years, and >1/3 of patients are immunocompromised. The clinical features mimic HME and HGA.

"*Candidatus* Neoehrlichia mikurensis" Ehrlichiosis

Seven European patients with "*Candidatus* Neoehrlichia mikurensis" infection presented with severe sepsis-like syndromes, some lasting weeks, and 1 patient died. In contrast, reported illnesses of 7 patients with "*Candidatus* Neoehrlichia mikurensis" infection in China were mild (45).

N. sennetsu Ehrlichiosis (Neorickettsiosis)

Named after the Japanese term for glandular fever, *Neorickettsia sennetsu* was first isolated from patients with suspected infectious mononucleosis in 1953 (99). The organism is rarely identified now. Patients develop a self-limited febrile illness with chills, headache, malaise, sore throat, anorexia, and generalized lymphadenopathy. Cases have been identified in Japan and possibly Malaysia, although at least one case was recognized in the Lao People's Democratic Republic (8). Laboratory findings include early leukopenia and atypical lymphocytes in the peripheral blood during early convalescence. No fatalities or severe complications have been reported.

Contributions of *Wolbachia* to Human Disease

Although *Wolbachia* spp. have not yet been shown to be directly pathogenic for humans or animals, evidence indicates a potential role for release of intracellular *Wolbachia* components from filarial helminths such as *Brugia malayi*, *Onchocerca volvulus*, and *Wuchereria bancrofti*. In theory, these microbial components act as potentiators of inflammation and contribute to lymphatic obstruction associated with parasitic infections (15, 100). At a minimum, the endosymbionts are mutualists on which the helminths depend to enable survival from stage to stage.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Currently, there are 3 methods for diagnosis of active infection with HME or HGA: (i) PCR amplification of nucleic acids from *Ehrlichia* or *Anaplasma* species, (ii) detection of morulae in the cytoplasm of infected leukocytes by nonspecific Romanowsky stains (e.g., Giemsa or Wright) or by specific immunocytologic or immunohistologic stains using *E. chaffeensis* or *A. phagocytophilum* antibodies, and (iii) culture of *Ehrlichia* or *Anaplasma* from blood or CSF. In contrast, testing of single serum samples (acute or convalescent phase) is rarely useful. However, concurrent testing of paired sera (obtained during the acute illness and in convalescence 2 to 4 weeks later) does provide definitive retrospective diagnosis.

EDTA-anticoagulated blood is a useful specimen for most tests (PCR, smears, and culture) and should be obtained during the active phase of illness before treatment (62, 83, 101). Acute-phase peripheral blood buffy coat smears or cytocentrifuged preparations of CSF cells should be prepared within hours of obtaining the samples since leukocytes degenerate rapidly. Once prepared, air-dried blood smears and cytocentrifuged CSF preparations are stable at room temperature for months or years.

Whole blood is the preferred sample for PCR since it contains infected leukocytes; serum should not be used. Samples for PCR should be tested promptly or frozen at −20 to −80°C.

The preferred specimen for culture of *Ehrlichia* and *Anaplasma* is peripheral blood. Samples should be obtained by sterile venipuncture or lumbar puncture and processed as soon as possible. The culture conditions for *Ehrlichia* and *Anaplasma* species are not optimized. Culture is currently performed only in a few public health and research laboratories. Samples should be maintained at 4°C during shipping but not frozen. *A. phagocytophilum* is easier to culture than *E. chaffeensis*, including from EDTA-anticoagulated blood stored for up to 18 days at 4°C (102); however, isolation can take days to weeks. *Ehrlichia*- and *Anaplasma*-infected cells can be stored frozen within infected host cells at −80°C for months. Storage of infected cells is best accomplished when more than 50 to 90% of the host cells are infected, and it is achieved by suspension of at least 10^6 cells per ml in tissue culture medium that contains 10% dimethyl sulfoxide and at least 30% fetal bovine serum.

LABORATORY CONFIRMATION OF *EHRLICHIA CHAFFEENSIS*

Direct Examination

Microscopy by Romanowsky Staining of Peripheral Blood

Patients with suspected HME should have Giemsa or Wright-stained peripheral blood or buffy coat leukocytes examined for the presence of morulae. However, the sensitivity is low (≤29%) compared to that of culture (58). *E. chaffeensis* is detected predominantly in monocytes and is more frequently detected in severe infection. When present, morulae are small (1- to 3-μm diameter), round to oval clusters of small bacteria that appear as basophilic to amphophilic stippling within cytoplasmic vacuoles (Fig. 2). Detection is accomplished more frequently for immunocompromised patients. Since the percentage of cells infected ranges from 0.2% to 10%, up to 500 cells should be examined.

Antigen Detection by Immunohistology

Immunohistochemistry can identify *E. chaffeensis* in bone marrow, liver, and spleen. However, the sensitivity of detecting active infection with examination of bone marrow is only 40% (103). A monoclonal antibody can specifically detect *E. chaffeensis* in human tissues (104), but most studies use polyclonal antibodies which react with other *Ehrlichia* species. Commercial assays are not currently available.

Nucleic Acid Detection Techniques

There are a large number of potential gene targets for molecular confirmation of infection with *E. chaffeensis*, with sensitivity of PCR ranging from 60% to 100% (58, 62, 105). The most widely used method is PCR amplification of DNA from *E. chaffeensis* in clinical samples using the HE1/HE3 primer set (58, 106, 107). This primer pair amplifies a 389-bp fragment of *rrs* (16S rRNA gene). The product can be detected by simple nucleic acid staining (e.g., ethidium bromide) after agarose gel electrophoresis, by Southern hybridization of the amplified products using an internal probe, or, preferably, by real-time PCR with either 5′ nuclease or molecular beacon probes that increase analytical sensitivity (108). A clinical evaluation of *E. chaffeensis* PCR using the HE1/HE3 system showed a sensitivity of 79 to 100% compared with that of serology; however, nucleic acids from *E. chaffeensis* were frequently detected in patients who never developed antibodies (58, 107). A similar sensitivity was shown with a nested PCR that employs broad-range "*Ehrlichia* genus" primers in an initial step followed by PCR with the HE1/HE3 primer pair (109). A nested PCR assay with broad-range *rrs* primers (8F and 1448R) followed by a second (nested) reaction with primers 15F and 208R on whole blood yielded results similar to those obtained by culture (58). Other targets for PCR that have not been fully evaluated for clinical sensitivity or specificity include the outer-surface variable-length PCR target protein gene present in *E. chaffeensis* (58, 68, 110), the TRP120 gene that encodes an immunodominant antigen with tandemly repeated subunits that vary among *E. chaffeensis* isolates, the quinolinate synthase A gene (*nadA*), the disulfide bond formation protein gene (*dsb*), and the p28 multigene family (3, 68, 111, 112). In a prospective study, the overall sensitivity and specificity of PCR were 56% and 100%, respectively, using the 16S rRNA subunit, *nadA*, and TRP120 genes (62). However, in this study, several samples had high titers of antiehrlichial antibodies by indirect immunofluorescence assay (IFA), suggesting that the pathogen was already cleared. When sensitivity was calculated using seroconversion as the "gold standard," it increased to 84%. Posttest probabilities for a positive and negative PCR result were 96% and 11.1%, respectively. Posttest probabilities depend critically on prevalence (62).

Real-time multicolor PCR and real-time multiplex reverse transcriptase PCR assays have been developed recently with extremely high analytical sensitivity and specificity comparable to those of nested PCR (3, 113). Advantages include improved specificity (lower risk of contamination), speed, cost, and the detection of multiple ehrlichia pathogens simultaneously. A broad-range real-time PCR assay that targets *groEL* of *E. chaffeensis*, *A. phagocytophilum*, and *E. ewingii* has shown excellent clinical sensitivity and specificity and also detects the *E. muris*-like ehrlichia (37, 114).

Isolation Procedures

Ehrlichia chaffeensis is classified as a biosafety level 2 pathogen. *Ehrlichia chaffeensis* has been isolated from the peripheral blood of a limited number of patients with HME, and an *E. canis*-like organism was recovered only once from an asymptomatic human (58, 67, 98, 115, 116). Culture remains a research tool that employs specialized methods and unique cell lines (58).The most frequently used cell for primary isolation is the canine histiocytic cell line DH82; however, *E. chaffeensis* has been successfully cultivated in other cells, including the human macrophage-like THP-1 cells, the fibroblast-like HEL-22 cells, Vero cells, and HL-60 cells (human promyelocytic cell line differentiated to the monocytic pathway), among others (117). Isolation may be successful even when infected leukocytes are not observed on peripheral blood examination (58). Isolation usually involves direct inoculation of leukocyte fractions or whole blood into flasks with confluent layers of adherent cells or into flasks that contain approximately 2 × 10^5 to

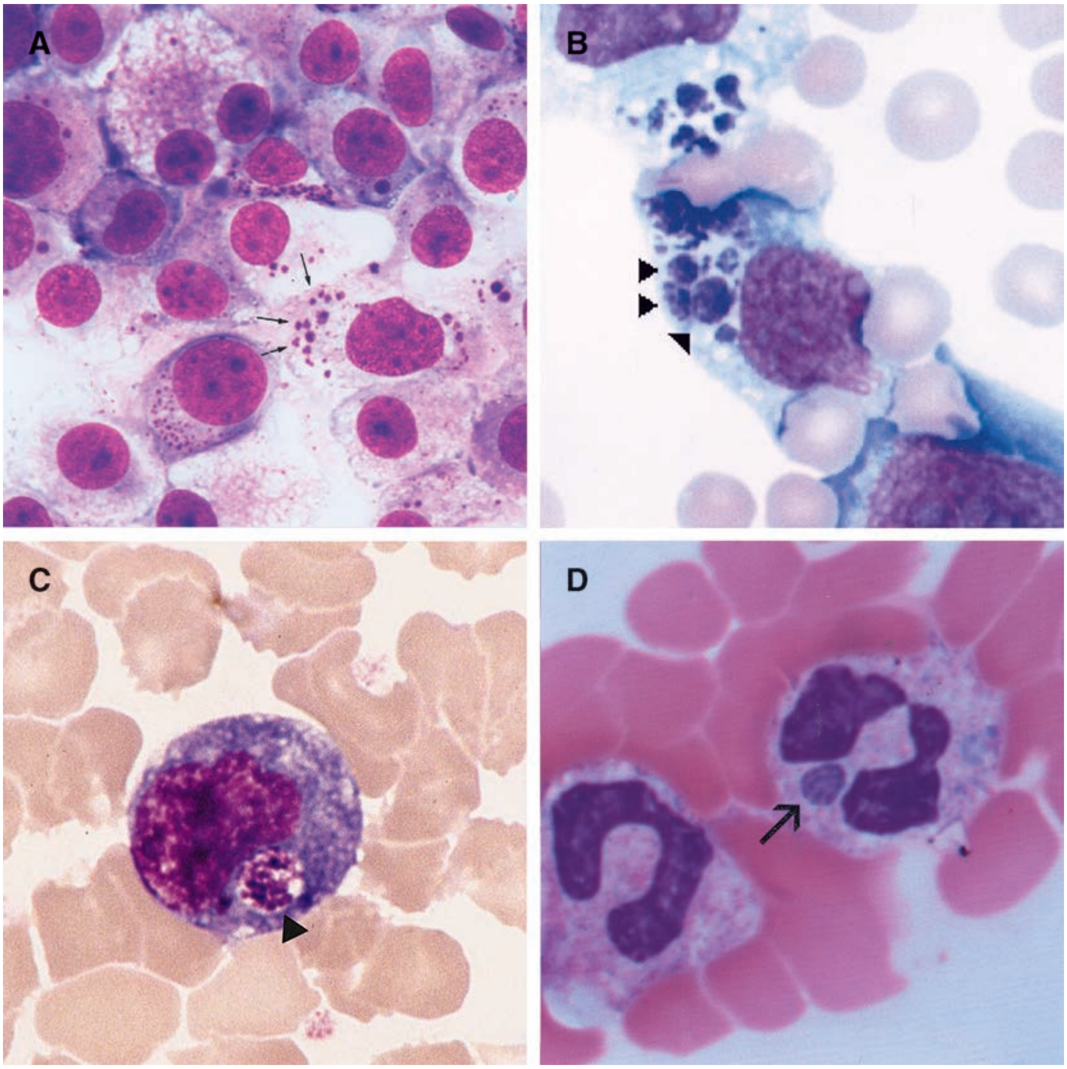

FIGURE 2 (A and C) Wright stains (original magnification, ×1,000) of *E. chaffeensis* (A) and *A. phagocytophilum* (C) in peripheral blood leukocytes. Note that an *E. chaffeensis* morula (arrowhead) is present in a monocyte (A) and that an *A. phagocytophilum* morula (arrow) is present in a neutrophil (C). (B) Romanowsky (Leukostat) stain (original magnification, ×1,000) of *E. chaffeensis* cultured in the canine histiocyte cell line DH82. Note the presence of basophilic, stippled, intracytoplasmic inclusions approximately 2 to 3 μm in diameter (arrows). The smaller intracytoplasmic granules may also be ehrlichial morulae. (D) Wright stain (original magnification, × 1,000) of *A. phagocytophilum* from the blood of an infected patient cultured in the human promyelocytic cell line HL-60. Note the presence of multiple basophilic, stippled, intracytoplasmic inclusions (arrowheads) in an HL-60 cell. doi:10.1128/9781555817381.ch65.f2

1×10^6 cells per ml of tissue culture medium. Macrophage-like cells that are highly phagocytic could be adversely affected by the presence of erythrocytes; thus, it is recommended that either (i) leukocytes be fractionated from erythrocytes by density gradient centrifugation (e.g., Ficoll-Paque), (ii) leukocytes be harvested after erythrocyte lysis (hypotonic lysis, NHCl$_4$ lysis, etc.), or (iii) confluent monolayers be reestablished after cultivation with erythrocyte-containing samples by addition of uninfected cells. Since *E. chaffeensis* can be present in very few peripheral blood leukocytes, it is advisable to inoculate cultures with as many peripheral blood leukocytes as possible (which may be difficult with leukopenia). Use of 2 to 3 ml of EDTA-anticoagulated blood diluted in 2 volumes of sterile Hanks' balanced salt solution followed by Histopaque (Sigma, St. Louis, MO) gradient separation of leukocytes has been effective (58).

The blood mononuclear cells are resuspended in a 2-ml volume of tissue culture medium supplemented with 5% fetal bovine serum and allowed to interact with adherent host cells in a 25-cm^2 flask for 3 h, usually enhanced by incubation with rocking at 37°C in 5% CO$_2$. The inoculum is removed if significant erythrocyte contamination is present and the monolayer is replenished with 5 ml of fresh tissue culture medium. Since *Ehrlichia* species are bacteria, antibiotics in the medium must be avoided. The generation time of *E. chaffeensis* is approximately 19 h (118), and thus, cultures must be maintained to allow a slow logarithmic or stable growth phase to avoid the host cells outgrowing the ehrlichiae.

Identification

The presence of infected cells is determined by sampling the medium (DH82 cells and THP-1 cells) or by lightly scraping part of the monolayer. Aliquots of the culture are cytocentrifuged, followed by Wright or Giemsa or immunofluorescent stains, and cells are examined for the presence of intracytoplasmic morulae or *Ehrlichia chaffeensis* antigen (Fig. 2). Culture can require 1 month or more but has been achieved in as short as a few days (58, 115, 116). Confirmation of the infectious agent is currently best achieved by PCR amplification using species-specific primers (119).

Serologic Tests

The gold standard for the diagnosis of HME is demonstration of a 4-fold rise in IgG titer or seroconversion by examination of paired (acute- and convalescent-phase) sera. Thus, diagnosis is at best retrospective. The most frequently used serologic method is the IFA. Other methods include enzyme-linked immunosorbent assay (ELISA) or enzyme immunoassay and protein (Western) immunoblotting, but none have been well validated. Ehrlichia antigens can be difficult to prepare. Commercial production and distribution now make these more readily available. Commercial sources of IFA serodiagnostic kits include Focus Technologies (Cypress, CA), Scimedx Corp. (Denville, NJ), and PanBio Diagnostics (not available in the United States).

Currently, there is little standardization for any method of ehrlichia serology, and cutoff titers are dependent upon validation in individual laboratories that perform these assays or are per manufacturer instructions. The algorithm for serologic testing by IFA includes an initial screen at a dilution of 1:64 or 1:80 for IgG antibodies to *E. chaffeensis*. Reactive samples are then titrated to endpoint. Of note, some patients with *Ehrlichia muris*-like agent have strong serologic reactivity with *E. chaffeensis* but not *A. phagocytophilum* antigens. However, when tested, these patients have even stronger reactivity with homologous antigens, although currently *Ehrlichia muris*-like antigens are not commercially available (37).

Ehrlichia chaffeensis IFA

E. chaffeensis IgG is detected by IFA using *E. chaffeensis* Arkansas strain-infected DH82 canine macrophage-like cells. Reactive sera are serially diluted starting at a dilution of 1:64. The presence of antibodies is detected after incubation with fluorescein isothiocyanate (FITC)-conjugated antihuman IgG. The test is positive if classic intracytoplasmic morulae are seen. It is important to identify the appropriate proportion of infected cells as determined by a positive control serum and the appropriate morphology for each antigen preparation to preclude false-positive interpretations. Prescreening for autoantibodies or routine removal of rheumatoid factors will lessen the risk of misinterpretation due to antibodies reactive with cellular components, including nuclear or cytoplasmic antigens that could have the morphologic appearance of morulae. A 4-fold increase in IgG antibody titer or seroconversion confirms the diagnosis of HME. A single high specific IgG titer, like identification of morulae in monocytes or macrophages for *E. chaffeensis* by microscopy, is suggestive of acute infection. However, antibody titers can be detected in a small proportion of subjects without HME owing to the presence of antigens that are highly conserved among bacterial species (120, 121). Acute-phase sera should be obtained at the time of presentation with acute illness, and convalescent-phase sera are best obtained 3 to 6 weeks later (120).

The sensitivity and specificity of IFA for the diagnosis of infection with *E. chaffeensis* are not known but are assumed to be high because of documented correlation between new or rising antibody titers against *E. chaffeensis* and characteristic clinical findings (50). In the early phase of infection, IFA testing is not sensitive compared to PCR (105). A systematic evaluation of the usefulness of IgM testing has not been conducted, but a preliminary evaluation based on nine culture-confirmed cases suggests that it might be slightly more sensitive than IgG for the diagnosis of HME during the acute phase (105). Previously, the serologically cross-reactive *E. canis* was used as a surrogate antigen; however, this serodiagnostic assay has a lower sensitivity than obtained using *E. chaffeensis* antigen, and its use should be discouraged (106). The role of immunoblots in diagnosis is not well established and cannot be advocated. Alternative methods based on recombinant proteins and synthetic peptides show promise, but these are not commercially available (112). Antibodies to *E. chaffeensis* can also be detected in patients diagnosed with Rocky Mountain spotted fever, Q fever, brucellosis, Lyme disease, and Epstein-Barr virus infections, suggesting that false-positive reactions do occur (120, 121). Antigenic diversity among *E. chaffeensis* isolates is well described (123) but may not affect the detection of polyclonal antibody responses generated with human infection. Several reports characterized patients with suspected HME who lacked antibody responses, even long after onset of symptoms (58, 107, 124). However, in the few cases in which *E. chaffeensis* infection was proven by isolation of the agent, patients who survived developed clear convalescent-phase serologic reactions by IFA (58, 68, 115, 116, 123). Hypothetical reasons for false-negative results include infection by antigenically diverse strains (unproven) and abrogation of antibody response by early therapy (58).

LABORATORY CONFIRMATION OF *ANAPLASMA PHAGOCYTOPHILUM*

Direct Examination

Microscopy by Romanowsky Staining of Peripheral Blood

Examination of Giemsa- or Wright-stained peripheral blood or buffy coat leukocytes for the presence of morulae is highly valuable in the diagnosis of HGA. Usually, 800 to 1,000 granulocytes are examined under magnifications of ×500 to ×1,000 for the presence of morulae (83–85). Since most patients presenting with positive smears have <1% of infected granulocytes and usually have leukopenia, buffy coat preparations will yield more than peripheral smears. Infection rates as high as 40% of granulocytes are described (125). As for HME, the presence of detectable infected granulocytes in peripheral blood correlates modestly with severity of infection (84, 86, 125). The sensitivity of the buffy coat smear examination in the acute phase of HGA is approximately 60% (86). When present, ehrlichia morulae are small (1- to 3-µm diameter), round to oval clusters of small bacteria that stain basophilic to amphophilic with Wright and Giemsa stains (Fig. 2). These clusters are present in the cytoplasm of neutrophils or eosinophils and have a stippled appearance owing to individual bacteria within the vacuole.

Immunohistology for Antigen Detection

Immunohistologic methods can also be used to identify *A. phagocytophilum* within human tissues, including bone marrow, liver, and spleen (93). Sensitivity and specificity have not been determined.

Nucleic Acid Detection Techniques

Multiple PCR assays for detection of *A. phagocytophilum* nucleic acids are published (113, 126–129). PCR is at least as sensitive as blood smear in the first 14 days after onset of illness and is more sensitive longer (>14 to ≤30 days) after illness onset (130). Most protocols utilize amplification of *rrs* (16S rRNA gene). The most frequently applied and evaluated method employs the primer set ge9f and ge10r, which amplify a 919-bp fragment, usually in a single-stage reaction with or without a hybridization probe to enhance sensitivity (5, 126). A previously popular alternative uses nested PCR with an outer set of primers to amplify eubacterial 16S rRNA genes, followed by PCR with internal nested *A. phagocytophilum*-specific primers (58, 127). PCR amplification of *groESL* using a nested reaction has also been useful. The size of the amplified product distinguishes *E. chaffeensis* from *A. phagocytophilum* (128). In studies conducted prior to the development of real-time PCR, the analytical sensitivity and specificity of several published primer sets were evaluated using DNA extracted from serial dilutions of *A. phagocytophilum*-infected HL-60 cells (127). Specificity was evaluated using DNA extracted from cultures of *E. chaffeensis*, *Rickettsia rickettsii*, and *Bartonella henselae*. The primer sets with the greatest sensitivity and specificity were those used in nested PCR to amplify *rrs*, ge3a-ge10 and ge9-ge2, and those amplifying the *msp2* gene, msp2-3f–msp2-3r (127). Both PCR assays detected as few as 0.25 infected HL-60 cells. Real-time PCR and 5′ nuclease (TaqMan) assays that target the >100-copy *msp2* gene family provide increased sensitivity to as few as 1 infected cell per microliter of blood (131). A multiplex assay to detect *Ehrlichia* and *Anaplasma* spp. by real-time reverse transcriptase PCR was developed and evaluated in peripheral blood of dogs suspected of ehrlichiosis (113). The assay has a sensitivity of 100 *rrs* transcripts (which corresponds to about 1 infected cell in a test sample), can detect single or multiple infections, and has the potential for automation. A broad-range real-time assay that targets *Anaplasmataceae groEL* can distinguish *E. chaffeensis*, *E. ewingii*, *A. phagocytophilum*, and *E. muris*-like agent when melt-curve analysis is applied, providing excellent clinical sensitivity and specificity (37, 114).

Isolation Procedures

A. phagocytophilum, a biosafety level 2 agent, is successfully cultivated more often from human patients than *E. chaffeensis*, *E. canis*, *E. muris*-like agent, or *N. sennetsu*, probably owing to the quantity of organisms present in the peripheral blood of infected patients (83). HGA is described in Europe and Asia, and success at isolating the organism is reported. Isolation is best achieved with the human HL-60 promyelocytic cell line (101) and has been accomplished even when morulae are not observed in peripheral blood smears. Optimal conditions for recovery have not been conclusively determined. Because erythrocytes do not adversely affect HL-60 cells, direct inoculation of EDTA-anticoagulated blood is effective. Fractionation of blood into buffy coat or granulocyte fractions by density gradient centrifugation is also effective (132). Approximately 100 to 500 µl of EDTA-anticoagulated blood, containing 10^2 to 10^4 infected granulocytes, is inoculated into 100-fold-more uninfected HL-60 cells. Cultures are subsequently maintained at a concentration between 2 × 10^5 and 1 × 10^6 cells per ml of tissue culture medium.

Identification

Cultures are examined every 2 to 3 days by Wright or Giemsa staining of cytocentrifuged preparations of 20 to 50 µl of culture suspensions. Ehrlichia morulae appear as small aggregates of basophilic bacteria in the cytoplasm of HL-60 cells (Fig. 2). Since HL-60 cells can contain cytoplasmic granules, immunocytochemistry or immunofluorescence is very helpful for inexperienced readers. Unfortunately, immunohistologic reagents are currently not commercially available for tissue sample examination. Cultures usually require between 5 and 10 days before morulae are clearly identified, but infected cells can be detected as early as 3 days postinoculation. Time to detection of organisms in culture correlates with the amount of bacteria present in blood at the time of culture (102). Definitive identification is achieved by PCR amplification using species-specific primers (5, 101) or by sequence analysis of PCR-amplified genes (5). The exact length of incubation before cultures are considered negative has not been determined, but they should be kept for at least 14 days, maintaining the cell density adjusted to about 2 × 10^5/ml.

Serologic Tests

Anaplasma phagocytophilum IFA

Although testing can be performed using *A. phagocytophilum* antigens prepared from infected circulating leukocytes of horses, the preferred method for testing human sera is the use of a human isolate propagated in the HL-60 promyelocyte cell line (36, 78, 101, 133–135). It is now well demonstrated that antigenic diversity exists among isolates of *A. phagocytophilum*, but such diversity has heretofore not been shown to affect detection in clinical specimens (135, 136). Recently, in 2 cases of anaplasmosis in humans in Japan, patient blood samples contained unique *p44/msp2* and antibodies bound to *A. phagocytophilum* antigens propagated in THP-1 rather than HL-60 cells, indicating that cases could go undetected if both cell lines are not used for serodiagnosis (137). Interpretation of immunofluorescent patterns is similar to that for *E. chaffeensis* and requires an experienced microscopist. Commercial sources of IFA serodiagnostic kits include Focus Technologies (Cypress, CA), Scimedx Corp. (Denville, NJ), and PanBio Diagnostics (not available in the United States).

Sera should be screened at a single dilution (1:64 or 1:80), and the presence of antibodies is determined after incubation with FITC-conjugated anti-human IgG. If specimens are reactive, they are serially diluted to determine the endpoint titer. A serologic confirmation diagnosis is achieved when a 4-fold rise in titer is demonstrated in convalescence with a minimum IgG titer of 80, or when a single high antibody titer is demonstrated for a patient with typical clinical features of HGA (84, 85, 138). Approximately 25 to 45% of infected patients have antibodies at the time of presentation (84, 85, 133, 138); however, in regions where HGA is endemic, up to 11 to 14% of the population possess antibodies, rendering a single serologic test less useful (80, 139). The typical response during acute infection is a rapid rise (within 2 weeks of onset) in antibody levels, reaching high titers (≥640) within the first month (133, 138). In treated patients whose diagnosis was confirmed by culture, antibody titers declined gradually over the several months, and about half had antibodies detectable by IFA 1 year after infection. However, many patients have antibodies detectable for months to years after initial infection (138).

The sensitivity and specificity of the HGA serologic tests are both believed to be high because of good correlation between typical clinical cases and serologic reactions to *A. phagocytophilum* group antigens (84, 133, 138). Seroconversion was documented for 21of 23 patients (91.3%) with culture-confirmed HGA from whom a convalescent-phase sample was available (133). In an inter- and intralaboratory evaluation, paired serology had a median sensitivity of 95% for the detection of acute HGA in a group of 28 patients diagnosed by culture, PCR, or the presence of morulae in blood smears (135). IgM testing appears to be a useful tool for identification of recent infection, but neither the sensitivity nor the specificity is as high as that for testing paired sera for IgG (135). Although ELISA and immunoblots are described (36, 134, 140), they are not routinely used for the serodiagnosis of HGA. Patients with HGA have serologic reactions to *E. chaffeensis* in up to 15% of cases but often show higher titers with homologous antigens (84, 135).Thus, when ehrlichiosis is clinically suspected, screening for antibodies against *E. chaffeensis* and *A. phagocytophilum* is recommended (141). Immunoblots can be used to differentiate among *A. phagocytophilum* and *E. chaffeensis* infections (121, 134, 140).

False-positive reactions can be observed in patients infected with other rickettsiae, the Q fever agent, and Epstein-Barr virus. Many patients with HGA develop antibodies that react with *B. burgdorferi* by ELISA and demonstrate diagnostic *B. burgdorferi* IgG or IgM immunoblots (142). Most of these likely represent false positives for *B. burgdorferi*, although some patients have been confirmed by culture to have concurrent infection with *A. phagocytophilum* and *B. burgdorferi* (121, 143). Another explanation is previous exposure to another tick-borne agent (sequential tick bites). Indeed, antibodies to multiple agents are common in individuals living in areas of high endemicity (80, 144). Autoantibodies to platelets and other leukocyte components also can cause false-positive IFA tests (145).

Technologies that use Western immunoblot confirmation or commercially prepared recombinant *A. phagocytophilum msp2* proteins or peptides in devices that enable rapid detection have been employed for serologic diagnosis in veterinary laboratories, but they have not been evaluated for diagnosis of human infections (146, 147).

ANTIMICROBIAL SUSCEPTIBILITIES

No prospective controlled treatment trials to determine antimicrobial effectiveness for ehrlichioses or anaplasmosis have been conducted. In retrospective studies and routine clinical practice, patients with either HME or HGA defervesce within 48 h of therapy with doxycycline, the drug of choice (50, 87). Tetracyclines are uniformly bactericidal for *Ehrlichia* and *Anaplasma* species, whereas the MICs of chloramphenicol cannot be safely achieved in humans with HME or HGA (118, 148–151). In contrast, many antibiotics prescribed for undifferentiated fever, such as penicillins, cephalosporins, aminoglycosides, and macrolides, do not inhibit the growth of ehrlichiae *in vitro*. The rifamycins (rifampin and rifabutin) can achieve effective inhibition or killing of *Ehrlichia* and *Anaplasma* species *in vitro*, and the fluoroquinolones (ofloxacin and levofloxacin) have very low MICs for human isolates of *A. phagocytophilum* (149, 151). However, at least one report documents recrudescence of infection with *A. phagocytophilum* after levofloxacin was discontinued and subsequent clinical response to doxycycline (152). Rifampin has been used successfully in patients who cannot receive tetracyclines (153, 154). *In vitro* susceptibility testing by real-time PCR found that *E. chaffeensis* was susceptible to doxycycline and rifampin and was partially susceptible to the fluoroquinolones. Resistance to macrolides, co-trimoxazole, and beta-lactam compounds was confirmed (118).

Whereas persistent infections with *Ehrlichia* and *Anaplasma* species can occur in naturally and experimentally infected animals even after treatment with tetracycline, persistence of ehrlichiae in humans is rarely documented and is not believed to have any clinical importance (122, 124, 155). Therapy is usually highly effective at eliminating ehrlichiae from the blood of infected humans.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Identification of infections with *Ehrlichia* and *Anaplasma* species requires clinical suspicion followed by laboratory confirmation. Since rapid specific diagnosis is infrequently possible, empirical therapy should be initiated when the diagnosis is suspected, since delays may lead to increased morbidity and perhaps mortality. Collection of diagnostic samples should ideally occur before therapy is initiated, and patients should be encouraged to return for clinical and serologic follow-up 2 to 4 weeks later.

The presence of intracytoplasmic inclusions within a leukocyte in peripheral blood is helpful when present but can be difficult to distinguish from overlying platelets, Döhle bodies, toxic granulation, nuclear fragments, Auer rods, other bacteria, yeasts, inorganic materials, or normal granules. If the typical morphology of an *Ehrlichia* or *Anaplasma* morula is observed, an assessment as to the hematopoietic lineage and the percentage of cells that contain morulae should be made and reported.

A positive PCR result should be reported as such, indicating the presence of *E. chaffeensis* or *A. phagocytophilum* DNA present, and it should be made clear that a positive PCR is not equivalent to the culture of ehrlichiae from blood. Laboratories that use a broad-range PCR to identify *Ehrlichia* or *Anaplasma* DNA in blood can also detect *E. ewingii* and *E. muris*-like agent infection that may mimic either HME or HGA (95). Broad-range PCR assays targeting *rrs* (16S rRNA gene) or *groEL* have also identified infection with "*Candidatus* Neoehrlichia mikurensis" in Europe (42).

IFA serologic results should be reported as the titer of antibodies determined to be reactive with *E. chaffeensis* or *A. phagocytophilum*, including the positive cutoff values determined in the laboratory. The gold standard for the diagnosis of *E. chaffeensis* or *A. phagocytophilum* infection by serology is a 4-fold increase in IgG titer or documentation of seroconversion. An interpretation should indicate whether the titers are considered "significant" or "positive" based upon a 4-fold increase or 4-fold decrease or only as a single high serum IgG titer. The use of IgM titer is not advocated to establish a diagnosis, since it is not as sensitive as IgG alone. It should be remembered that infections with *E. ewingii* yield serologic patterns considered diagnostic for *E. chaffeensis*.

Cases of ehrlichiosis and anaplasmosis should be reported using the CDC's Tick-Borne Rickettsial Disease Case Report form (http://www.cdc.gov/ticks/forms/2010_tbrd_crf.pdf). For the purpose of surveillance, the Council of State and Territorial Epidemiologists and CDC developed a case definition that was amended in 2008 to include HME, HGA, *E. ewingii* infection, and ehrlichiosis/anaplasmosis—undetermined (http://www.cdc.gov/NNDSS/script/casedef.aspx?CondYrID=667&DatePub=1/1/2008%2012:00:00%

20AM). According to this definition, a clinically compatible case is one in which the patient presents with fever, headache, myalgia, anemia, leukopenia, thrombocytopenia, or hepatic transaminase elevation. Definitive confirmation of a clinically compatible case requires (i) a 4-fold change in titer of IgG antibody to *E. chaffeensis* or *A. phagocytophilum* antigen by IFA in paired serum samples, (ii) positive PCR and confirmation of *E. chaffeensis* or *A. phagocytophilum* DNA, (iii) immunostaining of *E. chaffeensis* or *A. phagocytophilum* antigen in a biopsy or autopsy sample, or (iv) culture of *E. chaffeensis* or *A. phagocytophilum* from a clinical sample. A clinically compatible case with (i) an IFA IgG titer ≥64 to *E. chaffeensis* or *A. phagocytophilum* antigen or (ii) identification of morulae in monocytes (HME) or neutrophils (HGA) is classified as probable ehrlichiosis/anaplasmosis. Of note, the CDC does not use IgM test results as independent diagnostic support criteria. The diagnosis of *E. ewingii*, *E. muris*-like agent, and "*Candidatus* Neoehrlichia mikurensis" infection can be established only by nucleic acid amplification methods, since specific antigens and serologic methods are not available. Ehrlichiosis or anaplasmosis "undetermined" is used when serological tests cannot distinguish between infection by *E. chaffeensis* or *A. phagocytophilum*.

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Coxiella[*]

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TAXONOMY

Coxiella burnetii is a small, Gram-negative rod that grows within a parasitophorous vacuole located in the cytoplasm of a host eukaryotic cell (invertebrate or vertebrate animal). It is a member of the *Gammaproteobacteria*. The closest related bacterium is in the genus *Legionella*. *Bergey's Manual of Systematic Microbiology* (2005) classifies it under the order *Legionellales*, family *Coxiellaceae* (1). The only other member of the genus *Coxiella* is the proposed bacterium "*C. cheraxi*," a pathogen of the Australian freshwater crayfish *Cherax quadricarinatus* (2).

DESCRIPTION OF THE AGENT

C. burnetii consists of different morphological forms, depending on the stage of its life cycle. Large-cell variants (LCV) (0.4 to 1.5 μm by 0.2 to 0.5 μm) are metabolically active and divide by binary fission inside the parasitophorous vacuole of the host eukaryotic cell (Fig. 1). Small-cell variants (SCV) (0.5 μm by 0.2 μm) are electron dense and form when conditions are no longer conducive to active growth (3). SCV are filterable (0.22 μm), and this quiescent form of the cell differentially expresses certain proteins compared to LCV (4–7). SCV are functionally spores, although they are chemically different from Gram-positive bacterial spores in that they lack diaminopimelic acid. They act as the survival and transmissible form of the bacterium when it is extracellular and in the environment. However, they are not as heat stable as typical bacterial spores and can be inactivated by incubation at 63°C for 40 min. The cell wall, although Gram negative, stains poorly by Gram stain and better by Gimenez stain.

The complete genome sequences of Nine Mile and other strains of *C. burnetii* demonstrate a circular genome with approximately 2 million base pairs, including many insertion sequences; a single plasmid is found in most strains (8). The presence of many pseudogenes implies a process of ongoing gene degradation, presumably associated with a recent evolutionary adaptation to an intracellular lifestyle. Unlike the *Rickettsiaceae*, *C. burnetii* does not transport ATP across its cell membrane and has almost full biosynthetic capabilities. It is metabolically active in the extracellular phase, utilizes glucose and glutamate at low pH, and was recently grown in cell-free medium (9). Using microarray and whole-genome sequence analyses, variability in open reading frames and transposon-mediated genomic plasticity have been demonstrated (10).

The virulent form of *C. burnetii* is referred to as "phase I," because it is first isolated from humans with Q fever, infected vertebrate animals (especially cattle, sheep, and goats), and ticks. When these isolates are grown for an extended period in the laboratory in tissue culture or embryonated eggs, the population of bacteria can gradually change to a second form (phase II) and become avirulent. This population change can involve loss of genetic material (11) such that the microbe cannot synthesize a full-length polysaccharide, lacking a terminal sugar chain as part of its cell wall lipopolysaccharide (LPS) (12). However, in some strains, loss of virulence does not seem to involve genomic changes (13), presumably due to changes in the expression of genes coding for virulence determinants. The *in vitro* change from virulent phase I to avirulent phase II is analogous to the smooth-to-rough transition that occurs in bacteria of the *Enterobacteriaceae* group. In phase I cells, the terminal glycan chain of the LPS contains three unique sugars, L-virenose, dihydrohydroxystreptose, and galactosaminuronyl-α-(1, 6)-glucosamine, that are not present in phase II LPS (14). Phase I LPS appears to be a key virulence determinant of *C. burnetii*.

EPIDEMIOLOGY AND TRANSMISSION

Coxiella burnetii is associated with vertebrate animals, especially cattle, sheep, and goats. At parturition, when large concentrations of *C. burnetii* are present in the placenta, fetus, and associated membranes and fluids, the microbe readily contaminates the animal's environment. It can remain viable in soil, hay, etc., for many years (possibly decades), presumably in its "spore-like" form (15). Milk from infected cows and other lactating animals can contain *C. burnetii* (16), which is destroyed by the temperature reached during pasteurization.

A number of other vertebrate animals can be hosts for *C. burnetii*, especially native animals, for example, native rats, wombats, bandicoots, and kangaroos in Australia. Infections have been described for pets, including cats and dogs, with human outbreaks in North America linked to parturition by these animals (17–19). Birds are also hosts, and various other domestic and wildlife species, including

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[*] This chapter contains material presented by Philippe Brouqui, Thomas Marrie, and Didier Raoult in chapter 68 of the ninth edition of this *Manual*.

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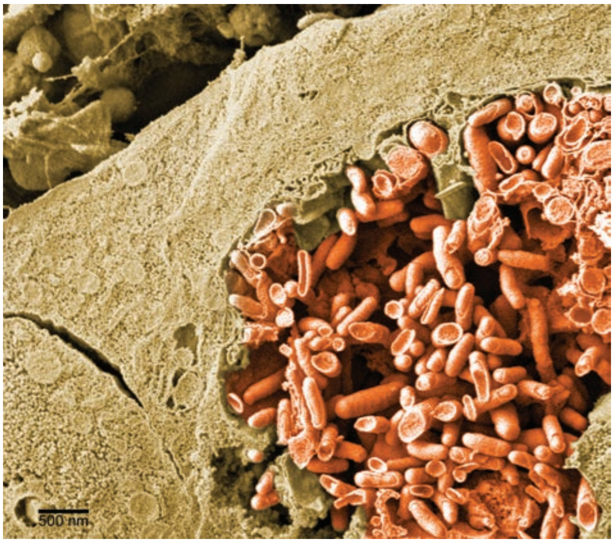

FIGURE 1 Scanning electron micrograph of *C. burnetii* (orange) in the parasitophorous vacuole of a cryo-prepared Vero cell infected with phase II *C. burnetii* strain Nine Mile. (Courtesy of Beth Fischer and Robert Heinzen, Rocky Mountain Laboratories, NIAID, NIH.)
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mice, horses, rabbits, and buffalo, have been reported as potential host species (20).

Numerous tick species either harbor or transmit *C. burnetii* and can be important for maintenance of the agent in veterinary populations, but tick transmission is not considered a major route of transmission to humans. Although there are a few reports of probable human infection by tick bite, the great bulk of human infections is by aerosol transmission from an infected animal focus, such as a herd of parturient goats. Infections in animals are almost always asymptomatic, with recrudescence only during parturition, usually followed by recovery of the maternal animal. Australian studies showed the importance of the bandicoot *Isoodon macrourus* (a marsupial) and its tick, *Haemaphysalis humerosa* (21), and the kangaroo and its tick, *Amblyomma triguttatum* (22), in sylvatic cycles of *C. burnetii* in Australia.

Derrick described Q fever in a large number of Australian patients, including some probably infected by tick bite, although most were infected by inhalation of dust associated with cattle (23). At present, Q fever is thought to be endemic in every country except New Zealand (24). In the United States, 22% of veterinarians are seropositive, compared to approximately 3% of the population overall (25, 26). Travelers and military personnel can also be infected (27). Transmission by aerosol and wind is well recognized (28). Nosocomial transmission, presumably by coughing with aerosol production, has been reported rarely (29), as has infection of surgical and obstetric staff during a cesarean section on an infected patient (30). Sexual transmission has also been noted, as presumably *C. burnetii* was present in semen, from a chronic focus in the prostate gland or testis (31).

The epidemiology of human Q fever is a combination of the worldwide ubiquitous distribution of *C. burnetii*; the extremely low infectious dose required for human infection (probably between 1 and 10 viable *C. burnetii* cells) (32); the environmental conditions favoring transmission, such as high concentrations of infected animals, high pregnancy rates, appropriate environmental conditions (transmission is greater under dry conditions), and the strength and direction of prevailing winds (28); differences between strains of *C. burnetii* (33–35); and the inherent variability in human susceptibility to *C. burnetii*. While some people are exposed and become sick, others are exposed and seroconvert asymptomatically or have only mild symptoms that are not sufficient to lead them to seek medical assistance. The proportion of persons that become ill after natural exposure can be as low as 50% (36).

The largest known outbreak of Q fever occurred in the Netherlands between 2007 and 2010 and was associated with dairy goat farming (37). Over 4,000 cases were recorded. Although multiple *C. burnetii* genotypes were identified among human and livestock samples (38), one founder genotype appears to have become established and diversified over time, eventually spreading from animals to humans due to intensive goat farming (39).

CLINICAL SIGNIFICANCE

Q fever can present in many forms: (i) as an acute, undifferentiated febrile illness; (ii) as a chronic infection, usually involving the cardiovascular system; or (iii) as a postinfection chronic fatigue syndrome. Q fever can be latent and recrudesce during periods of relative immunosuppression, such as late pregnancy, causing fetal infection. Infection can also result in asymptomatic seroconversion and complete clearance of the microbe, with the patient being unaware of infection or being only mildly ill.

Other features of Q fever include a higher incidence of symptomatic disease in men than in women and a higher incidence in middle-aged men than in those of other age groups. Although it is claimed that occupational and exposure differences explain the gender differences, this is probably not the complete picture. For example, female hormones appear to be protective in mice (40), and 86% of mouse genes modulated by *C. burnetii* infection were differentially expressed in males and females, with genes controlling circadian rhythm probably being protective in females (41).

Information on the diagnosis and management of Q fever in the United States has been published by the CDC and its Q fever working group (42).

Pathogenesis

Because *C. burnetii* is an intracellular pathogen, growing in a membrane-bound vacuole in the cytoplasm of a host cell, its survival in the host animal (or patient) depends on its ability to survive and grow intracellularly in the host cell (43). This in turn depends on its ability to keep the host cell alive, and this requires microbe-directed immunomodulation of the host (44). Normally a host cell deals with an invasion by an intracellular microbe by activating the host cell's self-destructing apoptotic process. Indeed, this is what happens in phase II (avirulent) *C. burnetii* infection. The microbe is rapidly internalized (involving receptor $\alpha V\beta 3$ integrin and complement receptor 3) (45), and it grows until the host's cell-mediated immune response, involving gamma interferon and other molecules, induces apoptosis and the infected host cell is destroyed, along with the microbe. Toll-like receptors, including Toll-like receptor 2, are involved in the initial microbe-host cell interaction (46). The silencing of certain host genes by small interfering RNAs increased the size and number of membrane-bound vesicles within *C. burnetii*-infected Vero cells (47).

However, in the case of phase I (virulent) *C. burnetii* infection, a different sequence of events occurs, leading to the survival and growth of the microbe (48, 49). The type 1 LPS fails to activate dendritic cells (50), and the initial contact between the host cell membrane and the bacterium bypasses complement receptor 3 (51). This leads to a different sequence of intracellular events. Genes of a type IV secretion system, similar to the Dot/Icm system of *Legionella pneumophila,* are expressed, leading to secretion of bacterial proteins into the cytoplasm of the host cell (52). These proteins divert the normal intracellular autophagy pathway, which results in the formation of a parasitophorous vacuole. This vacuole gradually enlarges by incorporating recycled endoplasmic reticulum membrane into its own vesicular membrane, allowing *C. burnetii* to grow. The inhibition of apoptosis is mediated by host kinases (53), which allow the microbe to survive by preserving the infected host cell. The metabolically active *C. burnetii* LCV continues to grow in the absence of any effective host immune response, despite the presence of circulating antibodies and T lymphocyte-mediated immunity. Eventually the conditions inside the parasitophorous vacuole become unsuitable for ongoing logarithmic-phase bacterial growth, presumably due to nutrient depletion, and *C. burnetii* converts to the SCV, the nonreplicating survival form. Even then, the host cell may not be destroyed, leading to a state of chronicity or latent infection. Host responses keep the microbe in check, but should immunosuppression develop, such as during pregnancy, the bacterium starts to grow again, causing a Q fever relapse. This is well recognized during the third trimester of pregnancy. In patients with chronic Q fever, there is an impaired maturation of phagolysosomes, permitting ongoing survival of *C. burnetii* (54). Pathogenesis and immunity in regard to Q fever were reviewed recently (55, 56).

Acute Q Fever

Q fever is a difficult disease to diagnose, as there are no pathognomic symptoms or signs that give health care providers a clue to the etiology. Many doctors rarely consider Q fever in the differential diagnosis of an acute febrile illness unless a link with animal contact is established from the patient's history. In fact, many patients without any significant animal contact or tick bite develop Q fever, due to its dispersal by wind (28). Living downwind of a herd of parturient animals, an animal holding yard, or an abattoir is a risk for Q fever. Presenting features can include fever, headache, myalgia, elevated liver transaminases, and interstitial pneumonia.

The great diversity in acute symptoms is probably due to differences in (i) strains of *C. burnetii,* (ii) human immune response genes and how they process and eliminate *C. burnetii* (57), (iii) the route of infection (patients infected by the respiratory route [the most common route] are more likely to develop pneumonia, and patients infected by other routes [e.g., tick bite, oral route, sexually transmitted route, or needle stick accident] can manifest the illness differently), and (iv) the infecting dose. An increased dose leads to a reduced incubation period (32). The infecting dose is likely to influence the symptoms and clinical course of the illness. Those with higher infecting doses are more likely to have severe symptoms. Reviews of Q fever from Australia (23, 58) and elsewhere (20, 59, 60) show the diverse symptoms of this disease.

Occasionally, acute Q fever can be fulminant (61). However, most patients survive acute Q fever and defervesce in about 10 to 14 days, at which time they develop either sterilizing or nonsterilizing immunity. It is the latter patients who can go on to develop chronic Q fever.

Chronic Q Fever

As a result of the early dissemination of *C. burnetii,* many organ systems are exposed and can become chronically infected. The cardiovascular system is particularly susceptible. Most cases of chronic Q fever involve endocarditis (62, 63), including infection of congenitally abnormal (e.g., bicuspid) or previously damaged aortic and mitral valves, aneurysms, and vascular grafts. Pericarditis, myocarditis, and splenic rupture have been reported. Other systems that are often involved in chronic Q fever include the gastrointestinal tract, with chronic granulomatous hepatitis (Fig. 2), acalculous cholecystitis, and diarrhea; the central nervous system, characterized by meningitis and meningoencephalitis; and bones and tendons. Many of these pathogenic features appear to involve autoimmunity, and the presence of autoantibodies is a feature of chronic Q fever (64, 65).

Chronic Q fever cases occurring in the Netherlands outbreak showed a significant risk for patients having had vascular surgery or with a vascular prosthesis or an aneurysm (66). In this series, patients who did not receive antibiotics for acute Q fever (due to mild symptoms) had an increased risk of developing chronic Q fever, indicating the importance of antibiotic treatment of acute Q fever. New diagnostic criteria have been proposed for chronic Q fever, with categories of "proven," "possible," and "probable" based on serology, PCR, clinical features, risk factors, and radiology (67).

Pregnancy and Q Fever

Q fever in pregnancy is an underrecognized problem rarely mentioned as a cause of congenital infection, yet it is clearly a problem in some countries where Q fever is significant. The impact of Q fever on pregnancy may well be dependent on the virulence of the circulating *C. burnetii* strain. In France (68) and Spain (69), it is significant, while in Germany (70), Denmark (71), and the Netherlands (72), it appears not to be so. As pregnancy develops, latent, viable *C. burnetii* starts to grow in the placenta and the fetus, leading to infection and fetal death. While not recognized as one of the classical

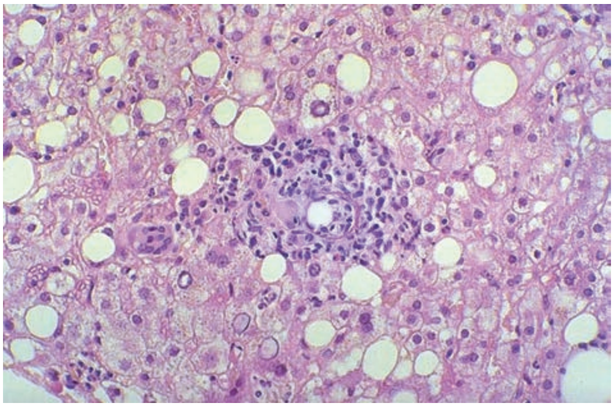

FIGURE 2 Hematoxylin and eosin stain of a liver biopsy specimen from a patient with acute Q fever. A doughnut ring granuloma is shown. Original magnification, ×400. (Courtesy of H. Lepidi, Marseille, France.)
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"TORCH" agents of congenital infection, it should be included under "O" (for "other").

Q Fever in Children

Children appear to be less susceptible than adults to symptomatic Q fever, as are young mice compared to older mice (73). Nevertheless, infections, mainly febrile, influenza-like illnesses or osteomyelitis, have been reported (74, 75).

Post-Q Fever Fatigue Syndrome

A postinfection fatigue syndrome lasting weeks to months after Q fever is now recognized. First described in 1996 (76, 77) in the United Kingdom and Australia, post-Q fever fatigue syndrome is defined as fatigue persisting for more than 12 months after the onset of acute Q fever.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Specimens for the diagnosis of Q fever in humans include blood and tissue, the latter most commonly from heart valves following valve replacement surgery. The sample collected depends on the diagnostic test(s) available. Whole blood can be used for isolation and nucleic acid detection methods, and serum or plasma can be used for serologic methods. Tissue samples are most commonly used for isolation, PCR, and immunohistochemistry (IHC). Whole blood should be collected in sodium citrate or EDTA tubes. Samples to be used for isolation should be collected aseptically and shipped promptly, while maintaining refrigeration. If storage of specimens prior to culture is necessary, samples should be kept frozen before and during shipment (to at least −20°C; −80°C is preferable). Blood or tissue samples to be tested by PCR or IHC should be frozen (−20°C) prior to and during shipment, although tissues fixed by the diagnostic laboratory for IHC may be shipped at room temperature.

For the diagnosis of a suspected acute infection by molecular methods or isolation, whole blood should be collected during the acute phase, preferably prior to antibiotic therapy. For serologic diagnosis of an acute infection, a serum sample should be collected during the acute phase, with a second sample collected 3 or 4 weeks after onset. While the same types of specimens can be used for the diagnosis of chronic infection and acute infection, the timing of collection is not as critical for chronic infection. Blood and tissue samples can be persistently positive by culture and/or PCR for chronic infections, and serum antibody levels are typically elevated (phase I and phase II immunoglobulin G [IgG] titers of >1,000) and sustained relative to those with acute infections, which generally have lower peak titers that decrease postinfection.

C. burnetii can be isolated from blood or tissue samples, and this has traditionally been done in cell culture or embryonated chicken eggs or by animal inoculation. However, the recent development of an axenic culture method may allow for isolation of *C. burnetii* without the use of host cells (78). Isolation must currently be performed in specialized high-containment biosafety level 3 (BSL-3) facilities, as the agent is highly infectious and classified as a U.S. Department of Health and Human Services select agent (79; http://www.selectagents.gov/SelectAgentsandToxinsList.html). If an isolate is propagated by a diagnostic laboratory, U.S. federal regulations require that it be reported to the Federal Select Agent Program within 7 days (http://www.selectagents.gov/form4.html). Diagnostic samples can be evaluated by PCR and serologic methods in BSL-2 facilities with the use of appropriate personal protective equipment.

DIRECT EXAMINATION

Microscopy and Antigen Detection

IHC is an excellent method for the detection of *C. burnetii* antigens in tissue samples (Fig. 3), particularly cardiac valve tissues that are colonized during chronic Q fever. Organisms in heart valve tissues have also been demonstrated by direct immunofluorescence methods or visualized by electron microscopy (Fig. 1). However, these methods are rarely used for the diagnosis of acute Q fever, as the appropriate tissue samples are not often collected and vary among patients and because other methods (PCR and serology) that are simpler to perform on blood samples are available.

Nucleic Acid Detection

PCR can be a useful diagnostic tool for acute and chronic Q fever infections (80). It is important that the sample be collected during the early period of an acute infection, while the patient is bacteremic, optimally within 4 weeks of onset of symptoms. A recent study showed that PCR can be more sensitive than serology during the first 2 weeks after the onset of symptoms and can provide an earlier diagnosis than that with serology alone (81). Whole blood is most commonly used for the analysis of acute infections, although enrichment of the white blood cell fraction (buffy coat) may increase sensitivity. Serum can be used if whole blood is not available, although it is less likely to be positive due to the lack of infected cells. For chronic Q fever with endocarditis, the valve tissue is typically positive, while blood can be positive or negative. It has been reported that PCR is generally positive for chronically infected patients with phase I IgG antibody titers between 800 and 6,400, but PCR is often negative for those with higher titers. A number of PCR assays have been described that amplify the multicopy IS*1111* insertion sequence (82), and these generally provide increased sensitivity compared to assays that amplify single-copy genes (*com1*, the 16S rRNA or 23S rRNA gene, etc.). However, the potential for false-positive

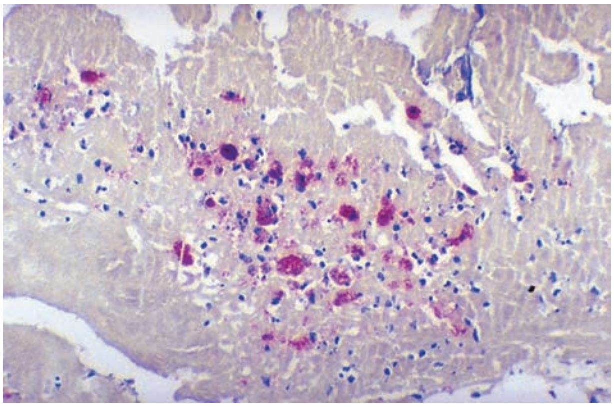

FIGURE 3 Alkaline phosphatase IHC on a heart valve from a patient with chronic Q fever endocarditis. *C. burnetii* microorganisms are stained pink within mononuclear cells. Original magnification, ×400. doi:10.1128/9781555817381.ch66.f3

results with PCR makes it imperative that these results be interpreted relative to those of other diagnostic assays, such as serology, and other clinical data.

ISOLATION PROCEDURES

Isolation of *C. burnetii* from human blood or tissue must be performed in a BSL-3 containment facility due to the low infectious dose of the agent and the potential for generating aerosols. Isolation can be accomplished in tissue culture cells or embryonated chicken eggs or by inoculation into animals, such as mice or guinea pigs. Any infected tissue sample can be used for isolation, and PCR assays are quite useful for screening tissue samples prior to isolation to determine those potentially containing organisms. The organism can be stable in tissue samples for months before isolation attempts. Animal inoculation is the most sensitive method for isolation. A mouse can be injected intraperitoneally with up to 0.5 ml of inoculum, and the spleen is then harvested at 10 to 14 days postinjection. The spleen is homogenized and injected into another mouse, inoculated onto uninfected tissue culture cells, or used to infect embryonated eggs for further propagation. Isolation by animal inoculation is particularly useful for tissue or environmental samples that may be contaminated with organisms other than *C. burnetii*, as the animal serves to amplify *Coxiella* while eliminating other agents. Aseptically collected tissues that likely contain only *C. burnetii* can be inoculated directly onto tissue culture cells or embryonated chicken eggs. A shell vial method that works well has been described for isolation in human embryonic lung fibroblast tissue culture cells (83) and can be used with many commonly available cell lines (e.g., Vero, RK13, THP1, and A549) susceptible to infection by *C. burnetii*. Egg inoculations work particularly well for propagating large amounts of *C. burnetii* for antigen preparation.

C. burnetii has now been cultivated in a cell-free liquid medium, as its metabolic pathways are largely intact. It generates ATP by substrate-level phosphorylation, has an oxidative capacity (is microaerophilic), and has oxygen-protective enzymes, such as peroxidases and superoxide dismutases. It should now be considered a facultative intracellular bacterium (84). It will also grow on agar and can be transformed genetically (78). It retains its phase I (virulent) character for 8 subcultures *in vitro*, and some strains grow to a concentration of 10^9/ml, reaching stationary phase by day 6 of culture (85).

IDENTIFICATION

Only reference laboratories are likely to isolate *C. burnetii* in pure culture and be required to confirm its identification by classical means. In recent years, identification has more often been accomplished by amplifying and sequencing key genes or sequences (e.g., *com1* and IS*1111*). A routine diagnostic laboratory that inadvertently isolates *C. burnetii* in tissue culture may identify it by a direct fluorescent-antibody assay (Fig. 4), although specific antisera would need to be obtained from a reference laboratory.

TYPING SYSTEMS

Typing systems based on genetic differences between isolates are not standardized and are still undergoing development. While no typing scheme is universally accepted, it is clear that there is considerable strain/isolate variability within the species *C. burnetii*. Restriction endonuclease-digested DNAs separated by SDS-PAGE yielded 6 groups from 32 isolates (33), while multispacer sequence typing of intergenic regions produced 30 different allelic combinations that grouped into 3 major clusters (86).

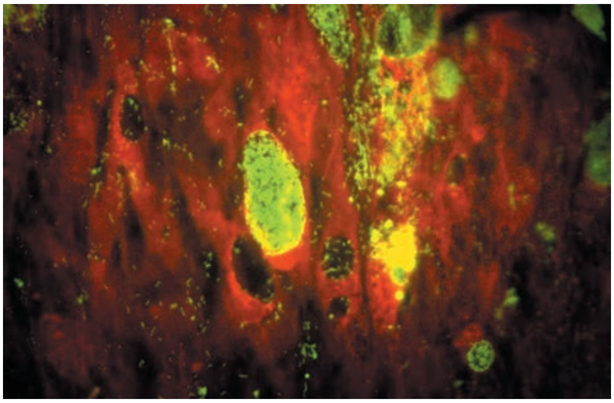

FIGURE 4 Identification of *C. burnetii* in shell vial culture at day 6 by use of a specific monoclonal antibody-based direct fluorescent-antibody test. Original magnification, ×1,000. doi:10.1128/9781555817381.ch66.f4

Single nucleotide polymorphism analysis methods, developed for typing of *C. burnetii* based on data from genome sequences and multispacer sequence typing, showed good correlation with the 6 genomic groups and 35 multispacer sequence typing genotypes previously identified (34). Use of microarrays yielded 10 genomotypes organized into 3 groups; some genomotypes were associated with acute human disease, all were associated with chronic human disease, and one was associated with hard tick isolates (35).

SEROLOGIC TESTS

The detection of antibodies to *C. burnetii* is the most commonly used and effective method for the diagnosis of Q fever. The primary serologic assays in use today are indirect immunofluorescent-antibody (IFA) assay, the complement fixation (CF) test, and enzyme-linked immunosorbent assay (ELISA) (87), with the IFA assay being the gold standard and most commonly used method. CF methods generally lack sensitivity and are used less commonly today, while ELISAs are growing in use and availability. The diagnosis of infection by any serologic assay can be complicated by the facts that *C. burnetii* has a worldwide distribution in nature and that many humans have been exposed and may be seropositive. Most serologic methods make use of *C. burnetii* antigen grown in either tissue culture cells or embryonated chicken eggs. Serologic methods also take advantage of the antigenic differences between naturally occurring virulent phase I *C. burnetii* and attenuated phase II isolates (88). Phase I strains contain intact LPS antigens, while phase II strains lack complete LPS antigens. The antibodies produced during natural human infection respond in a unique time sequence to the phase I and phase II forms, with the acute response directed primarily to phase II antigens, while the response in chronic infections is a mixture of phase I and phase II antibodies.

IFA Test

IFA tests detect IgG, IgM, and IgA antibodies for both phase I and phase II antigens and are useful for the detection of and discrimination between acute and chronic infections

(89). The IFA test has excellent specificity and sensitivity for the diagnosis of Q fever if the appropriate samples are available. The diagnosis of acute Q fever is dependent on seroconversion, defined as a fourfold increase in the IgG titer for phase II antigens between acute- and convalescent-phase samples. The diagnosis of chronic Q fever should not be based solely on an antibody titer. While earlier studies (20, 90) showed high phase I IgG levels in chronic Q fever, recent studies often regard this phenomenon as normal seroprogression (91, 92). Clinical features consistent with chronic Q fever and a high phase I IgG titer (e.g., ≥1,024) are necessary to make a diagnosis of chronic Q fever (42, 63, 67). However, the choice of cutoff titer for acute or chronic disease should be determined in each laboratory, as methods for antigen preparation, assays, and interpretation can vary. Ideally, reference laboratories should provide controls with known positive and negative samples that may be used for in-laboratory assay validation and cutoff determination. FDA-approved, commercial *C. burnetii* IFA tests are available for the diagnosis of acute Q fever (IgG and IgM assays for phase II antigens) and provide the best source for laboratories not equipped to prepare their own antigens (Focus Diagnostics, Cypress, CA). The diagnosis of chronic Q fever is best performed at reference laboratories that have BSL-3 facilities for the propagation and storage of phase I organisms, since *C. burnetii* is classified as a select agent.

Enzyme-Linked Immunosorbent Assay

ELISAs have been reported to be as sensitive and specific as IFA tests (93). FDA-approved, commercial ELISAs that detect IgG or IgM antibodies to phase II antigens are available (Inverness, Brisbane, Australia) and are useful for detection of IgM antibodies (94), although they are not currently marketed in the United States. A number of reference laboratories and research institutions have also developed in-house ELISAs. In general, ELISAs are qualitative and have not been evaluated as thoroughly as IFA tests. Commercial and in-house ELISAs can be automated to require minimal interpretation and are particularly useful for the detection of IgG antibodies to phase II antigens when employed in seroprevalence studies. However, their lack of standardization and quantification makes them less than ideal for the diagnosis of acute Q fever, and the lack of phase I antigens in commercial assays limits their use for the diagnosis of chronic Q fever.

CF Test

Whereas the CF test is still commonly used as a diagnostic assay for veterinary testing, the assay is rarely used for human diagnostics due to advances in IFA and ELISA methods and the demonstration that the CF test has a lower sensitivity, is more time-consuming, and detects seroconversion at a later date than the other assays. False-negative results with the CF assay have also been described for chronic infections with high titers, due to a prozone effect.

ANTIMICROBIAL SUSCEPTIBILITIES, TREATMENT, AND PREVENTION

In vitro antimicrobial susceptibility testing is not routinely performed. *C. burnetii* is an intracellular pathogen, and correlations between *in vitro* MICs of antibiotics in an infected tissue culture system and antibiotic activities in infected patients are uncertain.

The recommended treatment for acute Q fever is doxycycline, although strains with partial doxycycline resistance have been reported. Erythromycin and azithromycin are not recommended, as *in vitro* testing suggests that some strains are resistant (MICs of >8 μg/ml) (95, 96). Neither ciprofloxacin nor chloramphenicol is recommended, as these drugs do not inhibit *in vitro* growth (83, 97). Rifampin can be used, although it is not recommended in the United States. Treatment with cotrimoxazole during pregnancy is recommended, as infection adversely affects pregnancy outcomes (98).

Chronic Q fever, especially Q fever endocarditis, requires long-term antibiotics and often requires valve replacement. Dual therapy with doxycycline and hydroxychloroquine is recommended for a minimum of 1 year, and possibly longer. Hydroxychloroquine increases the pH of the phagolysosome from 4.8 to 5.7 and renders doxycycline bactericidal rather than bacteriostatic (99).

A human Q fever vaccine (QVAX) has been developed and shown to be efficacious (100, 101). Although the vaccine is commercially available only in Australia, it was used in the Netherlands outbreak in 2011 to prevent chronic Q fever in predisposed individuals.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Serological and nucleic acid amplification (PCR) reports for a patient evaluated for Q fever can be difficult to understand. Interpretation should always be provided and should include the following: (i) the significance of the antibody titer, particularly the difference between antibodies to phase I and phase II *C. burnetii* and the different antibody classes detected (IgM, IgG, and IgA); (ii) whether the serologic result supports recent Q fever (high phase II IgM titer), past Q fever (high phase II IgG titer), or chronic Q fever (high phase I IgG and IgA titers); (iii) the limitations if there is only one serum sample available for testing and the importance of receiving a second serum sample to detect a fourfold increase in antibody titer (for example, a raised phase II IgM titer in only one serum sample may represent very early acute Q fever or a false-positive result); (iv) in locales where vaccination occurs or where post-Q fever fatigue syndrome is considered, a comment stating that no antibody pattern can currently define either condition; and (v) an indication that detection of *C. burnetii* DNA in blood usually indicates acute, very early Q fever, often before seroconversion. Tissue specimens (e.g., heart valve, liver, and bone marrow) are most often positive in chronic Q fever, although peripheral blood leukocytes from such patients can be positive.

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Tropheryma whipplei

WALTER GEIßDÖRFER, ANNETTE MOTER, AND CHRISTIAN BOGDAN

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Tropheryma whipplei is the agent of classical Whipple's disease, a systemic infection originally described by the American pathologist George Hoyt Whipple in 1907 as intestinal lipodystrophy (1–4). Since then, *T. whipplei* has also been implicated in isolated infections of the central nervous system, the heart valves, joints, or other organs. A remarkable amount of research during the past 20 years has yielded new insights into the epidemiology, biology, pathogenicity, and immunological control of this Gram-positive bacterium. However, the early diagnosis of infections with *T. whipplei* still remains a challenge for physicians and microbiological laboratories due to the rarity of the disease, the diversity and nonspecificity of symptoms, the fastidious nature of the pathogen, and the lack of noninvasive serological tests (3).

TAXONOMY

In 1991, Wilson et al. identified the "Whipple's disease-associated bacterium" by amplification of a 16S rRNA gene fragment from a small-bowel biopsy specimen and sequence analysis (5). The preliminary name "Tropheryma whippelii" was derived from "trophe" and "eryma" (Greek words for nourishment and barrier) and was changed to *T. whipplei* in 2001 (6). *T. whipplei* is a member of the family of *Cellulomonadaceae*, which belongs to the phylogenetic subdivision of Gram-positive bacteria with high GC content (Class *Actinobacteria*, Order *Actinomycetales*). However, *T. whipplei* has an exceptional status within the *Actinobacteria*, owing to the low homology of its 16S rRNA gene sequence to other *Actinobacteria* (<92%), small genome size (approximately 930 kbp), and low GC content (46.3%) (7).

DESCRIPTION OF THE AGENT

Morphology

Although *T. whipplei* is categorized as a Gram-positive actinobacterium, it nevertheless stains poorly with Gram's method and is negative in acid-fast staining. *T. whipplei* can be detected as intracellular clusters within phagocytic vacuoles or as extracellular bacteria (8). It forms slender rods that react well to periodic acid-Schiff (PAS) staining due to glycoproteins in its unique trilaminar envelope (6, 9). After degradation of the bacteria by macrophages, remnants of the cell envelope are left as intracellular PAS-positive inclusions (9).

Culture

Since the first anecdotal report on the growth of *T. whipplei* in human macrophages deactivated by interleukin-4 (10) and the later documented culture and isolation of *T. whipplei* in a human fibroblast cell line (8), *T. whipplei* strains could be isolated from various clinical samples, including heart valves, blood, duodenal biopsy specimens (11), cerebrospinal fluid (7), synovial fluid (12), vitreous fluid (13), skin (14), saliva (15), and stool specimens (16). After genomic data revealed the absence of most genes necessary for amino acid synthesis, a cell-free culture method was developed using a medium supplemented with the missing amino acids (17). However, even optimized and standardized culture protocols of *T. whipplei* culture are insensitive, laborious, and slow and are therefore not applicable as routine methods for the diagnosis of *T. whipplei* infection.

Genome

Data from two sequenced *T. whipplei* genomes revealed a reduced genome (<1 Mbp) lacking key biosynthetic pathways. On the other hand, cell surface proteins seem to be abundant, including a new family of *T. whipplei* surface proteins (WiSP). Repetitive sequence elements may play a role in genetic and antigenic variation, suggesting host-dependent coevolution of *T. whipplei* (7).

EPIDEMIOLOGY AND TRANSMISSION

The estimated annual incidence of Whipple's disease is 1 case per 100,000 to 1,000,000 inhabitants (4), which makes Whipple's disease a rare condition. Infections with *T. whipplei* are mainly restricted to white Caucasians in Europe and North America (4) and primarily affect middle-aged (average age, 56 years) males (73%) (18).

T. whipplei DNA can be detected in wastewater from sewage plants, indicating an environmental occurrence of this bacterium (19, 20). *T. whipplei* can also be found in asymptomatic, healthy humans, suggesting that it can be a commensal bacterium and that human carriers may represent one of its reservoirs (2). Genotyping studies suggest interhuman transmission of *T. whipplei* (probably via fecal-oral or oral-oral routes), as the same strains have predominantly been found within families or contact persons in France (21) or rural villages in Senegal (2).

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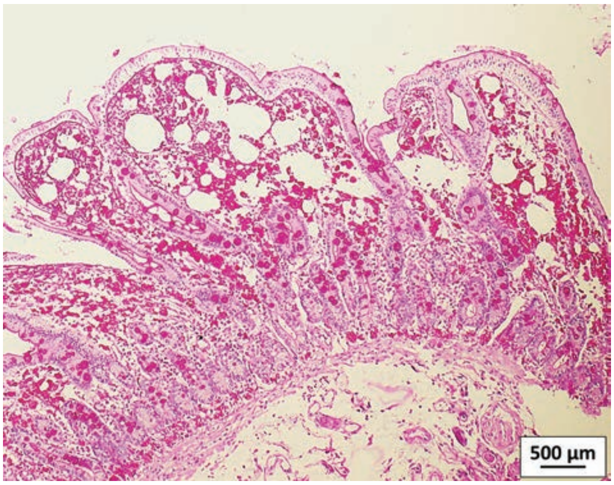

FIGURE 1 PAS staining of a duodenal biopsy specimen shows lymphangiectasia and massive infiltration of the lamina propria by strongly PAS-positive foamy macrophages containing *T. whipplei* (image kindly provided by Christoph Loddenkemper, Pathotres, Berlin, Germany).
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CLINICAL SIGNIFICANCE

Pathogenesis and Immunology

Local or systemic disease following *T. whipplei* infection is rare despite an abundance of this microbe in the environment and a high rate of human colonization. Thus, manifestations of clinical disease might require an immunological predisposition. According to the current model (2–4, 22), *T. whipplei* is taken up orally, primarily in early childhood, leading to temporary asymptomatic carriage, self-limiting gastroenteritis, fever, or cough. Usually, a protective humoral and cellular immune response will develop, resulting in the clearance of *T. whipplei*. In predisposed persons, *T. whipplei* may persist and spread systemically over the years, resulting in classical Whipple's disease with infiltration of the lamina propria by alternatively activated macrophages (Fig. 1 and 2), which cannot efficiently kill *T. whipplei* (23). In addition to a shift of T-cell subsets from Th1 and Th17 cells toward Th2 and regulatory T cells (24), there is also evidence for a reduced number of immunoglobulin-positive plasma cells and an impaired secretion of immunoglobulins in the duodenal lesions of Whipple's disease patients (25). The characterization of bacterial factors involved in disease pathogenesis and the modulation of immune response, as well as identification of host factors predisposing to symptomatic *T. whipplei* infections, are themes of current research.

Classical (Systemic) Whipple's Disease

In classical Whipple's disease, the infection with *T. whipplei* probably occurs years before clinical symptoms point to the diagnosis. Chronic diarrhea, as one of the leading signs, can start mildly; often migratory arthralgias of large joints are the prominent findings. After the prodromal state that can last for years, the condition of the patients may worsen rapidly with aggravated diarrhea, malabsorption, weight loss, abdominal pain, and lymphadenopathy, leading to the full picture of a wasting syndrome. Nonspecific symptoms like fever, (night) sweats, and asthenia may be present. Depending on the organ systems affected by the disease, a multitude of symptoms can emerge (Table 1). Involvement of the central nervous system is a serious complication of classical Whipple's disease that occurs in approximately one-fifth of patients (18). The prognosis is unfavorable because irreversible damage may occur, and antibiotic treatment failure has been described. In patients newly diagnosed with Whipple's disease, cerebrospinal fluid for PCR diagnosis should be obtained.

Isolated Organ Manifestations

Joints

Whereas arthropathy is the most common symptom in classical Whipple's disease, it may also be the only clinical manifestation (Table 1). When it appears as migrating oligo- or polyarthritis, it is often misdiagnosed as rheumatoid arthritis. This may result in erroneous immunosuppressive treatments with corticosteroids or tumor necrosis factor antagonists which then cause a dramatic deterioration of the disease. Therefore, it is prudent to test patients with presumed seronegative rheumatoid arthritis for Whipple's disease by PCR examination of synovial biopsy specimens or joint aspirates.

TABLE 1 Symptoms and signs of *T. whipplei* infections

Organ system	Location and/or material	Signs and symptoms
General condition		Weight loss, fever, impaired health condition
Orogastrointestinal tract	Esophagus, stomach, duodenum, jejunum, ileum, cecum	Acute and chronic diarrhea, abdominal pain
	Stool samples	Asymptomatic carrier; acute gastroenteritis?
	Saliva specimens	Asymptomatic carrier
Joints and bones	Joint fluid, biopsy specimens	Arthralgia
CNS	CSF	Headache, dementia, depression, seizures, ophthalmoplegia
Cardiovascular	Infective endocarditis; explanted heart valve	Vegetations on heart valves, symptoms and signs of valve stenosis or insufficiency
	Pericarditis, myocarditis	Pericardial effusion
Skin	Skin biopsy specimens	Cutaneous pigmentation
Eye	Vitreous humor	Uveitis, retinitis, vision loss, eye floaters
Lymphatic system	Lymph nodes	Lymphadenopathy
Lung	Bronchoalveolar lavage fluid, pleural effusion, lung biopsy specimen	Acute pneumonia
Kidney (rare)	Kidney biopsy	Secondary amyloidosis

Central Nervous System (CNS)

Nonspecific neuropsychiatric manifestations can be the only pathological findings of *T. whipplei* infection, ranging from psychiatric disorders such as depression, personality changes, dementia, or memory impairment to ocular movement disturbances, epileptic seizures, ataxia, and headache. In magnetic resonance imaging or computed tomography scans, solitary or multiple enhancing lesions can be present involving the temporal lobe, midbrain, hypothalamus, and thalamus region (26). Diagnosis is often delayed and is made by PCR detection of *T. whipplei* in cerebrospinal fluid (CSF) or brain biopsy specimens.

Eyes

Two studies on Whipple's disease with 696 or 113 patients revealed an ocular involvement in 2.7 or 6% of the cases, respectively (18, 27). Symptoms may be nonspecific, occurring bi- or unilaterally, and include mild vision loss or blurry vision, diplopia, eye floaters, or neuro-ophthalmic findings such as ophthalmoplegia, nystagmus, and papilledema (28, 29). Characteristic ocular findings are intraocular inflammation (uveitis, retinitis, and endophthalmitis) or crystalline keratopathy. In most cases, diagnosis of the typically chronic and corticosteroid-resistant infection is only made years after the onset of symptoms by PCR examination of vitreous humor. In general, ocular infection by *T. whipplei* points to classical Whipple's disease and, therefore, can also be diagnosed in other tissues (e.g., small bowel biopsy specimens). However, at the time of presentation, ocular involvement may be the only clinical sign of Whipple's disease (28, 29).

Endocarditis

Endocarditis (Fig. 3) might be the most frequent manifestation of *T. whipplei* infection and accounts for 8.5 and 6.3% of culture-negative endocarditis cases in France (Department Rhône-Alpes) (30) and Germany (31), respectively. Endocarditis may occur as a complication of classical Whipple's disease (6/113 cases in reference 18), but in the majority of cases, it is a localized infection (14/16 cases in reference 31). This suggests that most patients with isolated *T. whipplei* endocarditis might not exhibit the putative immunodeficiency which predisposes patients to systemic Whipple's disease. Valvular vegetations are detected by echocardiography in 78% of the patients. Arthralgias are the most frequent (75%) clinical symptom, whereas fever is present in only 39% of the cases. Duke criteria are not helpful in establishing a definitive diagnosis of Whipple's endocarditis (30, 31). PCR analysis of explanted heart valves is the diagnostic method of choice.

IRIS

Immune reconstitution inflammatory syndrome (IRIS), which was first reported as a complication following treatment of Whipple's disease in 2009 (32), has been diagnosed in up to 11% of treated patients. Prior immunosuppressive treatment, e.g., for suspected rheumatoid arthritis, is a risk factor for the development of an IRIS. After initial clinical improvement, immune reaction to nonviable *T. whipplei* antigen results in symptoms including fever, arthralgias, gastrointestinal symptoms, and skin irritation. PCR tests typically remain negative in this situation. After exclusion of opportunistic infections, corticosteroid treatment may be effective (see reference 33 and references therein).

Acute Gastroenteritis

Diarrhea and abdominal pain are frequent symptoms in classical Whipple's disease and may be a consequence of malabsorption and gut infection. Recently, large quantities of *T. whipplei* DNA have been detected in children with acute gastroenteritis, raising the question whether *T. whipplei* may also cause acute gastroenteritis in otherwise healthy people (34). However, it remains unclear whether this unusual finding reflects causality or an accidental association.

Skin

Manifestations like melanoderma, hyperkeratosis, peripheral edema, petechiae, and purpura may be present in late stages of the disease. Interestingly, *T. whipplei* DNA can often be detected in skin biopsy specimens of patients with Whipple's disease, even in the absence of cutaneous symptoms (14). Highly sensitive PCR methods may provide a mechanism to screen skin biopsy specimens for *T. whipplei* DNA, although negative PCR results do not exclude Whipple's disease (35).

Lungs

Pulmonary involvement (pleural effusion, pulmonary infiltration, and granulomatous mediastinal adenopathy) is estimated to occur in 30 to 40% of patients with classical Whipple's disease (1). Interstitial lung disease with detection of *T. whipplei* in biopsy specimens of pulmonary parenchymal nodules has been reported in only a few cases (18, 36). These patients presented usually with dry cough and shortness of breath, mostly accompanied by arthralgias and fever. In the absence of gastrointestinal symptoms, pulmonary manifestations may lead to the primary diagnosis of Whipple's disease. The relevance of increased *T. whipplei* colonization in human immunodeficiency virus-positive compared to human immunodeficiency virus-negative individuals (13 versus 1%) needs further investigation (37).

Healthy Carriers

T. whipplei can be detected in saliva, dental plaque, intestinal biopsy, gastric juice, and stool specimens of healthy individuals without signs of Whipple's disease. Although early reports may have overestimated the prevalence rate because of false-positive PCR results (38), colonization of asymptomatic adults is well established. In stool and saliva specimens, the estimated prevalence rates were 4% and <1%, respectively (39, 40). Higher prevalence rates were found in humans with increased risks for acquisition of *T. whipplei* (e.g., in sewage workers [40], homeless people [41], and family members of Whipple's disease patients [21]). As the prevalence and bacterial load is far higher in stool and saliva samples of Whipple's disease patients, PCR can be used as a diagnostic tool (14, 39). However, positive results are not proof of infection. A very high prevalence of *T. whipplei* DNA (75%) was reported in stool samples of children under 5 years of age in Senegal (2). As environmental specimens were largely negative for *T. whipplei* DNA and systemic Whipple's disease is restricted to Caucasians, this finding most likely reflects a human reservoir of *T. whipplei* and early colonization due to fecal-oral transmission (42).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Small bowel biopsy specimens should be examined by PAS staining and PCR whenever classical Whipple's disease is suspected (Fig. 4). To avoid sampling errors in cases of sparse or patchy prevalence of PAS-positive macrophages, at least five endoscopic biopsy specimens from the small bowel (distal duodenum and/or jejunum) should be evaluated. In the case of positive results, CSF should be analyzed

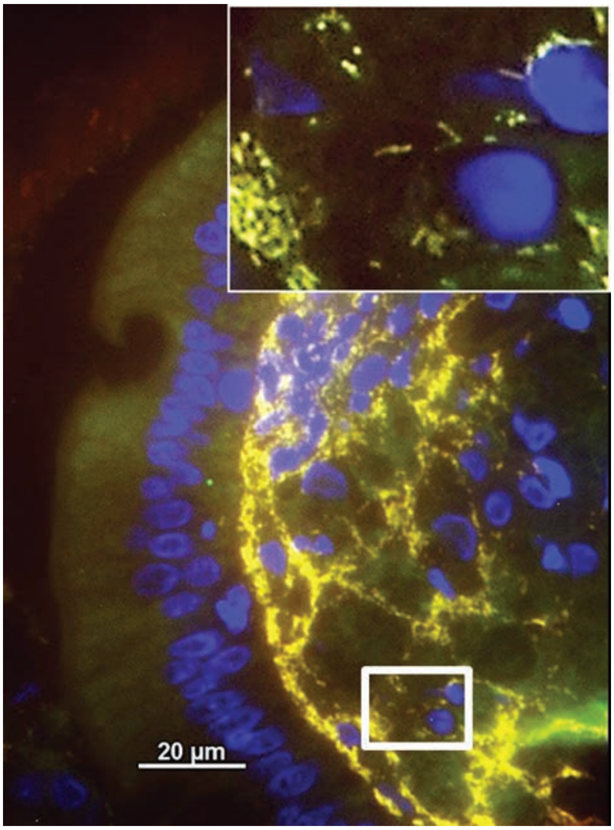

FIGURE 2 FISH of a duodenal biopsy of an untreated Whipple's disease patient using a *T. whipplei*-specific probe (48). The low-magnification image shows clusters of *T. whipplei* cells (yellow) against the slightly greenish autofluorescent background of the tissue. Host cell nuclei are stained blue with 4′, 6-diamidino-2-phenylindole (DAPI). Inset shows single *T. whipplei* bacteria at a higher magnification.
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by PCR to verify or exclude CNS involvement. A recently published alternative workflow proposes the screening of stool, saliva, and skin biopsy specimens using highly sensitive PCR methods as first steps in the diagnosis of classical Whipple's disease (35). To test for isolated *T. whipplei* infection, a sample of the affected organ should be examined by PCR and histological methods including PAS staining. When positive results are obtained, gastrointestinal and CNS involvement should be excluded.

Samples for PAS staining, immunohistochemistry (IHC) or fluorescence in situ hybridization (FISH) analysis should be fixed in 10% formalin and transported at room temperature. For PCR, the samples must not be fixed (i.e., no formalin pretreatment) and can usually be transported at room temperature within 1 day. If assays cannot be done within 1 day, fresh samples can be stored for a few days at 4°C and for long-term storage at −80°C. For culture, fresh samples should be frozen at −80°C and transported on dry ice.

DIRECT EXAMINATION

Microscopy

PAS Staining

PAS staining of biopsy specimens from the lower duodenum or jejunum typically shows an infiltration of the lamina propria with macrophages containing PAS-positive, diastase-negative granular inclusions of intracellular or ingested bacteria (Fig. 1). However, PAS staining can be both false-positive due to infection with other agents (e.g., nontuberculous mycobacteria) and false-negative as a consequence of low bacterial load (43). Therefore, every positive PAS result should be confirmed by an independent and specific method (PCR, IHC, or FISH). Similarly, in case of continuing clinical suspicion of Whipple's disease despite a negative PAS result, PCR should be performed due to its high sensitivity to detect small quantities of *T. whipplei* (3, 4) (Fig. 4).

In patients without gastrointestinal manifestations, specimens from the clinically affected sites, e.g., explanted heart valves, lymph nodes, synovial tissue, CSF, or brain biopsy specimens, may be PAS positive, whereas duodenal biopsy specimens remain negative (3).

For experienced pathologists, PAS staining is appropriate to assess the success of treatment based on the change of inclusions from intensely PAS-positive granular particles to faintly PAS-positive cytoplasm without granulation (44).

Transmission EM

Transmission electron microscopy (EM) can be used to identify *T. whipplei* in ultrathin histological sections from infected organs due to its unique trilamellar cell wall (9). As EM is laborious, time-consuming, and available in only a few specialized laboratories, its use is restricted to the analysis of special cases (43).

Antigen Detection

IHC

IHC with polyclonal rabbit anti-*T. whipplei* antibodies is able to specifically visualize the bacteria in circulating monocytes (45) and in histological slides from various tissues (46). Compared to PAS staining, IHC is more specific and sensitive in untreated Whipple's disease but less sensitive after treatment (46). As the antiserum is not yet commercially available, this method is restricted to a few specialized laboratories. Like FISH, IHC may be used to confirm positive results obtained by PAS staining.

Nucleic Acid Detection

FISH

FISH is a molecular technique using fluorescently labeled oligonucleotide probes to target *T. whipplei* 16S rRNA in histological sections. FISH is particularly useful to verify and localize *T. whipplei* in extraintestinal tissues (31, 47, 48) or to confirm PAS-positive staining of small bowel biopsy specimens (Fig. 2 and 3). FISH probes target the ribosomes which correlate with the activity of bacteria, whereas PAS staining stays positive for prolonged periods under therapy. However, so far, detection of *T. whipplei* by FISH is available only in specialized centers and is still regarded as a research rather than a routine technique.

PCR

Although PAS staining is still the first-line method for analysis of duodenal biopsy specimens, PCR outcompetes PAS staining with respect to total performance (sensitivity, specificity, time to result, and hands-on time) (43). Laboratories generally use in-house PCR protocols (for examples, see reference 49 and 50) and, therefore, are responsible for validation and accurate quality control procedures. This is important, as PCR is prone to laboratory contamination

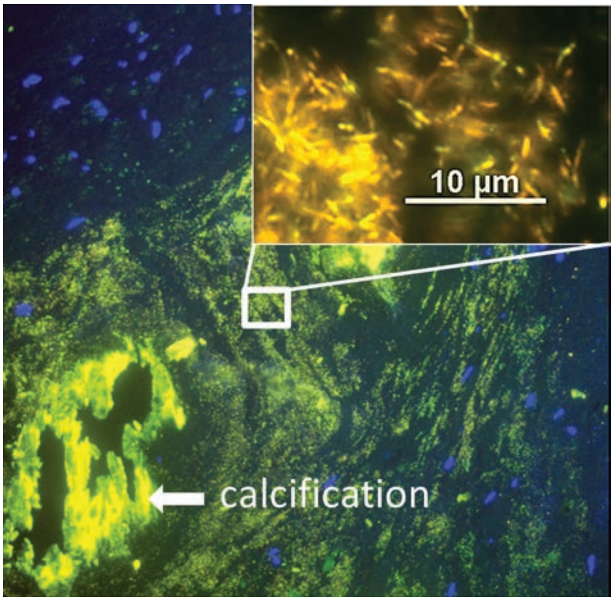

FIGURE 3 FISH of a heart valve section in a case of culture-negative endocarditis using a *T. whipplei*-specific probe (Cy3, orange) (48). The low-magnification image shows extensive areas with bacterial colonization (slightly orange glimmer) along with calcified tissue (bright yellow-green autofluorescence). Host cell nuclei are stained blue with 4′,6-diamidino-2-phenylindole (DAPI). Inset shows dense clusters of *T. whipplei* cells at a higher magnification (orange). doi:10.1128/9781555817381.ch67.f3

events (especially when using nested PCR) or false-positive results as a consequence of nonspecific reaction conditions or primer design. For instance, in the past, some positive PCR results were shown to be due to cross-reactivity with *Actinomyces odontolyticus* (38). Substantial improvement in terms of specificity and prevention of DNA contamination was achieved by using modern real-time PCR protocols with hybridization probes (40, 51). However, cross-reactions might still occur when analyzing polymicrobial samples (e.g., saliva or stool specimens) that contain numerous (unculturable) bacterial species with unknown DNA sequences. Therefore, PCR results should always be interpreted in the context of clinical and histopathological findings, and the initial diagnosis of Whipple's disease should not rely solely on one isolated PCR result. Moreover, a confirmatory PCR using a different target sequence, sequence analysis of 16S rRNA gene sequence, or genotyping PCR should be performed.

T. whipplei-specific PCRs can be performed with tissue specimens from any infected organ (duodenal biopsy tissue, lymph nodes, heart valves, vitreous humor, synovial fluid, or CSF). Stool and saliva specimens may be used for screening of *T. whipplei* carriage but do not prove infection. Analysis of peripheral blood samples is possible, but negative PCR results will not rule out infection (52). Universal bacterial ("eubacterial" or "panbacterial") PCR (16S rRNA gene amplification) followed by sequence analysis shows lower sensitivity than *T. whipplei*-specific PCR protocols. Therefore, it is only suitable for examination of specimens containing high bacterial loads, such as explanted heart valves in cases of *T. whipplei* endocarditis (30, 31), and may fail to detect the small amount of bacteria present in CSF.

ISOLATION PROCEDURES, IDENTIFICATION, AND TYPING SYSTEMS

Isolation of *T. whipplei* from clinical samples using long-term culture methods (8, 11, 17) is challenging and established only in very few laboratories worldwide.

Identification of *T. whipplei* and typing methods for epidemiological research are exclusively based on analyses of PCR-amplified DNA sequences and can be performed directly from sample material without cultivation. The most common target used is the 16S-23S rRNA intergenic spacer region with 7 known sequence types (reference 53 and references therein). Analysis of four highly variable genomic sequences (54) shows high discriminatory power and reveals more than 70 sequence types from 191 samples (55). No correlation between *T. whipplei* sequence types and pathogenicity, virulence, or specific organotropism has been described.

SEROLOGIC TESTS

Although several attempts have been made to identify *T. whipplei* proteins as antigens for serodiagnosis of Whipple's disease (56–58), no routine diagnostic assay with sufficient sensitivity and specificity is currently available. Indirect immunofluorescence assays using *T. whipplei* cell cultures as antigens proved to be problematic because control groups showed a high prevalence of reactive immunoglobulin G and a large proportion of patients was negative for *T. whipplei*-reactive immunoglobulin M (8, 58).

ANTIMICROBIAL SUSCEPTIBILITIES

T. whipplei was susceptible *in vitro* to many antibiotics, including penicillin G, macrolides, doxycycline, rifampin, chloramphenicol, and co-trimoxazole (59). Ciprofloxacin was found to be ineffective (60). Ceftriaxone and gentamicin showed high-level activity against extracellular but not intracellular bacteria. Susceptibility to imipenem was variable. *T. whipplei* is resistant to trimethoprim, as the gene encoding its target (dihydrofolate reductase) is absent from the *T. whipplei* genome (61). Consequently, sulfamethoxazole is the active compound of co-trimoxazole, but reduced susceptibility and resistance of clinical *T. whipplei* isolates to sulfonamides have recently been reported also (reference 62 and references therein).

If left untreated, systemic Whipple's disease is fatal. The former commonly used treatment with tetracycline resulted in high rates of CNS relapse (63). Current recommendations consist of an initial therapy with a parenteral expanded-spectrum cephalosporin, carbapenems, or high-dose penicillin G to achieve complete clearance of *T. whipplei* from the CNS, followed by 1 year of oral treatment with co-trimoxazole (Table 2) (64). Substantial clinical improvement within 7 to 21 days and subsequent complete recovery can be achieved in most patients. However, for trimethoprim-sulfamethoxazole, treatment failures and relapses have been reported (reference 65 and references therein). A highly efficient alternative regimen showing bactericidal activity consists of a combination of hydroxychloroquine and doxycycline for 1 year, followed by a life-long monotherapy with doxycycline (65) (Table 2). Hydroxychloroquine is thought to neutralize the low pH in acidic vacuoles, in which *T. whipplei* replicates (23). Relapse of Whipple's disease following therapy with doxycycline plus hydroxychloroquine has been reported (66).

For isolated organ manifestations, e.g., endocarditis or joint infection, there are no published recommendations for

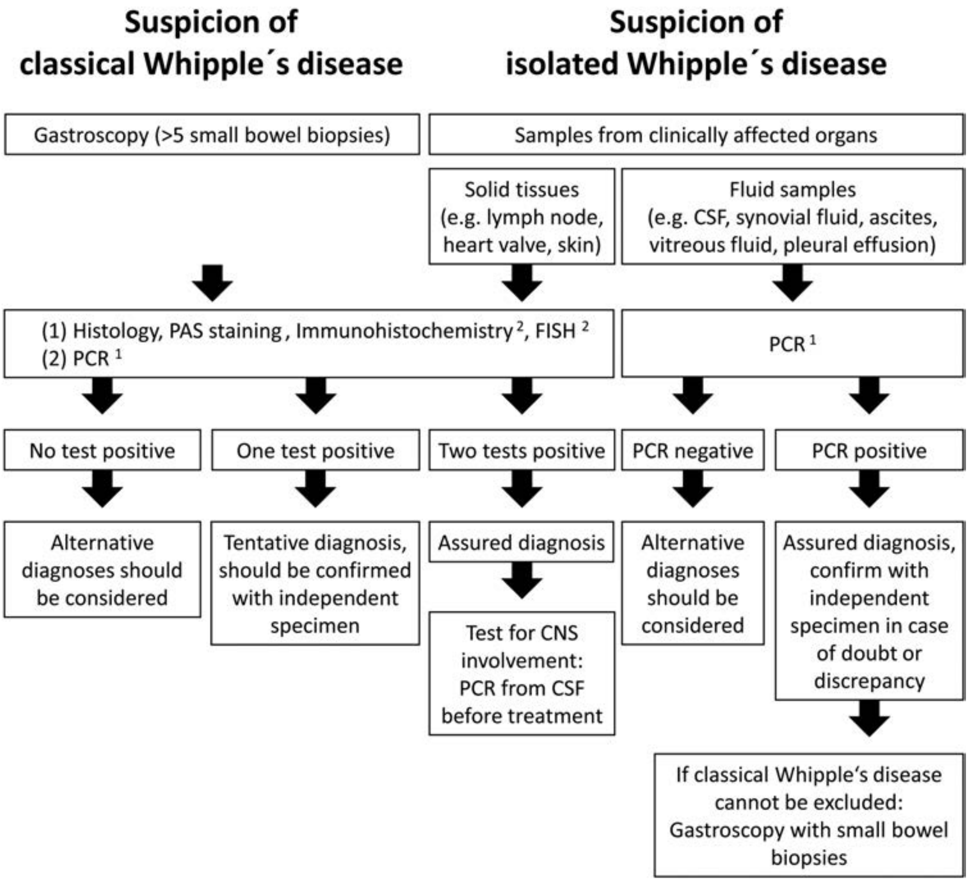
Suspicion of classical Whipple´s disease
Suspicion of isolated Whipple´s disease
Gastroscopy (>5 small bowel biopsies)
Samples from clinically affected organs
Solid tissues (e.g. lymph node, heart valve, skin)
Fluid samples (e.g. CSF, synovial fluid, ascites, vitreous fluid, pleural effusion)
(1) Histology, PAS staining, Immunohistochemistry 2, FISH 2
(2) PCR 1
PCR 1
No test positive
One test positive
Two tests positive
PCR negative
PCR positive
Alternative diagnoses should be considered
Tentative diagnosis, should be confirmed with independent specimen
Assured diagnosis
Test for CNS involvement: PCR from CSF before treatment
Alternative diagnoses should be considered
Assured diagnosis, confirm with independent specimen in case of doubt or discrepancy
If classical Whipple's disease cannot be excluded: Gastroscopy with small bowel biopsies

FIGURE 4 Hierarchical scheme for the diagnosis of Whipple's disease (modified from references 3 and 4). 1, positive PCR should be confirmed by 16S rRNA gene sequencing or a second specific PCR detecting a different *T. whipplei* target sequence; 2, IHC and FISH are available only in specialized laboratories. doi:10.1128/9781555817381.ch67.f4

treatment, but a shorter duration of the standard antibiotic treatment may be feasible. In the case of cerebral involvement, patients may benefit from adjunctive corticosteroid therapy. Corticosteroids have also been suggested in patients with IRIS (67). Immune therapy with gamma interferon was successfully used in a patient with relapsing disease (68).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Following the first diagnosis of Whipple's disease in a patient, the attending physicians and the responsible laboratory personnel (pathologist and microbiologist) should jointly review the test results and their correlation with the clinical presentation of the patient and decide on confirma-

TABLE 2 Recommended therapy for Whipple's disease (4, 65)[a]

Therapy type	Indication	Initial therapy (2 wk)	Maintenance therapy (≥1 yr)
1st line	Standard regimen	CRO, 1 × 2 g i.v./24h or Pen G, 6 × 2 MU i.v./24h or Procaine Pen, 1 × 1–2 MU/24h	TMP-SMX, 2 × 160/800 mg (p.o.)/24h
2nd line	Allergies to Pen or CRO	TMP-SMX, 3 × 160/800 mg p.o./24h plus STM, 1 × 1 g i.m./24h	TMP-SMX, 2 × 160/800 mg p.o./24h
	Alternative (see text)	DOX, 2 × 100 mg p.o./24h plus HCQ, 3 × 200 mg p.o./24h (followed by lifetime DOX)	

[a] Abbreviations: i.v., intravenously; i.m., intramuscularly; p.o., orally; Pen, penicillin; CRO, ceftriaxone; TMP-SMX, trimethoprim-sulfamethoxazole; STM, streptomycin; DOX, doxycycline; HCQ, hydroxychloroquine.

tory diagnostic approaches with a second, independent sample or method. A positive PCR should be confirmed by sequence analysis or by a different PCR test targeting another *T. whipplei*-specific DNA sequence. In each case, examination of small bowel biopsy specimens and CSF should be considered. For PCR, fresh tissue specimens are always preferable to paraffin-embedded samples, as the sensitivity is considerably higher and the risk of DNA contamination (as it might occur when extracting DNA from paraffin blocks) is much lower. Negative PCR results from blood samples do not rule out infection due to the generally low sensitivity observed with this specimen type. In the case of saliva and stool, positive PCR results do not differentiate between a carrier state and a florid infection.

Once the laboratory detection of *T. whipplei* is confirmed, a decision on the treatment regimen and follow-up strategy must be made. In the case of any doubts about the diagnosis, therapy, and/or follow-up of a patient or when complications occur (e.g., primary treatment failure or relapse), experts from reference centers or specialized laboratories should be contacted.

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section III ANTIBACTERIAL AGENTS AND SUSCEPTIBILITY TEST METHODS

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Antibacterial Agents*

JAMES S. LEWIS, II, AND KAREN BUSH

68

Antimicrobial chemotherapy has played a vital role in the treatment of human infectious diseases since the discovery of penicillin in the 1920s. Hundreds of antimicrobial agents have been developed, and multiple agents in a variety of classes are currently available for clinical use. However, the sheer numbers and continuing development of agents make it difficult for clinicians to keep up with progress in the field. Similarly, this variety presents significant challenges for clinical microbiologists, who must decide which agents are appropriate for inclusion in routine and specialized susceptibility testing.

This chapter provides an overview of the antibacterial agents currently marketed in the United States, with major emphasis on their mechanisms of action, spectra of activity, important pharmacologic parameters, and toxicities. Selected antibacterials not available in the United States are briefly mentioned as well. Antibiotics that have fallen into disuse or remained investigational are mentioned only briefly.

PENICILLINS

The penicillins (Table 1) are a group of natural and semisynthetic antibiotics containing the chemical nucleus 6-aminopenicillanic acid, which consists of a β-lactam ring fused to a thiazolidine ring (Fig. 1a). The naturally occurring compounds are produced by a number of *Penicillium* spp. The penicillins differ from one another in the substitution at position 6, where changes in the side chain may modify the pharmacokinetic and antibacterial properties of the drug.

Mechanism of Action

The major antibacterial action of penicillins is derived from their ability to inhibit multiple bacterial enzymes, namely, penicillin-binding proteins (PBPs), that catalyze the last steps of peptidoglycan synthesis (1). This ability to inhibit bacterial cell wall enzymes, such as the transpeptidases, usually confers bactericidal activity against Gram-positive bacteria. This activity of the penicillins is often related to their ability to trigger membrane-associated autolytic enzymes that destroy the cell wall.

Penicillins also can bind to bacterial endopeptidases, to bacterial carboxypeptidases, and to bifunctional PBPs with both transpeptidase and transglycosylase activities, enzymes involved in bacterial cell division (2).

Pharmacology

Levels of oral absorption differ markedly among the penicillins. As a natural congener of benzylpenicillin (penicillin G), phenoxymethyl penicillin (penicillin V) resists gastric acid inactivation and is better absorbed from the gastrointestinal tract than is penicillin G. Amoxicillin is a semisynthetic analog of ampicillin and has greater gastrointestinal absorption than ampicillin (95% versus 40% absorption). The isoxazolyl penicillins, also referred to as antistaphylococcal penicillins, cloxacillin, and dicloxacillin, are acid stable and are also absorbed from the gastrointestinal tract, allowing for oral administration. Nafcillin and oxacillin are administered only intravenously.

Repository forms of penicillin G, available in procaine or benzathine, delay absorption from an intramuscular depot. Procaine penicillin G provides detectable levels for 12 to 24 h, while benzathine penicillin G achieves only very low levels in blood for prolonged periods (3 to 4 weeks) and is useful for the therapy of syphilis and for prophylaxis of streptococcal pharyngitis and rheumatic fever.

Penicillins are well distributed to many body compartments, including lung, liver, kidney, muscle, bone, and placenta. Penetration into the eye, brain, cerebrospinal fluid (CSF), and prostate is poor in the absence of inflammation. These drugs are metabolized to a small degree and are rapidly excreted, essentially unchanged, via the kidney. With average half-lives of 0.5 to 1.5 h, they are usually administered every 4 to 6 h to maintain effective blood levels. The renal tubular excretion of penicillins can be blocked by probenecid; thus, their half-lives in serum are prolonged, though this is rarely done in clinical practice. Recommended dosage reductions for many penicillins begin in patients who demonstrate a creatinine clearance of less than 50 ml/min, with alterations being made to either the dose, the frequency of administration, or both. Dosages of all penicillins except nafcillin, oxacillin, and dicloxacillin are adjusted for hemodialysis. Many penicillins also require significant dosage adjustments for peritoneal dialysis.

Spectrum of Activity

The penicillins have antibacterial activity against most Gram-positive and many Gram-negative and anaerobic

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*This chapter contains material presented by Joseph D. C. Yao and Robert C. Moellering, Jr., in chapter 65 in the 10th edition of this *Manual* and is dedicated to the memory of Robert C. Moellering, Jr.

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TABLE 1 Penicillins

- Natural
  - Benzylpenicillin (penicillin G)
  - Phenoxymethyl penicillin (penicillin V)
- Semisynthetic
  - Penicillinase resistant
    - Cloxacillin
    - Dicloxacillin
    - Nafcillin
    - Oxacillin
    - Temocillin
  - Extended spectrum
    - Aminopenicillins
      - Amoxicillin
      - Ampicillin
      - Mecillinam
    - Carboxypenicillin
      - Ticarcillin
    - Ureidopenicillin
      - Piperacillin
- Penicillin–β-lactamase inhibitor combinations
  - Ampicillin-sulbactam
  - Ticarcillin-clavulanat1e
  - Amoxicillin-clavulanate
  - Piperacillin-tazobactam

organisms. Penicillin G is very effective against penicillin-susceptible *Staphylococcus aureus* (<10% of isolates), *Streptococcus pneumoniae*, *Streptococcus pyogenes*, viridans group streptococci, *Streptococcus bovis*, *Neisseria gonorrhoeae* (rarely susceptible to penicillin G), *Neisseria meningitidis*, *Pasteurella multocida*, anaerobic cocci, *Clostridium* spp., *Fusobacterium* spp., *Prevotella* spp., and *Porphyromonas* spp. However, the occurrence of penicillin-resistant pneumococci has increased worldwide (3–5). Penicillin G is the drug of choice for treatment of syphilis and *Actinomyces* infections. Penicillin V has a spectrum of activity similar to that of penicillin G. Both drugs are drugs of choice for the treatment of streptococcal tonsillopharyngitis and for the primary and secondary prevention of rheumatic fever (6). Penicillinase-resistant penicillins, of which oxacillin, nafcillin, and dicloxacillin are the three in clinical use, are effective primarily against penicillinase-producing staphylococci. The agents are at least 25 times more active than other penicillins against penicillinase-positive staphylococci. Although they are also active against fully penicillin-susceptible *S. pneumoniae* and *S. pyogenes*, their MICs for these organisms are higher than those of penicillin G. They are not active against enterococci, members of the family *Enterobacteriaceae*, *Pseudomonas* spp., or members of the *Bacillus fragilis* group. Mecillinam and temocillin, two penicillins not currently available in the United States, have increased stability to hydrolysis by β-lactamases, including some extended-spectrum β-lactamases (ESBLs). However, their increased enzymatic stability is balanced with the loss of antibacterial activity against Gram-positive bacteria.

Ampicillin and amoxicillin have spectra of activity similar to that of penicillin G, but they are more active against enterococci and *Listeria monocytogenes*. These are the drugs of choice for prevention of infective endocarditis in patients with high-risk cardiac conditions undergoing invasive dental, respiratory tract, gastrointestinal, and genitourinary procedures (7). Although they are also more active against *Haemophilus influenzae* and *Haemophilus parainfluenzae*, at least 25% of *H. influenzae* isolates are resistant, usually because of β-lactamase production. *Salmonella* and *Shigella* spp., including *Salmonella enterica* serovar Typhi, are suscep-

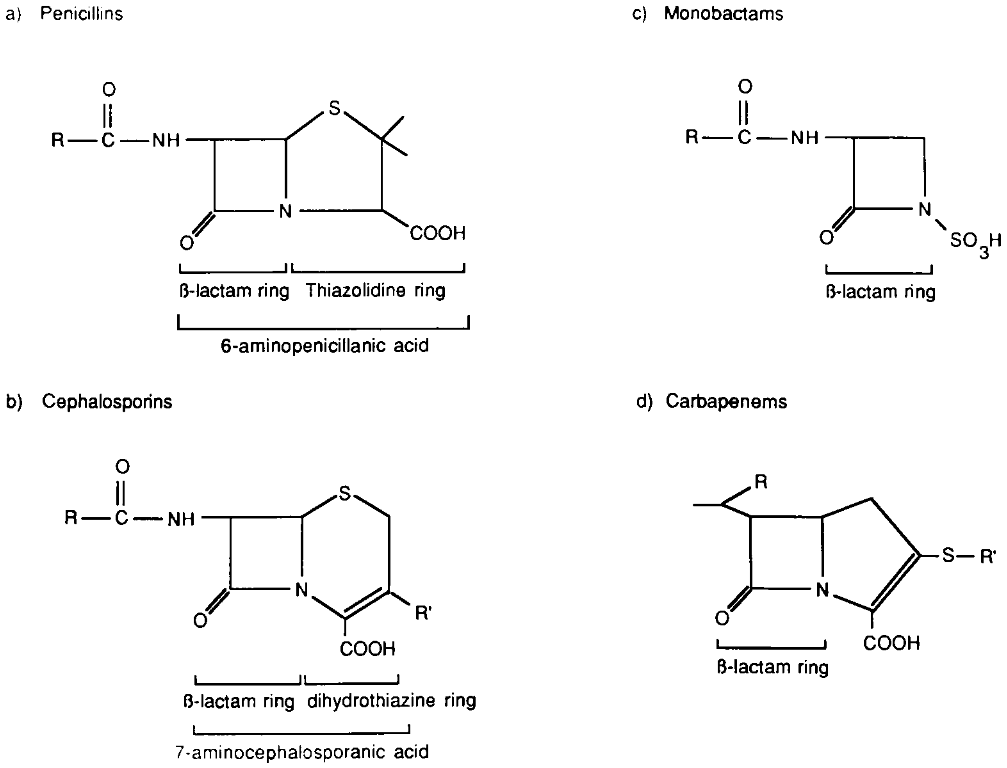

FIGURE 1 Chemical structures of β-lactam antibiotics. doi:10.1128/9781555817381.ch68.f1

tible to these agents. Ampicillin is more effective against shigellae, whereas amoxicillin is more effective against salmonellae. Both of these agents are degraded by β-lactamase and are inactive against *Pseudomonas* spp. and many *Enterobacteriaceae*.

The carboxypenicillins and ureidopenicillins have increased activity against Gram-negative bacteria that are resistant to ampicillin. Although these drugs are susceptible to staphylococcal penicillinase, they are more stable to hydrolysis by the β-lactamases produced by *Enterobacteriaceae* and *Pseudomonas aeruginosa*. Ticarcillin is the only commercially available carboxypenicillin and is available only in the United States as a product coformulated with clavulanic acid. Ticarcillin is relatively active against streptococci as well as against *Haemophilus* spp., *Neisseria* spp., and a variety of anaerobes. Ticarcillin inhibits many non-β-lactamase-producing *Enterobacteriaceae* but is inactive against *Klebsiella* spp. unless combined with clavulanic acid. Ticarcillin is not active against enterococci.

The ureidopenicillins, of which piperacillin is the only clinically available compound in the United States, have greater *in vitro* activity against streptococci and enterococci than do the carboxypenicillins, and they inhibit more than 75% of non-ESBL-producing *Klebsiella* spp. (8). When initially introduced, they exhibited excellent activity against many *Enterobacteriaceae* and anaerobic bacteria, including members of the *B. fragilis* group. Today their use has been compromised by plasmid-encoded β-lactamases (9). On a weight basis, piperacillin is considerably more potent than ticarcillin against *P. aeruginosa* (10). These agents may also act synergistically with aminoglycosides against *P. aeruginosa*. Piperacillin is currently available only in the United States as a product coformulated with the β-lactamase inhibitor tazobactam.

Adverse Effects

Common reactions to penicillins include allergic skin rashes, diarrhea, and drug fever. Severe anaphylactic reactions, which can be fatal, may occur in previously sensitized patients rechallenged with penicillins, but fortunately, such reactions are quite rare. At high doses (usually $>30 \times 10^6$ U/day), penicillin G can cause myoclonic twitching and seizures due to central nervous system toxicity. All of the penicillins may cause interstitial nephritis on an allergic basis. Hepatitis has been associated with prolonged use of oxacillin. High doses of many of the currently available penicillin formulations are associated with considerable sodium loads that have rarely been linked to hypokalemia. Neutropenia may occur with any of the penicillins, particularly when administered at high doses for longer than several weeks. Thrombocytopenia and Coombs test-positive hemolytic anemia are rare complications of penicillin therapy. Bleeding tendencies due to interference with platelet function can occur with the use of carboxypenicillins and ureidopenicillins (11). Although pseudomembranous colitis has been associated with all the penicillins, it occurs more frequently with ampicillin (12).

CEPHALOSPORINS

Cephalosporins are derivatives of the fermentation products of *Cephalosporium acremonium* (also designated *Acremonium chrysogenum*). They contain a 7-aminocephalosporanic acid nucleus, which consists of a β-lactam ring fused to a dihydrothiazine ring (Fig. 1b). Various substitutions at positions 3 and 7 alter their antibacterial activities and pharmacokinetic properties. Addition of a methoxy group at position 7 of the β-lactam ring results in a group of compounds called cephamycins, which are highly resistant to hydrolysis by a variety of class A β-lactamases, including many ESBLs.

Mechanism of Action

Like the penicillins, cephalosporins act by binding to PBPs of susceptible organisms, thereby interfering with the synthesis of peptidoglycan of the bacterial cell wall. In addition, these β-lactam agents may produce bactericidal effects by triggering autolytic enzymes in the cell envelope (1). Of note are the unique chemical structure-activity relationships for ceftaroline and ceftobiprole, which contain a thiazole moiety and a vinylpyrrolidinone moiety, respectively, at position 3 of the cephem ring. These chemical side chains promote binding of the drugs to the staphylococcal PBP 2a and the pneumococcal PBP 2x, thereby conferring bactericidal activity against methicillin-resistant *S. aureus* (MRSA) and against penicillin- and ceftriaxone-resistant pneumococci (13).

Pharmacology

Most cephalosporins require parenteral administration, but many are available in oral formulations (Table 2). Cefadroxil, cephalexin, cefaclor, cefprozil, cefuroxime axetil, cefdinir, cefditoren pivoxil, cefixime, cefpodoxime proxetil, and ceftibuten can be dosed orally with good gastrointestinal absorption (60 to 90% of the oral dose). Cefuroxime axetil is an acetoxyethyl ester of cefuroxime, and it is deesterified at the intestinal mucosa and absorbed into the bloodstream as cefuroxime. Cefditoren pivoxil and cefpodoxime proxetil are prodrugs that are absorbed and hydrolyzed by esterases *in vivo* to release the active drugs cefditoren and cefpodoxime, respectively. Relatively high concentrations of these agents are attained across the placenta and in synovial, pleural, pericardial, and peritoneal fluids. Levels in bile are usually high, especially with the injectable ceftriaxone, which has significant biliary excretion. The parenteral cephalosporins cefotaxime, ceftriaxone, ceftazidime, and cefepime penetrate into the CSF and may be useful for the treatment of meningitis. Cefuroxime penetrates inflamed meninges, but levels in CSF are inadequate in providing bactericidal activity against susceptible bacteria.

Cefotaxime is converted to the desacetyl form before excretion. All cephalosporins except ceftriaxone are excreted primarily by the kidney, and for these drugs, dosage adjustments are necessary in patients with renal insufficiency (creatinine clearance of <50 ml/min). Like that of the penicillins, the renal excretion of cephalosporins, except for ceftriaxone, is impeded by probenecid. In general, these agents are removed by hemodialysis but not by peritoneal dialysis. Of the cephalosporins, ceftriaxone has the longest elimination half-life, at 6.8 to 8.7 h, permitting once- or twice-daily drug administration in the treatment of serious infections.

Ceftaroline fosamil and ceftobiprole medocaril were developed as water-soluble prodrugs of ceftaroline and ceftobiprole, respectively, and they undergo rapid conversion (<1 h) to the respective active drugs after intravenous administration. With a half-life of 2.5 h, infusion of 600 mg of ceftaroline results in a maximum concentration of 27.9 μg/ml in serum (14). Following infusion of ceftobiprole medocaril at 500 mg, the maximum concentration achieved in serum is 36 μg/ml (15). With a terminal half-life of 3.4 h, the drug undergoes minimal hepatic metabolism and is primarily eliminated in the urine. Dosage adjustment is necessary for both drugs in patients with renal insufficiency.

TABLE 2 Cephalosporins

Class	Subclass[a]	Agent(s)
Cephalosporins (oral)	Narrow spectrum (cephalosporin I or 1st generation)	Cefaclor,[b] cefadroxil, cephalexin
	Broad spectrum (cephalosporin II or 2nd generation)	Cefprozil, cefuroxime (axetil)[c]
	Expanded-spectrum (cephalosporin III or 3rd generation)	Cefdinir, cefditoren (pivoxil),[c] cefixime, cefpodoxime (proxetil),[c] ceftibuten
Cephalosporins (parenteral)	Narrow spectrum (cephalosporin I or 1st generation)	Cefazolin
	Broad spectrum (cephalosporin II or 2nd generation)	Cefuroxime
	Expanded-spectrum (cephalosporin III or 3rd generation)	Cefoperazone,[b] cefotaxime, ceftazidime, ceftriaxone
	Cephalosporin IV (4th generation)	Cefepime
Cephamycins		Cefotetan, cefoxitin
Cephalosporins with anti-MRSA activity		Ceftaroline (fosamil), ceftobiprole (medocaril)[b]

[a] Based on nomenclature in the CLSI glossary (419).
[b] Not licensed for clinical use in the United States.
[c] The agent in parentheses indicates the ester that is cleaved to yield the microbiologically active cephalosporin.

Spectrum of Activity

Cephalosporins are classified by a somewhat arbitrary nomenclature scheme, based on either general features of their antibacterial activities or their chemical structures (Table 2). The commonly used designations of cephalosporin generations can be ambiguous, depending upon the properties that are used for the groupings. The narrow-spectrum cephems (cephalosporin I or first-generation cephalosporins), exemplified by cephalexin and cefazolin, have good Gram-positive activity and relatively modest Gram-negative activity. They are active against penicillin-susceptible *S. aureus* as well as penicillin-susceptible *S. pneumoniae*, *S. pyogenes*, and other aerobic and anaerobic streptococci. Methicillin-resistant staphylococci and enterococci are resistant, although penicillinase-producing non-MRSA (methicillin-susceptible *S. aureus* [MSSA]) isolates are susceptible. Some *Enterobacteriaceae*, including non-β-lactamase-producing strains of *Escherichia coli*, *Klebsiella* spp., and *Proteus mirabilis*, are susceptible. *Pseudomonas* spp., including *P. aeruginosa*, many *Proteus* spp., and *Serratia* and *Enterobacter* spp., are resistant. These agents are active against penicillin-susceptible anaerobes except members of the *B. fragilis* group. They have only modest activity against *H. influenzae*.

The broad-spectrum (cephalosporin II or second-generation) cephalosporins are stable against some β-lactamases found in Gram-negative bacteria and, as a result, have increased activity against Gram-negative organisms. The agents are more active than narrow-spectrum drugs against *E. coli*, *Klebsiella* spp., and *Proteus* spp. Their activity also extends to cover some *Enterobacter* and *Serratia* strains, and they have good activity against *Haemophilus* spp., *Neisseria* spp., and many anaerobes. However, they are hydrolyzed by plasmid-encoded ESBLs and carbapenemases now produced by many *Enterobacteriaceae*. Cefaclor, cefuroxime, and cefprozil are active against ampicillin-resistant *H. influenzae* and *Moraxella catarrhalis* (16, 17). None of these agents has useful antimicrobial activity against *Pseudomonas* spp.

Cefoxitin and cefotetan belong to a unique group of expanded-spectrum cephalosporins (cephamycins) that have marked activity against anaerobes, including members of the *B. fragilis* group, though resistance within the *B. fragilis* group to the cephamycins is increasingly problematic (18, 19). Cefotetan is two to four times less active than cefoxitin against Gram-positive cocci, but it is more potent than cefoxitin against susceptible *Enterobacteriaceae*. The two cephamycins are equally active against *H. influenzae*, *M. catarrhalis*, and *N. gonorrhoeae*, including penicillin-resistant strains. While these drugs are comparable in their activities against the *B. fragilis* group, cefoxitin is more active than cefotetan against *Prevotella* spp., *Porphyromonas* spp., and Gram-positive anaerobic cocci. Cefotetan has the advantage of a more prolonged half-life in serum. Both molecules are also stable against ESBLs produced by a variety of *Enterobacteriaceae*, though their clinical utility for infections due to these organisms remains uncertain.

Expanded-spectrum (cephalosporin III or third-generation) cephalosporins are generally less active than the narrow-spectrum agents against Gram-positive cocci, but they are much more active against the *Enterobacteriaceae*. Their expanded spectra of Gram-negative activity is due primarily to their stability to low-level production of chromosomal β-lactamases and to the early plasmid-encoded penicillinases (9). They are also notable for their ability to pass through the outer cell envelopes of Gram-negative bacilli (20, 21). There are two subclasses among these agents: those with potent activity against *P. aeruginosa* (ceftazidime and cefoperazone) and those without such activity (cefotaxime, ceftriaxone, and the oral agents in this subclass). Cefotaxime and ceftriaxone, however, are far more potent against streptococci than the other expanded-spectrum cephalosporins, while the Gram-positive activity of ceftazidime is clinically marginal. All the expanded-spectrum cephalosporins are hydrolyzed by ESBLs and carbapenemases that are found in Gram-negative pathogens. Ceftazidime, however, is currently in clinical development with the β-lactamase inhibitor avibactam to counteract the effects of many of these enzymes (see below).

Cefotaxime inhibits more than 90% of strains of non-ESBL- or non-carbapenemase-producing *Enterobacteriaceae*, including those resistant to aminoglycosides, with MIC_{90}s for *E. coli*, *Proteus* spp., and *Klebsiella* spp. of <0.5 μg/ml. Its activity against strains of *Serratia marcescens*, *Enterobacter cloacae*, and *Acinetobacter* spp. is limited, and it is inactive against *P. aeruginosa*.

Ceftriaxone and cefotaxime have similar spectra of activity. Both have high potency (MIC_{90}s, ≤0.12 μg/ml) against both penicillinase-positive and -negative strains of *N. gonorrhoeae* and are usually effective as single-dose therapy for infections caused by these organisms (22). However, *N. gonorrhoeae* strains with reduced susceptibility to these drugs have emerged (23). Because of its long half-life in serum (>6 h, the longest of the currently available cephalosporins), ceftriaxone is used frequently in outpatient antibiotic therapy of serious infections, including Lyme disease (24).

Cefoperazone is less active than cefotaxime against many *Enterobacteriaceae* and Gram-positive cocci. However, it has some clinically useful activity against *P. aeruginosa*, with an MIC$_{50}$ of ≤16 μg/ml. Its activity against anaerobes is similar to that of cefotaxime (25). Ceftazidime has more potent activity than other expanded-spectrum cephalosporins or the ureidopenicillins against *P. aeruginosa*, with an MIC$_{90}$ of <8 μg/ml (21). This cephalosporin has activity similar to that of cefotaxime against the *Enterobacteriaceae* but is not as active against Gram-positive cocci. It has little activity against Gram-negative anaerobes.

Cefdinir (26), cefditoren (16, 27, 28), cefixime (29), cefpodoxime (30), and ceftibuten (25) are expanded-spectrum oral cephalosporins that are more stable than the narrow- and broad-spectrum oral cephalosporins against Gram-negative bacterial β-lactamases. Compared with the earlier cephalosporins, the newer drugs are equally active against streptococci (MIC$_{90}$s, ≤0.06 μg/ml) but less active against methicillin-susceptible staphylococci (MIC$_{90}$s, 2 μg/ml). With activities similar to that of cefotaxime against many non-ESBL- or non-carbapenemase-producing *Enterobacteriaceae*, *H. influenzae*, *M. catarrhalis*, and *N. gonorrhoeae*, they are inactive against *Pseudomonas*, *Enterobacter*, *Serratia*, and *Morganella* spp. and anaerobes. None of the currently available cephalosporins is clinically useful against enterococci.

Cefepime is an expanded-spectrum (cephalosporin IV or fourth-generation) cephalosporin approved for clinical use in the United States. It tends to have greater potency against *Enterobacteriaceae* with high levels of AmpC cephalosporinases, due to greater penetrability and increased stability to the Bush and Jacoby group 1 cephalosporinases (9). Cefepime penetrates well through Gram-negative bacterial outer membranes, due to a quaternary nitrogen substitution that gives it a zwitterionic character (net neutral charge). It is more potent *in vitro* than cefotaxime and ceftriaxone against non-ESBL- or carbapenemase-producing *Enterobacteriaceae* (MIC$_{90}$s of ≤0.1 μg/ml) (31). Cefepime has activity comparable to that of ceftazidime against *P. aeruginosa*, with MIC$_{90}$s of ≤4 μg/ml, and is active against some ceftazidime-resistant strains (32). Against staphylococci (MIC$_{90}$ of ≤2 μg/ml) and streptococci (MIC$_{90}$ of ≤0.12 μg/ml), its activity is comparable to those of the narrow-spectrum cephalosporins (31). However, it is not clinically active against *Acinetobacter* spp., enterococci, or anaerobes.

Ceftolozane (CXA-101, FR264205) is a new cephalosporin currently undergoing phase 3 studies that possesses activity against many multidrug-resistant (MDR) *P. aeruginosa* isolates (MIC$_{90}$, ≤8 μg/ml) (33). This activity is against many isolates resistant to currently available antipseudomonal cephalosporins as well as carbapenems. However, ceftolozane is inactivated by metallo-β-lactamases, as well as by serine carbapenemases and many ESBLs. It is currently being developed in combination with tazobactam in a 2:1 ratio for clinical use (CXA-201) and has completed phase 3 clinical trials for complicated intra-abdominal infections as well as complicated urinary tract infections. Against the *Enterobacteriaceae*, ceftolozane is comparable in antimicrobial activity to ceftazidime and slightly less active than cefepime (MIC$_{90}$, ≤1 μg/ml). Activity against *Burkholderia* spp., *Acinetobacter* spp., enterococci, and streptococci appears similar to that of ceftazidime. No useful activity against oxacillin-susceptible staphylococci (MIC$_{50}$ = 32 μg/ml) is observed (34).

Ceftaroline and ceftobiprole are active against all staphylococci, including methicillin-susceptible *S. aureus* (MSSA), MRSA, heterogeneous vancomycin-intermediate *S. aureus* (hVISA), VISA, and vancomycin-resistant *S. aureus* (VRSA) at MIC$_{90}$s of ≤1 μg/ml and against MDR pneumococci at MIC$_{90}$s of 0.5 μg/ml (13, 35–37). Viridans group streptococci and beta-hemolytic streptococci are inhibited at MIC$_{90}$s of ≤1 μg/ml. While ceftaroline has minimal activity against enterococci, ceftobiprole exhibits good activity against *Enterococcus faecalis* but is largely inactive against *Enterococcus faecium* (38). The Gram-negative activity of ceftaroline is limited mainly to Gram-negative respiratory tract pathogens, including β-lactamase-producing *H. influenzae*, *M. catarrhalis* (MIC$_{90}$s of 0.125 and 0.25 μg/ml, respectively), and *N. gonorrhoeae* (MIC$_{90}$ of 0.25 μg/ml), as well as *Enterobacteriaceae* that do not produce ESBLs or carbapenemases. It is weakly active against *P. aeruginosa*, *Acinetobacter* spp., and Gram-negative bacilli with inducible AmpC β-lactamases and has no clinical utility for these pathogens. However, its activity against β-lactamase-producing strains is enhanced by the addition of avibactam; the combination is being studied for clinical utility. Ceftobiprole has *in vitro* antibacterial activity similar to that of cefepime against *P. aeruginosa* and *Enterobacteriaceae* with derepressed AmpC β-lactamases (MIC$_{90}$s of 8 to 16 μg/ml), but it is inactive against ESBL-producing *Enterobacteriaceae* and MDR *Acinetobacter baumannii* isolates (13). Both drugs display varied activities against anaerobes, with good activity against *Clostridium* spp. (except *Clostridium difficile*), *Fusobacterium*, *Lactobacillus*, *Peptostreptococcus*, *Porphyromonas*, *Propionibacterium acnes*, and *Veillonella*, but are inactive against *B. fragilis*, members of the *B. fragilis* group, and *Prevotella* (39). Ceftobiprole remains unavailable in the United States but received European regulatory approval in late 2013 for use in adults with hospital-acquired pneumonia (excluding those with ventilator-associated pneumonia) and community-acquired pneumonia.

Adverse Effects

Cephalosporins are generally very well tolerated. The most common side effects are diarrhea and hypersensitivity reactions, such as rash, drug fever, and serum sickness. Cross-reactions with these drugs occur in only 3% to 7% of penicillin-allergic patients (40). Other infrequent side effects include pseudomembranous colitis, elevated serum creatinine and transaminase levels, leukopenia, thrombocytopenia, and Coombs test-positive hemolytic anemia. These abnormalities are usually mild and reversible. Prolonged use of ceftriaxone has been associated with the formation of gallbladder sludge, which usually resolves after the drug is discontinued (41), and rarely cholecystitis.

Disulfiram-like reactions have been described in patients receiving cefotetan and cefoperazone. This reaction is attributed to the *N*-methylthiotetrazole side chains of these antibiotics, which are similar to the chemical structure of disulfiram. Hypoprothrombinemia and bleeding tendencies have been observed with these cephalosporins. Causes of the coagulopathy include (i) alteration of the healthy gut biota by the antibiotics, with inhibition of the synthesis of vitamin K and its precursors, and (ii) the *N*-methylthiotetrazole side chain, which inhibits the vitamin K-dependent carboxylase enzyme responsible for converting clotting factors II, VII, IX, and X to their active forms and also prevents regeneration of active vitamin K from its inactive form (42). Also of note has been the recent recognition of neurotoxicity, which manifests primarily as impaired consciousness in patients receiving high doses of cefepime with impaired renal function (43). In July 2012, the U.S. prescribing information was updated to reflect concerns regarding increased seizure risk with use of cefepime at high doses, again particularly in older patients with decreased renal function. Use of cefepime compared to other agents was also linked to an increased risk of mortality, though these reports have been refuted by the FDA (44).

OTHER β-LACTAM ANTIBIOTICS

■ Monobactams

Monobactams are monocyclic β-lactams with various side chains affixed to a monocyclic nucleus (Fig. 1c). Aztreonam is the only monobactam antibiotic currently in clinical use, although several other monocyclic agents are in early stages of clinical development.

Mechanism of Action

Aztreonam binds primarily to PBP 3 of Gram-negative aerobes, including *P. aeruginosa*, thereby disrupting bacterial cell wall synthesis. It is not hydrolyzed by many of the early Bush and Jacoby group 1 and 2 plasmid-encoded and chromosomally encoded β-lactamases (9) but is susceptible to hydrolysis by ESBLs and serine carbapenemases. It has high affinity for the group 1 cephalosporinases but does not induce the production of these enzymes (45). Metallo-β-lactamases are unable to hydrolyze aztreonam, leading to its potential clinical use against carbapenemase-producing Gram-negative pathogens (9). Aztreonam is currently in clinical development in combination with the broad-spectrum β-lactamase inhibitor avibactam (see below).

Pharmacology

Given intravenously, aztreonam is widely distributed to body tissues and fluids. Average drug concentrations in serum exceed the MIC_{90}s for most *Enterobacteriaceae* by four to eight times for 8 h and are inhibitory to *P. aeruginosa* for 4 h. It crosses inflamed meninges in an amount sufficient to be potentially therapeutic for meningitis caused by susceptible organisms. Its half-life in serum is about 1.7 h, and it is excreted mainly unchanged by the kidney. Dosage modification is necessary for patients with renal failure. The drug is removed by both hemodialysis and peritoneal dialysis.

Spectrum of Activity

The antibacterial activity of aztreonam is limited to aerobic Gram-negative bacilli, inhibiting susceptible *Enterobacteriaceae*, *Neisseria* spp., and *Haemophilus* spp., with MIC_{90}s of ≤0.5 μg/ml (46, 47). However, most *Acinetobacter* spp., *Burkholderia cepacia*, and *Stenotrophomonas maltophilia* are resistant. It can demonstrate *in vitro* synergism when combined with aminoglycosides against aztreonam-susceptible organisms, including *P. aeruginosa* and some aminoglycoside-resistant Gram-negative bacilli (48). Aztreonam is not active against Gram-positive bacteria or anaerobes.

Adverse Effects

Aztreonam is generally a safe agent, with a toxicity profile similar to those of other β-lactam drugs. Nausea, diarrhea, skin rash, eosinophilia, mild elevation of serum transaminase levels, and transiently elevated serum creatinine levels have occurred. It has minimal cross-reactivity with other β-lactams and can be used safely in patients allergic to penicillins or cephalosporins (49).

■ Carbapenems

Carbapenems are a unique class of β-lactam agents with the widest spectrum of antibacterial activity of the currently available antibiotics. Structurally, they differ from other β-lactams in having a hydroxyethyl side chain in the *trans* configuration at position 6 and possessing a carbon atom in place of a sulfur or oxygen atom in the bicyclic nucleus (Fig. 1d). The unique stereochemistry of the hydroxyethyl side chain confers stability against most β-lactamases. Doripenem, ertapenem, imipenem, and meropenem are the carbapenems currently available for clinical use (50).

Mechanism of Action

Carbapenems bind with high affinity to PBP 2 and then to PBPs 1a and 1b of Gram-negative bacteria, causing spherical cell formation and lysis (51). They display potent binding to all PBPs in staphylococci, with the exception of PBP 2a in MRSA, against which they have poor activity (52). They are stable toward most plasmid- or chromosomally mediated β-lactamases except for the more frequently occurring carbapenemases (9). Bacterial resistance arises from production of carbapenem-hydrolyzing enzymes that hydrolyze the carbapenem nucleus: the group 2d carbapenemases (OXA β-lactamases), group 2f serine carbapenemases (KPC, SME, NMC-A, IMI, and GES), and metallo-β-lactamases (plasmid-encoded IMP, VIM, and NDM, as well as chromosomal enzymes in *S. maltophilia* and *B. fragilis*). Resistance in *Enterobacteriaceae* can also occur from hyperproduction of group 1 cephalosporinases combined with outer membrane porin alterations that result in decreased uptake of the drugs. In *P. aeruginosa*, carbapenem resistance frequently occurs as a combination of decreased uptake (due to inactivation of the OprD porin) and increased AmpC production, although efflux can also be upregulated in this pathogen to give insufficient concentrations of drug for effective antibacterial activity (53).

Pharmacology

After intravenous administration, the carbapenems distribute widely in the body but undergo no significant biliary excretion. Imipenem is metabolized and inactivated in the kidneys by a dehydropeptidase I (DHP-I) enzyme found in the brush borders of proximal renal tubular cells. To achieve adequate concentrations in serum and urine, a DHP inhibitor, cilastatin, is combined with imipenem in a 1:1 dosage ratio for clinical use. Cilastatin has no antibacterial activity, nor does it alter the activity of imipenem. It has a renal protective effect by preventing excessive accumulation of potentially toxic imipenem metabolites in renal tubular cells. Meropenem, ertapenem, and doripenem contain a β-methyl group substitution at position C-1 of the bicyclic nucleus, which results in increased stability to inactivation by human renal DHP-I. These agents do not require concomitant administration of a DHP-I inhibitor.

The pharmacokinetics of doripenem, imipenem, and meropenem are very similar, with elimination half-lives in serum of about 1 h. Peak concentrations of the drugs in serum are about 25 to 35 μg/ml and 55 to 70 μg/ml following 0.5-g and 1-g doses, respectively. These drugs penetrate inflamed meninges well, with drug levels of 0.5 to 6 μg/ml in the CSF (50, 54). Ertapenem is highly (>95%) bound to human plasma proteins, with poor penetration into the CSF. Its relatively long plasma half-life of 4 h allows for once-daily dosing frequency. A peak serum concentration of 155 μg/ml is reached following a single intravenous dose of 1 g of ertapenem (55). Dosage adjustment of these carbapenem drugs is necessary for creatinine clearance of ≤50 ml/min. These agents, including cilastatin, are effectively removed by hemodialysis.

Spectrum of Activity

In general, all the carbapenems have similar antibacterial potencies, with minor differences. They have excellent *in vitro* activity against aerobic Gram-positive species: staphylococci (penicillin-susceptible and -resistant isolates); viridans group streptococci; group A, B, C, and G streptococci; *Bacillus* spp.; and *L. monocytogenes*. Doripenem and imipenem are two to four times more active than meropenem and ertapenem against streptococci and methicillin-susceptible staphylococci (MIC$_{90}$s of ≤0.5 μg/ml), but methicillin-resistant staphylococci are resistant to all clinically approved carbapenems. Although the MICs of carbapenems for penicillin-resistant pneumococci are elevated (MIC$_{90}$s of 0.25 to 2 μg/ml), most strains remain susceptible to these drugs, with doripenem and imipenem being most potent (56–58). Ertapenem has poor activity against *Enterococcus faecalis*, but these isolates are inhibited by other carbapenems at ≤4 μg/ml. However, *Enterococcus faecium* is usually resistant to all carbapenems.

More than 90% of *Enterobacteriaceae*, including those resistant to other β-lactams and aminoglycosides, are susceptible to carbapenems, with the following decreasing order of activity: doripenem, ertapenem, meropenem > biapenem > imipenem (59, 60). These agents are highly active against clinical isolates of ESBL-producing *K. pneumoniae* and *E. coli*, with MIC$_{90}$s of 0.015 to 0.125 μg/ml (59, 61). Most *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp. are inhibited by carbapenem concentrations of ≤1 μg/ml. Ertapenem is inactive against *Acinetobacter* and *Pseudomonas*. Most strains of *P. aeruginosa* are inhibited by other carbapenems at 2 to 4 μg/ml, with meropenem and doripenem being the most potent agents, including against many imipenem-resistant strains (53, 59). While they inhibit *B. cepacia* and *Pseudomonas stutzeri*, carbapenems are inactive against *S. maltophilia* due to its production of the chromosomal metallo-β-lactamase (62). Emergence of resistant *Pseudomonas* spp. has been observed during therapy with carbapenems.

Carbapenems are the most potent β-lactams against anaerobes, with activities comparable to that of metronidazole. The MIC$_{90}$s for anaerobic Gram-positive cocci, *Clostridium*, members of the *B. fragilis* group, *Fusobacterium*, *Porphyromonas*, and *Prevotella* are ≤4 μg/ml (63–65). This class of drugs is also active *in vitro* against *Actinomyces*, *Nocardia*, and atypical mycobacteria, with imipenem being substantially more active than the other carbapenems against these organisms (66, 67).

Adverse Effects

The side effects of carbapenems are similar to those of other β-lactam antibiotics. Nausea, vomiting, and diarrhea occur in up to 5% of patients, usually associated with parenteral administration of ertapenem and imipenem. Pseudomembranous colitis can occur with carbapenems. Allergic reactions, such as drug fever, skin rashes, and urticaria, are seen in about 3% of patients. Cross-reactivity with other β-lactam agents is possible but has not been fully studied. Seizures of unclear etiology have occurred in up to 5% of patients receiving imipenem, particularly in the elderly age group and in patients with renal insufficiency or underlying neurologic disorders, while other carbapenems have low seizure-inducing potential (<1%) (57). Reversible elevation of serum transaminases, leukopenia, and thrombocytopenia have been described for carbapenems, but coagulopathy has not been reported.

β-LACTAMASE INHIBITORS

Clavulanic Acid

Clavulanic acid is a naturally occurring weak antimicrobial agent found initially in cultures of *Streptomyces clavuligerus* (68). It inhibits penicillinases from staphylococci and many group 2 β-lactamases from Gram-negative bacteria. This agent acts primarily as a "suicide inhibitor" by forming an irreversible acyl enzyme complex with the β-lactamase, leading to loss of activity of the enzyme.

Clavulanic acid acts synergistically with various penicillins and cephalosporins against β-lactamase-producing staphylococci, klebsiellae, *H. influenzae*, *M. catarrhalis*, *N. gonorrhoeae*, *E. coli*, *Proteus* spp., members of the *B. fragilis* group, *Prevotella* spp., and *Porphyromonas* spp. (69, 70). Many plasmid-mediated TEM, SHV, and CTX-M β-lactamases, including ESBLs, present in cephalosporin-resistant strains of *K. pneumoniae* and *E. coli* can be inactivated by this drug (71). However, the role of clavulanic acid-containing products in the management of these infections remains unclear. Inducible and plasmid-encoded AmpC β-lactamases (chromosomal group 1 cephalosporinases) of *Enterobacter*, *Citrobacter*, *Proteus*, *Acinetobacter*, *Serratia*, and *Pseudomonas* spp. are not inhibited by clavulanic acid (9). With the currently common occurrence of multiple β-lactamases appearing in Gram-negative pathogens, clavulanic acid combinations may not provide sufficient concentrations of the inhibitor to be effective for serious infections.

In the United States, clavulanic acid is available for clinical use in combination with oral amoxicillin at dosage ratios of 1:2, 1:4, 1:7, and 1:16 and in a 1:15 or 1:30 parenteral combination with ticarcillin. Intravenous combinations of clavulanic acid and amoxicillin at ratios of 1:5 and 1:10 are also used outside North America. The pharmacologic parameters of amoxicillin and ticarcillin are not significantly altered when either drug is combined with clavulanic acid. Amoxicillin-clavulanate is moderately well absorbed from the gastrointestinal tract, with a half-life in serum of about 1 h for each component. One-third of a dose is metabolized, while the remainder is excreted unchanged in the urine. The drug is widely distributed to various body tissues and fluids, but it penetrates uninflamed meninges very poorly. Adverse reactions, with the exception of diarrhea, are similar to those reported for amoxicillin or ticarcillin used alone. Nausea, vomiting, abdominal cramps, and diarrhea occur in 5 to 10% of patients taking amoxicillin-clavulanate. The incidence of allergic skin reactions is similar to that of amoxicillin alone.

Sulbactam

Sulbactam is a semisynthetic 6-desaminopenicillin sulfone with weak antibacterial activity (72). It inhibits certain plasmid- and chromosomally mediated β-lactamases of *S. aureus*, many *Enterobacteriaceae*, *H. influenzae*, *M. catarrhalis*, *Neisseria* spp., *Legionella* spp., members of the *B. fragilis* group, *Prevotella* spp., *Porphyromonas* spp., and *Mycobacterium* spp. (73). The efficiency of sulbactam inhibition is less than for clavulanic acid (74). Sulbactam alone is active against *N. gonorrhoeae*, *N. meningitidis*, some *Acinetobacter* spp., and *B. cepacia* (73, 75). It acts synergistically with penicillins and cephalosporins against organisms that are otherwise resistant to the β-lactam drugs because of the production of β-lactamases. A combination of sulbactam (8 μg/ml) and ampicillin (16 μg/ml) inhibits most strains of staphylococci and many strains of *Klebsiella* spp., *E. coli*, *H. influenzae*, *M. catarrhalis*, *Neisseria* spp., the *B. fragilis* group, *Prevotella* spp., and *Porphyromonas* spp. that are ampicillin resistant (76, 77). Like clavulanic acid, sulbactam does not inhibit the cephalosporinases of *Enterobacter*, *Citrobacter*, *Providencia*, indole-positive *Proteus*, *Pseudomonas* spp., or *S. maltophilia*.

For clinical use, sulbactam is combined with ampicillin as a parenteral preparation in a 1:2 ratio. The pharmacologic properties of the drugs are not affected by each other in this combination. Ampicillin-sulbactam penetrates well into body tissues and fluids, including peritoneal and blister fluids. It enters the CSF in the presence of inflamed meninges. Like ampicillin, sulbactam has a half-life in serum of 1 h, and 85% of the drug is excreted unchanged via the kidneys. Since clearances of both sulbactam and ampicillin are affected similarly in patients with impaired renal function, dosage adjustments are similar for the two drugs.

The most common side effects of the ampicillin-sulbactam combination have been nausea, diarrhea, and skin rash. Transient eosinophilia and transaminasemia have been reported. Adverse reactions attributed to ampicillin may also occur with the use of ampicillin-sulbactam.

Tazobactam

Tazobactam is a penicillanic acid sulfone derivative structurally related to sulbactam. Like clavulanic acid and sulbactam, tazobactam acts as a suicide β-lactamase inhibitor. (73). Despite having very poor intrinsic antibacterial activity by itself, it is comparable to clavulanic acid and more potent than sulbactam in lowering the MICs up to 20-fold for many organisms when combined with various β-lactams against β-lactamase-producing organisms (74). Tazobactam actively inhibits the β-lactamases found in staphylococci, *H. influenzae*, *N. gonorrhoeae*, the *B. fragilis* group, *Prevotella* spp., and *Porphyromonas* spp. (74, 78, 79). It also has some activity against the group 1 β-lactamases of *Acinetobacter*, *Citrobacter*, *Proteus*, *Providencia*, and *Morganella* spp. (73, 74, 80). Like clavulanic acid, tazobactam displays inhibition of many ESBL-producing *Enterobacteriaceae*, especially *E. coli*, *Klebsiella* spp., and *P. mirabilis* (74). Of the penicillin-β-lactamase inhibitor combinations, piperacillin-tazobactam is the one most active (2- to 8-fold-lower MICs) against β-lactamase-producing aerobic and anaerobic Gram-negative bacilli (74, 81).

Available as a 1:8 ratio dosage combination with piperacillin, tazobactam is administered parenterally. The two drugs do not affect each other's metabolism or pharmacokinetics. High concentrations of both agents are achieved in the intestinal mucosa, lung, and skin, with relatively poor distribution to muscle, fat, prostate, and CSF (in the absence of inflamed meninges). With a half-life in serum of about 1 h, elimination of tazobactam is mainly via the renal route and is not affected by hepatic failure (82). Major adverse effects of the piperacillin-tazobactam combination are similar to those of piperacillin alone, such as diarrhea, skin rash, and allergic reactions. Mild elevation in serum transaminase levels may be encountered in about 10% of patients.

Tazobactam is currently under development as a 1:2 combination with ceftolozane. This combination has recently completed phase 3 trials for the treatment of complicated intra-abdominal infections and complicated urinary tract infections. The addition of tazobactam to ceftolozane improves the activity of ceftolozane against many organisms producing specific group 2 broad-spectrum β-lactamases, including the important CTX-M-14 and CTX-M-15 ESBLs commonly found in *E. coli*. Compared to the doses of tazobactam utilized in piperacillin-tazobactam, the higher dose of tazobactam utilized in combination with ceftolozane improves the activity of the combination against many AmpC-producing organisms, including *Enterobacter* spp. However, the combination still appears less active than cefepime against ceftazidime-resistant *Enterobacter* spp. (83).

Avibactam

Avibactam, formerly NXL104, is a novel diazabicyclooctane non-β-lactam inhibitor of class A and C β-lactamases as well as some class D enzymes (84). Avibactam inhibits these enzymes through the formation of reversible covalent carbamoyl linkages (85, 86). It is currently undergoing clinical studies in combination with ceftazidime, ceftaroline, and aztreonam for the treatment of nosocomial Gram-negative infections. When tested at a concentration of 4 μg/ml in combination with ceftazidime against *Enterobacteriaceae*, this drug potentiated the activities of the cephalosporins 4- to 8,000-fold, with MICs of ≤1.0 μg/ml for all organisms, including those producing AmpC β-lactamases, ESBLs of TEM, SHV, or CTX-M types, and KPC carbapenemases (84). Although it effectively restores the activity of imipenem against isolates producing class A carbapenemases, avibactam does not inhibit metallo-β-lactamases, such as NDM, IMP, and VIM (84).

MK7655

MK7655 is a novel β-lactamase inhibitor that is being developed in combination with imipenem. Like avibactam, it possesses a diazabicyclooctane structure (87). The addition of MK7655 to imipenem results in the protection of imipenem from hydrolysis by both class A carbapenemases, such as KPC, and hyperproduced AmpC cephalosporinases, resulting in the restoration of antibacterial activity against many multiresistant pathogens (88). MK7655 does not inhibit the class B metallo-β-lactamases in the NDM, IMP, or VIM families; its activity against OXA-type class D β-lactamases appears variable.

QUINOLONES

Quinolones belong to a group of potent antibiotics biochemically related to nalidixic acid, which was developed initially as a urinary antiseptic. Newer quinolones have been synthesized by modifying the original bicyclic 4-quinolone (or naphthyridone) nucleus with different side chain substitutions (89). Each of these newer agents, also known as fluoroquinolones, contains a fluorine atom attached to the nucleus at position 6. Quinolones that are currently available for clinical use in the United States are listed in Table 3. Grepafloxacin, temafloxacin, and trovafloxacin were with-

TABLE 3 Quinolones

Narrow spectrum
Nalidixic acid[a]
Broad spectrum
Ciprofloxacin
Levofloxacin
Norfloxacin
Ofloxacin
Pefloxacin[b]
Besifloxacin[c]
Gatifloxacin[c]
Gemifloxacin
Moxifloxacin

[a] Discontinued for clinical use in the United States.
[b] Not licensed for clinical use in the United States.
[c] Topical use only.

drawn from clinical use due to toxicities, while sparfloxacin, lomefloxacin, and gatifloxacin are no longer available for systemic use in the United States. However, gatifloxacin and besifloxacin are approved for ophthalmic use. Delafloxacin, finafloxacin, and nemonoxacin are currently entering or are in phase 3 clinical trials in the United States (90). Ozenoxacin is a topical nonfluorinated quinolone that has completed a phase 3 trial for impetigo (90).

Mechanism of Action

The primary bacterial target of the quinolones is DNA gyrase, a type II DNA topoisomerase enzyme essential for DNA replication, recombination, and repair (91). Newer fluoroquinolones also inhibit DNA topoisomerase IV. The DNA gyrase A subunit is the main target of quinolones in Gram-negative bacteria, whereas topoisomerase IV is the primary target in Gram-positive bacteria. Inhibition of these bacterial enzyme targets causes relaxation or decatenation of the supercoiled DNA, leading to termination of chromosomal replication and interference with cell division and gene expression. By inhibiting bacterial DNA synthesis, these agents are bactericidal. However, the antibacterial activities of quinolones are reduced in the presence of low pH, urine, and divalent cations (Mg^{2+} and Ca^{2+}).

Bacterial resistance to quinolones may occur by several mechanisms: (i) single-step chromosomal mutations in the structural genes (*gyr*A, *gyr*B, *par*C, and *parE*) encoding the DNA gyrase and topoisomerase IV, (ii) mutations in the regulatory genes governing bacterial outer membrane permeability to the drug, (iii) expression or overexpression of energy-dependent multidrug efflux pump AcrAB, (iv) acquisition of plasmid-mediated resistance genes (*qnr*A, *qnr*B, and *qnr*S) encoding proteins that prevent the binding of quinolones to bacterial DNA gyrase and topoisomerase IV, and (v) acquisition of a plasmid containing the resistance gene *aac(6′)-Ib-cr*, which encodes a variant aminoglycoside acetyltransferase capable of acetylating the amino nitrogen on the piperazinyl substituent of quinolones, such as ciprofloxacin and norfloxacin but not levofloxacin, and reducing their activity. The last two mechanisms confer low-level resistance to quinolones (92, 93).

Pharmacology

Fluoroquinolones are generally well absorbed from the gastrointestinal tract, with the exception of norfloxacin. The oral bioavailability varies from 60% to 95% for the various fluoroquinolones (94). After oral administration, serum concentrations peak in 1 to 2 h. The presence of food does not significantly alter the absorption of these drugs. However, coadministration with iron- or zinc-containing multivitamins or with antacids containing aluminum, magnesium, or calcium substantially reduces the gastrointestinal absorption and subsequent peak concentrations of quinolones in serum. The degree of serum protein binding is generally low, ranging from 8% for ofloxacin to 54% for moxifloxacin. The prolonged elimination half-lives of fluoroquinolones allow for twice- or once-daily dosing (95, 96). Ciprofloxacin, levofloxacin, and moxifloxacin are also available for intravenous use, while besifloxacin, ciprofloxacin, gatifloxacin, levofloxacin, moxifloxacin, and ofloxacin are available commercially as ophthalmic preparations.

Quinolones have good penetration into lung, kidney, muscle, bone, intestinal wall, and extravascular body fluids. Concentrations in prostate are about two times those in serum, and concentrations of 25 to 100 times above peak serum concentrations are achieved in urine. Moxifloxacin is eliminated to a significantly lesser extent in the urine than levofloxacin or ciprofloxacin, leading to recommendations that it not be used for urinary tract infections. In the presence of meningeal inflammation, only ofloxacin, levofloxacin, gatifloxacin, and moxifloxacin achieve concentrations of >1 μg/ml in the CSF, though ciprofloxacin at high doses (i.e., 400 mg intravenously every 8 h) has been successfully used in the treatment of Gram-negative meningitis (97, 98). Quinolones penetrate well into phagocytes, such that concentrations within neutrophils and macrophages are as high as 14 times concentrations in sera (99). This feature accounts for their excellent *in vivo* activity against such intracellular pathogens as *Brucella*, *Listeria*, *Salmonella*, and *Mycobacterium* spp.

Ofloxacin and levofloxacin exhibit little or no *in vivo* metabolism and are excreted mainly (90%) via the kidney. The other quinolones are cleared by both hepatic and renal routes in various proportions, with elimination primarily via the kidneys. This renal elimination is blocked by probenecid. Small amounts of these drugs are also excreted in the bile.

The clearance of other fluoroquinolones is significantly diminished in the presence of renal failure. All of these drugs are only partially removed by hemodialysis (<15%) and are minimally affected by peritoneal dialysis because of their marked extravascular penetration, as reflected in their very large volumes of distribution.

Spectrum of Activity

Quinolones may be categorized into groups with similar spectra of antibacterial activity (Table 3), with the fluoroquinolones exhibiting a broad spectrum of activity (89). The narrow-spectrum quinolones, represented primarily by nalidixic acid, are inactive against Gram-positive cocci, and their clinical utility is limited by widespread prevalence and the rapid emergence of bacterial resistance. Broad-spectrum fluoroquinolones are active against both Gram-positive and Gram-negative bacteria (100). Increased activity against Gram-positive cocci and favorable pharmacodynamic properties (high ratios of the area under the curve from 0 to 24 h to the MIC) are major features of the newer fluoroquinolones, such as gemifloxacin, with potencies 2- to 8-fold greater than those of earlier agents (100, 101). When organisms are susceptible, quinolone MICs for the staphylococci are in the range of 0.03 to 1 μg/ml; however, many methicillin-resistant staphylococci are resistant to these agents (102). Although gemifloxacin and moxifloxacin are less potent against enterococci than against the staphylococci, they are 4- to 8-fold (MIC_{90}s of 0.03 to 0.25 μg/ml) more active than levofloxacin against MDR *S. pneumoniae*.

Several fluoroquinolones are in late stages of clinical development. These agents include delafloxacin, which is active against many MRSA isolates (MIC_{90} = 0.12 μg/ml) and is currently undergoing phase 3 clinical trials for complicated skin and skin structure infections. Its activity against enteric Gram-negative organisms appears similar to that of ciprofloxacin, though it may be slightly more potent against *P. aeruginosa* (103). Delafloxacin is also being studied in phase 3 trials for the treatment of gonorrhea as a single-dose oral formulation (http://clinicaltrials.gov/ct2/show/NCT02015637). Nemonoxacin is active against many MRSA isolates for which the MIC_{90} is 1 μg/ml, and it has a Gram-negative spectrum similar to that of levofloxacin (104). Finafloxacin activity is impacted by pH in that it has improved activity in acidic environments, leading to its development for the treatment of infections caused by *Helicobacter pylori* (105). Finafloxacin was equivalent to levofloxacin against ciprofloxacin-resistant MRSA and was comparable to ciprofloxacin against many Gram-negative organisms, depending on the pH tested (105). Ozenoxacin is active against many quinolone-resistant MRSA isolates as well as other Gram-positive organisms likely to cause skin infections (106).

Of the quinolones currently available for systemic use, only moxifloxacin possesses reliable activity against anaerobes, including members of the *B. fragilis* group as well as *Prevotella*, *Porphyromonas*, *Fusobacterium*, *Clostridium*, and anaerobic Gram-positive cocci at concentrations of 0.06 to 2 μg/ml. However, there are increasing reports of resistance to moxifloxacin among many Gram-negative anaerobes (107–109).

The fluoroquinolones possess excellent activity *in vivo* against susceptible *Enterobacteriaceae*, *P. aeruginosa*, *Citrobacter*, *Serratia*, and *Acinetobacter* spp., *H. influenzae*, and Gram-negative cocci, such as *N. gonorrhoeae*, *N. meningitidis*, and *M. catarrhalis* (100). However, the emergence of resistance in many of these organisms over the past decade has markedly compromised the utility of these agents for severe infections. The emergence of resistance to the fluoroquinolones in *E. coli* has become a particularly significant challenge due to the emergence of ST131 *E. coli*, a MDR strain which is very often fluoroquinolone resistant (110, 111). Many U.S. medical centers now report rates of ciprofloxacin resistance in their *E. coli* isolates of >25%. Enteropathogenic Gram-negative bacilli, such as *Salmonella*, *Shigella*, *Yersinia enterocolitica*, *Vibrio* spp., *Aeromonas* spp., *Plesiomonas* spp., *Campylobacter jejuni*, and enteroinvasive and enterotoxigenic *E. coli*, are typically susceptible to the quinolones (102). Clinical studies have shown these drugs to be effective in the prophylaxis and treatment of infectious diarrheas. However, reduced susceptibility and resistance to quinolones have emerged in clinical isolates of *Salmonella*, *Shigella*, and *Campylobacter* spp. (112). *Legionella* spp. are susceptible to these agents, with the MICs of most fluoroquinolones being 0.12 to 1.0 μg/ml for these organisms (100). Ciprofloxacin and levofloxacin are the only oral antibiotics with clinical utility in nonurinary *P. aeruginosa* infections. Ciprofloxacin is the more active of the two, with MIC_{90}s of 0.5 to 1 μg/ml; however, resistance rates are substantial in many areas. *Burkholderia* spp. and *S. maltophilia* are variably resistant to quinolones, though *S. maltophilia* is often inhibited by moxifloxacin (113).

The fluoroquinolones, especially levofloxacin, gatifloxacin, and moxifloxacin, are active *in vitro* against the *Mycobacterium* species *M. tuberculosis*, *M. fortuitum* group, *M. chelonae*, *M. kansasii*, and *M. xenopi* (114–116). Moxifloxacin, in combination with other antituberculosis drugs, is currently being studied in late-stage clinical trials for the short-term treatment of drug-susceptible tuberculosis (http://www.tballiance.org/portfolio/node/53). Moxifloxacin is reliably the most active fluoroquinolone against members of the *Mycobacterium avium* complex, while other members of the class have fair to poor activity (117). They also exhibit activity against *Chlamydia trachomatis*, *Chlamydia pneumoniae*, and *Mycoplasma hominis*, with MIC_{90}s of 0.1 to 1 μg/ml, but are less potent against *Ureaplasma urealyticum* (102, 118). Fluoroquinolones reliably inhibit *Rickettsia conorii*, *Rickettsia rickettsii*, and *Coxiella burnetii* (119–121) as well as *Bartonella* spp. (122) and *Brucella melitensis* (123). Although quinolones possess *in vitro* activity against *Plasmodium falciparum* at achievable serum concentrations, they are relatively ineffective when used clinically for the treatment of malaria. *Nocardia* spp. is relatively resistant to the quinolones, though moxifloxacin and gatifloxacin appear to have markedly improved activities compared to those of older agents (124, 125).

No significant inoculum effect has been observed among the bacteria susceptible to quinolones. Combinations of quinolones with β-lactam drugs or aminoglycosides are usually indifferent or additive in their effects against Gram-negative and Gram-positive bacteria and mycobacteria (126). However, bactericidal activities of quinolones can be antagonized by rifampin or chloramphenicol.

Adverse Effects

Gastrointestinal symptoms, occurring in up to 10% of patients as nausea, vomiting, abdominal discomfort, and diarrhea, are the most common side effects (127–129). *C. difficile* colitis has increasingly been associated with the use of the fluoroquinolones, particularly some of the newer agents (130). Headaches, fatigue, insomnia, dizziness, agitation, and, rarely, seizures, can occur. These adverse neurologic effects are usually associated with high dosages in elderly patients or concurrent use of nonsteroidal anti-inflammatory drugs.

Allergic reactions are uncommon and often manifest as rash, urticaria, and generalized pruritus. Photosensitivity is rare but may be observed with the fluoroquinolones currently available for systemic use. Rare laboratory abnormalities occurring during fluoroquinolone therapy include elevations in serum transaminases, eosinophilia, leukopenia, and thrombocytopenia. Gatifloxacin for systemic use was removed from the U.S. market due to the sequelae hypoglycemia and hyperglycemia.

Ciprofloxacin can increase the levels of theophylline and caffeine in serum as a result of decreased hepatic clearance (127, 131). Other reported drug interactions include augmentation of the anticoagulant effects of warfarin and an increase in serum cyclosporine levels with ciprofloxacin (131).

Although irreversible cartilage erosions and skeletal abnormalities were observed in studies of quinolone toxicity in animals (127), such effects have not yet been documented unequivocally in humans. The use of quinolones is generally considered to be contraindicated in children and in pregnant or nursing mothers; however, several recent trials of fluoroquinolones for otitis media in children have been completed, and adverse events were no different than those of the comparator agent (132, 133). All of the systemic fluoroquinolones now carry a black box warning in their prescribing information for increased risk of tendonitis and tendon rupture in all ages of patients. It appears that the risk increases with age and with coadministration of corticosteroids. In addition, fluoroquinolones carry a black box warning for exacerbating muscle weakness in patients with myasthenia gravis (134). All of the fluoroquinolones available for systemic use in the United States carry a warning for QT interval prolongation and torsades de pointes. These cases are uncommon and occur mostly in patients with a history of cardiac dysrhythmias, abnormal serum electrolytes, and impaired renal function.

AMINOGLYCOSIDES AND AMINOCYCLITOLS

Since the first aminoglycoside (aminoglycosidic aminocyclitol), streptomycin, was introduced in 1944, this class of antibiotic has played a vital role in the treatment of serious Gram-negative infections. Among the unique features of the aminoglycosides are their bactericidal activities against aerobic Gram-negative bacilli (including *Pseudomonas* spp.)

and M. *tuberculosis*; there is also a relatively low incidence of bacterial resistance to them. The currently available aminoglycosides are derived from *Micromonospora* spp. (gentamicin) or from *Streptomyces* spp. (kanamycin, neomycin, streptomycin, and tobramycin). Streptomycin, neomycin, kanamycin, tobramycin, and gentamicin are naturally occurring aminoglycosides, whereas amikacin is a semisynthetic derivative of kanamycin. Structurally, each of these aminoglycosides contains two or more amino sugars linked by glycosidic bonds to an aminocyclitol ring nucleus.

Mechanism of Action

Aminoglycosides are bactericidal agents that inhibit bacterial protein synthesis by binding irreversibly to the bacterial 30S ribosomal subunit. The aminoglycoside-bound bacterial ribosomes then become unavailable for translation of mRNA during protein synthesis, thereby leading to cell death (135). The aminoglycosides also cause misreading of the genetic code, with resultant production of nonsense proteins. To reach the intracellular ribosomal binding targets, an aerobic energy-dependent process is necessary to enable successful penetration of the bacterial inner cell membrane by the aminoglycosides. The requirement of an aerobic environment for this step accounts for the aminoglycosides' lack of activity against anaerobic bacteria. Bacterial uptake of these agents is facilitated by inhibitors of bacterial cell wall synthesis, such as β-lactams and vancomycin. This interaction forms the basis of antibacterial synergism between aminoglycosides and β-lactam antibiotics. There are three known mechanisms of bacterial resistance to aminoglycosides: (i) decreased intracellular accumulation of the antibiotic by alteration of the outer membrane's permeability, decreasing inner membrane transport, or active efflux; (ii) modification of the target site by mutation of the ribosomal proteins or 16S RNA or posttranscriptional methylation of 16S RNA (136); and (iii) enzymatic modification of the drug (the most common resistance mechanism) (137).

Pharmacology

All aminoglycosides have similar pharmacologic properties. Gastrointestinal absorption of these agents is unpredictable and always low. Because of its severe toxicity with systemic administration, neomycin is available only for oral and topical use. After intravenous administration, aminoglycosides are freely distributed in the extracellular space but penetrate poorly into the CSF, vitreous fluid of the eye, biliary tract, prostate, and tracheobronchial secretions, even in the presence of inflammation.

In adults with normal renal function, the aminoglycosides have half-lives in serum of about 2 to 3 h. They are excreted, essentially unchanged, primarily via the kidneys. There is considerable variation in the means of elimination of aminoglycosides among individuals, especially in patients with impaired renal function. Monitoring of serum aminoglycoside levels in these patients is essential for providing adequate therapy and reducing toxicity. With their features of concentration-dependent killing and prolonged postantibiotic effect, aminoglycosides may be administered once daily to achieve maximum bactericidal activity at high concentrations in serum without increased risk of toxicities (138). In renal failure, the drugs accumulate and dosage reductions or lengthening of the dosing interval is necessary. Aminoglycosides are substantially removed by hemodialysis and to a lesser extent by peritoneal dialysis.

Spectrum of Activity

Aminoglycoside antibiotics are active primarily against aerobic Gram-negative bacilli and *S. aureus*. As a group, they are particularly potent against the *Enterobacteriaceae*, *P. aeruginosa*, and *Acinetobacter* spp. Certain differences in antimicrobial spectra among the various aminoglycosides do exist. Widespread resistance among *Enterobacteriaceae* has limited the usefulness of streptomycin. It is now most commonly used as part of a multidrug regimen in the treatment of *M. tuberculosis* (it is the most active of the aminoglycosides against this organism) or of high-level gentamicin-resistant but high-level streptomycin-susceptible enterococcal infections (139). As a single agent, streptomycin can be used in the therapy of infections due to *Francisella tularensis* (tularemia) and *Yersinia pestis* (plague) (140). It has historically been used in conjunction with doxycycline for the treatment of brucellosis but has largely been replaced by gentamicin in this role due to the difficulty in obtaining serum-level monitoring of streptomycin.

Although gentamicin and tobramycin have very similar antibacterial activity profiles, gentamicin is more active *in vitro* against *Serratia* spp., whereas tobramycin is more active against *P. aeruginosa* (141). However, these minor differences have not been correlated with greater efficacy of one agent over the other. For the most part, gentamicin and tobramycin are susceptible to inactivation by the same modifying enzymes produced by resistant bacteria, except that, in contrast to gentamicin, tobramycin can be inactivated by 6-acetyltransferase and 4′-adenyltransferase and has variable susceptibility to 3-acetyltransferase. Amikacin is resistant to many of these aminoglycoside-modifying enzymes and therefore is active against most *Enterobacteriaceae* that are resistant to gentamicin and tobramycin (142). Amikacin is often used as the aminoglycoside of choice when gentamicin and tobramycin resistances are prevalent. In addition, amikacin is active against many drug-susceptible *Mycobacterium* spp. (143). Aminoglycosides are only moderately active against *Haemophilus* and *Neisseria* spp. Of the agents active against *Bartonella* spp., aminoglycosides are the only drugs consistently bactericidal toward this group of organisms (140).

Although active against staphylococci, aminoglycosides are not recommended as single agents for the treatment of staphylococcal infections. Gentamicin is often combined with a penicillin or vancomycin for synergy in the treatment of serious infections due to staphylococci, enterococci, or viridans group streptococci (139, 144). However, this practice has fallen out of favor for native valve endocarditis due to *S. aureus* and is increasingly being questioned in enterococcal native valve endocarditis due to the toxicity associated with even short-term aminoglycoside use (145, 146). The aminoglycosides are not active against anaerobes.

Spectinomycin is not currently available for clinical use in the United States. It has historically been used primarily for uncomplicated anogenital infections due to *N. gonorrhoeae* in patients with penicillin or cephalosporin allergy and contraindications to fluoroquinolone therapy (22). However, spectinomycin is ineffective for pharyngeal gonococcal infections, syphilis, or chlamydial infections.

Adverse Effects

Considerable intrinsic toxicity, mainly in the form of nephrotoxicity and auditory or vestibular toxicity, is characteristic of all of the aminoglycosides. The nephrotoxic potential varies among the aminoglycosides, with neomycin being the most toxic and streptomycin the least. This effect is usually reversible when the drug is discontinued. Hypotension, prolonged duration of therapy, preexisting renal insufficiency, and possibly excessive trough serum aminoglycoside concentrations increase the risk of nephrotoxicity with these agents.

All aminoglycosides are capable of causing damage to the eighth cranial nerve in humans. Vestibular toxicity is more frequently associated with streptomycin, gentamicin, and tobramycin, whereas auditory toxicity is more typical of kanamycin and amikacin. This frequently irreversible side effect may occur even after discontinuation of the drug and is cumulative with repeated courses of the agent. The ototoxicity is a result of selective destruction of the hair cells in the cochlea. Clinically detectable auditory and vestibular dysfunction has been reported to occur in 3 to 5% of patients receiving gentamicin, tobramycin, or amikacin who underwent audiometric testing (147).

Neuromuscular paralysis, which is usually reversible, can occur after rapid intravenous infusion of aminoglycosides. This phenomenon occurs particularly in the setting of myasthenia gravis or concurrent use of succinylcholine during anesthesia. Other minor adverse reactions include local pain and allergic skin rashes. Serious adverse reactions have not been reported for spectinomycin.

MACROLIDES

Macrolides have been in use since the early 1950s, with erythromycin being the prototypical antibiotic of this class for over 30 years (148). Their chemical structures consist of a macrocyclic lactone ring attached to two sugar moieties, desosamine and cladinose. They differ from each other in the sizes (14 to 16 atoms) and substitution patterns of their lactone rings. Erythromycin is a naturally occurring 14-membered macrolide derived from *Streptomyces erythreus*, and other natural analogs include oleandomycin, spiramycin, and josamycin. Clarithromycin is a semisynthetic 14-membered-ring macrolide, while azithromycin is a 15-membered-ring derivative, also known as an azalide, with a nitrogen atom incorporated in its lactone ring. These newer macrolides offer significant advantages over erythromycin because of expanded antimicrobial spectra, improved pharmacokinetic parameters, and less frequent adverse effects and drug interactions.

Mechanism of Action

Macrolides are generally bacteriostatic agents that inhibit bacterial RNA-dependent protein synthesis. They may be bactericidal at high drug concentrations and against a low inoculum of bacteria. They bind reversibly to the 23S rRNA of the 50S ribosomal subunits of susceptible organisms, thereby blocking the translocation reaction of polypeptide chain elongation (149). The presence of *erm*(B) gene-encoded rRNA methyltransferases that modify the bacterial 23S rRNA target-binding site is the primary mechanism of macrolide resistance and confers macrolide-lincosamide-streptogramin B (MLS_B) coresistance (150, 151). Expression of *erm* may be constitutive or inducible. If inducible, the bacterial strains appear to be resistant to erythromycin (an inducer) but susceptible to clindamycin. However, treatment with clindamycin may lead to selection of resistant mutants and therapeutic failure (150). Other uncommon mechanisms of resistance to macrolides include production of macrolide-inactivating enzymes (esterases, phosphorylases, and glycosidases), *mef*- and *msr*-encoded active efflux of drug, *mtrR* promoter gene mutation-induced overproduction of an *mrtrCDE*-encoded efflux pump, and mutations in 23S rRNA (151, 152).

Pharmacology

Erythromycin is available in various topical, parenteral (lactobionate), and oral (base stearate, ethylsuccinate, and estolate) preparations. While clarithromycin is available only in oral forms, azithromycin is formulated for oral and intravenous administration. When administered orally, the erythromycin base is rapidly inactivated by gastric acid, whereas the newer macrolides are stable against acid degradation. Intestinal absorption of erythromycin (except for the estolate form) and azithromycin is reduced up to 50% in the presence of food. Peak levels in serum of 2 to 3, 1 to 2, and 0.4 μg/ml are reached at 3 h after oral doses of erythromycin (500 mg), clarithromycin (250 mg), and azithromycin (500 mg), respectively. Much higher concentrations of erythromycin and azithromycin are achieved with intravenous infusion. Tissue distributions of macrolides are excellent, with concentrations in various tissues 10- to 100-fold higher than those in serum (153). The high concentrations reached rapidly within neutrophils and macrophages account for their potent activity against intracellular pathogens (154). They penetrate poorly into the brain and CSF, but they do cross the placenta and are excreted in breast milk.

Erythromycin and clarithromycin are metabolized by the liver and excreted primarily in the bile. Azithromycin is excreted largely unchanged in the bile. Clarithromycin exhibits first-pass metabolism, producing a microbiologically active 14-hydroxy derivative that is two to four times more potent than the parent drug against some organisms. Erythromycin, clarithromycin, 14-hydroxy clarithromycin, and azithromycin have terminal half-lives in serum of 1.5, 5.0, 8.5, and 41 h, respectively. Because of its exceptionally high tissue penetration, azithromycin has a half-life in tissue of 2 to 4 days (154). Dosage adjustment of clarithromycin is necessary with moderate to severe renal failure (creatinine clearance of <30 ml/min). Except for clarithromycin, macrolides are removed minimally by hemodialysis or peritoneal dialysis.

Spectrum of Activity

Macrolides are relatively broad-spectrum antibiotics, with activity against Gram-positive and some Gram-negative bacteria, mycoplasmas, chlamydiae, treponemes, and rickettsiae (153, 154). Erythromycin shows good activity against staphylococci and streptococci, including *S. pneumoniae*, but the emergence of resistance among these isolates is increasingly common (3–5, 155). Clarithromycin is 2- to 4-fold more potent than the other macrolides, and azithromycin is less active than erythromycin against most staphylococci and streptococci (156). These drugs may be bactericidal against susceptible strains of streptococci but are generally thought to be bacteriostatic agents against most other organisms. Erythromycin-resistant strains display cross-resistance to clarithromycin and azithromycin. The vast majority of methicillin-resistant staphylococci and many enterococci are resistant to all macrolides. These drugs are also active against *Corynebacterium* spp., *L. monocytogenes*, and *Actinomyces israelii* (156). The antibacterial activities of macrolides against Gram-negative bacilli are influenced by pH, with increasing potency (lower MICs) as the pH rises to 8.5. *H. influenzae* and *M. catarrhalis* are more susceptible to azithromycin (MIC_{90} of 0.5 μg/ml) than to other macrolides (8- to 16-fold-higher MIC_{90}s) (157, 158). However, additive (and possibly synergistic) activity between clarithromycin and its 14-hydroxy metabolite reduces the MIC of clarithromycin for *H. influenzae* 2- to 4-fold

(159). Clarithromycin is the most active drug in this class against *C. pneumoniae* (MIC_{90} of 0.25 μg/ml) and *Legionella* isolates (MIC_{90} of 0.25 μg/ml) (156). All three macrolides are equally potent against *Bordetella pertussis* and *M. pneumoniae*; erythromycin has long been established as the drug of choice for the therapy of infections due to these pathogens and *Legionella* spp., though the newer macrolides are increasingly used clinically due to their superior tolerability, pharmacokinetics, and drug interaction profile. However, fluoroquinolones may be superior to macrolides for infections due to *Legionella* spp. (160). Macrolides are active against *Campylobacter* spp., *H. pylori*, *P. multocida*, *N. meningitidis*, and *Borrelia burgdorferi* (156, 158). Unlike other macrolides, azithromycin is also active *in vitro* against some strains of *E. coli*, *Shigella* spp., *Salmonella* spp., and *Y. enterocolitica*, although resistant strains are commonly encountered today (157, 159, 161).

Macrolide antibiotics are effective *in vitro* against many pathogens that cause sexually transmitted diseases. *N. gonorrhoeae*, *Haemophilus ducreyi*, *C. trachomatis*, and *U. urealyticum* are all susceptible, but only azithromycin is active against *Mycoplasma hominis* (156, 157). Azithromycin (2 g as a single dose) may be used for the treatment of gonorrhea in patients unable to receive ceftriaxone due to severe allergy (162). Azithromycin as a 2-g dose is suggested as a possible third-line therapy in early-syphilis patients who cannot tolerate penicillin G or doxycycline; however, resistance to azithromycin has been reported (22). Azithromycin is effective as an alternative to tetracyclines for the treatment of genital chlamydial infections (163). As a group, macrolides are among the most potent agents inhibitory toward *Bartonella* spp. (122). The macrolides have good activity against some anaerobic bacteria, such as *Actinomyces* spp., *Clostridium perfringens*, *Peptostreptococcus* spp., *Propionibacterium* spp., *Prevotella* spp., *Porphyromonas* spp., and some anaerobic Gram-positive cocci, with MIC_{90}s of 1 to 4 μg/ml (164). They are much less active against the *Bacteroides* spp., with MIC_{90}s in excess of 32 μg/ml. They also have poor activity against *Fusobacterium nucleatum*, *Clostridium innocuum*, and *Lactobacillus* spp., for which the macrolides exhibit MIC_{90}s in excess of 32 μg/ml.

Atypical mycobacteria are more susceptible than *M. tuberculosis* to macrolide antibiotics. Clarithromycin is widely considered the gold standard agent against these organisms (143, 157). The MIC_{90}s of clarithromycin and azithromycin for *M. avium* complex are in the range of 2 to 4 μg/ml, allowing additive or synergistic killing activity against these organisms within infected macrophages when these drugs are combined with other antimycobacterial drugs (156). Clarithromycin is used to treat infections due to *Mycobacterium chelonae* and many other nontuberculous mycobacteria (143).

Clarithromycin and azithromycin offer comparable *in vitro* activities against *Toxoplasma gondii* and are alternative therapies for this organism (165).

Adverse Effects

The incidence of serious side effects related to the use of the macrolides is relatively low. Gastrointestinal irritation, such as abdominal cramps, nausea, vomiting, and diarrhea, is common with oral administration of erythromycin and can occur when the drug is given intravenously. These side effects occur less frequently with clarithromycin and azithromycin. Thrombophlebitis is associated with intravenous erythromycin infusion and is one of the reasons that intravenous azithromycin has largely replaced intravenous erythromycin in most clinical settings. Hypersensitivity reactions may include skin rash, fever, and eosinophilia. Cholestatic hepatitis occurring in adults has frequently been associated with erythromycin estolate but has also been reported with other forms of erythromycin (166) and azithromycin (167). For this reason, erythromycin estolate is no longer recommended for use in adults.

Reversible hearing loss may occur with the use of large doses and very high serum concentrations of erythromycin (≥4 g/day), usually in elderly patients with renal insufficiency (168). Ototoxicity has also been reported with high doses of clarithromycin and azithromycin used to treat *M. avium* complex infections. Pseudomembranous colitis and superinfection of the gastrointestinal tract or vagina with *Candida* spp. or Gram-negative bacilli occur rarely. Erythromycin is a potent inhibitor of cytochrome P450 enzymes, leading to multiple drug-drug interactions with this agent (169). While significantly less so than erythromycin, clarithromycin remains a clinically important inhibitor of cytochrome P450 and also possesses a number of clinically significant drug interactions. Azithromycin minimally inhibits these hepatic enzymes and is much less likely to be associated with clinically significant drug interactions. In March of 2013, the FDA issued a drug safety communication regarding azithromycin and increased risk of sudden cardiac death due to cardiac dysrhythmias. The agency warned that azithromycin use may lead to potentially fatal irregular heart rhythms and stated that the risk was highest in patients with a history of cardiac dysrhythmias, electrolyte abnormalities, or concurrent use of cardiac antiarrhythmic medications.

Fidaxomicin

Fidaxomicin is a nonabsorbable macrocyclic 18-ring macrolide antibiotic that is FDA approved for the treatment of diarrhea due to *Clostridium difficile*. Fidaxomicin acts by inhibiting RNA polymerase, leading to impaired RNA chain synthesis and transcription (170). The drug is available only for clinical use as a tablet and is administered at a dose of 200 mg every 12 h, which results in high concentrations in the intestinal tract but concentrations in serum of less than 10 ng/ml. Its major metabolite, OP-1118, is also active against *Clostridium difficile* and also achieves very low serum concentrations. The MIC_{90} of fidaxomicin against *C. difficile* is 0.5 μg/ml, while stool concentrations of fidaxomicin are well in excess of 500 μg/g. The activity of fidaxomicin against *B. fragilis*, as well as other nonclostridial intestinal anaerobes, is significantly less, with MICs for *B. fragilis*, *Prevotella* spp., eubacteria, facultative Gram-negative rods, *Clostridium ramosum*, and other intestinal anaerobes typically in the 256- to 1,024-μg/ml range (171). The emergence of *C. difficile* with alterations in the *rpoB* and/or *rpoC* gene through serial passage, for which MICs of fidaxomicin are elevated, as well as in a clinical case has been reported, but its clinical significance remains uncertain due to MICs remaining 100 times less than fidaxomicin concentrations in the stool (172).

In clinical trials versus vancomycin administered at a dose of 125 mg every 6 h, fidaxomicin at 200 mg every 12 h was found to be noninferior to vancomycin for the treatment of *C. difficile* disease. Relapse rates were approximately 10% to 15% better in patients receiving fidaxomicin. The suggested mechanism for this advantage in relapse rates is fidaxomicin's relative sparing of the normal intestinal microbiota compared to what occurs with vancomycin (173). Though the drug appears to offer a therapeutic advantage over vancomycin with respect to relapse rates, clinical use of the compound has been slowed by the high cost of the medication.

KETOLIDES

Ketolides are semisynthetic derivatives of erythromycin A, having a ketone group instead of an L-cladinose moiety at the 3 position on the erythronolide A ring. This modification of the chemical structure results in increased stability in acid media, noninducibility of MLS_B resistance, and enhanced activity against Gram-positive cocci. The ketolides currently under clinical development also have a substituted carbamate link between carbon atoms 11 and 12 in the macrolide nucleus. This modification enables them to retain activity against bacteria whose ribosomes have been methylated at position A2058 as a result of acquired methylase genes (174). Telithromycin is the first and only ketolide currently approved for clinical use in the United States. However, its clinical use is severely limited due to cases of fulminant hepatic failure, which has resulted in a warning in its prescribing information for hepatotoxicity. In addition, telithromycin possesses a black box warning for respiratory failure in patients with myasthenia gravis. Another ketolide with similar antibacterial properties, cethromycin, completed phase 3 studies, but an FDA advisory committee voted that, while the drug appeared to be safe, the studies did not demonstrate sufficient efficacy in mild to moderate community-acquired pneumonia (CAP). The FDA refused to approve the agent and issued a complete response letter in August 2009, resulting in apparent discontinuation of clinical development. Solithromycin (CEM-101) is a fluoroketolide currently undergoing phase 3 clinical studies for community-acquired bacterial pneumonia (CABP). It has an antibacterial spectrum similar to telithromycin's, with MICs generally within 2-fold of that of the seminal ketolide, but is expected to demonstrate a more robust safety profile based on preclinical studies (175).

Mechanism of Action

Like the macrolide antibiotics, ketolides inhibit bacterial protein synthesis in susceptible organisms by binding to the bacterial 23S rRNA at domains II and V of the peptidyltransferase site in 50S ribosomal subunit 47 (176). These drugs may also inhibit the formation of the 30S ribosomal unit. Although ketolides do not induce MLS_B resistance, staphylococci with constitutively expressed MLS_B resistance encoded by *erm* genes are resistant to telithromycin. Although these drugs do not appear to be affected by efflux, mutations in the 23S rRNA and ribosomal proteins L4 and L22 (177) or *ermB* gene mutation-induced rRNA methylation (178) can lead to *in vitro* resistance to ketolides. This class of drugs has a low potential to select for resistance or induce cross-resistance among other MLS_B antimicrobials.

Pharmacology

Telithromycin is administered orally as a once-daily dose of 800 mg, with rapid gastrointestinal absorption, yielding a mean peak concentration in plasma of 2 μg/ml in 1 to 2 h and steady state in 2 days. A mean trough concentration in plasma of 0.07 μg/ml is attained at 24 h after dosing (179). The oral bioavailability of 57% is unaffected by food ingestion. With about 70% of the drug being protein bound, telithromycin exhibits biphasic elimination from plasma, with initial and terminal half-lives of 2 to 3 h and 9 to 10 h, respectively. The drug penetrates well into bronchopulmonary, tonsillar, and sinus tissues and into middle ear fluid, and it is accumulated by polymorphonuclear neutrophils with an intracellular concentration-to-plasma concentration ratio of >500 at 24 h. Hepatic metabolism with elimination via feces (~80%) is the main route of excretion, and <15% of the administered dose is eliminated in urine. Dosage adjustments are not necessary in patients with renal or hepatic impairment.

Spectrum of Activity

Ketolides possess potent activity against respiratory pathogens as well as intracellular bacteria, and all that have been developed have been designed specifically for the treatment of community-acquired respiratory tract infections. Telithromycin is more potent than macrolides against *S. pneumoniae* isolates, irrespective of penicillin susceptibility, with an MIC_{90} of ≤1 μg/ml; 90% of penicillin-resistant strains are inhibited at 0.25 μg/ml (180–183). Almost all macrolide-resistant strains of pneumococci are inhibited by the ketolides at ≤0.5 μg/ml, regardless of the underlying mechanism of macrolide resistance (3–5). Telithromycin is more active than erythromycin and clarithromycin and as potent as azithromycin against *H. influenzae* (MIC_{90}s of 2 to 4 μg/ml) and *M. catarrhalis* (MIC_{90}s of 0.06 to 0.125 μg/ml). The activity of telithromycin is unaffected by β-lactamase production in these strains, but the MICs are increased 2-fold in the presence of 5% CO_2. Significant postantibiotic effects may be observed for up to 9 h with this drug against the major respiratory pathogens (184).

Telithromycin is also active against staphylococci, with MIC_{90}s of 0.125 to 0.25 μg/ml for *S. aureus* and coagulase-negative staphylococci, regardless of the organism's susceptibility to oxacillin. However, isolates harboring the constitutive MLS_B mechanism of resistance are resistant to ketolides. Enterococci without underlying resistance to macrolides and clindamycin are susceptible to telithromycin, with MIC_{90}s of 0.125 μg/ml for *E. faecalis* and *E. faecium*. Higher MIC_{90}s (4 to 8 μg/ml) are observed with erythromycin- or clindamycin-resistant enterococci (185). Telithromycin displays good *in vitro* activity against beta-hemolytic streptococci and viridans group streptococci, regardless of their susceptibility to benzylpenicillin, with all isolates inhibited at ≤0.5 μg/ml. While streptococcal isolates with the *mefA* (gene-mediated) drug efflux mechanism of resistance to erythromycin remain susceptible to telithromycin, ketolide MICs for them are higher (2 to 16 μg/ml) among the strains with inducible or constitutive *erm* (gene-mediated) resistance to erythromycin. Other Gram-positive cocci, such as *Pediococcus*, *Leuconostoc*, *Stomatococcus*, and *Rhodococcus equi*, are susceptible to telithromycin, with MIC_{90}s for them of 0.03 to 0.25 μg/ml.

Ketolides are also very active against Gram-positive bacilli, inhibiting *Corynebacterium* (including *C. diphtheriae* and *C. jeikeium*), *Listeria*, *Lactobacillus*, *Actinomyces*, and *Erysipelothrix* at concentrations of ≤0.125 μg/ml (185). Telithromycin is inhibitory (MIC_{90}s of 0.125 to 0.5 μg/ml) to *Peptostreptococcus* spp., *Prevotella* spp., *Porphyromonas* spp., *Bilophila* spp., and *Clostridium perfringens*, but it is not active against other *Clostridium* spp., *Fusobacterium*, and members of the *B. fragilis* group (186). This drug has poor activity against other Gram-negative bacilli, including the *Enterobacteriaceae*, *Acinetobacter* spp., *P. aeruginosa*, and *Borrelia burgdorferi* (187).

Intracellular pathogens, such as *Legionella*, *Mycoplasma*, and *Chlamydia*, are highly susceptible to telithromycin, for which MIC_{90}s are 0.004 to 0.25 μg/ml (181). *Rickettsia* spp., *Bartonella* spp., *C. burnetii*, and *F. tularensis* are also susceptible to this agent. Telithromycin is comparable to the macrolides in its activity against *M. chelonae* and *M. avium*, with an MIC_{90} of 4 μg/ml. It is not active against *M. tuberculosis*, *M. bovis*, and other atypical mycobacteria (188).

Adverse Effects

Telithromycin's clinical utility has been severely hampered by the warnings for hepatotoxicity and respiratory failure in myasthenia gravis that have been added to the prescribing information. However, during registration trials, telithromycin was well tolerated by all patient populations, with gastrointestinal adverse effects, such as diarrhea (15%), nausea (9%), vomiting, and dizziness, as the most frequent adverse effects (189). Occurrence of *C. difficile*-associated diarrhea has not been reported in clinical trial studies. While elevation of serum transaminase levels is found in <10% of patients, rare cases of severe hepatotoxicity can occur (190). Since ketolides are substrates and inhibitors of the hepatic cytochrome P450 CYP3A4 isoenzyme pathway, their potential to lengthen the QT interval is augmented by concomitant administration of other CYP3A4 inhibitors, such as the triazole antifungal agents (191).

LINCOSAMIDES

The lincosamide antibiotics include lincomycin, which was initially isolated from *Streptomyces lincolnensis*, and clindamycin, which is a chemical modification of lincomycin. The chemical structure of each drug consists of an amino acid linked to an amino sugar. Compared with lincomycin, clindamycin has increased antibacterial activity and improved absorption after oral administration (192). Both drugs are available for parenteral and oral use, but lincomycin is very rarely used in the United States and will not be discussed further.

Mechanism of Action

Lincosamides bind to the 50S ribosomal subunits of susceptible bacteria and prevent elongation of peptide chains by interfering with peptidyl transfer, thereby suppressing protein synthesis. The ribosomal binding sites are the same as, or closely related to, those that bind macrolides, streptogramins, and chloramphenicol (193). Clindamycin can be bactericidal or bacteriostatic, depending on the drug concentration, bacterial species, and inoculum of bacteria.

Pharmacology

About 90% of an oral clindamycin dose is absorbed from the gastrointestinal tract, with no interference from the ingestion of food. A single oral dose of 150 mg yields a peak concentration in serum of 2 to 3 μg/ml in 1 h. Peak levels in serum of 10 to 12 μg/ml are obtained at 1 h after a 600-mg intravenous dose. Therapeutic serum drug levels are maintained for 6 to 9 h after these dosages (194).

Clindamycin distributes well into bone, lungs, pleural fluid, and bile, but it penetrates poorly into CSF, even in patients with meningitis. It readily crosses the placenta and enters fetal tissues. Clindamycin is actively concentrated in neutrophils and macrophages.

The normal half-life of clindamycin is 2.4 h. Most of the drug is metabolized by the liver and excreted in an inactive form in the urine. Its half-life is prolonged by severe liver dysfunction, necessitating dosage reduction in patients with severe liver disease. Although the serum drug levels are increased in patients with severe renal failure, dose modification is not essential. The drug is not removed significantly by hemodialysis or peritoneal dialysis.

Spectrum of Activity

Clindamycin has a broad spectrum of activity against the aerobic Gram-positive cocci and against anaerobes, which include methicillin-susceptible *Staphylococcus* spp., *S. pneumoniae*, and group A and viridans group streptococci (192, 194). The MIC_{90}s are in the range of 0.01 to 0.1 μg/ml for susceptible strains of these organisms. However, resistance to clindamycin has emerged in clinical isolates of these bacteria that are also resistant to erythromycin (3–5, 155). The prevalence of clindamycin-resistant *S. aureus* strains is 20% or higher in some institutions, with up to 85% coresistance in USA100 MRSA strains. USA300 community-associated MRSA strains often retain susceptibility to clindamycin. Resistance in beta-hemolytic streptococci, pneumococci, and viridans group streptococci is considerable as well. Enterococci and all *Enterobacteriaceae* are uniformly resistant to the lincosamides.

Clindamycin is one of the most active antibiotics available against anaerobes, including members of the *B. fragilis* group and *C. perfringens*, with MIC_{90}s of ≤2 μg/ml (194, 195). However, clindamycin resistance is increasing. In a recent study, more than 30% of *Bacteroides* spp. were resistant to clindamycin; MIC_{90}s for them were >128 μg/ml (196, 197). Thirty percent of all anaerobic clinical isolates in a more recent European series were resistant to clindamycin, with resistance highest in *Bacteroides* and *Parabacteroides* spp., excluding *B. fragilis* strains (54% resistant), *Prevotella* spp., and other Gram-negative anaerobes (31% resistant) (198). Ten to 20% of clostridial species, 10% of peptococci, and most *Fusobacterium varium* strains have also been found to be resistant to clindamycin (109, 199). Clindamycin has been used successfully as a single-agent therapy for actinomycosis (200), babesiosis (165, 201), and malaria (202). It is also effective in combination with pyrimethamine for toxoplasma encephalitis (203) and in combination with primaquine for *Pneumocystis jirovecii* pneumonia (204). Clindamycin phosphate and benzoyl peroxide (1.2%/2.5%) as a gel is also used for the topical treatment of acne vulgaris.

Adverse Effects

Clindamycin-associated diarrhea occurs in up to 20% of patients, and use of this drug has been commonly associated with pseudomembranous colitis caused by toxin-producing *C. difficile* (12). This complication is not dose related and may occur after oral or parenteral therapy. Prompt cessation of the antibiotic in conjunction with oral vancomycin or metronidazole is effective in reversing this complication.

Other uncommon side effects include skin rashes, fever, and reversible elevation of serum transaminases. Clindamycin can block neuromuscular transmission and may potentiate the action of neuromuscular blocking agents during anesthesia.

TETRACYCLINES AND GLYCYLCYCLINES

Tetracyclines are broad-spectrum bacteriostatic antibiotics with the hydronaphthacene nucleus, which contains four fused rings. The congeners form three groups based on their durations of action. Tetracycline is a short-acting drug, demeclocycline is an intermediate-acting drug, and doxycycline and minocycline are long-acting drugs. Demeclocycline is used mostly for the syndrome of inappropriate antidiuretic hormone secretion and is not routinely used for its antibacterial properties. Glycylcyclines are a group of semisynthetic tetracycline derivatives containing a glycylamido substitution at position 9. Tigecycline (a 9-*t*-butylglycylamido derivative of minocycline) is the first in this class of drugs available for clinical use (205). Omadacycline is an aminomethylcycline closely related to tigecycline that is entering phase 3 studies in the United States. Eravacycline, a C-7, C-9-disubstitution fluorocycline, is currently in phase 3 clinical trials for treatment of complicated intra-abdominal infections (cIAI).

Mechanism of Action

Tetracyclines and glycylcyclines act against susceptible microorganisms by inhibiting protein synthesis. They enter bacteria by an energy-dependent process and bind reversibly to the 30S ribosomal subunits, preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor A site in the RNA-ribosome complex (206, 207). Resistance to tetracyclines occurs among clinical isolates as a result of active efflux of the drug from the cell, an altered ribosomal target site that prevents binding of the drug (ribosomal protection) or production of modifying enzymes that inactivate the drug. With stearic hindrance from the bulky side group at position 9, glycylcyclines are unaffected by bacterial ribosomal protection proteins and evade most efflux pumps present in tetracycline-resistant strains. These drugs also have higher binding affinities for the bacterial ribosomes than tetracyclines (207). Reduced susceptibility to tigecycline has been found in clinical strains of *Enterobacteriaceae* (particularly problematic for *Proteus* spp., *Morganella* spp., and *Providencia* spp.), *A. baumannii*, *P. aeruginosa*, and *S. aureus* strains possessing chromosomally encoded multicomponent efflux pumps (208–211) and in some *Bacteroides* spp. with the *tet*(X) gene-mediated monooxygenase enzyme that degrades tetracyclines (212).

Pharmacology

Tetracyclines are incompletely absorbed from the gastrointestinal tract, but their absorption is improved in the fasting state. Ingestion of food, especially dairy products, and other substances, such as divalent- and trivalent-cation-containing antacids and iron preparations, impairs the absorption of these drugs. Less interference with absorption by foods occurs with doxycycline and minocycline. Minocycline and doxycycline are readily absorbed, and therefore lower doses are required. Peak concentrations in serum of 3 to 5 μg/ml are reached in 2 h after standard oral dosages. Intravenous preparations are available, and peak concentrations in serum of 3 to 9 μg/ml are reached in 1 h after intravenous administration, depending on the tetracycline infused and the dose (213). Tetracyclines are usually bacteriostatic at these clinically achievable concentrations in serum.

Tissue penetration of these drugs is excellent, but levels in CSF are low even in the presence of meningeal inflammation. Tetracyclines cross the placenta and are incorporated into fetal bone and teeth. They are excreted in high concentrations in human milk. Therefore, tetracyclines are not advised for pregnant or lactating women. Minocycline, the most lipophilic tetracycline at physiologic pH, reaches relatively high concentrations in saliva and tears (214).

Many tetracyclines are metabolized by the liver and concentrated in the bile. Biliary concentrations of tetracyclines are three to five times higher than concurrent levels in plasma, with significant drug accumulation in the blood of patients with hepatic insufficiency or biliary obstruction. Doxycycline is eliminated unchanged in the urine, and 35 to 60% of the dose is recovered in the urine. Minocycline is eliminated primarily through nonrenal routes, with only approximately 5 to 12% of the dose recovered in the urine. Renal failure prolongs the half-lives of the tetracyclines except doxycycline and minocycline (213). Therefore, doxycycline or minocycline should be considered the tetracycline of choice for extrarenal infections in the presence of renal failure.

Tigecycline is administered as an intravenous formulation because of limited oral bioavailability. After multiple doses of 50 mg infused every 12 h, the peak serum concentration is approximately 0.8 μg/ml (215, 216). Despite having plasma protein binding of 80%, the drug has a rapid and wide distribution into tissues, including bone, resulting in 3- to 5-fold-higher drug exposures in skin and soft tissue than in the blood. Penetration into CSF in patients with or without meningitis is marginal, with a drug level of 0.025 μg/ml in the CSF at 12 h after infusion of a single 100-mg dose (217). It is eliminated primarily by the liver via glucuronidation and biliary excretion of unchanged drug, and the mean elimination half-life is 36 h. With <30% of the drug excreted unchanged in the urine, dosage adjustment is not required for renal insufficiency, hemodialysis, or mild to moderate hepatic dysfunction.

Omadacycline and eravacycline are being developed in both oral and intravenous formulations (218). The half-life of omadacycline is approximately 17 h, with a 100-mg intravenous administration resulting in a time to maximum concentration of the drug in serum (C_{max}) of approximately 1.8 μg/ml (219). Like tigecycline, omadacycline and eravacycline evade the ribosomal protection resistance mechanism and most efflux pumps that are associated with tetracycline resistance. However, similarly to tigecycline, omadacycline is a substrate for the AcrAB multidrug efflux pump commonly found in *Proteus* spp. and *Klebsiella* spp. as well as the MexXY efflux system found in *P. aeruginosa*.

Spectrum of Activity

All tetracyclines have similar antimicrobial spectra, with activities against many Gram-positive and Gram-negative bacteria, mycoplasmas, chlamydiae, rickettsiae, and some protozoa. Many Gram-positive aerobic cocci, including *S. aureus*, *S. pyogenes*, and *S. pneumoniae*, are susceptible at concentrations achievable in the serum. However, tetracycline-resistant strains of *S. pneumoniae* are common (3–5). A substantial percentage of *E. coli* isolates are now doxycycline and minocycline resistant. Neither of these agents is recommended in the 2010 IDSA guidelines for cystitis and pyelonephritis (220, 221). Pseudomonads and many *Enterobacteriaceae* are also resistant. Most strains of *Shigella* and *Salmonella* spp. are resistant to these agents. With activity against *Burkholderia pseudomallei*, *Brucella* spp., *Vibrio* spp., and *Mycobacterium marinum* (222), they have been used successfully in the treatment of infections due to these bacteria. Their efficacy in the therapy of cholera is diminishing owing to the emergence of resistant *Vibrio cholerae* isolates (223). Minocycline is active against *Nocardia* spp. (224). There is also renewed interest in minocycline for the treatment of MDR *Acinetobacter* spp. and *S. maltophilia*. Minocycline is the most active tetracycline against these 2 problematic pathogens, and a large percentage of isolates remain susceptible despite resistance to a variety of other antibiotics (225, 226). Some anaerobic bacteria are susceptible to tetracyclines, though resistance is increasingly common (227). However, tigecycline retains activity against most anaerobes, as demonstrated in a recent study (228).

These drugs are useful in the treatment of urethritis and acute pelvic inflammatory diseases caused by *C. trachomatis*, *U. urealyticum*, and *M. hominis*. Resistance to tetracyclines is prevalent among *N. gonorrhoeae* strains (229). The drugs are effective for the treatment of other chlamydial infections (psittacosis, lymphogranuloma venereum, and trachoma)

(22). Other infections responsive to tetracyclines include granuloma inguinale, chancroid, relapsing fever, and tularemia. Tetracyclines are the drugs of choice for treating rickettsial infections (Rocky Mountain spotted fever, endemic and scrub typhus, and Q fever). Many pathogenic spirochetes, including *Treponema pallidum* and *B. burgdorferi*, are susceptible (22, 230). Protozoans, such as *Plasmodium falciparum* and *Entamoeba histolytica*, are also inhibited by these drugs (165, 230).

Tigecycline is indicated for the treatment of complicated skin and skin structure infections, cIAI, and CABP by the FDA. However, in October 2013, the prescribing information was updated with a black box warning to reflect increased mortality in patients who received tigecycline versus comparator agents. Although the cause of the increase in mortality is not clear, the FDA now recommends that tigecycline be used only when alternative treatments are not suitable. It is possible that this increased mortality may be related to the low concentrations in serum (i.e., <1.0 µg/ml) that are achieved with the dosing regimen of 50 mg every 12 h as stated in the package label (205, 231). Most staphylococci, including methicillin-resistant and vancomycin-intermediate strains, are inhibited by tigecycline at ≤1 µg/ml. The MIC_{90}s for vancomycin-susceptible and -resistant enterococci are typically 0.25 to 0.5 µg/ml, and *E. faecium* and *E. faecalis* are inhibited equally well by this drug. However, tigecycline exhibits no bactericidal activity against staphylococci and enterococci in time-kill studies. *P. aeruginosa* is uniformly resistant to tigecycline.

Viridans group streptococci, beta-hemolytic streptococci, and MDR pneumococci are highly susceptible to tigecycline; MIC_{90}s for them range from ≤0.25 to 0.5 µg/ml. The drug is also active against most *Enterobacteriaceae* and nonfermentative Gram-negative bacilli, including strains producing ESBLs, with MIC_{90}s of ≤2 µg/ml. It inhibits MDR *A. baumannii* and *S. maltophilia* at MIC_{90}s of 2 µg/ml (232, 233). For the fastidious respiratory tract pathogens *H. influenzae* and *M. catarrhalis*, MIC_{90}s are 0.5 µg/ml and 1 µg/ml, respectively. *Proteus*, *Morganella*, *Providencia*, and *P. aeruginosa* are generally resistant (MIC_{90}s for them are >16 µg/ml). Tigecycline also exhibits some activity against most *B. fragilis* group isolates (MIC_{90} of 8 µg/ml), peptostreptococci (MIC_{90} of 4 µg/ml), *Clostridium* spp. (MIC_{90} of 0.5 µg/ml), *Prevotella* spp., *Propionibacterium*, and *Fusobacterium* (64, 234).

Tigecycline exhibits a more potent activity against *M. pneumoniae*, *M. hominis*, *C. pneumoniae*, and *C. trachomatis* than tetracyclines, with MIC_{90}s in the range of 0.125 to 0.5 µg/ml. It is less active toward *U. urealyticum* and *Legionella* spp., with MIC_{90}s of 8 µg/ml. Rapidly growing mycobacteria, including *M. abscessus*, *M. chelonae*, and members of the *M. fortuitum* group, are 4- to 11-fold-more susceptible (MIC_{90}s for them are ≤0.25 µg/ml) to this drug than to tetracyclines (235). However, slowly growing nontuberculous mycobacteria, such as *M. kansasii*, *M. marinum*, and *M. xenopi*, are less susceptible to tigecycline than to minocycline.

Susceptibility testing with tigecycline should be done using freshly prepared media or media containing a biocatalytic oxygen-reducing reagent (e.g., Oxyrase), because the drug is prone to oxidative degradation. Testing using aged broth media (prepared >12 h prior to inoculation) yielded MIC results that were generally 1 to 2 dilutions higher than those obtained using fresh media (236). Commercial media high in Mn^{2+} (>2.5 ppm) have been reported to increase MICs of tigecycline during susceptibility testing (146).

The antibacterial spectrum of omadacycline appears comparable to that of tigecycline, but it is less potent than tigecycline against many Gram-negative pathogens. Reduced susceptibility is seen in *K. pneumoniae* isolates that express the AcrAB efflux pump (237).

Adverse Effects

Tetracyclines have irritative effects on the upper gastrointestinal tract, producing esophageal ulcerations, nausea, vomiting, and epigastric distress. Alterations in the enteric biota occur with the use of tetracyclines, often resulting in diarrhea, and pseudomembranous colitis can develop with prolonged use. Hypersensitivity reactions are unusual, generally manifesting themselves as urticaria, fixed drug eruptions, morbilliform rashes, and anaphylaxis. Cross-reactivity among tetracyclines is the rule. Photosensitivity reactions consist of an erythematous rash on areas exposed to sunlight and can occur with all analogs, especially demeclocycline (238).

Minocycline has been known to cause vertigo, and benign intracranial hypertension (pseudotumor cerebri) has been described with many of the analogs (239). Tetracycline can aggravate preexisting renal failure by inhibiting protein synthesis, increasing the azotemia from amino acid metabolism. Tetracyclines cause depression of bone growth, permanent discoloration of the teeth, and enamel hypoplasia when given during tooth and skeletal development (240). Therefore, these drugs are usually avoided in childhood (<8 years of age) and during pregnancy.Tigecycline is reasonably well tolerated; a notable exception is that the rates of both nausea and vomiting were significantly higher in the tigecycline arms of the registration studies than those of the comparator. However, only mild nausea was reported in clinical studies with omadacycline and eravacycline (218). Headache and diarrhea are the most commonly reported other side effects. Due to adverse effects similar to those of the tetracyclines on bone and tooth development, use of this drug is contraindicated during pregnancy, for nursing mothers, and for those <18 years of age. It may also show cross-hypersensitivity to tetracyclines (241).

GLYCOPEPTIDES AND LIPOPEPTIDES

Vancomycin, a bactericidal antibiotic obtained from *Streptomyces orientalis*, is the only nonlipoglycopeptide marketed for clinical use in the United States. Initially introduced for its efficacy against penicillin-resistant staphylococci, it has become most useful against methicillin-resistant staphylococci and in patients allergic to penicillins or cephalosporins. Teicoplanin (formerly teichomycin A), a complex glycopeptide chemically related to vancomycin (242), is currently available for clinical use in most countries of the world except the United States. Dalbavancin, oritavancin, and telavancin are semisynthetic lipoglycopeptides (glycopeptide derivatives with hydrophobic substituents); the last was approved for clinical use in the United States (243–246). Both dalbavancin and oritavancin have completed phase 3 studies for skin and skin structure infections. Both dalbavancin and oritavancin were approved by the FDA in mid-2014. Daptomycin is a unique, naturally occurring cyclic lipopeptide antibiotic found among the fermentation by-products of *Streptomyces roseosporus* and has more potent activity against Gram-positive bacteria than vancomycin.

Mechanism of Action

Glycopeptides inhibit peptidoglycan synthesis in the bacterial cell wall by complexing with the D-alanyl-D-alanine portion of the cell wall precursor. Resistance to vancomycin and teicoplanin can occur by one of two mechanisms: (i) a complex series of bacterial cytoplasmic enzymes present in vancomycin-resistant enterococci synthesize abnormal peptidoglycan precursors terminating in D-alanyl-D-lactate or D-alanyl-D-serine residues (instead of the usual D-alanyl-D-alanine residues), thereby markedly lowering the drug's binding affinity with the glycopeptides, or (ii) the accumulation of peptidoglycan precursors (murein monomers) increases, resulting in a thickened cell wall with "trapping" of drug molecules, which prevents further diffusion of the drug into the inner part of cell wall layers of VISA (247).

Daptomycin binds irreversibly to the cytoplasmic membrane of susceptible bacteria via a calcium ion-dependent insertion of the hydrophobic tail of the molecule and causes generalized disruption of membrane permeability (248). The end effect is cell death without cell lysis, providing this drug with potent bactericidal activity. It is unable to penetrate the outer membrane of Gram-negative bacteria. Reduced susceptibility and resistance to daptomycin have occurred in enterococci (249, 250) and *S. aureus* (251, 252), possibly due to the physical barrier of a thickened bacterial cell wall similar to those found in VISA, alteration of the charge of the outer cell envelope (253), or cumulative point mutations in genes encoding various bacterial enzymes (254).

Oritavancin and telavancin have a dual mechanism of action: (i) inhibition of the transglycosylation process of peptidoglycan cell wall synthesis by formation of a complex with the D-alanyl-D-alanine residues and (ii) depolarization of the bacterial cell membrane. The depolarization effect on membrane potential depends both on the presence of lipid II and on an interaction between telavancin and D-alanyl-D-alanine residues, affecting diverse *S. aureus* strains, including those with decreased susceptibility to vancomycin and daptomycin (255, 256).

Pharmacology

Vancomycin can be administered orally or parenterally. After oral administration, the drug is poorly absorbed, and high concentrations in stools are achieved, accounting for its efficacy in treating pseudomembranous colitis (257). Desirable peak and trough levels in serum of 20 to 50 μg/ml and 5 to 15 μg/ml, respectively, are obtained after a 1-g intravenous dose of vancomycin every 12 h in healthy subjects (258). Therapeutic levels of the drug are achieved in synovial, ascitic, pericardial, and pleural fluids, with variable penetration into the CSF only in the presence of inflamed meninges (259, 260).

Vancomycin has a half-life in serum of 6 h in patients with healthy renal function and is eliminated from the body almost exclusively by glomerular filtration. In severe renal insufficiency, its excretion is prolonged to about 9 days, and it may be removed by hemodialysis, depending on the type of membrane and dialyzer used. The true extent of removal by peritoneal dialysis remains controversial, though historically it was thought that glycopeptides were not removed.

Intravenous infusion of daptomycin at a dosage of 6 mg/kg of body weight results in peak and trough concentrations of 82 and 6 μg/ml, respectively, in serum. About 90% of the drug is bound to plasma proteins, with limited metabolism. The volume of distribution is low at 0.1 liter/kg, suggesting that penetration into some sites of infection may be an issue. Daptomycin shows poor penetration into the CSF and alveolar space, where it is bound by surfactant, precluding its use for the treatment of meningitis and pneumonia (261). Its elimination half-life is 9 h, and 80% of the drug is excreted via the kidney, with two-thirds as intact drug (261). The dosing interval is increased to every 48 h when creatinine clearance is ≤30 ml/min.

Telavancin is approximately 95% protein bound in human plasma, with good penetration into tissues and a prolonged half-life of 7 to 9 h. It exhibits concentration-dependent bactericidal activity *in vivo* (262). The drug is eliminated from the body via urinary excretion, with 75% of the drug unchanged. Hemodialysis removes ~6% of telavancin, and dosage reduction is necessary in cases of renal insufficiency, with a creatinine clearance of <50 ml/min (263).

Dalbavancin is approximately 93% bound to human plasma proteins, with a volume of distribution of approximately 0.15 liter/kg (264). The compound also possesses a very long terminal half-life of 240 h, with good tissue penetration in both animal and human studies (265). In contrast to vancomycin and teicoplanin, dalbavancin appears to have both renal and nonrenal clearance mechanisms, with only about 30% of an administered dose recovered in the urine (265).

Like telavancin and dalbavancin, oritavancin is highly bound to plasma proteins, with a free fraction of approximately 15% (266). Oritavancin undergoes a more complex 3-phase elimination than the other two agents and has the longest terminal half-life of any of the three lipoglycopeptides, at approximately 340 h. The drug is not metabolized, and only 1% and 5% of the drug is recovered in the feces and urine, respectively, 7 days after administration of a dose. Oritavancin also distributes widely and penetrates a variety of tissues.

Spectrum of Activity

Glycopeptides and lipopeptides are active mainly against aerobic and anaerobic Gram-positive organisms, including methicillin-susceptible and -resistant staphylococci, streptococci, enterococci, *Corynebacterium* spp., *Bacillus* spp., *L. monocytogenes*, *Clostridium* spp., and *Actinomyces* spp. The MICs of vancomycin against *S. aureus*, *Staphylococcus epidermidis*, streptococci, and enterococci are typically in the range of 0.25 to 2 μg/ml (267). Daptomycin (268, 269) is 2- to 4-fold more active than vancomycin against these Gram-positive cocci, with MICs usually at least 2-fold lower for telavancin, dalbavancin, and oritavancin than for daptomycin (270, 271). Resistance to vancomycin has emerged among clinical isolates of enterococci (272) and staphylococci, although vancomycin-intermediate staphylococcal strains are more prevalent than fully resistant strains (273). The cross-resistance of vancomycin with daptomycin and the lipoglycopeptides is varied in these strains.

Vancomycin is useful in the prevention and treatment of endocarditis due to Gram-positive bacteria in patients who are allergic to penicillin (139). It is the drug of choice for treating *Corynebacterium jeikeium* infections (274) and is useful for antibiotic-associated *C. difficile* colitis (130).

The glycopeptides and lipopeptides are not active against Gram-negative organisms or mycobacteria. They show no cross-resistance with other unrelated antibiotics. They act synergistically with aminoglycosides or rifampin against staphylococci, streptococci, and enterococci (144, 275, 276), and they are bactericidal with aminoglycosides against *Listeria* spp. Daptomycin exhibits concentration-dependent bactericidal activity *in vitro* against most Gram-positive bacteria in their growing and stationary phases (277). Daptomycin MIC_{90}s for MDR isolates of staphylococci (including VISA), pneumococci, and streptococci are usually ≤1 μg/ml (278, 279). Vancomycin-susceptible and -resistant enterococci are inhibited equally at MIC_{90}s of ≤4 μg/ml. Daptomycin is active (MIC_{90}s of ≤2 μg/ml) against *Listeria*, *Corynebacterium* spp., *Propionibacterium*, *C. difficile*, *C. perfringens*, and peptostreptococci (280, 281). While *Leuconostoc*, *Pediococcus*, and *Lactobacillus* spp. are susceptible, other *Clostridium* spp., *Actinomyces* spp., and *Eubacterium* spp. showed MIC_{90}s in the range of 4 to 16 μg/ml. Since the optimal *in vitro* activity of daptomycin depends on the calcium ion concentration in the growth medium, media used for susceptibility testing with this drug should contain the recommended calcium concentration of 50 μg/ml. Disk diffusion methods were found to be unreliable in testing daptomycin susceptibility and are no longer recommended. The agar dilution method has not been validated for testing this drug.

With antimicrobial spectra of activity similar to those of glycopeptides, lipoglycopeptides are more potent than vancomycin and teicoplanin against a broad range of Gram-positive bacteria (244, 282–285). MIC_{90}s for MSSA, MRSA, and coagulase-negative staphylococci range from 0.06 μg/ml for dalbavancin to 1 μg/ml for oritavancin. Although lipoglycopeptide MICs are higher than those for glycopeptide-susceptible strains, VISA, hVISA, and VRSA isolates are more susceptible to these drugs, with the MICs for them ranging from 0.5 to 4 μg/ml (286, 287). Lipoglycopeptides retain activity against staphylococci that exhibit reduced susceptibility to daptomycin and linezolid. Vancomycin-susceptible enterococci are inhibited by dalbavancin, oritavancin, and telavancin in the range of 0.06 to 1 μg/ml; MICs for *E. faecalis* are 2-fold higher than for *E. faecium*. Vancomycin-resistant enterococci of the VanA and VanB phenotypes are usually resistant; MIC_{90}s of dalbavancin and telavancin but not oritavancin are 4 to >64 μg/ml (282, 288). These drugs are most active against streptococci, including MDR pneumococci, with MIC_{90}s ranging from 0.015 to 0.125 μg/ml (289). Telavancin also exhibits potent activity against *Actinomyces* spp., *C. difficile*, *C. perfringens*, *Corynebacterium* spp. (including *C. jeikeium*), *Lactobacillus* spp., and *P. acnes*, with MIC_{90}s ranging from 0.06 to 1 μg/ml (290). Susceptibility testing for oritavancin has resulted in disparate MIC values over the drug's lengthy development history, which dates from the late 1990s. The propensity of the compound to stick to plastic was not initially recognized, resulting in artificially high MIC values. With the addition of surfactant to the testing media to prevent adsorption, oritavancin MICs for many organisms, particularly staphylococci, are now reported as lower values (266).

Adverse Effects

The most frequent side effects of vancomycin are fever, chills, and phlebitis at the site of infusion. Rapid or bolus infusion of vancomycin causes tingling and flushing of the face, neck, and thorax, known as the "red man syndrome," as a result of histamine release by basophils and mast cells (291). This phenomenon is not due to allergic hypersensitivity, and it may also occur with telavancin. Allergic maculopapular or diffuse erythematous rashes can occur in up to 5% of patients. Reversible leukopenia or eosinophilia can rarely develop with glycopeptide use.

High-frequency hearing loss due to ototoxicity has been reported in patients receiving high daily doses of vancomycin, especially among those who were >50 years of age (292). Vancomycin-induced nephrotoxicity is rare, but this risk increases with use of large doses of the drug at >4 g per day (293) and during combination therapy with vancomycin and aminoglycosides. The necessity to monitor vancomycin blood levels to reduce the risk for nephrotoxicity has diminished with the increased purity of this fermentation product that has been attained since its introduction.

Common adverse reactions of daptomycin include diarrhea, rash, dizziness, and dyspnea. Elevated serum creatinine phosphokinase levels (an effect that delayed the development of the drug), myalgias, and myopathy can occur but are reversible (294, 295) and are significantly reduced with lower dosages and once-daily dosing frequency. Nephrotoxicity has been consistently associated with televancin use during its clinical development. The current U.S. prescribing information, updated in June 2013, contains warnings about nephrotoxicity and increased mortality in patients with MRSA pneumonia who have creatinine clearances of less than 50 ml/min. The warning states that telavancin should be used for MRSA pneumonia only when other options are not acceptable. Taste disturbance, nausea, vomiting, and foamy urine were the most common adverse reactions (<10%) to telavancin during clinical development (263). Telavancin is labeled as a category C agent with regard to use in pregnant women; teratogenic effects were seen in three animal species at clinically relevant doses. Telavancin should be used in pregnant women only when no other options are available.

STREPTOGRAMINS

Streptogramins are natural cyclic peptides produced by *Streptomyces* spp. They are a unique class of antibiotics in which each member is a combination of at least two structurally unrelated components, group A and B streptogramins, acting synergistically against susceptible bacteria (296). Group A streptogramins are polyunsaturated macrolactones consisting of lactam and lactone linkages with an oxazole ring, and the main compounds in this group are pristinamycin II_A and pristinamycin II_B. Group B streptogramins are cyclic hexadepsipeptides, with pristinamycin I_A and pristinamycin I_C as the principal compounds. Quinupristin-dalfopristin is the first injectable streptogramin antibiotic combination developed for clinical use in the United States. It is a 30:70 mixture of the semisynthetic streptogramins quinupristin and dalfopristin, which are water-soluble derivatives of pristinamycin I_A and pristinamycin II_A, respectively.

Mechanism of Action

The streptogramins exert a synergistic bactericidal effect on susceptible organisms by inhibiting bacterial protein synthesis. They enter bacterial cells via passive diffusion and then bind specifically and irreversibly to the 50S subunits of the 70S bacterial ribosomes. Binding of group A streptogramins to the ribosome induces a conformational change in the ribosome that increases its affinity for group B compounds. Group A streptogramins prevent peptide bond formation during the chain elongation step, while group B components cause release of the incomplete peptide chains from the 50S ribosomal subunit (149).

Acquired bacterial resistance to the streptogramins, which may be chromosomal or plasmid mediated, is due mainly to modification of the drug target by methylation of the bacterial 23S rRNA, resulting in resistance to all macrolides, lincosamides, and group B streptogramins (MLS_B resistance phenotype) but not to group A streptogramins. Mutations in the L22 ribosomal protein gene (*rplV*), active efflux of group A and B streptogramins, and drug inactivation by streptogramin A acetylase and streptogramin B hydrolase have been described.

Pharmacokinetics

Quinupristin-dalfopristin is administered intravenously, with distribution into most tissues. Both components are highly protein bound (70 to 90%) and rapidly cleared from plasma via biliary excretion by hepatic conjugation processes (297). Less than 20% of the administered drug combination is excreted in the urine. Following intravenous doses of 7.5 mg/kg of body weight, peak concentrations in serum of quinupristin and dalfopristin reach 2.7 and 7.2 µg/ml, respectively, with elimination half-lives of 1 and 0.75 h. The two components penetrate and accumulate in macrophages, and the ratio of peak *in vitro* cellular-to-extracellular concentrations is 50:35. The drug combination does not cross a noninflamed blood-brain barrier or placenta to any significant degree. Dosage adjustment is needed for patients with renal insufficiency (creatinine clearance, <30 ml/min), and the drug combination is removed in modest amounts by dialysis.

Spectrum of Activity

Streptogramins are active mainly against Gram-positive bacteria, with modest activities against selected Gram-negative and anaerobic pathogens. Quinupristin-dalfopristin has potent bactericidal activity against MSSA, MRSA, coagulase-negative staphylococci, and streptococci, with MIC_{90}s of ≤1 µg/ml and minimum bactericidal concentrations (MBCs) within 2- to 4-fold of the MICs (298, 299). Staphylococci and streptococci, including *S. pneumoniae*, that are resistant to β-lactam drugs, macrolides, and fluoroquinolones usually remain susceptible to quinupristin-dalfopristin, but staphylococci with MLS_B resistance due to methylation of the 23S rRNA-binding site are inhibited but not killed by quinupristin-dalfopristin (298). While most *E. faecium* strains are susceptible (MIC_{90}s of ≤4 µg/ml), *E. faecalis* is intrinsically resistant (MIC_{90}s of ≥32 µg/ml) to the drug combination because of active efflux of dalfopristin. Although it is not bactericidal against enterococci, quinupristin-dalfopristin inhibits vancomycin-resistant *E. faecium* (VanA or VanB phenotype), including MDR strains, at MIC_{90}s of ≤2 µg/ml (297). This drug combination offers a therapeutic option for serious MDR Gram-positive bacterial infections (296, 300–302). *N. meningitidis*, *N. gonorrhoeae*, *M. pneumoniae*, *C. pneumoniae*, and *Legionella pneumophila* are all highly susceptible to the drug (MIC_{90}s of ≤2 µg/ml). Quinupristin-dalfopristin is also active against *M. catarrhalis* and *H. influenzae*, with MIC_{90}s of ≤4 µg/ml. *Enterobacteriaceae* and other nonfermenting Gram-negative bacilli are resistant. Among the anaerobes, *C. perfringens* and *C. difficile* are the most susceptible (MIC_{90}s of 0.25 µg/ml). Quinupristin-dalfopristin is active against the *B. fragilis* group (MIC_{90} of 4 µg/ml) as well as other anaerobic bacteria, including *Prevotella*, *Porphyromonas*, *Fusobacterium*, *P. acnes*, *Lactobacillus*, and peptostreptococci, with MIC_{90}s of 2 to 4 µg/ml.

Adverse Effects

Phlebitis at the site of intravenous infusion is the major local adverse reaction; the incidence and severity are dose and concentration related, which has led to the recommendation that the drug be administered only through a central line (297, 303). The most common systemic side effects, up to 47% of patients in one series, that may lead to discontinuation of therapy are arthralgias and myalgia, both of which are reversible upon discontinuation of the combination (87, 304). Elevated levels of serum transaminases and cutaneous reactions, such as itching, burning, and erythema of the face, neck, or upper body, have also been reported.

OXAZOLIDINONES

Oxazolidinones are chemically unique synthetic antimicrobials originally discovered in the 1970s (305). Linezolid is currently the only oxazolidinone available for clinical use (306), although a new-drug application (NDA) has been submitted for tedizolid for the treatment of acute bacterial skin and skin structure infections (ABSSSI). Other analogs are in preclinical or clinical development, including the oxazolidinones that are being investigated for the management of tuberculosis (307–309).

Mechanism of Action

Oxazolidinones inhibit bacterial protein synthesis by preventing the formation of a functional initiation complex consisting of $tRNA^{fMet}$, mRNA, initiation factors, and the ribosome (310).

Linezolid binds to the domain V region of 23S rRNA in the 50S ribosomal subunit, thereby distorting the binding site for $tRNA^{fMet}$ and inhibiting the formation of a functional 70S initiation complex, thus preventing initiation of mRNA translation. In this regard, this class of antimicrobials is unique, lacking cross-resistance to other antibiotics that also inhibit ribosomal protein synthesis. Resistance to linezolid has occurred in clinical isolates of MRSA, vancomycin-resistant enterococci, and pneumococci as a result of point mutations in domain V of the 23S rRNA gene (311, 312), mutations in the L3L4 ribosomal protein, and the methyltransferase coded by the *cfr* gene (313). The last mechanism is perhaps the most worrisome since it is found on mobile genetic elements. Oxazolidinones are generally inactive against Gram-negative bacteria because of endogenous efflux pumps present in these organisms (310).

Pharmacology

Linezolid is available in oral and parenteral forms. Rapid and extensive absorption occurs after oral administration (>95% bioavailability), reaching maximum serum concentrations of 15 to 20 µg/ml within 2 h after an oral dose of 600 mg (314). The drug is metabolized primarily in the liver, and the elimination half-life is about 5 h. With 30% of the drug being protein bound, it is well distributed in all body tissues, including the CSF (315). The drug is eliminated via the kidneys, with 30% being excreted unchanged in the urine. No dose adjustment is necessary in patients with renal insufficiency or mild to moderate hepatic impairment, while 20% of a dose is removed by hemodialysis (316).

Spectrum of Activity

As a group, oxazolidinones have varied activities against most Gram-positive bacteria and mycobacteria. Linezolid has excellent activity against staphylococci (including methicillin-resistant strains), streptococci, and MDR enterococci, with MIC_{90}s ranging from 1 to 4 µg/ml (317–319). The MIC_{90}s are in the range of 0.5 to 2 µg/ml for pneumococci.

Although the antibacterial effect of linezolid is generally bacteriostatic, the drug is bactericidal against most strains of pneumococci. Other bacteria that are inhibited by linezolid include *Actinomyces* spp., *Bacillus cereus*, *Corynebacterium* spp., *Leuconostoc*, *Pediococcus*, *R. equi*, *L. monocytogenes*, *Clostridium* spp., and Gram-positive anaerobic cocci (280). Both slowly and rapidly growing *Mycobacterium* spp. and *Nocardia* spp. are also susceptible to linezolid at MIC_{90}s of ≤8 µg/ml (320, 321). Linezolid is an important therapeutic option for skin and soft tissue infections (322), respiratory tract infections (323), and infections due to methicillin-resistant staphylococci (324, 325) and vancomycin-resistant enterococci (326–328).

Adverse Effects

The most common drug-related adverse events (≤5% incidence) are diarrhea, headache, and nausea (329, 330). Prolonged use of linezolid (usually of >28 days' duration) has led to rare optic neuropathy (331), peripheral neuropathy (332), and myelosuppression, including anemia, leukopenia, thrombocytopenia, and pancytopenia, which are reversible upon discontinuation of therapy (333, 334). All of these adverse events, as well as lactic acidosis, are thought to be related to the propensity of linezolid to interfere with mitochondrial RNA (335). As a mild nonselective inhibitor of monoamine oxidase, linezolid can interact with adrenergic or serotonergic drugs, and rare cases of serotonin syndrome have occurred with concomitant use of selective serotonin reuptake inhibitor drugs and other serotonergically active medications (336).

SULFONAMIDES AND TRIMETHOPRIM

Sulfonamides were the first effective systemic antimicrobial agents used in the United States during the 1930s. They are derived from sulfanilamide, which shares chemical similarities with *para*-aminobenzoic acid, a factor essential for bacterial folic acid synthesis. Various substitutions at the sulfonyl radical attached to the benzene ring nucleus enhance the antibacterial activity and also determine the pharmacologic properties of the drug.

Trimethoprim (TMP) is a pyrimidine analog that inhibits the enzyme dihydrofolate reductase, interfering with folic acid metabolism, subsequent pyrimidine synthesis, and one-carbon fragment metabolism in bacteria. Since TMP and sulfonamides block the bacterial folic acid metabolic pathway at different sites, they potentiate the antibacterial activity of one another and act synergistically against a wide variety of organisms. Such a combination, TMP-sulfamethoxazole (TMP-SMX), also called co-trimoxazole, was introduced clinically in 1968 and has proven to be very effective in the treatment of many infections (337)

Mechanism of Action

Sulfonamides competitively inhibit bacterial modification of *para*-aminobenzoic acid into dihydrofolate, whereas TMP inhibits bacterial dihydrofolate reductase. This sequential inhibition of folate metabolism ultimately prevents the synthesis of bacterial DNA (338). Since mammalian cells do not synthesize folic acid, human purine synthesis is not affected significantly by sulfonamides or TMP. The antibacterial effects of these agents may be reduced in patients receiving high doses of folinic acid.

Pharmacology

Sulfonamides are usually administered in oral and topical forms. The only intravenous formulation currently available in the United States is TMP-SMX. Mafenide acetate (Sulfamylon cream) and silver sulfadiazine are applied topically in burn patients and have significant percutaneous absorption. Sulfacetamide is available as an ophthalmic preparation.

The orally administered sulfonamides are absorbed rapidly and completely from the gastrointestinal tract. They are metabolized in the liver by acetylation and glucuronidation and are excreted by the kidney as free drug and inactive metabolites. Sulfonamides compete for bilirubin-binding sites on plasma albumin and increase levels of unconjugated bilirubin in blood. For this reason, they should not be given to neonates, in whom increased serum bilirubin levels may cause kernicterus.

Sulfonamides are well distributed throughout the body, with levels in the cerebrospinal, synovial, pleural, and peritoneal fluids being about 80% of the concentrations in serum. They readily cross the placenta and enter the fetal circulation. Sulfonamides may be used in renal failure, but the drugs may accumulate during prolonged therapy as a result of reduced renal excretion.

TMP is available only for oral use and is absorbed almost completely from the gastrointestinal tract. After the usual 100-mg dose, peak levels in serum reach 1 μg/ml in 1 to 4 h. This drug distributes widely in body tissues, including the kidney, lung, and prostate, and in body fluids (339). Concentrations in CSF are about 40% of levels in serum. Its half-life in serum is about 10 h in healthy subjects and is prolonged in those with renal insufficiency. Up to 80% of a dose is excreted unchanged in the urine by tubular secretion; the remaining fraction is excreted as inactive metabolites by the kidney or in the bile.

A fixed combination of TMP-SMX at a dose ratio of 1:5 is available for oral and intravenous use. An intravenous dose of 160 mg of TMP with 800 mg of SMX produces average peak levels in serum of 3.4 and 47.3 μg/ml, respectively, in 1 h. Similar peak levels are reached at 2 to 4 h after the same dose is taken orally. Widely distributed in the body, both drugs reach therapeutic levels in the CSF (40% of levels in serum). Excretion is primarily by the kidney; dosage reduction is necessary in patients with creatinine clearances of ≤30 ml/min. Both TMP and SMX are removed by hemodialysis and partially by peritoneal dialysis.

Spectrum of Activity

Sulfonamides are inhibitory to a variety of Gram-positive and Gram-negative bacteria, actinomycetes, chlamydiae, toxoplasmas, and plasmodia. Their *in vitro* antimicrobial activities are irregular, being strongly influenced by inoculum size and composition of the test media. Susceptibility testing end points are often difficult to determine because of the presence of hazy growth within zones of inhibition in disk diffusion tests and because of the phenomenon of "trailing" in dilution tests.

Sulfadiazine in combination with pyrimethamine has been used successfully to treat toxoplasmosis (165). Sulfonamides are active against *Nocardia asteroides* (224), and they show moderate activity against *M. kansasii*, members of the *M. fortuitum* group, *M. marinum*, and *Mycobacterium scrofulaceum* (340). Other uses of sulfonamides include therapy of melioidosis, dermatitis herpetiformis, lymphogranuloma venereum, and chancroid.

Among the Gram-negative bacilli, *E. coli* strains were initially susceptible to the sulfonamides, especially at levels achievable in urine. However, increasing bacterial resistance has limited their efficacy in recent years. *S. marcescens*, *P. aeruginosa*, enterococci, and anaerobes are usually resistant to the sulfonamides.

TMP is active *in vitro* against many Gram-positive cocci and most Gram-negative bacilli. *P. aeruginosa*, most anaerobes, *M. pneumoniae*, and mycobacteria are resistant. The MICs vary considerably depending on the test medium used. Like the sulfonamides, TMP was used primarily in the therapy of uncomplicated and recurrent urinary tract infections due to susceptible organisms (341). However, resistance to TMP is now prevalent among *Enterobacteriaceae*.

Combinations of TMP with other agents, such as rifampin, polymyxins, and aminoglycosides, have demonstrated *in vitro* synergistic antibacterial activity against various Gram-negative bacilli. TMP combined with dapsone is effective in the treatment of *P. jirovecii* pneumonia in immunocompromised patients.

Many Gram-positive cocci, including staphylococci and streptococci (except group A), and most Gram-negative bacilli except *P. aeruginosa* are susceptible to TMP-SMX (342). Staphylococci have surprisingly retained susceptibility to TMP-SMX, with recent surveillance data demonstrating >99% and 92% susceptibilities for methicillin-susceptible and methicillin-resistant *S. aureus* (343). However, 10% to 50% of strains of *S. pneumoniae* are resistant in many parts of the world. The drug combination has variable bactericidal effects on enterococci *in vitro*, depending on the test medium used for susceptibility testing (344). Unlike many bacteria that can utilize only thymidine for growth, enterococci can use thymidine, thymine, exogenous folinic acid, dihydrofolate, and tetrahydrofolate, resulting in higher MICs (25- to 50-fold increase) on media containing these compounds (345). This fact also explains the ineffectiveness of TMP-SMX against enterococci *in vivo*.

TMP-SMX is often active against *M. catarrhalis* and *H. influenzae*, including β-lactamase-producing strains; however, it is no longer recommended as empirical therapy for acute otitis media, sinusitis, acute bronchitis, and pneumonia due to the increasing resistance in *S. pneumoniae*. It has shown excellent results in the prophylaxis and therapy of acute and chronic urinary tract infections; however, these results have been compromised by resistance rates in *E. coli* that now exceed 20% in most centers (111, 341, 346). The drug combination is also useful in treating susceptible infections due to salmonellae, shigellae, enteropathogenic *E. coli*, and *Y. enterocolitica* (347). It has been used successfully for prophylaxis and treatment of traveler's diarrhea (348), but resistance to TMP-SMX in *Shigella* spp. and *E. coli* now severely limits its usefulness in many parts of the world.

Other microorganisms susceptible to TMP-SMX include *Brucella* spp., *B. pseudomallei*, *B. cepacia*, *S. maltophilia*, *M. kansasii*, *M. marinum*, and *M. scrofulaceum*. *M. tuberculosis* and *M. chelonae* are generally resistant. It is a valuable antibiotic for the treatment of *Nocardia* species infections (349), *B. cepacia* and *S. maltophilia* bacteremia, *L. monocytogenes* meningitis, gastroenteritis due to *Isospora belli* and *Cyclospora* spp. (165), and Whipple's disease. In immunocompromised hosts (e.g., those with leukemia or AIDS or organ transplant recipients), TMP-SMX is the drug of choice for the prophylaxis and treatment of *P. jirovecii* pneumonia (350).

Adverse Effects

Sulfonamides are known to cause nausea, vomiting, headache, and fever. Hypersensitivity reactions can occur as rashes, vasculitis, erythema nodosum, erythema multiforme, and Stevens-Johnson syndrome (351). Very high doses of less-water-soluble sulfonamides may result in crystalluria, with renal tubular deposits of sulfonamide crystals. Bone marrow toxicity with anemia, leukopenia, or thrombocytopenia can occur. Sulfonamides should be avoided in patients with glucose-6-phosphate dehydrogenase deficiency because of associated hemolytic anemia. Sulfonamides also potentiate the effects of warfarin, phenytoin, and oral hypoglycemic agents.

In general, TMP is well tolerated. With prolonged use, megaloblastic anemia, neutropenia, and thrombocytopenia can develop, especially in folate-deficient patients. Adverse reactions to TMP-SMX due to either the TMP or, more commonly, the SMX component can occur. Mild gastrointestinal symptoms and allergic skin rashes occur in about 3% of patients (352). Megaloblastic bone marrow changes may develop with leukopenia, thrombocytopenia, or granulocytopenia, usually in patients with preexisting folate deficiency. Nephrotoxicity usually occurs in patients with underlying renal dysfunction. Trimethoprim blocks apical membrane sodium channels in the kidney in a fashion similar to that of the potassium-sparing diuretic amiloride, resulting in hyperkalemia particularly when used at higher doses (353). Patients with AIDS have a much higher frequency (as much as 70%) of adverse reactions (354).

POLYMYXINS

Polymyxins are cationic cyclic polypeptides originally derived from *Bacillus polymyxa*. They consist of five different compounds (polymyxins A to E) and have limited spectra of antimicrobial activity and significant toxicity (355). Only polymyxins B and E (colistin) are used clinically in humans.

Mechanism of Action

Acting like detergents or surfactants, members of this group of antibiotics interact with the phospholipids of the bacterial cell membrane, thereby increasing cell permeability and disrupting osmotic integrity. This process results in leakage of intracellular constituents, leading to cell death. The bactericidal action is reduced in the presence of calcium, which interferes with the attachment of drugs to the cell membrane. With almost complete cross-resistance existing between polymyxin B and colistin, Gram-negative bacteria can become resistant by alterations of the outer cell membrane from reduced levels of lipopolysaccharides, reduced levels of specific outer membrane proteins, reduced cell envelope Mg^{2+} and Ca^{2+} contents, and lipid alterations. In addition, the presence of a polymyxin B efflux pump system and colistinase, which inactivates colistin, has been reported (356).

Pharmacology

Polymyxins are usually administered parenterally, orally, or topically. They are not significantly absorbed when given orally or topically, and intramuscular injections can be painful. Peak concentrations in serum of 5 μg/ml are obtained with a total daily dose of intravenous polymyxin B at 2.5 mg (or 25,000 U)/kg (357). Polymyxin E is available commercially as colistin sulfate and colistimethate sodium, an inactive sulfomethyl prodrug of colistin, with the former given orally for local antibacterial effect in the gut and the latter used for intravenous or intramuscular injections. The half-life of polymyxin B in serum is about 6 to 7 h, and that of colistin is 2 to 4 h. They do not penetrate well into pleural fluid, synovial fluid, or CSF even in the presence of inflammation (358). Excretion of colistimethate sodium is mostly via the kidneys by glomerular filtration; the mechanism of free colistin clearance after colistimethate sodium administration is less clear (359). Polymyxin B is only minimally excreted by the kidneys and undergoes extensive tubular reabsorption. Only 4% of the dose of polymyxin B is eliminated renally (360). These drugs are not removed by hemodialysis, but small amounts can be removed by peritoneal dialysis.

Polymyxin is often used topically as 0.1% polymyxin in combination with bacitracin or neomycin for treatment of skin, mucous membrane, eye, and ear infections. It is poorly absorbed from these surfaces. When the drug is used for irrigation of serous or wound cavities, systemic absorption can be significant enough to produce toxicity.

Spectrum of Activity

Polymyxins are active only against select Gram-negative bacilli, especially *Pseudomonas* spp. and *Acinetobacter* spp. The MIC$_{90}$s for *Pseudomonas* spp., including *P. aeruginosa*, and *Acinetobacter baumannii* are <8 μg/ml. *Proteus*, *Providencia*, *Serratia*, and *Neisseria* isolates are usually resistant (355). Emergence of resistance during therapy has been reported frequently, with only 93% and 97% susceptibility reported in *P. aeruginosa* and *A. baumannii*, respectively (343). No cross-resistance is seen with other classes of antibiotics. Polymyxins B and E have identical antimicrobial spectra and show complete cross-resistance to one another.

The combination of polymyxins with TMP-SMX may be synergistic in the treatment of serious infection due to multiply resistant *Serratia* spp., *P. aeruginosa*, *B. cepacia*, and *S. maltophilia* (62, 361). The polymyxins are usually reserved for serious, life-threatening *Pseudomonas* or Gram-negative bacillary infections caused by organisms resistant to all other antibiotics, such as carbapenemase-producing *Enterobacteriaceae* (356). Aerosolized polymyxins have been used successfully to treat respiratory tract colonization and nosocomial pneumonia due to MDR Gram-negative bacilli or *P. aeruginosa* in patients with or without cystic fibrosis or bronchiectasis (357, 362).

Adverse Effects

Neurotoxicity and nephrotoxicity are the two major side effects of polymyxins (357). Paresthesia with flushing, dizziness, vertigo, ataxia, slurred speech, drowsiness, or mental confusion occurs when levels in serum exceed 1 to 2 μg/ml (356). Polymyxins also have a curare-like effect that can block neuromuscular transmission. Dose-related renal dysfunction occurs in about 20% of patients receiving appropriate therapeutic dosages. Allergic reactions, such as fever and skin rashes, are rare, but urticaria and shock after rapid intravenous infusion have occurred.

CHLORAMPHENICOL

Chloramphenicol is a nitrobenzene cyclic antibiotic originally derived from *Streptomyces venezuelae*. It is a highly effective broad-spectrum antimicrobial agent with specific indications for use in seriously ill patients but is rarely used clinically in the United States due to its well-known toxicities.

Mechanism of Action

The drug is a bacteriostatic agent that inhibits protein synthesis by binding reversibly to the peptidyltransferase component of the 50S ribosomal subunit and preventing the transpeptidation process of peptide chain elongation. At therapeutic concentrations achievable in the serum, it can be bactericidal against common meningeal pathogens, such as *S. pneumoniae*, *N. meningitidis*, and *H. influenzae* (363, 364). Bacterial resistance occurs due to the plasmid-mediated production of *cat*, the gene encoding chloramphenicol acetyltransferase, an enzyme, which inactivates the drug (365).

Pharmacology

Chloramphenicol is available for topical, oral, or parenteral use. It is not absorbed in any significant amount when applied topically, but it is rapidly and completely absorbed from the gastrointestinal tract. After an oral or intravenous dose of 1 g, peak concentrations in serum at 2 h can reach 10 to 15 μg/ml. It diffuses well into many tissues and body fluids, including CSF, where levels are generally 30 to 50% of concentrations in serum even without meningeal inflammation (366). The antibiotic readily crosses the placental barrier and is present in human milk.

Chloramphenicol is metabolized and inactivated by glucuronidation in the liver, with a half-life of 4 h in adults. The active drug (5 to 10%) and its inactive metabolites are excreted by the kidneys. Careful monitoring of serum chloramphenicol levels, maintaining peak concentrations in serum in the therapeutic range of 10 to 20 μg/ml, is useful for ensuring therapeutic efficacy and reduced toxicity. Patients with hepatic failure have high levels of active drug in serum owing to the drug's prolonged half-life. Dosage modification is not necessary in the presence of renal insufficiency, since the metabolites are not as toxic as the active drug. Levels in serum are not affected by hemodialysis or peritoneal dialysis.

Spectrum of Activity

Chloramphenicol is active against many Gram-positive and Gram-negative bacteria, chlamydiae, mycoplasmas, and rickettsiae. MIC$_{90}$s for most Gram-positive aerobic and anaerobic cocci are ≤12.5 μg/ml (364). However, the drug is usually inactive against MRSA and methicillin-resistant *S. epidermidis* and is variably active against enterococci. *N. meningitidis*, *H. influenzae* (ampicillin-resistant and -susceptible strains), and most *Enterobacteriaceae* are susceptible. Its activities against *Serratia* and *Enterobacter* isolates are varied, and strains of *Pseudomonas* spp. are usually resistant. Salmonellae, including *Salmonella enterica* serovar Typhi, are also susceptible, but resistant isolates are being encountered with increasing frequency (367).

Chloramphenicol is also active against anaerobic bacteria, including members of the *B. fragilis* group. Almost all of these isolates are inhibited at concentrations of ≤10 μg/ml (199, 109). It is also active against *Rickettsia* spp. and *Coxiella burnetii*.

Adverse Effects

Bone marrow toxicity is the major complication of chloramphenicol use. This side effect may occur as either dose-related bone marrow suppression or idiosyncratic aplastic anemia. Reversible bone marrow depression with anemia, leukopenia, and thrombocytopenia occurs as a result of a direct pharmacologic effect of the drug on hematopoiesis. High doses (>4 g/day), prolonged therapy, and excessively high levels in serum (>20 μg/ml) predispose patients to develop this type of complication. The second form of bone marrow toxicity is a rare but usually fatal complication that manifests as aplastic anemia. This response is not dose related, and the precise mechanism is unknown. It can occur weeks to months after the use of chloramphenicol, and it can develop after the use of oral, intravenous, or topical preparations.

Gray baby syndrome, characterized by vomiting, abdominal distention, cyanosis, hypothermia, and circulatory collapse, may occur in premature infants and neonates treated with chloramphenicol. This toxicity results from the immature hepatic function of neonates, which impairs hepatic inactivation of the drug. In patients receiving prolonged chloramphenicol therapy, reversible optic neuritis causing decreased visual acuity can occur. Chloramphenicol can occasionally cause hypersensitivity reactions, including skin rashes, drug fevers, and anaphylaxis. It potentiates the actions of warfarin, phenytoin, and oral hypoglycemic agents by competitive inhibition of hepatic microsomal enzymes.

METRONIDAZOLE

Metronidazole is a 5-nitroimidazole derivative that was first introduced in 1959 for the treatment of *Trichomonas vaginalis* infections. It now has an important therapeutic role in the treatment of infections due to anaerobic bacteria and certain protozoan parasites. Tinidazole, a second-generation 5-nitroimidazole compound, is approved in the United States for treatment of trichomoniasis, giardiasis, and amebiasis.

Mechanism of Action

Metronidazole owes its bactericidal activity to the nitro group of its chemical structure. After the drug gains entry into the cells of susceptible organisms, the nitro group is reduced by a nitroreductase enzyme in the cytoplasm, generating certain short-lived, highly cytotoxic intermediate compounds or free radicals that disrupt host DNA (368). Resistance to nitroimidazoles may be due to decreased uptake of the drug or inducible production of 5-nitroimidazole reductase, which can scavenge the free-radical intermediates (369).

Pharmacology

Metronidazole can be administered via the topical, oral, or intravenous route. It is absorbed rapidly and almost completely when given orally. Peak levels in serum of 6 µg/ml are obtained 1 h after an oral dose of 250 mg. Intravenous doses of 7.5 mg/kg result in peak concentrations in serum of 20 to 25 µg/ml. The drug has a half-life in serum of 8 h. Therapeutic levels are achieved in all body tissues and fluids, including abscess cavities and CSF, even without meningeal inflammation. The drug crosses the placenta and is secreted in breast milk. It is metabolized mainly in the liver, and 60 to 80% is excreted in the kidney. With impaired hepatic function, plasma clearance of metronidazole is delayed and dosage adjustments are necessary. The pharmacokinetics is minimally affected by renal insufficiency. Metronidazole and its metabolites are removed completely by dialysis.

Spectrum of Activity

Metronidazole exhibits potent activity against almost all anaerobic bacteria, including members of the *B. fragilis* group, *Fusobacterium*, and *Clostridium* (195). It is the only antimicrobial agent with consistent bactericidal activity against members of the *B. fragilis* group. However, the susceptibilities of Gram-positive anaerobic cocci are somewhat varied; MIC_{90}s for these organisms are 16 µg/ml. Most strains of the genera *Actinomyces*, *Arachnia*, and *Propionibacterium* are resistant. Frequencies of metronidazole-resistant *B. fragilis* group isolates (MICs of >16 µg/ml) in the range of 2% to 5% have been reported from various institutions (199, 109).Tinidazole is somewhat more potent than metronidazole in its antianaerobic activities (370). Nitroimidazoles have no activity against aerobic bacteria, including the *Enterobacteriaceae*.

Metronidazole is effective in the treatment of antibiotic-associated colitis caused by *C. difficile* (12) and is equivalent to oral vancomycin in mild to moderate disease; however, recent data have shown it to be inferior to vancomycin in more severe disease (371). It is also useful for treating polymicrobial soft tissue infections and mixed aerobic-anaerobic intra-abdominal and pelvic infections.

Metronidazole and tinidazole are active against the protozoa *Trichomonas vaginalis*, *Giardia lamblia*, and *Entamoeba histolytica*. It is the drug of choice for the treatment of trichomoniasis, giardiasis, and intestinal and invasive amebiasis, including amebic liver abscess (165, 372).

Adverse Effects

Metronidazole is generally well tolerated, and adverse side effects are uncommon. It can cause mild gastrointestinal symptoms, such as nausea, abdominal cramps, and diarrhea. An unpleasant, metallic taste is often experienced with oral therapy. Metronidazole can potentiate the effect of warfarin and prolong the prothrombin time as well as cause a disulfiram-like reaction when patients receiving metronidazole therapy consume alcohol.

Although metronidazole is carcinogenic in mice and rats, there is no evidence for carcinogenicity in humans. However, use of this agent during pregnancy, especially during the first trimester, and in nursing mothers should be avoided.

RIFAMYCINS

Rifamycins are macrocyclic compounds produced by the mold *Streptomyces mediterranei*. Rifampin, also known as rifampicin, a semisynthetic antibiotic derived from rifamycin B, was the first of this class introduced for clinical use in 1968 as an effective antituberculous drug. A closely related compound, rifabutin, a derivative of rifamycin S, is another potent antimycobacterial agent, especially against *M. avium* complex (373). Rifapentine possesses a much longer half-life than rifampin and has been shown to be effective in shortening regimens for the treatment of latent *M. tuberculosis* infection (374). Rifaximin, a derivative of rifampin, possesses an additional pyridoimidazole ring and is a nonabsorbed oral drug that can be used for therapy of uncomplicated traveler's diarrhea but is most commonly prescribed in the United States for the management of hepatic encephalopathy.

Mechanism of Action

Rifamycins exert their bactericidal effects by forming a stable complex with bacterial-DNA-dependent RNA polymerase, preventing the chain initiation process of DNA transcription (375). Mammalian RNA synthesis is not affected, because the mammalian enzyme is much less sensitive to the drug. Rifampin-resistant isolates possess an altered RNA polymerase enzyme that arises easily from single-step mutations during monotherapy with rifampin.

Pharmacology

Rifampin is well absorbed after oral administration, reaching peak concentrations in serum of 5 to 10 µg/ml in 2 to 4 h following a 600-mg dose. A parenteral preparation is also available. Rifampin is deacetylated in the liver to an active metabolite and excreted in the bile, and it undergoes enterohepatic circulation. The normal half-life in serum varies from 1.5 to 5 h. The drug distributes well to almost all body tissues and fluids, reaching concentrations equal to or exceeding that in the serum. Levels in the CSF are highest in the presence of inflamed meninges. Rifampin is able to enter phagocytes and kill living intracellular organisms (376), and it crosses the placenta. About 30 to 40% of the drug is excreted in the urine, and it does not accumulate in patients with impaired renal function. Hemodialysis and peritoneal dialysis do not eliminate the drug. Dosage adjustments are necessary for patients with severe hepatic dysfunction.

Because it has an additional pyridoimidazole ring in its chemical structure, rifaximin is largely unabsorbed after oral administration, with >99% of the drug present in the stool, and <1% of an oral dose is detectable in the plasma of both healthy volunteers and persons with damaged intestinal mucosae (377). Average fecal concentrations of the drug reach 8,000 μg/g on the third day of therapy at an oral dose of 400 mg twice daily (378).

Spectrum of Activity

In addition to having well-known antimycobacterial effects (379), rifampin has a wide spectrum of antimicrobial activity. It is bactericidal against susceptible Gram-positive cocci, such as staphylococci (including methicillin-resistant strains), streptococci, and anaerobic cocci, with MICs in the range of 0.01 to 0.5 μg/ml. It remains an important adjunct in the combination therapy of serious and chronic staphylococcal infections (380–382). However, it is bacteriostatic against enterococci, with usual MICs of <16 μg/ml (275).

N. gonorrhoeae, *N. meningitidis*, and *H. influenzae*, including β-lactamase-producing strains, are susceptible to rifampin, which is used frequently in the prophylaxis of meningococcal and *H. influenzae* type b meningitis (383). MICs for *Enterobacteriaceae* are ≤12 μg/ml, while MICs for *S. marcescens* and *P. aeruginosa* are higher (275). Besides fluoroquinolones, rifampin is one of the most active agents against *L. pneumophila* and other *Legionella* spp., with MICs of ≤0.03 μg/ml. Because of its ability to enter phagocytes, rifampin inhibits the intracellular growth of *Brucella* spp. and *C. burnetii* (120), and it is used frequently in the combination therapy of infections due to these organisms. Although strains of *Chlamydia* spp. are susceptible to rifampin *in vitro*, resistance emerges rapidly when rifampin is used alone, as is seen in many bacterial species.

Rifaximin has broad-spectrum inhibitory activity against enteric bacterial pathogens, including enterotoxigenic and enteroaggregative strains of *E. coli*, *Aeromonas*, *Shigella*, and *Salmonella*, with MIC_{90}s ranging from 4 to 16 μg/ml (377, 384). Although it is less active against *C. jejuni* (MIC_{90} of 512 μg/ml), *Y. enterocolitica* (MIC_{90} of 128 μg/ml), *C. difficile* (MIC_{90} of 128 μg/ml), and *H. pylori* (MIC_{50} of 4 μg/ml), concentrations of rifaximin achieved in the intestinal lumen are >10-fold higher than the MICs of the drug for these pathogens. This drug has been used successfully to treat and prevent uncomplicated traveler's diarrhea (385). Rifaximin offered no benefit over a comparator when studied against *C. difficile* infections. Rapid emergence of resistance in *C. difficile* to this agent has been documented, and its role in therapy for *C. difficile* infections remains unclear (371).

Adverse Effects

Rifampin has many side effects, including gastrointestinal discomfort and hypersensitivity reactions, such as drug fever, skin rashes, and eosinophilia. It produces a harmless, orange-red coloration of saliva, tears, urine, and sweat. In up to 20% of patients, an influenza-like syndrome with fever, chills, arthralgias, and myalgias may develop after several months of intermittent therapy (386). This immunologic reaction may be associated with hemolytic anemia, thrombocytopenia, and renal failure. Rifampin-induced hepatitis occurs in <1% of patients and is more frequent during concurrent isoniazid therapy for tuberculosis. Rifampin is a potent inducer of cytochrome P450 enzymes; other rifamycins also possess this property but to a slightly lesser extent. This induction results in an impressive array of clinically significant drug-drug interactions that markedly complicate the therapeutic use of the rifamycins.

Headache, nausea, abdominal pain, and fatigue are the most frequent side effects reported for rifaximin. Rare hypersensitivity reactions, including allergic dermatitis, rash, angioneurotic edema, urticaria, and pruritus, can occur with rifaximin, which is contraindicated in those who are allergic to rifampin (377). However, unlike rifampin, rifaximin does not cause clinically relevant interactions with drugs because of the lack of systemic absorption of this drug.

NITROFURANTOIN

Nitrofurantoin belongs to a class of compounds consisting of a primary nitro group joined to a heterocyclic ring. Its role in human therapeutics is limited to treatment of urinary tract infections (341).

Mechanism of Action

Nitrofurantoin exerts its antibacterial effects via multiple mechanisms. At high concentrations, nitrofurantoin is converted by bacterial nitroreductases to highly reactive electrophilic intermediates that bind nonspecifically to bacterial ribosomal proteins and rRNA, causing complete cessation of synthesis of bacterial DNA, RNA, and proteins (387). It inhibits the inducible synthesis of essential bacterial enzymes (β-galactosidase and galactokinase) at concentrations near the MICs for susceptible organisms and disrupts bacterial metabolism in the absence of reductive activation of the drug (387).

Pharmacology

The drug is available in microcrystalline and macrocrystalline forms. It is administered orally and is well absorbed from the gastrointestinal tract. Very low levels of the drug are achieved in serum and most body tissues after the usual oral doses. With a half-life in serum of about 20 min, two-thirds of the drug is rapidly metabolized and inactivated in various tissues. The remaining one-third is excreted unchanged into the urine. An average dose of nitrofurantoin yields a concentration in urine of 50 to 250 μg/ml in patients with healthy renal function. In alkaline urine, more of the drug is dissociated into the ionized form, resulting in lowered antibacterial activity. Nitrofurantoin accumulates in the sera of patients with creatinine clearances of <60 ml/min. The drug is removed by hemodialysis. The risk of systemic toxicity increases in the presence of severe uremia. It is contraindicated in patients with significant renal impairment and hepatic failure.

Spectrum of Activity

Nitrofurantoin has a broad spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria, particularly the common urinary tract pathogens. It is active against Gram-positive cocci, such as *S. aureus*, *S. epidermidis*, *Staphylococcus saprophyticus*, and *E. faecalis*, with MICs in the range of ≤0.5 to 64 μg/ml (234, 343). *S. pneumoniae*, *S. pyogenes*, and *Corynebacterium* spp. are also susceptible, but they rarely cause urinary tract infections. Approximately 90% of *E. coli* strains and many coliform bacteria are susceptible to nitrofurantoin at MICs of <32 μg/ml. However, only one-third to one-half of *Enterobacter* spp. and *Klebsiella* isolates are susceptible. *Pseudomonas* spp. and most *Proteus* spp. are resistant. Susceptible organisms rarely become resistant to this drug during therapy. Nitrofurantoin is a first-line recommendation for cystitis in the recently updated IDSA guidelines (220).

Adverse Effects

Gastrointestinal irritation with anorexia, nausea, and vomiting is the most common side effect. Diarrhea and abdominal cramps may occur. Hypersensitivity reactions, such as drug fever, chills, arthralgia, skin rashes, and a lupus-like syndrome, have been observed (388). Pulmonary reactions are the most common serious side effects associated with nitrofurantoin use. Acute pneumonitis with fever, cough, dyspnea, eosinophilia, and pulmonary infiltrates present on chest radiographs can occur after a few days of therapy (388). This immunologically mediated reaction is more common in elderly patients and is rapidly reversible after cessation of therapy. Chronic pulmonary reactions with interstitial pneumonitis leading to irreversible pulmonary fibrosis can occur in patients on continuous therapy for 6 months or more (389).

Peripheral polyneuropathy is a serious side effect that occurs more often in patients with renal failure than in other patients. Hemolytic anemia, megaloblastic anemia, and bone marrow suppression with leukopenia can occur. Rare hepatotoxic reactions, such as cholestatic jaundice and chronic active hepatitis, have been reported (390).

FOSFOMYCIN

Fosfomycin, first isolated from cultures of *Streptomyces* spp. in 1969, is a phosphonic acid derivative originally named phosphonomycin (391). In the United States, it is used as single-dose therapy for uncomplicated urinary tract infections due to susceptible organisms, though it is increasingly being investigated and utilized in the therapy of MDR urinary tract infections (341, 392).

Mechanism of Action

Fosfomycin is bactericidal because it inhibits UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA), a bacterial cytoplasmic enzyme that catalyzes the formation of uridine diphosphate-*N*-acetylmuramic acid during the initial step of peptidoglycan synthesis (393). There is little cross-resistance between fosfomycin and other antibacterial agents, most likely because it differs from other agents in its chemical structure and site of action. Resistance to fosfomycin can occur by three mechanisms: (i) mutations of the structural or regulatory genes for the bacterial proteins (GlpT and UhpT) that transport the drug into the cell, (ii) plasmid-mediated production of a drug-inactivating enzyme (FosA), and (iii) overproduction of the target enzyme MurA (394, 395).

Pharmacology

Originally formulated as sodium and calcium salts for oral and intravenous use, fosfomycin is available in the United States as an oral, water-soluble tromethamine salt. Following oral administration, it is rapidly absorbed and converted to the free acid fosfomycin. With markedly improved oral bioavailability over earlier formulations (35 to 40%), fosfomycin has a mean elimination half-life of 5.5 h, and it is primarily excreted unchanged in the urine (396). Following a single oral dose of 3 g, peak serum concentrations (range, 22 to 32 μg/ml) are achieved in 2 h after administration, with peak urinary concentrations (1,000 to 4,400 μg/ml) occurring within 4 h and remaining high (>128 μg/ml) for 24 to 48 h, sufficient to inhibit most urinary tract pathogens. Peak urinary concentrations are reached later and lowered when the drug is administered with food or antiperistaltic agents. In patients with renal impairment (creatinine clearance, <30 ml/min), peak serum concentrations of fosfomycin are increased, with decreased urinary elimination and reduced urinary concentrations of the drug.

While not bound to plasma protein, it is widely distributed in various body fluids and tissues, including kidneys, prostate, and seminal vesicles, from which it is cleared slowly. Although it crosses the placental barrier, the drug can be used safely during pregnancy if clearly needed.

Spectrum of Activity

Fosfomycin has a broad spectrum of antibacterial activity against most Gram-positive and Gram-negative bacteria isolated from patients with lower urinary tract infections. *E. coli*, *Serratia*, *Klebsiella*, *Citrobacter*, *Enterobacter* spp., *S. aureus*, and enterococci are generally inhibited by fosfomycin at concentrations of <64 μg/ml (396, 397). MDR urinary tract isolates, including ESBL-producing *E. coli* isolates, are often susceptible to this agent (398). Fosfomycin is bactericidal at concentrations that are similar to the MIC values, with ≤2-fold differences. It is more active than TMP and nalidixic acid, while being similar to norfloxacin and co-trimoxazole, in its activity against these organisms (397). At a breakpoint concentration of ≤128 μg/ml, 60%, 20%, and 80% of isolates of *Pseudomonas* spp., *Morganella morganii*, and *S. saprophyticus*, respectively, are susceptible to fosfomycin (399). In multiple-dose use, bacterial resistance to fosfomycin emerges rapidly as the result of chromosomal mutations or, more rarely, by acquisition of mobile elements. However, cross-resistance with other antimicrobials has been uncommon (400). Resistance appears to be increasing in some parts of the world where the drug has been widely used for the treatment of urinary tract infections caused by ESBL-producing *E. coli* (401). The *in vitro* activity of fosfomycin is affected by test medium and conditions (396, 399). Fosfomycin has greater *in vitro* activity and closer correlation with *in vivo* activity when the test medium is supplemented with glucose-6-phosphate at 25 μg/ml, as recommended for susceptibility testing by the agar and broth dilution methods. The disk diffusion testing method utilizes disks containing 200 μg of fosfomycin tromethamine and 50 or 100 μg of glucose-6-phosphate.

Adverse Effects

Mild, self-limiting gastrointestinal disturbances, mainly diarrhea, are the most frequent side effects (3% to 5%). Other minor adverse events include headaches, dizziness, rash, and vaginitis.

BACITRACIN

Originally isolated from *Bacillus licheniformis* (formerly *B. subtilis*), bacitracin is a cyclic polypeptide antibiotic. Although it was introduced initially for the systemic treatment of severe staphylococcal infections, it is now restricted to topical use because of its systemic toxicity.

Bacitracin inhibits dephosphorylation of a lipid pyrophosphate, a step essential for bacterial cell wall synthesis. It also disrupts the bacterial cytoplastic membrane. Bacitracin is often used in various topical preparations, such as creams, ointments, antibiotic sprays and powders, and solutions for wound irrigation or bladder instillation. When used as a topical antibiotic, no significant amount of bacitracin is absorbed systemically. Large doses used to irrigate serous cavities may be associated with systemic toxicity.

This drug is active mainly against Gram-positive bacteria, especially staphylococci and group A beta-hemolytic streptococci. However, group C and G streptococci are less susceptible, while group B streptococci are resistant (402).

Strains of *Neisseria* spp. are also susceptible, but Gram-negative bacilli are resistant. Bacitracin is often combined with neomycin, polymyxin B, or both in topical preparations to provide broad-spectrum antibacterial coverage.

Systemic administration of bacitracin results in significant nephrotoxicity. Side effects are rare when the drug is given orally or applied topically. The drug is nonirritating to skin or mucous membranes. Allergic skin sensitization is rare.

MUPIROCIN

Mupirocin, formerly pseudomonic acid A, is a topical antibacterial agent derived from the fermentation products of *Pseudomonas fluorescens* (403). It contains a unique 9-hydroxynonanoic acid moiety in its chemical structure, and it inhibits isoleucyl-tRNA synthetase, resulting in cessation of bacterial protein synthesis (404). Low-level resistance (MICs of 4 to 256 μg/ml) results from an altered synthetase enzyme due to mutations in the bacterial chromosomal *ileS* gene, whereas high-level, transferable resistance (MIC of ≥512 μg/ml) is mediated by the plasmid-borne *mupA* and *mupB* genes, which encode an additional isoleucyl-tRNA synthetase (405).

Originally developed for the topical treatment of superficial soft tissue infections, particularly those due to staphylococci, mupirocin is available as a 2% ointment or cream in the United States. After topical application, <1% of the drug is absorbed systemically, with no detectable levels in the urine or feces. Penetration into deeper dermal layers of the skin is increased with traumatized skin or use of occlusive dressings. The drug is highly protein bound (95%), and its activity is lowered in the presence of serum. It is most active at moderately acid pH, with no inoculum effect (406). Mupirocin is slowly metabolized in the skin to the inactive monic acid.

In vitro activity is directed primarily against the Gram-positive cocci. *S. aureus*, including MRSA, and coagulase-negative staphylococci are uniformly susceptible, with MIC_{90}s of <0.5 μg/ml (407). Emergence of resistant strains of staphylococci can occur with widespread use of mupirocin (408–410). Most streptococci (including *S. pneumoniae*, beta-hemolytic streptococci of groups A, B, C, and G, and viridans group streptococci) are inhibited by mupirocin concentrations of ≤1 μg/ml. Resistant bacteria include enterococci, *Corynebacterium* spp., *Erysipelothrix* spp., *P. acnes*, Gram-positive anaerobes, and most Gram-negative bacteria. However, *H. influenzae*, *N. gonorrhoeae*, *N. meningitidis*, *M. catarrhalis*, *B. pertussis*, and *P. multocida* are susceptible; MICs for them are in the range of 0.02 to 0.25 μg/ml. There is no cross-resistance between mupirocin and other major groups of antibiotics. Clinically, mupirocin is efficacious in the therapy of superficial skin infections, such as impetigo, folliculitis, and burn wound infections that are caused by staphylococci or streptococci (411). Although this drug has been used successfully to eradicate nasal carriage of *S. aureus*, including methicillin-resistant strains (412), it may not prevent nosocomial *S. aureus* infections (413). No systemic toxic effects have been reported with mupirocin. Local irritation, such as burning, stinging, itch, and rash, which may be due to the polyethylene glycol base in the vehicle ointment, may occur.

RETAPAMULIN

Retapamulin belongs to a new class of antibiotics called pleuromutilins, which are derived from a fermentation product of *Pleurotus mutilus* (an edible mushroom). This class of agents selectively inhibits the elongation phase of bacterial protein synthesis by preventing normal formation of active 50S ribosomal subunits and interfering with peptide bond formation (414). Low-level resistance to retapamulin has been induced *in vitro* with stepwise mutations in the *rpl*C gene, which encodes the bacterial ribosomal protein L3 (415), but the frequency of such resistance is very low, and high-level mutational resistance is unlikely to occur. However, a recently described plasmid-mediated gene (*cfr*) encodes an enzyme that methylates the ribosomal binding site for pleuromutilins and leads to cross-resistance to lincosamides, oxazolidinones, chloramphenicol, and streptogramin B (313).

Retapamulin is active against *S. aureus*, *S. pyogenes*, *Streptococcus agalactiae*, and viridans group streptococci at very low concentrations (MIC_{90}s of 0.03 to 0.5 μg/ml), including MRSA and isolates resistant to β-lactams, macrolides, quinolones, and mupirocin, without target-specific cross-resistance to other antibacterial agents (416, 417). More than 50% of mupirocin- and fusidic acid-resistant strains of MRSA and *S. aureus* remain susceptible to this drug (418). It is also active against *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, and most anaerobes, including *P. acnes*. Retapamulin is licensed in the United States as a 1% ointment for the topical treatment of impetigo caused by MSSA and *S. pyogenes*. No systemic toxic effects have occurred with the topical use of retapamulin. Irritation, itching, pain, rash, and contact dermatitis at the application site were reported in <2% of patients in clinical studies of this drug.

APPENDIX

Approximate Concentrations of Antibacterial Agents in Serum

The concentrations of antimicrobial agents listed in the table below are approximations taken from various reports and publications. Several factors can influence the level of antimicrobial agent in individual patients, including inherent differences in the patients themselves, their physical condition, the dosages, and the routes of administration. The values can also be influenced by the assay methods used to obtain them. Therefore, these concentrations should be used only as approximate values, and clinicians should use their knowledge of the patient and the drugs, the recommendations from U.S. Food and Drug Administration-approved package inserts, or other reputable sources in planning their therapeutic regimens.

Approximate antibacterial concentrations in serum

Antimicrobial agent(s)	Serum half-life or half-lives (h)	Unit dose(s)[a]	Avg peak level(s) in serum (μg/ml) after the drug(s) is administered[b]:		
			p.o.	i.m.	i.v.
Amikacin	2–2.5	7.5 mg/kg		15–20	20–40
Amoxicillin	1	500 mg	6–8		
Amoxicillin-clavulanate	1.3, 1.0	250, 125 mg	3.3, 1.5		
		500, 125 mg	6.5, 1.8		
		875, 125 mg	11.6, 2.2		
		1,000, 62.5 mg	17, 2.1		
Ampicillin	1.1	500 mg	2.5–5	8–10	
		1 g			40
Ampicillin-sulbactam	1.1, 1.0	3 g			120, 60
		1.5 g			18, 13
Azithromycin	48	500 mg	0.4		3.5
Aztreonam	1.7	1 g		45	90–160
Cefaclor	0.6	500 mg	16		
Cefadroxil	1.5	500 mg	10		
Cefazolin	1.8	1 g		65	185
Cefdinir	1.7	300 mg	1.6		
Cefditoren	1.6	200 mg	3.1		
		400 mg	4.4		
Cefepime	2	1 g		30	82
Cefixime	3–4	400 mg	3.5		
Cefoperazone	2	1 g		65–75	153
Cefotaxime	1	1 g		20	40–45
Cefotetan	3–4.5	1 g		50–80	160
Cefoxitin	1	1 g		20–25	55–110
Cefpirome[c]	2	1 g		45	80
		2 g			180
Cefpodoxime	2.5	200 mg	2.3		
Cefprozil	1.5	500 mg	10.5		
Ceftaroline	2.6	600 mg		8.5	21
Ceftazidime	2	1 g		40	70
Ceftibuten	2.5	400 mg	15		
Ceftobiprole[c]	3–4	500 mg			44
		750 mg			60
Ceftriaxone	6–9	500 mg		40–45	
		1 g			150
Cefuroxime	1.5	750 mg		27	50
Cefuroxime axetil	1.5	500 mg	9		
Cephalexin	0.9	500 mg	18		
Cethromycin[c]	5	150 mg	0.2		
		300 mg	0.5		
Chloramphenicol	4	1 g	10–18		10–15
Ciprofloxacin	5–6	400 mg			4.5
		500 mg	3.0		
		500 mg XR	1.6		
		750 mg	4.0		
		1,000 mg XR	3.1		
Clarithromycin	5–7	250 mg	1–2		
		500 mg	3–4		
		1,000 mg XL	2–3		
Clindamycin	2.5	300 mg	3	6	
		600 mg			10–12
Cloxacillin	0.5	500 mg	10		
Colistimethate sodium	2	150 mg		5–6	
Dalbavancin[c]	149–198	1 g			285
Daptomycin	9	4 mg/kg			70
		6 mg/kg			82
Demeclocycline	12	300 mg	1–2		
Dicloxacillin	0.5–0.7	500 mg	15		
Doxycycline	18–22	100 mg	2.5		4
Ertapenem	4	1 g		70	155
Erythromycin	1.5	500 mg	2–3		
		1 g			10

(Continued on next page)

Approximate antibacterial concentrations in serum (*Continued*)

Antimicrobial agent(s)	Serum half-life or half-lives (h)	Unit dose(s)[a]	Avg peak level(s) in serum (μg/ml) after the drug(s) is administered[b]:		
			p.o.	i.m.	i.v.
Fosfomycin	5.7	3 g	25		
		50 mg/kg			275
Fusidic acid	13–19	500 mg	25–30		50
Gatifloxacin	7	400 mg	4		4.5
Gemifloxacin	7–8	320 mg	1.8		
Gentamicin	2–3	1.5 mg/kg	4–6		4–8
Imipenem	1	500 mg			25–35
Levofloxacin	6–8	500 mg	5.5		6.5
		750 mg	8.5		12
Lincomycin	5	500 mg	3.5		
		600 mg		10	16–21
Linezolid	5	400 mg	11		
		600 mg	13		13
Meropenem	1	500 mg			25–35
Metronidazole	8	500 mg	12		20–25
Minocycline	14–16	100 mg	1		
Moxifloxacin	12	400 mg	4.5		4.5
Nafcillin	0.5	500 mg			5–8
		1 g			20–40
Nalidixic acid	1.5	1 g	20–50		
Nitrofurantoin	0.3	100 mg	<2		
Norfloxacin	3.3	400 mg	1.5		
Ofloxacin	5	400 mg	4		
Oritavancin[c]	136–274	0.5 mg/kg			6.5
		200 mg			25–35
Ornidazole	13	500 mg	10		20
Oxacillin	0.5	500 mg	4–6	14–16	
		1 g			40
Pefloxacin[c]	10	400 mg	3		5.5
Penicillin G	0.5	500 mg	1.5–2.5		
Aqueous		1× 10^6 U		8–10	10
Benzathine		1.2 × 10^6 U		0.1–0.153	
Procaine		1.2 × 10^6 U			
Penicillin V	0.5	500 mg	3–5		
Piperacillin	1.1	2 g			36
		4 g			240
Piperacillin-tazobactam	1.1, 1.0	3.375 g			242, 24
		4.5 g			298, 34
Polymyxin B	6–7	2.5 mg/kg			5
Quinupristin-dalfopristin	1, 0.75	7.5 mg/kg			3, 7.5
Rifampin	2–5	600 mg	7–9		10
Streptomycin	2–3	1 g		25–50	
Sulfadiazine	17	2 g	100–150		
Sulfadoxine	150–200	1 g	50–75		
Sulfamethizole	4–7	2 g	60		
Sulfamethoxazole	10–12	1 g	40		
Teicoplanin[c]	45	200 mg		7	
		400 mg			20–40
Telavancin	6–8	7.5 mg/kg			88
		10 mg/kg			108
Telithromycin	9–10	800 mg	2		
Tetracycline	8	500 mg	4		8
Ticarcillin	1.2	1 g		20–30	
		3 g			190
Ticarcillin-clavulanate	1.2, 1.0	3.1 g			330, 8
Tigecycline	36	50 mg			0.8
Tinidazole	12–14	2 g	48		
Tobramycin	2–2.8	1.5 mg/kg		4–6	4–8
Trimethoprim	10–12	100 mg	1		
TMP-SMX		160, 800 mg	3, 46		9, 106
Vancomycin	6	500 mg			20–40

[a] XR or XL, extended-release formulation.

[b] p.o., *per os*; i.m., intramuscularly; i.v., intravenously (determined 30 min following intravenous infusion). If two values are present, doses are listed in the drug order in the stub of the table.

[c] Not licensed for clinical use in the United States.

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Mechanisms of Resistance to Antibacterial Agents*

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69

GENERAL CONCEPTS, INOCULUM EFFECTS, AND TOLERANCE

When the growing problem of antimicrobial resistance in bacteria is considered, it is worth remembering that resistance is not a new phenomenon, nor is it unexpected in an environment in which potent antimicrobial agents are used. The diversity of the microbial world and the relatively specific activities of our antimicrobial agents virtually ensure widespread resistance among bacteria. In many cases, this resistance is recognized when an antibiotic is first tested for development. For example, it is not considered a problem or threat that *Escherichia coli* is resistant to vancomycin. We simply understand that *E. coli* is not among vancomycin's spectrum of activity, and we avoid using vancomycin when *E. coli* infection is known or highly suspected (i.e., it has natural or intrinsic resistance to vancomycin). Conversely, the increasing resistance of *E. coli* to ciprofloxacin represents an important problem, since we frequently use the fluoroquinolone class of antimicrobial agents to treat infections in which *E. coli* is likely to be involved. So when we speak of the problem of resistance, we must recognize that most problems result from expression of resistance by bacteria that are intrinsically susceptible to the antibiotic in question (i.e., they have acquired resistance).

It is also important to recognize that resistance as a clinical entity is essentially a relative phenomenon, in many ways a problem only indirectly related to the microbiological techniques often used to detect it. For example, it is possible to incorporate enough ticarcillin into an agar plate to inhibit an ampicillin-resistant *Enterococcus faecium* (in many cases this requires about 10,000 μg/ml). So in one sense, ampicillin-resistant *E. faecium* is susceptible to high concentrations of ticarcillin. However, such concentrations cannot be achieved at the site of infection, so ampicillin-resistant *E. faecium* is not considered susceptible to ticarcillin. In the treatment of meningitis, relatively minor increases in the MIC can foil treatment, but in the treatment of simple urinary tract infections, in many cases, increases in the MIC are less relevant, given the tendency of many antibiotics to concentrate in the urine. It is the responsibility of the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards), the Food and Drug Administration, and other standard-setting bodies in different countries to establish breakpoints for antimicrobial agents that are based on *in vitro* susceptibility, pharmacokinetic/pharmacodynamic modeling, and clinical-outcome data. The dependence of cure on MIC and antimicrobial concentration at the site of infection is why the CLSI *Streptococcus pneumoniae* susceptible breakpoint for the treatment of meningitis with penicillin is lower than the breakpoint for parenteral therapy of pneumonia.

The relativity of resistance is no better exemplified than by considerations of susceptibility in bacterial strains that exhibit significant inoculum effects, such as those resistant by virtue of producing β-lactamase. β-Lactamase-mediated resistance results from a chemical interaction in which the β-lactamase molecule binds to the β-lactam antibiotic in a manner that ultimately results in the hydrolysis of the critical β-lactam ring structure (Fig. 1). The rapidity and efficiency with which binding and hydrolysis proceed are dependent upon the affinity with which the β-lactamase molecule binds the antibiotic and the efficiency of the subsequent hydrolysis. High-affinity and rapid hydrolysis mean that the cell wall synthesis machinery (penicillin-binding proteins [PBPs]) can be defended with relatively few β-lactamase molecules compared to the number of β-lactam molecules likely to be present in the vicinity of the PBPs. Low affinity and slow hydrolysis mean that more β-lactamase molecules are necessary for effective resistance but also that resistance can be more easily overcome by adding more antibiotic molecules. Increasing the inoculum of organisms in a solution with a fixed concentration of β-lactam antibiotic has the effect of increasing the number of β-lactamase molecules and can in some instances result in clinically important levels of resistance. A *Klebsiella pneumoniae* strain that produces the extended-spectrum β-lactamase (ESBL) TEM-26, for example, may exhibit a standard inoculum (ca. 10^5 CFU/ml) MIC for cefotaxime of 1 μg/ml. However, when the inoculum is increased to 10^7 CFU/ml, the MIC increases to >256 μg/ml (1).

A second, more amorphous and difficult-to-evaluate concept in considering antimicrobial resistance is that of tolerance, or resistance to killing, at antimicrobial concentrations sufficient to inhibit further growth. Tolerance is an unimportant concept for the treatment of most infections, since for therapeutic success it is usually sufficient to inhibit further growth of bacteria (bacteriostatic activity), allowing the patient's immune defenses to kill the growth-inhibited organisms and clean up the debris. In some instances, however, antimicrobial killing

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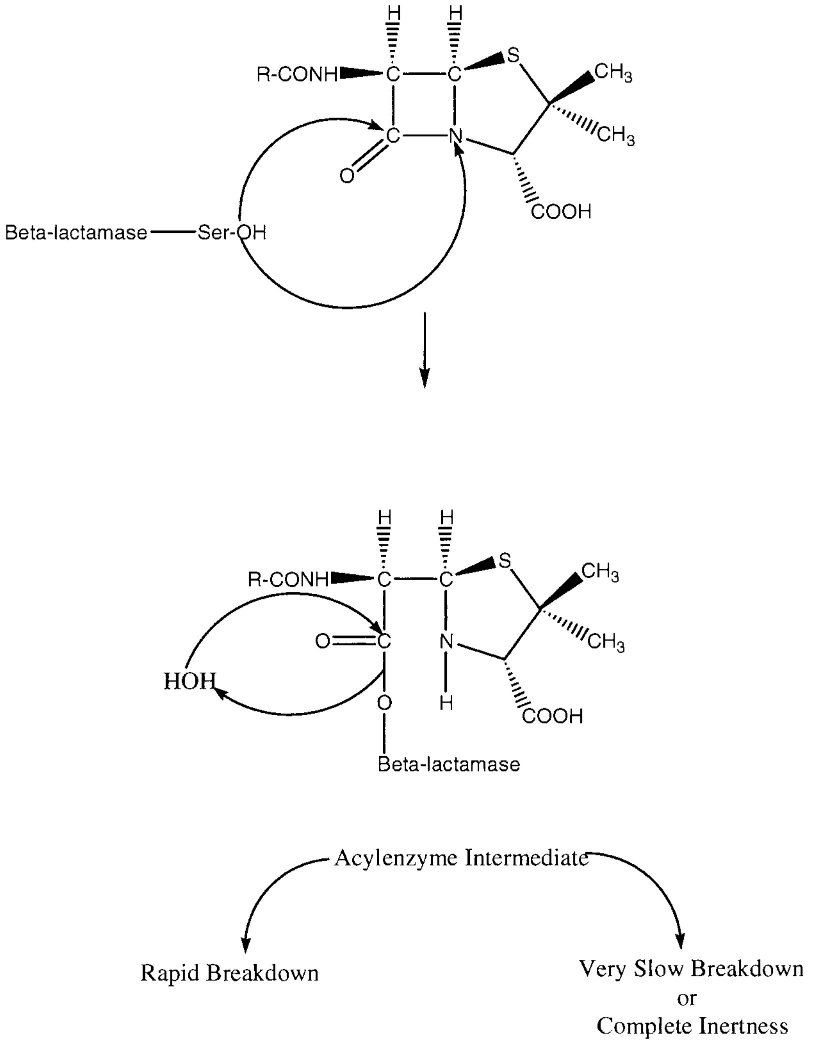

FIGURE 1 Serine β-lactamases and their reactions with β-lactam carbonyl donors. Modified from reference 344. doi:10.1128/9781555817381.ch69.f1

of the bacteria (bactericidal activity) is required to yield a high percentage of treatment success. Instances where bactericidal activity is preferred include endocarditis, meningitis, and osteomyelitis, in which the immune system has limited access to the infection site. They also include circumstances in which the immune system is severely compromised, such as in patients undergoing high-dose chemotherapy for hematologic malignancies. In these instances, antibiotics that are primarily bacteriostatic, such as the tetracyclines or the macrolides, are considered poor choices for therapy, whereas β-lactam antibiotics, which are primarily bactericidal, are preferred. Some bacteria are naturally tolerant to β-lactam antibiotics. Bactericidal activity against enterococci, for example, requires two agents, one active against cell wall synthesis and an aminoglycoside (2). Recognition of this bactericidal synergism raised cure rates for enterococcal endocarditis from about 40 to 70% or greater (3). Unfortunately, expression of aminoglycoside-modifying enzymes (AMEs) negates the synergism and appears to decrease the cure rates for enterococcal endocarditis. Although some level of tolerance to β-lactam antibiotics can be demonstrated for several different bacterial species, the impact on the treatment of clinical infections appears to be less dramatic than with enterococci.

Emergence and Spread of Antibiotic Resistance

The emergence of antimicrobial resistance phenotypes is inevitably linked to the clinical (or other) use of the antimicrobial agent against which resistance is directed. One reason for this association is trivial—we do not generally test for resistance to antibiotics that are not in clinical use. The second reason is that nature abhors a vacuum, so when an effective antibiotic eliminates a susceptible biota, resistant varieties soon fill the niche. Once a resistance phenotype has emerged within a previously susceptible species, the rapidity and efficiency with which it spreads are affected by a host of different factors, including the degree of resistance expressed, the ability of the organism to tolerate the resistance mechanism, linkage to other genes, site of primary colonization, and others. The rapidity and completeness of resistance gene spread are often unpredictable. For example, the staphylococcal β-lactamase gene (conferring resistance to penicillin) was first described shortly after the introduction of penicillin into clinical use and is now almost universally present within staphylococci in hospitals and communities. It was not until the early 1980s that this gene was described to occur in *Enterococcus*, and it has never spread widely in this genus. The reverse appears to be true with the vancomycin resistance genes, which are found widely in *E. faecium* but remain exceedingly rare in *Staphylococcus aureus*.

An important cause of the spread of antimicrobial resistance is the failure to adhere to appropriate infection control techniques, both within and outside hospitals. It is well established that strains of methicillin-resistant *S. aureus* (MRSA) within individual hospitals, and even within entire cities, are often clonally related, as determined by genetic techniques such as pulsed-field gel electrophoresis, multilocus sequence typing, and staphylococcal protein A typing (4). The spread of these problematic pathogens has been attributed to transmission from patient to patient, presumably by transiently or persistently colonized health care workers (5). The primary site of *S. aureus* colonization is the anterior nares. Colonization of the nares facilitates aerosol transmission of the resistant bacteria, particularly during periods of viral upper respiratory tract infection in colonized workers. It also facilitates direct transmission, given the frequent contact between hands and nose of many people and the frequently poor hand-washing practices of health care workers. The clinical consequences of patient colonization can be significant. Studies have shown a correlation between patient colonization with MRSA and subsequent infection during periods of high risk, such as the postoperative period (6).

Although antibiotic resistance is predominantly a nosocomial problem, resistant bacteria are also spread in the community setting. Sites in which resistant bacteria have been known to spread include day care centers and nursing homes (7, 8). Transmission probably reaches its peak in the winter months, when viral upper respiratory tract infections are prevalent. The prevalence of viral upper respiratory tract infections works in two ways to increase transmission: (i) it probably increases the inoculum of resistant organisms being spread by those already colonized and (ii) it makes those who are not colonized more likely to become colonized because of the increased likelihood that they will receive antimicrobial therapy. Nursing homes are predisposed to resistance for a variety of reasons, including the debilitated state of much of their population, frequent movement back and forth to tertiary care hospitals, and frequent use of antimicrobial agents in an effort to ward off infections that necessitate hospital admissions.

A final important source of the emergence and spread of antibiotic-resistant bacteria is nonhuman niches in which antibiotics are used. It is now well established that antimicrobial use in food animals is associated with both resistance in bacterial species that contaminate food and infect humans, primarily *Salmonella* and *Campylobacter*, and the transfer of resistance determinants to their human counterparts, such as *Enterococcus* (9). Compelling evidence that high rates of ciprofloxacin resistance in *E. coli* can be associated with the use of fluoroquinolones in poultry also exists (10). Finally, the European outbreak of vancomycin-resistant enterococci with the *van*A determinant in the 1990s was almost certainly fueled by the use of avoparcin (a glycopeptide antibiotic) as a growth promoter in food animals (11). The Alliance for the Prudent Use of Antibiotics and other groups support a ban on the nontherapeutic use of antimicrobial agents in animals to address rising levels of resistant bacteria present in food and the environment (12).

Genetic Basis of Resistance

Acquired antimicrobial resistance results from biochemical processes that are encoded by bacterial genes. A general list of mechanisms of resistance is presented in Table 1. In order to understand the biochemical processes, it is useful to first discuss the genetic underpinnings of resistance and its evolution. Antimicrobial resistance arises by (i) mutation of cellular genes, (ii) acquisition of exogenous resistance genes, or (iii) mutation of acquired genes.

Mutation of Cellular Genes

All antibiotics have targets, which are often (but not always) proteins with important functional responsibilities for cell growth or maintenance. Cellular genes encode these proteins. Interactions between antibiotics and target proteins are often quite specific, and changing a single amino acid, frequently as a result of a single base change in the gene, can sometimes alter these interactions. Perhaps the most familiar example of this mechanism is resistance to rifampin. Rifampin targets the cellular RNA polymerase (encoded by *rpoB*), and a single point mutation in this gene may confer complete resistance. These mutations occur in most bacterial species at a relatively high frequency (ca. 10^{-8}/CFU). Incubating enough cells with inhibitory concentrations of rifampin eliminates susceptible cells and allows the resistant mutants to proliferate. The rifampin in the medium does not actually cause resistance but, rather, selects mutants that occur naturally but which have no selective advantage for survival in the absence of rifampin in the environment. Other examples of mutational resistance include resistance to streptomycin by ribosomal mutation (13), resistance to fluoroquinolones through mutations of cellular topoisomerases (14), and resistance to linezolid by mutations in the rRNA (15), among others.

Resistance mutations may also be found in genes that regulate cellular processes. Perhaps the most completely studied example of regulatory mutation resulting in resistance is the derepression of the chromosomal β-lactamase of *Enterobacter* spp. (16). Mutations in a cellular amidase gene (designated *ampD*) result in buildup of a cell wall breakdown product that has the effect of dramatically increasing expression of a chromosomal β-lactamase gene (*amp*C). Other examples of regulatory changes include the downregulation of expression of the porin OMPD2 in *Pseudomonas aeruginosa* associated with resistance to imipenem (17) or the insertion of an insertion sequence (IS) element upstream of a chromosomal carbapenemase conferring imipenem resistance on *Bacteroides fragilis* (18).

Whether mutational resistance is likely to persist depends in some measure on whether the resistance mutation is tolerable to the cell. For example, although decreased expression of OMPD2 appears to be readily achievable for *P. aeruginosa*, the fact that these resistant strains have not spread widely in the nearly 20 years of carbapenem use probably reflects the fact that this porin has functions that are beneficial to the bacterium, favoring reexpression of the porin once the imipenem threat has been dissipated. Similarly, intermediate levels of susceptibility to vancomycin in *S. aureus* have thus far been attributed to marked changes in the composition of the cell wall (19). These changes are unlikely to be favored in an environment free of vancomycin, since *S. aureus* likely "decided" a long time ago the optimal size and composition of its cell wall. The deleterious effects of acquiring resistance are often referred to as fitness cost.

Disadvantageous resistance mutations do not always disappear. Although initial point mutations in the *rpoB* gene

TABLE 1 Common associations of resistance mechanisms

Antibiotic class	Resistance type(s)	Resistance mechanism(s)	Common example(s)
Aminoglycosides	Decreased uptake	Changes in outer membrane permeability	*P. aeruginosa*
	Enzymatic modification (AMEs)	Phosphotransferase	Wide range of enteric Gram-negative bacteria
		Adenyltransferase	Wide range of enteric Gram-negative bacteria
		Acetyltransferase	Wide range of enteric Gram-negative bacteria
		Bifunctional enzyme	*aac(6′)-aph(2″)* in *S. aureus*, *E. faecium*, and *E. faecalis*
β-Lactams	Altered PBP(s)	PBP 2a (additional PBPs)	*mecA* in *S. aureus* and coagulase-negative staphylococci
		PBP 2x, PBP 2b, PBP 1a (acquired from other streptococci by transformation)	*S. pneumoniae*
		PBP 5 (point mutation)	
	Enzymatic degradation (β-lactamases)	Ambler class A	*E. faecium* TEM-1 in *E. coli*, *H. influenzae*, and *N. gonorrhoeae*, SHV-1 in *K. pneumoniae*, K-1 (OXY-1) in *K. oxytoca*, ESBLs (TEM-3^+, SHV-2^+, and CTX-M types) in *K. pneumoniae* and *E. coli*, BRO-1 in *Moraxella catarrhalis*, PC1 in *S. aureus*, PSE-1 in *P. aeruginosa*, KPC β-lactamases
		Ambler class B	‘L-1 in *S. maltophilia* Ccr-A in *B. fragilis*
		Ambler class C	AmpC in *E. cloacae* and *C. freundii* and similar enzymes in *S. marcescens*, *M. morganii*, *P. stuartii*, and *Providencia rettgeri*
		Ambler class D	OXA-1 in *E. coli*
Chloramphenicol	Enzymatic degradation, efflux	CATs, membrane transporters	CAT in *S. pneumoniae*, *cmlA*- and *flo*-encoded efflux in *E. coli* and *Salmonella* spp.
Glycopeptides	Altered target	Altered peptidoglycan cross-link target (D-Ala–D-Ala to D-Ala–D-Lac or D-Ala–D-Ser) encoded by a complex gene cluster	VanA gene cluster in *E. faecium*, *E. faecalis*, and *S. aureus*, VanB gene cluster in *E. faecium* and *E. faecalis*
	Target overproduction	Excess peptidoglycan	Glycopeptide-intermediate strains of *S. aureus* and *Staphylococcus haemolyticus*
Oxazolidinones	Altered target	Mutation leading to reduced binding to active site	G2576U mutation in rRNA in *E. faecium* and *S. aureus*
MLSB	Altered target	Ribosomal active-site methylation with reduced binding	*erm*-encoded methylases in *S. aureus*, *S. pneumoniae*, and *S. pyogenes*
Macrolides	Efflux	Mef efflux pump	*mef*-encoded efflux in *S. pneumoniae* and *S. pyogenes*
Streptogramin A	Enzymatic degradation	Acetyltransferases	*vat*(A), *vat*(B), and *vat*(C)-encoded enzymes in *S. aureus*, *vat*(D) and *vat*(E)-encoded enzymes in *E. faecium*
Quinolones	Altered target	Mutation leading to reduced binding to an active site(s) (quinolone-resistance determining region)	Mutations in *gyrA* in enteric Gram-negative bacteria and *S. aureus*, mutations in *gyrA* and *parC* in *S. pneumoniae*
	Efflux	New membrane transporters	NorA in *S. aureus*
	Protection from DNA binding	Acquired protection protein	*qnr* gene and variants
	Enzymatic modification	Mutated aminoglycoside acetyltransferase	*aac6′-Ib* variant in *E. coli*
Rifampin	Altered target	Mutations leading to reduced binding to RNA polymerase	Mutations in *rpoB* in *S. aureus* and *M. tuberculosis*

(*Continued on next page*)

TABLE 1 Common associations of resistance mechanisms *(Continued)*

Antibiotic class	Resistance type(s)	Resistance mechanism(s)	Common example(s)
Tetracyclines	Efflux	New membrane transporters	*tet* genes encoding efflux proteins in Gram-positive bacteria (mainly group 2) and Gram-negative bacteria (mainly group 1)
	Altered target	Production of proteins that bind to the ribosome and alter the conformation of the active site (ribosomal protection proteins)	*tet*(M) in a variety of Gram-positive bacteria
Sulfonamides	Altered target	Mutation or recombination of genes encoding DHPS	Found in a wide range of species, e.g., *E. coli*, *S. aureus*, and *S. pneumoniae*
		Acquisition of new low-affinity DHPS genes	*sulI* and *sulII* in enteric Gram-negative bacteria
Trimethoprim	Altered target	Mutations in gene encoding DHFR	*S. aureus*, *S. pneumoniae*, and *H. influenzae*
	Overproduction of target	Acquisition of new low-affinity DHFR genes	*dhfrI* and *dhfrII* encoded, found in a wide range of species
		Promoter mutation leading to overproduction of DHFR	*E. coli*

that confer rifampin resistance on *Salmonella enterica* serotype Typhimurium appear to decrease the fitness of the organism for survival *in vivo*, persistence in a live host is frequently associated with compensatory mutations that at least partially restore fitness to the strain while retaining the resistance (rather than mutating back to susceptibility) (20). Similarly, transfer of mutated *pbp5* into *E. faecium* strains is often associated with decreases in the expression of ampicillin resistance, but growth on increased concentrations of ampicillin easily yields colonies that grow well at higher concentrations (21). Similar findings have been reported for *S. aureus* strains transformed with the *mecA* gene, encoding methicillin resistance (22). In summary, while mutational resistance often confers a fitness cost, subsequent adaptations may make expression of resistance less costly.

Acquisition of Resistance Genes

If resistance is not achievable through mutation, resistance determinants can be acquired. Most antimicrobial agents are natural products or derivatives of natural products. Therefore, resistance genes for most antibiotics must exist in the microbial world, either in the species that produce the antibiotic or within species that live in the same ecological niche as the antibiotic producers (23). The challenge for susceptible human pathogens is to find and acquire these resistance determinants. To assist in this acquisition, bacteria have evolved a range of mechanisms that promote gene exchange. Perhaps the simplest of these techniques is natural transformation, referring to the ability of some bacterial species to absorb naked DNA molecules from the environment under the appropriate circumstances (24). Once taken up by the susceptible bacterium, these foreign pieces of DNA enter the bacterial chromosome by recombining across regions of sufficient homology. In some cases, functional genes result from this recombination. If the acquired gene encodes a protein that is less susceptible to inhibition than the native protein, a reduction in susceptibility may result. Perhaps the best-studied example of the formation of these "mosaic" genes to confer resistance is penicillin and cephalosporin resistance in *Streptococcus pneumoniae* (24). A variety of mosaic *pbp* genes have been described to occur in resistant strains, with the level and degree of resistance determined by the number and nature of gene recombinations. Mosaic topoisomerase genes have also been described to occur in fluoroquinolone-resistant transformable bacteria (25).

Most bacteria are incapable of natural transformation and so have developed other mechanisms for acquiring useful genetic determinants. A commonly employed mechanism for genetic exchange is the transfer of conjugative plasmids. These extrachromosomal replicative DNA forms may bear a variety of important genes. Some plasmids are relatively narrow in their host range, while others transfer into and replicate within several different species. Transfer frequencies can be very high, as in the F factor of *E. coli* (virtual complete transfer in 1 h) or the pheromone-responsive plasmids found in *E. faecalis* (ca. 10^{-1} transconjugant/recipient CFU in 24 h), or can be more modest, as observed with the broad-host-range enterococcal plasmids, such as pAMβ1 (10^{-7} to 10^{-6} transconjugant/recipient CFU in 24 h) (26). Having entered into a new genus on broad-host-range plasmids, resistance determinants can readily transfer onto more frequently transferable plasmids to increase their movement through the new genus. In the first case of true vancomycin-resistant *S. aureus* described in the literature, it appears that vancomycin resistance transposon Tn*1546* entered into *S. aureus* from *Enterococcus faecalis* on a broad-host-range plasmid and then transposed to a conjugative plasmid native to the staphylococcus (27, 28). Plasmids may also integrate into the chromosome of the recipient strains, potentially increasing the stability of the genetic information that they carry.

Bacteria also take advantage of bacterial viruses (bacteriophages) for genetic exchange. These discrete packages deliver to uninfected cells a quantity of DNA approximating the size of their genome (in most cases roughly 40 kb). Designed to incorporate their own genome into the manufactured phage head, they sometimes incorporate bits of chromosomal DNA adjacent to the phage integration site (specialized transduction) and other times incorporate an appropriate-size plasmid or chromosomal DNA segment unrelated to the integrated phage genome (generalized transduction). Since the staphylococcal β-lactamase gene is frequently identified on nonconjugative plasmids of approximately 35 to 40 kb, and since bacteriophages have been well described for staphylococci for decades, it has been speculated that the high prevalence of β-lactamase production in staphylococci has resulted from bacteriophage-mediated transfer of these plasmids. Bacteriophages have also been implicated in the transfer of virulence determinants (29).

Nonreplicative mobile elements known as transposons have also been implicated in the transfer of resistance genes (30). Transposons encode their own ability to transfer between replicons (autonomously replicating DNA segments). In some cases, the transposons themselves encode conjugation functions which allow them to transfer from bacterial chromosome to bacterial chromosome. The best characterized of these conjugative transposons is Tn*916*, an 18-kb element originally described for *E. faecalis* but which has a very broad host range (31). Tn*916* encodes resistance to tetracycline and minocycline through the *tet*(M) resistance gene. Many different Tn*916*-like transposons have now been described for enterococci and other organisms, and some of them, such as Tn*1545* from *S. pneumoniae*, possess additional resistance genes (conferring resistance to erythromycin and kanamycin) (31). Some investigators have suggested that the conjugation events associated with Tn*916*-like transposons are akin to cell fusion events, in which portions of the genome distinct from that adjacent to the inserted transposon can exchange via homologous recombination (32). Transposons with structural similarity to Tn*916* have been implicated in the transfer of vancomycin resistance between *E. faecium* strains (33).

Transposons lacking conjugative functions may also transfer between strains. The most common mechanism by which this transfer is presumed to occur is either transient or more permanent integration into transferable plasmids. Among the more common classes of nonconjugative transposons are the Tn*3* family elements (including Tn*917*, conferring erythromycin resistance, and Tn*1546*, conferring VanA-type vancomycin resistance) (34, 35) and the composite elements formed by mobile IS elements flanking resistance genes (including Tn*4001*, conferring high-level gentamicin resistance on many Gram-positive species) (36).

In recent years, the importance of "common regions" has increasingly been recognized (37). These regions, which are often found near the 3′ conserved regions of integrons, have been implicated in the movement of a range of different antimicrobial resistance determinants. These regions represent an atypical type of IS element (IS*91*-like). They lack typical inverted repeats and transpose by a rolling-circle mechanism that allows them to transpose adjacent DNA without a flanking second copy of the element.

The precise origin of resistance genes is often difficult to discern, but in some cases it is at least possible to determine that acquired resistance determinants originated in other genera. The VanB-type vancomycin resistance gene in enterococci, for example, has a G+C content of nearly 50% (38). The enterococcal genome, in contrast, has a G+C content of approximately 35 to 38%. These differences virtually confirm the origin of the *vanB* determinant in a genus other than *Enterococcus*. The likely origin appears to be streptomycetes, probably species that manufacture glycopeptide antibiotics, with entry into the enterococcus facilitated by the incorporation of these resistance operons into transposons.

It is worth noting at this point that any concept of the bacterial genome as a fixed entity is untenable. Comparisons of *E. coli* genomes reveal striking differences between enteropathogenic and uropathogenic strains, with the different regions constituting pathogenicity islands that confer specific virulence traits that give the strains their clinical profiles (39, 40). Data emanating from a comparative study of 36 *S. aureus* genomes indicate that 22% of the genome is dispensable, with many of these variable regions constituting presumed pathogenicity islands and regions of antimicrobial resistance (41). Finally, it is estimated that 25% of the genome of *E. faecalis* strain V583 was acquired from outside the genus (42).

Mutation of Acquired Genes

As bacteria have responded to the challenge of antimicrobial agents, so have we responded to the challenge of antibiotic resistance. Our typical response to the appearance of antimicrobial resistance has been a concerted effort to develop novel antimicrobial agents that are active against resistant strains. The emergence of β-lactamase-mediated resistance to antibiotics is an instructive example of this interplay. Ampicillin was developed as the first penicillin with clinically significant activity against Gram-negative rods, primarily *E. coli*. Within a few years of the clinical introduction of ampicillin, strains of *E. coli* that were resistant to this antibiotic by virtue of production of a plasmid-mediated β-lactamase designated TEM (named after the patient from whom the resistant strain was isolated) were described. *S. aureus* expressed a similar β-lactamase, prompting a concerted effort on the part of the pharmaceutical industry to develop β-lactam antibiotics resistant to hydrolysis. Among the more successful compounds that were developed were methicillin (with activity against penicillinase-producing *S. aureus*), the cephalosporins and carbapenems (with widespread activities against many β-lactamase-producing species), and the β-lactamase inhibitors, which restored the activities of β-lactams susceptible to hydrolysis.

The most successful and widely developed class of β-lactamase-resistant β-lactam antibiotics is the cephalosporins. So many of these agents have been developed for clinical use that they are frequently lumped into "generations" to facilitate remembering their spectra of activity. The third-generation, or extended-spectrum, cephalosporins cefotaxime, ceftizoxime, ceftriaxone, and ceftazidime are particularly potent antibiotics that are resistant to hydrolysis by the original TEM enzyme. Unfortunately, increasing clinical use of these agents, particularly ceftazidime, was associated with the emergence of resistant Gram-negative rods, particularly *K. pneumoniae* (43). Molecular analysis of these resistant strains revealed that the resistance was mediated by β-lactamase and that many of these β-lactamases were derived from the native TEM enzyme through one or more point mutations in the bla_{TEM} gene.

Biochemical Mechanisms of Resistance

Modification of the Antibiotic

Many antibiotic-modifying enzymes, including the β-lactamases, the AMEs, and chloramphenicol acetyltransferases (CATs), have been described. Although these enzymes are in many cases acquired, some are intrinsic to certain genera. For example, chromosomal β-lactamases are intrinsic to almost all Gram-negative rods. Expression of these enzymes is often only at a very low level, conferring resistance to only very susceptible β-lactams, as with *K. pneumoniae* resistance to ampicillin through expression of the chromosomal SHV-1 enzyme (44), or to no β-lactams at all, as with wild-type *E. coli* strains. In some bacterial genera (notably *Enterobacter* and *Pseudomonas*), chromosomal β-lactamases are under regulatory control, with derangements in these regulatory mechanisms resulting in high-level, broad-spectrum β-lactam resistance (45). In some instances, AMEs are intrinsic to bacterial species as well, as with the chromosomal acetyltransferases of *Providencia stuartii* and *Serratia marcescens* (46, 47).

Modifying enzymes in general confer high levels of resistance to the antibiotics against which they have activity.

Expression of the TEM-1 β-lactamase by *E. coli*, for example, can increase the ampicillin MIC from 8 μg/ml to >10,000 μg/ml. Similarly, expression of the bifunctional aminoglycoside resistance enzyme in *E. faecalis* raises the gentamicin MIC from 32 to 64 μg/ml to >2,000 μg/ml. As effective as these mechanisms are, however, some antibiotics appear to be immune to inactivating enzymes. Vancomycin has been in clinical use since 1958, yet there are still no examples of vancomycin-modifying enzymes in bacteria.

Modification of the Target Molecule

Since antibiotic interaction with target molecules is generally quite specific, minor alterations of the target molecule can have important effects on antibiotic binding. There are numerous examples of antibiotic target modification as a mechanism of resistance, including the many erythromycin ribosomal methylases that confer resistance to the macrolide-lincosamide-streptogramin B (MLSB) class of antibiotics (48). Modifications of PBPs can affect the affinities of these molecules for β-lactam antibiotics, as noted above for *S. pneumoniae* and especially for ampicillin-resistant *E. faecium*, through mutations in PBP 5 (21, 24). Modifications of PBPs seem to be a favored mechanism of β-lactam resistance in Gram-positive bacteria, whereas β-lactamase production is favored in Gram-negative rods. Although the reason for this difference is unknown, it is interesting that β-lactamases produced by Gram-positive bacteria diffuse into the external medium once produced, whereas those produced by Gram-negative rods are kept within the periplasmic space by the outer membrane. The ability to concentrate β-lactamases enhances their efficacy and may help explain the preference for this mechanism among Gram-negative rods.

Other important examples of target modification include the altered cell wall precursors that confer resistance to glycopeptide antibiotics, mutated DNA gyrase and topoisomerase IV conferring resistance to fluoroquinolone antimicrobial agents, ribosomal protection mechanisms conferring resistance to tetracyclines, and RNA polymerase mutations conferring resistance to rifampin. The degree of resistance conferred by target modifications is variable and may be dependent upon the ability of the mutated target to perform its normal function. Mutations in PBPs of *S. pneumoniae*, for example, confer a relatively low level of resistance (although one that is significant in the treatment of meningitis) (24), whereas VanA-type vancomycin resistance confers a very high level of resistance to vancomycin in enterococci (49).

Restricted Access to the Target

It is axiomatic that an antibiotic must reach its target in order to be effective. Therefore, for targets for which barriers must be crossed by the antibiotic, strengthening these barriers can be a highly effective mechanism of resistance. All Gram-negative bacteria have an outer membrane that must be traversed before the cytoplasmic membrane can be reached. Reductions in the quantities of known or presumed porins (channels for movement of materials across the outer membrane) have been documented as important contributors to resistance to imipenem in *P. aeruginosa*, cefepime in *Enterobacter cloacae*, and cefoxitin or ceftazidime in *K. pneumoniae* (17, 50, 51). In most instances, this restricted entry must be in combination with the production of an at least moderately active β-lactamase to confer high-level resistance. Barriers to entry can also exist in the cytoplasmic membrane. Movement of aminoglycosides across the cytoplasmic membrane is an oxygen-dependent process, so these antibiotics are inactive in anaerobic environments (and hence against strictly anaerobic species) (52).

Efflux Pumps

Among the most active areas of research in antimicrobial resistance is the identification and characterization of pumps that extrude one or more antibiotic classes from bacterial cells. Several classes of pumps have been described for Gram-positive and/or Gram-negative bacteria. They may be quite selective, or they may have a broad substrate specificity. The majority of these pumps are located in the cytoplasmic membrane and use proton motive force to drive drug efflux. The major families of efflux transporters are (i) the major facilitator superfamily (MFS), which includes QacA and NorA/Bmr of Gram-positive bacteria and EmrB of *E. coli*; (ii) the small multidrug resistance family, including Smr of *S. aureus* and EmrE of *E. coli*; and (iii) the resistance-nodulation-cell division (RND) family, including AcrAB-TolC of *E. coli* and MexAB-OprM of *P. aeruginosa*. The structure of the AcrAB-TolC RND-type efflux pump is shown in Fig. 2. Deciphering the crystal structure of this pump was a major achievement (53). Among other things, it revealed that there was a periplasmic opening in the pump that could allow passage of molecules, explaining the previously confusing observation that RND pumps included β-lactam antibiotics (which do not enter the cytoplasm) among their substrates. In some instances, combinations of different types of pumps can result in higher levels of resistance than are achieved by the activity of a single pump alone (54).

Attribution of resistance to a specific mechanism may be difficult when more than one mechanism is involved. For example, resistance to imipenem in *P. aeruginosa* is contributed to by reduced access (through downregulation of OMPD2) and production of AmpC β-lactamase (17). Neither mechanism alone is sufficient to yield clinically significant levels of resistance, yet both are required for high levels of resistance to result.

RESISTANCE MECHANISMS FOR DIFFERENT ANTIMICROBIAL CLASSES

Aminoglycoside Resistance

In explaining resistance to aminoglycosides (amikacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin, and tobramycin), we first explain how aminoglycosides reach their target in bacterial cells and then review their mechanisms of action. The clinical indications for aminoglycoside therapy are also summarized.

The aminoglycosides contain an aminocyclitol ring (streptidine or 2-deoxystreptamine) and two or more amino sugars linked by glycosidic bonds. They are hydrophilic antibiotics whose antimicrobial activity is concentration dependent. They are particularly active against aerobic, Gram-negative rods. As a class, these agents have other highly desirable qualities: they are rapidly bactericidal, demonstrate a postantibiotic effect, and exhibit predictable pharmacodynamics.

Mechanisms of Action of Aminoglycosides

Aminoglycosides have unique effects on protein translation in prokaryotes. These effects may explain why aminoglycosides are bactericidal, whereas most other antibiotics that target the ribosome (chloramphenicol, macrolides, and tetracyclines) are bacteriostatic.

First, aminoglycosides penetrate bacteria by three main steps. The positively charged aminoglycoside binds to the

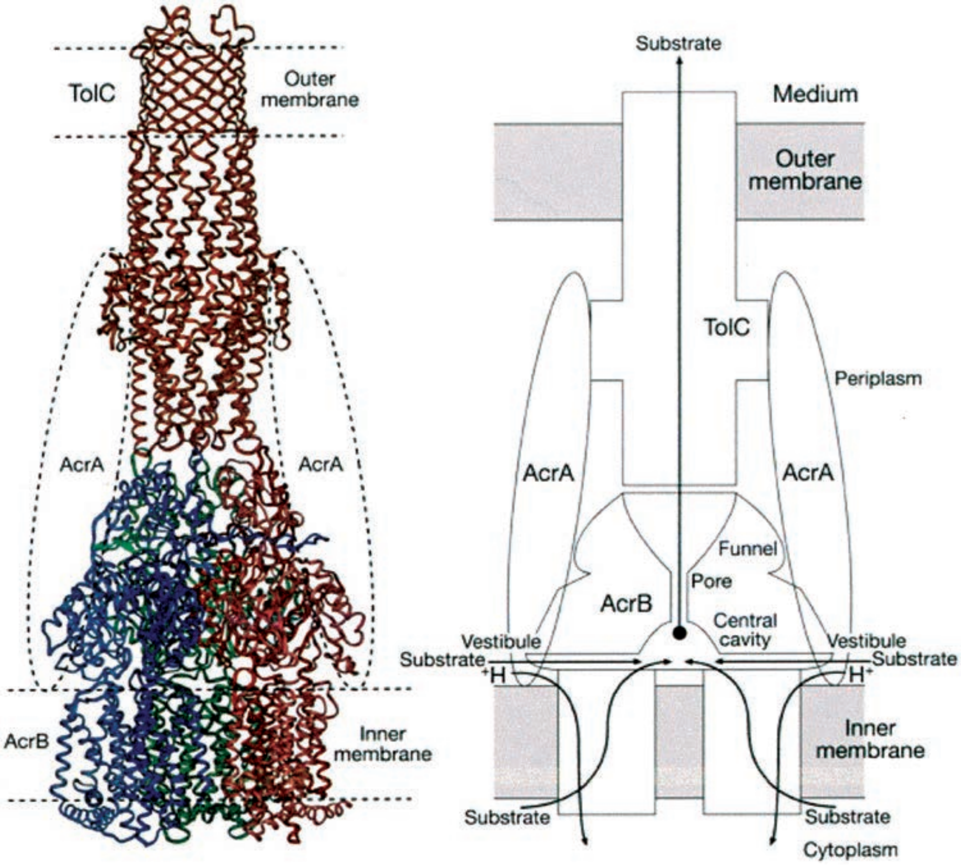

FIGURE 2 Representation of the crystal structure of the AcrAB-TolC three-component RND multidrug efflux pump. On the left are the three components of the pumps as they link the cytoplasmic (inner) membrane to the outer membrane. The periplasmic linker protein (AcrA) is shown only in outline to allow visualization of the linkage between AcrB and TolC. On the right, an outline of the pump shown at the left is presented, detailing the functional regions of the pump. Reprinted with permission from reference 53. doi:10.1128/9781555817381.ch69.f2

negatively charged bacterial cell. Next, in an energy-dependent process driven by the membrane-bound electron transport system present only in aerobic bacteria, a small fraction of the aminoglycosides that are attached to the bacterium are taken into the cell.

Once inside the cell, the principal target of aminoglycoside action is the 30S subunit of the ribosome. The molecular details of aminoglycoside binding to the ribosome are unique. Gentamicin attaches to the highly conserved tRNA acceptor A site of the *E. coli* 16S rRNA (55). This binding prevents the elongation of the growing peptide chain by causing misreading or premature termination of peptide synthesis. By interfering with translation of mRNA, protein production is altered, aberrant proteins are synthesized and inserted in the cell membrane, cell permeability is increased, more aminoglycosides are taken up into the cell, and cell death ensues.

Mistranslated proteins may also affect the bacterial cell. Paromomycin increases the error rate of protein translation by allowing the incorporation of the wrong tRNAs (the missense substitution rate of amino acids at specified positions in proteins is 1 in 3,000). Spectinomycin, a nonaminoglycoside aminocyclitol, inhibits translocation of the peptidyl-tRNA from the A site to the P site. Streptomycin also makes the ribosome error prone by allowing the binding of different tRNAs (56).

Resistance to aminoglycosides can occur by four mechanisms: (i) loss of cell permeability (decreased uptake), (ii) alterations in the ribosome that prevent binding, (iii) expulsion by efflux pumps, and (iv) enzymatic inactivation by AMEs.

Resistance due to Decreased Uptake and Altered Electrical Potential, Divalent Cations, and Efflux

It is well known that bacterial respiration generates an electrical potential across the membrane. Hence, anaerobes and facultative anaerobes (e.g., enterococci or certain small-colony variants of staphylococci) do not allow movement of aminoglycosides across their cell membranes. This intrinsic resistance confers low-level cross-resistance to all aminoglycosides.

Studies of *P. aeruginosa* and *E. coli* indicate that movement across the outer membrane may be due to a self-promoted uptake mechanism. The cationic aminoglycosides displace divalent cations (e.g., Mg^{2+}) that cross-bridge adjacent lipopolysaccharide (LPS) molecules, thereby permeabilizing the outer membrane and allowing entry of aminoglycosides (57). Consistently with this model, divalent cations have long been known to antagonize the activities of aminoglycosides against Gram-negative bacteria (58).

Recent data indicate that *P. aeruginosa* possesses an inducible RND-type pump (MexXY-OprM) that extrudes aminoglycosides (59, 60). In *E. coli*, aminoglycosides are captured from both periplasm and cytoplasm by the AcrD multidrug efflux transporter (61). Evaluating a multidrug-resistant (MDR) strain of *Acinetobacter baumannii*, BM4454, that was resistant to aminoglycosides, Manges et al. found an RND-type efflux pump in *A. baumannii* (62) and showed that it was under stringent control by a two-component regulatory and sensor system, *adeRS* (63).

Modification of the Ribosome

Bacterial cells have multiple copies of the rRNA genes. Mutations that affect aminoglycoside binding to the ribosome include alterations in ribosomal proteins and 16S rRNA and enzymatic methylation of the rRNA. Ribosomal mutations have been demonstrated to confer resistance to spectinomycin and streptomycin (64) and to other aminoglycosides as well (65). An emerging resistance problem is the 16S rRNA methylases found in Gram-negative rods. The first enzyme was found in *K. pneumoniae* BM4536 and designated *arm*A (aminoglycoside resistance methylase A) (66, 67). Subsequently, the enzymes named Rmt for rRNA methyltransferase were described. These currently include RmtA, RmtB, RmtC, RmtD, RmtE, and RmtF (68–70). These enzymes confer broad-spectrum resistance to the aminoglycosides, including new drugs like plazomicin, and they are increasingly identified on plasmids with broad-spectrum β-lactamases like CTX-M, NDM, and KPC (71, 72).

AMEs

As in the case with β-lactamases (see below), aminoglycoside inactivation by AMEs is the most important mechanism in terms of frequency and level of resistance (73). AMEs are believed to originate from actinomycetes that synthesize these antibiotics (*Streptomyces* and *Micromonospora* spp.). It is also possible that AMEs originated from enzymes involved in normal cellular respiration (housekeeping functions) (74). These enzymes are aminoglycoside phosphotransferases (APHs), nucleotidyltransferases or adenyltransferases (ANTs), and acetyltransferases (AACs). AMEs covalently modify specific amino or hydroxyl groups, resulting in aminoglycosides that bind poorly (with higher K_ms and less affinity) to the ribosome. AMEs can be passed from one bacterium to another by mobile genetic elements. These resistance determinants are frequently carried on specialized transposable genetic elements called integrons (75, 76).

Sites of AME modification of aminoglycosides are delineated by a standard numbering system. The streptidine or 2-deoxystreptamine nucleus forms the center for the numbering scheme (from 2-deoxystreptamine derive all aminoglycosides except streptomycin and spectinomycin). The first sugar moiety at the 4 position has positions numbered with a single prime (1′ to 6′); the second sugar moiety at the 6 position gets positions numbered with a double prime (1″ to 6″). Hence, AAC(6′) acetylates an amino group at the 6′ position (via an acetyltransferase) on the amino sugar attached to position 4 (Fig. 3). Each AME class consists of numerous enzymes that can modify different OH or NH$_2$ groups. These are divided into subclasses. In each subclass, there are different enzyme types that are designated by a roman numeral, e.g., AAC(3)-I. Isoenzymes are also described and are designated by a lowercase letter, a or b, etc. These isoenzymes are functionally identical and confer identical resistance phenotypes.

Currently, there are seven major phosphotransferases [APH(3′), APH(2″), APH(3″), APH(6), APH(9), APH(4), and APH(7″)], four nucleotidyltransferases [ANT(6), ANT(3″), ANT(4′), and ANT(2″)], and four acetyltransferases [AAC(2′), AAC(6), AAC(1), and AAC(3)]. A bifunctional AME able to acetylate and phosphorylate [AAC(6)-APH(2″)] also exists. This enzyme is found in *Staphylococcus*, *Streptococcus*, and *Enterococcus* and is responsible for high-level resistance to aminoglycosides (77). The gene *aac(6′)-aph(2″)*, which encodes the synthesis of this enzyme, is present in Tn*4001*-like transposons which are inserted in both plasmids and the chromosomes of aminoglycoside-resistant isolates. Most aminoglycoside-resistant health care-associated MRSA strains express this enzyme.

β-Lactam Resistance

PBP-Mediated Resistance

β-Lactam antibiotics (penicillins, cephalosporins, monobactams, and carbapenems) are the safest and mostly widely used class of antibiotics ever developed. They act by inhibiting the PBPs, the transpeptidases that manufacture peptidoglycan. The specific functions of different PBPs have been identified for some bacteria, but the precise ways in which they interact with each other and with cell wall precursors remain largely a mystery. Some are clearly essential for cell viability (generally the high-molecular-weight transpeptidases and their partner transglycosylases), while others appear to be dispensable, with no apparent deleterious effects on cellular structure or function resulting from their absence

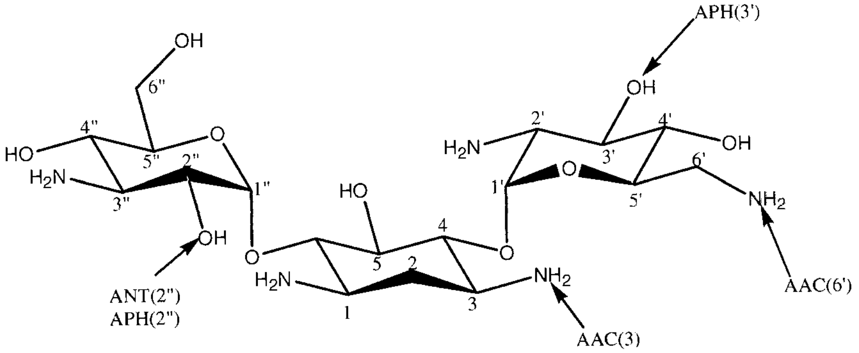

FIGURE 3 Sites of modification on kanamycin B by various AMEs. The arrows point to the sites of modification by the specific enzymes, namely, acetyltransferases, phosphotransferases, and nucleotidyltransferases. Reprinted with permission from reference 345.
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(most commonly the low-molecular-weight carboxypeptidases). It has long been suspected that some PBPs are redundant and can perform the functions of others. For example, *E. faecium* strains in which all of the PBPs except low-affinity PBP 5 are saturated grow normally, implying that PBP 5 can perform all of the penicillin-inhibitable functions required for cell wall synthesis (78). On the other hand, *E. faecium* strains in which *pbp5* has been deleted grow normally as well, implying that the other PBPs can provide all of these functions (21).

PBPs are all members of a larger family of serine peptidases that includes most of the β-lactamases. PBPs and β-lactamases interact with β-lactam molecules (themselves structural analogues of the peptidyl-D-alanyl–D-alanine termini of peptidoglycan precursors) by catalytically disrupting the β-lactam bond, resulting in a serine ester-linked acyl enzyme derivative (Fig. 1). In the case of β-lactamases, a water molecule then hydrolyzes the ester linkage of the acyl enzyme intermediate, releasing the irreversibly damaged penicilloyl (or cephalosporyl) moiety and regenerating the active enzyme. PBP–β-lactam acyl enzyme derivatives are in general less accommodating to nucleophilic attack by water molecules, resulting in a persistence of the covalent bond and inactivation of the PBP. The stability of this interaction allows identification of these proteins by binding to radiolabeled penicillin and is the genesis of their designation as PBPs. Because β-lactamases have no definable function in the cell other than to interact with β-lactam molecules, their affinity for the β-lactam and the rapidity with which the reaction proceeds determine their effectiveness and hence the level of resistance. Conversely, since PBPs have a very important alternative function (manufacture of the cell wall), the affinity of the interaction between PBPs and the β-lactam is a measure of distraction from their primary functions and thereby often defines the level of susceptibility of the strain.

Inhibition of PBPs interrupts cell wall synthesis, which by itself should inhibit cell growth rather than kill the cell. However, the interaction of β-lactam molecules with PBPs triggers the activity of cell wall-degrading molecules known as autolysins, which rupture the cell, leading to cell death (79). The extent to which these autolytic enzymes are activated correlates in most cases with the killing activity of a β-lactam against a particular bacterial strain.

Some bacterial species are intrinsically resistant to some β-lactam antibiotics by virtue of decreased PBP affinity. For example, enterococci are resistant to clinically achievable levels of cephalosporin antibiotics because of the presence of low-affinity PBP 5. Similar low affinity is demonstrated for the semisynthetic antistaphylococcal penicillins nafcillin and oxacillin, as well as for the antipseudomonal penicillins carbenicillin and ticarcillin. Enterococcal PBP 5 is bound with a diminished affinity by ampicillin and the ureidopenicillins mezlocillin and piperacillin, resulting in MICs that are higher than for streptococci but within the concentration range achievable in human serum.

PBP-mediated resistance for normally susceptible bacteria takes several forms, including (i) overproduction of a PBP, (ii) acquisition of a foreign PBP with low affinity, (iii) recombination of a susceptible PBP with more resistant varieties, and (iv) point mutations within PBPs that lower affinity for the β-lactam antibiotic. PBP-mediated resistance is predominantly found in Gram-positive bacteria. However, there are examples of Gram-negative bacteria with PBP-mediated resistance, and we point these out as they come up.

PBP Overexpression

Increased expression of a PBP as a mechanism of conferring resistance is relatively uncommon. Clear examples of settings in which increased quantities of a PBP are associated with resistance include the increased levels of methicillin resistance found in *S. aureus* strains overexpressing PBP 4 (80) and increased levels of penicillin resistance in *Enterococcus hirae* and *E. faecium* strains that overexpress PBP 5 (21, 81). The existence of this mechanism serves as a reminder that, as with β-lactamases, susceptibility or resistance depends on the number of β-lactam molecules relative to the number of targets. Increasing the number of target molecules can, under the correct circumstances, result in resistance. Conversely, imipenem's effectiveness against *E. coli* has been partially attributed to the fact that its primary target is PBP 2, which is present in roughly 200 copies, in contrast to PBP 3, estimated at 2,000 copies. In any case, overproduction of PBPs is a rare mechanism of resistance to β-lactam antibiotics.

Acquisition of Foreign PBPs

Acquisition of a foreign PBP as a mechanism of resistance is best exemplified by the expression of methicillin resistance in *S. aureus* strains by virtue of the expression of PBP 2a, a low-affinity PBP not native to *S. aureus* and encoded by *mecA* (82). Isolates with the *mecA* gene are resistant to all β-lactams except for the new cephalosporins (e.g., ceftaroline and ceftobiprole) that can bind to PBP 2a (83). PBP 2a is classified as a class B PBP, meaning that it has a functional C-terminal transpeptidase domain but an N-terminal domain whose function has yet to be determined. This contrasts with class A PBPs, which have N termini with glycosyltransferase activities and C termini with transpeptidase activities. As a result, the transglycosylase domain of *S. aureus* PBP 2 is required for expression of β-lactam resistance by PBP 2a (84). The cooperation of specific class A PBPs with low-affinity class B PBPs has also been shown for *E. faecalis* and, more recently, for *E. faecium* (85, 86).

The origin of the *mecA* gene is unknown. A *mecA* homologue has been identified in *Staphylococcus sciuri*, a primitive staphylococcal species associated with rodents and primitive mammals (87). The deduced amino acid sequences of the two enzymes exhibited 88% similarity across the entire protein and 91% identity within the transpeptidase domain. *S. sciuri* strains are not methicillin resistant, however, which may be due to the lack of an effective promoter upstream of the gene. *S. sciuri mecA* homologues in which the promoter has spontaneously mutated express higher levels of methicillin resistance both in *S. sciuri* and when cloned on a high-copy-number vector in *S. aureus* (88). Expression of methicillin resistance in *S. aureus* is commonly under regulatory control, either by the product of the upstream *mecI* gene or in *trans* by the homologous *blaI* gene, which regulates the expression of β-lactamase (the promoter regions of *mecA* and the *blaZ* β-lactamase genes are similar) (89). The *mecI*/*blaI* repressors are, in turn, controlled by the *mecR1*/*blaR1* sensors/transducers, although the precise mechanism of this interaction is incompletely understood. The efficiency of induction varies with the mechanism (*blaR1-blaI*-mediated induction is faster), leading to complications in detecting the methicillin resistance phenotype. In fact, only a small minority of a resistant population may express high levels of resistance. Several techniques are used in the laboratory to “bring out” the resistance (prolonged incubation, increased salt in the media, and cefoxitin susceptibility testing), and more recently, techniques have been developed to bypass phenotypic expression in favor of directly identifying the *mec*A gene or directly detecting PBP 2a.

Expression of PBP 2a-mediated resistance to β-lactams in *S. aureus* is also influenced by the expression of other genetic loci called the *fem* (factors essential for methicillin resistance) or *aux* (auxiliary) genes (82). The *fem* and *aux* genes were first identified by transposon mutagenesis of MRSA strains to search for insertions that would reduce the expression of resistance. Many *fem* and *aux* factors have now been identified, and all are involved in the formation of the staphylococcal cell wall (82). These data indicate that minor perturbations of the normal processes prevent PBP 2a from functioning, suggesting that it is a very particular enzyme. However, when cloned into a heterologous (*E. faecalis*) background, PBP 2a was found to be capable of synthesizing peptidoglycan polymers using precursors distinctly different from those found in *S. aureus* (90).

The *mec*A gene is located within a larger (ca. 21- to 67-kb) region of the chromosome known as the staphylococcal chromosomal cassette (SCC*mec*) region (91). SCC*mec* is a mobile element, with mobility conferred by the presence of the *ccrA*/*ccrB* or *ccr*C gene. The basic elements of SCC*mec* are the *mecR1*/*mecI*/*pbp2a* region and *ccrA*. In recent community-acquired *S. aureus* isolates, little else is included in the SCC*mec* complexes (types IV to VII) (92). Hence, the SCC*mec* region in these isolates is relatively small (20 to 30 kb), and the isolates themselves tend to be resistant only to β-lactams. As of this writing, 11 different *mec* regions (SCC*mec* I to XI) have been described (92–94). Nosocomial isolates have larger SCC*mec* regions, owing to the accumulation over time of integrated plasmids or transposons that contribute to the multiresistance of these isolates (91). Although the SCC*mec* element has never been shown to be transferable *in vitro*, compelling data that the type IV SCC*mec* element has been recently acquired have emerged from the study of clinical isolates (95). Transfer of the *mec* region between staphylococcal strains has never been conclusively documented. The spread of MRSA within institutions is therefore largely due to the transmission of resistant organisms from patient to patient, probably on the hands of transiently colonized health care workers (5). Single strains have spread through entire hospitals and even cities (4). *mec*A stabilities differ among different *S. aureus* clones (96), suggesting one potential explanation for the limited lineages within which the resistance determinant has been found. In 2011, human and animal MRSA isolates from Europe with a divergent *mec*A homologue termed *mec*C were first reported (97). Isolates harboring *mec*C were detected by susceptibility testing but not by commercial assays targeting *mec*A or PBP 2a.

Resistance Mutations by Recombination with Foreign DNA

Resistance through recombination between native, susceptible PBPs and those of less susceptible species is largely restricted to species that are capable of natural transformation or of taking up naked DNA from the environment. Prominent among these species are *S. pneumoniae*, viridans group streptococci, *Neisseria gonorrhoeae*, and *Neisseria meningitidis* (24). *S. pneumoniae* contains six PBPs (1a, 1b, 2a, 2b, 2x, and 3), all of which are subject to recombination, with foreign PBPs taken up by transformation. In most cases, resistant *pbp* genes demonstrate mosaic patterns (individual segments of foreign *pbp* genes integrated with the native *pbp* gene) with the foreign DNA in the less penicillin-susceptible viridans group streptococci (24). In fact, genetic exchange appears common between these closely related species, with mosaic patterns demonstrable even in PBPs from susceptible *Streptococcus mitis* strains (98). Penicillin resistance in *S. pneumoniae* can be established by alterations in PBPs 2x, 2b, and 1a, whereas only alterations in PBPs 2x and 1a are required for cephalosporin resistance (98). Penicillin resistance has been described to occur in *S. pneumoniae* for some time, with cephalosporin resistance emerging more recently, most commonly in strains already resistant to penicillin. However, strains resistant to cephalosporins but susceptible to penicillin have also been reported (99). High-level resistance (≥2 μg/ml) usually implies modification of more than one PBP, sometimes with several mosaic regions in each one (98).

As with PBP 2a-mediated resistance to methicillin in *S. aureus*, expression of penicillin and cephalosporin resistance in *S. pneumoniae* is dependent on the proper functioning of auxiliary genes. The *fib* locus of *S. pneumoniae* (*fib*A and *fib*B) is analogous to *femA* and *femB* in *S. aureus*. It is involved in the formation of interpeptide bridges, and inactivation of the *fib* locus is associated with reduction of cross-linked muropeptides and loss of penicillin resistance even in the presence of low-affinity mosaic PBPs (100). The *murM*-*murN* operon encodes enzymes involved in the biosynthesis of branched structured cell wall muropeptides commonly found in penicillin-resistant pneumococci (101). Inactivation of *murM*-*murN* results in loss of branched-chain muropeptides as well as loss of penicillin resistance.

Among Gram-negative species, *Neisseria* organisms are well known to be naturally transformable. It is therefore not entirely surprising that strains of both *Neisseria gonorrhoeae* and *Neisseria meningitidis* in which mosaic PBP genes are associated with decreased susceptibility to penicillin have been described (24). Similarly to the *S. pneumoniae* picture, the resistant portions of the PBP genes have been acquired from closely related commensal *Neisseria* species that are more resistant to penicillin (102). While penicillin resistance in *N. meningitidis* remains, thankfully, extremely rare, both β-lactamase-mediated resistance and PBP-mediated resistance to β-lactams in *N. gonorrhoeae* are quite common.

Point Mutations

The final mechanism of PBP-mediated β-lactam resistance results from point mutations within the *pbp* genes that result in lower affinity for the β-lactam in question. This form of mutational resistance is seen most commonly in PBP 5 of *E. faecium* strains, raising penicillin MICs from 4 to 16 μg/ml to as high as >1,000 μg/ml. High-level penicillin-resistant strains now represent the majority of clinical *E. faecium* isolates in the United States (103). These mutations further reduce the affinity for cephalosporins and other β-lactams, with lower affinity for the nonmutated version, leading to an increase in resistance that may have implications for the likelihood that antibiotic use will promote colonization with MDR *E. faecium* (104). Most of the mutations occur in the vicinity of one or more of several conserved boxes important for β-lactam binding (105). A systematic study of several common mutations of PBP 5 has revealed that resistance increases with increasing number of mutations, with some loci conferring higher levels of resistance than others (106).

Reduced penicillin susceptibility in group B streptococci (GBS) is uncommon and usually associated with point mutations in PBP 2x (107, 108). Mutations in PBP 1a and PBP 2a have also been detected in a non-penicillin-susceptible GBS isolate (109). In rare instances, point mutations of the *S. aureus pbp2* gene have been associated with methicillin resistance, but the importance of this mechanism pales in comparison to that of expression of PBP 2a (110). Alterations in PBP 2a of MRSA isolates have been associated with increased ceftaroline MICs (111).

Among Gram-negative species, point mutations of the *ftsI* gene of *Haemophilus influenzae* (encoding the transpeptidase domain of PBP 3a and/or PBP 3b) have been associated with non-β-lactamase-mediated resistance to ampicillin and cephalosporins in this species (112). Although mutations have also been noted within PBP 4 in this species, it is not clear that these have a significant impact on β-lactam susceptibility.

β-Lactamase-Mediated Resistance

Classification of β-Lactamases

Two major schemes are currently used to classify β-lactamases: the Ambler (AMB) classification system and the Bush-Jacoby classification system (113). The Ambler classification separates β-lactamases into four distinct classes (A to D) based on similarities in amino acid sequence. Classes A, C, and D are serine β-lactamases, whereas class B enzymes are metallo-β-lactamases that require one or two zinc atoms for activity (see above). The Bush-Jacoby classification system (formerly referred to as the Bush-Medeiros-Jacoby scheme [114–116]) classifies β-lactamases according to functional similarities (substrate and inhibitor profiles). There are four categories (groups) and multiple subgroups in the updated Bush-Jacoby system (groups 1, 2 [2a, 2b, 2br, 2d, 2be, 2c, and 2f], etc.) (115). A comparison of the two classification systems is summarized in Table 2. Both classification systems are referred to in this chapter. More than 1,000 β-lactamases have been reported (http://www.lahey.org/studies). The most recent increases are mostly the result of diversification within well-established β-lactamases. The largest increases in numbers occurred in the class A and class D families. To illustrate, there are currently more than 217 TEM β-lactamases, 183 SHV β-lactamases, more than 400 OXA enzymes, 152 CTX-M ESBLs, and 120 CMY β-lactamases. Most worrisome is the increase in the number of carbapenemases. Numbers of IMP, VIM, and NDM metallo-β-lactamases have increased to 48, 41, and 10, respectively. There are now 18 variants of KPC. A complete listing can be found at http://www.lahey.org/studies.

β-Lactamase Mechanism

β-Lactamases are members of a superfamily of active-site serine proteases or D,D-peptidases (117). The mechanism of hydrolysis of β-lactams by β-lactamases is best studied for TEM-1. TEM-1 β-lactamase disrupts the amide bond of a β-lactam in a two-step reaction. First, the negatively charged carboxylate group of the β-lactam antibiotic is attracted to the active site by the enzyme's positively charged residues. There, the β-lactam is properly positioned, making key hydrogen bonding interactions with the enzyme (118). The residues in the active site that facilitate this attraction in the serine β-lactamases are often called the oxyanion hole or electrophilic center. Next, the β-lactam is acylated (Fig. 1). A conserved serine, Ser70, in the active site of TEM-1 serves as the reactive nucleophile in this acylation reaction. Recent ultrahigh-resolution X-ray crystallography studies of TEM-1 (0.85 Å) indicate that Glu166, acting through the catalytic water, activates Ser70 for nucleophilic attack of the β-lactam ring. Then, a strategically positioned water molecule is activated by a general base (e.g., again Glu166 and the same water molecule). This water molecule deacylates the β-lactam and regenerates the active β-lactamase. This symmetric mechanism is also supported by the ultrahigh-resolution structure (0.90 Å) of another common class A β-lactamase, SHV-2, found in *K. pneumoniae* (119). There is general consensus on the mechanics of deacylation by the class A TEM-1 β-lactamase (attention to Glu166), but the details of acylation still remain contentious. Debate centers on which residue in the active site (Lys73 or Glu166) deprotonates the reactive Ser70 or whether either pathway is in competition with the other (120).

β-Lactamase Processing

Generally speaking, β-lactamases are secreted into the periplasmic space in Gram-negative bacteria or into the surrounding medium by Gram-positive organisms. Membrane-associated enzymes have rarely been reported (*Bacillus licheniformis*, *Bacillus cereus*, and *Bacteroides vulgatus*). β-Lactamases are synthesized as precursor proteins. As in other bacteria, the export of proteins into the periplasmic space is mediated by an amino-terminal signal peptide. After transport, the signal peptide of the β-lactamase is cleaved by a processing enzyme, signal peptidase I.

TABLE 2 β-Lactamase classification

Bush-Jacoby system classification	Major subgroup	Ambler system classification	Main attributes
Group 1 cephalosporinases		C (cephalosporinases)	Usually chromosomal; resistance to all β-lactams except carbapenems; not inhibited by clavulanate
Group 2 penicillinases (clavulanic acid susceptible)	2a	A (serine β-lactamases)	Staphylococcal penicillinases
	2b	A	Broad spectrum; TEM-1, TEM-2, and SHV-1
	2be	A	Extended spectrum; TEM and SHV variants, predominantly
	2br	A	IRT β-lactamases
	2c	A	Carbenicillin hydrolyzing
	2e	A	Cephalosporinases inhibited by clavulanate
	2f	A	Carbapenemases inhibited by clavulanate
	2d	D (oxacillin hydrolyzing)	Oxacillin-hydrolyzing (OXA)
Group 3 metallo-β-lactamase	3a	B (metalloenzymes)	Zinc-dependent carbapenemases
	3b	B	
	3c	B	
Group 4		Not classified	Miscellaneous enzymes, most not yet sequenced

Genetic Environment of β-Lactamases

β-Lactamases can be chromosome-, plasmid-, or transposon-encoded enzymes that are produced in a constitutive or inducible manner. An increasing number of β-lactamases that are encoded on integrons have been found.

Integrons are genetic elements of variable length that contain a 5′ conserved integrase gene (*int*), mobile antibiotic resistance genes (called cassettes), and an integration site for the gene cassette, *attI* (*att*, attachment). To date, five distinct integron classes have been found to be associated with cassettes that contain antibiotic resistance genes. Three main classes of integrons (classes 1 to 3) have been described for Gram-negative bacteria. Integrons capture antibiotic resistance gene cassettes by using a site-specific recombination mechanism. In the class 1 integrons, the 3′ conserved segment includes three open reading frames, *qacE*Δ*1*, a deletion derivative of the antiseptic resistance gene *qacE*, and *sul1*, a sulfonamide resistance gene. As integrons carry multiple resistance determinants and can be readily mobilized, their impact on antibiotic resistance is significant. In the words of Hall and Collis, "integrons thus act both as natural cloning systems and as expression vectors" (121). The capture and spread of antibiotic resistance determinants by integrons underlie the rapid evolution of multiple-antibiotic resistance among diverse Gram-negative clinical isolates (122). As reviewed by Stokes and Hall (123), integrons were originally found on mobile elements from pathogenic bacteria and were found to be a major reservoir of antibiotic resistance genes. Analysis of their gene content suggests that integrons are phylogenetically diverse and have been with bacteria for a long time. Interestingly, integrons have been found in approximately 9% of sequenced bacterial genomes. It is maintained that the integron/gene cassette system has a function in evolution beyond just conferring resistance to antibiotics. Consequently, integrons may be the agents of change that drive bacterial evolution and adaptation. It should be noted that gene cassettes are mobile and can also exist in free circular form. However, these cassettes do not include all functions required for their mobility. Cassettes are formally part of the integron only when they are integrated at the integron receptor site. The genes within the cassette do not have promoters.

Integrons are an important source for the spread of *bla* genes. Integrons containing β-lactamases have been found in *A. baumannii*, *P. aeruginosa*, and other species of Gram-negative bacteria encompassing Ambler class A, B, and D β-lactamases (124). The β-lactamase enzymes/families found on integrons are VEB-1, VEB-2, GES-1, GES-2, IBI-1, IBI-1, CTX-M-2, CTX-M-9, PSE-1, and numerous OXA β-lactamases (125–127). OXA and metallo-β-lactamases that confer resistance to carbapenems (IMP-1 to IMP-4, IMP-6 to IMP-8, IMP-12, VIM-1, VIM-2, and GIM-1) are also included in integron-encoded β-lactamases (124, 128).

Class A β-Lactamases (Bush Group 2b Penicillinases)

Class A β-lactamases possess four important structural motifs that create a complex hydrogen-bonding network to fix the β-lactam in the substrate-binding pocket. Residues Ser70-Xaa-Xaa-Lys73 (where Xaa designates 100% random amino acid substitutions), Ser130-Asp131-Asn132 (SDN loop), and Lys/Arg234-Thr/Ser235-Gly236 define the conserved residues critical for β-lactam binding and hydrolysis (117). The Ω loop (amino acids Arg164 to Asn179) is unique in class A β-lactamases. A highly conserved Glu166 that functions as a general base (electron donor) in the catalytic process is located in the Ω loop (see above). The salt bridge formed between Arg164 and Asn179 defines the limits, or "neck," of the Ω loop.

The two commonly encountered class A β-lactamases found in *Enterobacteriaceae* are designated TEM-1 and SHV-1. TEM-1 and SHV-1 β-lactamases are primarily penicillinases with diminished activity against cephalosporin substrates. These two families of β-lactamases have received considerable attention over the past 3 decades since they are the progenitors of the ESBLs and IRT β-lactamases, now common in many hospitals.

Bush Group 2be

ESBLs are generally class A β-lactamases that have "expanded" or changed their substrate profile as a result of amino acid substitutions. Normally, extended-spectrum cephalosporins are very poor substrates for hydrolysis by Bush group 2be enzymes (high K_m and low k_{cat}). Extended-spectrum cephalosporins are extremely potent β-lactams. Mutations at critical amino acids expand the spectra of these enzymes and allow the hydrolysis of extended-spectrum cephalosporins (128). In most cases, ESBL mutations render the enzymes more susceptible to inhibition by mechanism-based inactivators (clavulanic acid, sulbactam, and tazobactam). An explanation for this increased susceptibility can be found by studying reactions of β-lactamases with β-lactamase inhibitors using Raman spectroscopy and stopped-flow kinetics. Increased amounts of certain key intermediates of SHV-2 and SHV-5 are formed (i.e., the enamine intermediate). As a result, the K_i values of the mechanism-based inhibitors are reduced (increased susceptibility to inhibitors) up to 50-fold in SHV-2 and SHV-5 enzymes. The impact of enteric Gram-negative rods possessing ESBLs on the choice of empirical and definitive antimicrobial therapy has been substantial (129–133).

Among the TEM family enzymes, five amino acid residues appear to be most important for conferring the ESBL phenotype: Gly238 and Ala237 (located on the b3 β-pleated sheet), Arg164 and Asp179 (located on the neck of the Ω loop), and Asp104 (located directly across from G238 and A237 at the opening of the active-site cavity) (118, 134). Of note, the replacement of Gly with Ser, Ala, or Asp at ABL position 238 is a common mutation in both TEM and SHV ESBLs (http://www.lahey.org/studies/).

CTX-M ESBLs

CTX-M β-lactamases were first reported in the 1980s, but isolates with these enzymes were less common than isolates with TEM or SHV enzymes (135). However, CTX-M ESBLs have demonstrated a global dissemination, and now these are the most common ESBLs. These enzymes can be divided into distinct clusters (see http://www.lahey.org/studies/). Unlike most (but not all) TEM- and SHV-derived ESBLs, CTX-M β-lactamases hydrolyze cefotaxime and ceftriaxone better than they do ceftazidime. CTX-M enzymes are readily inhibited by tazobactam and clavulanic acid. They are also inhibited by the new β-lactamase inhibitor avibactam (136).

CTX-M β-lactamases are commonly found in *K. pneumoniae* and *E. coli* but can also be found in several other species of *Enterobacteriaceae*, including typhoidal and nontyphoidal *Salmonella*, *Shigella* spp., *Citrobacter freundii*, *Enterobacter* spp., and *Serratia marcescens* (137–139). A comprehensive review delineates the lineage of many CTX-M enzymes resulting from *Kluyvera* spp. (135). Of note, different genetic elements are involved in the mobilization of *bla*$_{\text{CTX-M}}$ genes. In addition, CTX-M enzymes have spread via some successful *E. coli* lineages, such as ST131.

Many clinically important ESBLs other than TEM, SHV, and CTX-M (K1, GES, PER, VEB, BES, IBI, and OXA-type) have been described (140–142).

Structural Biology of ESBLs

Important insights have emerged from the study of a number of atomic structures of class A ESBLs. The common theme that emerges is that the active site is selectively remodeled and expanded to accommodate the bulky R1 side chain of extended-spectrum cephalosporins. Although the details of this modification are different for many of the ESBLs, the remodeling comes at a price. Many of these ESBLs are not as catalytically efficient (e.g., reduced k_{cat}/K_m) as the wild-type progenitors against certain substrates. With the expanded substrate spectrum, one uniformly observes decreases in penicillin MICs and in k_{cat}/K_m ratios. In addition, these enzymes are less stable to proteolysis and heat. The structures of Toho-1, TEM-52, TEM-64, the Gly238Ala ESBL in TEM, SHV-2, and K1 β-lactamases all reveal insights into why expanded-spectrum cephalosporins fit in the active site (119, 143–149).

Serine Carbapenemases of Bush Group 2f or Class A type

In the past, β-lactamases able to hydrolyze carbapenems were rare. It is regrettable that this is no longer the case. Representatives of these carbapenemases are SME, NMC, IMI, GES, and KPC (125, 148, 150). *bla*$_{\text{NMC-A}}$ and *bla*$_{\text{IMI-1}}$ are chromosomally located genes in *Enterobacter cloacae* and are induced by cefoxitin and imipenem (151, 152). *bla*$_{\text{NMC-A}}$ is regulated by a LysR-type regulatory protein (153). It is felt that *bla*$_{\text{IMI-1}}$ is also regulated in the same manner (152). *bla*$_{\text{SME}}$ is a chromosomally located gene encoding serine carbapenemase of class A in *E. cloacae* and *Serratia marcescens*.

A notable increase in bacteria expressing class A carbapenemases, specifically KPC, has occurred in the United States. First found in *K. pneumoniae*, these β-lactamases have been detected in nearly all species of *Enterobacteriaceae* and in non-*Enterobacteriaceae*, such as *Acinetobacter* spp. and *Pseudomonas aeruginosa* (154), among others. The gene coding KPC is found on plasmids, some of which are conjugative. In addition, the gene has disseminated by transmission of successful bacterial lineages, such as *K. pneumoniae* ST258 (155). Bacteria bearing KPC enzymes are now found in nearly all parts of the world, including North and South America, the Caribbean, Europe, Israel, and Asia. These isolates are highly resistant to penicillins, cephalosporins, and commercially available β-lactam–β-lactamase inhibitor combinations and carbapenems. The new β-lactamase inhibitor avibactam is active against KPCs, suggesting that β-lactam drugs combined with avibactam may be therapeutic options for treating infections caused by KPC-producing bacteria (136).

Detection of KPC β-lactamases has been a problem for clinical laboratories, but by using the revised CLSI carbapenem breakpoints for *Enterobacteriaceae* (i.e., the breakpoints recommended in 2010 and after) and by implementing newly available tests for carbapenemase production, the detection of this important resistance mechanism is becoming easier. Using the imipenem, meropenem, and doripenem susceptibility breakpoint of ≤1 μg/ml and ertapenem breakpoint of ≤0.5 μg/ml, the vast majority of carbapenemase-producing isolates will test nonsusceptible to the carbapenems (154). Both the CLSI and the European breakpoint-setting organization, EUCAST, recommend that susceptibility data alone be used for making breakpoint decisions and that tests for resistance mechanisms, like carbapenemases, be used for infection control and epidemiological purposes (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/Breakpoint_table_v_4.0.pdf) (137).

Tests for carbapenemase production are improving. Initially, tests with β-lactamase inhibitors, such as clavulanic acid or boronic acid, were used, but these tests can result in inaccurate results if other enzymes are present (156). The CLSI recommended the modified Hodge test (MHT). In this test, a lawn of susceptible *E. coli* cells (e.g., strain ATCC 25922), a carbapenem disk (ertapenem or meropenem), and a streak of the suspected KPC producer are placed upon an agar plate and incubated overnight. Growth up to the disk is checked the next day and, if present, suggests carbapenemase production. This test suffers from false-positive results and false-negative results for carbapenemases other than KPC, specifically NDM (157). More recently, the Carba NP test was described (158). This is a colorimetric, rapid test for carbapenemase production which can be employed in multiple laboratory settings, but it can fail to detect OXA-48 carbapenemases (see chapter 73). Several molecular assays have been developed to detect multiple carbapenemase-resistant determinants (see chapter 77 for more information).

The crystal structure of only KPC has been determined. KPC has an overall structure similar to that of other class A enzymes, and interestingly, this β-lactamase has only 50% protein sequence conservation compared to CTX-M-1, 39% compared to SHV-1, and 35% compared to TEM-1 (159). The KPC β-lactamase possesses a large and shallow active site, allowing it to accommodate "bulkier" β-lactams. As a result of these structural changes, KPC is regarded as a versatile β-lactamase (160).

Among these serine carbapenemases exists a notable curiosity. *bla*$_{\text{GES-2}}$ is a plasmid-borne β-lactamase gene found in *P. aeruginosa* (161). *bla*$_{\text{GES-2}}$ is a point mutation mutant of *bla*$_{\text{GES-1}}$, which encodes a clavulanic acid-inhibited ESBL (a non-TEM, non-SHV ESBL). It is curious that GES-1 is an ESBL and that a single point mutation (Gly→Asp at position 170 in the Ω loop) can add an imipenemase activity to GES-2 (162). The OXA carbapenemases are discussed below.

Inhibitor-Resistant Class A β-Lactamase: Bush Group 2br

Amino acid mutations within TEM and SHV that confer resistance to inhibition by β-lactamase inhibitors have also been characterized at the following amino acid positions: Met69, Ser130, Arg244, Arg275, and Asn276 (163–171). Most inhibitor-resistant β-lactamases are variants of the TEM-1 enzyme; there were only two descriptions of clinical isolates expressing SHV β-lactamases that are resistant to inhibitors at the time of this writing (SHV-10 and SHV-49) (166, 172). It is possible that the frequency of inhibitor-resistant β-lactamases in TEM and SHV family enzymes is underestimated, since many laboratories in the United States do not identify these strains routinely (173). Cephalosporins or high-dose piperacillin-tazobactam may be effective for the treatment of *E. coli* expressing some inhibitor-resistant β-lactamases.

Mutants of TEM β-lactamases that maintain the ESBL phenotype but also demonstrate inhibitor resistance are being recovered. These are referred to as CMTs. There are several reports of CMTs (174–177). It is still unknown how these enzymes will affect empirical treatment. At this time, clinical microbiology laboratories do not have the resources to detect these complex phenotypes.

Class B β-Lactamases (Bush Group 3 Enzymes)

In contrast to the serine-dependent β-lactamases (classes A, C, and D), class B β-lactamases are metalloenzymes. These enzymes contain an αββα motif with a central β sandwich and two α helices on each side. Consequently, class B β-lactamases require zinc or another heavy metal for catalysis, and their activities are inhibited by chelating agents (EDTA). This zinc atom is held in place by three histidines and a water molecule. Some metallo-β-lactamases contain a second Zn binding site. These two sites function separately, with the primary Zn binding site assisted by the secondary site. A coordinated water molecule also plays a critical role in catalysis (178).

With few exceptions (see below), class B β-lactamases confer resistance to a wide range of β-lactam compounds, including cephamycins and carbapenems. The class B β-lactamases are resistant to inactivation by clavulanate, sulbactam, tazobactam, and avibactam. Many of these enzymes poorly hydrolyze aztreonam (136, 178).

Class B β-lactamases can be grouped into three different subclasses (B1, B2, and B3) depending on their requirements for zinc. B1 enzymes (IMP-1, VIM-2, and CcrA) are fully active with one or two zinc ions, B3 enzymes (e.g., L1) require two zinc ions, and B2 enzymes (e.g., CphA) are inhibited by the addition of a second zinc ion. Amino acid positions can be assigned according to a specific numbering system, designated BBL, that is based upon structural alignment (179). Although the genes encoding metallo-β-lactamases show very little primary-structure sequence identity (17 to 37%), the three-dimensional structures of the known metallo-β-lactamases appear to be similar (180–183).

Because of the metal ion, the catalytic pathway of metallo-β-lactamases does not involve an acyl enzyme intermediate as it does in classes A and C. The catalytic pathway in class B also incorporates a hydrolytic water molecule (the "bridging" water molecule) that possesses enhanced nucleophilicity due to the proximity to the metal ion. The zinc ions coordinate two water molecules. The addition of the hydroxide to the carbonyl carbon of the β-lactam leads to the formation of a transient, noncovalent reaction intermediate. The mechanistic details of this pathway have been summarized by Crowder et al. (184) and are beyond the scope of this chapter.

The majority of metallo-β-lactamases are chromosomally encoded, and their expression may be constitutive or inducible. The metallo-β-lactamases of *B. cereus*, *Stenotrophomonas maltophilia*, *Aeromonas hydrophila*, and *Aeromonas jandaei* are inducible. In *A. jandaei*, regulation of the metallo-β-lactamase appears to involve two-component signal transduction systems (185).

The metallo-β-lactamases of the VIM and IMP types are now established as important threats to our antimicrobial armamentarium. These metallo-β-lactamases are broad-spectrum enzymes and are active against most β-lactams, including carbapenems, and have been found in various Gram-negative clinical isolates mostly in the Far East and the Mediterranean regions. *bla*$_{\mathrm{VIM}}$ is an integron-borne metallo-β-lactamase that is usually found in *P. aeruginosa* isolates. Unfortunately, the VIM metallo-β-lactamase has spread to species of *Enterobacteriaceae* and also *Acinetobacter* spp. (186). *bla*$_{\mathrm{VIM}}$ has now spread to more than 20 countries (187). The majority of metallo-β-lactamases are mobilized on integrons, transposons, and mobile common regions (178). Similarly, IMP metallo-β-lactamases are very widespread. IMP metallo-β-lactamases have been found as part of integrons in the following bacteria: *P. aeruginosa*, *Pseudomonas putida*, *Serratia marcescens*, *Pseudomonas stutzeri*, *Acinetobacter baumannii*, *Pseudomonas fluorescens*, *K. pneumoniae*, *Klebsiella oxytoca*, *Enterobacter aerogenes*, *Achromobacter xylosoxidans*, and *Escherichia coli* (178).

A novel class B carbapenemase has raised significant concern because of its widespread dissemination in a relatively short period of time (188). NDM (New Delhi metallo-β-lactamase) is a class B β-lactamase encoded by a very mobile genetic element, and the pattern of spread is proving to be more complex and apparently more unpredictable than that of the gene encoding KPC. Moreover, the number of patients possessing bacteria containing *bla*$_{\mathrm{NDM}}$ is growing. The gene has moved from India and Pakistan to the United Kingdom, the United States, Kenya, Japan, Canada, Belgium, The Netherlands, Taiwan, Oman, and Australia. *bla*$_{\mathrm{NDM}}$ has been found on plasmids of different sizes and has been located near a pathogenicity island. The resistance determinants flanking this *bla* gene seem to be numerous, including chromosomal β-lactamases (*bla*$_{\mathrm{CMY}}$ and *bla*$_{\mathrm{DHA}}$) and chloramphenicol and aminoglycoside resistance genes. The global impact of this gene and its spread must be carefully monitored.

As stated above, the atomic structures of a number of class B β-lactamases have been solved (179–181, 189). The structures are being used to design novel inhibitors of class B β-lactamases.

Class C β-Lactamases

Ambler class C (Bush-Jacoby-Medeiros group 1) chromosomal β-lactamases are produced to a greater or lesser degree by almost all Gram-negative bacteria (*Salmonella*, *Klebsiella*, *Proteus mirabilis*, *Proteus vulgaris*, and *Stenotrophomonas maltophilia* being the major exceptions) (190). Chromosomally encoded (and inducible) enzymes are particularly important in clinical isolates of *C. freundii*, *E. aerogenes*, *E. cloacae*, *Morganella morganii*, *P. aeruginosa*, and *S. marcescens*. Although class C β-lactamases hydrolyze cephalosporins (including extended-spectrum cephalosporins) more effectively than they do penicillins, it should be kept in mind that these enzymes have a great efficiency for hydrolysis of penicillins (the K_m is very low). Most class C enzymes are resistant to inhibition by clavulanate, sulbactam, and tazobactam (in the case of tazobactam, the resistance to inhibition is generally less), but avibactam, a new inhibitor, demonstrates activity for class C enzymes (136).

Class C β-lactamases have larger active-site cavities than do class A enzymes, which may allow them to bind the bulky extended-spectrum cephalosporins (oxyimino-β-lactams) (191, 192). It is claimed that this conformational expansion and flexibility facilitate hydrolysis of oxyimino-β-lactams by making the acyl enzyme intermediate more open to attack by water (191–193).

The important structural elements described for class A enzymes are also present in class C β-lactamases. The active-site serine (Ser64) is located near the N terminus of a long helix and is followed on the next helix turn by a lysine (Ser64-Xaa-Xaa-Lys67). The second element contains a Tyr-Xaa-Asn (Tyr150) or Ser-Xaa-Asn pattern corresponding to the Ser130-Asp131-Asn132 loop of class A β-lactamases. The opposite side of the active site is marked by Lys315/Arg/His-Thr/Ser-Gly, corresponding to the KTG motif of class A enzymes.

Class C cephalosporinases acylate β-lactams in the same manner as class A enzymes do. In many ways, the reaction mechanism of class C β-lactamases remains enigmatic (194, 195). First, the catalytic Ser64 attacks the β-lactam carbonyl carbon and the acyl enzyme forms. The approaches of the activated water molecule are different between AmpC and class A enzymes (class C from the β-face). This implies that the deacylation mechanism is distinct. Unlike with class A enzymes, the ring amine of the acyl enzyme of class C facilitates deacylation. This "substrate-assisted catalysis" generally distinguishes class C from class A (although there are reports of substrate-assisted catalysis occurring in class A TEM enzymes as well) (196). In an examination of the catalytic mechanism, a "Glu166 equivalent" in class C β-lactamases is not readily apparent; this role may be filled by the tyrosine (Tyr150) in the Tyr-Xaa-Asn motif. The current thinking is that the conserved residue, Tyr150, acts as a general base in the acylation mechanism by increasing the nucleophilicity of Ser64. However, Tyr150 may stay protonated during the reaction and is thus not able to serve as an anionic base during hydrolysis. The proton on Tyr150 helps stabilize the water's developing negative charge. However, mutagenesis studies do not rule out a role for Lys67 in the coordinate base mechanism as well (197).

The first class C β-lactamase structure determined was for the AmpC cephalosporinase of *C. freundii*, determined by Oefner et al. (198). The structures of the P99 β-lactamase of *E. cloacae*, AmpC β-lactamase from *E. coli*, and *E. cloacae* GC1 and *E. cloacae* 908R β-lactamases have ensued (191, 192) (http://www.rcsb.org). The GC1 β-lactamase of *E. cloacae* has improved hydrolytic activity for oxyimino-β-lactam antibiotics because of a tripeptide insertion in the Ω loop (a tandem repeat, Ala211-Val212-Arg213). In a strict sense, this is a class C ESBL. As a result of this addition, the width of the opening of the active-site binding cavity is larger and the substrate spectrum has expanded (191). As stated in the paragraph above, a number of other structures of the *E. coli* class C β-lactamases have been solved in an attempt to decipher the mechanism of catalysis. In the clinically important class C enzyme-producing Gram-negative rods, β-lactamase production is normally repressed. The details of the repression have mostly been elucidated for *Enterobacter* spp. (16). Repression and activation are closely linked to the processes of cell wall synthesis and breakdown. The molecule that serves as both the repressor and the activator of *ampC* transcription is AmpR, a transcriptional regulator of the LysR family. AmpR is present as a repressor by virtue of its interaction with UDP-MurNAc pentapeptide, a peptidoglycan precursor molecule. In this form, AmpR is incapable of activating *ampC* and in fact serves as a repressor of *ampR* expression. In the setting of high concentrations of the cell wall breakdown product anhydro-MurNAc tripeptide (or anhydro-MurNAc pentapeptide), however, UDP-MurNAc pentapeptide is displaced from its site in AmpR, resulting in the conversion of AmpR to an activator of *ampC* transcription.

Increases in *ampC* expression may result from the actions of β-lactam antibiotics, certain of which cause the release of significant quantities of anhydro-MurNAc tripeptide and/or pentapeptide from the peptidoglycan. This anhydro-UDP-MurNAc tripeptide enters the cell through a channel (AmpG) and overwhelms the recycling ability of the cytosolic amidase (AmpD) specific for recycling of muropeptides. Under these circumstances (induction), β-lactamase is produced only as long as the antibiotic is present in the medium.

Constitutive high-level production of AmpC β-lactamase most commonly results from a mutation in the *ampD* gene, reducing the quantity of (or eliminating) AmpD from the cytoplasm. Under these circumstances, a constant high level of anhydro-MurNAc tripeptide is present in the cytoplasm, and AmpR serves as a constitutive activator of *ampC* transcription. Constitutive production can also result from deletion of *ampR*, but in this circumstance, β-lactamase production is generally at a low level.

The widespread dissemination of *ampC*-type β-lactamase genes on transferable plasmids is a continuing challenge. These plasmid-encoded AmpC cephalosporinases are separated into four general groups. Group 1 plasmid-encoded AmpC cephalosporinases consist of those which originated from the chromosomal AmpC of *C. freundii* (BIL-1, CMY-2, LAT-1, and LAT-2). Members of group 2 are related to the chromosomal cephalosporinase of *E. cloacae* (MIR-1 and ACT-1), group 3 β-lactamases belong to the AmpC enzyme of *P. aeruginosa* (CMY-1, FOX-1, and MOX-1), and group 4 enzymes belong to the CMY-1 β-lactamase (CMY-1 cluster). Plasmid-mediated class C β-lactamases have been described to occur in many Gram-negative organisms from all parts of the world. Host strains harboring these enzymes include *K. pneumoniae*, *E. aerogenes*, *Salmonella enterica* serotype Senftenberg, *E. coli*, *Proteus mirabilis*, *M. morganii*, and *K. oxytoca*. The loss of porin proteins in clinical isolates with plasmid-encoded AmpC enzymes may result in resistance to carbapenems (199, 200).

Class D β-Lactamases

The OXA-type (oxacillin-hydrolyzing) β-lactamases have been most commonly described for *Enterobacteriaceae*, *Acinetobacter* spp., and *P. aeruginosa* (201). In terms of their genetic background in these Gram-negative organisms, many class D β-lactamase genes are associated with class 1 integrons or with ISs (202). OXA enzymes confer resistance to a wide variety of penicillins. The activities of inhibitors for class D enzymes vary. They are only weakly inhibited by clavulanic acid. However, OXA-2 and OXA-32 are inhibited by tazobactam. OXA-48 is inhibited by avibactam, but other OXA enzymes are not. In an unusual manner, OXA β-lactamases are inhibited by sodium chloride (50 to 75 mM NaCl). Mutagenesis studies suggest that susceptibility to inhibition by NaCl is related to the presence of a Tyr residue at position 144. Overall, the amino acid identities between class D and class A or class C β-lactamases are less than 20%. Their frequent location on mobile genetic elements (plasmids or integrons) facilitates spread (124, 203, 204).

Several OXA β-lactamases (OXA-11 and OXA-14 to OXA-20) are associated with an ESBL phenotype. These OXA types have been found exclusively in *P. aeruginosa*. A comparison of the crystal structure of the OXA-10 β-lactamase with those of the class C enzyme from *E. cloacae* P99 and of the class A TEM-1 enzyme from *E. coli* shows that the class D and class A enzymes have a common fold (α helices and β-pleated sheets), although the distributions of secondary-structure elements are different. A remarkable feature is the nearly perfect symmetry of all atoms that constitute the catalytic machinery for acylation (Ser67, Lys70, Ser115, and Lys205 in OXA-10 and Ser70, Lys73, Ser130, and Lys234 in TEM-1). There seems to be an extension of the substrate-binding site (tripeptide strand) in OXA-10. The role of the peptide extension is not known. The "oxyanion hole" is provided by the main-chain nitrogen atoms of Ser67 and Phe208. In class D, the same residue (Lys70) is involved in acylation and deacylation (205, 206).

OXA enzymes are assuming greater importance due to the ability of members of this class to hydrolyze carbapenems. The first description of a serine carbapenemase in *A. baumannii* was ARI-1 (OXA-23) in 1985 (207). Although

OXA carbapenemases hydrolyze imipenem inefficiently, their presence in an organism with an active efflux pump or a porin mutation may confer clinically significant levels of resistance (208). It is notable that *A. baumannii* isolates possess a chromosomally encoded oxacillinase, OXA-69, that confers very low level imipenem resistance. This gene is ubiquitous in *A. baumannii* and is referred to as a housekeeping gene (209).

Our understanding of the hydrolytic mechanism of class D β-lactamases is based on the careful study of OXA-10, -13, and -1. OXA β-lactamases are unique because of the direct role of carboxylation of the active-site Lys70. The carbamic acid on Lys70 can ionize to yield a carbamate that hydrogen bonds with the nucleophilic Ser67 residue. In this manner, the carboxylated Lys70 residue may serve as the general base by activating both Ser67 for acylation and the hydrolytic water for deacylation.

More relevant to the issue of carbapenem resistance are the crystal structures of OXA-24/40 and OXA-48. OXA-24/40 is one of the most widespread carbapenemases found in *A. baumannii* (210). This enzyme was originally part of a clinical epidemic in Spain that involved a 10-month-long outbreak affecting 29 patients, 23 of them hospitalized in five intensive care units. The work by Santillana et al. showed that OXA-24/40 has a hydrophobic barrier formed by Tyr112 and Met223 side chains, which define a tunnel-like entrance to the active site (211). Docquier et al. (212) found that OXA-48 is similar to OXA-10 in structure and not like OXA-24/40. Molecular-dynamics simulation showed that meropenem may position itself between Leu158 and Thr213, with the C-6 ethoxy group approaching Val120. In this manner, H_2O gets near Lys73 and can attack C=O at the amide bond.

OXA-48 was initially isolated from *K. pneumoniae* in Turkey, but now it has been identified in a number of different species of *Enterobacteriaceae* and in patients all over the world. This enzyme weakly hydrolyzes carbapenem, but when it is combined with permeability defects, isolates can demonstrate high-level carbapenem resistance. OXA-48 is an unusual carbapenemase because it does not hydrolyze cephalosporins, so isolates with this enzyme can test carbapenem resistant but cephalosporin susceptible. However, bacteria harboring OXA-48 often carry another β-lactamase as well, like an ESBL, so that the isolate tests resistant to both cephalosporins and carbapenems (213).

Chloramphenicol Resistance

Acetyltransferases

Chloramphenicol is a broad-spectrum antimicrobial agent whose use has waned in recent years due to its well-characterized hematologic toxicity and a wealth of less toxic therapeutic options. The most common mechanism of resistance to chloramphenicol is the elaboration of CATs. A large number of CAT genes have been reported, and these determinants generally confer extremely high levels of resistance on the organisms expressing them. Substantial structural similarities exist among the different CAT variants, although their nucleotide sequences may be quite divergent (214). Relationships among the different CATs have been described in detail in a review by Schwarz and colleagues (215). Chloramphenicol contains two hydroxyl groups that are acetylated in a reaction catalyzed by CAT in which acetyl coenzyme A serves as the acyl donor. Initial acetylation occurs at the C-3 hydroxyl group to yield 3-acetoxy-chloramphenicol (216). Following nonenzymatic rearrangement to 1-acetoxy-chloramphenicol and reacetylation, the 1,3-diacetoxy-chloramphenicol product is formed. Neither the mono- nor the di-acetoxy derivatives are able to bind to the 50S ribosomal subunit and inhibit prokaryotic peptidyltransferase (216).

CATs are generally divided into two types: type A (classical) CATs and type B (xenobiotic) CATs (215). For *S. aureus*, five structurally similar type A CATs (A, B, C, D, and that encoded by the prototypic plasmid pC194) have been described (217). The *cat* genes encoding these enzymes are commonly located on small, multicopy plasmids, and expression is inducible by a translational attenuation mechanism (215). *E. faecalis* and *S. pneumoniae* also express inducible CAT genes that are similar to the type D gene of *S. aureus*. Two *cat* genes encoding constitutive CAT expression have been described to occur in *Clostridium perfringens*. *catP* is generally found within transposon Tn*4451*, whereas *catQ* (nearly identical to *catD* of *Clostridium difficile*) is chromosomal (215).

Three types of type A CATs (I, II, and III) have been identified in Gram-negative bacteria. The widely prevalent type I enzymes are distinguished by their ability to bind and inhibit (without acetylation) the activity of fusidic acid. These enzymes are frequently found to be associated with transposon Tn9 or related elements. Type II CATs are notable for their sensitivity to inhibition by thiol-reactive agents and by their association with *H. influenzae* (218). Most knowledge of the structural features of the type A CAT enzymes comes from the study of the type III enzyme, for which the tertiary structure is known at high resolution (214). The structural determinants of binding for each substrate are also known for this enzyme.

Type B (xenobiotic) acetyltransferases (214) are structurally unrelated to classic CATs, and those that have been demonstrated to acetylate chloramphenicol confer only low levels of chloramphenicol resistance even when present in high copy numbers. Their natural substrate is likely something other than chloramphenicol, explaining their limited ability to acetylate this antibiotic. First described to occur in *Agrobacterium tumefaciens*, they have now been identified in a wide range of species (216). Included among this class of agents are the virginiamycin acetyltransferases found in *S. aureus* and *E. faecium* (see Quinupristin-Dalfopristin Resistance below). In fact, although they are members of this class, the *vat* genes do not confer resistance to chloramphenicol, nor have they been demonstrated to be able to acetylate chloramphenicol *in vitro* (217). They are, however, quite adept at acetylating streptogramins. The crystal structures of two trimeric type B CATs have been determined (219, 220).

Decreased Accumulation of Chloramphenicol

It is now well recognized that chloramphenicol serves as a substrate for many of the MDR efflux pumps that exist in Gram-positive and Gram-negative bacteria, including those found in *E. coli*, *P. aeruginosa*, *Bacillus subtilis*, and *S. aureus* (221). In addition, there are efflux systems that are specific for chloramphenicol. The first chloramphenicol-specific efflux gene that was described was *cmlA* within the In*4* integron of Tn*1696* (222). *cmlA* encodes an efflux mechanism that uses chloramphenicol but not florfenicol (a chloramphenicol derivative licensed for use in animals in 1996 in the United States for the treatment of bovine respiratory pathogens) as a substrate. Gram-negative bacteria also express efflux genes specific for both chloramphenicol and florfenicol (*floPp* and *floSt*). These resistance genes are being reported with increasing frequency for animal-derived *E. coli* and *Salmonella* isolates (223, 224). In fact, the chloramphenicol resistance expressed by MDR *Salmonella enterica* serovar Typhimurium DT104 is most commonly encoded by *floSt* (223), emphasizing again the potential negative impact of using similar antimicrobial agents in humans and animals. Very recently, chloramphenicol resistance in *Acinetobacter baumannii* has been attributed to the activity of an MFS-type pump designated CraA (225).

Daptomycin Resistance

Daptomycin is a cyclic lipopeptide antibiotic with activity exclusively against Gram-positive bacteria. It has bactericidal activity against most strains and acts by a cooperative interaction in the presence of physiological concentrations of calcium that result in the formation of pores in the cytoplasmic membranes of target bacterial cells. The result is leakage of ions from the cell and cell death. Resistance to daptomycin is rare but has been found in both enterococci and staphylococci.

The setting where *S. aureus* strains with reduced susceptibility to daptomycin usually occur is during prolonged treatment of deep-seated infections (226). The mechanisms of daptomycin resistance involve complex changes in the cell membrane, including phospholipid content, fluidity, and surface charge, that have not been completely defined (227). Whole-genome sequencing of non-daptomycin-susceptible *S. aureus* strains revealed deletions in *mprF*, *pgsA*, or *cls2* genes, which encode phospholipid synthesis and may reduce the cell membrane negative charge, causing electrorepulsion of daptomycin (228). The *vraSR* (vancomycin resistance-associated sensor/regulator) signaling pathway (229) plays a role in the expression of daptomycin resistance in *S. aureus* isolates with *mprF* mutations (230). A thickened cell wall has been noted in many non-daptomycin-susceptible *S. aureus* strains (228, 231) as well as isolates with intermediate-level resistance to vancomycin (232), but the mechanisms may differ since nonsusceptibility to only one of these agents may occur (230, 233).

In *E. faecalis*, daptomycin resistance has been associated with deletions in phospholipid metabolism genes (*gdpD* and *cls*) as well as a gene (*liaF*) thought to be part of the LiaFSR system, which regulates a stress-sensing response to antimicrobial therapy (234). Whole-genome sequencing of a daptomycin-resistant *E. faecium* strain demonstrated changes in the YycFG regulator system (similar to the LiaFSR system) and mutations of genes involved in phospholipid metabolism (235).

Glycopeptide Resistance

Glycopeptide antibiotics (vancomycin and teicoplanin) inhibit cell wall synthesis by binding to the pentapeptide peptidoglycan precursor molecule as it exits the cytoplasmic membrane. This binding prevents the cross-linking (transpeptidation) of peptidoglycan precursors necessary for the formation of normal, stable cell walls. The large size of the glycopeptide molecules also appears to inhibit the other major peptidoglycan linkage reaction (transglycosylation) by steric hindrance. The specific moiety bound by vancomycin, the terminal D-alanine–D-alanine of the peptidoglycan precursors, is present in most bacteria. However, the large size of vancomycin exceeds the exclusion limits of the porins in the outer membranes of Gram-negative bacteria, so vancomycin cannot access the target in these species. Hence, vancomycin is active only against bacteria lacking outer membranes, which are predominantly Gram positive.

Acquired resistance to vancomycin in Gram-positive bacteria comes in three varieties largely defined by the species within which they have been described: (i) altered pentapeptide precursors terminating in D-lactate or D-serine rather than the D-alanine in enterococci, (ii) mutational cell wall changes in staphylococci, and (iii) tolerance in pneumococci. The importance of the first type of resistance is characterized by both its prevalence and the importance of the species as a cause of infection, whereas the other two are defined more by the importance of the species than by their prevalence.

To date, nine varieties of enterococcal glycopeptide resistance that alter the vancomycin-binding target have been described. Four operons (VanA, VanB, VanD, VanM) encode terminal D-lactate precursors, and five operons (VanC, VanE, VanG, VanL, VanN) produce precursors ending in D-serine (236–238). Of these, the most clinically important are VanA and VanB (50). VanA and VanB are encoded by similar operons in which three genes (*vanH*, *vanA*, and *vanX* or *vanHB*, *vanB*, and *vanXB*) are required for expression of resistance (50). Two other genes (*vanY* and *vanZ* or *vanYB* and *vanW*) serve to amplify resistance but are not required for its expression (239, 240), and two more genes (*vanS* and *vanR* or *vanSB* and *vanRB*) regulate the transcription of the three essential genes (240, 241). The ultimate purpose of these genes is to alter the structure of the pentapeptide precursor from terminating in D-alanine–D-alanine to D-alanine–D-lactate, in so doing reducing the binding affinity of vancomycin to its target roughly 1,000-fold. The sequence of reactions resulting in this structure is outlined in reference 242. Since the terminal amino acid is cleaved from the pentapeptide in the transpeptidation reaction, the final composition of the cell wall is indistinguishable from that of strains lacking the resistance determinant. Apparently, the enterococcal PBPs, which facilitate transpeptidation, have no trouble processing the altered precursors.

VanA enterococci are phenotypically resistant to vancomycin and teicoplanin, whereas VanB strains are resistant to vancomycin but appear to be susceptible to teicoplanin. This susceptibility results from the fact that teicoplanin does not induce expression of resistance (241). Once the VanB operon is expressed, however, resistance to teicoplanin results. Consequently, teicoplanin has been disappointing as a therapy for infections caused by VanB enterococci, since mutations in the VanB regulatory apparatus resulting in either inducibility by teicoplanin or constitutive expression occur readily during therapy (243–245).

Both VanA and VanB operons are carried by transposons. VanA is found exclusively within transposon Tn*1546*, a 10.4-kb Tn*3* family element that is presumed to disseminate among enterococci by integrating into conjugative plasmids (240). The genes of the VanA operon are found to be highly conserved in their sequence when different strains are compared, but the restriction maps of the operons and of Tn*1546* often differ markedly among clinical strains (246). These differences result from insertions of a variety of IS elements with or without subsequent deletions of parts of the mobile element and have been used by some investigators to establish lineages of strains within defined clinical settings. The VanB operon is most commonly encoded on highly similar transposons designated Tn*5382* or Tn*1549* (33, 247). These transposons exhibit significant homology to prototype enterococcal conjugative transposon Tn*916*. In contrast to the *vanA* gene, three allelic variants of *vanB* (*vanB1*, *vanB2*, and *vanB3*) have been described. The *vanB2* gene is associated with Tn*5382* (248).

The overwhelming majority of clinical vancomycin-resistant enterococcal strains are *E. faecium*, a predilection that remains unexplained (103). The vast majority of vancomycin-resistant *E. faecium* strains that cause clinical infection are also resistant to ampicillin, owing to the expansion of a group of hospital-adapted clones (referred to as clonal complex 17) that have emerged around the world. Clonal complex 17 strains are found worldwide and are characterized by their resistance to ampicillin and by the fact that they frequently harbor putative virulence determinants, such as *esp* and *hyl* of *E. faecium* (249, 250).

Despite *in vitro* transfer of the VanA determinant to *S. aureus* (251) and at least 14 case reports of patient cultures with VanA-expressing *S. aureus* (252–254), vancomycin-resistant *S. aureus* (VRSA) remains exceedingly rare. In all cases, resistance has been conferred by the VanA operon, and in one well-characterized case transfer appears to have been facilitated by the presence of Tn*1546* on a broad-host-range plasmid in *E. faecalis*, with transposition of Tn*1546* to a staphylococcal plasmid once entry into the staphylococcus occurred (27, 28).

The VanC operon is intrinsic to the cell wall synthesis machinery of the minor enterococcal species *Enterococcus casseliflavus* (including the biotype formerly classified as *Enterococcus flavescens*) and *Enterococcus gallinarum* (255, 256). The peptidoglycan precursor in VanC strains terminates in D-alanine–D-serine, reducing vancomycin affinity about 7-fold and resulting in low levels of resistance, with vancomycin MICs of 2 to 32 µg/ml. The cell wall precursors encoded by VanE, VanG, VanL, and VanN also terminate in D-alanine–D-serine and facilitate low-level glycopeptide resistance (257). In 2011 and 2012, epidemiologically unrelated *Streptococcus agalactiae* isolates with VanG elements for which nonsusceptible vancomycin MICs were 4 µg/ml were recovered from 2 patients in the United States (New York and New Mexico) (258). Both strains were positive by a PCR assay targeting the VanG sequence of *Enterococcus faecalis* (258), but the *van*G sequences of only the New York strain shared complete sequence homology (253). A *Streptococcus anginosus* isolate for which the vancomycin MIC was 4 µg/ml and with VanG elements nearly identical to those of the non-vancomycin-susceptible *S. agalactiae* New York strain was recovered from the urine of a Minnesota patient in 2012 (B. Beall, personal communication).

The modified pentapeptide precursor of *E. faecium* VanD terminates in D-alanine–D-lactate, and these strains have moderate levels of glycopeptide resistance (vancomycin MIC, 64 to 128 µg/ml). The failure to observe dissemination of VanD may be explained in part by the fact that the VanX-equivalent enzyme in *E. faecium* BM4339 appears to be ineffective in an enterococcal background. Resistance was expressed in BM4339 because that strain lacked a functional cellular ligase (*ddl*) gene, eliminating the need for VanX activity to express resistance (259).

In *S. aureus*, intermediate-level resistance to glycopeptides (vancomycin-intermediate and glycopeptide-intermediate *S. aureus* [VISA/GISA]) occurs more frequently than full resistance. Human VISA (hVISA) (heterogeneous VISA) strains are susceptible to vancomycin at MICs of ≤2 µg/ml by the CLSI broth microdilution method (260). However, within these cultures are smaller populations of cells that express intermediate levels of resistance requiring special techniques (population analysis profiling with area under the curve [PAP-AUC] analysis) for accurate detection. The hVISA and VISA phenotypes are characterized by thickened cell walls, which may decrease glycopeptide susceptibility by providing an excess of false targets for glycopeptide binding (228). In many, but not all, cases, conversion to the hVISA or VISA phenotype is associated with the *vraSR* two-component regulatory circuit, which responds to cell wall damage in *S. aureus* and regulates more than 40 genes, some of which are associated with the biosynthesis of peptidoglycan (260–262). Interestingly, recent work suggests that the hVISA phenotype can be selected by exposure to β-lactam antibiotics as well as by exposure to glycopeptides (263). Animal studies suggest that the level of resistance expressed by hVISA strains reduces the effectiveness of vancomycin therapy (264).

Glycopeptide resistance has also been reported for coagulase-negative species of staphylococci. Unlike in *S. aureus*, resistance in *Staphylococcus haemolyticus* has been associated with changes in the composition of the cross-links of the peptidoglycan (265). The mechanism by which this may lead to vancomycin resistance is incompletely understood.

Vancomycin is, in general, a less bactericidal antibiotic than are the β-lactams. Evidence for the importance of this observation can be found in several species. The bacteremia associated with *S. aureus* endocarditis, for example, takes roughly twice as much time to clear with vancomycin treatment as with treatment by the β-lactam oxacillin (266). Recent clinical data also suggest that vancomycin treatment is associated with higher rates of failure and relapse than is nafcillin treatment for bacteremia due to methicillin-susceptible *S. aureus* (267). Vancomycin efficacy appears to be particularly poor against some strains of MRSA. In a recent clinical trial, vancomycin successfully treated left-side endocarditis in only 2 of 9 cases, a success rate that was similar to that of daptomycin (2 of 10 cases) (226). Reports of vancomycin tolerance in *Streptococcus pneumoniae* first appeared in 1990 (268). *S. pneumoniae* is the most common cause of bacterial meningitis in most patient populations, and bactericidal therapy is optimal for treatment of this condition. At least one case of presumed recrudescence of meningitis after treatment of a case of vancomycin-tolerant pneumococcal meningitis has been reported (269). Tolerance appears to involve mutations within an operon (*vex123*) encoding an ABC transporter, but the mechanism by which this occurs remains undefined (270). Further work will be required before we understand the true importance of pneumococcal tolerance for the treatment of clinical infections.

Linezolid Resistance

The oxazolidinone antibiotic linezolid inhibits bacterial protein synthesis by interacting with the *N*-formylmethionyl-tRNA–ribosome–mRNA ternary complex commonly referred to as the initiation complex (271). Linezolid exerts excellent bacteriostatic activity against a wide range of Gram-positive pathogens, including methicillin-resistant staphylococci and MDR enterococci. Clinical use of this agent has been associated with the emergence of resistant strains, most commonly after prolonged therapy of difficult-to-eradicate bacteria. Resistance has now been described for both enterococci and staphylococci, but overall rates several years after clinical introduction of this agent remain very low (272). Resistance is most often associated with a G2576U (*Escherichia coli* numbering scheme) point mutation in the 23S rRNA, although mutations at other positions may also contribute to resistance (273). Resistance to linezolid, macrolides, and chloramphenicol has been attributed to a 6-bp deletion in the gene encoding riboprotein L4 in *S. pneumoniae* (274). Since the 23S subunit genes exist in multiple copies in different bacteria (four in *E. faecalis* and *S. pneumoniae*, six in *E. faecium*, and five or six in *S. aureus*), more than one copy of the genes must be mutated to confer resistance, with strains having a higher percentage of mutated 23S genes expressing greater levels of resistance (275). Gene conversion, or recombination between mutated genes and wild-type genes, can rapidly increase the levels of resistance once the first gene mutation has occurred (276). In this fashion, persistent selective pressure exerted by linezolid can lead to rapid development of high-level (MIC > 128 μg/ml) resistance.

Plasmid-mediated resistance to linezolid through the expression of the *cfr* rRNA methylase gene has been reported for staphylococci (276), but the prevalence of this type of linezolid resistance remains very low (272).

Macrolide Resistance

Erythromycin (the first macrolide) was initially isolated from *Streptomyces erythraeus*, a soil organism found in the Philippines. There are currently four macrolides in common use: erythromycin, clarithromycin, azithromycin, and roxithromycin. Macrolides inhibit protein synthesis in susceptible organisms by binding reversibly to the peptidyl-tRNA binding region of the 50S ribosomal subunit, inhibiting translocation of a newly synthesized peptidyl-tRNA molecule from the acceptor site on the ribosome to the peptidyl (or donor site). Erythromycin does not bind to mammalian ribosomes. Most Gram-negative organisms are resistant to erythromycin because entry of erythromycin into the cell is restricted.

Resistance to macrolides occurs by several mechanisms. Among the more important of these mechanisms is methylation of the ribosome, preventing erythromycin binding (277). This methylation is most commonly accomplished by different *erm* (erythromycin ribosomal methylase) genes. Methylated ribosomes confer resistance to macrolides, the related lincosamides (clindamycin and lincomycin), and streptogramins B (MLSB resistance). Many *erm* genes have been described—*erm*(A) and the related *erm*(TR), plus *erm*(B) and the related *erm*(AM)—and resistance is frequently inducible by macrolides but not by clindamycin (iMLSB). In some strains, *erm*-type resistance is expressed constitutively (cMLSB), resulting in resistance to clindamycin as well.

The second major mechanism of resistance to macrolides is expression of efflux pumps encoded by *mef* genes (Mef in Gram-positive bacteria and Acr-AB-TolC in *H. influenzae* and *E. coli*) (278). The efflux pumps confer resistance to the macrolides but not to clindamycin, hence the phenotypic description of this resistance as of the "M" type. *mef* genes have been studied most extensively for *Streptococcus pneumoniae* [*mef*(E)] and *Streptococcus pyogenes* [*mef*(A)], but similar genes have been described for a variety of Gram-positive genera. The prevalences of *mef*-mediated resistance versus that mediated by MLSB-type mechanisms in *S. pneumoniae* vary in different parts of the world. Minor mechanisms of resistance to macrolides include esterases that hydrolyze the antibiotics and point mutations within the 50S rRNA gene.

Ketolide Resistance

Ketolides belong to a new class of semisynthetic 14-membered-ring macrolides, which differ from erythromycin by having a 3-keto group instead of the neutral sugar L-cladinose. Ketolides bind to an additional site on the bacterial ribosome, increasing their binding affinity relative to that of other macrolides (279). Telithromycin, a ketolide, is uniformly and highly active against pneumococci (regardless of their susceptibility or resistance to erythromycin and/or penicillin), erythromycin-susceptible *S. pyogenes*, and erythromycin-resistant *S. pyogenes* strains of the M phenotype or iMLSB or cMLSB phenotype [in which resistance is mediated by a methylase encoded by the *erm*(TR) gene] (280). Ketolides are less active against erythromycin-resistant *S. pyogenes* strains with the cMLSB phenotype or the iMLSA subtype (in which resistance is mediated by a methylase encoded by the *ermB* gene); these strains range in phenotype from the upper limits of susceptibility to resistant. Methicillin-resistant staphylococci, which commonly express a cMLSB phenotype, are not susceptible to telithromycin (281).

Quinupristin-Dalfopristin Resistance

Quinupristin-dalfopristin is a mixture of semisynthetic streptogramins A and B licensed in Europe and the United States. A related streptogramin A and B combination, virginiamycin, has been used for years as a growth promoter in animal feed. Resistance to these mixtures can result from resistance to streptogramin A alone and was first described for staphylococci conferred by genes encoding streptogramin A acetyltransferases [*vat*(A), *vat*(B), and *vat*(C)] or ATP-binding efflux genes [*vga*(A) and *vga*(B)]. Quinupristin-dalfopristin's excellent activities against *E. faecium* and MRSA make it an alternative for the treatment of MDR *E. faecium* and health care-associated MRSA infections, especially since the combination retains *in vitro* activity against streptogramin B-resistant strains. Two acetyltransferase-encoding resistance genes that confer resistance to quinupristin-dalfopristin in *E. faecium*, *vat*(D) [previously *sat*(A)] and *vat*(E) [previously *sat*(G)], have now been described. In most cases, these resistance genes are found along with an *erm* gene (281), suggesting that resistance to both streptogramins A and B may be necessary to confer clinically significant levels of resistance to quinupristin-dalfopristin in *E. faecium*. These resistance genes are frequently present on transferable plasmids. Although quinupristin-dalfopristin remains active against the majority of human *E. faecium* strains, the use of virginiamycin in animal feeds has been associated with high percentages of resistance in isolates derived from animals (282). In many cases, the known mechanisms of resistance to quinupristin-dalfopristin are not present in these isolates (282), indicating that there is still much to be learned about resistance to quinupristin-dalfopristin in *E. faecium*.

Metronidazole Resistance

Metronidazole is a member of the nitroimidazole family of bactericidal antimicrobials. The 5-nitroimidazole molecule is a prodrug whose activation depends upon reduction of the nitro group in the absence of oxygen. An exception to this rule occurs in *Helicobacter pylori*, in which the RdxA protein reduces metronidazole in a microaerophilic environment (283). The nitro group of metronidazole accepts a single electron from electron transport proteins (ferredoxins) in bacteria, yielding a toxic radical anion. Metronidazole's activity appears to result in DNA damage and cell death (284). Resistance to metronidazole is rare. Decreased uptake and/or a reduced rate of reduction is believed to be responsible for metronidazole resistance in some cases (285). Five *Bacteroides* genes, *nimA* to *nimE*, have been implicated in resistance to 5-nitroimidazole antibiotics. Analysis of the NimA-susceptible and -resistant *Bacteroides* strains and recent crystal structure analysis suggest that the enzyme utilizes pyruvate for a two-electron reaction resulting in an amine that prevents the formation of the toxic anion radical (286, 287). Expression of *nim* genes varies depending on the positioning of a variety of IS elements that supply active promoters (288). Recent data indicate that the enzyme thioredoxin reductase is responsible for the reduction of metronidazole in *Trichomonas vaginalis* (289).

Nitrofurantoin Resistance

The antibiotics nitrofurazone and nitrofurantoin are used in the treatment of genitourinary infections and as topical antibacterial agents. Nitrofurazone is used primarily as a topical antiseptic (290). Nitrofurantoin {1-[(5-nitrofurfurylidene)amino]hydantoin} is a synthetic antibacterial agent used primarily in the treatment of urinary tract infections. The mechanism of action of nitrofurazone and nitrofurantoin has not been fully elucidated. Investigators have reported that the ability of nitrofurantoin to kill bacteria correlates with the presence of bacterial nitroreductases which convert nitrofurantoin to highly reactive electrophilic intermediates (291). These intermediates are believed to attack bacterial ribosomal proteins nonspecifically, causing complete inhibition of protein synthesis. In *E. coli*, nitroreductases are type 1 oxygen-insensitive enzymes encoded by the *nfnA* (*nfsA*) and *nfnB* (*nfsB*) genes. Strains of bacteria that are resistant to nitrofurantoin have been shown to possess diminished nitroreductase activity (292), which may seriously compromise their fitness (293). Resistance to nitrofurantoin from reduced nitroreductase activity seems to be present in other genera as well.

Polymyxin B and Polymyxin E (Colistin) Resistance

Clinical and scientific interest in the cationic polypeptides is increasing. Although they were first used in the early 1960s, colistin (polymyxin E) and polymyxin B are now often used as first-line therapy of infections caused by MDR Gram-negative bacteria. The polymyxins are polycationic peptide antibiotics isolated from *Bacillus polymyxa* (294). They exert their bactericidal activity by binding to the cell membrane of Gram-negative bacteria and disrupting its permeability, resulting in leakage of intracellular components. They also disrupt bacterial biofilm formation. In mechanistic terms, polymyxin binds to phosphorylated head groups of lipid A. Hence, by disrupting cell membranes, these agents become rapidly bactericidal against certain Gram-negative bacteria (295, 296).

Not all Gram-negative bacteria are susceptible to polymyxins. Organisms that are resistant to polymyxins have cell walls that prevent access of the drug to the cell membrane. In general, polymyxins are bactericidal against *P. aeruginosa*, *Acinetobacter* spp., some *Proteus mirabilis* strains, and some strains of *Serratia marcescens*. *Proteus* spp., *Providencia* spp., *Neisseria* spp., and Gram-positive bacteria are resistant to polymyxins (294).

Polymyxin-resistant mutants and bacteria exhibit a modified lipopolysaccharide (LPS) or loss of LPS production. Modification of the phosphate groups of lipid A with positively charged groups, such as 4-amino-4-deoxy-L-arabinose, is the most common resistance mechanism in *E. coli*, *Salmonella* serovar Typhimurium, *K. pneumoniae*, *P. aeruginosa*, *Acinetobacter* spp., and many other pathogenic Gram-negative bacteria (297). This modification of lipid A is mediated by two-component regulatory systems, such as PhoB-PhoQ and the downstream regulatory system PmrA-PmrB. Several mutations that affect these regulatory systems have been described in polymyxin-resistant isolates. LPS modifications include alteration of the fatty acid content of lipid A, and complete loss of LPS production was identified in a resistant strain of *Acinetobacter* spp. (298). There are non-LPS mechanisms of resistance, including changes in outer membrane protein expression and the overexpression of efflux pumps (299).

Quinolone Resistance

The fluoroquinolones are among the most widely used antimicrobial agents in both hospital and community settings. Quinolone antibiotics all act by directly inhibiting DNA synthesis. Their targets include two type 2 topoisomerases: DNA gyrase and topoisomerase IV. These two enzymes are structurally related in that both exist as tetramers composed of two different subunits (GyrA and GyrB of DNA gyrase and ParC and ParE of topoisomerase IV). DNA gyrase acts to maintain negative supercoiling of DNA, whereas topoisomerase IV separates interlocked daughter DNA strands formed during replication, facilitating segregation into daughter cells. Fluoroquinolones bind to the topoisomerase-DNA complexes and disrupt various cellular processes involving DNA (replication fork, transcription of RNA, and DNA helicase) (299–301). The result is cellular death by unclear mechanisms.

The affinities of fluoroquinolones for the two targets vary, explaining to some degree the differing potencies of the various agents against different bacterial species. The enzyme for which a particular fluoroquinolone exerts the greatest affinity is referred to as the primary target (302–304). In general, the primary target of fluoroquinolones in Gram-negative bacteria is DNA gyrase, whereas in Gram-positive bacteria it is topoisomerase IV.

Alterations in Target Enzymes

The most common mechanism of clinically significant levels of fluoroquinolone resistance is through alterations of the topoisomerase enzymes. These alterations are created by spontaneous mutations that occur within the respective genes. In GyrA and ParC, resistance-associated mutations are often localized to a region in the amino terminus of the enzyme containing the active-site tyrosine that is covalently linked to the broken DNA strand. This 130-bp region of *gyrA* has been referred to as the quinolone resistance-determining region. X-ray crystallographic studies of a fragment of the GyrA enzyme suggest that these mutations are clustered in three dimensions, lending support to the hypothesis that the region constitutes a part of the quinolone binding site (305). Particularly frequent sites for resistance-associated mutations are serine 83 and aspartate 87 of GyrA and serine 79 and aspartate 83 of ParC (306).

Experimental data suggest that point mutations occur singly in roughly 1 in 10^6 to 10^9 cells. The level of resistance conferred by a single point mutation in the primary target enzyme depends upon the reduction of enzyme affinity created by the mutation, as well as the affinity of the fluoroquinolone for the secondary target. In this scenario, it is expected that fluoroquinolones exhibiting strong affinity for both target enzymes are less likely to be associated with the emergence of resistant strains, since the retained activity against the secondary target would be enough to inhibit the bacterium even in the presence of a primary target mutation. Fluoroquinolone-species combinations for which single mutations result in significantly higher MICs (such as ciprofloxacin and *S. aureus* or *P. aeruginosa*) are expected to be readily selected in clinical settings (307).

Most highly resistant strains exhibit more than one mutation in both the GyrA and ParC enzymes, a phenomenon that can be reproduced in the laboratory by serial passage of strains on progressively higher concentrations of fluoroquinolones. It is noteworthy in this context that fluoroquinolone resistance conferred by enzyme mutations is essentially class resistance. In other words, the activities of all fluoroquinolones are affected by mutations that result in resistance. Therefore, while single point mutations that confer resistance to one fluoroquinolone may not result in MICs conferring clinical resistance to another, the MICs of the second fluoroquinolone will inevitably be increased. In the setting of such preexisting mutations, the second fluoroquinolone could then select for an additional mutation that would result in clinically significant levels of resistance. This reasoning has led to the recommendation that the most potent and broadly active fluoroquinolone always be used first to prevent the emergence of resistance. The wisdom of this recommendation remains to be tested.

Mutations in GyrB and ParE are far less common than in their companion subunits and tend to cluster in the midportion of the subunit (308). A clear understanding of the impact that these mutations have on enzyme structure or function awaits detailed crystallographic studies of enzyme-fluoroquinolone complexes.

Resistance due to Decreased Intracellular Accumulation

Fluoroquinolones penetrate the outer membrane of Gram-negative bacteria through porins, so the absence of specific porins can affect the level of susceptibility. However, their ability to diffuse through outer and cytoplasmic membranes is sufficient to retain activity against strains solely lacking porins (309). More important in reducing intracellular accumulation of fluoroquinolones is the expression of MDR pumps (306). The intrinsic efflux pump complexes in Gram-negative bacteria extend from the cytoplasmic membrane through the outer membrane, whereas Gram-positive pumps need only traverse the cytoplasmic membrane. These pumps move compounds across the bacterial membranes by proton motive force and are presumed to represent systems by which bacteria rid themselves of toxic materials. Resistance results when expression of pumps is increased due to mutations within their regulatory genes (310). By themselves, pumps generally confer only a low level of resistance to fluoroquinolones. However, their expression may amplify the level of resistance conferred by point mutations within the topoisomerase genes. By so doing, they may increase the risk that use of a given fluoroquinolone will select for resistant mutants through single point mutations. In recent years, a plasmid-mediated efflux pump (QepA) has been recognized among strains of *Enterobacteriaceae* (311). This pump extrudes the hydrophilic fluoroquinolones (ciprofloxacin, enrofloxacin, and norfloxacin).

The major type of plasmid-mediated fluoroquinolone resistance present in Gram-negative bacteria is conferred by the Qnr proteins (312, 313), which protect DNA from quinolone binding (314, 315). In general, only low levels of resistance are conferred by this mechanism, but as with other accessory mechanisms, the presence of Qnr can facilitate the clinical emergence of strains resistant by virtue of point mutations in the topoisomerase genes. Five variants of Qnr have now been described (A, B, C, D, and S). There are several alleles within the A, B, and S variants (http://www.lahey.org/qnrStudies/). The prevalence of this mechanism is increasing, which may be partly explained by the frequent presence of *qnr* within complex *sulI*-type integrons (313) often associated with insertion elements (37).

Plasmid-mediated fluoroquinolone resistance can also be conferred by the AAC(6′)-Ib-cr protein, which is a mutant of the AAC(6′)-Ib AME (311). This confers low levels of resistance to ciprofloxacin and norfloxacin.

Rifampin Resistance

Rifampin is particularly active against Gram-positive bacteria and mycobacteria. It acts by inhibiting bacterial-DNA-dependent RNA polymerase. Point mutations in the chromosomal *rpoB* gene confer resistance to rifampin (316). The frequency with which these point mutations occur precludes using rifampin as a single agent for the treatment of bacterial infections.

Tetracycline Resistance

The tetracyclines are a group of bacteriostatic antibiotics that act by inhibiting attachment of aminoacyl-tRNA to the ribosome acceptor site, thereby preventing elongation of the peptide chains of nascent proteins (317). In order to gain access to the bacterial ribosome, tetracyclines need to enter the cell. In *E. coli* and presumably other Gram-negative bacteria, they enter the periplasmic space through outer membrane porins OmpC and OmpF, probably chelated to magnesium ions (318). Once in the periplasmic space, the weakly lipophilic tetracycline molecule dissociates from the magnesium ion and crosses into the cell by diffusing though the lipid bilayer in an energy-dependent process. Once inside the cell, tetracycline-ion complexes bind to the ribosome at a single, high-affinity binding site on the 30S subunit, blocking access of the aminoacyl-tRNA to the ribosome acceptor site. Although of high affinity, binding of tetracycline to the ribosome is reversible (319).

Tetracyclines are broad-spectrum and effective antimicrobial agents. Unfortunately, widespread use of tetracyclines to treat clinical infections and for promotion of growth in livestock has been associated with the emergence and dissemination of a variety of resistance determinants. As a consequence, the number of infections for which tetracyclines are recommended as first-line therapy has been limited for many years (319). The vast majority of tetracycline resistance determinants fall into one of two classes: (i) efflux or (ii) ribosomal protection. The designations of the different resistance determinants and their classes can be found in detail in an excellent review of tetracyclines by Chopra and Roberts (318). Initial designations of tetracycline resistance determinants used the prefix *tet* or *otr*, with letters (A, for example) designating the different determinants. Since the number of resistance determinants now exceeds the number of letters in the alphabet, a system using numbers has been devised (320).

Tetracycline efflux proteins are all membrane associated and members of the MFS proteins. They expel tetracycline from the cell by exchanging a proton for a tetracycline-cation complex. In general, the efflux proteins confer resistance to tetracyclines but tend to spare minocycline (318). The single exception to this rule is the Tet(B) protein of Gram-negative organisms, which confers resistance to both tetracycline and minocycline. The efflux proteins have been divided into six groups based on amino acid identity. Group 1 consists of Tet efflux proteins that are found primarily in Gram-negative species [with the exception of Tet(Z)], whereas group 2 [consisting only of Tet(K) and Tet(L)] is found primarily in Gram-positive species. Groups 3 through 6 are small groups consisting of one or two efflux proteins each.

Ribosome protection proteins comprise the other major mechanism of tetracycline resistance. These proteins exhibit homology to elongation factors EF-Tu and EF-G and exhibit ribosome-dependent GTPase activity (321). They act by binding to the ribosome, thereby changing its conformation and inhibiting binding of tetracycline. Tet(M) and Tet(O) are the best characterized of these proteins. Ribosome protection genes are widespread in bacteria, in many cases as a result of their incorporation into broad-host-range conjugative transposons.

Both efflux proteins and ribosomal protection proteins are regulated in ways that allow their expression to be increased in the presence of tetracyclines. The efflux proteins of Gram-negative organisms are regulated by repressors that are divergently transcribed relative to the efflux proteins (322). Binding of the repressors to tetracycline changes the conformation of the repressor so that it can no longer bind to the operator region, resulting in increased transcription of both the efflux protein and the repressor genes. The Gram-positive efflux genes are not associated with specific protein repressors; sequence analysis suggests that these determinants may be regulated by mechanisms similar to translational attenuation, but study of this area has been limited (323). Transcription of ribosomal protection genes is augmented by growth in the presence of tetracycline.

Intrinsic mechanisms of tetracycline resistance exist in many, if not all, Gram-negative bacteria. Among the best characterized of these systems is the *mar* (multiple antibiotic resistance) operon (324). This locus consists of a repressor (MarR) that represses transcription of *marA*, which encodes a transcriptional activator of a variety of genes. Overexpression of MarA results in decreased expression of OmpF, a porin through which tetracycline enters the periplasmic space, and increased expression of multidrug efflux pump AcrAB, a member of the RND family of efflux proteins, which includes tetracyclines among its substrates. Several similar pump systems have been described for *P. aeruginosa* and other Gram-negative bacteria (325). As our knowledge of the genomes of different bacterial species becomes more complete, we will no doubt discover several other pump systems that affect levels of susceptibility to tetracyclines and other antibiotics.

The remarkable diversity of species within which tetracycline resistance determinants are found owes much to the inclusion of these resistance genes within broad-host-range transferable genetic elements. These include transferable plasmids in Gram-negative species, where *tet* genes may be found included within integrons, and conjugative transposons. Among the best studied of the conjugative transposons is the Tn*916* family, originally described for *E. faecalis* (31). The complete sequence of Tn*916* has been determined and is remarkable for its dearth of restriction enzyme digestion sites [except in the region of the *tet*(M) gene, which appears to be a late arrival to the element] (326). This lack of restriction sites likely facilitates its entry into a variety of different bacterial species. Transfer of Tn*916*-like elements from enterococci into many other species has been demonstrated *in vitro* and in animal models, and the remnants of Tn*916*-like sequences in *N. gonorrhoeae* are impressive testimony to its ability to travel widely (31). Transfer of Tn*916*-like elements, which is increased after exposure to tetracycline, has also been suggested to facilitate transfer of unlinked genes, further amplifying the risks of overexposure to tetracycline in the environment.

Tigecycline Resistance

The FDA approval of the glycylcycline tigecycline in 2005 provided a broad-spectrum antimicrobial alternative for treating infections due to resistant pathogens, including MRSA and carbapenem-resistant *Enterobacteriaceae*. Tigecycline's broad spectrum of antimicrobial activity is due to its resistance to the common efflux or ribosomal protection mechanisms that confer resistance to older tetracyclines. Some bacterial species, notably *P. aeruginosa* and *Proteus* spp., are intrinsically resistant to tigecycline because they express RND-type efflux pumps that effectively extrude the antibiotic (327). Resistance to tigecycline in other Gram-negative species has also been reported, generally resulting from activation of normally repressed AcrAB-type RND efflux pumps (328). Mutations in the S10 ribosomal protein previously demonstrated in tetracycline-resistant *N. gonorrhoeae* and RamR mutations associated with increased expression of the AcrAB efflux system have been associated with tigecycline resistance in KPC-producing *K. pneumoniae* (329).

Trimethoprim-Sulfamethoxazole Resistance

Biosynthesis of several amino acids and purines depends upon the availability of tetrahydrofolate. With few exceptions, bacteria are unable to absorb preformed folic acid and hence rely upon their ability to synthesize it. Sulfamethoxazole and trimethoprim are inhibitors of two enzymes (dihydropteroic acid synthase [DHPS] and dihydrofolate reductase [DHFR]) that act sequentially in the manufacture of tetrahydrofolate. It is thought that the two inhibitors act synergistically to inhibit folate synthesis, although the mechanism for possible synergism (since sequential blockage of a fully inhibited pathway should not augment resistance) is not clear.

Intrinsic Resistance

Trimethoprim-sulfamethoxazole is a remarkably broad spectrum antimicrobial agent. Intrinsic resistance is relatively rare and may occur by decreased access to the target enzymes (*P. aeruginosa*) (330) or low-affinity DHFR enzymes (*Neisseria* spp., *Clostridium* spp., *Brucella* spp., *Bacteroides* spp., *Moraxella catarrhalis*, and *Nocardia* spp.) (331) or by the ability to absorb exogenous folate (*Enterococcus* spp. and *Lactobacillus* spp.) (332) or thymine (*Enterococcus* spp.) (333). The decreased access to the target enzyme in *P. aeruginosa* appears to be due to both a permeability barrier and active efflux from the cell (334, 335). The percentage contribution of each of these mechanisms to resistance remains unclear.

Acquired Resistance to Trimethoprim

Mutational resistance to trimethoprim has been described for several species and involves promoter mutations leading to overproduction of DHFR (in *E. coli*), point mutations within the *dhfr* gene leading to resistance (in *S. aureus* and *S. pneumoniae*), or both mechanisms (in *H. influenzae*) (336). More common is the acquisition of low-affinity *dhfr* genes, of which approximately 20 have been described (337). Expression of the *dhfrI* gene and variants of the *dhfrII* gene, which are most commonly found on plasmids in Gram-negative bacteria, increases resistance to levels greatly exceeding clinically achievable concentrations.

Acquired Resistance to Sulfonamides

Point mutations or small insertions of DNA segments within chromosomal *dhps* genes conferring resistance to sulfonamides have been reported for many different species (337, 338). More-extensive changes within *dhps* genes resulting in resistance have been reported for *N. meningitidis* and *S. pyogenes*. In these instances, the extensive changes have suggested acquisition of at least some parts of the *dhps* genes from other species via transformation and recombination (339). Plasmid-mediated, transferable resistance to sulfonamides has been reported for Gram-negative bacteria (338). Unlike with the diversity in *dhfr* genes, only two acquired low-affinity *dhps* genes (*sulI* and *sulII*) have been described. Genes conferring resistance to sulfonamides are frequently incorporated into MDR integrons, which are themselves frequently integrated into transferable plasmids. The transferability of these resistance plasmids and the frequent association with other resistance genes explain in part the widespread nature and persistence of resistance to this antimicrobial combination. One trimethoprim-sulfamethoxazole-resistant *E. coli* strain was reported to have spread widely in the United States, causing urinary tract infections in young women in at least two states (340), although more-recent data suggest that this widespread prevalence may owe more to parallel emergence of related strains than to direct spread of an outbreak isolate (341).

Mupirocin Resistance

Mupirocin is a topical antimicrobial agent that can be used to treat skin and soft tissue infections, such as impetigo, or secondary wound infections caused by *Streptococcus pyogenes* or *S. aureus*. This drug is also used with increasing frequency to decolonize nasal passages of MRSA patients in an effort to reduce health care-associated MRSA infections or outbreaks of community associated MRSA (342). The drug exerts antimicrobial activity by inhibiting the bacterium's isoleucyl tRNA synthetase and thereby inhibiting RNA and protein synthesis. Both low-level and high-level mupirocin resistance has been described for staphylococci. Low-level-resistance strains (MICs ≥ 8 µg/ml and ≤ 64 µg/ml) have acquired mutations in the native gene encoding isoleucyl tRNA synthetase. High-level-resistance strains (MIC ≥ 512 µg/ml) have acquired a gene encoding a novel isoleucyl tRNA synthetase, such as *mupA*. The clinical significance of low-level resistance is not well understood, but failure to decolonize or achieve sustained decolonization have been associated with *mupA*-mediated resistance. The *mupA* gene is commonly found on a conjugative plasmid, and rates of high-level mupirocin resistance tend to increase with increasing use of the topical agent. A new resistance determinant conferring high-level mupirocin resistance was identified in *S. aureus*. The gene, *mupB*, has low identity to *mupA* but encodes a protein with structural motifs common to isoleucyl tRNA synthetase. So far, only a single *mupB*-positive strain has been identified (343).

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Susceptibility Test Methods: General Considerations

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Determination of the antimicrobial susceptibilities of significant bacterial isolates is one of the principal functions of clinical microbiology laboratories. From the physician's pragmatic point of view, the results of susceptibility tests are often considered important or more important than the identification of the pathogen involved. This is particularly true in an era of increasing antimicrobial resistance in which treatment options are at times limited to newer, more costly antibacterial agents. As a result, the laboratory must give high priority not only to producing technically accurate data but also to reporting those data to physicians in an easily interpretable manner.

The main objective of susceptibility testing is to predict the outcome of treatment with the antimicrobial agents tested. Results are generally reported as categories of susceptibility. The implication of the result category "susceptible" is that there is a high probability that the patient will respond to treatment with the appropriate dosage regimen for that antimicrobial agent. The result "resistant" implies that treatment with the antimicrobial agent is likely to fail. One group has coined the term "90-60 rule"; that is, for many infections, we can expect treatment success about 90% of the time when the organism tests as "susceptible" to that treatment, and success may still occur in around 60% of cases when the organism tests as "resistant" to the agent used (1). The 60% apparent response rate to ineffective antimicrobials is said to reflect the natural response to many bacterial infections in immunologically healthy hosts (2).

Most test methods also include an "intermediate" category of susceptibility, which can have several meanings. With agents that can be safely administered at higher doses (e.g., penicillins, cephalosporins), this category may imply that higher doses may be required to ensure efficacy or that the agent may prove efficacious if it is normally concentrated in an infected body fluid, e.g., urine. Conversely, for body compartments where drug penetration is restricted even in the presence of inflammation (e.g., cerebrospinal fluid), it suggests that extreme caution should be taken in the use of the agent. It may also represent a buffer zone that prevents truly resistant strains from being incorrectly categorized as susceptible and vice versa. Recently, one of the standard-setting bodies (the Clinical and Laboratory Standards Institute [CLSI]) added an additional interpretive category: "susceptible dose dependent." This is designed to replace the "intermediate" interpretation where the agent is likely to be efficacious if approved dosing regimens resulting in higher exposures can be safely used (as, e.g., with many β-lactams).

A further aim of susceptibility testing is to guide the clinician in the selection of the most appropriate agent for a particular clinical problem. In most clinical settings, susceptibility test results are usually obtained 24 to 48 h or more after the patient has been given empirical treatment. The test results may confirm the susceptibility of the organism to the drug initially prescribed or may indicate resistance, in which case alternative therapy will likely be required. Where possible, the report describing the susceptibility testing results should provide the clinician with alternative agents to which the organism is susceptible. These alternatives also may be useful if the patient subsequently develops an adverse reaction to the initial antimicrobial agent. There is a growing emphasis from the professional societies, managed care organizations, and institutional stewardship programs on the use of susceptibility test results to direct therapy toward the most-narrow-spectrum, least-expensive agent to which the pathogen should respond. This is particularly true for hospitalized patients, in whom the rate of antimicrobial resistance tends to be higher, and it is easier to make therapeutic changes for inpatients than for outpatients. This makes the accuracy of susceptibility testing even more critical for effective patient care.

Clinical microbiology laboratories should perform susceptibility testing only with pathogens for which well-standardized methods are available and whose resistance is known or suspected of being a clinical problem; susceptibility testing should not be performed on normal microbiota or colonizing organisms. Currently, routine susceptibility testing methods are best standardized for common aerobic and facultative bacteria and systemic antibacterial agents. For some uncommon or highly fastidious bacteria and for most topical antibacterial agents, simple routine test methods have not been standardized. Taking into account this limitation, the CLSI has released recommendations on how some uncommon and fastidious bacteria may be tested and the results interpreted (3). With some pathogens (e.g., *Mycobacterium tuberculosis* and invasive fungi other than *Candida* spp.), routine testing is important for patient management, but testing is best performed by specialized laboratories in which test volumes are sufficient to maintain technical proficiency and where unusual or inaccurate results are likely to be recognized. Susceptibility testing methods for certain other pathogens (e.g., mycoplasmas, chlamydiae, legionellae, spirochetes, viruses, protozoa, and helminths)

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may not be well established at present and/or are limited to a few specialty laboratories. A number of choices exist in antibacterial susceptibility testing with respect to methodology and selection of agents for routine testing.

SELECTING AN ANTIMICROBIAL SUSCEPTIBILITY TESTING METHOD

Clinical microbiology laboratories can choose from among several conventional or novel methods for performance of routine antibacterial susceptibility testing. These include the broth microdilution, disk diffusion, antimicrobial gradient, and automated instrument methods. In recent years, there has been a trend toward the use of commercial broth microdilution and automated instrument methods instead of the disk diffusion procedure. However, there remains ongoing interest in the disk diffusion test because of its inherent flexibility in drug selection, its ability to respond quickly to the introduction of new agents or changes in interpretive breakpoints, and its low cost. The availability of numerous antibacterial agents and the diversity of antimicrobial agent formularies of different institutions have made it difficult for manufacturers of commercial test systems to provide standard test panels that fit everyone's needs. Thus, the inherent flexibility in drug selection that is provided by the disk diffusion test is an undeniable asset of the method. The test is also one of the most established and best proven of all susceptibility tests and continues to be updated and refined through frequent (usually annual) CLSI (4, 5) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (6, 7) publications. Instrumentation is now available for reading and interpreting zone diameters, as well as for storing this information, and may reduce interobserver reading errors (8–11).

Advantages of the microdilution and agar gradient diffusion methods include the generation of a quantitative result (i.e., an MIC) rather than a category result, the ability to test accurately some anaerobic or fastidious species that may not be tested by the disk diffusion method (3, 12–15), and the ancillary benefits of computer systems that accompany many of the microdilution and automated systems (14). Indeed, computerized management systems are very important in laboratories that may have limited or inflexible laboratory information systems. However, an MIC method should not be chosen on the basis that MICs are routinely more useful to physicians. There is no clear evidence that MICs are more relevant than susceptibility category results to the selection of appropriate antibacterial therapy for most infections (16, 17), although the integration of MIC results into a more detailed and patient-focused report has been suggested (18).

A laboratory may choose to perform rapid, automated antibacterial susceptibility testing in order to generate results faster than manual methods can generate them. The provision of susceptibility results 1 day sooner than that provided by conventional methods seems a logical advance in patient care, especially in cases of bacteremia (19–23). The impetus for more-rapid susceptibility tests has been boosted by the introduction of very rapid (same-day) mass-spectroscopic identification of bacterial pathogens into routine laboratories (24). Also emerging are rapid commercial PCR technologies capable of detecting critical resistances with same-day impact on antimicrobial treatment (25, 26). Several studies have demonstrated both the clinical and economic benefits derived from the use of rapid susceptibility testing and reporting (27–30), while a further study has not shown such a benefit (31). However, rapid susceptibility testing results may not have substantial impact unless the laboratory uses more-aggressive means of communication to make physicians aware of the results (32). A previously cited shortcoming of rapid susceptibility testing methods was the failure to detect some inducible or subtle resistance mechanisms (33–36). However, the instruments most notorious for such problems are no longer marketed, and the manufacturers of the remaining instruments have made substantial efforts to correct earlier problems (37–40) or to extend testing to include fastidious organisms (41). It is important to emphasize that accuracy should not be sacrificed in an effort to generate a rapid susceptibility testing result.

SELECTING ANTIBACTERIAL AGENTS FOR ROUTINE TESTING

Laboratories have the responsibility of testing and reporting the antimicrobial agents that are most appropriate for the organism isolated, the site of infection, and the clinical practice setting in which the laboratory functions. The battery of antimicrobial agents routinely tested and reported on by the laboratory will depend on the characteristics of the patients under care in the institution and the likelihood of encountering highly resistant organisms (14). A laboratory serving a tertiary-care medical center that specializes in the care of immunosuppressed patients may need to test routinely agents that are broader in spectrum than those tested by a laboratory that supports a primary-care outpatient practice in which antibiotic-resistant organisms are less commonly encountered.

When a laboratory's routine susceptibility testing batteries are determined, several principles should be followed. First, the antimicrobial agents that are included in the institution's formulary and that physicians prescribe on a daily basis should be tested. Second, the species tested strongly influences the choice of antimicrobial agents for testing. The CLSI publishes tables that list the antimicrobial agents appropriate for testing various groups of aerobic and fastidious bacteria (5). The guidelines indicate the drugs that are most appropriate for testing against each organism group and for treatment based upon the specimen source (e.g., cerebrospinal fluid, blood, urine). The lists also include a few agents that may be tested as surrogates for other agents because of the greater ability of a particular agent to detect resistance to closely related drugs (e.g., the use of the cefoxitin disk test to predict overall β-lactam resistance in staphylococci) (5). This initial list of agents must be tailored to an individual institution's specific needs through discussions with infectious disease physicians, pharmacists, and committees concerned with infection control and the institutional formulary (14).

A third important step in defining routine testing batteries is ascertaining the availability of specific antimicrobial agents for testing by a laboratory's routine testing methodology. Certain methods (e.g., the disk diffusion, gradient diffusion, and in-house-prepared-broth and -agar dilution methods) allow the greatest flexibility in the selection of test batteries. In contrast, some commercial systems may have less flexibility or may experience delays in adding the latest antimicrobial agents approved for clinical use. However, practicality limits the maximum number of drugs that can be tested simultaneously with an isolate by any susceptibility testing method. For example, a maximum of 12 disks can be placed on a 150-mm-diameter Mueller-Hinton agar plate, and a similar number can ordinarily be accommodated in a microdilution panel if full concentration ranges of each agent are to be included for routine determination of MICs. Most commercial test panels attempt to resolve this problem by testing a larger array of antimicrobial agents, although in a very limited concentration range (perhaps 2 to 4 dilutions for each agent).

ESTABLISHING SUSCEPTIBILITY BREAKPOINTS

There is general agreement that the MIC is the most basic laboratory measurement of the activity of an antimicrobial agent against an organism. It is defined as the lowest concentration that will inhibit the growth of a test organism over a defined interval related to the organism's growth rate, most commonly 18 to 24 h. The MIC is the fundamental measurement that forms the basis of most susceptibility testing methods and against which the levels of drug achieved in human body fluids may be compared to determine breakpoints for defining susceptibility.

The conventional technique for measuring the MIC involves exposing the test organism to a series of 2-fold dilutions of the antimicrobial agent in a suitable culture system, e.g., broth or agar for bacteria. The 2-fold-dilution scheme was originally used because of the convenience of preparing dilutions from a single starting concentration in broth or agar dilution methods. Subsequently, this system proved to be meaningful because an antimicrobial's MICs for a single bacterial species in the absence of resistance mechanisms have a statistically normal distribution when plotted on a logarithmic scale. This provides investigators with the opportunity to examine the distributions of MICs for bacterial populations and distinguish strains for which the MICs are abnormally high (above the epidemiological cutoff value [42]) from those for which MICs are normal (susceptible strains) (43; http://mic.eucast.org/Eucast2/).

MIC measurements are influenced *in vitro* by a number of factors, including the composition of the medium, the size of the inoculum, the duration of incubation, and the presence of resistant subpopulations of the organism. The *in vitro* test conditions also do not encompass other factors that can have an influence on *in vivo* antimicrobial activity. These include sub-MIC effects, postantibiotic effects, protein binding, effects on organism virulence or toxin production, variations in redox potential at sites of infection, and the pharmacokinetic changes resulting from different drug levels in blood and at the site of infection over time. Nevertheless, if determined under standardized conditions, MIC measurements provide a fixed reference point for the setting of pharmacodynamic target values with the power to predict efficacy *in vivo*. Pharmacological target values can be applied directly to routine dilution testing methods that generate MICs, such as the broth microdilution, agar dilution, and gradient methods and some automated instruments. MICs also provide reference values for deriving breakpoints for disk diffusion methods.

Breakpoints (or interpretative criteria) are the values that determine the categories of susceptible, intermediate, and resistant. The approach to setting breakpoints varies by organization or regulatory body. Depending on the approach taken, up to four sources of data can be examined in establishing breakpoints (44).

MIC Distributions

Examination of MIC distributions can indicate the range of MICs of a population of strains that lack any known mechanisms of resistance to the particular drug (wild-type population). These distributions may aid in the recognition of new resistance mechanisms by highlighting strains for which the MICs fall outside the normal distribution. However, distributions of MICs have limited direct application since they vary between species, and for some strains for which the MICs are above the normal range, the MICs may be below clinically derived breakpoints. Recent evidence suggests that such strains are still likely to respond to treatment, a position agreed to by both the CLSI and EUCAST, although this position is still controversial for some resistance mechanisms (45).

Pharmacokinetics and Pharmacodynamics

Pharmacokinetics examines the absorption, distribution, accumulation, and elimination (metabolism and excretion) of a drug in the body over time. These parameters are usually determined using healthy volunteers. A drug's MICs can be compared with the concentration of the drug achievable in blood or other body fluids (e.g., cerebrospinal fluid). In the past, breakpoints were chosen generally such that the MICs for susceptible pathogens would be exceeded by the drug level for most or all of the dosing interval. Newer data now considered when establishing breakpoints include pharmacodynamic calculations. Pharmacodynamics is the study of the time course of drug action against the microorganism. For antimicrobial agents, the desired action is pathogen eradication. *In vitro* pharmacodynamic studies have revealed that agents fall into three classes: those with principally time-dependent antimicrobial action and no or short postantibiotic effects, those with time-dependent action and long postantibiotic effects, and those with prominent concentration-dependent action (16). For drugs with time-dependent action and no or short postantibiotic effects, the critical determinant of bacterial killing *in vivo* is the percentage of time in a dosing interval that the drug concentration is above the MIC. For the other two classes, the important determinant is the ratio of the area under the concentration-time curve to the MIC and/or the ratio of the peak concentration to the MIC. For β-lactams, short-acting macrolides, and clindamycin, the relevant measure is the percentage of time that the drug concentration is above the MIC, and for aminoglycosides, long-acting macrolides, tetracyclines, glycopeptides, and fluoroquinolones, the ratio of the area under the concentration-time curve or of the peak concentration to the MIC is the relevant parameter (46, 47). These values can be used to calculate the maximum MICs or breakpoints that would allow the achievement of optimum efficacy with standard drug-dosing schedules.

Clinical and Bacteriological Response Rates

During clinical trials, the clinical and/or bacteriologic eradication response rates of organisms for which the MICs of new antimicrobial agents have been determined give an indication of the relevance of breakpoints selected by using the MIC distributions and the pharmacokinetic/pharmacodynamic properties of the drug. Response rates of at least 80% may be expected for organisms classified as susceptible, although the rates can be lower depending on the site and type of infection. While in some countries breakpoints are determined primarily from clinical and bacteriological response rates, the CLSI and EUCAST evaluate clinical and bacteriological response rates in conjunction with population distributions, pharmacokinetics, and pharmacodynamics in establishing the breakpoints in an attempt to provide the best correlation between *in vitro* test results and clinical outcome (48, 49).

Inhibition Zone Diameter Distributions for Disk Diffusion Methods

Once the MIC breakpoints are selected, disk diffusion breakpoints can be chosen by plotting the inhibition zone diameters against the MICs derived from the testing of a large number of strains of various species. A statistical approach that uses the linear-regression formula has been used in the past to calculate the appropriate zone diameter intercepts for the predetermined MIC breakpoints, but this is no longer recommended. The usual, pragmatic approach to deriving disk diffusion breakpoints is the use of the error rate-bounded method, in which the zone diameter criteria are selected on the basis of the minimization of the disk interpretive errors, especially the very major errors (50, 51) (Fig. 1). Newer statistical techniques are being studied to improve the correlation with MICs (52). The newest CLSI approach focuses on the rate of interpretive errors near the proposed breakpoint versus those with MICs more than a single $\log_2$ dilution from the MIC breakpoints (48). The concept is that errors that occur with isolates for which MICs are very close to the MIC breakpoints are less of a concern than errors with more highly resistant or susceptible strains.

Breakpoints derived by professional groups or regulatory bodies in various countries are often quite similar. For instance, there is a small number of breakpoint discrepancies between the CLSI and the U.S. Food and Drug Administration, and these are under review by both groups. However, there can be notable differences in the breakpoints used in different countries or regions for the same agents. The reasons for the differences may be that certain countries use different dosages or administration intervals for some drugs. In addition, some countries are more conservative in assessing the susceptibility to antimicrobial agents and place greater emphasis on the detection of emerging resistance, noted primarily by examination of microorganism population distributions. Technical factors, such as the inoculum density, atmosphere of incubation, and test medium, can also affect MICs and zone diameters, thereby justifying different interpretive criteria in some countries. These technical differences are summarized in chapter 71 of this *Manual*. Two non-U.S. methods minimize or avoid the use of an intermediate category of susceptibility, based on the rationale that such results are of little value to clinicians (53, 54). The lack of a buffer between susceptible and resistant categories can result in higher rates of incorrect categorization. It may be safer for a laboratory to employ a method that uses an intermediate category or, if not, to report intermediate results as resistant.

Information on a range of international susceptibility testing methods that are known to conform to the International Organization for Standards reference method and their associated breakpoint documents can be downloaded or purchased from the following websites: the CLSI website at http://www.clsi.org and the EUCAST website at http://www.eucast.org (EUCAST now sets or harmonizes breakpoints for all its member countries).

MOLECULAR DETECTION OF RESISTANCE

As highlighted in chapter 77, there is now a range of molecular techniques for the detection of many resistance genes. While none are currently recommended for routine testing, some have found a place in larger laboratories, where detection of certain important resistance genes can be implemented in a cost-effective manner. Examples include the detection of *mecA* in *Staphylococcus* species, especially *Staphylococcus aureus*, and the detection of *vanA* and *vanB* genes in *Enterococcus* species. In addition, molecular techniques are now available for the rapid detection of methicillin-resistant *S. aureus* and coagulase-negative staphylococci, vancomycin resistance genes in enterococci, and certain resistance genes in Gram-negative bacteria from positive

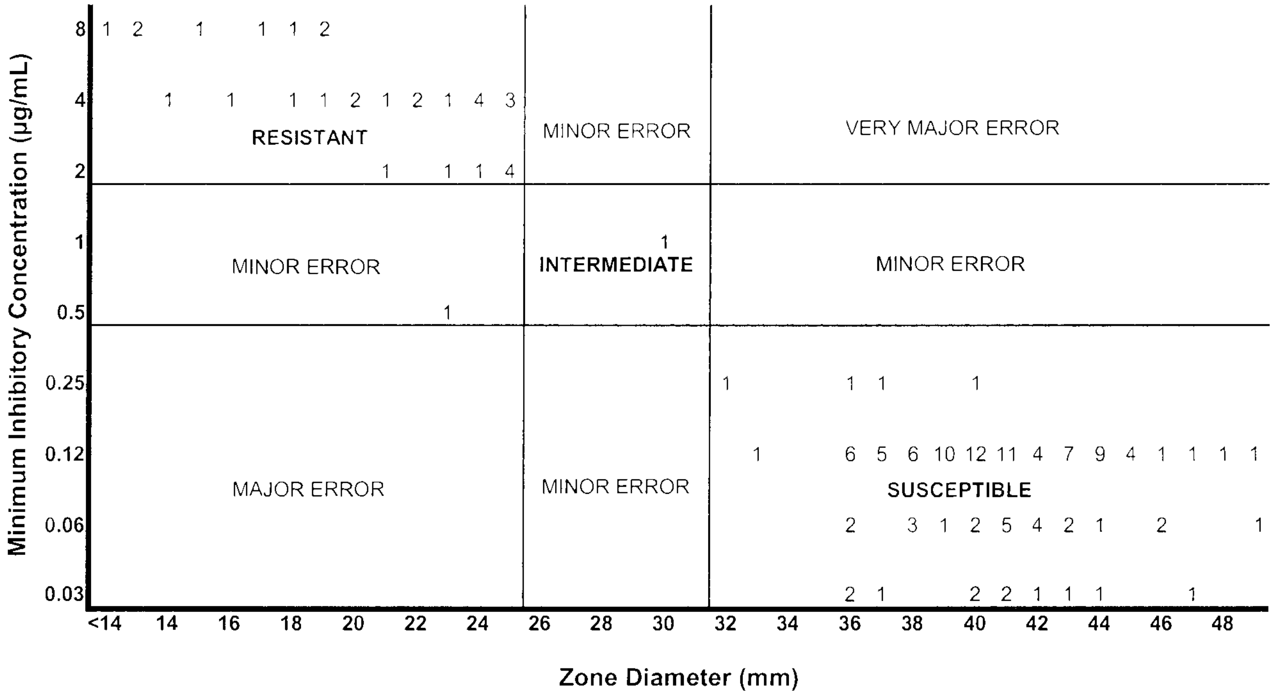

FIGURE 1 Comparison of zone diameters with MICs of a hypothetical antimicrobial agent. doi:10.1128/9781555817381.ch70.f1

blood culture bottles (55, 56). These molecular tests have been increasingly valuable for infection control purposes (57). Molecular methods are also valuable for confirming unusual resistances and for determining which mechanism of resistance is present when this has epidemiological significance. For example, there is currently great interest in the emergence and spread of plasmid-mediated varieties of carbapenem resistance (58), plasmid-mediated quinolone resistance (59), and resistance to aminoglycosides attributable to ribosomal methylases (60) in enteric Gram-negative bacteria.

SELECTED USE OF CONFIRMATORY AND SUPPLEMENTARY TESTS

While performing routine testing, a laboratory will encounter isolates or tests results (i) that are unexpected, (ii) for which there is no testing guidance available, or (iii) which are considered to be of major epidemiological importance. The CLSI and EUCAST provide some guidance on what resistances might be considered unexpected (either uncommon or never reported (5, 61). Recommendations on how to proceed vary, but if results are not attributable to simple laboratory errors and are reproducible, then testing by an alternative method and, if necessary, referral to a reference laboratory are warranted. For some key resistances, e.g., carbapenem resistance in *Enterobacteriaceae*, the most effective confirmatory method is resistance gene detection, as described in chapter 77. In other circumstances, one of the special phenotypic tests described in chapter 73 will suffice.

Inducible resistance is considered to be clinically important for a small number of antimicrobial-bacterial combinations. At present, only methods for detecting inducible resistance to clindamycin in *Staphylococcus* and *Streptococcus* species have been sufficiently evaluated and standardized to be recommended for routine laboratory use (5).

For some clinical conditions, e.g., bacterial meningitis caused by *Streptococcus pneumoniae*, susceptibility test interpretation and clinician guidance can be enhanced by performing MIC measurements if these data were not generated by the laboratory's routine method. Customized or locally prepared microtiter trays may be used or, alternatively, tested by a commercial gradient diffusion method (62).

REPORTING OF RESULTS

The reporting of results is the crucial final step in susceptibility testing. There are no universally agreed upon practices for generating reports, but the following elements should be considered. Categorical reporting, that is, reporting the test results as susceptible (S), intermediate (I), or resistant (R), is standard practice and widely understood by clinicians. When available, MIC data may be reported but should appear only along with a categorical interpretation. Susceptibility test reports should be formatted in such a way that the results are unambiguous in either a printed or an electronic form, especially if reporting more than one organism. Most importantly, so-called cascade reporting is recommended to reduce the chance of the clinician choosing a broader-spectrum antimicrobial agent inappropriately (18, 63). Cascade reporting involves the withholding of results for broader-spectrum antimicrobials from the report when the isolate tested is susceptible to narrower-spectrum agents, e.g., withholding a vancomycin result when an isolate of *Staphylococcus aureus* tests as susceptible to oxacillin or cefoxitin. Such reporting is considered to be an essential part of hospital antimicrobial stewardship programs (64, 65), as is the production of annual reports that summarize overall susceptibility and resistance patterns (antibiograms) (66).

FUTURE DIRECTIONS AND NEEDS IN ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial resistance is becoming widespread among a variety of clinically significant bacterial species (67). Therefore, microbiology laboratories play a key role in the patient management process by providing accurate data on which physicians can base therapy decisions. Susceptibility testing results, however, are also used by investigators in surveillance studies and by infection control practitioners to detect and control the spread of antibiotic-resistant organisms (66, 68). Surveillance can be performed at the laboratory, local, regional, national, and international levels through direct interchange of data from laboratory information systems to centralized databases (68). Thus, the accuracy of stored results becomes almost as important as the accuracy of test performance and interpretation.

To meet these challenges and responsibilities, clinical microbiologists must continuously assess and update their susceptibility testing strategies. The first priority is to use accurate and reliable methods, whether they are conventional or perhaps newer molecular methods. Then, careful monitoring of test performance with well-characterized control strains that challenge the capability of the testing methods becomes essential. Today, laboratories must use a variety of testing methods, each tailored specifically to a particular species or group of organisms. It is not likely that a single method, whether conventional or commercial, will be optimal for all antimicrobial agents, organisms, and resistance mechanisms. This will require increased education and training for clinical microbiologists in the future. Some assistance may be sought from the computer-based "expert" systems that allow a rapid and accurate view of antimicrobial susceptibility profiles and recognition of potentially aberrant results or novel resistance mechanisms (61). Rapid progress is also being made on molecular methods that are starting to have practical application in routine clinical laboratories (chapter 77).

More-effective means of conveying critical antimicrobial susceptibility testing information to clinicians in a time frame that allows efficient and effective management of patients and in a format that is unambiguous to clinicians in various practice specialties are still needed. Clinical microbiologists should become more proactive in the reporting of antimicrobial susceptibility results and in cross-linking that information to other databases (e.g., those for pharmacy prescriptions) to ensure that patients receive the most efficacious cost-effective therapy.

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Susceptibility Test Methods: Dilution and Disk Diffusion Methods*

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There are a number of methods for antimicrobial susceptibility testing of bacteria, which can be categorized into (i) dilution methods that generate MIC results and (ii) disk diffusion methods that generate a zone diameter result. Susceptibility testing methods can also be categorized as generic reference methods, which are described by standards-setting organizations (e.g., The Clinical and Laboratory Standards Institute [CLSI], The European Committee on Antimicrobial Susceptibility Testing [EUCAST], the British Society for Antimicrobial Chemotherapy [BSAC], and the International Organization for Standardization [ISO, Geneva, Switzerland]), and commercial methods, which are mostly mechanized or automated (e.g., MicroScan [Siemens Healthcare Diagnostics, Deerfield, IL], Vitek 2 [bioMérieux, Durham, NC], Phoenix [BD, Sparks, MD], and Sensititre ARIS [Thermo Fisher Scientific, Cleveland, OH]) or gradient diffusion methods (e.g., Etest [bioMérieux, Durham, NC] or M.I.C.Evaluators [Oxoid, Basingstoke, United Kingdom]). Generic reference methods are those in which the reagents for testing can be obtained from multiple sources and prepared in a laboratory without the need for sophisticated manufacturing processes. The CLSI reference methods are broth macrodilution, broth microdilution, agar dilution, and disk diffusion (1, 2). EUCAST advocates both broth microdilution and disk diffusion methods (http://www.EUCAST.org), and the global standard reference method described by ISO is broth microdilution (3).

The choice of methods to be used in individual laboratories is based on factors such as relative ease of performance, cost, flexibility in selection of drugs for testing, availability of automated or semiautomated devices to facilitate testing, and the perceived accuracy of the methodology (4). Reference dilution methods are typically used by pharmaceutical companies to establish MIC data for new antimicrobial agents, by research laboratories and device manufacturers as a standard by which new susceptibility testing methods are evaluated, and by reference laboratories for confirming unusual susceptibility test results. Although it has become increasingly uncommon, some clinical microbiology laboratories use reference dilution methods for routine diagnostic testing, including preparing their own broth microdilution panels or agar dilution plates. More frequently, clinical microbiology laboratories use automated systems or a combination of MIC and disk diffusion methods for routine susceptibility testing.

Interpretive categories for antimicrobial agent test results (i.e., susceptible, intermediate, susceptible-dose dependent, resistant, and nonsusceptible) are established for MIC values and then disk diffusion methods based on MIC data so that interpretive errors between methods are minimized (5). Briefly, interpretive categories, or breakpoints, are first established for MIC results generated by either the broth or agar reference method. These breakpoints are based upon the normal MIC distributions for a wild-type bacterial species, MIC distributions for strains with known resistance mechanisms affecting the drug class, pharmacokinetic/pharmacodynamic (PK/PD) data from animal models of infection and modeling of human PK/PD data, and data from clinical outcome studies. Subsequently, disk diffusion breakpoints are set by comparing disk diffusion zone diameters on a large collection of strains to MIC data and choosing breakpoints so that interpretive errors between methods are within acceptable limits (a more detailed description is provided later in this chapter). Interpretive categories for MIC methods and disk diffusion are established after both intralaboratory and interlaboratory reproducibility are verified for these methods. Since interpretive criteria of disk diffusion data are set so that there is optimal correlation with MIC results, in most cases, one method of susceptibility testing is not superior to the other. However, there are specific examples of when one method may be preferred. Daptomycin, polymyxin B, and colistin are antimicrobial agents that do not diffuse well in agar, so in most cases, disk diffusion is not an accurate method for these agents and MIC methods are recommended (6, 7). MIC tests are also recommended for susceptibility testing of staphylococci to vancomycin because vancomycin susceptibility testing by disk diffusion is not an accurate method for distinguishing vancomycin-intermediate from vancomycin-susceptible staphylococci (8). Also, MIC testing is necessary for accurate category assignment of *Streptococcus pneumoniae* isolates to penicillin, several cephalosporins, and carbapenems (9). For some bacteria, there are limited or no disk diffusion data available from well-controlled studies; thus, establishing interpretive criteria for most drugs of interest is not feasible (e.g., *Stenotrophomonas maltophilia*, *Bacillus* spp.,

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Corynebacterium spp., *Lactobacillus* spp., *Leuconostoc* spp.). An example of a disk diffusion test being preferred to MIC testing is the cefoxitin disk test to predict *mecA*-mediated β-lactam resistance in coagulase-negative staphylococci (10). The disk test is more accurate than cefoxitin or oxacillin MIC testing for detecting *mecA*-mediated resistance in those species.

When MIC testing is performed, CLSI recommends reporting the MIC value along with the interpretive category, but for disk diffusion testing, only the interpretive category should be reported. In most instances, the actual MIC value does not significantly impact patient management, except for cases of endocarditis and meningitis when MIC values direct the appropriate choice of therapy and dosage (11). However, most clinicians typically use only the interpretive category to make their treatment decisions. MIC results also can be informative for epidemiological purposes for multiply resistant isolates. For epidemiological purposes, emerging resistance mechanisms may be identified among those isolates where the antimicrobial agent MIC is above the normal MIC distribution for isolates of the same species (sometimes called epidemiologic cutoff values) but may be within the clinically susceptible or intermediate category for that agent. A valuable source of normal MIC distributions for various bacterial/antimicrobial agent combinations is the EUCAST website (http://www.escmid.org/research_projects/eu_cast/) that describes epidemiologic cutoff values separating wild-type MIC distributions from those of strains that contain a known resistance mechanism.

There are several instances where elevated, but susceptible, MIC information is useful. For example, isolates of *Enterobacteriaceae* that show elevated fluoroquinolone MICs but that are still in the susceptible category may posses a first-step fluoroquinolone mutation (of *gyrA*) or possibly a plasmid-mediated fluoroquinolone resistance mechanism that may either reduce the effectiveness of the drug, as with *Salmonella enterica* serovar Typhi or *Salmonella enterica* serovar Paratyphi infections (12), or allow the organism to survive long enough to develop high-level resistance (13). Similarly, an elevated, but susceptible-range, cephalosporin MIC in an isolate of the *Enterobacteriaceae* using the older breakpoints (pre-2010) may indicate production of an extended-spectrum β-lactamase (ESBL). When using the revised CLSI cephalosporin breakpoints for the *Enterobacteriaceae*, tests for ESBL detection are no longer required for the care of individual patients, but ESBL production is useful information for epidemiological and infection control purposes. When treating an infection caused by a multi-drug-resistant isolate, which may be susceptible only to a single antimicrobial agent, clinicians may consider using agents that give intermediate or even resistant results at an alternative dose, a different route of administration to optimize drug concentrations at the site of infection, or combinations of agents to try and effect a cure (14). An MIC result combined with agent-specific PK/PD data may help to guide this decision.

The selection of antibacterial agents for testing is complicated by the large number of agents available today and the diversity of institutional formularies. A few of these compounds, however, exhibit similar if not identical activities *in vitro*, so that in some cases, one compound can be tested as a surrogate to represent one or more closely related compounds. The number of such extrapolations has diminished over time as resistance mechanisms have complicated the predictive value of testing surrogate markers. Use of drug surrogates can reduce the number of agents required for testing and, in some cases, provide necessary flexibility in adapting commercial test systems for routine use in a different institution. For instance, the susceptibility of *Staphylococcus* spp. to oxacillin (or cefoxitin) is extrapolated to apply to all currently available penicillinase-stable penicillins, most cephalosporins (with the exception of the anti-MRSA [methicillin-resistant *Staphylococcus aureus*] cephalosporins, e.g., ceftaroline and ceftobiprole), penicillin–β-lactamase inhibitor combinations, and all current carbapenems. Thus, it is unnecessary to test any of the agents in these chemical classes other than penicillin, oxacillin, cefoxitin, and ceftaroline (1).

It is important that microbiologists work with members of the institution's pharmacy and therapeutics committee to ensure that the antibacterial agents being tested in the laboratory reflect those in the institution's current formulary (15). Doing so can contribute to the hospital's efforts to improve antimicrobial stewardship (16). Guidelines for the selection of antibacterial agents to be tested routinely are published annually by the CLSI (10) and are summarized in Table 1. While this listing is sometimes regarded rigidly as the standard for selecting the agents that must be tested, it is a list of agents that should be considered for routine testing only; many variables go into the final decision as to which agents should be tested in any particular setting (17). The CLSI also cautions that the decision about testing and reporting of some agents selectively should be made by the clinical microbiologist in conjunction with the infectious disease practitioners, the pharmacy, and/or the infection control committees (1, 10).

DILUTION METHODS

Broth and agar dilution susceptibility testing methods are used to determine the minimal concentration, usually in micrograms per milliliter, of an antimicrobial agent required to inhibit or kill a microorganism. Antimicrobial agents are usually tested at $\log_2$ (2-fold) serial dilutions, and the lowest concentration that inhibits visible growth of an organism is designated the MIC. The concentration range used may vary with the drug, the organism being tested, and the site of infection (e.g., cerebrospinal fluid in meningitis). Ranges should encompass the concentrations defining the interpretive categories (i.e., susceptible, intermediate, susceptible-dose dependent, and resistant) and also the ranges that include the expected MICs for quality control reference strains. Other dilution methods include those that test a single concentration or a selected few concentrations of antimicrobial agents (i.e., breakpoint susceptibility tests and single-drug concentration screens) (see below).

Dilution methods offer flexibility in the sense that the standard medium used to test frequently encountered organisms (e.g., staphylococci, enterococci, members of the family *Enterobacteriaceae*, and *Pseudomonas aeruginosa*) may be supplemented or even replaced with another medium to allow accurate testing of certain fastidious bacterial species that may not be reliably tested by disk diffusion (see chapters 74 and 75). Broth dilution methods form the basis of testing performed by the current automated commercial test systems (see chapter 72).

DILUTION TESTING: AGAR METHOD

Dilution of Antimicrobial Agents

The solvents and diluents needed to prepare stock solutions of most commonly used antimicrobial agents are listed in the CLSI document on dilution testing (1).

TABLE 1 Antimicrobial agents recommended for routine dilution and disk diffusion susceptibility testing[a]

Antimicrobial agent	Group[b] recommended for testing with:							
	Enterobacteriaceae	*P. aeruginosa*	Other non-*Enterobacteriaceae*[c,d]	*Acinetobacter* spp.[d]	*Burkholderia cepacia*[d]	*Stenotrophomonas maltophilia*[d]	Staphylococci[d]	Enterococci[d]
Penicillins								
Penicillin G							A	A[e,f]
Ampicillin	A							A[e,f]
Oxacillin[g] or methicillin							A	
Ticarcillin		B						
Piperacillin	B	A	A*	B				
Ampicillin-sulbactam	B			A				
Amoxicillin-clavulanic acid	B							
Piperacillin-tazobactam	B	B	B*	B				
Ticarcillin-clavulanic acid	B		B*	B	B*	B*		
Cephalosporins								
Cefazolin[h]	A							
Cefuroxime	B							
Cefoxitin	B						A[i]	
Cefotetan	B							
Cefotaxime	B		C*	B				
Ceftaroline	C						B	
Ceftriaxone	B		C*	B				
Ceftazidime	C	A	A*	A	B	B*		
Cefepime	B	B	B*	B				
Carbapenems								
Doripenem	B	B						
Ertapenem	B							
Imipenem	B	B	B*	A				
Meropenem	B	B	B*	A	B			
Other β-lactams								
Aztreonam	C	B	B*					
Aminoglycosides								
Gentamicin	A	A	A*	A			C	C[j]
Tobramycin	A	A	A*	A				
Amikacin	B	B	B*	B				
Streptomycin								C[j]
Macrolides								
Azithromycin							A	
Clarithromycin							A	
Erythromycin							A	

(*Continued on next page*)

TABLE 1 Antimicrobial agents recommended for routine dilution and disk diffusion susceptibility testing[a] *(Continued)*

Antimicrobial agent	Group[b] recommended for testing with:							
	Enterobacteriaceae	*P. aeruginosa*	Other non-*Enterobacteriaceae*[c,d]	*Acinetobacter* spp.[d]	*Burkholderia cepacia*[d]	*Stenotrophomonas maltophilia*[d]	Staphylococci[d]	Enterococci[d]
Quinolones								
Ciprofloxacin	B	B	B*	A			C	U
Levofloxacin	B	B	B*	A	B*	B	C	U
Lomefloxacin	U	U	U*				U	
Moxifloxacin							C	
Norfloxacin	U	U	U*				U	U
Ofloxacin	U	U	U*				C	
Miscellaneous								
Chloramphenicol	C		C*		B*	B*	C	
Clindamycin							A	
Daptomycin							B*	B*
Doxycycline				B			B	
Linezolid							B	B
Minocycline				B	B	B		
Nitrofurantoin	U						U	U
Rifampin							B	
Sulfisoxazole	U		U*				U	
Tetracycline	C		U*[k]	B			B[k]	U
Telithromycin								
Trimethoprim-sulfamethoxazole	B		B*	B	A	A	A	
Trimethoprim	U						U	
Vancomycin							B	B[f]

[a] Modified from the CLSI M100 document (10) with permission. Current standards and supplements to them may be obtained from the CLSI, 950 West Valley Rd., Suite 2500, Wayne, PA 19087-1898. www.clsi.org

[b] Group A comprises primary drugs to be tested and reported, group B comprises those to be tested as primary drugs but reported selectively, group C comprises supplemental drugs to be reported selectively, and group U comprises drugs to be tested with urinary isolates only.

[c] Non-*Enterobacteriaceae* include *Pseudomonas* spp. and other nonfastidious, non-glucose-fermenting Gram-negative bacilli but exclude *P. aeruginosa*, *Acinetobacter* spp., *Burkholderia cepacia*, *B. mallei*, *B. pseudomallei*, and *Stenotrophomonas maltophilia*, since there are separate listings of suggested drugs to test and report for them.

[d] Those agents marked with an asterisk (*) should be tested only by using an MIC method and not by using disk diffusion.

[e] Results of tests with penicillin apply to other penicillins (e.g., ampicillin, amoxicillin, carboxypenicillins, and ureidopenicillins) against β-lactamase-negative enterococci.

[f] Combination therapy consisting of penicillin, ampicillin, or vancomycin and an aminoglycoside is recommended.

[g] Staphylococci resistant to the penicillinase-resistant penicillins should also be considered resistant to penicillins, β-lactam–β-lactamase inhibitor combinations, cephalosporins (except cephalosporins with anti-MRSA activity, e.g., ceftaroline and ceftobiprole), and carbapenems.

[h] Cefazolin-intermediate category test results can be used to reflect susceptibility to oral agents, i.e., cefadroxil, cefpodoxime, cephalexin, and loracarbef.

[i] Cefoxitin is used as a surrogate for oxacillin for staphylococci.

[j] For use of aminoglycosides to screen enterococci for synergy resistance, see the sections "Breakpoint Susceptibility Tests" and "Resistance Screens."

[k] Doxycycline or minocycline may be tested on a supplemental basis because of their greater activities against some nonfermentative Gram-negative bacilli and staphylococci.

Preparation, Supplementation, and Storage of Media

Mueller-Hinton agar is the recommended medium for testing most commonly encountered aerobic and facultatively anaerobic bacteria (1). The dehydrated agar base is commercially available and should be prepared as described by the manufacturer. Before sterilization, the molten agar is usually distributed into screw-cap tubes in exact aliquots sufficient to dilute the desired antimicrobial concentrations 10-fold. Tubes of agar, one for each drug concentration to be tested, are sterilized by autoclaving at 121°C for 15 min, and the agar is allowed to equilibrate to 48 to 50°C in a preheated water bath. Once the molten agar has equilibrated, the appropriate volume of antimicrobial agent is added, the tube contents are mixed by gentle inversion and poured into 100-mm-diameter round or square sterile plastic petri plates set on a level surface, and the agar is allowed to solidify. For growth controls, agar plates without antimicrobial agents are also prepared. All plates should be filled to a depth of 3 to 4 mm (20 to 25 ml of agar per round plate and 30 ml per square plate), and the pH of each batch should be checked to confirm the acceptable pH range of 7.2 to 7.4 (1).

After sterilization and temperature equilibration of the molten agar, any necessary supplements are aseptically added to the Mueller-Hinton agar at the time of addition of the drug solutions. For testing of streptococci, supplementation with 5% defibrinated sheep or horse blood is recommended (1). However, sheep blood supplementation may antagonize the activities of sulfonamides and trimethoprim with some organisms (18). The presence of blood also affects results with novobiocin and nafcillin as well as the *in vitro* activities of cephalosporins against enterococci (19, 20); therefore, blood supplementation should not be used unless necessary for bacterial growth (see chapter 74 for acceptable methods for testing of fastidious bacterial species). Performance standards and the ionic composition of Mueller-Hinton agar have been defined and do not require calcium or magnesium supplementation to achieve expected results (21). The agar should be supplemented with 2% NaCl if testing of oxacillin against staphylococci is being performed (22).

Once prepared, plates should be sealed in plastic bags and stored at 4 to 8°C. In general, they should be used within 5 days of preparation or as long as the MICs for control strains that are tested concomitantly are within the acceptable ranges. However, certain agents are sufficiently labile that plates may not be stored prior to use, e.g., carbapenems, cefaclor, and clavulanic acid. Before inoculation, plates that have been stored under refrigeration should be allowed to equilibrate to room temperature, and the agar surface should be dry prior to inoculation.

Inoculation Procedures

Variations in inoculum size may substantially affect MICs; therefore, careful inoculum standardization is required to obtain accurate results. The recommended final inoculum for agar dilution is 10^4 CFU per spot (1). This may be achieved in either of two ways. Four or five colonies are picked from overnight growth cultures on agar-based medium and inoculated into 4 to 5 ml of suitable broth that will support good growth (usually tryptic soy broth). Broths are incubated at 35°C until visibly turbid, and then the suspension is diluted until it matches the turbidity of a 0.5 McFarland, barium sulfate ($BaSO_4$), or latex particle turbidity standard (ca. 10^8 CFU/ml). The 0.5 McFarland standard may be purchased or the barium sulfate standard may be prepared as described in the CLSI document (1). The accuracy of the standard should be verified by using a spectrophotometer with a 1-cm light path; for the 0.5 McFarland standard, the absorbance at 625 nm should be 0.08 to 0.13 (1). An alternative inoculum standardization method, one that is preferred by many microbiologists, utilizes direct suspension of colonies from overnight growth cultures on a nonselective agar medium in saline or broth to a turbidity that matches the 0.5 McFarland standard. This approach eliminates the time needed for growing the inoculum in broth (1). In either case, normal saline or sterile broth is used to make a 1:10 dilution of the suspension to give an adjusted concentration of 10^7 CFU/ml (1).

Once the adjusted bacterial inoculum suspension is prepared, inoculation of the antimicrobial agent plates should be accomplished within 15 min, since longer delays may lead to changes in inoculum size. By using a pipette, a calibrated loop, or more commonly, an inoculum-replicating device, 0.001 to 0.002 ml (1 to 2 μl) of the 10^7-CFU/ml suspension is delivered to the agar surface, resulting in the final desired inoculum of approximately 10^4 CFU per spot. For convenience, use of a replicator is preferred, because consistent inoculum volumes for up to 36 different isolates are delivered simultaneously (1, 23). To use this device, an aliquot of the adjusted inoculum for each isolate is pipetted into the appropriate well of an inoculum seed plate and a multipronged inoculator is used to pick up and gently transfer 1 to 2 μl from the wells to the agar surfaces. Replicators are also available that deliver only 0.1 to 0.2 μl per spot and that do not require the 0.5 McFarland standard suspension to be diluted prior to delivery to the agar surface (1). The surfaces of the agar plates must be dry before inoculation, which should begin with a growth control plate that does not contain drug. Then inoculation continues from plates with the lowest drug concentration to plates with the highest drug concentration. Finally, a second growth control plate that does not contain drug is inoculated to check for contamination or significant carryover of the antimicrobial agent. All plates should be clearly marked so that the locations of the different isolates being tested on each plate are known.

Incubation

Inoculated plates are allowed to stand for several minutes until the inoculum drops have been completely absorbed by the medium; then they are inverted and incubated in air at 35°C for 16 to 20 h before results are read. To facilitate detection of vancomycin-resistant enterococci and methicillin-resistant or vancomycin-resistant or -intermediate staphylococci, plates containing vancomycin or oxacillin should be incubated for a full 24 h before results are read (1). Incubation should not be carried out in the presence of an increased CO_2 concentration unless a fastidious organism is being tested (see chapter 74).

Interpretation and Reporting of Results

Before reading and recording the results obtained with clinical isolates, those obtained with applicable quality control strains tested at the same time should be checked to ensure that their values are within the acceptable ranges (see "Quality Control" below) and the drug-free control plates should be examined for isolate viability and purity. Endpoints for each antimicrobial agent are best determined by placing plates on a dark background and examining them for the lowest concentration that inhibits visible growth, which is recorded as the MIC. A single colony or a faint haze left by the initial inoculum should not be regarded as growth. If two or more colonies persist at antimicrobial concentrations beyond an otherwise obvious endpoint or if there is no growth at lower concentrations but there is growth at higher concentrations, the isolate should be subcultured to confirm purity and the test should be repeated. Substances that may antagonize the antibacterial activities of sulfonamides and trimethoprim may be carried over with the inoculum and cause "trailing," or less definite endpoints (18, 19). Therefore, the MICs of these antimicrobial agents should be interpreted as the endpoints at which 80% or more diminution of growth occurs. Although much less pronounced, trailing endpoints may also occur for some organisms with bacteriostatic drugs such as chloramphenicol, the tetracyclines, linezolid, and quinupristin-dalfopristin (1).

The MIC of each antimicrobial agent is usually recorded in micrograms per milliliter, although in Europe and in the international (ISO) standard reference method, the values are expressed as milligrams per liter (4, 24, 25). These quantitative results should be reported with the appropriate corresponding interpretive category (susceptible, intermediate, or resistant), or the interpretive category may be reported alone. The MIC interpretive standards for these susceptibility categories, as currently recommended by the CLSI (1), are provided in Table 2. For detailed instructions concerning the use of these criteria and categories, the latest CLSI standards for dilution testing methods should be consulted (10).

The five interpretive categories are defined as follows. Susceptible indicates that an infection caused by the tested microorganism may be appropriately treated with the usually recommended regimen of the antimicrobial agent (i.e., the appropriate dose for the recommended period of time). Intermediate indicates that the isolate may be inhibited by attainable concentrations of certain drugs (e.g., the β-lactams) if higher dosages or prolonged infusions can be used safely or if the infection involves a body site where the drug is physiologically concentrated (e.g., the urinary tract). The intermediate category also serves as a buffer zone that prevents slight technical artifacts from causing major interpretive discrepancies. Susceptible-dose dependent focuses specifically on those agents that can be safely administered in higher dosages than those used to set the susceptible breakpoint or by prolonged infusions to increase exposure times at the site of infection. Resistant isolates are not inhibited by the concentration of antimicrobial agent normally achievable with the recommended dose and/or yield results that fall within a range indicating that specific resistance mechanisms are likely to be present (1). The term "nonsusceptible" is used when no resistance breakpoint has been defined for an organism-drug combination because of the absence or rare occurrence of resistant strains (e.g., daptomycin and staphylococci).

Advantages and Disadvantages

Dilution testing by the agar method is a well-standardized, reliable susceptibility testing technique that may be used as a reference method for evaluating other testing methods. In addition, the simultaneous testing of a large number of isolates with a few drugs is efficient (such as when new agents are evaluated in the pharmaceutical industry). Microbial contamination or population heterogeneity is more readily detected by the agar method than by broth methods. The agar dilution method has been considered the reference test method in some areas of Europe (26), while broth microdilution has been much more widely used for research and clinical testing in North America (17) and is now considered the international reference method for determining MICs (3). The major disadvantages of the agar method are associated with the time-consuming and labor-intensive tasks of preparing the plates, especially if the number of different antimicrobial agents to be tested for each isolate is high or if only a few isolates are to be tested. Also, agar dilution is not always evaluated as a susceptibility testing method for newer antimicrobial agents. For example, agar dilution has not been validated for susceptibility testing of ceftaroline, daptomycin, and doripenem (10).

DILUTION TESTING: BROTH METHODS

The general approaches for broth methods include broth macrodilution, in which the broth volume for each antimicrobial concentration is ≥1.0 ml (usually 2 ml) contained in test tubes, and broth microdilution, in which antimicrobial dilutions are most often in 0.1-ml volumes in wells of 96-well microdilution trays.

Broth Macrodilution Methods

Dilution of Antimicrobial Agents

Stock solutions are prepared as discussed in the CLSI document on dilution testing (1) and are the same as those used for agar dilution tests. As in the agar method, the actual volumes used for the dilutions would be proportionally increased according to the number of tests being prepared, with a minimum of 1.0 ml needed for each drug concentration. Because addition of the inoculum results in a 1:2 dilution of each concentration, all final drug concentrations must be prepared at twice the actual desired testing concentration (see "Inoculation Procedures" below).

Preparation, Supplementation, and Storage of Media

Cation-adjusted Mueller-Hinton broth (CAMHB) is recommended for routine testing of commonly encountered nonfastidious organisms (1). Adjustment of the cations Ca^{2+} (20 to 25 mg/liter) and Mg^{2+} (10 to 12.5 mg/liter) is required to ensure acceptable results when *Pseudomonas aeruginosa* isolates are tested with aminoglycosides and when tetracycline is tested with other bacteria (27). However, for convenience and consistency, cation adjustment of Mueller-Hinton broth is now recommended for testing of all species and antimicrobial agents (1). Some manufacturers provide Mueller-Hinton broth that already has appropriate concentrations of divalent cations, so the cation content of commercial dehydrated media must be ascertained and care must be taken to supplement only those commercial broths that have not already been adjusted. If adjustment is necessary, it can be accomplished by the addition of suitable volumes of filter-sterilized, chilled $CaCl_2$ stock (3.68 g of $CaCl_2 \cdot 2H_2O$ dissolved in 100 ml of deionized water for a concentration of 10 mg of $Ca^{2}+$ per ml) and $MgCl_2$ stock (8.36 g of $MgCl_2 \cdot 6H_2O$ in 100 ml of deionized water for a concentration of 10 mg of $Mg^{2}+$ per ml) to the cooled broth (1). Insufficient cation concentrations result in increased aminoglycoside activity (28), and excess cation content results in decreased aminoglycoside activity against *P. aeruginosa* (28, 29). While the effects of inappropriate calcium and magnesium ion contents are well recognized, other ions, including zinc and manganese, may adversely affect the activities of some drugs, e.g., carbapenems (30). Other supplements to CAMHB may be required for accurate susceptibility results of specific agents. For example, accurate daptomycin testing requires a calcium supplement so that the final concentration is 50 mg/liter (1, 31) and detection of staphylococcal resistance to oxacillin requires that the CAMHB be supplemented with 2% NaCl (1, 32). Accurate

TABLE 2 Interpretive criteria (breakpoints) for dilution and disk diffusion susceptibility tests[a] for commonly prescribed antimicrobial agents

Antimicrobial agent and organism	MIC (μg/ml)			Zone diam (mm)		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Penicillins						
Penicillin G						
Staphylococci[b]	≤0.12		≥0.25	≥29		≤28
Enterococci[c]	≤8		≥16	≥15		≤14
Oxacillin[d]						
S. aureus (oxacillin tested)	≤2		≥4			
S. aureus (cefoxitin tested)	≤4		≥8	≥22		≤21
Coagulase-negative staphylococci (oxacillin tested)	≤0.25		≥0.5			
Coagulase-negative staphylococci (cefoxitin tested)				≥25		≤24
Ampicillin						
Enterococci[c]	≤8		≥16	≥17		≤16
Enterobacteriaceae	≤8	16	≥32	≥17	14–16	≤13
Amoxicillin-clavulanic acid						
Enterobacteriaceae	≤8/4	16/8	≥32/16	≥18	14–17	≤13
Ampicillin-sulbactam						
Enterobacteriaceae	≤8/4	16/8	≥32/16	≥15	12–14	≤11
Mecillinam						
Enterobacteriaceae	≤8	16	≥32	≥15	12–14	≤11
Piperacillin						
Enterobacteriaceae	≤16	32–64	≥128	≥21	18–20	≤17
P. aeruginosa	≤16	32–64	≥128	≥21	15–20	≤14
Piperacillin-tazobactam						
Enterobacteriaceae	≤16/4	32/4–64/4	≥128/4	≥21	18–20	≤17
P. aeruginosa	≤16/4	32/4–64/4	≥128/4	≥21	15–20	≤14
Ticarcillin						
Enterobacteriaceae	≤16	32–64	≥128	≥20	15–19	≤14
P. aeruginosa	≤16	32–64	≥128	≥24	16–23	≤15
Ticarcillin-clavulanic acid						
Enterobacteriaceae	≤16/2	32/2–64/2	≥128/2	≥20	15–19	≤14
P. aeruginosa	≤16/2	32/2–64/2	≥128/2	≥24	16–23	≤15
Cephalosporins						
Cefazolin						
Enterobacteriaceae	≤2	4	≥8	≥23	20–22	≤19
Enterobacteriaceae (oral cephalosporins for UTI)	≤16		≥32	≥15		≤14
Cefepime						
Enterobacteriaceae	≤2	4–8[e]	≥16	≥25	19–24[e]	≤18
Cefotaxime						
Enterobacteriaceae	≤1	2	≥4	≥26	23–25	≤22
Cefotetan						
Enterobacteriaceae	≤16	32	≥64	≥16	13–15	≤12
Cefoxitin						
Enterobacteriaceae	≤8	16	≥32	≥18	15–17	≤14
Cefpodoxime						
Enterobacteriaceae	≤2	4	≥8	≥21	18–20	≤17
Ceftaroline						
S. aureus	≤1	2	≥4	≥24	21–	≤20
Enterobacteriaceae	≤0.5	1	≥2	≥23	20–22	≤19
Ceftazidime						
Enterobacteriaceae	≤4	8	≥16	≥21	18–20	≤17
P. aeruginosa	≤8	16	≥32	≥18	15–17	≤14
Ceftriaxone						
Enterobacteriaceae	≤1	2	≥4	≥23	20–22	≤19
Cefuroxime axetil						
Enterobacteriaceae	≤4	8–16	≥32	≥23	15–22	≤14
Cefuroxime sodium						
Enterobacteriaceae	≤8	16	≥32	≥18	15–17	≤14

(Continued on next page)

TABLE 2 Interpretive criteria (breakpoints) for dilution and disk diffusion susceptibility tests[a] for commonly prescribed antimicrobial agents *(Continued)*

Antimicrobial agent and organism	MIC (µg/ml)			Zone diam (mm)		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Other β-lactams						
Aztreonam						
Enterobacteriaceae	≤4	8	≥16	≥21	18–20	≤17
P. aeruginosa	≤8	16	≥32	≥22	16–21	≤15
Doripenem						
Enterobacteriaceae	≤1	2	≥4	≥23	20–24	≤19
P. aeruginosa	≤2	4	≥8	≥19	16–18	≤15
Acinetobacter spp.	≤2	4	≥8	≥19	16–18	≤15
Ertapenem						
Enterobacteriaceae	≤0.5	1	≥2	≥22	19–21	≤18
Imipenem	≤4	8	≥16	≥16	14–15	≤13
Enterobacteriaceae	≤1	2	≥4	≥23	20–24	≤19
P. aeruginosa	≤2	4	≥8	≥19	16–18	≤15
Meropenem	≤4	8	≥16	≥16	14–15	≤13
Enterobacteriaceae	≤1	2	≥4	≥23	20–24	≤19
P. aeruginosa	≤2	4	≥8	≥19	16–18	≤15
Aminoglycosides						
Amikacin	≤16	32	≥64	≥17	15–16	≤14
Gentamicin	≤4	8	≥16	≥15	13–14	≤12
Enterococci (high-level resistance)	≤500		>500	≥10	7–9	6
Tobramycin	≤4	8	≥16	≥15	13–14	≤12
Streptomycin						
Enterococci (high-level resistance)						
Broth microdilution method	≤1,000		>1,000			
Agar-based method	≤2,000		>2,000	≥10	7–9	6
Glycopeptides						
Teicoplanin	≤8	16	≥32	≥14	11–13	≤10
Vancomycin						
Enterococci	≤4	8–16	≥32	≥17	15–16	≤14
S. aureus	≤2	4–8	≥16	≥15		
Coagulase-negative staphylococci	≤4	8–16	≥32	≥15		
Lipopeptide						
Daptomycin						
Enterococci	≤4					
Staphylococci	≤1					
Macrolides						
Azithromycin	≤2	4	≥8	≥18	14–17	≤13
Clarithromycin	≤2	4	≥8	≥18	14–17	≤13
Dirithromycin	≤2	4	≥8	≥19	16–18	≤15
Erythromycin	≤0.5	1–4	≥8	≥23	14–22	≤13
Quinolones						
Ciprofloxacin	≤1	2	≥4	≥21	16–20	≤15
Levofloxacin						
Staphylococci	≤1	2	≥4	≥19	16–18	≤15
Other organisms	≤2	4	≥8	≥17	14–16	≤13
Lomefloxacin	≤2	4	≥8	≥22	19–21	≤18
Moxifloxacin	≤0.5	1	≥2	≥24	19–23	≤20
Nalidixic acid[f]	≤8	16	≥32	≥19	14–18	≤13
Norfloxacin[f]	≤4	8	≥16	≥17	13–16	≤12
Ofloxacin						
Staphylococci	≤1	2	≥4	≥18	15–17	≤14
Enterobacteriaceae	≤2	4	≥8	≥16	13–15	≤12
Tetracyclines						
Doxycycline	≤4	8	≥16	≥16	13–15	≤12
Minocycline	≤4	8	≥16	≥19	15–18	≤14
Tetracycline	≤4	8	≥16	≥19	15–18	≤14

(Continued on next page)

TABLE 2 *(Continued)*

Antimicrobial agent and organism	MIC (μg/ml)			Zone diam (mm)		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Other						
Chloramphenicol	≤8	16	≥32	≥18	13–17	≤12
Clindamycin	≤0.5	1–2	≥4	≥21	15–20	≤14
Fosfomycin	≤64	128	≥256	≥16	13–15	≤12
Linezolid						
Staphylococci	≤4		≥8	≥21		≤20
Enterococci	≤2	4	≥8	≥23	19–22	≤20
Nitrofurantoin	≤32	64	≥128	≥17	15–16	≤14
Rifampin	≤1	2	≥4	≥20	17–19	≤16
Sulfonamide	≤256		≥512	≥17	13–16	≤12
Trimethoprim[f]	≤8		≥16	≥16	11–15	≤10
Trimethoprim-sulfamethoxazole	≤2/38		≥4/76	≥16	11–15	≤10
Tigecycline[g]						
S. aureus	≤0.5			≥19		
Enterobacteriaceae	≤2	4	≥8	≥19	15–18	≤14

[a] Adapted from CLSI data (10) with permission. The interpretive data are valid only if the methodologies in documents M2-A11 (2) and M7-A9 (1) are followed. Breakpoints for some agents apply only to certain genera or species. The CLSI annually updates the interpretive tables through new editions of the standards and supplements to them. Users should refer to the most recent editions. The current standards and supplements to them may be obtained from the CLSI, 950 West Valley Rd., Suite 2500, Wayne, PA 19087-1898. www.clsi.org

[b] Penicillin should be used as the class representative for all penicillins (e.g., ampicillin, amoxicillin, mezlocillin, piperacillin, and ticarcillin). Isolates for which MICs are ≤0.03 μg of penicillin per ml generally do not produce β-lactamase, whereas those for which MICs are ≥0.25 μg/ml do and should be regarded as resistant to penicillins. Isolates for which MICs of penicillin are 0.06 or 0.12 μg/ml should be tested for β-lactamase.

[c] Therapy for serious enterococcal infections requires high doses of penicillin or ampicillin in combination with an aminoglycoside. Vancomycin may be substituted for the penicillin in instances of penicillin hypersensitivity or of penicillin or ampicillin resistance.

[d] Oxacillin or methicillin may be tested; however, oxacillin is preferred because of its greater stability *in vitro*. The results from the testing of oxacillin apply also to other penicillinase-resistant penicillins. Oxacillin-resistant staphylococci should be considered resistant to all penicillins, cephalosporins (except cephalosporins with anti-MRSA activity), carbacephems, carbapenems, and β-lactam–β-lactamase inhibitor combinations. Disk testing with cefoxitin is the most sensitive and specific method for phenotypic detection of *mecA*-mediated oxacillin resistance in staphylococci (10, 51).

[e] For cefepime, the category "susceptible dose-dependent" applies rather than "intermediate."

[f] For the treatment of urinary tract infections (UTI) only.

[g] Interpretive criteria are from the Food and Drug Administration drug label.

susceptibility testing of tigecycline requires that the CAMHB be prepared fresh on the day of testing or frozen within 12 h of preparation (10).

To minimize evaporation and deterioration of antimicrobial agents, tubes should be tightly capped and stored at 4 to 8°C until needed. With most agents, the dilutions should be used within 5 days of preparation or as long as quality control ranges are maintained (see "Quality Control" below). As in agar dilution testing, certain β-lactam agents are too labile for prolonged storage at final test concentrations.

Inoculation Procedures

The recommended final inoculum for broth dilution testing is 5×10^5 CFU/ml. Isolates are inoculated into a broth that will support good growth (such as tryptic soy broth) and incubated until turbid. The turbidity is adjusted to match that of a 0.5 McFarland standard (approximately 10^8 CFU/ml). Alternatively, four or five colonies from overnight growth cultures on a nonselective agar plate may be directly suspended in broth to match the turbidity of the 0.5 McFarland standard (1). A portion of the standardized suspension is diluted approximately 1:100 (to 10^6 CFU/ml) with broth or saline. When 1 ml of this dilution is added to each tube containing 1 ml of CAMHB containing the 2× concentrated drug dilutions, a final inoculum of 5×10^5 CFU/ml is achieved. Broth not containing an antimicrobial agent is inoculated as a control for organism viability (growth control). All tubes should be inoculated within 30 min of inoculum preparation, and an aliquot of the inoculum should be plated to check for purity.

Incubation

Tubes are incubated in ambient air at 35°C for 16 to 20 h before MICs are determined. Incubation should be extended to a full 24 h for the detection of vancomycin-resistant enterococci and oxacillin-resistant or vancomycin-resistant or -intermediate staphylococci (1). An atmosphere with increased CO_2 should not be used.

Interpretation and Reporting of Results

Before MICs for the test strains are read and recorded, the growth controls should be examined for viability, inoculum subcultures should be checked for contamination, and appropriate MICs for the quality control strains should be confirmed (see "Quality Control" below). Growth or lack thereof in the antimicrobial agent-containing tubes is best determined by comparison with the growth control. Generally, growth is indicated by turbidity, a single sedimented button of >2 mm in diameter, or several buttons with smaller diameters. As with the agar method, trailing endpoints may be seen when trimethoprim or sulfonamides are tested, and the concentration at which 80% or greater diminution of growth, compared with that of the growth control, should be recorded as the MIC (1). Other interpretation problems include the "skipped tube" phenomenon, in which growth is not observed at one concentration but is observed at lower and higher drug concentrations. Most authorities suggest that when this occurs, the skipped tube should be ignored and the concentration that finally inhibits growth at serially higher concentrations should be recorded as the MIC. If more than one skipped tube occurs or if there is growth at higher antimicrobial concentrations but not at lower ones, the results should not be reported and the test for that drug should be repeated.

The lowest concentration that completely inhibits visible growth of the organism as detected by the unaided eye is recorded as the MIC. The CLSI MIC interpretive standards in effect as of the date of this writing (10) for the susceptibility categories are provided in Table 2. The definitions of and comments concerning these categories that were given for the agar method also pertain to the broth macrodilution method.

Advantages and Disadvantages

The broth macrodilution method is a well-standardized and reliable method that may be useful for research purposes or for testing of one drug with a bacterial isolate. However, because of the laborious nature of the procedure and the availability of more-convenient dilution systems (e.g., microdilution), this procedure is generally not useful for routine susceptibility testing in most clinical microbiology laboratories.

Broth Microdilution Method

The convenience afforded by the availability of dilution susceptibility testing in microdilution trays has led to the widespread use of broth microdilution methods. In fact, the broth microdilution method is now considered the international reference susceptibility testing method (3). The disposable plastic trays, containing a panel of several antimicrobial agents to be tested simultaneously, may be prepared in-house or obtained commercially either frozen or freeze-dried. When commercial systems are used, the manufacturer's recommendations concerning storage, inoculation, incubation, and interpretation should be followed. The primary focus of this section will be the in-house preparation and use of broth microdilution panels. However, most of the principles and practices discussed here are pertinent to the broth microdilution method regardless of the source of the antibiotic panels.

Dilution of Antimicrobial Agents

Antimicrobial stock solutions are prepared as outlined in the CLSI document on dilution testing (1). The dilution scheme for preparing broth microdilution panels is the same as that described for agar and broth macrodilution methods. Automated dispensing systems for preparation of microdilution panels use tubes that contain from 10 to 200 ml or more of broth containing each antimicrobial concentration. From the master tube dilutions, aliquots of 0.05 or 0.1 ml are simultaneously dispensed into the corresponding wells of each broth microdilution tray by using a mechanized dispenser. If 0.05-ml volumes are dispensed, allowances must be made for the 1:2 dilution of the final drug concentration that will occur when the 0.05 ml of inoculum is added (see "Inoculation Procedures" below). When 0.1-ml aliquots are dispensed, the volume of inoculum normally used is sufficiently small (≤0.01 ml) that adjustments in the antimicrobial dilution scheme are not needed. As a general rule, when the inoculum volume is less than 10% of the broth volume in the well, dilution of the antimicrobial concentration by the inoculum does not have to be taken into account (1).

Preparation, Supplementation, and Storage of Media

CAMHB is the recommended medium for broth microdilution testing of nonfastidious organisms and should be prepared as discussed above for the broth macrodilution method. Also, supplementation of the broth with 2% NaCl is required if oxacillin is to be tested on staphylococci (if testing oxacillin rather than cefoxitin) and daptomycin testing requires that the broth be adjusted to 50 mg/liter of Ca^{2+} (1). After the antimicrobial dilutions have been dispensed into the plastic trays, the panels are stacked in groups of 5 to 10, with a tray lid or an empty tray placed on top to minimize contamination and evaporation. Each stack is sealed in a plastic bag and frozen immediately, preferably at −60°C or colder, or at −20°C if a lower temperature freezer is not available. At −20°C, preservation is ensured for at least 6 weeks with most drugs, but the shelf life may be extended to months if the trays are stored at −60°C or colder. Care must be taken in storing highly labile compounds such as cefaclor, clavulanic acid, and the carbapenems, which may lose potency during storage. Trays with these agents should not be stored at temperatures warmer than −60°C. If thawed, panels must be used or discarded but not refrozen, since freeze-thaw cycles cause substantial deterioration of β-lactam antibiotics. For this reason, −20°C household type freezers with self-defrosting units must not be used.

Inoculation Procedures

As with the macrodilution procedure, the final desired inoculum concentration is 5×10^5 CFU/ml. The isolates may be grown in broth to match the turbidity of a 0.5 McFarland standard (ca. 1×10^8 to 2×10^8 CFU/ml), or a suspension of that density can be made from colonies grown overnight on a nonselective agar medium (33). For broth microdilution procedures that require 0.05 to 0.010 ml volumes to inoculate wells containing 0.1 ml of broth, a portion of the 0.5 McFarland standard suspension is diluted 1:20 (5×10^6 CFU/ml) in sterile saline or broth. Multipoint metal or disposable plastic inoculum replicators designed to collect and deliver appropriate volumes are used to transfer the inoculum from the diluted suspension to the wells of the broth microdilution tray, resulting in further dilutions ranging from 1:10 to 1:20. Final inoculum concentrations should be 4×10^5 to 6×10^5 CFU/ml (4×10^4 to 6×10^4 CFU per well). For protocols that use an inoculum volume of 0.05 ml to inoculate 0.05 ml of broth, a 1:100 dilution of a 0.5 McFarland standard suspension (ca. 10^6 CFU/ml) is used. When the inoculum is added to the wells, the 1:2 dilution of the 10^6-CFU/ml inoculum results in a final inoculum concentration of 5×10^5 CFU/ml (5×10^4 CFU per well) and also halves the antimicrobial concentration in each well. Special care should be taken to confirm the inoculum density by performing viable counts on a periodic basis to ensure that the appropriate density of inoculum is achieved. Moreover, slight deviations from the initial 1:10 dilution described above may be necessary to provide the target inoculum density with some species or organism groups. Insufficient inoculum can be a significant problem with the inducible resistance mechanisms of some organisms (such as β-lactamases) that may not be recognized as a problem based on the MICs obtained for the very susceptible quality control strains.

Broth microdilution trays should be inoculated within 30 min of inoculum preparation, and an aliquot should be subcultured to check the purity of the isolates. Finally, one well of each panel not containing an antimicrobial agent should be inoculated and used as a growth control, and a second uninoculated well serves as a sterility control.

Incubation

After inoculation, each tray should be covered with plastic tape or sealing film, sealed in a plastic bag, or tightly fitted with a lid or an empty tray to prevent evaporation during incubation. Trays are incubated in ambient air at 35°C for

16 to 20 h before results are read and should not be incubated in stacks of more than four trays for uniform temperature distribution. The incubator should be kept sufficiently humid to avoid evaporation but not so humid that condensation results in contamination problems. A full 24 h of incubation is recommended for the detection of vancomycin-resistant enterococci and oxacillin-resistant or vancomycin-resistant or -intermediate staphylococci (1). Incubation in an atmosphere with increased CO_2 should not be used with nonfastidious organisms.

Interpretation and Reporting of Results

Before MICs for the clinical isolates are read and recorded, the growth control wells should be examined for organism viability and the inoculum purity should be checked. The appropriateness of the MICs obtained for the quality control strains should be confirmed if tests of these strains were set up simultaneously with those of clinical isolates (see "Quality Control" below). Various viewing devices are available and should be used to facilitate examination of the broth microdilution wells for growth. The simplest and most reliable method is the use of a parabolic magnifying mirror and tray stand that allows clear visual inspection of the undersides of the microdilution trays. Growth is best determined by comparison with that in the growth control well and generally is indicated by turbidity throughout the well or by buttons, single or multiple, in the well bottom. The occurrence of trailing endpoints with trimethoprim or sulfonamides should be ignored, and the MIC endpoint should be based on ≥80% growth inhibition. Results for drugs with more than one skipped well should not be reported, as with the broth macrodilution test.

The CLSI MIC interpretive criteria (10) for susceptibility categories are given in Table 2. It should be noted that these values are published each year and only the most recent tables should be used for interpretation of results. The definitions of the interpretive categories and the comments concerning the use of these standards for agar and broth macrodilution methods are also applicable to broth microdilution methods.

Advantages and Disadvantages

Broth microdilution is a well-standardized reference method for antimicrobial susceptibility testing. Inoculation and reading procedures allow convenient simultaneous testing of multiple antimicrobial agents with individual isolates. Because few laboratories have the facilities required for preparation of broth microdilution trays, several sources of commercially prepared antimicrobial agent panels are available. Such products provide either frozen or freeze-dried trays with wells containing prepared antimicrobial dilutions. Frozen trays must be stored at least at −20°C in the laboratory, whereas dried panels can be stored at room temperature. Most of these products are accompanied by multipoint inoculating devices; however, the trays may be inoculated with multichannel pipetters. Results of testing may be determined by visual examination or by use of semiautomated or automated instrumentation.

Breakpoint Susceptibility Tests

While no longer very popular due to difficulties in performing adequate quality control, breakpoint susceptibility methods conserve reagents and space. Breakpoint testing refers to methods by which antimicrobial agents are tested only at the specific concentrations necessary for differentiating between the interpretive categories of susceptible, intermediate, and resistant rather than in a range of five or more doubling-dilution concentrations used to determine MICs. When two drug concentrations are selected adjacent to the breakpoints defining the intermediate and resistant categories, any one of the interpretive categories may be determined. Growth at both concentrations indicates resistance, growth at only the lower concentration signifies an intermediate result, and no growth at either concentration indicates susceptibility.

Like full-range dilution testing, breakpoint methods require the use of appropriately adjusted and supplemented Mueller-Hinton broth or agar. In addition, the standard inoculation, incubation, and interpretation procedures recommended for the full-range dilution methods should be followed.

Considering the limited range of drug concentrations tested, a greater number and variety of antimicrobial agents can be incorporated into a broth microdilution panel for breakpoint testing than into panels designed for full-range dilution testing (34). However, quality control procedures to ensure that appropriate concentrations of each antimicrobial agent are present are lacking for breakpoint panels.

Resistance Screens

In some circumstances, testing a single drug concentration may be a convenient method for detecting antimicrobial resistance. The most clinically useful resistance screens are those for high-level resistance to gentamicin and streptomycin in enterococci, resistance to vancomycin in *Enterococcus* spp. and *S. aureus* (MIC > 8 μg/ml), and resistance to oxacillin in *S. aureus* (1). These practical and reliable methods are described in chapter 73 of this *Manual*.

Gradient Diffusion Method

The Etest (bioMérieux) and the M.I.C.Evaluator (Oxoid) are commercial methods for quantitative antimicrobial susceptibility testing that incorporate a preformed antimicrobial gradient applied to one side of a plastic strip to provide drug diffusion into an agar medium. The test is performed in a manner similar to disk diffusion testing, in that a 0.5 McFarland standard suspension of a test isolate is generally swabbed onto the agar surface for inoculation. Following incubation, the MIC is read directly from a scale on the top of the strip at the point where the ellipse of organism growth inhibition intercepts the strip (4). Several strips, each containing a different antimicrobial agent, can be placed on the surface of a large round Mueller-Hinton agar plate, or they can be placed in opposing directions on large rectangular plates. MICs determined by this method generally agree well with MICs generated by standard broth or agar dilution methods (35, 36, 37). The Etests and M.I.C.Evaluators combine the simplicity and flexibility of the disk diffusion test with the ability to determine MICs of up to five antimicrobial agents on a single 150-mm-diameter round agar plate. However, both agar gradient diffusion strips are more expensive than the paper disks used for diffusion testing. Strengths of these methods include the simplicity of the procedure itself and the ability to determine an MIC of an infrequently tested drug and to test fastidious or anaerobic bacteria by applying the strips onto specialized enriched media (see chapters 74 and 75).

QUALITY CONTROL

Quality control recommendations are designed for evaluation of the precision and accuracy of test procedures, monitoring of reagent performance, and evaluation of the performance of the individuals who are conducting the tests.

Reference Strains

A critical element of quality control is the selection and use of reference bacterial strains that are genetically stable and for which MICs are in the midrange of MIC concentrations of each antimicrobial agent tested (1). That is, the dilutions in a series should ideally encompass at least two concentration increments above and below the previously established MIC for the reference strain. If there are four or fewer dilutions in a series or if nonconsecutive dilutions are tested (e.g., in breakpoint susceptibility testing), quality control for the correct interpretive category only rather than actual MIC ranges can be accomplished. *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *S. aureus* ATCC 29213 are the recommended reference strains for both agar and broth dilution MIC methods (1). The β-lactamase-producing strain *E. coli* ATCC 35218 is recommended only for penicillin–β-lactamase inhibitor combinations (1). These organisms may be obtained from the American Type Culture Collection or other reliable commercial sources. For proper storage and subculture procedures, the recommendations of either the CLSI (1) or the commercial provider should be followed.

MIC Ranges

The acceptable quality control MIC ranges for the various reference strains are given in the annual CLSI M100 supplements (10) and should be readily available in each clinical laboratory. An out-of-control result is defined as an MIC not within the acceptable range of values. Certain out-of-control results can be directly related to the medium used for testing. High MICs of gentamicin for *P. aeruginosa* ATCC 27853 suggest an inappropriately high divalent cation content or excessively low pH of the Mueller-Hinton medium, and low MICs indicate an insufficient divalent cation concentration or elevated pH. Although trimethoprim-sulfamethoxazole is not recommended for therapy of *Enterococcus faecalis* infections, results obtained with the ATCC 29212 strain can be useful for detecting excessive amounts of substances such as thymidine in the testing medium that interfere with the *in vitro* activity of antifolate drugs. Trimethoprim-sulfamethoxazole MICs of >0.5/9.5 μg/ml indicate the presence of such interfering substances (1). Extensive quality control (QC) troubleshooting guidance is available in the annual CLSI supplement (10).

Batch and Lot Quality Control

Representative plates, panels, or trays from each new reagent batch, if prepared in-house, or from each new lot, if obtained from a commercial source, should be subjected to quality control and sterility testing. Antimicrobial agent MICs obtained by testing reference quality control strains should be within acceptable CLSI ranges (10). If such accuracy is not achieved, the batch or lot should be rejected or patient results obtained with the antimicrobial agent(s) in question should not be reported (see below). Similarly, if selected uninoculated plates or trays fail the sterility check after incubation, the batch or lot should be rejected. In addition to these formal quality control procedures that use reference strains, careful review of susceptibility results obtained during daily testing of clinical isolates is important to identify aberrant or unusual susceptibility patterns possibly indicative of reagent or technical problems.

Quality Control Frequency

In addition to batch and lot testing, quality control tests should be performed daily, or at least every day that the plates or trays are being used to test clinical isolates. When quality control is performed on each day of testing, performance is considered satisfactory if no more than 3 of 30 consecutive results for each drug-reference strain combination are outside the acceptable limits. If this frequency is exceeded, the laboratory must perform corrective action to determine the source of the error and to correct it as described below. However, if daily quality control testing does not reveal an excessive rate of errors, daily testing may be replaced by weekly testing as outlined below (1, 10).

To convert to a weekly quality control testing interval, each drug-reference strain combination is tested for 20 or 30 consecutive testing days to obtain a total of 20 to 30 MIC values for each combination. If no more than 1 of 20 or 3 of 30 MICs per combination are outside the accuracy range, weekly testing may replace daily testing. Alternatively, a streamlined 15-replicate series may be performed by testing three replicates (separate inocula) of each relevant QC strain for 5 days (1). If 0 or 1 value is out of range, conversion to weekly QC testing is permitted. If 2 or 3 are out of range, testing of an additional 15 replicates is required. Recently, CLSI has recommended that the 20 to 30 tests do not have to be conducted prospectively. Instead, results of consecutive individual tests performed in the last 12 months may be tallied and the above calculation performed (1). During weekly testing, a single MIC outside the acceptable range requires that the test be repeated once. If the repeat value is within the approved range, no further action will be needed. If the single repeat value is again out of range, daily testing must be performed for five consecutive days to document a return to in control values or measures taken to resolve the problem (1, 10).

DISK DIFFUSION TESTING

The disk diffusion method of susceptibility testing allows categorization of most bacterial isolates as susceptible, intermediate, or resistant to a variety of antimicrobial agents. To perform the test, commercially prepared filter paper disks impregnated with a specified single concentration of an antimicrobial agent are applied to the surface of an agar medium that has been inoculated with the test organism. The drug in the disk diffuses through the agar. As the distance from the disk increases, the concentration of the antimicrobial agent decreases logarithmically, creating a gradient of drug concentrations in the agar medium surrounding each disk. Concomitant with the diffusion of the drug, the bacteria that were inoculated onto the surface and were not inhibited by the concentration of the antimicrobial agent in the agar continue to multiply until a lawn of growth is visible. In areas where the concentration of the drug is inhibitory, no growth occurs, forming a zone of inhibition around each disk.

The disk diffusion procedure has been standardized primarily for testing common, rapidly growing bacteria (2, 38). This method should not be used to evaluate antimicrobial susceptibilities of bacteria that show marked strain-to-strain variability in growth rates, e.g., some fastidious or anaerobic bacteria. The test, however, has been modified to allow reliable testing of certain fastidious bacterial species by incorporation of enriched media and modified incubation conditions (detailed in chapter 74).

The diameter of the zone of inhibition is influenced by the rate of diffusion of the antimicrobial agent through the agar, which may vary among different drugs depending upon the size of the drug molecule and its hydrophilicity. The zone size, however, is inversely proportional to the logarithm of the MIC, measured as discussed earlier in this chapter. Criteria currently recommended for interpreting zone diameters and MIC results for commonly used antimicrobial agents are listed in Table 2 and published annually by the CLSI (10).

Establishing Zone-of-Inhibition Diameter Interpretive Criteria

The first step in determining interpretive criteria for the disk diffusion test is selection of MIC breakpoints that define susceptibility and resistance categories for each antimicrobial agent. Zone of inhibition diameters that correspond to these breakpoints are initially established by testing 300 or more bacterial isolates by both dilution and disk diffusion methods and correlating the diameters of the zones of inhibition with the MIC values determined for each drug tested (5). Isolates tested should include not only those commonly encountered in clinical laboratories but also those with resistance mechanisms pertinent to the class of antimicrobial agent being tested (5). Organisms evaluated should be those most likely to be tested with the antimicrobial agent in question. The data from these studies are analyzed by preparing a scattergram of values (see the example in chapter 70). By convention, each MIC value ($\log_2$ scale) is plotted on the y axis, and the corresponding zone of inhibition diameter (arithmetic scale) is plotted on the x axis. Regression analysis can be performed, and a straight regression line showing the best fit is drawn. From this line, an approximation of the MIC can be inferred from any zone diameter. For antimicrobial agents to which isolates are either susceptible or resistant and only infrequently intermediate, regression analysis is not valid. In such cases, the data are plotted as a scattergram, and the interpretive standards are selected so as to allow optimal separation of resistant and susceptible populations (5, 39, 40). This approach, often called the error-rate-bounded method, may also be employed to minimize interpretive errors that can ensue from strictly applying the linear regression formula to a data set (5).

Antimicrobial Agent Disks

The amounts of antimicrobial agents in the disks used for the disk diffusion method are standardized, and in the United States, only a single concentration of each drug is recommended (10). The optimal amount of an antimicrobial agent per disk is determined early in the development of a new drug by testing disks with several different drug contents that can be evaluated by using scattergrams and regression lines (5). The most desirable concentration of a drug per disk is that which produces a zone-of-inhibition diameter of at least 10 mm with resistant isolates and a zone diameter of no larger than 30 mm with susceptible isolates.

Commercially prepared antimicrobial disks usually are supplied in separate containers, each with a desiccant. They must not be used beyond the specified expiration date and should be stored under refrigeration (2 to 8°C) or frozen in a non-frost-free freezer at −20°C or colder until needed. Disks containing a β-lactam agent should always be stored frozen to ensure that they retain their potency, although a small supply may be stored in the refrigerator for up to 1 week. Unopened disk containers should be removed from the refrigerator or freezer 1 to 2 h before use. This allows the disks to equilibrate to room temperature before the container is opened, thus minimizing the amount of condensation that will occur when warm air contacts the cold disks. A commercially available, mechanical disk-dispensing apparatus can be used and should be fitted with a tight cover, supplied with an adequate desiccant, stored in the refrigerator when not in use, and warmed to room temperature before being opened.

Agar Medium for Disk Diffusion

The recommended medium for disk diffusion testing in the United States is Mueller-Hinton agar (2). This unsupplemented medium has been selected by the CLSI for several reasons: (i) it demonstrates good batch-to-batch reproducibility for susceptibility testing; (ii) it is low in sulfonamide, trimethoprim, and tetracycline inhibitors; (iii) it supports the growth of most nonfastidious bacterial pathogens; and (iv) years of data and clinical experience regarding its performance have been accrued. Fastidious bacteria, such as *Haemophilus* species, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and streptococci, do not grow satisfactorily on unsupplemented Mueller-Hinton agar but can be tested by the disk method by using supplemented or modified test media as discussed in chapter 74.

Plates of Mueller-Hinton agar may be purchased, or the agar may be prepared from a commercially available dehydrated base according to the manufacturer's directions. If the agar is prepared, only formulations that have been tested according to, and have met, acceptance limits recommended by the CLSI should be used (21). The prepared medium is autoclaved and immediately placed in a 45 to 50°C water bath. When cool, it is poured into round plastic flat-bottomed petri dishes on a level surface to give a uniform depth of about 4 mm (67 to 70 ml of medium for 150-mm-diameter plates and 26 to 30 ml for 100-mm-diameter plates) and allowed to cool to room temperature. Agar deeper than 4 mm may cause false resistance results (excessively small zones), whereas agar less than 4 mm deep may be associated with excessively large zones and false susceptibility.

Each batch of Mueller-Hinton agar should be checked when the medium is prepared to ensure that the pH is between 7.2 and 7.4 at room temperature, which means that the pH must be measured after the medium has solidified. This can be done by allowing a small amount of agar to solidify around the tip of a pH electrode in a beaker or a cup, by macerating a sufficient amount of agar in neutral distilled water, or by using a properly calibrated surface electrode. A pH outside the range of 7.2 to 7.4 may adversely affect susceptibility test results. If the pH is too low, drugs such as the aminoglycosides, macrolides, and fluoroquinolones will appear to lose potency, whereas others (for example, the penicillins and tetracyclines) may appear to have excessive activity. The opposite effects are possible if the pH is too high.

Freshly prepared plates may be used the same day or stored in a refrigerator (2 to 8°C); if refrigerated, they should be wrapped in plastic to minimize evaporation. Just before use, if excess moisture is visible on the agar surface, plates should be placed in an incubator (35°C) or, with lids ajar, in a laminar-flow hood at room temperature until the moisture evaporates (usually 10 to 30 min). At the time the medium is to be inoculated, no droplets of moisture should be visible on the agar surface or on the petri dish lid.

Various components of or supplements to Mueller-Hinton medium may affect susceptibility test results; therefore, appropriate quality control procedures (see "Quality Control" below) must be performed and zone diameters must be within acceptable limits. For example, media containing excessive amounts of thymidine or thymine can reverse the inhibitory effects of sulfonamides and trimethoprim, causing zones of growth inhibition to be smaller or less distinct. Organisms may therefore appear to be resistant to these drugs when in fact they are not. Variation in the concentrations of divalent cations, primarily calcium and magnesium, affects results of aminoglycoside and tetracycline tests with *P. aeruginosa* isolates (2). A cation content that is too high reduces zone sizes, whereas a cation content that is too low has the opposite effect. Sheep blood should not be added to Mueller-Hinton medium for testing of nonfastidious organisms because the blood can significantly alter the zone diameters with several agents and bacterial species (19).

Inoculation Procedure

To ensure reproducibility of disk diffusion susceptibility test results, the inoculum must be standardized (2, 38). The inoculum may be prepared by the growth method or by direct suspension from colonies on the agar plate, as described above for dilution testing.

When trimethoprim-sulfamethoxazole is tested by the direct inoculum suspension method, colonies from blood agar medium may carry over enough trimethoprim or sulfonamide antagonists to produce a haze of growth inside the zones of inhibition with susceptible isolates.

The Mueller-Hinton agar plate should be inoculated within 15 min after the inoculum suspension has been adjusted. A sterile cotton swab is dipped into the suspension, rotated several times, and gently pressed onto the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The swab is then streaked over the entire surface of the agar plate three times, with the plate rotated approximately 60° each time to ensure even distribution of the inoculum. A final sweep of the swab is made around the agar rim. The lid may be left ajar for 3 to 5 min, but no longer than 15 min, to allow any excess surface moisture to be absorbed before the drug-impregnated disks are applied.

Antimicrobial Disks

Within 15 min after the plates are inoculated, selected antimicrobial agent disks are distributed evenly onto the surface, with at least 24 mm (center to center) between them. Disks are placed individually with sterile forceps or, more commonly, with a mechanical dispensing apparatus and then gently pressed down onto the agar surface to provide uniform contact. No more than 12 disks should be placed onto one 150-mm-diameter plate, and no more than 5 disks should be placed onto a 100-mm-diameter plate to avoid overlapping zones. Some of the antimicrobial agent in the disk diffuses almost immediately; therefore, once a disk contacts the agar surface, the disk should not be moved.

Incubation

No longer than 15 min after disks are applied, the plates are inverted and incubated at 35°C in ambient air. A delay of more than 15 min before incubation permits excess prediffusion of the antimicrobial agents. The interpretive standards for nonfastidious bacteria are based on results of test samples incubated in ambient air, and the zone-of-inhibition diameters for some drugs, such as the aminoglycosides, macrolides, and tetracyclines, are significantly altered by CO_2; therefore, plates should not be incubated in atmospheres with increased CO_2. Testing isolates of some fastidious bacteria, however, requires incubation in 5% CO_2, and zone diameter criteria for those species have been established on that basis (see chapter 74).

Interpretation and Reporting of Results

Each plate is examined after incubation for 16 to 18 h for all nonfastidious bacterial isolates except staphylococci and enterococci, which should be incubated for a full 24 h if oxacillin and vancomycin, respectively, are tested (2). If plates are inoculated correctly, the diameters of the zones of inhibition are uniformly circular and the lawns of growth are confluent. Growth that consists of individual isolated colonies indicates that the inoculum was too light, and the test must be repeated. The diameters of the zones of complete inhibition, including the diameter of the disk, are measured to the nearest whole millimeter with calipers or a ruler. With unsupplemented Mueller-Hinton agar, the measuring device is held on the back of the inverted petri dish, which is illuminated with reflected light located a few inches above a black, nonreflecting background.

The zone margin is the area where no obvious growth is visible with the naked eye. When isolates of staphylococci or enterococci are tested, any discernible growth (especially a haze of pinpoint colonies) within the zone of inhibition around the oxacillin disk (for staphylococci) or vancomycin disk (for enterococci) is indicative of likely resistance. For other bacteria, discrete colonies growing within a clear zone of inhibition may indicate testing of a mixed culture that should be subcultured, reidentified, and retested. However, the presence of colonies within a zone of inhibition may also indicate selection of high-frequency mutants predicting eventual resistance to that agent, e.g., *Enterobacter* spp. with penicillins and cephalosporins. Such small in-growth colonies are frequently seen when testing fosfomycin but do not appear to reflect pending resistance to that agent. With *Proteus* species, if a thin film of swarming growth is visible in an otherwise obvious zone of inhibition, the margin of heavy growth is measured and the film is disregarded. With trimethoprim, the sulfonamides, and the combination of the two agents, antagonists in the medium may allow some minimal growth; therefore, the zone diameter is measured at the obvious margin, and slight growth (20% or less of the lawn of growth) is disregarded.

The zone diameters measured around each disk are interpreted on the basis of guidelines published by the CLSI, and the organisms are reported as susceptible, intermediate, susceptible-dose dependent, or resistant (or in some cases "nonsusceptible" when no resistance breakpoint has been defined) to the antimicrobial agents tested (Table 2) (10). The clinical interpretation of the categories of susceptible, intermediate, susceptible-dose dependent, and resistant has already been provided above under "Dilution Methods." Computer programs are available that accompany some automated zone size reading devices to allow MICs to be derived from the linear regression equation with selected antimicrobial agents and bacterial isolates (41, 42) (see also chapter 72).

Advantages and Disadvantages

The disk diffusion test has several advantages: (i) it is technically simple to perform and very reproducible, (ii) the reagents are relatively inexpensive, (iii) it does not require any special equipment, (iv) it provides susceptibility category results that are easily understood by clinicians, and (v) it is flexible regarding selection of antimicrobial agents for testing. The primary limitation of the disk diffusion test is the spectrum of organisms for which it has been standardized. There have not been adequate studies to develop reliable interpretive standards for disk testing of bacteria not listed in the CLSI disk diffusion document (2) or the CLSI guideline for infrequently isolated or fastidious bacteria (43). It is also important to note that only certain drugs have been validated for disk diffusion testing of *Stenotrophomonas maltophilia* and *Burkholderia cepacia* (10). The disk test is inadequate for detection of vancomycin-intermediate *S. aureus* (2, 44, 45) and is no longer recommended for testing staphylococci, does not detect daptomycin resistance in staphylococci and enterococci (24, 46) or colistin resistance in Gram-negative bacilli other than possibly *P. aeruginosa* (6), and in the past, prior to the recommendation to test cefoxitin, was reported to have difficulties in the detection of oxacillin-heteroresistant staphylococci (47) and enterococci with low-level (Van B-type) vancomycin resistance (45, 48). A potential disadvantage of disk diffusion susceptibility testing is that it provides only a qualitative result, and a quantitative result indicating the degree of susceptibility (MIC) may be needed in some cases, e.g., those involving penicillin and cephalosporin susceptibilities of *S. pneumoniae* and certain viridans group streptococci (see chapter 74).

Quality Control

The goals of a quality control program for disk diffusion tests are to monitor the precision and accuracy of the procedure, the performance of the reagents (medium and disks), and the performance of persons who do the testing and read, interpret, and report results. To best achieve these goals, reference strains are selected for their genetic stability and their usefulness in the disk diffusion test.

Reference Strains

Reference strains recommended by the CLSI for quality control of the disk diffusion procedure when nonfastidious bacteria are tested are *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923 (not the same strain used for quality control of MIC tests), *Enterococcus faecalis* ATCC 29212, and *E. coli* ATCC 35218 (10, 49). *E. coli* ATCC 35218 is recommended as a control only for β-lactamase inhibitor combinations containing clavulanic acid, sulbactam, or tazobactam. *Enterococcus faecalis* ATCC 29212 can be used to ensure that the levels of inhibitors of trimethoprim or sulfonamides in Mueller-Hinton agar do not exceed acceptable limits and can also be used to control disks containing a high concentration of gentamicin or streptomycin (see chapter 73).

The reference strains listed above should be obtained from a reliable source, and stock cultures should be maintained in such a way that viability is ensured and the opportunity for selection of resistant variants is minimal (49). The procedures for maintaining and storing working stock cultures are described in the CLSI standard (2). If an unexplained result indicates that the inherent susceptibility of the strain has been altered, a fresh subculture of that organism should be obtained.

Zone-of-Inhibition Diameter Ranges

The ranges of zone diameters for reference strains used to monitor performance of the disk diffusion test are updated frequently and published annually; therefore, readers should refer to the most recent CLSI document for this information (10). Generally, results of 1 in every 20 tests in a series of tests might be out of the accepted limits. If a second result falls outside the stated limits, corrective action must be taken. The action taken and the results of that action must be documented.

Frequency of Testing

Each new batch or lot of Mueller-Hinton agar must be tested with the reference strains listed above before the medium is released for use with clinical specimens, and quality control must be done before or at the same time as a new lot of antimicrobial disks is placed in use. Appropriate reference strains also should be tested each day that the disk diffusion test is performed. The frequency of testing, however, may be reduced if satisfactory performance is documented for 20 or 30 consecutive days of testing: for each combination of drug and reference strain, no more than 1 of 20 or 3 of 30 zone-of-inhibition diameters may be outside the accepted limits published by the CLSI (10). The streamlined 15-replicate protocol described above for MIC testing is also an acceptable alternative approach for demonstrating proper performance prior to converting to weekly QC testing (10). When either of these criteria are fulfilled, each reference strain need be tested only once per week and any time a reagent component of the test is changed. However, if the diameter of a zone of inhibition falls outside the acceptable control limits, corrective action must be taken. If the problem appears to be caused by an obvious error such as use of the wrong disk or the wrong reference strain, contamination of the reference strain, or incubation in an incorrect atmosphere, or even if there is no obvious reason for an out-of-control value, repeating the test one time is acceptable. However, if the repeat value is again out of control, quality control must be performed daily for a period that will allow discovery of the source of the aberrant result and documentation of how the problem was resolved. This may be accomplished by the same approach described above under “Quality Control” in “Dilution Methods.”

Special Disk Tests

Two specialized applications of the disk test are described in detail in chapter 73. In brief, disk testing with cefoxitin is now the recommended method for detection of *mecA*-mediated oxacillin resistance in both *S. aureus* and coagulase-negative staphylococcal species (2, 10, 50, 51). Cefoxitin disk testing serves as a surrogate marker for the principal mechanism of oxacillin resistance in staphylococci and provides more reliable results than oxacillin disk testing itself. Secondly, inducible clindamycin resistance is not reliably detected by standard dilution or disk diffusion susceptibility testing without induction of the expression of *erm*-mediated macrolide-lincosamide-streptogramin B resistance in staphylococci, hemolytic streptococci, and *S. pneumoniae* (10, 52, 53). Such strains can be accurately detected only by induction of resistance expression by exposure to a macrolide. A disk approximation test in which erythromycin and clindamycin disks are placed in close proximity allows recognition of inducible resistance by truncating the clindamycin zone and giving rise to a positive “D-zone test” (10, 54). Alternatively, single-well microdilution tests have been described that include fixed concentrations of erythromycin and clindamycin in the same well for testing staphylococci or streptococci (53, 55, 56). When recognized through either method of induction testing, such strains should be regarded as resistant to clindamycin (10, 52).

ANTIBACTERIAL SUSCEPTIBILITY TESTING AND INTERPRETAIVE METHODS USED OUTSIDE THE UNITED STATES

The CLSI is best known for developing laboratory testing standards for use in the United States, including those for antimicrobial susceptibility testing (1, 2, 10). The CLSI standards are recognized as U.S. national standards by the American National Standards Institute and by federal regulations, including the Clinical Laboratory Improvement Amendments (25), and as standard reference procedures by the Food and Drug Administration. However, the CLSI procedures are also used by an increasing number of laboratories outside the United States, including countries in North and South America and in several areas of Europe, Asia, and Australia. Some countries have national standards or professional committees comprising their own expert microbiologists that establish methods of susceptibility testing for their countries and interpretive criteria for those tests that may or may not be the same as those of the CLSI (57, 58), as described further in chapter 72.

Several variations on dilution and diffusion methods are used for routine susceptibility testing outside the United States (Table 3). Most non-U.S. methods are specific to individual countries, having developed and evolved locally over many years. Many of the non-U.S. methods differ from the CLSI procedures in the choice of media, inoculum preparation procedures, and, for diffusion methods, disk contents. There are also some variations between these methods in breakpoints and the approaches to establishing the breakpoints (59). Variation in test methods can cause considerable confusion in laboratories, especially if both CLSI and non-CLSI methods are used for different organisms. Thus, it is important that any method should be followed in all its detail for valid application of specific breakpoints.

There are continuing efforts to harmonize breakpoints internationally. In Europe, EUCAST has become a major force for developing and harmonizing methods and breakpoints for aerobic and facultative organisms. EUCAST has defined reference agar and broth microdilution MIC methods (www.eucast.org) for use in Europe (60) and is in the process of defining disk diffusion methods. The establishment of a global standard reference MIC susceptibility method (broth microdilution) developed through the ISO (Geneva, Switzerland) (3) in concert with the European Committee on Standardization (Brussels, Belgium) has also occurred recently. The global reference method is in essence the CLSI and EUCAST broth microdilution methods with a few differences harmonized in the ISO standard (3). This will hopefully provide a degree of international standardization for reference MIC determinations and allow a benchmark for assessment of the performance of commercial devices for susceptibility testing. In a second document, the ISO has established criteria for acceptable performance of susceptibility testing devices (61).

International Dilution Methods

Both broth and agar dilution methods have been developed in multiple countries outside the United States. In the past, methods using a limited range of concentrations (often one or two), or so-called breakpoint methods, have been advocated (62). They are the standard form of susceptibility testing advocated by the Japanese Society for Chemotherapy (63, 64) and as an alternative to disk diffusion testing by the BSAC (65). Other MIC methods and standards include those of the BSAC (66) (see also http://www.bsac.org.uk), the Société Française de Microbiologie (CA-SFM—Comité de l'Antibiogramme de la SFM; http://sfm-microbiologie.org), the Deutsches Institut für Normung (DIN; documents are available through http://www.beuth.de), and the Swedish Reference Group for Antibiotics (SRGA; http://www.srga.org).

Breakpoint MIC methods (or more commonly, MIC methods with limited concentration ranges) are often used in larger laboratories because large numbers of isolates can be tested cost-effectively using replicators and the methods provide qualitative endpoints (i.e., susceptible, intermediate, and resistant). Optical readers are available to facilitate reading of agar dilution plates (e.g., Mastascan Elite; Mast Laboratories, Bootle, United Kingdom [http://www.mastascan.com]). However, there are considerable difficulties with quality control, including the lack of appropriate control strains for which the MICs are near the breakpoints, and the complexity of quantifying drug concentrations prior to use (67). In addition, problems have been reported in the past with the use of Iso-Sensitest agar (68, 69) and with the incorporation of inhibitors to prevent swarming of *Proteus* spp., such as *p*-nitrophenylglycerol (70), or increased agar content for the same purpose (71). In turn, the choice of Mueller-Hinton by the CLSI has been criticized for not providing luxuriant growth of all organisms (72), suggesting

TABLE 3 Non-United States disk diffusion methods for susceptibility testing

Method (reference)	Location	Society	Agar medium	Comments
EUCAST[a]	Europe	EUCAST/ECDC/EMEA	MH[b] and MH + 5% defibrinated horse blood and 20-mg of β-NAD/liter	Published 2009 on EUCAST website; correlates to European clinical breakpoints
BSAC working party on antimicrobial susceptibility testing (66)	United Kingdom	BSAC	Iso-Sensitest	Very similar to the SRGA method; EUCAST clinical breakpoints
CA-SFM (76)	France	CA-SFM	MH + semiconfluent inoculum	EUCAST clinical breakpoints
DIN (77, 78)	Germany	DIN	MH	EUCAST clinical breakpoints
SRGA (79, 82)	Sweden	SRGA	Iso-Sensitest	Very similar to the BSAC method; EUCAST clinical breakpoints
CDS (83, 84)	Australia		Sensitest	Dichotomous (no intermediate category); disk strengths chosen to give annular radius of inhibition of ~17 mm where possible

[a] www.eucast.org.
[b] MH, Mueller-Hinton agar.

that there is no ideal medium. As the best-studied, most widely used medium for susceptibility testing, Mueller-Hinton broth was chosen for the ISO reference method (3) and is supported by the EUCAST (see below).

International Diffusion Methods

A wide variety of diffusion methods have been developed in different countries over the years. They are quite diverse in their approaches. With the exception of the Danish method, which uses 9-mm tablets containing the antimicrobial agents, the other international diffusion methods use disks similar to CLSI. Almost all have been maintained primarily because of the cost-effectiveness and flexibility of diffusion testing. Many, including CA-SFM and the newly developed EUCAST disk diffusion method (www.eucast.org) are similar to the modified Kirby-Bauer method (38) advocated by the CLSI (2). As pointed out above, disk methods are inexpensive and flexible and have become more popular with the use of automated zone readers and interconnected computers for interpretation of zone diameters (42, 68). Some of the automated readers also contain "expert" software for antibiogram interpretation.

The Stokes' method, which had previously been recommended by the BSAC, differed from other diffusion methods in that susceptibility categorization was achieved by comparison with a control strain rather than by reference to a defined set of zone diameters (73). This technique attracted criticism because it was not based upon or derived from correlations with MICs (74) and has now been replaced by a correlated diffusion method (66), which is now well developed and updated periodically (http://www.bsac.org.uk). For the new BSAC method, Iso-Sensitest (Oxoid, Basingstoke, United Kingdom) is the recommended agar, supplemented for the growth of fastidious bacteria with whole defibrinated horse blood, with or without NAD. Mueller-Hinton supplemented with 5% sodium chloride is recommended for the detection of methicillin and oxacillin resistance in staphylococci. The inoculum is prepared to produce semiconfluent growth only, rather than the confluent lawn of growth as recommended in the CLSI method. One important change in the newer British method is the elimination of an intermediate category for most organism-antimicrobial agent combinations (75).

Since 1980, the CA-SFM has put considerable effort into standardization of susceptibility testing, including regular updates with breakpoints for new drugs that are published frequently (76) (see also http://sfm-microbiologie.org). Like the CLSI, the CA-SFM has selected Mueller-Hinton as the testing medium. For diffusion testing, plates can be inoculated either by flooding or swabbing with a standardized inoculum of cells. In most other aspects, this method resembles that of the CLSI, including the use of control organisms and the choice of disk strength. The CA-SFM provides zone diameter breakpoints for drugs available in France and elsewhere that are not approved for clinical use in the United States, e.g., fusidic acid and pristinamycin (76). The French breakpoints often differ from those advocated by CLSI and place greater emphasis on the detection of emerging resistance. Thus, they are more likely to classify organisms as resistant (or nonsusceptible) than CLSI methods.

The German standards organization, DIN, published methods for diffusion susceptibility testing as early as 1979, with intermittent updates since then (77, 78). They too use Mueller-Hinton agar but will include the use of other media provided that the MIC-zone diameter relationships have been determined for those media. Like the CA-SFM method, the DIN method has much in common with the CLSI method.

The SRGA (79, 80) method uses Iso-Sensitest, and it is based on the methodology developed by the original International Collaborative Study (81) that was the first to provide a sound theoretical basis to diffusion susceptibility testing. The breakpoints for susceptibility were restructured in 1981 into the more conventional susceptible, intermediate, and resistant categories (80) and were subsequently updated (82).

A method developed by a commercial firm in Denmark differs technically if not in principle from the other methods. As noted above, this method employs Neo-sensitabs that are compressed tablets, 9-mm in diameter, into which the antimicrobial agent has been incorporated (Rosco Diagnostica, Taarstrup, Denmark; http://www.rosco.dk). The method and the interpretive zone diameter criteria are updated and published by the manufacturer periodically. Not only are the tablets larger and thicker than conventional 6-mm-diameter paper disks, they usually contain larger amounts of antimicrobial agent to be tested, resulting in significantly larger zones of inhibition with most drugs. This has the disadvantage of reducing the number of tablets that can be put on a single plate and still produce readable zones. However, the system does have the advantage that the tablets can be stored at room temperature for up to 4 years, obviating the need for storage under refrigeration or freezing. This is an obvious benefit for laboratories in developing countries where reliable refrigeration and power can be a problem.

The calibrated dichotomous sensitivity (CDS) disk diffusion method developed in Australia in 1975 (83, 84) is still widely used in that country and has a number of unique features. The CDS method employs Sensitest agar and an unusual method for inoculum preparation and is unique in defining just two categories of susceptibility: susceptible and resistant. To simplify test result reading, each new drug is calibrated against the MIC breakpoint to yield, wherever possible, a zone diameter of 18 mm. This is achieved by adjusting disk strengths, which in most cases are substantially lower than those used with other methods. The lack of an intermediate category, which increases the risk of serious interpretive errors (e.g., susceptible instead of resistant), the absence of some common drugs from the test range, and some unusual use of surrogate drugs for testing have restricted the adoption of this method outside Australia. The interpretative criteria are updated regularly and can be found on the Internet (http://web.med.unsw.edu.au/cdstest/).

EUCAST Methods

Outside CLSI, EUCAST is probably the most widely recognized breakpoint setting organization. EUCAST is a standing committee of the European Society for Clinical Microbiology and Infectious Diseases (ESCMID) and the European Centre for Disease Prevention and Control (ECDC). It consists of two bodies: the EUCAST Steering Committee and the EUCAST General Committee. EUCAST emerged as a concerted effort to harmonize susceptibility testing across Europe into a single unified approach (60). Although there are many similarities between EUCAST and CLSI methods, there are also some differences. Both methods are based on the use of cation-adjusted Mueller-Hinton broth for broth microdilution and Mueller-Hinton agar for disk diffusion. For more fastidious organisms, EUCAST has developed a single medium, MH-F (Mueller-Hinton-Fastidious), Mueller-Hinton supplemented with 5% mechanically defibrinated horse blood and 20-mg/liter β-NAD in place of CLSI's *Haemophilus* test medium, and Mueller-Hinton broth and agar supplemented with 5% sheep or horse blood for other fastidious organisms (e.g., streptococci). For disk diffusion, EUCAST has also selected lower disk masses for certain organism-antimicrobial agent combinations. Quality control procedures are very similar, with essentially the same control strains and quality control ranges, except for disk diffusion, where the test medium and/or disk masses differ.

In recent years, there has been a growing consensus between EUCAST and CLSI about data sets and methods for breakpoint setting. Both organizations agree that microbiological, pharmacokinetic-pharmacodynamic, and clinical outcome data are major considerations when selecting breakpoints. In addition, EUCAST has formalized its approach to establishing epidemiological (wild-type) cutoff values (59) (see http://www.eucast.org), while this is implicit in CLSI's approach. However, while there has been a slow convergence of breakpoints themselves, there are still a number of differing breakpoints for a range of reasons apart from methodological ones (such as different disk masses or test medium). These reasons include (i) differing standard dosing regimens within Europe and between Europe and the United States, (ii) access to different data sets during the breakpoint setting process, and (iii) differences in the time that the breakpoint setting process occurred (some CLSI breakpoints were set before the full suite of data sets were available, especially the more recent information on pharmacokinetics-pharmacodynamics). It is likely that breakpoints will continue to converge as methods for breakpoint setting become more standardized internationally.

COMMON SOURCES OF ERROR IN ANTIBACTERIAL SUSCEPTIBILITY TESTING

Potential sources of error in antibacterial susceptibility testing may be categorized as those that relate to the test system and its components, those associated with the test procedure, those peculiar to certain organism and drug combinations, and those that relate to reporting (16, 85, 86). The most common sources of error encountered in clinical microbiology laboratories are reviewed in the following paragraphs.

Various components of the susceptibility test system may be a source of error. First, the system itself may have limitations regarding the organisms that should be tested. For example, the disk diffusion method should be used only to test rapidly growing bacterial pathogens that have consistent growth rates (those for which interpretive criteria have been developed and published by the CLSI). Second, the medium used may be a source of error if it fails to conform to recommended composition and performance. Factors common to both agar-based and broth-based systems are the pH of the medium, which for Mueller-Hinton agar or broth should be between 7.2 and 7.4, and their cation content. The concentration of magnesium and calcium in the broth medium should be that recommended by the CLSI to ensure reliable results (1). The Mueller-Hinton agar should be 3 to 4 mm deep for agar dilution and 4 mm deep for disk diffusion (1, 2). Third, the components of the system (antimicrobial disks, agar plates, and trays) must be stored properly, and they should not be used beyond the stated expiration dates.

Steps in the susceptibility test procedure that may be a source of error if they are not performed correctly include inoculum preparation, incubation (conditions and duration), endpoint interpretation, and performance of appropriate quality control. The inoculum must be a pure culture, and it must contain an adequate density of bacteria. With rare exceptions, all systems should be incubated in ambient air at 35°C. The incubation time, however, varies. For conventional dilution and disk diffusion systems, incubation for 16 to 20 h and 16 to 18 h, respectively, is recommended, except in tests of staphylococci with oxacillin and vancomycin (MIC only) and enterococci with vancomycin, in which systems must be incubated for a full 24 h (1, 2, 87). The endpoints for all susceptibility tests must be measured accurately, following guidelines published by the CLSI (1, 2). If endpoints are interpreted by an instrument, the reliability of that instrument must be monitored and calibrated periodically. Moreover, with all susceptibility test systems, appropriate reference strains must be tested at regular intervals, any problems that occur must be thoroughly investigated, and corrective action must be well documented.

Testing of certain antimicrobial agents with some bacteria may yield misleading results because *in vitro* results do not necessarily correlate with *in vivo* activity. Examples include aminoglycosides and narrow- and expanded-spectrum cephalosporins tested with *Salmonella* spp. and *Shigella* spp.; all β-lactam agents except penicillin, oxacillin, cefoxitin, and ceftaroline with staphylococci; cephalosporins, aminoglycosides (except concentrations used to detect high-level resistance), clindamycin, and trimethoprim-sulfamethoxazole tested with enterococci; and cephalosporins tested with *Listeria* spp. (1, 2, 10). Therefore, for these combinations of organisms and drugs, results should not be reported. Other errors associated with reporting include possible transcriptional errors for laboratories that use a manual recording and reporting system and possible errors in transmission of data for laboratories in which an automated susceptibility test system is interfaced with the laboratory and/or hospital information system.

For several species of bacteria, resistance to a commonly tested antimicrobial agent is expected. For example, several species of *Enterobacteriaceae*, including *Klebsiella*, *Citrobacter*, and *Enterobacter* spp., among others, should test as ampicillin resistant (10). Likewise, *Stenotrophomonas maltophilia* should test as resistant to carbapenems, and *Pseudomonas aeruginosa* should test as resistant to trimethoprim-sulfamethoxazole (10). A susceptible result for any of these bacterium-antimicrobial combinations could indicate an error in antimicrobial susceptibility testing or bacterial identification, and the tests (including identification when necessary) should be repeated.

PROBLEM ORGANISMS AND RESISTANCE MECHANISMS

The dilution and diffusion methods described in this chapter have been developed through careful studies and standardized by national professional organizations and diagnostic device manufacturers. Despite this, there are still some organisms for which methods have not yet been standardized (e.g., coryneform bacteria and some fastidious bacteria in the case of disk testing) or which fail to provide reliable results with some of the standard tests (e.g., *Stenotrophomonas maltophilia* and *Burkholderia cepacia* in the case of disk diffusion testing of some drugs).

Other problems occur with detecting resistance mechanisms in isolates that may possess inducible resistance (e.g., clindamycin resistance in staphylococci and some streptococci, Van B-type resistance in some enterococci or AmpC-type β-lactamase in some Gram-negative species). Similarly, there are issues with detection of resistance mechanisms that result in subtle phenotypic expression under standard inoculum and test conditions (e.g., extended-spectrum β-lactamase [ESBL] or carbapenemase expression in some *Enterobacteriaceae*). Special methods to detect these resistance mechanisms are outlined in chapter 73. Controversy has existed regarding the need to identify the resistance mechanisms such as ESBLs or carbapenemases for making treatment decisions. For example, revised CLSI cephalosporin, aztreonam, and carbapenem interpretive criteria that are consistent with recent pharmacokinetic and pharmacodynamic data and the most common dosing regimens obviate the need for ESBL or carbapenemase confirmation before a treatment decision is made (10). It is the MIC that seems most predictive of therapeutic outcome in an individual patient as opposed to the resistance mechanism present in the patient's infecting strain. However, there may still be a need to identify certain resistance mechanisms (especially those that are plasmid mediated) for epidemiological and infection prevention purposes.

There has not previously been uniform agreement regarding what level of accuracy is acceptable when selecting a testing method or system for performing antimicrobial susceptibility testing (17). However, general guidelines for acceptable performance (e.g., rates of essential agreement and category agreement) now have been developed through an international consensus effort (ISO) (3). However, it is important to keep in mind that new resistance mechanisms or decreases in susceptibility to important therapeutic agents can arise at any time to challenge our methods of susceptibility testing, e.g., the emergence of carbapenemase-mediated resistance in *Enterobacteriaceae* (88). Thus, susceptibility testing methods must continue to evolve and develop over time as new challenges are presented.

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Antimicrobial Susceptibility Testing Systems*

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Commercial antimicrobial susceptibility testing (AST) systems were introduced into clinical microbiology laboratories during the 1980s and have been used in the majority of laboratories since the 1990s (1). Manual and semiautomated broth microdilution systems are utilized for small volumes of susceptibility testing, while larger laboratories often choose an automated broth microdilution system. Most AST systems also perform organism identification as described in chapter 4. Only one semiautomated system for disk diffusion testing is marketed in the United States; additional systems are available in other countries. The AST systems include data management software that may be interfaced with a laboratory information system (LIS) and offer various levels of expert system and epidemiological analyses (2).

The U.S. Food and Drug Administration (FDA) provides regulatory oversight for AST systems marketed in the United States. Susceptibility test systems are classified as class II medical devices (subject to general controls) and require premarket notification with a 510(k) submission for FDA clearance (3, 4). Automated short-incubation (<16 h) AST systems are subject to additional special controls (4).

A 510(k) submission must demonstrate that a device is substantially equivalent to other devices marketed in the United States. The FDA (4) recommends a multicenter comparison of an AST system to the Clinical and Laboratory Standards Institute (CLSI) reference method (5, 6). The level of performance considered acceptable for each antimicrobial agent-organism combination is >89.9% categorical agreement (same susceptible, intermediate, or resistant classification using FDA breakpoints), >89.9% essential agreement (MIC results within 1 dilution of the reference method), ≤1.5% very major errors (VME; false susceptibility based on the number of resistant organisms), and ≤3% major errors (ME; false resistance based on the number of susceptible isolates) (4). Any antimicrobial agent-organism combination not meeting these standards must be listed as a limitation in the package insert with a recommendation to use an alternative method. Limitation statements are also required if the evaluation did not include a sufficient number of resistant organisms, showed unacceptable (<95%) reproducibility, or showed an elevated "no growth" rate (>10%) for an organism group (4). It is important to consult individual instrument/panel package inserts for specific antimicrobial agent-organism limitations, as significant differences exist between systems.

The reporting of AST results for antimicrobial agents without proven clinical efficacy against the specific organisms listed in the pharmaceutical package insert is discouraged (4). Manufacturers apply FDA interpretive standards when evaluating AST results (4). When FDA interpretive criteria change, manufacturers must perform a comparative study, and if the new breakpoints affect device performance, a new 510(k) submission is required (7). Changes to existing FDA interpretative criteria are generally driven by CLSI interpretative criteria changes and, subsequently, clinical laboratory expectations. If an appropriate validation is completed, laboratories may report results using interpretive criteria other than those published in the AST device label (8). Current information describing FDA regulations and a list of approved devices may be found at http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance.

This chapter focuses primarily on commercial susceptibility testing systems currently available in the United States. The broth microdilution AST systems are manufactured by four companies: bioMérieux (Durham, NC; http://www.biomerieux-usa.com), Siemens Healthcare Diagnostics (Deerfield, IL; http://www.siemens.com), Becton Dickinson Diagnostics (Sparks, MD; http://www.bd.com), and Thermo Scientific (Cleveland, OH; http://www.thermoscientific.com; formerly TREK Diagnostic Systems). The BIOMIC V3 system, manufactured by Giles Scientific (Santa Barbara, CA; http://www.biomic.com), has FDA clearance to read disk diffusion zone sizes and broth microdilution panel wells. Readers should be aware that susceptibility testing system components are constantly changing in response to new technology and problems that are discovered.

SEMIAUTOMATED INSTRUMENTATION FOR DISK DIFFUSION TESTING

The advantages of the disk diffusion method of susceptibility testing include simplicity, reliability, low cost, and a high degree of flexibility in selection of agents tested (1). Semiautomated systems available for reading and interpreting disk diffusion inhibition zones are listed in Table 1. For all systems, agar plates are manually inserted into an instrument after incubation for image acquisition and measurement of the zone of inhibition. Despite advances in imaging technol-

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TABLE 1 Overview of manual and semiautomated susceptibility testing instrumentation

Type	Features	Manufacturer[a]	System	Reference(s)
Semiautomated disk diffusion	Assistance in reading, recording, and interpreting zones of inhibition; data management with expert and epidemiology software	Giles Scientific	BIOMIC V3	10, 114
		i2a	Sirscan[b]	11
		Oxoid	Aura Image[b]	12, 115
		Mast	Mastacan Elite[b]	116
		Bio-Rad	Osiris[b]	11, 117
Manual broth microdilution	Devices to facilitate visual interpretation, recording, and reporting	Siemens	MicroScan data management (LabPro)	
		Thermo Scientific	Sensititre Vizion	
Semiautomated broth microdilution	Automated devices read and report results after offline incubation of tray or strip	Siemens	MicroScan AutoSCAN-4	118
		Thermo Scientific	Sensititre OptiRead	
		bioMérieux	Mini API[b]	
		Giles Scientific	BIOMIC V3	

[a] Bio-Rad, Hercules, CA, http://bio-rad.com; i2a, Montpellier, France; Mast, Bootle, United Kingdom, http://www.mastascan.com; Oxoid, Basingstoke, United Kingdom, http://www.oxoid.com. See text for other manufacturers.
[b] Not currently available in the United States.

ogy, a visual review of plates for faint growth or pinpoint colonies within the zone is recommended to assess the need for manual adjustment of the diameter measurement. Data management software determines the categorical interpretation (susceptible, intermediate, or resistant) and may be interfaced with an LIS. Although linear regression may be used to generate an MIC from a zone measurement, the validity for some antimicrobial agent-bacteria combinations has been questioned (9). Expert system analysis and epidemiology software are available for the systems. The primary advantages of these instruments are (i) less variability in zone measurement (in comparison to caliper or ruler readings by different technologists), (ii) reduced transcription errors, (iii) labor savings, (iv) improved data management capabilities, and (v) expert review to ensure correct reporting of results that are consistent with known resistance phenotypes. In general, these instruments provide reproducible and accurate results. In evaluations of the systems, bacteria with faint growth accounted for most discrepancies (10, 11, 12).

MANUAL BROTH MICRODILUTION SYSTEMS

The manual broth microdilution systems listed in Table 1 facilitate the visual reading and recording of MICs. The panels are frozen (Thermo Scientific custom plates) or dehydrated (MicroScan and Sensititre microwell trays). Devices for rehydration and inoculation of dehydrated trays include the manual RENOK device (MicroScan), the microprocessor-controlled Sensititre automated inoculation delivery system, and the Sensititre multichannel electronic pipette.

After offline incubation, the MicroScan data management system (LabPro) displays an image of the tray configuration for recording manual results directly on the computer. The Sensititre Vizion system magnifies a digital image of the 96-well susceptibility plate on a computer screen with superimposed templates to guide the reading of endpoints that are transferred to the data management system (SWIN, Sensititre Windows software) with expert analysis. The SWIN data management system is also available for recording of manual results without the Sensititre Vizion instrument.

Most manufacturers offer standard Gram-positive, Gram-negative, *Streptococcus* species, and extended-spectrum β-lactamase (ESBL) confirmatory panels. Thermo Scientific offers a more extensive menu of FDA-cleared Sensititre plates (*Haemophilus influenzae* and yeast [YeastOne]) and "research use only" panels (mycobacteria [rapid and slow growers], *Campylobacter*, and anaerobic) that can be read with the Vizion system or manually.

SEMIAUTOMATED BROTH MICRODILUTION SYSTEMS

The semiautomated broth microdilution systems listed in Table 1 utilize automated devices to read susceptibility and identification tests after offline incubation. The results are transferred to a data management system that may include expert system analysis using the same software as available for the automated systems. Further information regarding MicroScan and Sensititre panels is presented in the section on automated systems.

AUTOMATED BROTH MICRODILUTION SYSTEMS

Automated AST systems do not require further manual intervention to obtain results after placement of the test panel in an instrument where incubation and reading of endpoints occur. An overview of the automated systems currently available in the United States is presented in Table 2. The VITEK, MicroScan WalkAway, and Phoenix systems provide AST results after short-term incubation (<16 h); the currently available Sensititre ARIS panels and some MicroScan WalkAway panels require overnight incubation. Manufacturers should be consulted regarding the current antimicrobial agents available for each system.

VITEK Systems

The first VITEK instrument developed for the provision of rapid MIC results was introduced in the 1980s. The VITEK 1 is still used in a number of laboratories; however, bioMérieux has stopped further development of new cards and software updates and no longer supports the maintenance of this instrument. The more automated VITEK 2 received FDA clearance in 2000. The AST panels are thin plastic 64-well cards with one to six concentrations of 9 to 20 antimicrobial agents. An identification card for common Gram-positive or Gram-negative bacteria may be run simultaneously with each AST card. The Smart Carrier Station includes a bar code scanner and base unit with microproces-

TABLE 2 Overview of automated broth microdilution susceptibility testing instrumentation[a]

Manufacturer	System(s)	Panel capacity	Panels	Types of panels (no.)	Instrument features	Software
Becton Dickinson	BD Phoenix	100	Two-sided panels: 85-well AST/51-well ID or 136-well AST (Emerge)	Gram pos (4) Gram neg (12) *Streptococcus* (1) Emerge Gram neg (1)	Automated adjustment of inoculum and AST dilution. AST panels available as MIC ± ID substrates. Turbidimetric and redox indicator readings every 20 min. Full-range MICs.	BDXpert BD EpiCenter
bioMérieux	VITEK 2, VITEK 2 XL	60, 120	64-well cards	Gram pos (2) Gram neg (14) *S. pneumoniae* (1) Yeast (1)	Automated AST dilution and filling/sealing of cards. Turbidimetric readings every 15 min. MICs derived from 1–6 antimicrobial agent dilutions.	AES Myla Observa
	VITEK 2 Compact	15, 30, 60	64-well cards	See VITEK 2	Less automated, more affordable than VITEK 2	See VITEK 2
Siemens	MicroScan WalkAway *plus*	40 or 96	Standard 96-microwell trays	ON (32) *Streptococcus* (1) ESBL (1) Rapid (4) Synergies *plus* (10)	Panels available as full-range MIC or breakpoint MIC. Combination panels include ID substrates. MIC readings: ON, turbidimetric; "read when ready," turbidimetric; rapid panels (3.5–15 h), fluorometric.	LabPro LabPro Alert
Thermo Scientific	Sensititre ARIS 2X	64	Standard 96-microwell trays	Gram pos (2) Gram neg (4) *Streptococcus* (1) ESBL (1) Yeast (1)	Fluorometric readings after ON incubation of full-range MIC trays. *Haemophilus*/*S. pneumoniae*, RUO (mycobacteria, anaerobic, campylobacter, Gram neg, yeast), and custom (frozen, dried) plates also available.	SWIN epidemiology module

[a] neg, negative; ON, overnight; pos, positive; RUO, research use only.

sor that holds a cassette with a capacity of 15 cards. After manual preparation of the inoculum, cards are placed in the cassette. A memory chip on the cassette allows the transfer of scanned information to the reader-incubator unit. Work flow for large laboratories can be optimized by placement of Smart Carrier Stations at multiple locations. After placement of a cassette into the VITEK 2 loading station, it is automatically moved through stations for bar code reading, AST dilution preparation, card inoculation, and card sealing. The transport system then places cards on a carousel with a 60-card capacity for incubation. The VITEK 2XL instrument has a capacity of 120 cards. Each card is moved to an optic station every 15 minutes for measurement of light transmittance that is proportional to growth. Linear regression analysis is used to determine algorithm-derived MICs that are reported in 4 to 16 h. The Advanced Expert System (AES) is discussed in a later section.

The VITEK 2 Compact was introduced in 2005 for smaller laboratories and uses the same cards as the VITEK 2 system. The instrument is available in three sizes that accommodate 15, 30, or 60 cards. The VITEK 2 Compact is less automated and more affordable than the VITEK 2. The initial inoculum and dilution preparation is manual. Cards are initially placed into the vacuum unit (left side of instrument) for filling, followed by manual transfer to the right side of the instrument for automated sealing and transfer to the incubator-reader unit.

The VITEK 2 data management system (Observa) is Windows based, with better visual aesthetics and a more layered presentation of the same expert system (AES) analysis originally offered with the VITEK 2. Observa software may be used to create epidemiological reports for data generated by VITEK 2 and the manufacturer's blood culture instrument, BacT/ALERT. Myla middleware has recently been added by bioMérieux to manage the connectivity of multiple instruments with the LIS, allowing comprehensive monitoring of test results and quality indicators. Functions of Myla software include linking mass spectrometric identifications from VITEK MS to bioMérieux VITEK 2 antimicrobial susceptibility results, remote validation of antimicrobial susceptibility testing results, entry of manual susceptibility test results, and generation of reports including antibiograms.

MicroScan WalkAway

The MicroScan WalkAway system was developed in the late 1980s and initially offered two major types of AST panels: conventional panels read turbidimetrically after overnight incubation and rapid panels read fluorometrically after 3.5 to 15 h of incubation. These panels, all conventional 96-well microdilution trays, include (i) MIC panels (a broad range of antimicrobial agent dilutions), (ii) MIC combination panels (some wells used for identification), and (iii) breakpoint combination panels (identification with a limited range of antimicrobial agent dilutions for a categorical result of susceptible, intermediate, or resistant). A third type of MicroScan panel, Synergies *plus*, became available in 2005. Synergies *plus* combines three methods in one panel: "read-when-ready" AST results available as quickly as 4.5 h (turbidimetric reading), overnight results for drugs requiring longer incubation (turbidimetric reading), and identification (fluorometric results within 2.5 h).

The fourth-generation WalkAway *plus* system includes an incubator-reader unit, a personal computer with LIS interface, and a printer. Fluid level sensors, a directional light-emitting diode system, and larger-capacity reservoirs have reduced the time needed to perform maintenance. Two instrument sizes accommodate 40 or 96 panels. The Prompt Inoculation System (available for overnight panels) incorporates a wand to prepare a standard inoculum without turbidity adjustments that is stable for 4 h. A manual device (RENOK) rehydrates and inoculates panels. The humidified incubator-reader unit has a bar code scanner, rotating carousel, and robotics to position panels under a central photometer or fluorometer for readings.

The data management system, LabPro, interprets results, generates patient reports, and archives data to allow production of user-defined reports (antibiograms, trend analysis, and epidemiology reports). Since 2002, the data management system has been able to be coupled with an expert system (LabPro Alert) that incorporates >100 rules and may be customized.

BD Phoenix

The BD Phoenix System has been available in Europe since 2001 and the United States since 2004. The instrument holds up to 100 test panels. The panels are polystyrene trays containing 136 wells dedicated entirely to AST (Emerge panels; 28 antimicrobial agents plus ESBL test wells) or divided into a 51-well identification (ID) side and an 85-well AST side with 14 to 22 antimicrobial agents.

The Phoenix AP instrument was introduced in 2008 to reduce the hands-on time required to set up Phoenix panels and to standardize inoculum preparation. Placement of EpiCenter workstations with bar code printers at each technologist bench maximizes work flow efficiency. Each ID broth is labeled with an EpiCenter-generated bar code label and then inoculated with a heavy isolate suspension. The ID broth and corresponding AST broth for five isolates are placed in a rack that is loaded on the AP instrument for automated standardization of the ID broth inoculum, inoculation of AST broth, and addition of the AST redox indicator. The rack is manually transferred to an inoculation station where the ID broth bar code label and a Phoenix panel are scanned. After the ID and/or AST broth is poured into the appropriate side, each panel is sealed with a plastic cap and placed on the Phoenix instrument.

The Phoenix instrument reads panels every 20 min using both a redox-indicator colorimetric change and turbidity to determine bacterial growth. Growth (metabolic activity) causes the redox indicator to change from an oxidized (blue) state to a reduced (pink) form. A full range of antimicrobial agent concentrations and a "growth" or "no growth" reading for each well allow the system to provide direct rather than calculated MICs. Susceptibility results are completed in 6 to 16 h.

The BDXpert system applies rules that incorporate CLSI or other standards to interpret AST results. Results can be finalized at the Phoenix instrument, the BD EpiCenter or the laboratory LIS. The BD EpiCenter is data management software for analyzing epidemiological trends and generating reports using information from multiple BD instruments (Phoenix, BACTEC blood culture, MGIT 960, and Bruker matrix-assisted laser desorption–ionization Biotyper systems). Features of BD EpiCenter include a bidirectional LIS interface, software that allows BDXpert system analysis of manual offline AST results, and tools to create user-specific rules for analyzing AST results.

Sensititre ARIS 2X

The Sensititre ARIS (automated reading and incubation system) was introduced in the United States in 1992 and provides overnight AST results (13, 14). The latest ARIS 2X version with hardware and software upgrades was released in 2004. The ARIS 2X instrument fits on the Sensititre AutoReader and holds up to 64 plates (standard 96-microwell trays) available as MIC panels or separate ID plates. Plates are rehydrated and inoculated with the Sensititre AIM automated inoculation system or a handheld multichannel electronic pipette before placement in the instrument's carousel. In 2012, the inoculum transferred from McFarland suspensions to 11-ml Sensititre Mueller-Hinton broth tubes used to inoculate MIC susceptibility plates and JustOne strips for testing Gram-negative and Gram-positive nonfastidious isolates was increased from 10 µl to 30 µl to improve the detection of resistant subpopulations of bacteria. The Sensititre MycoTB plate uses a microtiter plate MIC format for susceptibility testing of *Mycobacterium tuberculosis* complex isolates against first- and second-line antituberculosis agents and produces results approximately 1 week earlier than the 1% indirect agar proportion reference method (15). An internal bar code scanner identifies the plate type to assign the appropriate time of incubation. After 16 to 24 h of incubation, each AST plate is transported to the OptiRead automated fluorometric plate reading system for reading of endpoints. The data management software (SWIN) provides expert analysis of results. The SWIN epidemiology module enables laboratories to monitor trends and generate antibiogram reports.

Advantages of Automated Systems

Advantages of automated AST systems include labor savings, reproducibility, data management with expert system analysis, and the opportunity to generate results more rapidly. A work flow and performance evaluation of the VITEK 2 and Phoenix systems reported a longer mean setup time per isolate for Phoenix (3 min) than for VITEK 2 (1.5 min) but more monthly maintenance time for VITEK 2 (63.2 min) than for Phoenix (21.2 min) (16). The new Phoenix AP instrument reduced hands-on time by 50%, with a manual manipulation time per isolate that was 11.5 s less than for VITEK 2 work flow ($P < 0.001$) for batches of 14 isolates (17).

The overall mean time to generate AST results was higher for Phoenix (12.1 ± 2.7 h) than for VITEK 2 (8.4 ± 2 h) ($P < 0.001$) (16). Ligozzi et al. reported that the time required for VITEK 2 AST for Gram-positive cocci was 6 to 17 h, with 90% of results available as follows: 8 h, *Staphylococcus aureus*; 11 h, coagulase-negative staphylococci (CoNS); 9 h, enterococci; 7 h, *Streptococcus agalactiae;* and 9 h, *Streptococcus pneumoniae* (18). There are limited data showing financial and clinical benefits in association with the rapid provision of AST results. Doern et al. reported lower mortality rates and cost savings (fewer diagnostic tests and days in intensive care) associated with the rapid reporting of AST results (19). Barenfanger et al. also demonstrated reduced lengths of stay and cost savings for patients with rapid reporting of AST results that were attributed to earlier adjustments in antimicrobial therapy (20).

The time required to complete AST testing may eventually be reduced further with the application of molecular techniques. Rolain et al. described a real-time quantitative PCR method to measure the effect of antimicrobial agents on bacterial growth with a shorter time needed to generate results (4 h for Gram-positive cocci and 2 h for Gram-negative rods) (21). Additional research, increased automation, and lower cost are needed to make this molecular technology available for clinical laboratories (21). Direct inoculation of automated AST systems with positive blood culture bottles has also been reported and may be validated for laboratory reporting in conjunction with rapid identification (22, 23).

Effective communication of the results to clinicians and pharmacists is essential to realizing the potential benefits of rapid testing. Communication may be enhanced by software packages that interface with medication records and alert clinicians or pharmacists when adjustments in antimicrobial therapy are needed.

Disadvantages of Automated Systems

Disadvantages of automated systems include a higher cost for equipment and consumables than with manual methods, predetermined antimicrobial panels, an inability to test all clinically relevant bacteria and antimicrobial agents, and problems with detection of heteroresistant isolates and some resistance phenotypes (24, 25). In addition, laboratories must validate reformulated antimicrobial agents and use additional testing and reporting methods for agents where FDA breakpoints have not kept pace with changes to CLSI guidelines. Reports of AST performance for detecting problematic resistance phenotypes are discussed below. Current performance of a system may not be accurately reflected by studies utilizing panels and software that are no longer available. A higher error rate should be accepted for evaluations using challenge strains with difficult-to-detect phenotypes than for studies that test populations of isolates usually encountered in the clinical laboratory.

Ability of Automated Systems To Detect Resistance

Vancomycin Resistance in Enterococci

Problems with the detection of low-level vancomycin resistance (*vanB* and *vanC*) among enterococci by automated systems have been demonstrated in multiple studies (26). A VITEK 2 evaluation only reported difficulty detecting *vanC2* (*Enterococcus casseliflavus*) strains (27). Other VITEK 2 and Phoenix studies have demonstrated accurate detection of vancomycin-resistant enterococci, but rigorous studies comparing systems are lacking (28–31). An FDA limitation for *E. casseliflavus* and *Enterococcus gallinarum* requires Phoenix users to determine vancomycin susceptibility for those species with an alternate method (29). MicroScan overnight panel studies reported detection of all isolates except those containing *van*C that are difficult for all AST systems since their MICs (4 to 16 μg/ml) span susceptible and intermediate categories (32).

High-Level Aminoglycoside Resistance in *Enterococcus* spp.

The detection of high-level aminoglycoside resistance in enterococci by overnight and short-incubation AST systems has been improved by changes in growth medium and extended incubation (33). Initial problems detecting high-level streptomycin resistance (HLSR) in MicroScan overnight panels appear to have been resolved after a broth reformulation (34). A study reporting a higher VME rate for MicroScan detection of HLSR (32) may not have performed the recommended 48-h read for isolates that appear streptomycin susceptible after overnight incubation. Separate VITEK 2 and Phoenix evaluations testing different strains reported VME rates of 0 to 5.2% and ME rates of 0 to 7.3% for the detection of HLSR or high-level gentamicin resistance (28–31).

Linezolid Resistance in Enterococci and Staphylococci

Reading endpoints when testing linezolid can be difficult due to trailing growth. The low prevalence of linezolid resistance limits the ability of manufacturers to optimize detection with AST systems. Linezolid susceptibility testing of 50 enterococcal and 50 staphylococcal challenge isolates (included 32 non-linezolid-susceptible strains) demonstrated categorical agreements of 96.0% (MicroScan), 93.0% (VITEK 2), 89.6% (Phoenix), and 85.9% (VITEK 1) in comparison to CLSI broth microdilution results (35). Brigante et al. reported 89.0% categorical agreement of Phoenix linezolid results compared to Etest determinations for 100 enterococci (28).

Penicillin Resistance in Staphylococci

Staphylococcal isolates for which the penicillin MIC is ≤0.12 μg/ml require an induced β-lactamase test (coagulase-negative staphylococci) or a penicillin zone edge test (*S. aureus*) to be negative before being reported as penicillin susceptible (8). The sensitivity of phenotypic methods for penicillinase detection performed on 197 *S. aureus* isolates for which the VITEK 2 penicillin MICs were ≤0.12 μg/ml ranged from 39% (induced nitrocefin test) to 71% (zone edge determination method) when *blaZ* PCR was used as the reference standard (36). Since the prevalence of penicillin-susceptible staphylococcal isolates is low and β-lactamase production may not be detected using phenotypic methods, testing of subsequent isolates from the patient (8) or use of a molecular method (36) should be considered before relying on penicillin for treatment of serious infections.

Oxacillin Resistance in Staphylococci

Most of the studies discussed below used *mec*A PCR as the gold standard when evaluating the accuracy of a system for detection of oxacillin resistance in staphylococci. Multiple studies have demonstrated excellent sensitivity and specificity of automated systems for detecting methicillin-resistant *S. aureus* (MRSA) (18, 29, 30, 37, 38). However, heterogeneously resistant populations may not be detected with routine oxacillin testing (39, 40). Manufacturers of the MicroScan, Phoenix, and VITEK 2 systems have improved MRSA detection by adding cefoxitin to panels (40–44).

For the detection of oxacillin resistance among CoNS, MicroScan, Phoenix, and VITEK 2 evaluations have demonstrated excellent sensitivities (96 to 99.4%), with lower specificities (66 to 96%) (18, 30, 45–49). Isolates with false-resistant results often have oxacillin MICs of 0.5 to 2 μg/ml that would have been considered susceptible under previous CLSI CoNS oxacillin breakpoints that were lowered to ≤0.25 μg/ml in 1999 (50). Some of the ME were for *Staphylococcus lugdunensis* isolates with oxacillin MICs now considered susceptible based on the CLSI 2005 decision to apply *S. aureus* breakpoints (≤2 μg/ml) to this species of CoNS (45, 46). The lower CoNS oxacillin breakpoint is most accurate for detecting *mec*A carriage in *Staphylococcus hominis*, *Staphylococcus haemolyticus*, and *Staphylococcus epidermidis* isolates, but may overcall oxacillin resistance for other CoNS species (50). An evaluation of the VITEK 2 cefoxitin screen found it to be more specific than the VITEK 2 oxacillin test for detection of *mec*A-mediated resistance in CoNS (51).

Reduced Glycopeptide Susceptibility in Staphylococci

The initial failure of automated AST systems to reliably detect vancomycin-resistant *S. aureus* led to a temporary requirement for a supplemental vancomycin agar screening plate until the systems were optimized for vancomycin-resistant *S. aureus* detection (52, 53). An evaluation of four commercial AST systems for the detection of vancomycin-intermediate *S. aureus* reported sensitivities of 64 to 100%; the two systems with 100% sensitivity incorrectly categorized 12% (MicroScan) and 24% (Phoenix) of susceptible isolates as vancomycin-intermediate *S. aureus* (54). Reports of vancomycin MIC "creep" among MRSA appear to depend upon the method of testing and storage of isolates (55). Unreliable AST system detection of CoNS with reduced glycopeptide susceptibility has also been reported (56). CLSI encourages laboratories to confirm nonsusceptible vancomycin results with a second method and to send any *S. aureus* isolate with a vancomycin MIC of ≥8 μg/ml or isolate of CoNS with a vancomycin MIC of ≥32 μg/ml to a reference laboratory (8).

Inducible Clindamycin Resistance

Staphylococci and beta-hemolytic streptococcal isolates that are macrolide resistant and clindamycin susceptible may be assessed for inducible clindamycin resistance using the CLSI disk diffusion or broth microdilution test (8). The inoculum purity plates from an automated broth AST system may be used to perform the disk induction (D-zone) test (57). Recently, FDA-cleared tests for inducible clindamycin resistance in staphylococci have become available for all of the automated systems in Table 2. An evaluation of the Phoenix test for inducible clindamycin resistance performed on 194 macrolide-resistant, clindamycin-susceptible staphylococcal isolates reported a sensitivity of 99% and a specificity of 97% (58). A multicenter study of the VITEK 2 inducible clindamycin resistance test using challenge and local strains of *S. aureus* with 244 tests performed reported a sensitivity of 98% and a specificity of 99% (59). A more recent comparison using fresh clinical isolates reported Phoenix (100% sensitivity; 99.6% specificity) to be more sensitive than VITEK 2 (91.1% sensitivity; 99.8% specificity) at detecting inducible clindamycin resistance (60). Another study found the inducible clindamycin resistance test on the VITEK 2 AST-P612 card to generate false-negative results at a rate of one in four for bacteria not susceptible to erythromycin (61).

Streptococcus spp. Resistance

Evaluations of the Phoenix and VITEK 2 panels for testing *Streptococcus* species demonstrated reliable results despite the shorter incubation (28, 62–64). Overnight *S. pneumoniae* trays available from MicroScan and Sensititre provided accurate results for agents other than trimethoprim-sulfamethoxazole (discrepancies were attributed to trailing endpoints) (65). The detection of emerging resistance by commercial AST systems is often suboptimal. For example, a VITEK 2 study in Japan, using AST-P546 cards, was only able to detect 50% of 28 strains of *Streptococcus agalactiae* with reduced susceptibility to penicillin, as tested by agar dilution, suggesting that these rare isolates of *S. agalactiae* may be misclassified as susceptible to penicillin G (66).

Other Resistance in Gram-Positive Bacteria

Daptomycin results generated by MicroScan, using panel PC29, for *Enterococcus faecium*, *Enterococcus faecalis*, and *S. aureus* have a high rate of false nonsusceptibility that cannot be confirmed by Etest or reference broth microdilution testing (67). These and other unusual resistance phenotypes require confirmation prior to reporting (8).

ESBL-Producing *Enterobacteriaceae*

Implementation of revised (2010) cephalosporin (cefazolin, cefotaxime, ceftriaxone, ceftizoxime, and ceftazidime) and aztreonam CLSI breakpoints for *Enterobacteriaceae* makes ESBL testing unnecessary unless there is consideration of using a cephalosporin for which interpretive criteria were not recently evaluated (cefonicid, cefamandole, cefoperazone, or moxalactam) (8). Some laboratories may continue to perform ESBL testing for infection control purposes. Confirmatory ESBL tests that typically measure the inhibitory effect of clavulanate on ceftazidime and cefotaxime are available for all of the automated systems listed in Table 2. Two comparative evaluations of ESBL detection among *Escherichia coli* and *Klebsiella* spp. revealed sensitivities of 74 to 91% (VITEK 2) and 92 to 96% (Phoenix), with specificities of 81 to 85% (68, 69). Evaluations of a single AST system (VITEK 2 or Phoenix) have reported sensitivities of 54 to 100% and specificities of 96 to 99.7% for ESBL detection (70–75). Evaluations of the MicroScan ESBL panels have demonstrated sensitivities of 88 to 100% and specificities of 77 to 98% (76–79). False-positive ESBL results for K1-hyperproducing *Klebsiella oxytoca* isolates have been reported for MicroScan (79) and Phoenix (72, 80) systems. An evaluation performed on a collection of *E. coli* isolates with CTX-M phenotypes found that ESBL detection by VITEK 2 occurred for only 68% of the 137 isolates tested (81).

Carbapenem Resistance

Failure of automated AST systems to recognize emerging carbapenem resistance among *Klebsiella pneumoniae* and non-*Klebsiella pneumoniae Enterobacteriaceae* isolates has been reported primarily in studies that applied CLSI MIC interpretative guidelines from 2009 or earlier (71, 82–85). The addition of ertapenem to AST system panels improved detection of bla_{KPC}-positive *Enterobacteriaceae* isolates due to relatively lower CLSI breakpoints (in comparison to those for meropenem and imipenem) with a higher sensitivity for carbapenemase production. The 2009 CLSI recommendation to perform the modified Hodge test for detection of carbapenemase production in *Enterobacteriaceae* isolates with elevated carbapenem MICs still in the susceptible range also enhanced bla_{KPC} detection (8). Subsequent studies have reported that this issue may be largely, although not entirely, rectified by application of the 2010 CLSI update to carbapenem breakpoints and to be highly test system dependent (83, 86, 87). The meropenem wells on VITEK 2 cards have been reformulated since a study showed problems with the detection of resistance for meropenem (AST-GN28 card) tested against KPC-producing *K. pneumoniae* (88). Difficulties in identifying carbapenem resistance in *Acinetobacter baumannii* have been reported for Phoenix, MicroScan, and VITEK 2 (84, 89, 90).

Problems with false resistance when determining carbapenem susceptibility have also been noted (91, 92). The CDC could only confirm 9% of 123 *Enterobacteriaceae* and 74% of 325 *Pseudomonas aeruginosa* isolates initially reported as non-imipenem susceptible by 44 U.S. hospital laboratories during the period from 1996 to 1999 (91). The lack of a clear explanation for this overdetection of carbapenem resistance led the authors to recommend that laboratories consider using a second AST method to confirm non-carbapenem-susceptible results (91). Antimicrobial agent deterioration in test panels and technical errors are factors that may contribute to the overdetection of carbapenem resistance by AST systems (91, 92).

Other Resistance in Gram-Negative Bacteria

Two evaluations directly comparing the accuracies of automated AST systems for testing clinical and challenge isolates of *P. aeruginosa* found unacceptable levels of error for imipenem, piperacillin-tazobactam, cefepime, ceftazidime, and aztreonam (93, 94). Susceptibility testing of clinical isolates of *P. aeruginosa* using only VITEK 2 identified three agents with categorical agreement of <90% (cefepime, cefotaxime, and gentamicin) that were predominantly minor errors (95). A VITEK 2 study utilizing *P. aeruginosa* isolates selected to represent specific β-lactam resistance phenotypes revealed elevated error rates for all β-lactam agents tested (96). A VITEK and MicroScan study concluded that automated commercial AST systems were contraindicated for testing isolates of *P. aeruginosa* from cystic fibrosis patients and attributed the poor correlation with reference methods to slow growth and mucoid strains (97). Phoenix studies have reported low categorical agreement for nonfermenting Gram-negative bacillus isolates primarily due to minor errors and ME with β-lactams, ciprofloxacin, and trimethoprim-sulfamethoxazole (75, 98, 99).

A study from Korea comparing commercial methods used to test colistin against 213 bloodstream isolates of *Acinetobacter* spp. found, compared to agar dilution, excellent categorical agreement for VITEK 2 (99.1%) compared to 87.3% using MicroScan (100). VITEK 2 has also been reported to provide high (97%) essential and categorical agreement with broth microdilution for tigecycline tested against clinical isolates of *E. coli* but not other species of *Enterobacteriaceae* (essential agreement, 81%; categorical agreement, 59%) (101).

COMPUTERIZED EXPERT SYSTEMS

Expert systems to assist in the critical review of AST results are available for all commercial susceptibility systems currently marketed in the United States. Expert systems can enhance work flow by identifying the subset of results that require human expert attention and may also improve the quality of AST results reported from smaller laboratories that may lack a human expert (2, 102). By continuous monitoring, the algorithms allow more rapid recognition of incorrect results and more uniform reporting. However, the software must be frequently updated to reflect the emergence of new resistance and changes in reporting guidelines recommended by national organizations such as CLSI. Users must be aware of what rules and comments are activated in their system and work closely with manufacturer-provided specialists to customize the expert system for their laboratory. The degree of customization allowed varies among AST systems. Ideally, an expert system will report actual MICs with categorical interpretation before and after recommended changes. Expert systems may also deduce the susceptibility of an isolate to agents not tested and detect inconsistencies between bacterial ID and AST results (2, 102).

Most expert systems use a rules-based approach focusing on AST results for one drug at a time without considering results for other agents tested simultaneously. The VITEK 2 AES differs by performing an "interpretive reading" that compares the MICs for multiple agents to a large database of known resistance phenotypes and MIC distributions for different species (2, 103, 104). The rationale for interpretive reading with phenotype assignment is that a single mechanism typically mediates resistance to multiple agents (105). Excellent concordance of VITEK 2 AES interpretive reading with resistance genotypes has been reported in multicenter studies (103, 106). When the AES was compared to human expert analysis of VITEK 2 results for 259 consecutive clinical isolates in a university-based microbiology laboratory, there was disagreement for only 5 of the 65 isolates (8%) with AES corrections (102). A limitation that has been noted for the AES is an inability to interpret multiple inconsistent results as being caused by a single problem (102, 107). Nakasone et al. reported inaccurate resistance genotype reporting by the AES for vancomycin-resistant enterococci and macrolide-resistant *S. pneumoniae* (108). In 2011, Winstanley and Courvalin published a detailed review of VITEK 2, Phoenix, MicroScan, and other expert systems (2).

CRITICAL REVIEW OF AST RESULTS

Regardless of whether a laboratory is using a commercial expert system, it is important to be aware of unusual "resistant" and "susceptible" results that require verification of the organism's ID and repetition of the susceptibility test by the same or a different method (8, 105). An example of an unprecedented phenotype that should prompt retesting is an *Enterobacteriaceae* or *P. aeruginosa* isolate that appears more resistant to piperacillin-tazobactam than to piperacillin (109). There are a number of antimicrobial agents that may appear active *in vitro* but lack clinical efficacy (Table 3); therefore, the isolates should be reported as resistant (8). The most recent CLSI M100 document should be consulted for current recommendations regarding agents to test for specific bacteria, methodology, interpretive criteria, results that may be inferred without testing a specific agent, antimicrobial agents to report based on the site of infection, and unusual results requiring verification (8). Expert rules

TABLE 3 Antimicrobial agents that may appear active *in vitro* but lack clinical efficacy

Organism(s)	Antimicrobial agents to which the organism should be reported as resistant
Oxacillin-resistant staphylococci	All β-lactam agents except ceftaroline
Enterococcus spp.	Aminoglycosides (other than high level), cephalosporins, clindamycin, trimethoprim-sulfamethoxazole
ESBL-producing *E. coli*, *Klebsiella* spp., and *Proteus mirabilis*[a]	Cefonicid, cefamandole, cefoperazone, moxalactam
Listeria spp.	Cephalosporins
Salmonella and *Shigella* spp.	Aminoglycosides, narrow- and expanded-spectrum cephalosporins
Yersinia pestis	All β-lactam agents

[a] Implementation of the revised (2010) cephalosporin and aztreonam CLSI breakpoints for *Enterobacteriaceae* makes routine ESBL testing unnecessary unless there is consideration of using the agents listed. If ESBL testing is performed and results are positive, the organisms should be reported as resistant to these agents.

to assist with the interpretation of AST results are also available at the European Committee on Antimicrobial Susceptibility Testing (EUCAST) website (http://www.eucast.org/expert_rules/).

SELECTING AN AST SYSTEM

Factors to consider when selecting an AST system include cost, performance, work flow, data management capabilities, ability to interface with other LIS and hospital information system components, and manufacturer technical support (110). Performance may be assessed by comparing dilutions of FDA-cleared antimicrobial agents and limitations (antimicrobial agent or bacteria listed in package inserts that require an alternative method) of panels from different manufacturers. Current users of systems and publications in peer-reviewed journals are important resources for assessing performance. Manufacturers should be asked if problems reported for particular antimicrobial agent-bacterium combinations have been resolved and what is under development. Manufacturer exhibits at national meetings offer demonstrations of systems and convenient access to industry representatives. Poster presentations at conferences provide the opportunity to interact with recent system users and acquire new information regarding performance. Another method of assessing the performance of AST systems is participation in proficiency testing surveys such as those of the College of American Pathologists (111), whose final critiques of susceptibility testing challenges provide information regarding AST methods used and problem antimicrobial agent-bacterium combinations with high error rates.

An AST system's ability to perform ID is also important because expert rules are linked to bacterial identity. Additional information regarding selection of an AST system and laboratory verification of performance as required by the Clinical Laboratory Improvement Amendments of 1988 (112) is in the *Clinical Microbiology Procedures Handbook* (110) and Cumitech 31A (113).

SUMMARY

AST systems provide accurate and reproducible results for many antimicrobial agent-bacterium combinations. Expert analysis may improve work flow as well as the quality of reported results. The labor savings attributed to automated AST systems are particularly important for laboratories in regions with current or projected technologist shortages. In addition, the provision of more rapid AST results with a short-incubation system may improve patient care and lower health care costs. Future advances in the development of AST systems may increase their clinical impact with the incorporation of molecular techniques that dramatically shorten the time required for results.

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Special Phenotypic Methods for Detecting Antibacterial Resistance*

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Most of the tests described in this chapter are phenotypic tests that characterize an organism's susceptibility or resistance to an antimicrobial agent by screening for a specific resistance mechanism or phenotype. Some of these can be used to guide therapy, whereas others are used for epidemiology or infection control purposes. We have tried to describe the appropriate use of each test in its description. As screening tests, these tests do not provide an MIC of the agent; however, some of these tests have sufficient sensitivity and specificity that confirmation of the result is unnecessary and the results of the screening test can be reported without additional testing. For example, screening tests for inducible clindamycin resistance in *Staphylococcus aureus* and *Streptococcus* spp. and MIC screening tests for high-level aminoglycoside resistance in enterococci have been shown to have reliability comparable to that of standard methods for detecting clinically significant resistance, so additional confirmatory tests are unnecessary. The tests described here may either supplement or replace traditional testing methods, depending on the organism and the assay. This chapter describes details of tests for detection of the following: high-level aminoglycoside resistance and acquired vancomycin resistance in enterococci; inducible clindamycin resistance in streptococci; penicillin, oxacillin, vancomycin, inducible clindamycin, and high-level mupirocin resistance in staphylococci; extended-spectrum-β-lactamase (ESBL) and carbapenemase production in *Enterobacteriaceae;* and β-lactamases in multiple organisms. The tests included are listed in Table 1. Other special phenotypic tests described in previous versions of this chapter, including those for determining the bactericidal activity or combined activities of antimicrobial agents, have not changed substantially and are thus not discussed here. If guidance on such testing is needed, please refer to previous editions of this *Manual* which included this section (1).

Quality control information is given for many of the tests in each section; however, guidelines for the frequency of quality control testing are not provided, because they either have not been defined or vary depending on laboratory circumstances. A practical approach would be to perform quality control testing each day that clinical isolates are tested, or less frequently (e.g., weekly) once a laboratory has thoroughly documented that less frequent quality control testing can verify the reliability of the procedures. The College of American Pathologists recommends that quality control testing be done daily for β-lactamase tests. Quality control tests should be performed each time new lots of material are put into use.

TESTS TO DETECT RESISTANCE IN ENTEROCOCCI

Serious invasive enterococcal infections, such as endocarditis, are commonly treated with a cell wall-active agent (either a penicillin or a glycopeptide, such as vancomycin) and an aminoglycoside (usually gentamicin or streptomycin). These agents act synergistically to enhance killing (2, 3). However, when an enterococcal strain is resistant to the cell wall-active agent or has high-level resistance (HLR) to the aminoglycoside, there is no synergism and combination therapy will not provide a bactericidal effect (2). Because of this, it is important to determine the susceptibility to both the aminoglycoside and the cell wall-active agent individually in order to predict the likelihood of synergy. Methods for detection of aminoglycoside and vancomycin resistance are discussed here. Discussions of methods for detection of high-level penicillin and ampicillin resistance can be found in the relevant Clinical and Laboratory Standards Institute (CLSI) documents (4, 5).

Detection of HLR to Aminoglycosides

Because aminoglycosides have poor activity against enterococci (MICs range from 8 to 256 μg/ml), they cannot be used as single agents for therapy (2, 6). This intrinsic, moderate-level resistance is due to poor uptake of aminoglycosides by the cell (7). Acquired aminoglycoside resistance in enterococci is due either to mutations resulting in decreased binding of the agent to the ribosome, as occurs with streptomycin (called ribosomal resistance), or, more commonly, to the acquisition of new genes that encode enzymes that modify aminoglycosides (called acquired resistance). Acquired aminoglycoside resistance usually corresponds to MICs that are significantly above the concentrations normally tested in routine susceptibility tests, e.g., ≥2,000 μg/ml for streptomycin and ≥500 μg/ml for gentamicin, and is designated HLR (7) (see also chapters 23 and 69).

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*This chapter contains information presented by Jana M. Swenson, Jean B. Patel, and James H. Jorgensen in chapter 70 of the 10th edition of this *Manual.*

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TABLE 1 Special phenotypic tests for detecting antibacterial resistance described in this chapter

Organism group(s)	Resistance mechanism	Test method(s) described	Further testing or confirmation required?
Enterococci	High-level aminoglycoside resistance	Broth microdilution, disk diffusion, agar dilution	No for MIC; yes for disk diffusion, if inconclusive
	Vancomycin MIC of ≥8 μg/ml	Vancomycin agar screening test using BHI agar	Yes
	β-Lactamase production	Direct β-lactamase test	
Staphylococcus aureus	Oxacillin resistance	Oxacillin-salt agar screening test	No
	mecA-mediated oxacillin resistance	Cefoxitin broth microdilution, cefoxitin disk diffusion	No
	Vancomycin MIC of ≥8 μg/ml	Vancomycin agar screening test using BHI agar	Yes
	Inducible clindamycin resistance	Broth microdilution using a clindamycin-erythromycin combination well, D-zone test (disk diffusion)	No
	High-level mupirocin resistance	Broth microdilution, disk diffusion	No
	β-Lactamase production	Penicillin disk diffusion zone edge test, induced direct β-lactamase test	No for zone edge test; yes for direct β-lactamase test
CoNS	*mecA*-mediated oxacillin resistance	Cefoxitin disk diffusion	No
	Inducible clindamycin resistance	Broth microdilution using a clindamycin-erythromycin combination well, D-zone test (disk diffusion)	No
	β-Lactamase production	Direct β-lactamase test	Yes
Enterobacteriaceae	ESBL production	Broth microdilution with and without clavulanate inhibitor, disk diffusion with and without clavulanate inhibitor	No
	Carbapenemase production (general)	MHT	Yes
Pneumococci	Penicillin resistance	Oxacillin disk diffusion	Yes, if nonsusceptible
Other organism groups	β-Lactamase production	Direct β-lactamase test	See Table 4

Synergy between an aminoglycoside and a cell wall-active agent can be determined directly by performing complex time-kill studies (8, 9) or can be predicted by using less cumbersome screening tests. Gentamicin and streptomycin are the only two agents that should be tested on a routine basis. Enterococci that are resistant to gentamicin are considered resistant to tobramycin and amikacin as well, because gentamicin resistance is most commonly due to the bifunctional enzyme encoded by *aac(6′)-Ic-aph(2″)-Ia*, which also confers resistance to other aminoglycosides except streptomycin. Resistance to streptomycin is mediated by a different resistance mechanism, and consequently, streptomycin resistance must be determined separately from gentamicin resistance. Isolates of *Enterococcus faecium* are intrinsically resistant to the synergistic actions of amikacin, kanamycin, tobramycin, and netilmicin with cell wall-active agents, irrespective of *in vitro* testing results for HLR (10). *Enterococcus faecalis* strains that are susceptible to gentamicin may be resistant to tobramycin, netilmycin, kanamycin, and amikacin (11) by means of various other aminoglycoside-modifying enzymes, but optimal methods for testing have not been determined.

The following genes for three additional enzymes that mediate gentamicin resistance have been described: *aph(2″)-Ic* (33), *aph(2″)-Id* (238), and *aph(2″)-Ib* (12). All of these genes confer resistance to ampicillin-gentamicin synergism; however, the first *[aph(2″)-Ic]* may not be detected using the screening methods described below, since its presence seems to lead to intermediate, not high-level, resistance. The second gene, *aph(2″)-Id,* if detected by the screening methods described here, would be assumed to be resistant to synergy with amikacin, although this may not be the case. Only time-kill or molecular studies for the specific enzymes will detect their presence. Methods for detection of HLR to aminoglycosides are summarized in Table 2 and discussed below.

Agar Dilution Screening Method

Agar plates are prepared with brain heart infusion (BHI) agar with addition of 500 μg of gentamicin per ml or 2,000 μg of streptomycin per ml. The plates are inoculated by spotting 10 μl of a suspension that is equivalent to a 0.5 McFarland standard, prepared from growth on an 18- to 24-h agar plate, giving a final inoculum of 10^6 CFU per

TABLE 2 Screening methods for detecting vancomycin and high-level aminoglycoside resistance in enterococci

Parameter	Value or description for screening procedure[c]			
	Vancomycin agar dilution	Aminoglycoside agar dilution	Aminoglycoside broth microdilution	Aminoglycoside disk diffusion
Medium	BHI agar	BHI agar	BHI broth	MHA
Inoculum	10^5–10^6 CFU/spot	10^6 CFU/spot	5 × 10^4 CFU/0.1 ml	0.5 McFarland standard[a]
Incubation (h) in ambient air	24	24[b]	24[b]	18–24
Drug concn				
Gentamicin	NA	500 μg/ml	500 μg/ml	120 μg/disk
Streptomycin	NA	2,000 μg/ml	1,000 μg/ml	300 μg/disk
Vancomycin	6 μg/ml	NA	NA	NA
Endpoint	>1 colony	>1 colony	Any growth	6 mm = resistant, 7–9 mm = inconclusive,[d] ≥10 mm = susceptible

[a] CLSI disk diffusion method (5).

[b] If streptomycin is negative at 24 h, incubate for an additional 24 h.

[c] NA, not applicable.

[d] If the zone is 7 to 9 mm, then the test is inconclusive, and an agar or broth microdilution test should be performed to confirm susceptibility or resistance.

spot. The plates are incubated for a full 24 h in ambient air. The presence of more than one colony of growth should be read as presumptive resistance. For streptomycin, the plates should be incubated for an additional 24 h if there is no growth at 24 h. Mueller-Hinton agar (MHA), MHA plus 5% sheep blood, or dextrose phosphate agar may be substituted for BHI agar, but because growth is better on BHI agar, this is the preferred medium. Commercially prepared agar screening plates are available and have performed well (13–15).

Broth Microdilution Screening Method

Broth dilution tests are prepared using a single well or a tube containing BHI broth with the addition of 500 μg of gentamicin per ml or 1,000 μg of streptomycin per ml. The final inoculum concentration is that recommended for routine broth microdilution testing, i.e., 5 × 10^5 CFU/ml. The plates are incubated for 24 h in ambient air. For streptomycin, the test samples should be incubated for an additional 24 h if there is no growth at 24 h. Any growth is interpreted as denoting resistance.

The recommended streptomycin concentration for use in the broth microdilution screen is half that used in the agar dilution screening test. Because this test is often included as part of a routine Gram-positive MIC panel, the inoculum is that commonly used in broth microdilution testing (5 × 10^5 CFU/ml). The total number of cells tested in the agar dilution screening procedure (10^6 CFU/spot) is 20-fold larger than that normally used in the broth microdilution test (5 × 10^4 CFU/0.1-ml well). In order to provide a test that uses a small inoculum and at the same time maximizes the detection of HLR to streptomycin, it was necessary to lower the concentration recommended for testing streptomycin from 2,000 to 1,000 μg/ml in the broth microdilution test. Because of poorer growth and the smaller inoculum, Mueller-Hinton broth is inadequate for use in the broth microdilution screening test (16). The performance of other aminoglycosides in this test has not been evaluated.

Disk Diffusion Screening Method

The standard disk diffusion procedure (5) described in chapter 71 (with unsupplemented MHA) is used, except that special high-content disks (gentamicin at 120 μg and streptomycin at 300 μg) are required (17). Zones are measured after 18 to 24 h of incubation in ambient air at 35°C. Isolates for which the zone diameters are ≥10 mm are categorized as susceptible. The absence of a zone of inhibition corresponds to the presence of HLR. Strains for which the zones of inhibition are 7 to 9 mm usually display HLR, but a few are strains for which the MICs are only moderately elevated (16). Therefore, strains for which the zone diameters are 7 to 9 mm should be tested by either the standard agar or broth microdilution screening method to determine susceptibility or resistance. High-content gentamicin and streptomycin disks are available commercially.

Quality Control

For both gentamicin and streptomycin, *E. faecalis* ATCC 29212 is used as the susceptible control strain and *E. faecalis* ATCC 51299 is used as the resistant control strain (18). Only *E. faecalis* ATCC 29212 is used for control of disk diffusion tests. The expected quality control limits are 16 to 23 mm for gentamicin (120 μg) disks and 14 to 20 mm for streptomycin (300 μg) disks (19).

Detection of Vancomycin Resistance in Enterococci

As defined by CLSI, the MIC interpretive criteria for vancomycin are ≤4 μg/ml for susceptible, 8 to 16 μg/ml for intermediate, and ≥32 μg/ml for resistant. The three most common phenotypes of resistance are (i) high-level vancomycin resistance (MICs, ≥64 μg/ml) with accompanying teicoplanin resistance (MICs, ≥16 μg/ml) (VanA phenotype); (ii) moderate- to high-level vancomycin resistance (MICs, 16 to 512 μg/ml), most commonly without teicoplanin resistance (VanB phenotype); and (iii) intrinsic low-level resistance associated with *Enterococcus gallinarum* and *Enterococcus casseliflavus* (MICs, 2 to 32 μg/ml) (VanC phenotype) (20, 21). Both the VanA and VanB phenotypes are most commonly seen in *E. faecalis* and *E. faecium* but have been found in other species, including *E. casseliflavus* and *E. gallinarum* (20, 22). Additional genes conferring vancomycin resistance have also been described, including *vanD* (23, 24), *vanE* (25), *van*G (26), *vanL* (17), *vanN* (27), and *van*M (28). Detailed information that explains the phenotypes associated with these, including the usual MICs for vancomycin and teicoplanin and species in which they are found, is described in chapter 23.

Many methods commonly used by clinical laboratories, including disk diffusion and the Vitek (bioMérieux, Durham, NC) and MicroScan (Siemens Healthcare Diagnostics, Deerfield, IL) systems, originally had problems detecting low-level vancomycin resistance (both VanB and VanC types) (29–35). However, systems have improved (34, 36–39). The Vitek 2 and Phoenix systems perform very well for detection of vancomycin resistance in enterococci (40–46); however, a recent study cautioned that *vanA*- or *vanB*-containing *E. casseliflavus* and *E. gallinarum* may not be differentiated from the usual wild-type *vanC* resistance (43) with the Vitek 2 expert system. Disk diffusion testing requires 24 h of incubation and examination of zones under transmitted light.

The vancomycin agar screening test first described by Willey et al. (35) was adopted by CLSI in 1993 (47, 48) (Table 2). Sensitivity and specificity levels of 96 to 99% and 100%, respectively, were noted at that time. Commercially prepared plates also perform well (29, 49, 50). However, there may be some confusion about the characterization of susceptibility or resistance for the *vanC*-containing enterococci, *E. gallinarum* and *E. casseliflavus*, because their growth is variable on agar screening plates. Both of these species intrinsically contain a *vanC* gene, but the MICs of vancomycin for them range from 2 to 32 μg/ml (20). Whether the presence of this gene is associated with therapeutic failures is not known. Since the vancomycin MICs for these strains are often >4 μg/ml, the strains are likely to grow on agar screening plates, where a larger inoculum and a richer medium may promote growth (14, 48, 49). Most strains of *vanC*-containing enterococci are motile at 30°C; *E. casseliflavus* is typically yellow pigmented. These characteristics have been used to distinguish *vanC*-containing enterococci from other species (49, 51, 52). However, some *E. gallinarum* and *E. casseliflavus* strains may be nonmotile. Because of this, a better test to differentiate them from *E. faecalis* and *E. faecium* is fermentation of 1% methyl-α-D-glucopyranoside (MGP). All *vanC*-containing enterococci acidify MGP, whereas *E. faecium* and *E. faecalis* do not (53) (see also chapter 23).

Vancomycin Agar Screening Test

Agar plates are prepared with BHI agar supplemented with 6 μg of vancomycin per ml (BHI-V6). Using growth from an 18- to 24-h agar plate, a suspension equivalent in turbidity to a 0.5 McFarland standard is made and used to inoculate the plates by spotting 1 to 10 μl or swabbing an area of 10 to 15 mm in diameter. The final inoculum is 10^5 to 10^6 CFU per spot. After inoculation, the plates are incubated for a full 24 h in ambient air at 35°C. The presence of more than one colony of growth indicates presumptive resistance.

Quality Control

For quality control, *E. faecalis* ATCC 29212 (no growth, i.e., susceptible) and *E. faecalis* ATCC 51299 (growth, i.e., resistant) should be tested (216). Plates made with BHI agars from certain manufacturers may allow light growth of *E. faecalis* ATCC 29212, especially if the larger inoculum (10 μl) is used or the plates are held for longer than 24 h.

Reporting Resistance in Enterococci

For any serious enterococcal infection (e.g., endocarditis), results of the screen for HLR to gentamicin and streptomycin should be reported in concert with the results of the testing of the cell wall-active agent (penicillin, ampicillin, or vancomycin), because synergy would not be expected if any one of the agents was reported as resistant. High-level aminoglycoside resistance determined by an MIC screening method can be reported without further confirmation. With the disk diffusion test, results can be reported unless the test is inconclusive, in which case an agar dilution or broth microdilution test should be done to clarify the result. Helpful suggestions on reporting the results of enterococcal tests are given by Hindler and Sahm (6).

TESTS TO DETECT RESISTANCE IN STAPHYLOCOCCI

Because some of the phenotypic tests described here for *S. aureus* are not recommended for coagulase-negative staphylococci (CoNS), the two groups are discussed separately, as *S. aureus* and *Staphylococcus lugdunensis* and as CoNS (except *S. lugdunensis*), for detection of oxacillin resistance. Although *S. lugdunensis* is coagulase negative, it tends to behave more like *S. aureus* than like other CoNS with regard to oxacillin tests (54–56). This grouping may also work for other tests, although possible exceptions are noted in the discussions that follow. Tests for detection of resistance in this group are summarized in Table 3.

Detection of Penicillin Resistance in Staphylococci

Penicillin resistance in staphylococci is due to production of penicillinase, encoded by the *blaZ* gene, which is usually plasmid mediated and inducible. Most staphylococci are now resistant to penicillin, so its use for treatment is rarely considered. The susceptibility breakpoint for penicillin resistance is ≤0.12 μg/ml; however, because rare isolates with penicillin MICs in the susceptible range may produce β-lactamase, current recommendations state that a β-lactamase test should be performed on staphylococcal isolates that test susceptible to penicillin before reporting of the isolate as penicillin susceptible (19, 57).

In the past, commercial β-lactamase tests utilizing a chromogenic cephalosporin, such as nitrocefin, were widely used. However, a recent study comparing phenotypic methods for detecting penicillinase in *S. aureus* strains that test susceptible to penicillin showed that nitrocefin-based methods lack sensitivity (58). In that study, *blaZ* was detected by PCR in 28 of 197 *S. aureus* isolates with penicillin MICs of ≤0.12 μg/ml by a Vitek 2 system. When a nitrocefin-based method was used, only 11 of 28 (39%) isolates were detected as β-lactamase positive. The most sensitive nonmolecular β-lactamase detection method studied was the appearance of the penicillin zone edge as either sharp (β-lactamase positive) or tapered (β-lactamase negative) (59), which detected 20 of 28 (71%) *blaZ*-positive strains.

Both CLSI and EUCAST recommend that the zone edge test be performed for *S. aureus* isolates with penicillin MICs or zone diameters within the susceptible range before reporting them as susceptible (19, 57). The zone edge test is not recommended for CoNS, but CLSI recommends that a nitrocefin-based test be performed before reporting of CoNS, including *S. lugdunensis*, as penicillin susceptible (19). Furthermore, CLSI also recommends that MIC and β-lactamase tests be performed on all subsequent *Staphylococcus* isolates from the same patient because of the possibility that rare strains that contain *blaZ* but test as penicillin susceptible may become phenotypically resistant by both MIC and β-lactamase testing (19).

Detection of Oxacillin Resistance in *S. aureus* and *S. lugdunensis*

Strains of *S. aureus* resistant to oxacillin are still referred to as methicillin-resistant *S. aureus* (MRSA), even though

TABLE 3 Screening methods for detection of resistance in *Staphylococcus aureus*

Parameter	Value or description for resistance mechanism or phenotype									
	Oxacillin resistance[a]	*mecA*-mediated oxacillin resistance[b]		Vancomycin resistance[b]		Inducible clindamycin resistance[b]		High-level mupirocin resistance[a]		β-Lactamase production[a,c]
Test method	Agar dilution (oxacillin-salt agar screening test)	Disk diffusion (cefoxitin)	Broth microdilution (cefoxitin)	Agar dilution (BHI-V6 agar screen)	Disk diffusion	Disk diffusion (D-zone test)	Broth microdilution	Disk diffusion	Broth microdilution	Penicillin disk diffusion zone edge test
Medium	MHA + 4% NaCl	MHA	CAMHB	BHI agar	MHA	MHA or BAP[d]	CAMHB	MHA	CAMHB	MHA
Inoculum	0.5 McFarland standard; 1-µl loop or swab	Routine disk diffusion method	Routine MIC method	0.5 McFarland standard; 10-µl loop or swab	Routine disk diffusion method	Routine disk diffusion method or heavy inoculum on purity plate	Routine MIC method	Routine disk diffusion method	Routine MIC method	Routine disk diffusion method
Drug	6 µg of oxacillin/ml	30-µg cefoxitin disk	Cefoxitin	6 µg of vancomycin/ml	30-µg vancomycin disk	15-µg erythromycin disk and 2-µg clindamycin disk spaced 15–26 mm apart	4 µg of erythromycin and 0.5 µg of clindamycin/ml in same well	200-µg mupirocin disk	256 µg of mupirocin/ml	10-U penicillin disk
Incubation conditions	35°C; 24 h	35°C; 16–18 h	35°C; 16–20 h	35°C; 24 h	35°C; 16–18 h	35°C; 16–18 h	35°C; 18–24 h	35°C; 24 h	35°C; 24 h	35°C; 16–20 h
Endpoint	>1 colony	≤21 mm = *mecA* positive; ≥22 mm = *mecA* negative	>4 µg/ml = *mecA* positive; ≤4 µg/ml = *mecA* negative	>1 colony or light film of growth[e]	6 mm = resistant (MIC ≥ 16 µg/ml)	Flattening of clindamycin zone adjacent to erythromycin zone = inducible clindamycin resistance	Any growth in well containing 4 µg of erythromycin/ml and 0.5 µg of clindamycin/ml = inducible clindamycin resistance	No zone = *mupA* positive; >6 mm = *mupA* negative	Any growth in 256-µg/ml well = *mupA* positive	Sharp zone edge = β-lactamase positive; fuzzy zone edge = β-lactamase negative
Confirmation needed?	No	No	No	Yes[e]	Yes	No	No	No	No	No

[a] Applies to *S. aureus* only.
[b] Applies to both *S. aureus* and *S. lugdunensis*.
[c] Commercial β-lactamase tests may also be used, but if the test is negative, the penicillin disk diffusion zone edge test should be performed before reporting the isolate as susceptible.
[d] BAP, blood agar plate.
[e] Isolates with a vancomycin MIC of 8 µg/ml (intermediate) will also grow.

methicillin is no longer available; the abbreviation MRSA has persisted and therefore is also used here. At least four different resistance mechanisms can confer resistance to oxacillin in *S. aureus*: (i) expression of the acquired chromosomal *mecA* gene to produce a supplemental penicillin-binding protein (PBP), designated PBP 2a, with decreased affinity for penicillins; (ii) production of a different PBP variant by the recently described *mecC* gene, found in isolates recovered from livestock; (iii) inactivation of the drug by increased production of β-lactamase; and (iv) production of modified intrinsic PBPs with altered affinity for the drug (60–65).

Resistance mediated by *mecA* is by far the most common mechanism of oxacillin resistance worldwide, and it is the most-studied and most-characterized type. Rapid and reliable detection of *mecA*-mediated resistance is recognized as a crucial component of laboratory testing, and numerous laboratory methods for this purpose have been described. Since its description in 2011, *mecC*-mediated resistance has been identified in *S. aureus* from human, livestock, and other animal isolates, but it appears to have a low prevalence overall, even among persons with high rates of animal exposure (66–69). Although studies to determine the prevalence of other resistance mechanisms have not been done, it is assumed that resistance that is not mediated by a *mec* gene occurs only rarely in *S. aureus*, and such mechanisms have not been found at all in *S. lugdunensis*. MRSA isolates have historically demonstrated resistance to multiple classes of antimicrobial agents, including erythromycin, clindamycin, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole, older fluoroquinolones, and aminoglycosides. However, some contemporary MRSA isolates, especially community-associated strains (CA-MRSA), are frequently not multidrug resistant (70–72).

Strains that possess *mecA* (classic resistance) are either heterogeneous or homogeneous in their expression of resistance. With homogeneous expression, virtually all cells express resistance when tested by standard *in vitro* test methods. However, testing of a heteroresistant isolate results in a subpopulation of cells that appear to be susceptible and others that appear to be resistant. Often, only 1 in 10^4 to 1 in 10^8 *mecA*-positive cells in the test population express resistance (73–75). Oxacillin MIC interpretive criteria for *S. aureus* and *S. lugdunensis* are ≤2 μg/ml for susceptible and ≥4 μg/ml for resistant (19, 57). Heterogeneous expression occasionally results in MICs near the breakpoint (oxacillin MICs of 2 to 8 μg/ml) and consequently may be misinterpreted as susceptible.

Non-*mecA*-mediated resistance due to hyperproduction of β-lactamase (borderline oxacillin-resistant *S. aureus* [BORSA]) or production of modified PBPs (MOD-SA resistance) also results in oxacillin MICs near the breakpoint. BORSA-type resistance can usually be distinguished from the classic type (*mecA* positive) of resistance or MOD-SA resistance by the addition of a β-lactamase inhibitor (e.g., clavulanic acid) to the oxacillin MIC test, which lowers the MIC by 2 dilutions or more. Isolates that are resistant by either the BORSA or the MOD-SA mechanism usually do not have multiple-drug resistance, similar to CA-MRSA.

Incomplete induction of *mecA* expression by oxacillin can result in light or hazy growth within the zone of inhibition, which in turn affects interpretation of oxacillin zone diameters, leading to performance concerns with both sensitivity and specificity (56, 76, 77). Therefore, if oxacillin is tested against staphylococci, only MIC-based methods are recommended for use (19). Phenotypic tests described below for the *S. aureus* group include (i) the use of cefoxitin as a surrogate for oxacillin in routine susceptibility testing methods and (ii) the oxacillin-salt agar screening test.

Detection Using Cefoxitin as a Surrogate for Oxacillin

The use of cefoxitin has been validated extensively since it was first proposed by investigators in France (78, 79). When used as a surrogate for prediction of oxacillin resistance in *S. aureus*, it has been shown to be equal in sensitivity to oxacillin-based tests, but with better specificity, because it is easier to read than tests with oxacillin, especially for disk diffusion tests (56, 80).

Both cefoxitin MIC and disk diffusion methods are described for *S. aureus* and *S. lugdunensis* (Table 3). The cefoxitin disk test is performed using routine disk diffusion for *S. aureus* and *S. lugdunensis*: results of ≤21 mm are reported as oxacillin resistant, and those of ≥22 mm are considered oxacillin susceptible (19). The test is easy to read using reflected light and does not require careful examination of the disk diffusion zones for light growth or small colonies. Following validation in a multilaboratory study (80), cefoxitin MIC breakpoints for prediction of *mecA*-mediated oxacillin resistance in *S. aureus* and *S. lugdunensis* were included in CLSI document M100 (81); cefoxitin MICs of ≤4 μg/ml are interpreted as oxacillin susceptible, and those of ≥8 μg/ml are considered oxacillin resistant. The cefoxitin screen for oxacillin resistance is also endorsed by EUCAST (57). Cefoxitin MIC and disk diffusion tests are read after 16 to 18 h of incubation (19).

Oxacillin-Salt Agar Screening Test for *S. aureus*

The oxacillin-salt agar screening test (Table 3) has been used widely, although it has been shown to be less sensitive than the cefoxitin disk diffusion test (78, 82, 83). Its use for detection of the presence of *mecA* in *S. lugdunensis* has not been studied, but theoretically it should work because oxacillin MICs for *mecA*-positive strains are ≥16 μg/ml (84, 85). Some investigators examined the use of 4 μg/ml cefoxitin in an agar dilution screen similar to the oxacillin-salt agar screen (77, 86, 87), and they demonstrated sensitivities and specificities equivalent to or better than those obtained with oxacillin-salt agar (87–89), although this method has not been endorsed by CLSI as a screening test.

MHA supplemented with 4% sodium chloride and 6 μg of oxacillin per ml is used for the agar screening method recommended by CLSI (4). For this test, inoculum suspensions are prepared by selecting colonies from overnight growth on a nonselective agar plate. The colonies are transferred to broth (e.g., tryptic soy broth) or saline to produce a suspension that matches the turbidity of a 0.5 McFarland standard. This suspension is used to inoculate the oxacillin agar screening plate by either (i) dipping a cotton swab into the test suspension, expressing the excess liquid from the swab, and inoculating an area of 10 to 15 mm in diameter (or streaking the swab onto a quadrant of the agar surface); or (ii) spotting an area of 10 to 15 mm in diameter with a 1-μl loop that has been dipped in the suspension (90). Test plates are incubated for a full 24 h at 33 to 35°C in ambient air and examined for growth of more than one colony, which indicates resistance. It is important that the incubation temperature not exceed 35°C, as MRSA may not be detected with this method if plates are incubated at warmer temperatures (4).

Quality Control

S. aureus ATCC 29213 (oxacillin susceptible) and *S. aureus* ATCC 43300 (oxacillin resistant) are the recommended quality control strains.

Limitations of Methods for Detection of Oxacillin Resistance in *S. aureus*

For *S. aureus,* growth of an isolate on an oxacillin agar screening plate generally means that the isolate is *mecA* positive. When performed properly, the oxacillin agar screening method will detect most *mecA*-positive *S. aureus* strains. Occasionally a heteroresistant *mecA*-positive strain is not detected, which may in part be due to a low frequency of resistance expression (91, 92) or to lot-to-lot or manufacturer-to-manufacturer variation in the test medium (93, 94). The test may also not detect borderline-resistant strains with non-*mecA*-mediated resistance mechanisms.

Early comparisons of the oxacillin agar screening test and the cefoxitin disk diffusion test for *S. aureus* found an increased sensitivity for detecting *mecA*-mediated resistance for the cefoxitin disk diffusion test (82, 95). In more recent comparisons of the two methods, using the revised disk diffusion breakpoints, the tests performed equally, with sensitivities of 98 to 100% for cefoxitin disk diffusion and 100% for the oxacillin-salt agar screen (96–98). Cefoxitin disk diffusion testing has been shown to perform well for detection of borderline resistance caused by *mecA* (76, 78), but it will not detect other types of borderline oxacillin resistance, i.e., MOD-SA or BORSA strains. In one study, reference and commercial methods for detection of *mecA* were studied with a collection of *S. aureus* strains expressing either borderline oxacillin MICs (oxacillin MICs of 1 to 4 µg/ml) or for which previous results with oxacillin tests were discrepant by disk and MIC testing (77). In that study, MIC and disk diffusion tests using cefoxitin performed significantly better than those using oxacillin, including the oxacillin-salt agar screening test and oxacillin MIC tests for detection of *mecA*-mediated resistance.

Although the cefoxitin-based tests demonstrate the best performance for detection of *mecA*-mediated resistance in *S. aureus,* only tests using oxacillin will work for the detection of resistance due to MOD-SA or BORSA (mechanisms not mediated by *mec* genes). The rarity of this type of resistance (typically associated with oxacillin MICs of 2 to 16 µg/ml) precludes a clear understanding of its clinical relevance, but experimental studies suggest that it may not have a significant impact on outcomes (99–101).

Recently, a novel *mec* gene, *mecC,* was described among *Staphylococcus* species in Europe. This was originally described as a novel *mecA* allele, termed *mecA*$_{\mathrm{LGA251}}$, but since it shares only 69% nucleotide identity and 63% amino acid similarity to *mecA*, it has now been designated a distinct gene (102). Like *mecA*, *mecC* encodes an alternate PBP that confers resistance to β-lactam agents. MRSA strains carrying *mecC* were originally identified among *S. aureus* strains associated with bovine mastitis, but subsequent studies have identified such strains among humans and animals in Europe, as both colonizers and causes of infection (69, 103–105). Although *mecC* appears to be most common in *S. aureus,* it has also been described for *Staphylococcus xylosus* (106).

As with MOD-SA and BORSA, *mecC*-mediated resistance is reliably detected by antimicrobial susceptibility tests, but unlike the case with MOD-SA and BORSA, *mecC* is more reliably detected with cefoxitin than with oxacillin (107, 108). The discrepancy between oxacillin and cefoxitin resistance among *mecC*-producing *S. aureus* isolates is so striking that some have even suggested using the oxacillin-susceptible but cefoxitin-resistant phenotype as a first-line screen for the presence of *mecC* (108). As one would expect, *mecC* cannot be detected with assays targeting *mecA* or PBP 2a (107). Since *mecC* is both clinically significant and conferred by a single gene, molecular tests have been developed for *mecC* surveillance and detection (109–111), and additional mechanism-specific diagnostic methods will likely be developed in the future.

Using only cefoxitin-based methods for detection of oxacillin resistance may eliminate the possibility of finding strains that are either phenotypically oxacillin resistant but *mecA* and *mecC* negative (such as BORSA or MOD-SA) or phenotypically oxacillin susceptible but *mecA* positive (112, 113). However, until it is shown that these patterns are clinically significant, using a cefoxitin-based test alone is a reasonable approach, with the understanding that should therapy with a β-lactam agent be ineffective, additional testing, including testing of the specific agent used for treatment or appropriate molecular tests, might be required.

Detection of Oxacillin Resistance in CoNS except *S. lugdunensis*

Of the different resistance mechanisms that have been described for *S. aureus,* only *mecA*- and *mecC*-mediated oxacillin resistance has been described to occur in CoNS. However, detection of oxacillin resistance by using standard methods can be more difficult than it is for *S. aureus,* due in part to the more heteroresistant expression of resistance in CoNS (114). With CLSI reference methods, the sensitivity of detection of oxacillin resistance in this group improved when the MIC breakpoints were adjusted downward in 1999 by CLSI (115), to ≤0.25 µg/ml for susceptible and ≥0.5 µg/ml for resistant. However, correlation of these interpretive criteria with the presence or absence of *mecA* is optimum only for *Staphylococcus epidermidis*, *Staphylococcus haemolyticus,* and, possibly, *Staphylococcus hominis* (116–118). For other species of CoNS (e.g., *Staphylococcus saprophyticus* and *Staphylococcus sciuri*), oxacillin breakpoints tend to be less specific, i.e., many *mecA*-negative strains are categorized as resistant by oxacillin disk and MIC methods (55, 117–121).

In 2009, CLSI removed oxacillin disk diffusion breakpoints for CoNS from document M100 (81) because the test lacks specificity and the cefoxitin disk diffusion test more accurately predicts the presence or absence of *mecA*. The oxacillin-salt agar screening test cannot be used for detecting oxacillin resistance in CoNS because it uses 6 µg of oxacillin per ml, which is higher than the breakpoint for CoNS (5, 122, 123). Therefore, the only methods now recommended by CLSI and EUCAST for detection of oxacillin resistance in CoNS are the standard oxacillin MIC test and the cefoxitin disk diffusion test as a surrogate for oxacillin (19, 57).

With CoNS, the cefoxitin disk diffusion test generally provides equal sensitivity but greater specificity than that of the oxacillin MIC test for detection of strains harboring the *mecA* gene (76, 124, 125), although there is some evidence that performance may be somewhat species dependent. The cefoxitin disk diffusion test fails to reliably classify *mecA*-positive strains of *Staphylococcus simulans* as resistant (76, 126); the reason for this is unknown. For *S. saprophyticus,* cefoxitin disk diffusion testing reliably separates *mecA*-positive from *mecA*-negative isolates, but one study suggests that specificity is improved if *S. aureus* breakpoints rather than CoNS breakpoints are used for interpretation (127).

The cefoxitin disk diffusion test is performed using the routine disk diffusion procedure, with breakpoints of ≤24 mm for oxacillin resistant and >25 mm for oxacillin susceptible. The use of a cefoxitin MIC breakpoint for prediction of *mecA*-mediated resistance in CoNS was recently investigated in a multilaboratory study, but because of performance differences between broths from different manufacturers and overlap of the oxacillin-susceptible and -resistant populations, its performance for MIC testing was not better than that of oxacillin (80), so cefoxitin MIC breakpoints were not adopted. Quality control is the same as that recommended for standard disk diffusion and MIC methods (19).

Limitations of Methods for Detection of Oxacillin Resistance in CoNS except *S. lugdunensis*

Except for *S. simulans* (see above), cefoxitin disk diffusion testing provides good sensitivity and greater specificity than that of oxacillin tests for prediction of *mecA*-mediated resistance in CoNS.

Oxacillin MIC tests lack specificity for non-*S. epidermidis* isolates, so CLSI recommends that for serious infections with CoNS other than *S. epidermidis*, isolates with oxacillin MICs of 0.5 to 2 μg/ml (i.e., MICs that are resistant using the CoNS breakpoints but susceptible using those for *S. aureus*) be tested for *mecA* or PBP 2a. However, this would require that identification of CoNS to the species level be performed.

To improve detection of oxacillin resistance in CoNS, several methods have been proposed, including lowering the concentration of oxacillin to 4 μg/ml in the salt agar screening test (87); using cefoxitin in an agar dilution screen (87); and using a combination of results, for example, oxacillin-salt agar screening and cefoxitin disk diffusion test results (124), oxacillin agar dilution and cefoxitin disk diffusion test results (128), and oxacillin MIC and moxalactam MIC results (129). Other investigators have recommended that CoNS strains with oxacillin zone diameters of 17 to 27 mm or cefoxitin zone diameters of 24 to 31 mm be tested for *mecA* or PBP 2a (130).

Clearly, methods for detection of oxacillin resistance in this group are less than ideal and deserve further study. Since there is discrepancy between the appropriate tests for *S. epidermidis* and non-*S. epidermidis* isolates, species identification, MIC testing of the specific agent being used, and molecular testing for *mecA* or PBP 2a should be considered for CoNS strains isolated from patients for whom therapy choices are limited.

Other Tests for Detection of Oxacillin Resistance in Staphylococci

Other commercial methods for the rapid detection of oxacillin resistance in staphylococci include (i) direct culture of patient specimens (usually nasal swabs) onto agar media containing antimicrobial agents with a chromogenic indicator, (ii) detection of the presence of PBP 2′ by latex agglutination tests, and (iii) automated PCR assays.

Several selective chromogenic agars are now FDA cleared for detection of MRSA from surveillance specimens, including MRSA *Select* (Bio-Rad, Redmond, WA), Spectra MRSA (Remel, Lenexa, KS), CHROMagar MRSA II (BD, Sparks, MD), ChromID MRSA (bioMérieux, Hazelwood, MO), and, most recently, HardyCHrom MRSA (Hardy Diagnostics, Santa Maria, CA). The exact components of these media are proprietary and vary by manufacturer, but they typically contain both chromogenic and selective components. The chromogenic indicator results in *S. aureus* colonies demonstrating a characteristic color that differentiates them from other *Staphylococcus* species on the medium, and the selective component inhibits growth of bacteria that are not oxacillin resistant. These media can be used for direct plating from surveillance swabs and interpreted as early as 24 hours postinoculation; in this way, they can reduce the time to MRSA detection compared to that of traditional culture by 1 to 3 days. Although the performances of specific chromogenic agars vary, numerous studies have demonstrated good correlations compared to both conventional culture-based methods and PCR screening tests for detection of MRSA colonization (131–133) and similar performances between different commercial chromogenic agars (132, 134–136). However, when their use was compared to the use of molecular methods, molecular methods were found to have increased sensitivity and rapidity of detection, but at a higher cost (136–138).

The impact of such agars has been especially dramatic for surveillance cultures performed for specific patient populations, such as cystic fibrosis patients, in whom cocolonization with methicillin-susceptible *S. aureus* or multiple bacterial species is likely. In two recent studies, the inclusion of chromogenic agar improved the sensitivity by 9% to 50% and decreased the turnaround time by 24 to 48 hours (131, 139). However, some have pointed out that addition of a broth enrichment step prior to plating on MRSA-selective agar is important to maximize the sensitivity of selective chromogenic agars, especially when the colonization burden is low (132, 140).

Although not all chromogenic agars are FDA cleared for clinical use in the United States, studies have validated the performance of these media for detection of MRSA in clinical specimens. In one study of specimens that are typically polymicrobial, including superficial wounds, endotracheal aspirates, and sputa, inclusion of chromogenic agar improved detection of MRSA by 21% overall and decreased the turnaround by 1 day (141). Another study of clinical specimens demonstrated better performances of chromogenic selective agar than of traditional methods across multiple specimen types, including wounds, respiratory specimens, stools, and positive blood cultures (142). In studies focusing only on positive blood cultures containing Gram-positive cocci, MRSA-selective chromogenic agar demonstrated excellent sensitivity and specificity (142–145) and enabled detection of MRSA 12 to 24 h earlier than by conventional methods (143–145). Only Spectra MRSA (Remel) has FDA approval for direct detection of MRSA from positive blood culture bottles containing Gram-positive cocci (http://www.accessdata.fda.gov/cdrh_docs/reviews/k092407.pdf).

Commercial rapid methods that detect the presence of PBP 2a in staphylococci by using latex agglutination include the MRSA-Screen test (Denka Seiken Co., Ltd., Tokyo, Japan), the PBP 2′ latex agglutination test (Oxoid Limited, Basingstoke, United Kingdom), the Mastalex test (Mast Diagnostics, Bootle, United Kingdom) (146), and the Slidex MRSA detection test (bioMérieux). The first three tests have been cleared by the FDA. The MRSA-Screen test, cleared for use only with *S. aureus*, has been evaluated widely and has a high sensitivity and specificity for that species (83, 147–149). Detection of resistance in CoNS by the MRSA-Screen test has been less successful, requiring induction, an increased inoculum, or an increased agglutination time for adequate sensitivity (118, 149–151). The Oxoid PBP 2′ latex agglutination test was approved by the FDA for testing of both *S. aureus* and CoNS, with the latter requiring induction with oxacillin or cefoxitin. Although not extensively evaluated, its use for same-day reporting of MRSA from blood cultures (152) and of teicoplanin-resistant CoNS (153) has been described.

In addition to these, automated commercial methods that allow the detection of oxacillin resistance genes by PCR include the GeneOhm MRSA (BD, Franklin Lakes, NJ) and Xpert MRSA (Cepheid, Sunnyvale, CA) tests. Molecular analysis may also be performed by standard PCR when confirmation of the presence of the *mecA* gene is required (see chapter 77).

Reporting Results of Tests for Oxacillin Resistance

CLSI recommends that oxacillin-resistant staphylococci be reported as resistant to all β-lactam agents, including penicillins, cephems except cephalosporins with anti-MRSA activity (i.e., all cephalosporins, including cephamycins), β-lactam–β-lactamase inhibitor combination agents, and carbapenems. These agents are clinically ineffective against oxacillin-resistant staphylococcal infections, even though they may demonstrate activity *in vitro* (4, 5, 19). Newer β-lactam agents were recently developed that have activity against MRSA (154–156). Only ceftaroline has received FDA approval, and both ceftaroline and ceftobiprole are included in CLSI quality control tables and are listed in the CLSI document M100 glossary, in a new class labeled "cephalosporins with anti-MRSA activity" (19). Such agents should not be reported as resistant based on the oxacillin result but must be tested directly if they are reported for isolates that test resistant to oxacillin or cefoxitin (19).

Isolates of *S. aureus* that appear oxacillin resistant by an alternative test method that uses oxacillin but fail to grow on the agar screening plate are probably borderline resistant and lack *mecA* (92, 157, 158). Much less is known about borderline resistance than about *mecA*-positive resistance because there have been few clinical studies (159). In animal model studies, isolates with β-lactamase-mediated oxacillin resistance appeared to be treated effectively with β-lactam agents (61, 99, 101, 160); therefore, if a phenotypically oxacillin-resistant *S. aureus* strain isolated from a seriously ill patient does not contain *mecA*, this information should be conveyed to the patient's medical provider. The incidence of phenotypically susceptible *mecA*-positive strains of *S. aureus* is not known. However, should phenotypically susceptible strains be isolated from serious infections in patients with a history of MRSA infection, confirmation by another method, such as detection of PBP 2a by latex agglutination testing, should be considered (113, 148, 161).

Most automated susceptibility panels now use both oxacillin and cefoxitin for determination of oxacillin resistance, but the final results are determined by algorithms within the expert system; commercial systems are validated by the device manufacturers and cleared by the FDA (see chapter 72). In general, if both oxacillin and cefoxitin tests are used and discrepancies occur, oxacillin-susceptible, cefoxitin-resistant results should be assumed to indicate *mecA*-mediated resistance. Oxacillin-resistant, cefoxitin-susceptible results, on the other hand, are likely not due to *mecA* but should occur only rarely. Current CLSI standards recommend a conservative approach to handling these discrepancies by suggesting that if both agents are tested and either one yields a resistant result, the isolate should be reported as MRSA. This is also true for isolates that test as *mecA* positive but oxacillin or cefoxitin susceptible (19).

Detection of Vancomycin Resistance in Staphylococci

In January 2006, CLSI vancomycin interpretive categories for *S. aureus* were changed to ≤2 μg/ml for susceptible, 4 to 8 μg/ml for intermediate, and ≥16 μg/ml for resistant. Interpretive categories for CoNS were not changed and are ≤4 μg/ml for susceptible, 8 to 16 μg/ml for intermediate, and ≥32 μg/ml for resistant (19, 162). Unlike the case for oxacillin and cefoxitin testing, *S. lugdunensis* results for vancomycin testing should be interpreted using the breakpoints for CoNS. The rationale for lowering the *S. aureus* intermediate breakpoint to include 4 μg/ml was that (i) isolates with a vancomycin MIC of 4 μg/ml, although rare, likely represent a population of organisms with heteroresistance; and (ii) limited outcome data suggested that infections with these isolates were likely to fail vancomycin monotherapy (163). Vancomycin-intermediate *S. aureus* strains (MICs of 4 to 8 μg/ml) are commonly referred to as VISA, and those that are vancomycin resistant (MICs of ≥16 μg/ml) are known as VRSA. CoNS with reduced susceptibility to vancomycin are referred to here as VISS, for vancomycin-intermediate *Staphylococcus* species other than *S. aureus*.

As of January 2014, 13 VRSA strains have been documented in the United States; 8 were from Michigan, 3 from Delaware, 1 from New York, and 1 from Pennsylvania (25, 164, 165). There are also reports of VRSA from countries outside the United States, including India, Pakistan, Iran, Portugal, and Brazil (166–170). All VRSA isolates identified to date contain the *vanA* gene and were isolated from patients who had underlying medical conditions, including prior infection or colonization with MRSA and/or vancomycin-resistant enterococci (VRE); most patients also had received previous vancomycin therapy. The vancomycin MICs for VRSA strains range from 32 to 1,024 μg/ml; all display no zone of inhibition with a 30-μg vancomycin disk.

Resistance in VISA is not due to acquisition of a *van* gene but is thought to be due to changes in the cell wall that render such strains less susceptible to vancomycin (171). At present, no single genetic change has been linked to the VISA phenotype, and it is believed that several different mutations can lead to VISA. The incidence of VISA strains is greater than that of VRSA strains, although the true prevalence is difficult to know because when VISA first began to appear, some routine susceptibility testing methods failed to detect vancomycin-intermediate staphylococci (172–174).

No *van* gene-mediated vancomycin resistance has been reported for VISS, but there are reports of CoNS isolates with elevated vancomycin MICs (175–180) that are also thought to be due to cell wall changes (181). An early study that reviewed the prevalence of VISS found it to be very low (i.e., <1%), except in one institution (171). In a 2009 report of a large surveillance study of isolates from 33 countries, the incidence of VISS was documented to still be <1.0% (182).

The CLSI reference broth microdilution method is reliable for detection of VISA and VRSA, and presumably VISS. However, the disk diffusion test cannot detect VISA or VISS because these isolates produce zone diameters within the susceptible range (≥15 mm). Because of this, all parameters for the disk diffusion test were removed from document M100 in 2014. However, a vancomycin disk diffusion test result of 6 mm (no zone) can still detect *S. aureus* isolates that contain *vanA*; if such results are encountered, both the purity and the identity of the isolate should be confirmed, and a vancomycin MIC test should be performed. The use of BHI-V6 screening agar, used to detect VRE, is a sensitive method for detection of *S. aureus* isolates for which the vancomycin MICs are ≥8 μg/ml, but its performance is variable for detection of *S. aureus* isolates for which the vancomycin MIC is 4 μg/ml (183), and it cannot be used to detect VISS. Most available susceptibility testing methods can now detect VRSA strains, although accurate detection of VISA strains is still problematic (see below).

Although reduced susceptibility to vancomycin can occur in both *S. aureus* and CoNS, the occurrence in *S.*

aureus is more concerning because this species is more frequently a cause of serious infections. It is for this reason that the Centers for Disease Control and Prevention (CDC) recommends the use of BHI-V6 screening agar for detection of *S. aureus* with reduced susceptibility to vancomycin (http://www.cdc.gov/HAI/settings/lab/visa_vrsa_lab_detection.html). This recommendation is for laboratories that may miss these isolates by using their primary susceptibility testing method (i.e., those that routinely use disk diffusion testing). Laboratories may consider limiting this testing to MRSA strains, since these are the isolates in which resistance is of greatest clinical relevance.

BHI-V6 Screening Agar Test Method

The recommended method for inoculating the agar is using a 10-µl drop of a suspension equivalent to a 0.5 McFarland standard delivered with a micropipette. Alternatively, a swab can be used to spot inoculate an area of 10 to 15 mm in diameter. The agar plate should be read after 24 h of incubation and carefully examined with transmitted light. The presence of more than one colony or a light film of growth is considered positive (4, 19).

Quality Control

As mentioned above, BHI-V6 agar is the same screening agar as that used to detect VRE, and the quality control strains recommended for the VRE test can also be used for the *S. aureus* test (4). These strains are vancomycin-susceptible *E. faecalis* ATCC 29212 and *vanB*-mediated vancomycin-resistant *E. faecalis* ATCC 51299. Since the amount of inoculum applied for the VRE test (1 to 10 µl of a suspension equivalent to a 0.5 McFarland standard) spans the recommended inoculum for the *S. aureus* test, a laboratory that uses this medium for both purposes need perform quality control only once for both tests, either daily or weekly.

Limitations of Methods for Detection of Vancomycin Resistance

Standard broth microdilution MIC tests are reliable for detection of any reduced vancomycin susceptibility (i.e., intermediate or resistant susceptibility) in staphylococci. Most commercial methods and routine disk diffusion tests also detect vancomycin resistance (i.e., MICs of ≥16 µg/ml). The detection of intermediate vancomycin resistance by other methods is more challenging. In a recent study looking at the ability of commercial and reference susceptibility testing methods to detect VISA strains, the essential agreement (percentage of results within ±1 dilution) of all methods compared to the reference broth microdilution method was excellent except for disk diffusion testing, which detected no intermediate strains, and use of BHI-V6 screening agar, which failed to identify 12 of 33 strains with a vancomycin MIC of 4 µg/ml (183). This finding was also noted in an earlier study (174). However, in the more recent study, although the essential agreement was excellent, the categorical agreement was not, ranging from 64.8 to 92.2% for the commercial methods tested, with some methods missing VISA strains and others overcalling resistance in susceptible strains (183). This may have been due to differences in the media used in the systems, as it has been shown that recognition of VISA may be medium dependent (174).

Recent studies have shown that the Etest (bioMérieux) method tends to result in slightly higher vancomycin MICs than those obtained by broth microdilution (183, 184), and it may characterize susceptible strains as VISA strains. This was also true of some other commercial systems in the study mentioned above, which compared commercial and reference methods for detection of VISA strains. However, all methods in that study generated vancomycin MICs of ≥2 µg/ml for the VISA isolates tested; therefore, to increase detection of VISA strains, a laboratory might consider further testing of any isolate with an MIC of 2 µg/ml by an alternate method. Etest might be considered for use as the alternate method, since it was successful in detecting 44 of 45 (98%) of the VISA isolates; however, it may also categorize some susceptible strains as intermediate (183, 184).

Reporting Results of Vancomycin Testing

Growth of *S. aureus* on BHI-V6 agar is presumptive for either VISA or VRSA (4, 19; http://www.cdc.gov/HAI/settings/lab/visa_vrsa_algorithm.html). Additional testing is needed to confirm vancomycin resistance. First, the identity and purity of the isolate should be confirmed. The MIC of the isolate should then be determined using a validated method. For many laboratories, the most available method is Etest. It is recommended that any *S. aureus* isolate for which the vancomycin MIC is ≥8 µg/ml be sent to a reference laboratory for confirmation. In the United States, several states request that VISA and/or VRSA be reported to the public health authority.

Detection of hVISA

Heteroresistant vancomycin-intermediate *S. aureus* (hVISA) isolates are isolates of *S. aureus* that normally test susceptible by standard methods but contain a subpopulation of cells (typically 1 in every 10^5 to 10^6 cells) for which the vancomycin MICs are nonsusceptible, i.e., ≥4 µg/ml. This phenomenon was first described in 1997 (185). Some hVISA isolates are detected by standard methods because the population MIC is 4 µg/ml; some, however, continue to be missed because the population MIC is ≤2 µg/ml. There is no agreement in recent reports about the incidence of hVISA or their clinical significance (163). The "gold standard" for detection of hVISA is population analysis/area-under-the-curve analysis, a technique that is unsuitable for routine use (186). No standardized technique that will detect these isolates is available to clinical laboratories. However, there are two Etest methods that have been developed: (i) the macro Etest and (ii) Etest GRD. The macro Etest uses a large inoculum spread onto BHI agar plates and separate vancomycin and teicoplanin strips; MICs of ≥8 µg/ml for either agent or ≥12 µg/ml for teicoplanin alone are considered positive (187). Several studies evaluating the macro Etest for hVISA detection found it to be relatively insensitive (range of 57 to 89% sensitivity, with positive predictive values ranging from 21 to 71%) (188–190). Etest GRD is performed by using MHA with 5% sheep blood, an inoculum equivalent to a 0.5 McFarland standard, and a single strip impregnated with both vancomycin and teicoplanin; specific MICs of the two agents are then interpreted as positive or negative (191). Published sensitivities of Etest GRD range from 57 to 100%, with positive predictive values of 16 to 80% (188–190, 192).

Detection of Inducible Clindamycin Resistance in Staphylococci

Although erythromycin and clindamycin are in separate antimicrobial agent classes, i.e., macrolides and lincosamides, respectively, their mechanisms of action (inhibition of protein synthesis) and mechanisms of resistance are similar. The two main mechanisms of macrolide resistance are (i) an efflux pump that affects only macrolides and (ii) a methylase that alters the ribosomal binding site for both antimicrobial agents. The efflux mechanism of resistance, which confers resistance to macrolides only (designated M-type resistance, for macrolide), is mediated in staphylococci by *msrA*. The target modification type of resistance, which confers resistance to macrolides, lincosamides, and streptogramin B agents (designated MLS_B-type resistance, for macrolide-lincosamide-streptogramin B), is mediated by an *erm* gene (usually *ermA* or *ermC*). In staphylococci, MLS_B-type resistance can be either constitutive or inducible; if it is inducible, the isolate appears to be susceptible to the lincosamide (i.e., clindamycin) by routine testing methods unless it is induced by a macrolide (i.e., erythromycin). It is important to determine if resistance (whether inducible or constitutive) to clindamycin exists when it is being considered for therapy. A detailed explanation of these resistance mechanisms can be found in chapter 69.

Phenotypically, if an isolate has M-type resistance, it is resistant to erythromycin but susceptible to clindamycin. If an isolate has MLS_B-type resistance, it is erythromycin resistant and may test as susceptible or resistant to clindamycin, depending on whether expression is constitutive or inducible for that drug. For strains that are erythromycin resistant but clindamycin susceptible, it is important to determine whether inducible clindamycin resistance exists (due to an *erm* gene) or if the strain remains clindamycin susceptible (due to an efflux gene).

There are two test methods recommended for detecting inducible clindamycin resistance in staphylococci: (i) the D-zone test and (ii) a broth dilution test that uses a single well or tube containing 4 µg of erythromycin per ml and 0.5 µg of clindamycin per ml (Table 3). The test methods are described below.

Test Methods

D-Zone Test

Detection of inducible clindamycin resistance can easily be accomplished using a disk diffusion procedure by placing a 15-µg erythromycin disk adjacent to a 2-µg clindamycin disk and looking for a flattening of the clindamycin zone, which looks like the letter D and is therefore referred to as a D zone. For laboratories that are already performing disk diffusion testing, this may be done by placing the disks 15 to 26 mm apart (193), along with the other disks tested on an MHA plate. Some disk dispensers may position disks more than 26 mm apart even if the disks are placed in adjacent positions. Therefore, because dispensers may vary and the distance is critical, the distance should be verified before the test is adopted as a standard procedure. Placing the disks 15 to 20 mm rather than 26 mm apart may make the test more sensitive and easier to interpret (194–196), although to accomplish this one of the disks must be placed on the plates by hand instead of with a disk dispenser.

For laboratories that routinely perform antimicrobial susceptibility test methods other than disk diffusion testing, Jorgensen et al. have shown that the test can be performed on a standard blood agar plate used for purity checks (197) by streaking one-third of the plate for confluent growth and then streaking for isolation on the rest of the plate. In that study, the investigators showed that dilutions of up to 1:250 of inoculum equivalent to a 0.5 McFarland standard can be used. The disks are placed 15 mm apart on the portion of the plate where confluent growth would occur. In a later study, it was determined that the BBL Prompt system (BD, Sparks, MD) should not be used to inoculate the purity plate for D-zone determination (196). In that study, the investigators suggested that no more than a 1:20 dilution of a suspension equivalent to a 0.5 McFarland standard should be used to inoculate the purity plate. However, if the Prompt inoculum wand was used directly to inoculate the plate, it may have delivered an inadequate inoculum. More recently, the Prompt system was successfully used for this purpose (94) by transferring inoculum from the Prompt reservoir tray by using a 10-µl loop and streaking one-third of the plate to obtain confluent growth. For either method, organisms that show a blunting or flattening of the clindamycin zone are considered D-zone test positive; those that show no flattening are D-zone test negative.

Single-Well Broth Dilution Method

The single-well broth dilution test is performed by preparing microdilution wells (containing no less than 100 µl per well) or tubes of cation-adjusted Mueller-Hinton broth (CAMHB) with a combination of 4 µg of erythromycin per ml and 0.5 µg of clindamycin per ml. The combination is prepared by combining equal amounts of 2× concentrations of the agents, i.e., 8 µg of erythromycin per ml and 1 µg of clindamycin per ml, and transferring the appropriate volume to a microdilution well or tube. The final concentrations in the well or tube are 4 µg/ml of erythromycin and 0.5 µg/ml of clindamydin. The wells or tubes are then inoculated by use of routine inoculation procedures for broth microdilution or macrodilution (38). The test should be interpreted only for those staphylococcal strains that are erythromycin resistant, i.e., with MICs of ≥8 µg/ml, and clindamycin susceptible or intermediate, i.e., with MICs of ≤2 µg/ml. Growth indicates inducible clindamycin resistance; no growth indicates no inducible clindamycin resistance.

Quality Control and Quality Assessment

Two strains have been designated for quality assessment purposes (e.g., for training, competency assessment, or test evaluation): *S. aureus* BAA-977, which has inducible *ermA*-mediated resistance, and *S. aureus* BAA-976, which has *msrA*-mediated resistance to erythromycin only. *S. aureus* ATCC 25923 should be used as the routine quality control strain for daily or weekly quality control testing of clindamycin and erythromycin disks, using MHA. If the test is performed as part of the purity check procedure, CLSI recommends that the disk content be verified using *S. aureus* ATCC 25923 on MHA (4). However, this would require that laboratories which do not routinely do disk diffusion testing have a supply of MHA plates on hand for quality control purposes only. An alternative to this would be to use the two quality assessment strains, BAA-977 and BAA-976, as quality control organisms at daily or weekly intervals, based on CLSI recommendations, when using the purity plate method.

For adequate quality control of the broth microdilution test, it is necessary to test both an inducibly clindamycin-resistant strain and one that is not inducibly resistant so that it can be confirmed that both the agents are in the well and performing as they should. The best way to do this to avoid having to test both strains every time a test is performed is to test BAA-976 and BAA-977 once when the tubes or microdilution panels are prepared (or once for each new lot). For subsequent daily or weekly testing, *S. aureus* ATCC 29213 can be used as a susceptible control and BAA-977 as a resistant control. For further discussion, see reference 198.

Reporting Results

The incidence of inducible clindamycin resistance in staphylococci can be highly variable, both by geographic area and by organism group (i.e., hospital-associated MRSA, CA-MRSA, and CoNS). CLSI now recommends that isolates that are D-zone test positive or grow in the combination well be reported as clindamycin resistant (4, 5, 19). Although some have suggested that clindamycin may be effective in some situations where inducible resistance is demonstrated (199), CLSI recommends that inducibly clindamycin-resistant strains be reported as resistant, with a comment stating that "this isolate is presumed to be resistant based on detection of inducible clindamycin resistance." If the test is not offered routinely, it should be available by request for cases in which clindamycin is being considered for therapy.

Detection of High-Level Mupirocin Resistance in Staphylococci

Mupirocin is a topical antibacterial agent that is used for treatment of skin infection and eradication of nasal carriage of *S. aureus* (200). Resistance to mupirocin is either low level, associated with MICs of 8 to 256 μg/ml, or high level, associated with MICs of ≥512 μg/ml (41). HLR usually implies the presence of the *mupA* gene, although strains with HLR have occurred without *mupA* (201) or have been created in the laboratory (202). More recently, HLR to mupirocin has been attributed to a different allele, *mupB*. The predicted MupB protein shares limited homology with MupA, but expression of *mupB* from a plasmid is sufficient to confer HLR to a naïve *S. aureus* strain (203). It has been shown that elimination of *S. aureus* colonizing strains with mupirocin HLR is not possible (204); whether colonizing strains with low-level resistance can be eliminated with mupirocin is debatable (41, 205).

Methods for the detection of HLR to mupirocin have been suggested by many investigators (206–211), and the British Society for Antimicrobial Chemotherapy recommends methods for testing mupirocin (http://www.bsac.org.uk). Based on data from a multilaboratory study (201), CLSI included methods for prediction of mupirocin HLR by use of disk diffusion or a single-well broth microdilution screen (4, 5, 19) (Table 3).

For disk diffusion testing, the standard disk diffusion procedure is used with a 200-μg mupirocin disk. The test plate is incubated at 35°C for 24 h; the absence of a zone of inhibition is indicative of mupirocin HLR, whereas any zone indicates the absence of HLR. (Note that disks for the test may not yet be available commercially but can be prepared in-house by the following method. Using a stock solution of mupirocin at 8,000 μg/ml and a micropipettor, add 0.025 ml to sterile 6-mm disks that have been spread out on a sterile surface. Allow the disks to dry in a laminar flow hood. When the disks are dry, transfer them to a small tube with desiccant and store at −20°C.) For the broth microdilution procedure, a single well containing 0.1 ml of 256-μg/ml mupirocin is inoculated using a standard inoculum (4) and incubated at 35°C for 24 h. Growth in the well indicates high-level mupirocin resistance; no growth indicates the absence of HLR.

Quality control parameters are given in CLSI document M100 (19). For both tests, *S. aureus* ATCC BAA-1708, a *mupA*-positive strain, is used as the resistant control and should show no zone of inhibition. For disk diffusion tests, *S. aureus* ATCC 25923 is used as the negative control (zone range, 29 to 38 mm), and for the MIC test, *S. aureus* ATCC 29213 (mupirocin MIC, 0.06 to 0.5 μg/ml) or *E. faecalis* ATCC 29212 (MIC, 16 to 128 μg/ml) can be used as the susceptible control.

TESTS TO DETECT RESISTANCE IN STREPTOCOCCI

Oxacillin Disk Screening Test for Detection of Penicillin Resistance in Pneumococci

A screening test in which a 1-μg oxacillin disk is used to detect penicillin resistance in pneumococci was first described following an outbreak caused by *Streptococcus pneumoniae* strains resistant to multiple antimicrobial agents in South Africa in the 1970s (212–214). Since then, this test has been used extensively and was shown to be highly sensitive but not specific for detection of penicillin-nonsusceptible pneumococci (215). Strains identified as nonsusceptible by this method may be penicillin susceptible, intermediate, or resistant. Penicillin MIC tests must be performed on any strain that produces a zone diameter of ≤19 mm to determine if it is resistant (215). For nonmeningitis strains that produce zone diameters of ≥20 mm, CLSI now recommends that they can be considered susceptible to ampicillin, ampicillin-sulbactam, amoxicillin, amoxicillin-clavulanic acid, cefaclor, cefdinir, cefepime, cefotaxime, cefpodoxime, cefprozil, ceftaroline, ceftizoxime, ceftriaxone, cefuroxime, doripenem, ertapenem, imipenem, meropenem, and oral and parenteral penicillins, with no further testing (19). MIC tests rather than the oxacillin disk screen should be used routinely on strains isolated from cerebrospinal fluid and blood.

Detection of Inducible Clindamycin Resistance

The importance of determining erythromycin and clindamycin resistance phenotypes and detecting inducible clindamycin resistance in streptococci follows the same logic as that described for staphylococci (see "Detection of Inducible Clindamycin Resistance in Staphylococci," above); however, the genes responsible for the resistance differ from those in staphylococci and can occur in β-hemolytic streptococci, *S. pneumoniae*, and viridans group streptococci. The M phenotype (i.e., erythromycin resistance and clindamycin susceptibility) in streptococci is due to *mefA*, and the gene responsible for the MLS$_B$ type (constitutive or inducible) is usually *ermB* or, more rarely, *ermTR* (216). Although routine testing of β-hemolytic streptococci is not required because penicillin- or ampicillin-nonsusceptible isolates are extremely rare (or in fact do not exist, for *Streptococcus pyogenes*), a recent publication documented the clinical significance of inducible clindamycin resistance in β-hemolytic streptococci by using an animal model (217). The authors recommended that tests to detect inducible resistance in streptococci be performed and reported routinely. The CDC also recommends that when susceptibility testing is clinically indicated, colonizing isolates of group B streptococci from penicillin-allergic pregnant women should be tested for inducible clindamycin resistance (218).

As for staphylococci, there are two test methods recommended for detecting inducible clindamycin resistance in β-hemolytic streptococci and *S. pneumoniae:* (i) the D-zone test and (ii) a broth dilution test that uses a single well or tube containing 1 μg of erythromycin per ml and 0.5 μg of clindamycin per ml (4, 5, 19). These are described below. A variation of the D-zone test has also been used successfully for detection of viridans group streptococci (219, 220) but is not described here.

Test Methods

D-Zone Test

Detection of inducible clindamycin resistance in β-hemolytic streptococci and pneumococci can be accomplished using a disk diffusion procedure similar to that for staphylococci by placing a 15-µg erythromycin disk adjacent to a 2-µg clindamycin disk and looking for a flattening of the clindamycin zone, which looks like the letter D and is therefore referred to as a D zone, as stated above. For laboratories that are already performing disk diffusion testing, the disks must be placed by hand at a distance of 12 mm from each other (221) on an MHA plate with 5% sheep blood, along with other disks being tested. A disk dispenser cannot be used to place the two disks, because they must not be separated by more than 12 mm (221). For laboratories that routinely perform other antimicrobial susceptibility test methods, the purity plate method suggested by Jorgensen et al. can also be performed on a standard blood agar plate used for purity checks (197, 221) by streaking one-third of the plate for confluent growth and then streaking for isolation on the rest of the plate. The disks are then placed 12 mm apart on the portion of the plate where confluent growth would occur. Only the use of a suspension equivalent to a 0.5 McFarland standard has been evaluated for inoculation of the purity plate (221). For either method, after incubation at 35° for 20 to 24 h in 5% CO_2, organisms that show a blunting or flattening of the clindamycin zone are considered D-zone test positive; those that show no flattening are D-zone test negative.

Single-Well Broth Dilution Method

The single-well broth dilution test is performed by preparing microdilution wells (containing no less than 100 µl per well) or tubes of CAMHB with 3% lysed horse blood with a combination of 1 µg/ml of erythromycin and 0.5 µg/ml of clindamycin. The original studies used only microdilution wells (222, 223), although theoretically tubes should also work if the test conditions are kept the same. The combination is prepared by combining equal amounts of 2× concentrations of the agents, i.e., 2 µg/ml of erythromycin and 1 µg/ml of clindamycin, and transferring the appropriate volume to a microdilution well or tube. The final concentrations in the well or tube are 1 µg/ml of erythromycin and 0.5 µg/ml of clindamydin. The wells or tubes are then inoculated using routine inoculation procedures for broth microdilution or macrodilution (4, 19). The test should be interpreted only for those streptococcal strains that are erythromycin resistant, i.e., with MICs of ≥1 µg/ml, and clindamycin susceptible or intermediate, i.e., with MICs of ≤0.5 µg/ml. Growth indicates inducible clindamycin resistance; no growth indicates no inducible clindamycin resistance.

Quality Control and Quality Assessment

Two strains have been designated as supplemental quality control strains for quality assessment (e.g., for training, competency assessment, or test evaluation) of the D-zone test: *S. aureus* BAA-977, which has inducible *ermA*-mediated resistance to clindamycin, and *S. aureus* BAA-976, which has *msrA*-mediated resistance to erythromycin only. *S. pneumoniae* ATCC 49619 should be used as the routine quality control strain for daily or weekly quality control testing of clindamycin and erythromycin disks, using MHA with 5% sheep blood. If the test is performed as part of the purity check procedure, ideally the disk content should be verified using *S. pneumoniae* ATCC 49619 on MHA with 5% sheep blood. However, this would require that laboratories which do not routinely do disk diffusion testing have a supply of MHA with 5% sheep blood on hand for quality control purposes only. An alternative to this would be to use the two quality assessment strains, BAA-977 and BAA-976, as quality control organisms, with testing daily or weekly.

For adequate quality control of the broth microdilution test, it is necessary to test both an inducibly clindamycin-resistant strain and one that is not inducibly resistant so that it can be confirmed that both the agents are in the well and performing as they should. The best way to do this to avoid having to test both strains every time a test is performed is to test BAA-976 and BAA-977 once when the tubes or microdilution panels are prepared (or once for each new lot). For subsequent daily or weekly testing, *S. pneumoniae* ATCC 49619 can then be used as a susceptible control, with BAA-977 as a resistant control.

Reporting Results

The incidence of inducible clindamycin resistance in β-hemolytic streptococci can be highly variable, both by geographic area and by organism group (216, 224–226). However, when inducible clindamycin resistance is detected, CLSI recommends that, as for staphylococci, isolates shown to be D-zone test positive or that exhibit growth in the microdilution well be reported as clindamycin resistant. As a conservative approach, the CLSI has suggested that inducibly clindamycin-resistant strains may be reported as resistant, with a comment stating that "this isolate is presumed to be resistant based on detection of inducible clindamycin resistance." Based on the recent publication by Lewis et al. establishing the clinical significance of inducible clindamycin resistance in streptococci, consideration should be given to routine testing and reporting (217). If the test is not offered routinely, it should be available by request for cases in which clindamycin is being considered for therapy.

TESTS TO DETECT RESISTANCE IN *ENTEROBACTERIACEAE*

Antimicrobial resistance among *Enterobacteriaceae*, and especially resistance to β-lactam agents, is of increasing concern worldwide. Whereas infections with *Enterobacteriaceae* were once very treatable, resistance to multiple classes of antibiotics among this family of bacteria, and even panresistance to all available therapeutic agents, has been described (227–231). Preserving effective antibiotic agents for treatment and preventing the spread of these organisms are increasingly recognized as high priorities throughout the continuum of patient care, and reliable detection of resistance in this group of organisms is essential for guiding therapeutic decisions and infection control interventions. This is complicated, however, because β-lactam resistance in *Enterobacteriaceae* is not a straightforward issue; resistance occurs through multiple mechanisms, can be intrinsic or acquired, can be induced from low- to high-level expression, and can spread both through clonal expansion and through horizontal transmission of resistance genes.

In 2010, CLSI lowered the *Enterobacteriaceae* MIC interpretive criteria for cephalosporins and carbapenems. One impact of this change is that clinical laboratories using the lowered breakpoints are no longer instructed to perform tests for specific mechanisms of resistance and to change susceptibility testing reports accordingly. Instead, with the lower breakpoints in place, laboratories are instructed to report the interpretation for each antimicrobial agent according to the observed MIC (19, 57, 232). As such, the mechanism-specific tests described in this chapter may not be necessary for routine testing to guide reporting of susceptibility tests but are useful for purposes of surveillance and targeted infection prevention.

Tests for ESBLs

Enterobacteriaceae can produce β-lactamases capable of hydrolyzing penicillins and cephalosporins, including the extended-spectrum cephalosporins (e.g., cefotaxime, ceftriaxone, ceftizoxime, and ceftazidime). These enzymes are referred to as ESBLs (233, 234) and are discussed in chapter 69. More than 200 different types of ESBLs have been identified among Gram-negative bacteria, and they are associated with a variety of *in vitro* antimicrobial susceptibility profiles (233, 234; http://www.lahey.org/Studies/). Although all ESBLs hydrolyze extended-spectrum cephalosporins, the activity against specific antimicrobial agents varies by enzyme type. For example, some of the TEM and SHV ESBLs demonstrate greater activity for ceftazidime than for ceftriaxone, whereas the CTX-M enzymes demonstrate greater activity for ceftriaxone and cefotaxime than for ceftazidime (234, 235). ESBLs do not hydrolyze carbapenems, and therefore carbapenems have been the treatment of choice for infections with ESBL-producing isolates.

CLSI guidelines describe screening criteria and confirmatory tests for ESBL detection in *Klebsiella* spp., *Escherichia coli*, and *Proteus mirabilis* (19); EUCAST guidelines also include *Salmonella* spp. and *Shigella* spp. (57). Although ESBLs do occur in bacteria other than these species, the phenotypic ESBL confirmatory test can produce a false-negative result in the presence of an AmpC-type enzyme (236), so validation of the ESBL test in bacteria with a chromosomally encoded AmpC enzyme (e.g., *Enterobacter* spp. and *Serratia* spp.) is difficult. When ESBL testing was performed routinely, it was important to establish screening criteria for ESBL detection, because routine disk diffusion and MIC tests did not always identify isolates that produced ESBLs (237–240). Therefore, MIC and disk diffusion screening breakpoints were developed for aztreonam, cefotaxime, cefpodoxime, ceftazidime, and ceftriaxone for aid in detecting ESBL-producing isolates (57, 241). ESBL-producing clinical isolates may demonstrate HLR to one or more of the screening drugs (239, 242–246). Thus, the sensitivity of the screening test increases when more than one screening drug is used (240, 247, 248).

ESBLs are inhibited by clavulanic acid, and this property is used in laboratory tests to confirm ESBL production. These tests are based on enhanced drug activity when a cephalosporin (usually ceftazidime or cefotaxime) is tested with clavulanic acid compared to its activity when tested alone. Both a disk diffusion test and a broth microdilution MIC test have been described for confirmation of ESBL production. For broth microdilution, cefotaxime and ceftazidime are tested with and without 4 μg of clavulanic acid per ml. A decrease in the MIC of ≥3 doubling dilutions for the agents tested in combination with clavulanic acid compared to the values obtained for the agents tested alone indicates the presence of an ESBL. For disk diffusion testing, the same agents incorporated into disks with and without 10 μg of clavulanic acid are tested. An increase in the zone diameter of ≥5 mm for either of the disks with clavulanic acid indicates the presence of an ESBL. *Klebsiella pneumoniae* ATCC 700603 should be included for quality control purposes; accepted ranges are given in the current CLSI document M100 tables and at the EUCAST website (19, 57).

There are multiple explanations for a screen-positive, confirmatory test-negative result for an isolate. The isolate may be ESBL negative but demonstrate reduced susceptibility to the screening agents because of decreased porin production, hyperproduction of a normal-spectrum β-lactamase, such as TEM-1 or SHV-1, or production of another cephalosporin-hydrolyzing enzyme, such as an AmpC-type enzyme, which is not inhibited by clavulanic acid. Alternatively, the isolate may be ESBL positive but produce an additional β-lactamase, such as the AmpC-type enzymes, which can interfere with ESBL inhibition by clavulanic acid (236, 249). The addition of an AmpC inhibitor (i.e., cloxacillin) appears to improve the sensitivity of ESBL detection among strains that also produce an AmpC enzyme (250). In one version of such a test, the same disk diffusion method and interpretive criteria used for ESBL detection are performed on MHA containing cloxacillin to inhibit AmpC enzymes, thereby improving detection of ESBLs in the presence of both types of resistance (251).

In recent years, both CLSI and EUCAST revised the recommendations for when to perform ESBL screening and confirmation tests. These revised recommendations were made because the interpretive criteria or breakpoints for cephalosporins and aztreonam were also revised (57, 241). The new breakpoints, based upon pharmacokinetic/pharmacodynamic data and limited clinical outcome data, are meant to more accurately predict treatment outcomes by using the most commonly used dosages for the cephalosporins. When the current breakpoints are used, ESBL screening and confirmatory tests should be performed for infection control and epidemiological purposes rather than for changing interpretive criteria (e.g., changing a cephalosporin report from susceptible to resistant). In other words, it is possible for an isolate to produce an ESBL but still to test susceptible to one or more extended-spectrum cephalosporins. This recommendation seems to contradict previous recommendations to report all cephalosporins as resistant if an ESBL is detected (81), but the new pharmacokinetic/pharmacodynamic data suggest that previous practices may have resulted in reporting of false resistance (254).

Tests for Detection of Plasmid-Mediated AmpC-Type β-Lactamases

Genes encoding inducible AmpC-type β-lactamases occur on the chromosomes of several Gram-negative bacilli, such as *Enterobacter cloacae*, *Citrobacter freundii*, *Serratia marcescens*, and *Pseudomonas aeruginosa* (255). These enzymes confer resistance to a wide spectrum of β-lactams, and they are resistant to the commonly used β-lactamase inhibitors (see details in chapter 69). Genes encoding AmpC-type β-lactamases may also be located on transmissible plasmids in several bacterial species that lack an inducible chromosomally mediated enzyme, including *E. coli*, *Klebsiella* spp., *Salmonella* spp., and *P. mirabilis* (256). Like plasmids encoding ESBLs, plasmids encoding AmpC-type enzymes often carry resistance determinants for multiple classes of antimicrobial agents, and the combination of an AmpC-type enzyme with porin loss can result in carbapenem resistance (257, 258). None of the phenotypic tests described below for AmpC detection can differentiate between genes located on the chromosome and those on a plasmid. Detection of an AmpC enzyme in members of the *Enterobacteriaceae* that do not typically carry a chromosomal enzyme gene is assumed to indicate a plasmid-mediated enzyme; in *E. coli*, which carries a chromosomally located but typically unexpressed AmpC enzyme gene, molecular studies are necessary to distinguish between plasmid and chromosomal enzyme genes. The detection of plasmid-mediated AmpC-type enzymes is helpful for infection control and epidemiological investigations (259–261), and most large studies evaluating methods for AmpC detection have focused on plasmid-mediated enzymes (262).

Several tests have been proposed for detection of AmpC-mediated resistance. Two assays, the modified three-dimensional extract test and the cefoxitin-agar medium-based test, are labor-intensive and difficult to implement in the clinical laboratory (263, 264). The more promising assays incorporate an inhibitor of AmpC enzymes incorporated into one of several confirmatory AmpC assays, described below. Two inhibitors that have been evaluated widely are cloxacillin and boronic acid, although proprietary inhibitors have also been evaluated for this purpose (265, 266). It is important that the boronic acid inhibitor is not specific for AmpC enzymes; boronic acid affects both class A (e.g., KPC) and class C (e.g., AmpC) enzymes, so additional testing must be performed to differentiate these (267, 268).

Phenotypic tests for AmpC production are generally performed on isolates that screen positive for potential AmpC production by testing nonsusceptible to the cephamycins (i.e., cefoxitin or cefotetan). Cefoxitin resistance demonstrates better sensitivity for this purpose, although cefotetan resistance is a more specific predictor of AmpC production (269–271). Others have suggested that a combination of resistance to cefoxitin and resistance to ceftazidime or cefotaxime can improve specificity without decreasing sensitivity (272, 273). Isolates that screen as potential AmpC producers can be confirmed with a molecular test (see chapter 77) or one of the phenotypic confirmatory tests described below.

The double-disk synergy test has been evaluated widely for detection of both plasmid- and chromosome-encoded AmpC enzymes. This test involves a paper disk or tablet containing an antibiotic (typically cefoxitin, ceftazidime, or cefotaxime) placed near an AmpC inhibitor (typically cloxacillin or boronic acid) on a lawn of the organism to be tested. The inhibitor inactivates the β-lactamase, resulting in a zone of inhibition around the antibiotic disk that is enlarged and distorted toward the inhibitor disk (274, 275). Numerous studies have reported this test to have good sensitivity (range of 85 to 96%), although its specificity is much less reliable (272, 276, 277). Ruppé et al. developed a modification of this approach that can be used to detect the AAC-1 β-lactamase, which is inhibited by oxacillin. Essentially, the double-disk synergy test is set up in duplicate with ceftazidime and cefotaxime, using cloxacillin as an inhibitor for one set and cefoxitin as the inhibitor for the other. Organisms producing an AAC-1 enzyme will demonstrate inhibition in both assays, while those producing other AmpC β-lactamases will demonstrate inhibition only with cloxacillin (275).

Another test incorporates a disk containing both a test drug (typically cefoxitin, but also cefotaxime or ceftazidime) and an AmpC inhibitor (cloxacillin or boronic acid) in a standard disk diffusion assay. The zone diameter of the test drug alone is compared to that obtained in the presence of the inhibitor; an increase in zone size (typically of ≥4 mm) is interpreted as positive (277, 278). Numerous studies have evaluated different combinations of β-lactams and inhibitors with this assay, and each has concluded that it demonstrates good sensitivity and specificity for confirmation of both plasmid- and chromosome-encoded AmpC enzymes (269–271, 278, 279). In one study evaluating the cloxacillin and boronic acid inhibitors, each compound performed well, but the best assay performance was observed when both compounds were combined with cefoxitin (278).

At least two commercial manufacturers have developed test systems based on various tests described here. Rosco NeoSensitabs and Mast antibiotic resistance detection sets both incorporate several disks or tablets into panels that can be used to decipher various β-lactam resistance mechanisms; these systems have generally performed well in a limited number of published studies (269, 277, 280). Etest also produces an AmpC assay (Etest AmpC) that follows a similar logic. Etest AmpC uses a double-ended strip containing a gradient of cefotetan on one end and a gradient of cefotetan plus cloxacillin on the other; the MIC for cefotetan alone is compared to the MIC with the inhibitor, and a ratio of ≥8 is considered positive, as is a deformation of the ellipse or formation of a phantom zone (http://www.ilexmedical.com/files/E-test-Package-Insert/Product_supplement.pdf).

Tests for Detection of Carbapenemases

Carbapenems are broad-spectrum β-lactam agents used for empiric treatment of very serious infections and for treatment of infections with multidrug-resistant organisms. As such, carbapenems are viewed as "agents of last resort," and resistance to them is a significant clinical and public health concern. Carbapenemases are β-lactamases that can hydrolyze carbapenem antimicrobial agents (e.g., ertapenem, doripenem, imipenem, and meropenem). Typically, carbapenemases can also hydrolyze other β-lactam agents, such as the penicillins, β-lactam–β-lactamase inhibitor combinations, and cephalosporins, so isolates producing a carbapenemase can be resistant to all β-lactam drugs. Although carbapenemases can be chromosomally encoded, most are encoded on a plasmid that can also contain other resistance determinants. It is these features of carbapenemases that make identifying isolates harboring such resistance mechanisms a significant infection control concern.

Carbapenemases fall within the following three classes of β-lactamases: class A enzymes, including KPC, SME, IMI, NMC, and GES; class B enzymes, also known as the metallo-β-lactamases (MBLs), with NDM, VIM, and IMP being the most common types; and class D OXA enzymes (281, 282). These enzymes are described in chapter 69. Carbapenemases have been identified in many different Gram-negative bacilli, but their recent emergence among the *Enterobacteriaceae* represents a significant escalation of their clinical relevance. The KPC enzyme is the most commonly identified class A carbapenemase in the United States; it usually occurs in *Enterobacteriaceae* but has also been identified in *Pseudomonas* spp. (281, 283). In the past, MBLs were most often found in *P. aeruginosa* and *Acinetobacter* spp., and only occasionally in *Enterobacteriaceae* (284). In contrast to historic MBLs, the NDM mechanism has emerged primarily among *Enterobacteriaceae*, although it has also been described for *Acinetobacter* spp., *Pseudomonas* spp., and *Stenotrophomonas maltophilia* (285–287), and its presence was documented in numerous countries across several continents within the first few years of its description (285, 287). OXA carbapenemases are most commonly found in *Acinetobacter* spp., but the OXA-48 family of carbapenemases is found among *Enterobacteriaceae* (288).

In January 2009, the CLSI guidelines recommended use of the modified Hodge test (MHT) for detection of carbapenemase activity in *Enterobacteriaceae* (81). This test is performed by inoculating a carbapenem-susceptible isolate, *E. coli* ATCC 25922, onto MHA as described for disk diffusion testing, with the exception that an inoculum equivalent to a 0.5 McFarland standard is diluted 1:10 prior to inoculation. A carbapenem disk (either 10 μg meropenem or 10 μg ertapenem) is placed on the plate, and then a loopful of the test isolate is streaked from the disk to the edge of the plate. After overnight incubation, the test is read at the intersection of the zone of inhibition for *E. coli*

ATCC 25922 and the test isolate. Indented growth of *E. coli* ATCC 25922 toward the carbapenem disk is interpreted as a positive result; no indentation of *E. coli* ATCC 25922 growth from the zone of inhibition toward the disk is interpreted as a negative result. When a carbapenemase-producing isolate is streaked three or four times around one carbapenem disk, the multiple distortions of the zone of inhibition can resemble a clover leaf, and hence this test is sometimes called the cloverleaf test.

Initially, the MHT was recommended for the detection of carbapenemase production in *Enterobacteriaceae* isolates that demonstrated an elevated but susceptible carbapenem MIC (i.e., 2 µg/ml for ertapenem and 2 to 4 µg/ml for imipenem or meropenem) or zone diameter (i.e., 16 to 21 mm for ertapenem or meropenem) (81, 289–292). Additionally, it was recommended to report isolates that tested MHT positive as "carbapenemase-producing" isolates in the laboratory report, with a warning that the carbapenem therapeutic outcome was unknown. In 2010, the carbapenem breakpoints were revised; now the susceptible breakpoints are ≤1 µg/ml for doripenem, imipenem, and meropenem and ≤0.50 µg/ml for ertapenem (19). When these breakpoints are used, the susceptibility of the β-lactam agents is reported according to the interpretive criteria for the MIC obtained with each agent; the MHT is no longer recommended for routine reporting, and interpretive criteria are not changed according to the presence of carbapenem resistance. However, some laboratories may still choose to perform the MHT for special infection prevention strategies or to address specific epidemiologic questions related to the mechanism of resistance.

The advantages of the MHT are that it is easy to perform, multiple isolates can be tested on one agar plate, and carbapenemases of different classes can be detected using the same test (289, 293). The disadvantages are that reading the test is subjective and the test cannot differentiate between different carbapenemases, which can be useful epidemiological data. However, false-positive results have been reported for some isolates that produce AmpC-type enzymes or ESBLs when these noncarbapenemase mechanisms combine with chromosomal porin mutations (267, 294, 295). What is even more concerning is that NDM-producing *Enterobacteriaceae* may produce false-negative results (294, 296). Although the MHT performs well for class A and D enzymes and for detection of some MBLs, its poor performance for detection of isolates carrying the rapidly emerging NDM MBL is a significant limitation.

Another assay that is not specific for any particular class of carbapenemase is the Carba NP test, which relies on a pH-sensitive indicator to detect hydrolysis of the imipenem β-lactam ring by a carbapenemase (297). Several studies have demonstrated excellent sensitivity and specificity for detection of many different carbapenemase enzymes in both *Enterobacteriaceae* and *P. aeruginosa* (297, 298), although this test performs less well for the GES and OXA-48 families of carbapenemases (299). The Carba NP test is inexpensive, quick (1 to 3 h), and relatively easy to perform and interpret (297–299), all of which suggest that it may be a valuable tool for detection of carbapenemases. At least one commercial supplier provides reagents for the Carba NP test (Rosco Diagnostica, Taastrup, Denmark), but this test has not been FDA cleared and is not available commercially in the United States. Therefore, U.S. laboratories that wish to perform the Carba NP test must validate its performance and prepare testing reagents in-house, thus increasing the test complexity.

Researchers have also described carbapenemase detection through the use of matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. This technology is being introduced widely in clinical microbiology laboratories for the purpose of rapid and accurate bacterial identification, but it can also detect carbapenemases by detecting the hydrolyzed and decarboxylated metabolites of specific carbapenems (300, 301). This approach has been described as inexpensive, rapid, and applicable to several different types of carbapenemase, but the current state of this assay requires that the user be relatively savvy about operation of the MALDI-TOF instrument, as the systems placed in microbiology laboratories are typically optimized for bacterial identification rather than detection of drug metabolites.

In addition to the general tests described above, several tests have been described for detection and differentiation of specific carbapenemase types, including class A (e.g., KPC) and class B (i.e., MBL) carbapenemases. These tests may be useful for epidemiological studies but would likely require additional validation before they could be used for making treatment decisions. Class A and C enzymes are inhibited by boronic acid, and class B MBLs are inhibited by chelators, such as EDTA and 2-mercaptopropionic acid. Many tests that incorporate one or more of these inhibitors with a carbapenem have been described for differentiating the activity of one enzyme from that of another. The test formats fall into three general categories: a double-disk zone distortion test, a two-disk zone enhancement test, and a broth microdilution or gradient diffusion MIC reduction test.

Kim et al. (302) described a double-disk distortion test for detection of MBLs which uses a carbapenem disk (e.g., imipenem) and another disk containing Tris-EDTA and 2-mercaptopropionic acid chelators. These disks were placed 10 mm apart, edge to edge, on MHA inoculated with the test organism. After overnight incubation, MBL production of the test isolate was identified by distortion of the inhibition zone around the carbapenem disk toward the chelator disk, indicating that the removal of metal ions by the chelators inhibited carbapenemase activity. Doi et al. (303), Pasteran et al. (267), and Tsakris et al. (268) each described a two-disk potentiation test for detection of class A carbapenemases. In this test, one disk contains a carbapenem and the other disk contains a carbapenem plus 3-aminophenylboronic acid (APB), both of which are placed on MHA inoculated with the test organism. Production of a class A carbapenemase is indicated by an increase in the zone diameter around the carbapenem disk with APB compared to that around the carbapenem-only disk.

Tests for carbapenem MIC reduction in the presence of APB or a chelator have been described for detection of both class A carbapenemases and MBLs (267, 304), in addition to the use of the commercial Etest MBL strip. These tests are similar to the CLSI MIC confirmatory test for ESBLs in that they are positive for carbapenemase production if the carbapenem MIC is significantly lower in the presence of the inhibitor than the MIC without the inhibitor. The commercial Etest MBL strip, which has been evaluated in numerous studies, demonstrates substantial performance variability (305–307), but it is both convenient and familiar to most clinical laboratories. Broth microdilution-based MIC reduction tests have been evaluated less widely but have demonstrated good performance for sensitive and specific detection of common MBL enzymes in *Enterobacteriaceae* (267, 308).

These tests for detection of specific carbapenemases are generally convenient and easy to perform, and some have very favorable performance characteristics. However, significant disadvantages of the tests are that multiple tests would have to be performed routinely to detect carbapenemases of different classes, and none of these inhibitor-based tests can detect OXA carbapenemases (288).

DIRECT TESTS FOR β-LACTAMASES

In the clinical laboratory, β-lactamase tests can be used for two purposes. The first is to detect an underlying mechanism of resistance that may not be detected using routine susceptibility testing methods, and the second is to detect a mechanism of resistance that is an infection control concern and is epidemiologically important. These purposes are not mutually exclusive. Identification of a β-lactamase is often epidemiologically important, and in the health care setting, it can be an infection control concern. However, the use of a special test for enzyme detection does not always mean that the β-lactamase test should be used to predict treatment outcomes. For example, a β-lactamase-positive result for a *Neisseria gonorrhoeae* isolate means that the isolate is resistant to penicillin and that penicillin would not be an appropriate treatment choice (19). In contrast, the CLSI test for detection of ESBLs is a useful test for detecting a resistance mechanism with epidemiological and infection control significance, but with the recently revised CLSI cephalosporin breakpoints, this test is not recommended for prediction of cephalosporin treatment outcomes (19). Instead, it is recommended that laboratories use the results of routine susceptibility testing and apply the revised interpretive criteria. A list of the organisms for which β-lactamase tests are useful is given in Table 4.

In the direct β-lactamase test, a positive reaction indicates that the isolate is resistant to the β-lactam agents noted in Table 4, but a negative reaction is inconclusive, because other mechanisms of β-lactam resistance are possible. For example, most ampicillin-resistant *Haemophilus influenzae* isolates produce β-lactamases which can be detected by direct β-lactamase tests; however, rare strains are ampicillin resistant but β-lactamase negative (309–311). For the latter, conventional disk diffusion or dilution tests are needed to detect the resistance (see chapter 71). Three direct β-lactamase assays, the acidimetric, iodometric, and chromogenic methods, have been used widely in the past (312, 313), although the penicillin zone edge test is now recommended for *S. aureus*. Each method involves testing bacteria grown on nonselective media, and the results are available within 1 to 60 min. The acidimetric and iodometric methods use a colorimetric indicator to detect the presence of penicilloic acid in the reaction vessel following β-lactamase hydrolysis of penicillin. In the acidimetric method, the substrates are citrate-buffered penicillin and a phenol red indicator. A decreasing pH associated with the presence of penicilloic acid results in a color change from red (negative result) to yellow (positive result) (314). The substrates in the iodometric test are phosphate-buffered penicillin plus a starch-iodine complex. Penicilloic acid, if present, reduces the iodine and prevents it from combining with starch, resulting in a colorless reaction (positive); a bluish purple color corresponds to a negative result (315).

The chromogenic cephalosporin nitrocefin can be used in a test tube assay (316) but has been incorporated into several filter paper-type disk or strip products that are commercially available and widely used in clinical laboratories. β-Lactamase hydrolysis of the chromogenic cephalosporin molecule causes an electron shift that results in a colored product (316). Although the acidimetric and iodometric methods have varied in performance, perhaps due in part to a lack of experience with these methods, the chromogenic method has been reliable for detecting β-lactamases produced by all of the organisms indicated in Table 4 (317, 318).

The colorimetric β-lactamase tests rely on visualization of a colored product that presumably results from β-lactamase destruction of the substrate β-lactam molecule. However, these tests are not 100% specific, and other substances may yield colored endpoints. Serum may cause a colored reaction with the nitrocefin test (316), and if reagents are not stored properly, spontaneous degradation of penicillin may produce false-positive acidimetric or iodometric β-lactamase reactions. The penicillin zone edge test described above, in "Detection of Penicillin Resistance in Staphylococci," demonstrated much better sensitivity for detecting β-lactamase production in *S. aureus* than any of the colorimetric tests decribed here; therefore, it is now the preferred phenotypic method for use on *S. aureus* (19, 57). The zone edge test has not been evaluated extensively for other bacterial species and is not recommended for routine use for species other than *S. aureus*.

While some bacteria (e.g., *H. influenzae*, *N. gonorrhoeae*, and enterococci) constitutively produce β-lactamases, others (e.g., staphylococci) may produce detectable amounts of enzyme only after exposure to an inducing agent, which is generally a β-lactam (319). If staphylococci produce a positive β-lactamase result without induction, the results can be reported. However, if no β-lactamase is detected, then the test must be performed on cells that have been exposed to an inducing agent before a negative result is reported. This can be done by testing organisms that have been grown in the presence of subinhibitory concentrations

TABLE 4 Bacteria for which β-lactamase tests can be used in the clinical laboratory

Species	Method(s) commonly used[a,b]
Bacteroides spp. and other Gram-negative anaerobes, except the *B. fragilis* group	Direct β-lactamase tests
Enterococcus spp.	Direct β-lactamase tests
Haemophilus influenzae	Direct β-lactamase tests
Moraxella catarrhalis	Direct β-lactamase tests (nitrocefin only)
Neisseria gonorrhoeae	Direct β-lactamase tests
Staphylococcus spp.	Penicillin disk diffusion zone edge test (*S. aureus* only); direct β-lactamase tests with prior induction

[a] A positive result indicates resistance to all penicillinase-labile penicillins, including amoxicillin, ampicillin, azlocillin, carbenicillin, mezlocillin, piperacillin, and ticarcillin; however, a negative result is inconclusive, since other resistance mechanisms may occur.

[b] Direct β-lactamase tests include chromogenic cephalosporin, acidimetric, and iodometric tests.

of a β-lactam agent (e.g., 0.25 μg of cefoxitin per ml) in a broth or agar system. Alternatively, growth from around the periphery of the zone surrounding a β-lactam disk (e.g., a 1-μg oxacillin or 30-μg cefoxitin disk) can be tested. A positive result may take longer to develop for staphylococci than for other organisms, and the test should not be considered negative until it has been allowed to react for at least 60 min. Although a nitrocefin-based test can be performed for *S. aureus*, a negative result should be confirmed by the more sensitive zone edge test described above (19).

Although penicillin resistance in enterococci is not reliably detected with standard susceptibility tests, direct β-lactamase tests using nitrocefin-based assays are effective. However, because β-lactamase-producing enterococci are rare, it is not necessary to perform such testing routinely (19).

β-Lactamase testing by the chromogenic nitrocefin method with anaerobic Gram-negative bacilli other than those from the *Bacteroides fragilis* group may be performed prior to susceptibility testing (320). It is not necessary to test members of the *B. fragilis* group because they characteristically produce β-lactamases and should be considered penicillin resistant. As with aerobes, resistance to β-lactam drugs among anaerobic bacteria is not always mediated by β-lactamase production (e.g., in some strains of *Parabacteroides distasonis* and *B. fragilis*) (252, 253, 320).

The *S. aureus* strains recommended by CLSI for quality control of routine disk diffusion and dilution tests (19) can be used for quality control of β-lactamase tests. *S. aureus* ATCC 25923 is β-lactamase negative, whereas *S. aureus* ATCC 29213 is β-lactamase positive (19).

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Susceptibility Test Methods: Fastidious Bacteria*

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Most fastidious bacteria do not grow satisfactorily in standard *in vitro* susceptibility test systems that use unsupplemented media. For certain fastidious species that are more frequently encountered, such as *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, and other *Streptococcus* spp., slight modifications have been made to standard Clinical and Laboratory Standards Institute (CLSI) disk diffusion and MIC methods to allow reliable testing of these bacteria. The modifications generally involve the use of a test medium with added nutrients and sometimes extended incubation times and/or incubation in an atmosphere with increased levels of CO_2 (Table 1). Specific zone diameter and MIC interpretive criteria have been developed by the CLSI for these bacteria, as have acceptable ranges for recommended quality control (QC) strains (1). The CLSI also published an approved guideline for testing infrequently isolated or fastidious bacteria, including *Abiotrophia* spp., *Granulicatella* spp., *Aeromonas* spp., *Plesiomonas* spp., *Bacillus* spp. (not *Bacillus anthracis*), *Campylobacter jejuni*/*coli*, *Corynebacterium* spp., *Erysipelothrix rhusiopathiae*, the HACEK group (*Aggregatibacter* [formerly the aphrophilus group of *Haemophilus* and *Actinobacillus*], *Cardiobacterium*, *Eikenella*, and *Kingella* spp.), *Helicobacter pylori*, *Lactobacillus* spp., *Leuconostoc* spp., *Listeria monocytogenes*, *Moraxella catarrhalis*, *Pasteurella* spp., *Pediococcus* spp., and *Vibrio* spp. Standard MIC methods for testing potential agents of bioterrorism (*Bacillus anthracis*, *Yersinia pestis*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Francisella tularensis*, and *Brucella* spp.) are also included in CLSI document M45 (2), and conditions for testing these are listed in Table 2. Interpretive criteria for the organisms in the CLSI M45 guideline were primarily adapted from those for organisms included in CLSI standards (1), taking into consideration information in the literature and the experiences of authors of the guideline. This is in contrast to the extensive microbiological, clinical, and pharmacodynamic databases normally used for development of interpretive criteria that appear in CLSI antimicrobial susceptibility testing standards. The classification of M45 as a guideline rather than a standard was based on this fact. A summary of the testing conditions for these organisms appears in Table 3.

There are no specific recommendations for other fastidious bacteria such as *Bordetella* spp. and *Legionella* spp. This is because in part (i) infections caused by these bacteria usually respond to drugs of choice, (ii) isolates are infrequently recovered from patients infected with these organisms, and (iii) isolates are often difficult to grow and special media are required.

In addition to conventional MIC test methods (e.g., agar dilution or broth dilution methods), the Etest MIC determination method (bioMérieux, Marcy L'Etoile, France) has been used to test many types of fastidious bacteria. The Etest approach allows placement of strips on special media and the use of various incubation conditions. The limitations of this method include its cost and lack of clearance by the U.S. Food and Drug Administration (FDA) for testing many less commonly encountered fastidious bacteria. Prior to use of the Etest for clinical testing in the United States, the FDA clearance status for the particular organism-antimicrobial agent combination should be determined. If FDA clearance has not been granted, laboratories should perform a verification study to meet the requirements of the Clinical Laboratory Improvement Amendments to allow use of this test for organisms off-label, and the results should be qualified on the patient report. However, for organisms not included in CLSI document M100 or M45, this is complicated by the absence of a defined method with which to compare results. Laboratories may consider exchanging isolates with a reputable laboratory as a means by which to perform such a verification study, in this instance.

This chapter summarizes the standard methods recommended by the CLSI for antimicrobial susceptibility testing of *Streptococcus* spp. (including *S. pneumoniae*), *H. influenzae*, *N. gonorrhoeae*, and *N. meningitidis*. Methods for testing the infrequently isolated or fastidious bacteria included in CLSI document M45 are summarized to include testing potential agents of bioterrorism. Test methods and indications for testing and the reporting of results are provided.

In the past, regional and global surveillance studies for antimicrobial resistance among respiratory pathogens, including *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis*, were conducted regularly. However, the numbers of these studies have declined, and it is somewhat difficult to get an accurate global picture of the incidence of resistance in these organisms. Similarly, for more infrequently encountered and fastidious bacteria, surveillance studies are rarely performed, and resistance rates are predominantly reported only in single institutional studies and case series and reports. Nonetheless, the incidence of resistance among contemporary isolates is also provided, as available in the cur-

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*This chapter contains information presented by Janet A. Hindler and James H. Jorgensen in chapter 71 of the 10th edition of this *Manual*.

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TABLE 1 Disk diffusion and MIC testing conditions and recommended QC strains for selected fastidious bacteria

Organism(s)	Method	Medium	Inoculum source[a]	Incubation atmosphere[b]	Incubation length (h)	Recommended QC strain(s)
S. pneumoniae and *Streptococcus* spp.	Disk diffusion	MHA + 5% sheep blood	18- to 20-h growth (from SBA)	5–7% CO_2	20–24	*S. pneumoniae* ATCC 49619
	Broth microdilution	CAMHB-LHB	18- to 20-h growth (from SBA)	Ambient air	20–24	*S. pneumoniae* ATCC 49619
H. influenzae and *Haemophilus parainfluenzae*	Disk diffusion	HTM agar	20- to 24-h growth (from CHOC)	5–7% CO_2	16–18	*H. influenzae* ATCC 49247, *H. influenzae* ATCC 49766[c]
	Broth microdilution	HTM broth	20- to 24-h growth (from CHOC)	Ambient air	20–24	*H. influenzae* ATCC 49247, *H. influenzae* ATCC 49766[c]
N. gonorrhoeae	Disk diffusion	GC agar base + supplement	20- to 24-h growth (from CHOC)	5–7% CO_2	20–24	*N. gonorrhoeae* ATCC 49226
	Agar dilution	GC agar base + supplement	20- to 24-h growth (from CHOC)	5–7% CO_2	20–24	*N. gonorrhoeae* ATCC 49226
N. meningitidis	Disk diffusion	MHA + 5% sheep blood	20- to 24-h growth (from CHOC)	5–7% CO_2	20–24	*S. pneumoniae* ATCC 49619
	Broth microdilution	CAMHB-LHB	20- to 24-h growth (from CHOC)	5–7% CO_2	20–24	*S. pneumoniae* ATCC 49619
	Agar dilution	MHA + 5% sheep blood	20- to 24-h growth (from CHOC)	5–7% CO_2	20–24	*S. pneumoniae* ATCC 49619

[a] Inoculum suspension is in Mueller-Hinton broth or saline standardized to a 0.5 McFarland standard. For broth dilution, the final organism concentration is 5×10^5 CFU/ml; for agar dilution, the final organism concentration is 10^4 CFU/spot. CHOC, chocolate agar; SBA, sheep blood agar.

[b] Incubation temperature, 35°C.

[c] In addition, *H. influenzae* ATCC 10211 can be used to assess growth-supporting capabilities of HTM. *H. influenzae* ATCC 49766 is used for QC of selected cephalosporins (e.g., cefaclor, cefamandole, and cefuroxime).

rent published literature for those organisms described in this chapter.

S. PNEUMONIAE

Incidence of Resistance

Penicillin resistance in pneumococci is caused by alterations to the penicillin binding proteins (PBPs), in particular PBPs 2a and 2x and to a lesser extent PBPs 1a and 2b, that result in reduced binding affinity for the penicillins and other β-lactams (3). These altered PBPs are the result of recombination events with homologous genes from related *Streptococcus* species. Since it was first reported in 1967, penicillin resistance in pneumococci has increased steadily worldwide (4). This trend was interrupted for several years by the introduction of the protein-polysaccharide-conjugated 7-valent pneumococcal vaccine (PCV-7) in the United States and other developed countries (5, 6). However, penicillin resistance among pneumococci has since returned to the pre-PCV-7 trajectory (7, 8), as penicillin- and multidrug-resistant, nonvaccine serotypes (e.g., 6C, 15A, 19A, and 23A) have emerged (4, 7). Regardless, the incidence of invasive pneumococcal disease caused by penicillin-nonsusceptible isolates remains lower than that in the pre-PCV-7 era (9). In 2010, the 13-valent conjugate pneumococcal vaccine (PCV-13) was introduced, which includes serotype 19A, the serotype most commonly associated with penicillin resistance in the United States (7, 10). The long-term impact of PCV-13 use on antimicrobial resistance remains uncertain. While the incidence of serotype 19A started to decline in 2007, before release of PCV-13, in the United States (11), it remains the most commonly identified pneumococcal serotype in colonized children (10).

Three sets of penicillin breakpoints exist for pneumococci: one for meningitis, one for nonmeningitis, and one applicable to therapy with oral penicillin G (1, 12). The breakpoints for pneumococcal meningitis are ≤0.06 µg/ml for susceptibility and ≥0.12 µg/ml for resistance, with no intermediate category. For nonmeningitis, ≤2 µg/ml is considered susceptible, 4 µg/ml is considered intermediate, and ≥8 µg/ml is considered resistant. The breakpoints for oral penicillin are ≤0.06 µg/ml for susceptibility, 0.12 to 1 µg/ml for the intermediate category, and ≥2 µg/ml for resistance (1). The SENTRY antimicrobial surveillance program recently reported on the 14-year antimicrobial susceptibility trends for more than 18,900 *S. pneumoniae* isolates from patients with community-acquired respiratory infections and bacteremia at 9 U.S. medical centers between 1998 and 2011 (8). In 2011, 85% of isolates were susceptible to penicillin by the nonmeningitis breakpoints and 56% by the meningitis breakpoints; in contrast, 97% and 72% susceptibility, respectively, was identified by this study in 1998 (8). In parts of the world where the PCV-7 vaccine has not been widely used, penicillin resistance rates remain high and serotype 19A has increased in incidence for reasons other than serotype replacement disease among a vaccinated population (5). In particular, penicillin susceptibility was found to be only 54% by the nonmeningitis breakpoint in an international study of more than 3,300 isolates recovered from various specimen sources among pediatric patients between 2004 and 2011 (13). In a study of isolates collected from 20 hospitals in Taiwan between 2006 and 2010, only 11.7% of isolates were susceptible to penicillin by the

TABLE 2 Broth microdilution MIC testing conditions, recommended QC strains, and drugs recommended for testing potential agents of bioterrorism

Organism(s)	Medium	Inoculum source[a]	Incubation atmosphere[b]	Incubation length (h)	Recommended QC strain(s)	Drugs recommended for testing
B. anthracis	CAMHB	16- to 18-h growth (from SBA)	Ambient air	16–20	*Escherichia coli* ATCC 25922, *S. aureus* ATCC 29213	Ciprofloxacin, doxycycline, levofloxacin, penicillin, tetracycline
Brucella spp.	Brucella broth, pH adjusted to 7.1 ± 0.1	48-h growth (from SBA)	Ambient air[c]	48	*E. coli* ATCC 25922, *S. pneumoniae* ATCC 49619	Doxycycline, gentamicin, tetracycline, TMP-SMX, streptomycin[d]
B. mallei, *B. pseudomallei*	CAMHB	16- to 18-h growth (from SBA)	Ambient air	16–20	*E. coli* ATCC 25922, *E. coli* ATCC 35218,[e] *P. aeruginosa* ATCC 27853	Amoxicillin-clavulanic acid (*B. pseudomallei* only), ceftazidime, doxycycline, imipenem, tetracycline, TMP-SMX (*B. pseudomallei* only)
F. tularensis	CAMHB with 2% defined growth supplement, pH adjusted to 7.1 ± 0.1	24-h growth (from CHOC)	Ambient air	48	*E. coli* ATCC 25922, *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853	Chloramphenicol, ciprofloxacin, doxycycline, gentamicin, levofloxacin, streptomycin, tetracycline
Y. pestis	CAMHB	24-h growth (from SBA)	Ambient air	24[f]	*E. coli* ATCC 25922	Chloramphenicol, ciprofloxacin, doxycycline, gentamicin, levofloxacin, streptomycin, tetracycline, TMP-SMX

[a] Inoculum suspension is in CAMHB standardized to a 0.5 McFarland standard. The final organism concentration is 5 × 10^5 CFU/ml. CHOC, chocolate agar; SBA, sheep blood agar.

[b] Incubation temperature, 35°C.

[c] Some isolates may require incubation in 5% CO_2.

[d] Different breakpoints for incubation in 5% CO_2 versus ambient air.

[e] *E. coli* ATCC 35218 is used for QC when testing β-lactam–β-lactamase inhibitor combination drugs.

[f] If unacceptable growth is in the control well, reincubate for an additional 24 h.

meningitis breakpoints, and 63.2% of isolates were susceptible by the nonmeningitis breakpoints (14). Among 616 isolates collected in 2010 from the Asia-Pacific region and South Africa, the rates of penicillin resistance by the nonmeningitis breakpoints were found to be ≥50% in Hong Kong, Korea, South Africa, and Thailand (15).

Strains of *S. pneumoniae* that are susceptible to penicillin generally are susceptible to other β-lactam agents; however, as the penicillin MIC increases, the MICs of other β-lactams also increase (16). Current rates of resistance to the extended-spectrum cephalosporins vary by location (6, 8, 13, 15). In the international survey of pediatric isolates mentioned above, 93% of all isolates were susceptible to ceftriaxone (MIC, ≤1.0 μg/ml) (13). Ceftaroline, an antimicrobial recently approved by the FDA for the treatment of community-acquired bacterial pneumonia, displays the most potent activity among the β-lactams against *S. pneumoniae*. The SENTRY U.S. study documented 94.5% susceptibility in 2011 by the FDA breakpoint, which at the time was ≤0.25 μg/ml, among penicillin-resistant (MIC, ≥2 μg/ml) isolates (8). The current CLSI and FDA susceptibility breakpoint for ceftaroline is ≤0.5 μg/ml (1).

Resistance has been described for all other classes of antimicrobial agents that are usually considered for treating pneumococcal infections, except for the glycopeptides. Resistance to macrolides and clindamycin has increased in the United States in the post-PCV-7 era (6) and appears to be influenced by macrolide use. The SENTRY study found that 82% of isolates were macrolide susceptible (MIC, ≤0.25 μg/ml) just prior to the introduction of PCV-7, whereas in 2011 macrolide susceptibility had declined to 55.2%. In the same study, clindamycin susceptibility declined from 96.2% pre-PCV to 78.2% in 2011 (8). Resistance to macrolides in Asian-Pacific countries is much higher than in the United States, exceeding 75% in some areas (15, 17). Erythromycin MICs for pneumococci with macrolide-lincosamide-streptogramin B (MLS)-type resistance (encoded by the *ermB* gene) are usually ≥64 μg/ml, and clindamycin MICs are ≥8 μg/ml, whereas for isolates with the M phenotype (encoded by the *mefA* gene), erythromycin MICs are 1 to 32 μg/ml and clindamycin MICs are ≤0.25 μg/ml. The clinical significance of the lower-level macrolide resistance expressed by the M phenotype was once debated; however, treatment failures have been documented with such strains (18). Erythromycin resistance but clindamycin susceptibility may also be an indicator of the presence of an inducible *ermB* gene (19). There is a theoretical risk of spontaneous conversion from inducible to constitutive resistance in these isolates during extended clindamycin therapy, although this has not specifically been demonstrated for pneumococci. Susceptibility and resistance to azithromycin, clarithromycin, and dirithromycin among *S. pneumoniae* isolates can be predicted by testing erythromycin (1). Telithromycin, a ketolide, is considerably more active than macrolides against *S. pneumoniae*, with less than 1% of isolates showing resistance in

TABLE 3 Disk diffusion and MIC testing conditions, recommended QC strains, and agents to consider for primary testing for infrequently isolated or less common fastidious bacteria

Organism(s)	Method	Medium	Inoculum source[a]	Incubation atmosphere[b]	Incubation length (h)	Recommended QC strain(s)	Agents to consider for primary testing
Abiotrophia spp., *Granulicatella* spp.	Broth microdilution	CAMHB-LHB + 0.001% pyridoxal HCl	20- to 24-h growth (from CHOC containing cysteine)	Ambient air	20–24	*S. pneumoniae* ATCC 49619	Cefotaxime or ceftriaxone, penicillin, vancomycin
Aeromonas hydrophila complex, *Plesiomonas shigelloides*	Broth microdilution	CAMHB	16- to 18-h growth (from SBA)	Ambient air	16–20	*E. coli* ATCC 25922, *E. coli* ATCC 35218[c]	Amoxicillin-clavulanic acid, broad-spectrum or extended-spectrum cephalosporins, fluoroquinolones, TMP-SMX
	Disk diffusion	MHA	16- to 18-h growth (from SBA)	Ambient air	16–18	*E. coli* ATCC 25922, *E. coli* ATCC 35218	Amoxicillin-clavulanic acid, broad-spectrum or extended-spectrum cephalosporins, fluoroquinolones, TMP-SMX
Bacillus spp. (not *B. anthracis*)	Broth microdilution	CAMHB	16- to 18-h growth (from SBA)	Ambient air	16–20	*S. aureus* ATCC 29213	Clindamycin, fluoroquinolones, vancomycin
C. jejuni, *C. coli*	Broth microdilution	CAMHB-LHB	24- to 48-h growth (from SBA)	10% CO_2, 5% O_2, 85% N_2 (microaerobic)	48 at 36°C or 24 at 42°C	*C. jejuni* ATCC 33560	Ciprofloxacin, erythromycin
Corynebacterium spp., coryneforms	Broth microdilution	CAMHB-LHB	24- to 48 h growth (from SBA or CHOC)	Ambient air	24–48	*S. pneumoniae* ATCC 49619	Erythromycin, gentamicin, penicillin, vancomycin
E. rhusiopathiae	Broth microdilution	CAMHB-LHB	18- to 24-h growth (from SBA)	Ambient air	20–24	*S. pneumoniae* ATCC 49619	Penicillin or ampicillin
HACEK group	Broth microdilution	CAMHB-LHB	24- to 48-h growth (from CHOC)	5% CO_2	24–48	*S. pneumoniae* ATCC 49619, *E. coli* ATCC 35218	Ampicillin, amoxicillin-clavulanic acid, ceftriaxone or cefotaxime, ciprofloxacin or levofloxacin, imipenem, TMP-SMX
H. pylori	Agar dilution	MHA + 5% aged (≥2-wk-old) sheep blood	72-h growth (from SBA)[e]	Microaerobic; produced by gas-generating system for campylobacters	~72	*H. pylori* ATCC 43504	Clarithromycin
Lactobacillus spp.	Broth microdilution	CAMHB-LHB	18- to 24-h growth (from SBA)	5% CO_2	20–24	*S. pneumoniae* ATCC 49619	Penicillin or ampicillin, gentamicin (for combined therapy)
Leuconostoc spp.	Broth microdilution	CAMHB-LHB	18- to 24-h growth (from SBA)	Ambient air	20–24	*S. pneumoniae* ATCC 49619	Penicillin or ampicillin, gentamicin (for combined therapy)
L. monocytogenes	Broth microdilution	CAMHB-LHB	18- to 24-h growth (from SBA)	Ambient air	20–24	*S. pneumoniae* ATCC 49619	Penicillin or ampicillin, TMP-SMX
M. catarrhalis	Broth microdilution	CAMHB	18- to 24-h growth (from SBA)	Ambient air	20–24	*S. aureus* ATCC 29213, *E. coli* ATCC 35218	Amoxicillin-clavulanic acid, cefaclor or cefuroxime, TMP-SMX
	Disk diffusion	MHA	18- to 24-h growth (from SBA)	Ambient air	20–24	*S. aureus* ATCC 25923, *E. coli* ATCC 35218	Amoxicillin-clavulanic acid, TMP-SMX

(*Continued on next page*)

TABLE 3 Disk diffusion and MIC testing conditions, recommended QC strains, and agents to consider for primary testing for infrequently isolated or less common fastidious bacteria *(Continued)*

Organism(s)	Method	Medium	Inoculum source[a]	Incubation atmosphere[b]	Incubation length (h)	Recommended QC strain(s)	Agents to consider for primary testing
Pasteurella spp.	Broth microdilution	CAMHB-LHB	18- to 24-h growth (from SBA)	Ambient air	18–24	*S. pneumoniae* ATCC 49619, *E. coli* ATCC 35218	β-Lactam–β-lactamase inhibitor combinations, cephalosporins, fluoroquinolones, macrolides, penicillins, tetracyclines, TMP-SMX
	Disk diffusion	MHA with 5% sheep blood	18- to 24-h growth (from SBA)	Ambient air	16-18	*S. pneumoniae* ATCC 49619, *E. coli* ATCC 35218, *S. aureus* ATCC 25923	β-Lactam–β-lactamase inhibitor combinations, cephalosporins, fluoroquinolones, macrolides, penicillins, tetracyclines, TMP-SMX
Pediococcus spp.	Broth microdilution	CAMHB-LHB	18- to 24-h growth (from SBA)	Ambient air	20–24	*S. pneumoniae* ATCC 49619	Penicillin, gentamicin (for combined therapy)
Vibrio spp. (not *V. cholerae*)[e]	Broth microdilution	CAMHB	16- to 18-h growth (from SBA)	Ambient air	16–20	*E. coli* ATCC 25922, *E. coli* ATCC 35218	For *Vibrio* spp. (not *V. cholerae*): cefotaxime, ceftazidime, tetracycline, fluoroquinolones For *V. cholerae*: ampicillin, azithromycin, chloramphenicol, doxycycline, sulfonamides, tetracycline, TMP-SMX
	Disk diffusion	MHA	16- to 18-h growth (from SBA)	Ambient air	16–18	*E. coli* ATCC 25922, *E. coli* ATCC 35218	For *Vibrio* spp. (not *V. cholerae*): cefotaxime, ceftazidime, tetracycline, fluoroquinolones For *V. cholerae*: ampicillin, azithromycin, chloramphenicol, doxycycline, sulfonamides, tetracycline, TMP-SMX

[a] Suspension is in Mueller-Hinton broth or saline standardized to a 0.5 McFarland standard. For broth dilution, the final organism concentration is 5 × 10^5 CFU/ml. CHOC, chocolate agar; SBA, sheep blood agar.

[b] Incubation temperature, 35°C (except for *C. jejuni* and *C. coli*).

[c] *E. coli* ATCC 35218 is used for QC when testing β-lactam–β-lactamase inhibitor combination drugs.

[d] Suspension is in saline standardized to a 2.0 McFarland standard.

[e] For halophilic species, prepare inoculum in 0.85% NaCl (normal saline).

North America in 2003 and 2004, the most recent year for which susceptibility data for this agent are available (20).

Resistance rates for trimethoprim-sulfamethoxazole (TMP-SMX) range from 35 to 47% in the United States in the pre- and post-PCV-7 eras and are similar outside the United States (8, 21). Resistance rates for fluoroquinolones have remained low (6, 8, 22), but resistance is more frequent in elderly patients (22, 23). Resistance has only rarely been documented by surveillance studies for linezolid, tigecycline, and quinupristin-dalfopristin (6, 24).

Reference Test Methods

The CLSI describes both a broth microdilution method and a disk diffusion procedure for testing pneumococci. Details of these methods are listed in Table 1. The broth microdilution method may be used to test all of the antimicrobial agents recommended by the CLSI for pneumococci. With the exception of the oxacillin disk screening test for penicillin susceptibility, the disk diffusion method does not work reliably for testing β-lactam agents, including the cephalosporins. When used to predict penicillin susceptibility, oxacillin disk diffusion zone diameters of ≥20 mm indicate that the isolate is susceptible to penicillin using the more conservative meningitis or oral penicillin breakpoints (1). However, strains with zone diameters of ≤19 mm cannot be readily categorized as resistant, and at a minimum the MICs of penicillin and an extended-spectrum cephalosporin or meropenem should be determined. It should be noted that the oxacillin screening procedure should be used only for isolates from patients with non-life-threatening infections.

If clindamycin testing is performed, it should include a screen for inducible clindamycin resistance. This can be accomplished via either the D-zone disk diffusion test or by a single-well broth microdilution test. For the D-zone test, a 15-μg erythromycin disk and a 2-μg clindamycin disk are placed 12 mm apart on Mueller-Hinton agar (MHA) supplemented with sheep blood (5%, vol/vol); a flattening of the clindamycin zone of inhibition adjacent to the erythromycin disk is indicative of inducible clindamycin resistance. For the broth microdilution test, 1 μg/ml of erythromycin and 0.5 μg/ml of clindamycin are combined in a single well (1); growth is indicative of inducible clindamycin resistance.

Increased use of tetracyclines, in particular doxycycline, as alternative agents for the treatment of community-acquired pneumonia in ambulatory and penicillin-allergic patients prompted the CLSI to revise the *S. pneumoniae* tetracycline susceptibility breakpoint from ≤2 μg/ml to ≤1 μg/ml, concomitant with introduction of doxycycline-specific breakpoints in 2013 (1). Prior to this change, doxycycline susceptibility was inferred from tetracycline testing, although doxycycline is more active than tetracycline against *S. pneumoniae* (25, 26). Currently, the CLSI has not assigned minocycline breakpoints for *S. pneumoniae*, and susceptibility is inferred from tetracycline testing (1).

Commercial Methods for Testing

Several options are available for determining MICs of various antimicrobial agents with pneumococci. The Etest method has been extensively evaluated (27, 28). Etest strips have been cleared by the FDA for all drugs recommended by the CLSI for testing of *S. pneumoniae* except doxycycline and telithromycin. The accuracy of the Etest has been reported to be >90% for most relevant drugs (27, 28), although the number of minor errors with penicillin is relatively high and Etest penicillin MICs are slightly lower than those determined by reference broth microdilution (27). Since it is recommended that the Etest be incubated in CO_2, the MICs of the macrolides and ketolides tend to be 1 or 2 dilutions higher than those by the reference broth method, in which incubation is conducted in ambient air. This is because these agents are less active at lower pH, which occurs with CO_2 incubation.

Other commercially available FDA-cleared panels or systems specifically designed for testing pneumococci include MicroScan (Siemens Healthcare Inc., West Sacramento, CA), Phoenix (BD Diagnostic Systems, Sparks, MD), Sensititre (Trek Diagnostic Systems, Inc., Cleveland, OH), and VITEK 2 (bioMérieux, Inc., Durham, NC). The MicroScan MICroSTREP, Sensititre, VITEK 2, and Phoenix systems have been evaluated and found to show MICs that are comparable to those obtained with the CLSI broth microdilution reference method (28–30).

Strategies for Testing and Reporting of Results

The CLSI stipulates that *S. pneumoniae* isolates from cerebrospinal fluid (CSF) should be routinely tested by a reliable MIC method to determine susceptibilities to penicillin and cefotaxime, ceftriaxone, or meropenem (1). For CSF isolates, as with penicillin, only interpretations using the meningitis breakpoints should be reported for cefotaxime and ceftriaxone; for isolates from other sites, both meningitis and nonmeningitis interpretations should be reported for these agents (1). In addition, vancomycin can be tested by either an MIC or a disk diffusion method on isolates from CSF. Along with penicillin, the CLSI recommends primary testing and reporting of erythromycin and TMP-SMX for isolates from non-CSF sources; these drugs can be tested by either an MIC or a disk diffusion method (1). MIC testing of penicillin, cefotaxime or ceftriaxone, and/or meropenem, and MIC or disk diffusion testing of vancomycin, a fluoroquinolone, and tetracycline or doxycycline would also be appropriate for strains isolated from non-CSF sources. Other drugs that might warrant testing if being considered for treatment of infections other than meningitis include cefepime, clindamycin, and telithromycin. For isolates that are resistant to erythromycin and susceptible or intermediate to clindamycin, screening for inducible clindamycin resistance should be performed prior to reporting an isolate as clindamycin susceptible (1).

STREPTOCOCCI OTHER THAN PNEUMOCOCCI

Incidence of Resistance

Because there are significant differences in susceptibility of β-lactam agents in viridans group versus β-hemolytic streptococci, there are separate ampicillin, penicillin, cefotaxime, ceftriaxone, and cefepime interpretive criteria for the two organism groups (1). The viridans group of streptococci includes the mutans group, salivarius group, bovis group, anginosus group (previously "*Streptococcus milleri*" group), and mitis group. The anginosus group includes small-colony-forming β-hemolytic strains with group A, C, F, or G antigens. The β-hemolytic group includes the large-colony-forming pyogenic strains with group A (*Streptococcus pyogenes*), C, or G antigens and strains with group B (*Streptococcus agalactiae*) antigen.

Although β-hemolytic streptococci have been uniformly susceptible to penicillin (MIC ≤ 0.12 μg/ml), two reports have described penicillin MICs of 0.25 to 0.5 μg/ml for *S. agalactiae* (31) and group C streptococci (32). Kimura et al. from Japan reported 14 isolates of *Streptococcus agalactiae* collected from 1995 to 2005 with penicillin MICs of 0.25 to 1.0 μg/ml. These isolates harbor a point mutation in the PBP 2x gene, and introduction of this organism's PBP 2x into penicillin-susceptible strains through allelic exchange resulted in elevated penicillin MICs (33). The clinical significance of the elevated penicillin MICs in these β-hemolytic streptococci is not known. These isolates also demonstrated fluoroquinolone nonsusceptibility and were more frequently macrolide resistant than fully penicillin-susceptible isolates (34). Similar isolates with altered PBP 2x and elevated penicillin MICs have subsequently been reported in the United States (35) and Canada (36, 37). High-level aminoglycoside resistance has been described on rare occasions for *S. agalactiae*.

The MLS-type macrolide resistance due to *erm* genes in β-hemolytic streptococci may be either inducible or constitutive. If the resistance is inducible, the strains will be resistant to erythromycin (and the other 14- and 15-membered macrolides) but appear susceptible to clindamycin unless resistance is induced. The rate of erythromycin resistance was recently reported to be 23% and that of clindamycin resistance to be 12.6% from an international surveillance study of 763 β-hemolytic streptococci isolated from skin and soft tissue, respiratory tract, and bloodstream infections in 2010 (38). Rates of resistance to erythromycin and clindamycin among 155 *S. pyogenes* isolates from U.S. patients in 2011 were 13.5% and 3.9%, respectively (39). In contrast, rates of erythromycin and clindamycin resistance among 153 *S. agalactiae* U.S. isolates were 54% and 33%, respectively (39). Resistance to erythromycin and clindamycin among contemporary isolates of *S. agalactiae* is significantly lower in Canada (40) and Europe. *S. agalactiae* strains colonizing pregnant women and isolated as part of prenatal screening are more likely to be erythromycin and clindamycin susceptible than those isolated from clinical infections (41).

Resistance rates among β-hemolytic streptococci vary for tetracyclines and remain low for fluoroquinolones. In several recent large surveillance studies, some international, resistance was not observed for daptomycin or linezolid (40, 42).

Penicillin resistance in viridans group streptococci has been described, and resistance rates among bloodstream isolates can be >50% (43, 44), particularly for *S. mitis*, *S. oralis*, and *S. sanguinis*. Resistance has been described for some viridans group streptococcal isolates for macrolides, lincosamides, and tetracyclines (43–45), quinupristin-dalfopristin (44, 45), and fluoroquinolones (45). Single reports of resistance to vancomycin have been noted for *S. gallolyticus* (46) and *S. mitis* (47), and resistance to linezolid has been reported on rare occasions (48). In a European surveillance study, two viridans group *Streptococcus* isolates showed a daptomycin MIC of 2 μg/ml, which indicates nonsusceptibility according to CLSI criteria (49), and a case of daptomycin-nonsusceptible *S. anginosus* bacteremia was recently described for a patient with prior daptomycin exposure (50). Among a large international collection of bloodstream isolates, all were susceptible to tigecycline (44).

Reference Test Methods

The disk diffusion test may be used to determine the penicillin susceptibility of β-hemolytic streptococci; however, it is unreliable and should not be used for viridans group streptococci (51). For either group, carbapenems and daptomycin should be tested only by an MIC method, whereas other agents may be tested by either the MIC or disk method (1). Aminoglycoside MICs of >1,000 μg/ml for streptococci have been described; however, there are no published methods for screening for high-level aminoglycoside resistance in streptococci.

CLSI standards describe a D-zone test for inducible resistance to clindamycin in β-hemolytic streptococci, which is performed in the same manner as the D-zone test described above for *S. pneumoniae*. Alternatively, a single-well erythromycin (1 μg/ml) plus clindamycin (0.5 μg/ml) combination broth microdilution test can be performed, which displays >95% sensitivity and specificity compared to those of the D-zone test, for the detection of inducible clindamycin resistance (52).

Commercial Test Methods

There have been several reports demonstrating that the BD Phoenix performs reliably for susceptibility testing of *Streptococcus* spp. (30). Two studies have evaluated the performance of the MicroScan MICroSTREP plus antimicrobial panel for testing antimicrobial susceptibility of streptococci (53); one study demonstrated reliable performance of the system for the β-hemolytic streptococci. However, numerous minor errors were noted in the second study with viridans group streptococci for ampicillin and penicillin (54). For the other test systems, there are no peer-reviewed publications, although it might be expected that systems capable of testing pneumococci would also perform adequately for other streptococci. The MicroScan, Phoenix, and Sensititre systems are cleared by the FDA for the testing of *Streptococcus* spp. The VITEK 2 system received FDA clearance in 2013 for the AST-ST01 card, with expanded species coverage that includes *S. pneumoniae*, as well as the viridans group and β-hemolytic streptococci. Etest has not been extensively evaluated but does appear to be a possible alternative to the reference methods for testing *Streptococcus* spp. (55, 56).

Strategies for Testing and Reporting of Results

Routine testing of β-hemolytic streptococci is unnecessary since there have been only sporadic reports of decreased susceptibility to penicillin, primarily in *S. agalactiae* (31–33, 35–37). However, if erythromycin is being used to treat infections caused by *S. pyogenes* and treatment failure is suspected, testing might be considered. Similarly, if clindamycin is being considered for prophylaxis of pregnant women who are highly allergic to penicillin, in an effort to prevent perinatal group B streptococcal disease, testing for erythromycin and clindamycin, including inducible resistance, should be performed. Erythromycin is not reported but tested only to determine if an isolate has the erythromycin-resistant and clindamycin-susceptible phenotype, in which case a test for inducible clindamycin would be required prior to reporting the isolate as clindamycin susceptible. For viridans group streptococci, penicillin MICs should be determined for strains isolated from blood, especially for patients with infective endocarditis.

H. INFLUENZAE

Incidence of Resistance

Ampicillin resistance in *H. influenzae* is primarily due to a plasmid-borne β-lactamase. TEM-1 is the most common enzyme, and ROB-1 contributes to less than 5% of β-lactamase-producing strains (57). It was recently shown among 11 *H. influenzae* isolates from several countries containing *bla*$_{\text{ROB-1}}$ that the plasmid on which *bla*$_{\text{ROB-1}}$ is commonly found is mobilized at a low frequency, which might account for the infrequency of $bla_{\text{ROB-1}}$ compared to the rate of occurrence of $bla_{\text{TEM-1}}$ (58). Ampicillin resistance can also result from altered penicillin-binding proteins in β-lactamase-negative, ampicillin-resistant (BLNAR) strains. Resistance in BLNAR strains is primarily associated with mutations of the *ftsI* gene that encodes PBP 3 (59, 60), which is the sole essential PBP in *H. influenzae* (59). A singular N526K mutation has been shown to result in a 2-fold increase in the MIC of ampicillin and a 2- to 8-fold increase in resistance to various cephalosporins. This mutation is relatively common in BLNAR isolates, although other mutations have been described (61–63). Isolates of BLNAR *H. influenzae* express lower levels of resistance to ampicillin, with MICs generally ranging from 0.5 to 8 µg/ml. Compared with β-lactamase-producing or ampicillin-susceptible *H. influenzae*, BLNAR isolates are slightly less susceptible to amoxicillin-clavulanic acid and to various cephalosporins, such as cefaclor and cefotaxime (64–66). Some strains may possess this β-lactam resistance mechanism and also produce β-lactamase; these strains are referred to as β-lactamase-producing amoxicillin-clavulanic acid-resistant (BLPACR) (61). Ampicillin MICs for β-lactamase-producing *H. influenzae* and BLPACR isolates are as high as 512 µg/ml (61, 63).

A study in the United States showed that 27% of 1,545 isolates collected from 2008 to 2010 were β-lactamase positive (67). Similar rates were noted in a global surveillance study in 2010 (38) in which 24% of isolates were β-lactamase positive and in another study of isolates from the Asia-Pacific region and South Africa in which 22% were reported as β-lactamase positive (15). From 2009 to 2010, 43% of 174 isolates from Taiwan were reported to be β-lactamase positive (68). However, the reported incidences of *H. influenzae* strains that are BLNAR and BLPACR vary considerably. From 2009 to 2012, 9.8% of *H. influenzae* isolates (n = 266) from patients with community-acquired pneumonia in 6 cities in China were β-lactamase producing and 9.0% were BLNAR (66). A study conducted in a single hospital in Thailand between September 2005 and March 2008 reported rates of β-lactamase-positive and BLNAR *H. influenzae* isolates of 40.9% and 18.2%, respectively (69). Investigators in Sweden noted a significant increase in β-lactam-resistant invasive isolates of *H. influenzae* over the last decade, mainly due to an increase in BLNAR isolates (70). Among 2,206 isolates collected from children aged ≤5 years in France between 2001 and 2008, 27.5% were β-lactamase positive, while 16.9% were BLNAR and 6.5% were BLPACR. Authors of that study noted a statistically significant decrease in β-lactamase-producing strains ($P < 0.0001$) and a statistically significant increase in BLNAR in isolates from 2001 to 2008 (64). A decrease in β-lactamase-producing isolates has also been documented in North America and Europe. In Japan, BLNAR strains are prevalent and more common than β-lactamase-producing strains (17, 65).

Resistance among *H. influenzae* isolates to broad-spectrum oral cephalosporins (e.g., cefixime and cefpodoxime) (64, 71) and to extended-spectrum cephalosporins (e.g., ceftriaxone) (15, 38, 64, 67, 72) is rare. All 453 *H. influenzae* isolates from the Asia-Pacific region and Africa were susceptible to the newer extended-spectrum cephalosporin ceftaroline when a CLSI susceptibility breakpoint of ≤0.5 µg/ml was used; however, when a EUCAST breakpoint of ≤0.03 µg/ml was used, only 93.4% were susceptible (15). For 515 *H. influenzae* isolates from Europe, 100% were susceptible to ceftaroline using CLSI breakpoints and 97.7% were susceptible with EUCAST breakpoints (72). In a U.S. study of 1,545 isolates, 99.9% were susceptible to ceftaroline using the FDA breakpoint at that time of ≤0.12 µg/ml (67). All 3 studies included at least 25% β-lactamase-producing isolates. Use of the various breakpoints emphasizes the need to check this detail when evaluating antimicrobial surveillance data.

Of 1,545 isolates of *H. influenzae* collected from 2008 to 2010 in the United States, 77.2% were susceptible to TMP-SMX (67), and 68% of 360 isolates in a global study were TMP-SMX susceptible (38). Resistance to fluoroquinolones remains rare in *H. influenzae* (15, 67, 72); however, investigators in Taiwan recently described the emergence of polyclonal levofloxacin- and moxifloxacin-resistant *H. influenzae* isolates among residents of a nursing home where nearly half of the 48 isolates recovered from 30 patients were resistant to fluoroquinolones (73). A surveillance study in Asia noted nearly 6% levofloxacin resistance in Taiwan but rates of less than 1% fluoroquinolone resistance in several other Asian countries (68). Resistance to newer macrolides is uncommon, with less than 3% of isolates examined in various studies showing resistance to azithromycin (15, 38, 67).

Reference Test Methods

β-Lactamase production in *H. influenzae* can easily be detected by the chromogenic cephalosporin, acidometric, or iodometric β-lactamase test methods (see chapter 73). The CLSI has developed broth microdilution MIC and disk diffusion methods for testing *H. influenzae*, and these methods can also be used to test *H. parainfluenzae*. Testing of the more unusual *Haemophilus* spp., including *Aggregatibacter aphrophilus* (formerly *H. aphrophilus* and *H. paraphrophilus*) and *Aggregatibacter segnis* (formerly *H. segnis*), is now addressed in CLSI document M45 (2). Specific variables related to each of the methods for testing *H. influenzae* and *H. parainfluenzae* are listed in Table 1. *Haemophilus* test medium (HTM) is recommended and consists of a Mueller-Hinton base, 15 µg of hematin per ml, 15 µg of NAD per ml, and 5 mg of yeast extract per ml. Cation-adjusted Mueller-Hinton broth (CAMHB) is used with the components listed above for the preparation of HTM broth, which contains 0.2 IU of thymidine phosphorylase per ml when testing sulfonamides or trimethoprim. Although HTM agar is transparent, difficulties in measuring zones and poor growth of some strains have been noted. Broth microdilution tests with HTM generally give clearer endpoints. The problems most often noted with both the disk diffusion and the broth microdilution methods are equivocal endpoints with BLNAR strains with several β-lactams. Because of this, the CLSI recommends that BLNAR strains (which are best detected by tests with ampicillin) be considered resistant to amoxicillin-clavulanic acid, ampicillin-sulbactam, cefaclor, cefamandole, cefetamet, cefonicid, cefprozil, cefuroxime, loracarbef, and piperacillin-tazobactam and that the activities of these agents against BLNAR strains not be tested (1). Some have suggested the use of disks with an ampicillin concentration lower (63, 74) than the 10 µg recommended by the CLSI (1). In addition to the CLSI, the EUCAST (http://www.eucast.org/) describes methods and interpretive criteria for both MIC and disk diffusion testing of *Haemophilus influenzae*. The methods are nearly identical to the CLSI methods, with the exception of the test media, which are MHA with 5% defibrinated horse blood and 10 mg/liter of β-NAD for disk diffusion and Mueller-Hinton broth supplemented with 5% lysed horse blood and 20 mg/liter of β-NAD for MIC testing. Recently, investigators tested 89 *H. influenzae* isolates with various ampicillin resistance mechanisms by disk diffusion with 12 β-lactam drugs. The main genotypes of β-lactam-resistant strains were separated by using the EUCAST disk diffusion method; however, there was some overlap among ampicillin-susceptible and BLNAR strains (74).

Commercial Test Methods

Currently, the only FDA-cleared broth microdilution system for testing *Haemophilus* spp. is Sensititre (Trek). The Etest has been cleared by the FDA for testing *H. influenzae* with most drugs that would be used for treating *Haemophilus* infections, and Etest generally demonstrates results comparable to those obtained by CLSI reference methods. However, Billal et al. reported that Etest produces considerably higher MICs than broth microdilution when testing ampicillin, amoxicillin-clavulanic acid, and cefixime and perhaps other β-lactams (76). This is a particular concern with BLNAR and BLPACR strains.

Strategies for Testing and Reporting of Results

β-Lactamase testing detects the most common type of clinically significant resistance in *H. influenzae*. β-Lactamase-positive isolates are ampicillin and amoxicillin resistant. To detect BLNAR strains, an ampicillin disk diffusion or MIC test is required. To detect BLPACR strains, both a β-lactamase test and amoxicillin-clavulanic disk diffusion or MIC test are required, as these strains are β-lactamase positive and show decreased susceptibility to amoxicillin-clavulanic acid. Since the incidence of BLNAR is currently low in many geographic areas, such tests may not be routinely needed and for practical purposes, a negative β-lactamase test result translates into ampicillin susceptibility. However, for laboratories where the incidence of BLNAR is unknown because routine susceptibility tests are not performed on *H. influenzae* isolates, it would be reasonable to perform susceptibility tests, at a minimum, on isolates from sterile sources with ampicillin (for β-lactamase negative strains) and ceftriaxone or any other agent that might be considered for therapy. Testing should be considered for TMP-SMX, and the CLSI suggests that this agent be tested and reported routinely (1). Other oral agents that might be considered, such as amoxicillin-clavulanic acid, the oral cephalosporins, newer macrolides, and fluoroquinolones, are predictably active against *H. influenzae* and are often prescribed empirically. Consequently, routine testing of these drugs is generally not useful. However, these and other agents may be tested for surveillance or epidemiological purposes (1).

N. GONORRHOEAE

Incidence of Resistance

Antimicrobial resistance among *N. gonorrhoeae* isolates is a significant concern, and resistance to penicillin, tetracycline, and the fluoroquinolones has precluded the use of these drugs as primary agents for the treatment of gonococcal infections in most countries. In 2012, emerging resistance to the oral cephalosporins among *N. gonorrhoeae* isolates in the United States led the CDC to recommend combination therapy with ceftriaxone and azithromycin or doxycycline as the first-line regimen for the treatment of uncomplicated gonorrhea (77).

Penicillin resistance in *N. gonorrhoeae* occurs via either the production of a plasmid-associated TEM-1 or TEM-135 β-lactamase (penicillinase-producing *N. gonorrhoeae* [PPNG]) or multifactorial mutations to the chromosome (chromosomally mediated resistant *N. gonorrhoeae* [CMRNG]). CMRNG arises through stepwise acquisition of mutations to various genes that result in penicillin resistance. These include mutation to the primary penicillin targets, PBP 1 and PBP 2, the membrane porin PorB1b, and the promoter and coding sequence of the transcriptional repressor of the MtrCDE efflux pump, *mtrR*. While the spectrum of activity for the TEM β-lactamases expressed by PPNG is restricted to the penicillins (78), CMRNG may show decreased susceptibility to other β-lactams.

Strains of *N. gonorrhoeae* with reduced susceptibility to the cephalosporins (e.g., cefixime [MIC ≥ 0.25 μg/ml] and ceftriaxone [MIC ≥ 0.125 μg/ml]) emerged in Japan in 2007 (79) and have now spread to many parts of the world (80–82). Whereas penicillin-resistant CMRNG isolates harbor only 4 to 8 mutations to *penA*, the gene that encodes PBP2, isolates with elevated cephalosporin MICs harbor a mosaic *penA* gene that encodes more than 60 amino acid polymorphisms compared to the wild-type PBP 2 (83). The mosaic *penA* gene is thought to have arisen from DNA recombination with the *penA* genes of multiple saprophytic *Neisseria* spp. Overexpression of the MtrCDE efflux pump system and mutation of the PorB1b porin result in additional increases in extended-spectrum cephalosporin MICs, and at least one unknown resistance determinant also exists in these isolates (83). Oral cephalosporin treatment failures have been confirmed for patients infected with these strains (84), but injectable ceftriaxone appears to maintain activity. The Gonococcal Isolate Surveillance Program (GISP), which tests urethral isolates from male clients visiting sexually transmitted disease clinics throughout the United States, reported 133 isolates with decreased susceptibility to cefixime (MIC ≥ 0.25 μg/ml) between 2005 and 2010, and in 2011, 1.3% of more than 5,500 isolates tested had a cefixime MIC of ≥0.125 μg/ml, which is significantly above the wild-type MIC. Only 57 isolates with reduced susceptibility to ceftriaxone (MIC ≥ 0.125 μg/ml) were identified in the GISP in the United States during this period (85). In contrast, decreased gonococcal susceptibility to ceftriaxone was observed much more frequently by the World Health Organization's Gonococcal Antimicrobial Surveillance Program (GASP) in 2010 in China (55.8% of 1,398 isolates tested), Korea (29.3% of 82 isolates tested), Japan (20.3% of 403 isolates tested), Hong Kong (23.3% of 947 isolates tested), and India (10.8% of 37 isolates tested) (86).

Disturbingly, high-level cefixime-resistant (MIC, 8 μg/ml) and ceftriaxone-resistant (MIC, 4 μg/ml) *N. gonorrhoeae* strains have been reported in Japan (87), France (88), and Spain (89) and were associated with likely ceftriaxone treatment failures. These strains harbor a novel mosaic *penA* allele, with several unique mutations not previously described for isolates with elevated extended-spectrum cephalosporin MICs (88, 90). In particular, the PBP 2_{A510P} mutation was found to be an important determinant of ceftriaxone resistance (90). The mosaic PBP 2 from one of these strains, when introduced into a wild-type *N. gonorrhoeae* isolate, resulted in a 500-fold increase in ceftriaxone MIC (90). These strains are the first extensively drug-resistant *N. gonorrhoeae* isolates, as, in addition to ceftriaxone and cefixime, they are resistant to the tetracyclines and fluoroquinolones (90). To date, these strains have not been identified in the United States. *In vitro*, ertapenem appears to have activity against these isolates, with MICs ranging from 0.016 to 0.064 μg/ml, although the ertapenem MIC parallels that of ceftriaxone in isolates with mosaic *penA* alleles other than the ceftriaxone-resistant PBP 2_{A510P} (91).

Tetracycline resistance in *N. gonorrhoeae* can be plasmid or chromosomally mediated, but plasmid-mediated resistance results in higher MICs. Gonococcal fluoroquinolone resistance occurs primarily via spontaneous mutations to the quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC*. Efflux-mediated resistance can also occur, again by mutation to *mtrR*; overexpression of the MtrCDE efflux system is also associated with elevated MICs for tetracyclines and macrolides (including azithromycin). In the United States in 2011, 22.7% of GISP isolates were resistant to tetracycline. Ciprofloxacin resistance peaked in the United States in 2007 at 14.8%, and in 2011, 13.3% of isolates in the GISP tested resistant to ciprofloxacin. However, ciprofloxacin resistance is much more commonly encountered in isolates recovered from men who have sex with men; for such isolates, the rate of ciprofloxacin resistance was 26% in 2011. The Western Pacific and Southeast Asian regions in the GASP reported an overall 59% fluoroquinolone resistance rate among 9,744 *N. gonorrhoeae* isolates in 2010 (86). In many countries, including Cambodia, Hong Kong, Korea, Philippines, Vietnam, Bhutan, India, and Thailand, >95% of isolates were fluoroquinolone resistant (86).

Isolates with azithromycin MICs of ≥2 μg/ml (reduced susceptibility) were uncommon in 2011 in the United States, documented for 0.3% of GISP isolates. Five of these isolates had MICs of 8 μg/ml and 1 had an MIC of 16 μg/ml. In the Western Pacific and Southeast Asian regions in the GASP, azithromycin resistance was low in all countries (e.g., <1%), with the exception of Mongolia, where azithromycin resistance was documented for 34% of gonococcal isolates in 2010 (86).

Reference Test Methods

Routine β-lactamase tests readily detect PPNG and can reliably be performed by either the chromogenic cephalosporin, acidimetric, or iodometric method.

The CLSI recommends the use of GC agar base for disk diffusion and agar dilution MIC testing. For both tests, a 1% defined growth supplement must be added; however, in agar dilution tests with imipenem or clavulanic acid, the growth supplement must be free of cysteine to avoid inhibition of the activities of these two agents (1, 92, 93). The agar dilution method is preferred to the broth dilution method for MIC testing because *N. gonorrhoeae* has a tendency to autolyze in liquid media. For other details of testing, see Table 1. Liao and colleagues evaluated the performance of disk diffusion on chocolate agar for 163 unique *N. gonorrhoeae* isolates, as compared to CLSI reference GC agar dilution (94). While performances for ciprofloxacin and ceftriaxone were equivalent on both media, improved categorical agreement with agar dilution was found when chocolate agar was used as opposed to GC agar for penicillin disk diffusion testing (84.1% versus 65% categorical agreement).

Recently, the use of cefpodoxime disk diffusion on chocolate Columbia blood agar was proposed as a screen for the detection of mosaic PBP 2 isolates with decreased cefixime and ceftriaxone MICs. A zone diameter of ≤11 mm was predictive of a mosaic PBP 2 with 98.7% sensitivity and 65% specificity (95).

Commercial Test Methods

The only commercial method currently available for susceptibility testing of *N. gonorrhoeae* is Etest, which has been shown to produce results comparable to those of CLSI reference agar dilution methods. One study demonstrated several major errors for tetracycline, although essential agreement with CLSI reference disk diffusion was 94.6% for 295 isolates of *N. gonorrhoeae* tested (96). Another study of 163 *N. gonorrhoeae* isolates demonstrated better Etest performance for ceftriaxone when chocolate agar as opposed to GC agar was used (94); in that study, the values for essential agreement with reference agar dilution were 55.8% for GC agar and 82.8% for chocolate agar.

Strategies for Testing and Reporting Results

Generally, routine antimicrobial susceptibility testing is not necessary for clinical isolates of *N. gonorrhoeae*. However, the CDC recently recommended performing culture and susceptibility testing for *N. gonorrhoeae* isolated from patients with suspected treatment failures with the recommended combination therapy regimens (77). Thus, laboratories should, at a minimum, identify a reliable reference laboratory for this testing, if culture and susceptibility testing of *N. gonorrhoeae* is infrequently performed. Primary agents for testing include ceftriaxone, cefixime, ciprofloxacin, and tetracycline.

Complicating *N. gonorrhoeae* antimicrobial susceptibility testing is the widespread use of nucleic acid amplification tests (NAATs) for the diagnosis of *N. gonorrhoeae*. NAATs remain the test of choice for the diagnosis of *N. gonorrhoeae* from genital sources because they are much more sensitive than culture, but these tests do not yield an isolate for susceptibility testing. Several investigators have evaluated molecular methods by which to detect important resistance determinants, such as the mosaic PBP 2 sequence and mutations to *gyrA* and *parC*, directly from patient specimens that tested positive for *N. gonorrhoeae* by a NAAT (97–99). These assays do not yet have widespread use, although the ability to rapidly detect key resistance determinants in *N. gonorrhoeae* may soon enhance both culture-based antimicrobial resistance surveillance and allow for tailored patient treatment regimens.

N. MENINGITIDIS

Incidence of Resistance

The modal MIC for penicillin and *N. meningitidis* is 0.06 μg/ml. However, *N. meningitidis* isolates from the United States, Europe, and elsewhere with reduced susceptibility to penicillin (MICs, 0.12 to 1.0 μg/ml) have been recognized for nearly two decades, and the incidence of these strains is increasing (100–107). A study of 4,735 *N. meningitidis* isolates from 19 Latin American countries and the Caribbean between 2006 and 2010 noted that 29.1% of isolates were penicillin intermediate (MICs, 012 to 0.25 μg/ml). There were also 14 isolates categorized as fully penicillin resistant (MIC ≥ 0.5 μg/ml), and these were associated with an outbreak of disease in a Mexican prison (100). In contrast, only 2.8% of penicillin-intermediate isolates among 1,447 strains from invasive infections were reported during a similar time frame in South Africa (104). Diminished penicillin and ampicillin susceptibility in meningococci is due to polymorphisms in PBP 2 that result from point mutations of the *penA* gene encoding that PBP (107). Recently, 296 nonsusceptible meningococcal strains isolated between 2000 and 2010 in Belgium were examined for their genetic diversity, and 41 different alleles of *penA* were identified (101). The clinical significance of isolates with elevated penicillin MICs is uncertain, and infections caused by isolates with reduced susceptibility to penicillin have successfully been treated with high doses of penicillin in several cases, although rare reports cited clinical failure in meningitis when a dose of penicillin lower than recommended was used. Two independent groups examined outcomes in patients infected with isolates with reduced susceptibility to penicillin and concluded that the serogroup rather than the penicillin MIC is the microbiological parameter most predictive of mortality (103, 108). High-level resistance due to β-lactamase production was observed in a few isolates in the 1980s and 1990s; additional isolates have not been documented for more than a decade (107).

The extended-spectrum cephalosporins (e.g., ceftriaxone and cefotaxime) remain highly active against isolates with elevated penicillin MICs (101–103, 105, 106, 109) and are often used as first-line therapy for meningococcal meningitis in developed countries. However, a report of eight ceftriaxone-nonsusceptible serogroup A isolates from India is concerning. Ceftriaxone MICs ranged from 0.25 to 8 μg/ml in these isolates (110).

Regarding agents used for prophylaxis, resistance to sulfonamides occurs frequently (104–106, 111), and the incidence of resistance to rifampin is low but has been documented on several occasions following exposure to that agent (100, 105, 111–113). A comprehensive study of 392 isolates from diverse geographic locations collected over 25 years, including 161 isolates with rifampin MICs of >0.25 μg/ml, showed that isolates of *N. meningitidis* displaying rifampin MICs of >1 μg/ml possess *rpoB* alleles with mutations that are absent in the alleles found in all isolates with rifampin MICs of ≤1 μg/ml (112).

Of the greatest concern is the increasing frequency of diminished fluoroquinolone susceptibility in meningococci from several countries. These isolates generally have ciprofloxacin MICs of 0.125 to 0.5 μg/ml, rather than demonstrating high-level resistance. Resistance has been attributed to mutations in the *gyrA* gene, similar to the situation in gonococci (114, 115). A single isolate from Venezuela has been reported to be resistant to both ciprofloxacin and a second agent that could be used for prophylaxis, azithromycin (116), and three meningococcal isolates recovered in Spain were ciprofloxacin nonsusceptible and rifampin resistant (117).

Strategies for Testing and Reporting of Results

The CLSI has published standards for MIC testing of *N. meningitidis* that utilize either the broth microdilution method and CAMHB with 2.5 to 5% lysed horse blood (CAMHB-LHB) or the agar dilution method and MHA with 5% sheep blood, both with incubation in 5% CO_2 (1, 92).

Interpretive criteria are listed for agents such as penicillin, cefotaxime, and ceftriaxone that might be prescribed for treating a meningococcal infection. In addition, interpretive criteria are listed for agents such as ciprofloxacin, rifampin, and azithromycin that might be used for prophylaxis of meningococcal case contacts. Strains with diminished fluoroquinolone susceptibility may best be detected by testing with nalidixic acid by MIC or disk methods (105). The CLSI describes a disk diffusion method for testing *N. meningitidis* that utilizes MHA with 5% sheep blood and CO_2 incubation. As with MIC tests, interpretive criteria are listed for several therapeutic and prophylactic agents. Unfortunately, these do not include breakpoints for ampicillin and penicillin, as disk testing results obtained with these agents were unreliable (1, 93). Several investigators have examined modified penicillin disk diffusion methods by using 2- and 10-IU penicillin disks and 1-μg oxacillin disks and also determined that these cannot reliably distinguish *N. meningitidis* isolates that are susceptible from those that have decreased ampicillin and penicillin susceptibility (118). Therefore, disk diffusion testing should not be used for these drugs. One possible exception might be testing of mecillinam (amdinocillin) by the disk method as a screen for isolates with *penA* polymorphisms (105).

Because of the lack of clinical failures with the drugs of choice for the treatment of meningococcal infections, susceptibility testing is not warranted in most situations. Although the Etest has not been cleared by the FDA for testing *N. meningitidis*, several studies have shown it to be suitable for testing meningococci and to perform best by using MHA with 5% sheep blood incubated in CO_2 (118, 119). However, in a 14-laboratory study, Vazquez et al. noted some difficulties in obtaining reliable Etest results with rifampin (119).

POTENTIAL BACTERIAL AGENTS OF BIOTERRORISM

Several bacterial agents are identified as potential agents of bioterrorism by the CDC (http://www.bt.cdc.gov/agent/index.asp). These include *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, *Burkholderia mallei*, *Burkholderia pseudomallei*, and *Brucella* spp. The antimicrobial susceptibility patterns for most of these organisms are predictable, so susceptibility testing of naturally occurring isolates is often not necessary. However, it is possible for antimicrobial resistance to spontaneously occur among these organisms, and in the case of a bioterrorism event, there is the possibility of engineered resistance. To detect any potential resistance, standardized broth microdilution susceptibility testing methods and interpretive criteria were established and published by the CLSI (2).

Acquired resistance to antimicrobial agents commonly used for treatment of infections caused by *B. anthracis*, *Y. pestis*, *F. tularensis*, *B. mallei*, *B. pseudomallei*, and *Brucella* spp. is rare. In contrast to other species of *Bacillus* which produce *bla1* and *bla2* β-lactamases that confer resistance to penicillin, most isolates of *B. anthracis* are susceptible to penicillin. Penicillin-susceptible strains of *B. anthracis* produce an anti-sigma factor, RsiP, that sequesters the sigma factor required for transcription of the β-lactamase genes. In a naturally occurring penicillin-resistant strain, a single nucleotide deletion in the RsiP gene results in a truncated protein. As a result, sigma factor sequestration does not occur, and the β-lactamase genes are transcribed (120). However, amoxicillin is recommended for anthrax prophylaxis only when the isolate has been determined to be susceptible to penicillins, and this recommendation is limited to young children and pregnant women (121). Laboratory-generated fluoroquinolone resistance in *B. anthracis* was described, but fluoroquinolone resistance in clinical isolates has not been reported (122, 123).

There are reports of two spontaneously occurring drug-resistant *Y. pestis* isolates recovered in Madagascar (124, 125). Both isolates acquired conjugative plasmids. In one isolate, the plasmid carried resistance determinants for ampicillin, chloramphenicol, tetracycline, kanamycin, streptomycin, and sulfonamide. In the other isolate, the plasmid conferred resistance to streptomycin. The concern for multidrug resistance encouraged a recent study of 392 *Y. pestis* isolates from 17 countries in the Americas, Africa, and Asia. Eight antimicrobial agents used for treatment or prophylaxis of plague were tested, and no resistance was reported (126). In another study, screening of 713 isolates of *Y. pestis* from North America failed to demonstrate the backbone plasmids that conferred resistance in the Madagascar strains (127).

B. pseudomallei is intrinsically resistant to many antimicrobial agents, including gentamicin, streptomycin, rifampicin, erythromycin, and many β-lactams (128). Susceptibility studies of *B. pseudomallei* and *B. mallei* indicate that using CLSI interpretive criteria (2), most isolates are susceptible to amoxicillin-clavulanic acid, ceftazidime, imipenem, tetracycline, doxycycline, and TMP-SMX (129–131). However, some isolates of *B. pseudomallei* have elevated ceftazidime MICs (i.e., MIC ≥8 μg/ml) (130–132) and elevated amoxicillin-clavulanic acid MICs (i.e., MIC ≥8 μg/ml) (130–132). Resistance to ceftazidime and amoxicillin-clavulanic acid can be due to class A PenA β-lactamase (130).

Clinical isolates of *Brucella* spp. with reduced susceptibility to rifampin and TMP-SMX (133–136) have been reported. In these publications, the susceptibility testing methods used were either not clearly defined or different from the methods currently recommended by the CLSI. Also, resistant isolates were not characterized for resistance determinants or mutations. However, mutations in the *rpoB* gene, the target for rifampin activity, have been observed *in vitro* in *Brucella* mutants (137, 138).

Isolates of *F. tularensis* demonstrate β-lactamase activity, and they are resistant to β-lactams, including cephalosporins and carbapenems (139–142). Resistance to aminoglycosides, tetracyclines, fluoroquinolones, and chloramphenicol has not been reported.

B. anthracis, *B. mallei*, *B. pseudomallei*, and *Y. pestis* demonstrate sufficient growth with commonly used broth microdilution MIC testing medium and incubation conditions (Table 2). However, the recommended testing medium for *F. tularensis* is CAMHB with a 2% concentration of a defined growth supplement. This is the same cysteine-containing defined growth supplement as is added to GC agar base for susceptibility testing of *Neisseria gonorrhoeae*. Some isolates of *Brucella* spp., particularly isolates of *B. abortus*, require CO_2 incubation. However, incubation in CO_2 may increase the MICs of aminoglycosides and decrease the MICs of tetracyclines. The streptomycin susceptibility breakpoint is one doubling dilution higher (i.e., increased from 8 μg/ml to 16 μg/ml) when panels are incubated in CO_2 (2, 143). For the slower-growing organisms, *F. tularensis*, *Y. pestis*, and *Brucella* spp., 48 h of incubation may be needed to achieve sufficient growth.

There are few studies comparing alternative susceptibility testing methods to the standard CLSI reference broth microdilution methods. One study compared Etest to broth microdilution for testing *B. anthracis* (144). In that study, Etest MICs were within a single doubling dilution of the broth microdilution MICs for ceftriaxone, chloramphenicol, ciprofloxacin, clindamycin, erythromycin, rifampin, tetracycline, and vancomycin but not for penicillin. Penicillin MIC values for Etest tended to be lower than those obtained by broth microdilution. A similar trend for penicillin MICs was noted for *B. anthracis* in a study comparing Etest to agar dilution (145). These results suggest that Etest is acceptable for susceptibility testing of *B. anthracis* with antimicrobial agents other than penicillin. More recently, Etest was shown to be reliable for testing 26 diverse strains of *Y. pestis* with ciprofloxacin, doxycycline, gentamicin, levofloxacin, streptomycin, and tetracycline but not with chloramphenicol and TMP-SMX (146).

In addition to routine phenotypic methods, Weigel et al. combined a modified broth microdilution susceptibility testing method with real-time quantitative PCR for testing for ciprofloxacin and doxycycline with *B. anthracis*, which allowed reliable results to be available following 6 h versus the standard 16 to 20 h of incubation (147). Others have examined molecular methods, including pyrosequencing and single nucleotide polymorphism (SNP)-based PCR assays, to detect ciprofloxacin resistance in *Bacillus anthracis*, *Yersinia pestis*, and *Francisella tularensis* (148, 149). SNP-based PCR assays have also been used for characterization of doxycycline resistance in the three species (149).

Susceptibility testing of potential agents of bioterrorism raises safety concerns for clinical microbiology laboratories. All of the above-mentioned organisms require at least biosafety level 2 (BSL2) practices, containment, and facilities. BSL3 conditions are recommended for activities with a high potential for aerosol production (150). Since the inoculum preparation for susceptibility testing has the potential for aerosol production, all susceptibility testing procedures must be performed in a BSL3 facility. Laboratories without BSL3 facilities should send isolates to an appropriately equipped reference laboratory that has select-agent clearance status, such as reference laboratories within the Laboratory Response Network and at the CDC (http://www.selectagents.gov/).

ABIOTROPHIA SPECIES AND *GRANULICATELLA* SPECIES

For *Abiotrophia* spp. and *Granulicatella* spp., resistance to agents from several different antimicrobial classes, including penicillins, cephalosporins, carbapenems, macrolides, lincosamides, fluoroquinolones, and tetracyclines, has been reported. In a study of 20 blood isolates by Murray et al., MIC_{90}s for specific agents were as follows: penicillin, 0.5 μg/ml; cefotaxime, 4 μg/ml; cefepime, >8 μg/ml; meropenem, 0.5 μg/ml; erythromycin, 16 μg/ml; clindamycin, 0.25 μg/ml; and tetracycline, 2 μg/ml (151). Murray et al. also noted an isolate of levofloxacin-resistant *Abiotrophia elegans* (currently known as *Granulicatella elegans*) which was obtained from a patient who had received 10 days of levofloxacin prophylaxis (151). Among isolates from 28 patients with infections with nutritionally variant streptococci (19 *Granulicatella adiacens*, 8 *Abiotrophia defectiva*, and 1 *G. elegans* isolate) treated at a university hospital in Taiwan, 50% had penicillin MICs of 0.25 to 2 μg/ml and 10 isolates were not susceptible to cefotaxime, with 7 fully resistant at MICs of ≥4 μg/ml. Three isolates had cefepime MICs of ≥4 μg/ml; the MIC_{90} for imipenem was 0.12 μg/ml, and that for meropenem was 0.5 μg/ml (152). When CAMHB-LHB with 0.001% pyridoxal was used to test 27 *Granulicatella* (*Abiotrophia*) *adiacens* and 12 *Abiotrophia defectiva* isolates, 41%, 51%, and 8% were found to be penicillin susceptible, intermediate, and resistant, respectively. Interestingly, *G. adiacens* was substantially more susceptible to penicillin than was *A. defectiva* (55% versus 8%) in this study (153). Little is known about mechanisms of resistance in these bacteria. There is one report of an *Abiotrophia defectiva* isolate with a conjugative, Tn*916*-like transposon, Tn*3872* (154). The transposon carried *ermB* and *tetM* resistance determinants, which conferred resistance to erythromycin and tetracycline, respectively. In another study that examined 15 isolates from pediatric patients (6 *Abiotrophia* and 9 *Granulicatella* isolates), 8 were resistant to erythromycin. Five of these were clindamycin susceptible and harbored *mefA*, and three that were clindamycin resistant were positive for *ermB* and also *tetM*. One of the *ermB*-positive *Granulicatella* spp. also had *mefA* (155). These findings demonstrate that these bacteria are able to acquire and transfer mobile genetic elements which confer antimicrobial resistance.

Because *Abiotrophia* spp. and *Granulicatella* spp. are fastidious, it is recommended that these be tested in CAMHB-LHB supplemented with 0.001% pyridoxal hydrochloride and incubated in ambient air (Table 3) (2). In a report by Namdari et al., a procedure for adding pyridoxal to an inoculum prepared with the Prompt system and a MicroScan dried Gram-positive panel is described (156). It should be noted that susceptibility testing of aminoglycosides against *Abiotrophia* spp. and *Granulicatella* spp. is not recommended even though aminoglycosides are commonly used in combination therapy with an agent active against the cell wall, such as penicillin or vancomycin, for treating serious infections caused by these organisms. Since the aminoglycoside acts synergistically with the agent, *in vitro* susceptibility to aminoglycosides does not predict *in vivo* susceptibility.

AEROMONAS HYDROPHILA COMPLEX (*A. CAVIAE, A. HYDROPHILA, A. JANDAEI, A. SCHUBERTII*, AND *A. VERONII*) AND *PLESIOMONAS SHIGELLOIDES*

Aeromonas spp. are typically resistant to ampicillin, show variable results with β-lactam–β-lactamase inhibitor combinations, and are resistant to narrow-spectrum cephalosporins (157–160). Inducible β-lactamases in *Aeromonas* spp. have been noted, and resistance may emerge during therapy with a β-lactam agent (161). Among 172 isolates of *Aeromonas* spp. tested as part of a global surveillance program conducted from 2003 to 2007, >90% were susceptible to extended-spectrum cephalosporins, 98.8% were susceptible to meropenem, but only 63.7% were susceptible to piperacillin-tazobactam (162). In contrast, among 260 isolates of *Aeromonas* spp. isolated in the Asia-Pacific region, 78% were susceptible to ceftazidime, the only expanded-spectrum cephalosporin tested, and 68.8% were susceptible to imipenem (163). *Aeromonas* isolates that express metallo-β-lactamases have been recently described, including an *Aeromonas hydrophila* isolate carrying an integron-borne *bla*$_{\text{VIM-4}}$ gene isolated from a patient in Hungary (164) and an *Aeromonas caviae* isolate carrying a plasmid-borne *bla*$_{\text{IMP-19}}$ isolated from a patient in France (165). The metallo-β-lactamase CphA is also frequently found in *Aeromonas* spp.

In Taiwan, where *Aeromonas* spp. are frequently encountered, results from 234 isolates in 1996 found species-based differences in overall antimicrobial susceptibility, noting that *Aeromonas sobria* was more susceptible than either *A. hydrophila* or *A. caviae* (158). Similar results were noted by Vila et al. from Spain in 2002 (159). Tobramycin susceptibility may be more common in *Aeromonas veronii*, with one study finding less than 30% susceptibility, whereas other species tested susceptible to this agent (157). *Aeromonas* spp. are generally considered susceptible to fluoroquinolones (158), although *Aeromonas caviae* isolated from the stools of patients with diarrhea was shown in two independent studies to be more frequently fluoroquinolone resistant than other *Aeromonas* spp. (166, 167). Resistance to fluoroquinolones appears to be increasing in the Asia-Pacific region, more than doubling, from 8% to 17%, between the periods of 2003 to 2006 and 2007 to 2010 (163). Fluoroquinolone resistance in *Aeromonas* is associated with mutations to the QRDR of *gyrA* and *parC* (166, 167). In the United States, two reports have documented ciprofloxacin-resistant *A. hydrophila* infections following leech therapy when patients were treated prophylactically with ciprofloxacin (168, 169).

Plesiomonas spp. are typically resistant to ampicillin and other penicillins, including piperacillin, and susceptible to β-lactamase inhibitor combinations and several other classes of antimicrobial agents (170, 171).

Although *Aeromonas* spp. and *Plesiomonas shigelloides* cause diarrheal disease, otherwise healthy individuals with diarrhea due to these bacteria usually recover spontaneously without treatment (159, 170), and routine antimicrobial susceptibility testing of isolates from fecal specimens is not recommended (2). However, both genera can cause a variety of other infections for which treatment may be necessary (157, 158, 160, 161, 170, 171). Consequently, for isolates from extraintestinal sources, antimicrobial susceptibility testing may be warranted. These organisms show growth characteristics similar to those of *Enterobacteriaceae*, which led the CLSI to recommend disk diffusion or broth microdilution MIC testing using the same methods as recommended for *Enterobacteriaceae*.

BACILLUS SPECIES

Bacillus spp. other than *B. anthracis* are common contaminants in the clinical laboratory, but these bacteria can cause serious disease, including catheter-associated infections, traumatic wound infections, and corneal ulcers. Since the susceptibility profiles of *Bacillus* spp. are quite variable, susceptibility testing should be considered when *Bacillus* spp. are isolated from normally sterile body sites or serious or refractory infections. Isolates of *Bacillus* spp. can express β-lactamases that confer resistance to penicillins and possibly cephalosporins. *Bacillus cereus* is the most common species found in clinical specimens, and most isolates are resistant to penicillin and extended-spectrum cephalosporins, although penicillin resistance does not always predict cephalosporin resistance (145, 172). Penicillin and cephalosporin resistance also occurs in other species of *Bacillus*, but it is less common. Resistance to other agents has been reported. These agents include aminoglycosides, erythromycin, clindamycin, chloramphenicol, tetracycline, fluoroquinolones, TMP-SMX, and even vancomycin (145, 172, 173). When 70 isolates of *Bacillus* spp. from a variety of clinical specimens were examined, daptomycin was found to have activity against vegetative cells but not against spores (173). In the same study, 2 strains (vegetative cells) had ciprofloxacin MICs of >4 μg/ml and 10 strains were resistant to erythromycin. A case of fatal septicemia caused by *Bacillus cereus* resistant to carbapenems was reported for a patient treated with meropenem (174); the resistance mechanism was not determined for this isolate.

Standard susceptibility testing methods for nonfastidious bacteria can be used for testing *Bacillus* spp. The CLSI provides guidelines for broth microdilution testing (Table 3) (2). Agar dilution and Etest methods using MHA were compared in a study by Turnbull et al. (145). In this study, Etest MICs were similar to agar dilution MICs for several agents, including cefotaxime, ciprofloxacin, erythromycin, gentamicin, penicillin, tetracycline, and vancomycin. Two commercial β-lactamase tests, nitrocefin (Oxoid, Basingstoke, England) and Intralactam strips (Mast Diagnostics, Merseyside, United Kingdom), were evaluated in a study by Andrews and Wise (175); both tests failed to detect β-lactamase activity in four of five penicillin-resistant isolates of *B. cereus*. Therefore, β-lactamase testing is not recommended for predicting penicillin susceptibility in *Bacillus* spp.

CAMPYLOBACTER

Resistance to the fluoroquinolones, which are agents commonly used for treating gastrointestinal infections caused by *Campylobacter jejuni* and *Campylobacter coli*, has been reported with increasing frequency in the United States among both human and animal isolates of these two species over the past two decades. The National Antimicrobial Resistance Monitoring System (NARMS) noted no ciprofloxacin resistance among *C. jejuni* human isolates in 1990, but rates of 18% resistance in 2001 and 24% resistance in 2011 were noted (176). Only 1.8% of human isolates were erythromycin resistant in 2011, and 45% were tetracycline resistant. Similar resistance was noted by the NARMS for *C. coli* in 2011, with 23.5% resistance to ciprofloxacin, 1.7% resistance to erythromycin, and 46% resistance to tetracycline (176). Resistance among animal isolates, which may subsequently be transmitted to humans, has been associated with the addition of macrolides and fluoroquinolones to animal food as growth-promoting agents (177). In the United States in 2004, the FDA reversed its approval for the therapeutic use of the veterinary fluoroquinolone enrofloxacin because of concern of rising fluoroquinolone resistance in human isolates. Studies in other countries have demonstrated that fluoroquinolone-resistant *Campylobacter* persists in poultry populations after withdrawal of veterinary fluoroquinolones (178), suggesting little fitness cost to fluoroquinolone resistance in *Campylobacter* (179). Since its peak in 2007 at nearly 26%, ciprofloxacin resistance in human *C. jejuni* isolates in the United States has declined slightly, to 24% in 2011 (176); however, whether this trend will continue, or whether fluoroquinolone resistance has plateaued among human isolates of *Campylobacter*, remains to be determined.

Unlike *Escherichia coli* and *Salmonella*, which require multiple mutations to the QRDR of *gyrA* to achieve high-level quinolone resistance, a single mutation, T85I, in *gyrA* yields high-level resistance to both nalidixic acid and fluoroquinolones in *Campylobacter* (180). Despite reports of enhanced fitness associated with this mutation in *Campylobacter* in chicken models of infection (179), Wassenaar and colleagues concluded that fluoroquinolone-resistant *Campylobacter* infections are no more severe than infections caused by susceptible isolates following an extensive review of previous studies and nearly 11,000 new cases (181).

Because of the increasing incidence of resistance, testing of isolates from individual patients with severe illness or prolonged symptoms may be warranted, and isolates might also be tested for epidemiological purposes. Susceptibility testing by broth microdilution in CAMHB-LHB and incubation in a microaerobic atmosphere (Table 3) is suggested by the CLSI (2). Interpretive criteria for ciprofloxacin, erythromycin, doxycycline, and tetracycline are provided. The CLSI also describes a disk diffusion method for ciprofloxacin and erythromycin in which the absence of a zone indicates resistance. However, due to wide variations in measured zones that occur as a result of hazy or film-like growth, criteria for interpreting results other than resistant are not provided (2). Gaudreau et al. suggested that ciprofloxacin, erythromycin, and tetracycline disk diffusion tests can be used to identify both resistance and susceptibility to these three agents in *C. jejuni* and *C. coli*. However, for the ciprofloxacin results, a nalidixic acid disk must also be tested to ensure detection of fluoroquinolone resistance (182, 183). The Etest correlated well with agar dilution in a study by Baker when ciprofloxacin, erythromycin, and tetracycline were tested (184).

Currently, no testing methods have been validated, or interpretive criteria developed, for *Campylobacter* spp. other than *C. jejuni* or *C. coli*.

CORYNEBACTERIUM SPECIES AND CORYNEFORMS

The many *Corynebacterium* spp. may exhibit a variety of susceptibility profiles, and many strains are highly susceptible (185–189). However, most isolates of *C. amycolatum*, *C. jeikeium*, *C. resistens*, and *C. urealyticum* are multiresistant to antimicrobial agents often considered for therapy of Gram-positive infections, including penicillins, cephalosporins, macrolides, aminoglycosides, fluoroquinolones, tetracyclines, and clindamycin. Resistance to daptomycin was reported recently for a *C. striatum* isolate causing native valve endocarditis in a patient with 12 weeks of prior daptomycin therapy for the treatment of methicillin-resistant *Staphylococcus aureus* bacteremia and osteomyelitis (190), but as a whole the *Corynebacterium* spp. remain susceptible to teicoplanin, vancomycin, linezolid, daptomycin, and tigecycline (185, 187). Most isolates of *C. urealyticum* are resistant to fluoroquinolones, which has important implications for empirical therapy for urinary tract infections due to this species (187, 191). Mutation to *gyrA* has also been associated with quinolone resistance in *C. striatum*, *C. amycolatum*, and *C. macginleyi* (192). Macrolide resistance is frequently found in clinical isolates of *Corynebacterium* and is mainly associated with the presence of an *ermX* methylase gene (193).

Significant antimicrobial resistance is rare for *C. diphtheriae*, and very few reports of nonsusceptibility to either penicillin or macrolides exist (194–196). In a study of 410 isolates from around the world, 5 showed reduced susceptibility to macrolides and ketolides (194). Using Etest to evaluate 47 isolates of *C. diphtheriae*, investigators in Brazil reported that the penicillin MICs ranged from 0.002 to 0.38 μg/ml, the penicillin MIC_{90} was 0.19 μg/ml, and the penicillin resistance rate was 14.8%. However, in that study, a penicillin breakpoint of ≤0.12 μg/ml was used to categorize isolates as penicillin susceptible, rather than the breakpoint of ≤1 μg/ml for susceptibility that is currently recommended by the CLSI (2, 195). Poor bactericidal activity of penicillins for *C. diphtheriae* has been reported. von Hunolstein demonstrated penicillin tolerance among 17 of 24 *C. diphtheriae* isolates from patients with pharyngitis or tonsillitis (197). A paradoxical effect (e.g., decreasing bactericidal activity with increasing antibiotic concentration) with amoxicillin has also been demonstrated with two strains of nontoxigenic *C. diphtheriae* isolated from patients with endocarditis (198). The authors of the study suggested that poor bactericidal activity of penicillins at high doses may contribute to poor outcomes when penicillins are included in the therapeutic regimen for *C. diphtheriae* endocarditis (198). Monoresistance to the folate pathway inhibitors trimethoprim, sulfamethoxazole, and co-trimoxazole has been reported, most recently for a *C. diphtheriae* bv. mitis isolated from a patient in Mayotte (199). This isolate harbored a class 1 integron with the *dfrA* gene that conferred resistance to trimethoprim.

Growth characteristics among the *Corynebacterium* spp. may vary, and previous studies have used a variety of methods, media, and incubation conditions for antimicrobial susceptibility testing. Prior to the publication of CLSI document M45, the absence of interpretive criteria specifically for *Corynebacterium* spp. led to inconsistencies in interpreting results when CLSI interpretive criteria for other Gram-positive organisms (e.g., streptococci and staphylococci) were applied (186, 188, 194). This continues to be a problem if investigators are unaware of CLSI M45 (195). The CLSI recommends broth microdilution with CAMHB-LHB and incubation in ambient air (Table 3). Some strains grow satisfactorily after 24 h of incubation, but others require 48 h of incubation for sufficient growth. The 48-h incubation period is particularly important for detecting resistance to β-lactams, and it is recommended, if growth is not satisfactory or if an isolate appears susceptible to β-lactams at 24 h, that the test be reincubated and final β-lactam results be read after 48 h of incubation (2). Currently, disk diffusion testing of *Corynebacterium* spp. is not recommended by the CLSI. Several investigators have used Etest satisfactorily for testing *Corynebacterium* spp. (185, 195), and Engler et al. used Etest to confirm the reduced susceptibility to macrolides and ketolides that was initially identified by agar dilution testing of *C. diphtheriae* (194).

Testing and reporting recommendations in CLSI M45 are applicable to other coryneforms, including the genera *Arcanobacterium*, *Brevibacterium*, *Cellulomonas*, *Dermabacter*, *Leifsonia*, *Microbacterium*, *Oerskovia*, *Rothia*, and *Turicella* (2). Scarce data are available in the literature regarding antimicrobial susceptibility of these organisms, and methods used to test for this vary between studies. However, macrolide resistance has been noted for isolates of *Brevibacterium*, *Dermabacter*, and *Turicella*, and fluoroquinolone resistance has been noted for *Brevibacterium* and *Dermabacter* (187). Some isolates of *Arcanobacterium haemolyticum* may display tetracycline resistance (200) but are reportedly susceptible to other agents that might be considered for treatment. One study has reported *A. haemolyticum* and *Oerskovia turbata* harboring the *vanA* gene on a plasmid; both isolates were vancomycin resistant (201). A study of 50 clinical isolates of *Microbacterium* spp. demonstrated variable susceptibility profiles, but notably, only 56% of isolates were susceptible to ciprofloxacin; 1 isolate, a *Microbacterium resistens*, was resistant to vancomycin, which is typical for this species (202). *Turicella otitidis* is susceptible to most β-lactams, ciprofloxacin, rifampin, tetracyclines, and gentamicin, but significant resistance to macrolides and lincosamides has been reported (203, 204). Susceptibility testing of *Corynebacterium* spp. and other coryneforms is warranted when the organisms are isolated from normally sterile sites.

ERYSIPELOTHRIX RHUSIOPATHIAE

Erysipelothrix rhusiopathiae is an intrinsically vancomycin-resistant Gram-positive bacillus. Other agents that have little to no activity against isolates of this species are aminoglycosides and TMP-SMX. Antimicrobial susceptibility studies of isolates from a number of sources have shown all isolates of *E. rhusiopathiae* to be susceptible to penicillins, cephalosporins, carbapenems, and fluoroquinolones (205–207). Resistance to clindamycin, erythromycin, and tetracycline does occur. In one study of 66 isolates from swine in Japan, 94% were susceptible to erythromycin (206). In that study, the authors found that 71% of the isolates had an MIC of ≥8 μg/ml to tetracycline, which they assigned as resistant, although no CLSI interpretive criteria exist to date for *E. rhusiopathiae* and the tetracyclines (206). Isolates with elevated tetracycline MICs were previously shown to be positive for *tetM* (208). Recently, all four clinical isolates tested with daptomycin had MICs of ≤0.125 (209).

Isolates of *E. rhusiopathiae* are fastidious, so CAMHB-LHB is recommended for broth microdilution MIC testing (2). There are also reports of agar dilution using either MHA supplemented with 5% horse blood or unsupplemented MHA (205–207). The CLSI provides susceptible-only breakpoints for the penicillins, cephems, carbapenems, and fluoroquinolones, whereas resistance and intermediate-status interpretive criteria are provided for erythromycin and clindamycin. When organisms are isolated from sterile sites, laboratories should primarily test ampicillin or penicillin (2).

HACEK GROUP

There are limited antimicrobial susceptibility test data on the HACEK group of organisms (i.e., *Aggregatibacter* [formerly the *aphrophilus* group of *Haemophilus* and *Actinobacillus*], *Cardiobacterium*, *Eikenella*, and *Kingella* spp.), in part because these organisms are infrequently encountered and often difficult to grow. Members of the HACEK group are susceptible to extended-spectrum cephalosporins and fluoroquinolones and are often susceptible *in vitro* to ampicillin and penicillin (51, 210, 211), although occasional isolates of *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella* spp. produce β-lactamase (211). Coburn and colleagues noted that 22.9% of 68 isolates belonging to the HACEK group were nonsusceptible to penicillin, but 95.8% of isolates remained susceptible to ampicillin and all but two isolates were susceptible to amoxicillin-clavulanic acid (211). One *E. corrodens* isolate tested resistant to penicillin and ampicillin but tested negative for the presence of β-lactamase by the Cefinase disk method. American Heart Association recommendations for treatment of patients with endocarditis caused by HACEK organisms suggest that HACEK organisms be considered ampicillin resistant, largely because of difficulties in performing antimicrobial susceptibility testing and the potential failure by the laboratory to identify ampicillin-resistant strains (51).

Broth microdilution with CAMHB-LHB can be used for testing HACEK organisms. Some isolates may require 48 h of incubation to obtain adequate growth, as evidenced by substantial turbidity in the positive growth control well. Some isolates may not grow satisfactorily in CAMHB-LHB; in a recent study of 174 HACEK isolates, 59.8% failed to grow sufficiently in CAMHB-LHB to allow susceptibility testing (211). In particular, poor growth was noted for *A. actinomycetemcomitans* (83.3% of isolates), *Aggregatibacter aphrophilus* (60.7%), and *C. hominis* (60%). Susceptibility testing may be warranted for isolates from normally sterile sites, and β-lactamase testing should be performed on HACEK isolates (2), although laboratories should be aware that not all ampicillin resistant isolates may be detected by this testing (211).

H. PYLORI

Incidence of Resistance

H. pylori is intrinsically resistant to glycopeptides, polymyxins, nalidixic acid, trimethoprim, and sulfonamides. The rates of resistance for *H. pylori* vary considerably among the first-line agents recommended for therapy, with resistance to metronidazole the highest, followed by clarithromycin. Resistance to amoxicillin, tetracyclines, and quinolones is low (212–214).

Routine surveillance for *H. pylori* resistance is no longer performed in the United States; however, between 1998 and 2002, the CDC conducted the *Helicobacter pylori* Antimicrobial Resistance Monitoring Program, which evaluated antimicrobial susceptibility of *H. pylori* isolated over this period. Among 347 isolates tested, 25% were resistant to metronidazole, 13% to clarithromycin, and less than 1% to amoxicillin; no resistance to tetracycline was found (215). More recently, a study of 531 *H. pylori* isolates obtained between 2000 and 2008 from an Alaskan Native population with high seroprevalence of infection documented 42% metronidazole resistance, 30% clarithromycin resistance, 19% levofloxacin resistance, and less than 2% amoxicillin and tetracycline resistance (214). Studies in other developed countries reveal much higher rates of metronidazole and clarithromycin resistance among contemporary *H. pylori* isolates. A German study of 5,296 *H. pylori* isolates collected between 2006 and 2011 found 67.1% resistance to metronidazole and clarithromycin and 24.9% resistance to ciprofloxacin and levofloxacin, whereas resistance to tetracycline and rifampin was rare and amoxicillin resistance was not detected (212). A surveillance study in the United Kingdom of isolates collected between 2009 and 2010 revealed 88% metronidazole resistance and 68% clarithromycin resistance among 109 *H. pylori* isolates from patients evaluated at a reference laboratory; 61% of the isolates were resistant to both drugs (213). In contrast, that study documented 22% and 3% resistance to metronidazole and clarithromycin, respectively, when isolates from patients seen at a general hospital were tested. Prior treatment with metronidazole and clarithromycin was associated with increased risk of resistance to these drugs in both patient populations. Similarly, <4% of 3,707 *H. pylori* isolates in Japan between 2002 and 2005 were resistant to metronidazole, which may be related to infrequent use of metronidazole in this country (216).

Reference Test Method

The CLSI describes an agar dilution MIC method for testing *H. pylori*. The test medium is MHA supplemented with 5% (vol/vol) aged (≥2-week-old) sheep blood. The inoculum is prepared from 72-h-old growth on a blood agar plate to obtain a final concentration of bacteria approximating 10^5 CFU/spot. Incubation is for 3 days at 35°C in a microaerobic atmosphere produced by a gas-generating system typically used for campylobacters. *H. pylori* ATCC 43504 has been designated a QC strain, and currently there are interpretive criteria only for clarithromycin. Other antimicrobial agents that have been studied and for which there are CLSI QC ranges include amoxicillin, metronidazole, telithromycin, and tetracycline (2). The EUCAST has defined clinical interpretive criteria, based on epidemiological cut-off values, for metronidazole, amoxicillin, levofloxacin, tetracycline, and rifampin; these are similar to those used in many studies that have evaluated *H. pylori* antimicrobial resistance. The exception is amoxicillin, for which the EUCAST defines MICs of >0.12 μg/ml as resistant (http://www.EUCAST.org), whereas many studies utilize >1 μg/ml to denote amoxicillin resistance. Although there are currently no CLSI interpretive criteria for metronidazole, many investigators have used an MIC of >8 μg/ml for resistance. The European *Helicobacter pylori* Study Group has published an agar dilution method that is similar to the CLSI method; however, they recommend horse blood-supplemented MHA and a higher inoculum (217). Although there is no standard method for disk diffusion testing, Grignon et al. have demonstrated that this method is reliable for testing clarithromycin (218), the drug that is usually of greatest interest. Because point mutations in specific genes have been implicated in resistance, genotypic methods are also used, especially for clarithromycin (219).

Commercial Test Methods

Although no commercial methods are FDA cleared for antimicrobial susceptibility testing of *H. pylori*, several investigators have examined the performance of Etest. Drawing a conclusion for the performance of Etest across studies is complicated by the fact that significant differences in methods, including medium types, inoculum concentration, and incubation conditions, exist. One study found an essential agreement (i.e., results within ±1 doubling dilution) between Etest and agar dilution of 84.6% for amoxicillin, 94.1% for clarithromycin, 89.9% for metronidazole, and 89.1% for tetracycline (220). In a multilaboratory study, categorical agreement between Etest and agar dilution was 93% for both clarithromycin and metronidazole (221); a second multilaboratory found >98% agreement between Etest and agar dilution for clarithromycin (217). However, two large studies reported significant discrepancies between agar dilution and Etest MICs for metronidazole (222). The Etest manufacturer now recommends alternative incubation conditions for *H. pylori* when testing metronidazole, e.g., incubation in an anaerobic environment for 24 h, followed by 48 h of incubation in a microaerophilic environment (Etest package insert, 2012, bioMérieux). This preincubation in anaerobic environment was shown to significantly improve Etest agreement with agar dilution (223), although MHA supplemented with 5% horse blood as opposed to aged sheep blood was used for agar dilution in this study. Several studies have evaluated Etest determination of clarithromycin resistance compared to detection of 23S mutations. These studies demonstrate an imperfect relationship between phenotypic and genotypic resistance. In particular, a study by De Francesco and colleagues found 74% concordance between clarithromycin resistance determined by Etest and the presence of a 23S mutation. That study demonstrated an 85.7% cure rate in patients with phenotypic susceptibility but genetic resistance, in contrast to a 71.4% cure rate when phenotypic resistance but genetic susceptibility was documented. Cure rates were significantly poorer if a resistant result was documented by either method (224).

Strategies for Testing and Reporting of Results

Growth requirements and complex antimicrobial susceptibility testing recommendations for only a limited number of drugs make testing of *H. pylori* impractical for the routine clinical laboratory. However, because of the significant resistance noted for metronidazole and clarithromycin, testing may be required in selected situations, in which case a reliable reference laboratory should be used. Kim et al. identified discordant susceptibility results for clarithromycin, metronidazole, and tetracycline when testing multiple isolates from different locations in the stomachs of individual patients (225). A similar finding was reported by Osato et al. (222) for metronidazole susceptibility. These results suggest that resistant isolates are not evenly distributed throughout the stomach, and susceptibility results for isolates from a single biopsy sample may not be representative of the entire population, an important consideration when evaluating results of susceptibility testing for *H. pylori*. Testing of isolates from multiple gastric sites may be indicated, although no studies have demonstrated this to correlate with patient outcomes.

LACTOBACILLUS, *PEDIOCOCCUS*, AND *LEUCONOSTOC* SPECIES

Pediococcus spp., *Leuconostoc* spp., and most *Lactobacillus* spp. are intrinsically vancomycin resistant. *Lactobacillus gasseri*, *L. delbrueckii*, and *L. acidophilus* may be vancomycin susceptible (226–229).

For *Lactobacillus* spp. the MICs of piperacillin-tazobactam, imipenem, erythromycin, and clindamycin are generally low, but the MICs of penicillin and cephalosporins for these organisms can vary (226–230). In a study of 85 blood isolates, MICs ranged from 0.06 to 4 μg/ml for penicillin and from 0.25 to ≥256 μg/ml for ceftriaxone, with *L. casei* and *L. rhamnosus* being among the species with the least susceptibility to these agents (229). Fluoroquinolones have poor activity against most *Lactobacillus* spp. (226, 227, 229, 230).

Leuconostoc spp. are usually susceptible to clindamycin and erythromycin, and penicillin MICs are generally less than 0.5 µg/ml (230–232). Ceftriaxone MICs have been shown to vary from 1 to >128 µg/ml, and elevated imipenem MICs (>0.5 µg/ml) and meropenem MICs (>8 µg/ml) have been described (230–233). There is one report of an imipenem therapeutic failure for a central nervous system infection due to *Leuconostoc* with an imipenem MIC of 4 µg/ml (234). Daptomycin MICs for *Leuconostoc* spp. are low (<0.5 µg/ml), and linezolid MICs range from 2 to 8.0 µg/ml (235).

Pediococcus spp. are generally susceptible to clindamycin and erythromycin (228, 230, 231, 236) but can be resistant to both of these (231). A penicillin MIC_{90} of 0.5 µg/ml was reported for 49 isolates (228), but penicillin MICs as high as 2 µg/ml have been noted (231). MICs for ceftriaxone are generally ≥16 µg/ml; however, most isolates are very susceptible to imipenem (MICs, <0.25 µg/ml) (230, 231). *Pediococcus* spp. are resistant to ciprofloxacin (230, 231, 236). Gentamicin, which would only be used in combination therapy, has variable activity against isolates of all three genera (228, 230, 231, 236).

The medium recommended by the CLSI for broth microdilution testing of *Lactobacillus* spp., *Pediococcus* spp., and *Leuconostoc* spp. is CAMHB-LHB (Table 3) (2). Some have performed susceptibility testing by agar dilution using either unsupplemented MHA or MHA with 5% sheep blood (230). If testing by agar dilution, incubation in 5% CO_2 may be necessary to achieve sufficient growth.

L. MONOCYTOGENES

Clinical isolates of *L. monocytogenes* remain susceptible to the drugs of choice, including ampicillin (or penicillin) and TMP-SMX. With the exception of occasional resistance to tetracyclines, expressed by a *tet*M or *tet*S gene, and ciprofloxacin, mediated by active efflux (237), *L. monocytogenes* is typically susceptible *in vitro* to other agents active against Gram-positive bacteria, including chloramphenicol, vancomycin, and macrolides (237, 238). Daptomycin MICs are elevated (MIC_{90}, 4 µg/ml), and linezolid MICs range from 1 to 2 µg/ml (238). Although *L. monocytogenes* may appear susceptible to cephalosporins *in vitro*, these agents are not effective clinically. Antimicrobial susceptibilities of 4,668 clinical isolates of *L. monocytogenes* were evaluated in France between 1989 and 2007 (237). Only 61 isolates were found to be resistant to at least one clinically relevant antibiotic, most commonly tetracycline (0.7% of isolates) and ciprofloxacin (0.4% of isolates), which emerged in the 1980s and 1990s, respectively. In addition, 1 isolate was resistant to trimethoprim, 1 to erythromycin, and 1 to chloramphenicol, and 2 were resistant to streptomycin. However, some studies have recently reported an increased rate of resistance to one or more clinically relevant antibiotics in *L. monocytogenes* isolated from food and food processing environments (239). Overexpression of efflux systems has been shown to be selected for by the disinfectant benzalkonium chloride, used in commercial food production facilities (240).

Listeria infections are generally treated empirically, and antimicrobial susceptibility testing is usually not necessary. Only criteria for susceptibility (MIC, ≤2.0 µg/ml) are listed for ampicillin and penicillin in the CLSI guidelines because clinical isolates of *L. monocytogenes* have not been noted to have results other than those indicating susceptibility (2). *L. monocytogenes* is not truly fastidious, and testing in Mueller-Hinton broth without the blood supplement has been done satisfactorily. However, the CLSI recommends using CAMHB-LHB. It is important to remember that cephalosporins should not be tested or reported for *L. monocytogenes* because some isolates may, for example, have ceftriaxone MICs as low as 8 µg/ml, which could suggest false susceptibility (241). This cautionary measure is emphasized in CLSI documents (2) and illustrates why it is inappropriate to indiscriminately report susceptibility results for any agent without knowing if it would be a reasonable therapeutic option. Because cephalosporins are frequently used empirically for the treatment of meningitis, the laboratory should quickly communicate smear or culture findings suspicious for *Listeria* whenever they occur.

M. CATARRHALIS

M. catarrhalis has maintained a high degree of susceptibility to all antimicrobial agents that might be used to treat infections caused by them, with the exception of penicillinase-susceptible penicillins. More than 90% of *M. catarrhalis* isolates produce β-lactamase and are resistant to amoxicillin, ampicillin, and penicillin (66–68, 72, 242). These isolates remain susceptible to amoxicillin-clavulanic acid, which is often prescribed for *M. catarrhalis* infections.

Most clinical isolates produce one of two types of β-lactamases: BRO-1 or BRO-2 (242–247). Both enzymes are encoded by chromosomal genes and are phenotypically identical and membrane associated. BRO-1-producing strains are up to10-fold more prevalent than BRO-2-producing strains, and ampicillin and penicillin MICs for BRO-1-producing strains are often higher (e.g., ≥4.0 µg/ml) than those for BRO-2-producing strains (e.g., ≤0.5 µg/ml) (242–247). Because of the low MICs for the latter strains, the clinical significance of their response to β-lactamase-labile penicillins is questionable. When 1,440 isolates of *M. catarrhalis* from children and adults were examined as part of a global surveillance study, BRO-negative isolates were more commonly isolated from adults than from children and oral cephalosporins had consistently higher MICs for isolates from the Far East than for isolates from the rest of the world (243). Resistance rates for TMP-SMX are less than 5% in most studies (67, 72, 242–244, 246). Hsu et al. reported that 18.5% of 314 isolates from 11 hospitals in Taiwan were resistant to TMP-SMX (242). Resistance to macrolides and tetracyclines is generally low (<1% from isolates in the Western Hemisphere from 2009 to 2011) (15, 38, 66, 68, 242). In the Asia-Pacific region, rates of clarithromycin and tetracycline resistance were 7.6% and 3.2%, respectively (248).

Only the chromogenic cephalosporin method has reliably detected the β-lactamases produced by *M. catarrhalis*. Routine β-lactamase testing may not be necessary because of the high incidence of β-lactamase-positive strains. Nevertheless, some advocate reporting of β-lactamase results to highlight the fact that this pathogen is generally unresponsive to some agents (e.g., amoxicillin) commonly prescribed for the treatment of respiratory tract infections. MIC testing without β-lactamase testing can be problematic, as there is some overlap in ampicillin and amoxicillin MICs in β-lactamase-positive and -negative strains. Since *M. catarrhalis* typically responds to the drugs of choice, testing beyond the β-lactamase test is rarely indicated. CLSI document M45-A2 addresses MIC testing of *M. catarrhalis*, and the recommended test medium is CAMHB (2). Recommendations for disk diffusion testing of amoxicillin-clavulanic acid, azithromycin, clarithromycin, erythromycin, tetracycline, and TMP-SMX using MHA and incubation in 5% CO_2 are included in CLSI document M45-A2 (Table 3). These were adopted following studies performed by Bell et al. which demonstrated a high correlation of disk diffusion results with results obtained by MIC testing (244).

PASTEURELLA SPECIES

Human isolates of *Pasteurella* spp. are generally susceptible to penicillin, with MICs of ≤0.5 µg/ml (249–252). Sporadic reports of human infections with β-lactamase-producing isolates demonstrated positive reactions with a nitrocefin-impregnated disk test, amoxicillin MICs of 8 to 64 µg/ml, and amoxicillin-clavulanic acid MICs of 0.25 µg/ml to 1 µg/ml (253, 254). For one of these isolates, the β-lactamase was characterized as a plasmid-borne TEM-1 (254), whereas two other cases were associated with plasmid-borne ROB-1 β-lactamases (253). *Pasteurella* spp. are generally very susceptible to antimicrobial agents commonly used for the treatment of acute bacterial skin and skin structure infections: a recent study documented very low MICs for cefazolin, ceftriaxone, ampicillin-sulbactam, ertapenem, azithromycin, doxycycline, and TMP-SMX among 156 *Pasteurella* spp. isolated from patients with animal bite wounds (255). No studies have documented resistance among clinical isolates for parenteral cephalosporins, fluoroquinolones, tetracyclines, chloramphenicol, or TMP-SMX (249–252). Resistance to erythromycin can occur, and there is at least one report of failure of erythromycin to cure a cat bite victim who subsequently developed meningitis (256).

Broth microdilution and disk diffusion methods are described by the CLSI for testing *Pasteurella* spp. (Table 3). Due to the absence of nonsusceptible strains, there are only susceptibility interpretive criteria for agents that might be tested, with the exception of erythromycin. Susceptibility testing may be warranted for isolates from normally sterile sites, and β-lactamase testing should be done on these isolates as well as those from respiratory sources (2). Testing of isolates from bite wounds is not necessary since bite wound infections are generally treated empirically with agents (e.g., amoxicillin-clavulanic acid) that would cover a variety of organisms likely to be implicated in the infection.

VIBRIO SPECIES

Both *Vibrio cholerae* and the noncholera *Vibrio* spp. are often susceptible to most antimicrobial agents, including newer cephalosporins, aminoglycosides, fluoroquinolones, and tetracyclines; however, resistance to one or more of these can occur (257).

In parts of the developing world, rates of resistance among *V. cholerae* vary considerably both geographically and year to year. Resistance to tetracyclines, fluoroquinolones, and TMP-SMX, drugs commonly prescribed when therapy is needed, has been documented (258). Multidrug-resistant *V. cholerae* has been attributed to the presence of genetic elements harbored on an R plasmid (259), whereas the integrating conjugative resistance element SXT encodes resistance to trimethoprim, sulfamethoxazole, and streptomycin and has been observed among *V. cholerae* strains from multiple cholera outbreaks (257, 260). Wang et al. recently reported the antimicrobial susceptibilities of 550 *V. cholerae* El Tor biotype O1 isolates collected in China between 1961 and 2010; these isolates demonstrated significant increases in resistance to TMP-SMX (from 0 to 38.5%), tetracycline (from 0 to 89%), and nalidixic acid (0 to 45.9%) over this period (261). In contrast, a study of 340 *V. cholerae* O139 isolates collected in China between 1993 and 2009 displayed high rates of resistance to nalidixic acid (83.1%), tetracycline (83.4%), azithromycin (50.3%), and TMP-SMX (90.7%) (262). Recently, a *V. cholerae* El Tor Ogawa strain was isolated from a child in India that harbored both the bla_{DHA} and $bla_{\mathrm{NDM\text{-}1}}$ genes, with resistance to third-generation cephalosporins by Etest and agar dilution (263).

When a double-blind randomized study was conducted to determine the equivalence of azithromycin and ciprofloxacin, 78% of 97 patients had a bacteriologic cure with azithromycin, compared with only 10% of 98 patients who received ciprofloxacin. The 168 isolates from these patients were found to be susceptible to both azithromycin and ciprofloxacin when tested by disk diffusion and Etest, but the median ciprofloxacin MIC for 91 *V. cholerae* serogroup O1 isolates was 0.25 µg/ml, which is significantly above the wild-type MIC (264). The elevated MICs and poor outcomes suggest the possibility of a resistance mechanism not detectable when an MIC of ≤1 µg/ml is used to define ciprofloxacin susceptibility. Indeed, isolates from the 2010 Haitian *V. cholerae* O1 outbreak were found to have reduced susceptibility to ciprofloxacin (0.25 to 1.0 µg/ml), which was associated with mutation to the QRDR of *gyrA* and *parC* (75).

Since 2009, the CDC has been annually tracking antimicrobial resistance in *Vibrio* spp. other than *V. cholerae*. In each year studied, *V. parahaemolyticus* represented the largest number of isolates submitted to the CDC for testing, followed by *V. alginolyticus* and *V. vulnificus*. Fewer than 50 isolates of other species were submitted for testing. In 2011, 95.1% of *V. alginolyticus* isolates were resistant to ampicillin, in contrast to 40.3% of *V. parahaemolyticus* and 4.8% of *V. vulnificus* isolates. No isolates of *Vibrio* tested were resistant to quinolones or tetracycline, and 0.3% of isolates were resistant to TMP-SMX (176).

Antimicrobial therapy is not required to manage cholera, but such therapy may shorten the duration and reduce the severity of disease. Similarly, although the noncholera *Vibrio* spp. cause diarrheal disease, otherwise healthy individuals with diarrhea due to these bacteria usually recover spontaneously without treatment. When *Vibrio* spp. are isolated from sources associated with serious infections, antimicrobial susceptibility testing is warranted. The CLSI suggests use of broth microdilution or disk diffusion using methodology similar to that for testing *Enterobacteriaceae*. In 2010, an MIC test for detecting azithromycin in *Vibrio* spp. was added to the CLSI guidelines, and an MIC of ≤2 µg/ml is considered susceptible; no criteria for interpreting resistance are described. For testing of the halophilic *Vibrio* spp., some have suggested use of MHA containing 1% NaCl. However, the CLSI recommends preparation of the inoculum suspension in 0.85% NaCl solution for both disk diffusion and broth microdilution MIC testing with MHA and CAMHB (both without NaCl supplementation), respectively (2).

CONCLUSION

With the exceptions of serious streptococcal infections, extraintestinal *Aeromonas* or *Vibrio* infections, and device-associated coryneform infections, infections caused by many of the fastidious bacteria discussed in this chapter are treated empirically, as these bacteria are often susceptible to the drugs of choice. Consequently, susceptibility testing is infrequently needed for many of these organisms. In certain circumstances testing may be warranted, such as when there appears to be clinical failure, patient intolerance to the drug(s) of choice, or serious infections for which there are limited appropriate drugs that might be prescribed. Additionally, susceptibility testing may aid in species identification (e.g., differentiating *C. jeikeium* from other *Corynebacterium* species). If a physician seems unsure about requesting antimicrobial susceptibility testing on a fastidious organism, he or she should be encouraged to seek assistance from an infectious diseases clinician or pharmacist.

The Etest has been examined for testing a variety of fastidious bacteria. For the less common species, many of the data generated have been obtained from comparisons with nonstandardized *in vitro* test methods, with limited examination of the clinical correlation of the results. Consequently, the results for Etest on many of the organisms addressed in this chapter should be interpreted with caution in the clinical setting. Requests for susceptibility testing of fastidious bacteria are often for isolates associated with serious infections, and MIC results are likely to be more useful than qualitative results. The disk diffusion test should be used only for bacteria for which there are CLSI or EUCAST interpretive criteria. It is important for laboratory workers to maintain an awareness of the methods available for testing fastidious bacteria and their strengths and limitations. If testing must be performed, it should be done by a laboratory familiar with these methods and their limitations.

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Susceptibility Test Methods: Anaerobic Bacteria*

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Infections due to anaerobic bacteria are common and can be serious or even life threatening. Severe infections due to anaerobic bacteria may lead to high morbidity and mortality if appropriate antibiotic therapy is not started (1). Since anaerobes are part of the normal human microbiota, infections due to anaerobic bacteria are typically endogenous in nature. Anaerobic infections are usually polymicrobic. For instance, three to five anaerobes, on average, can be involved in a single case of suppurative anaerobic surgical infection (2).

Susceptibility testing for anaerobes can be problematic for many reasons, including the fastidious nature and slow growth of the organism. For this reason, empirical therapy for infections has often been instituted long before results of susceptibility testing are available. Additionally, anaerobic susceptibility testing can be difficult to perform due to the manipulation required under anaerobic conditions. The use of empirical broad-spectrum antimicrobial therapy has led some clinicians and laboratories away from performing susceptibility testing. Thus, a correlation between clinical outcomes and decreased susceptibility of isolates may be difficult to document.

However, anaerobic bacterial resistance is increasing globally, as demonstrated by multiple surveys in Europe, the United States, Canada, New Zealand, and other areas of the world (3–6). Moreover, resistance patterns differ by geographic location, not only between countries but also within countries and even among hospitals located within the same city. Resistance patterns in one area of the world cannot always be predicted by published reports of resistance from another area. Differences in susceptibility among individual reports may be due to various testing methods, selective antibiotic pressures on the bacteria secondary to patterns of antimicrobial usage in distinct areas, and lack of uniformity in adoption of interpretive breakpoints.

Certain resistance patterns are well known and well spread across geographic areas, such as β-lactamase production by almost all *Bacteroides* spp. and two-thirds of *Prevotella* spp. (5). Yet other resistance mechanisms are currently being recognized, and certain organisms are becoming more resistant to various classes of drugs, such as the decreasing susceptibility to clindamycin in the *Bacteroides fragilis* group (3). Clinicians and laboratorians can no longer assume susceptibility of anaerobes without performing the testing.

The Clinical and Laboratory Standards Institute (CLSI) has provided laboratories with procedural guidelines for anaerobic susceptibility testing as well as various antimicrobial choices for testing on certain organisms (7, 8). This chapter outlines the CLSI and other susceptibility testing methodologies available, emphasizing the advantages and disadvantages of each. As current resistance patterns guide the choice of antimicrobials to test, the main categories of resistance are emphasized for Gram-negative and Gram-positive anaerobes. Pertinent trends of increasing resistance among each group are noted. Finally, specific recommendations for antibiotic testing and reporting are reviewed.

SUSCEPTIBILITY TESTING METHODS AND QC

Reference Test Methods

Agar Dilution

The CLSI reference agar dilution procedure (also called the Wadsworth method) is the recommended reference testing method for anaerobic organisms (7). The agar dilution method is the method by which other susceptibility testing methods are compared to assess their validity. Briefly, 2-fold dilutions of each antimicrobial are added to molten agar, poured into a petri dish, and allowed to solidify (9). Standardized inocula of microorganisms are applied onto the surface of each plate. Within 30 min of inoculation of the plates and after the bacteria have absorbed into the media, the plates are inverted and incubated in an anaerobic atmosphere. After approximately 48 h of incubation, the plates are examined visually. The MIC is defined as the lowest concentration of each antimicrobial agent at which growth of the organism is inhibited. Although it is the "gold standard" of susceptibility testing for anaerobes, the agar dilution method requires considerable time, labor, and expertise. For these reasons, it can be difficult for routine clinical microbiology laboratories to perform as the sole susceptibility testing method for an individual isolate recovered from an anaerobic infection.

Medium

The recommended medium is brucella agar supplemented with hemin (5 μg/ml), vitamin K_1 (1 μg/ml), and 5% (vol/vol) laked (lysed) sheep blood. This medium supports the growth of essentially all anaerobes (10). Details

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*This chapter contains information presented by Diane M. Citron and David W. Hecht in chapter 72 of the 10th edition of this *Manual*.

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concerning the preparation of this agar and its composition can be found in the appendixes of CLSI document M11-A8 (7). Stock solutions of hemin, vitamin K_1, and laked sheep blood can be prepared and stored ahead of time. Hemin and vitamin K_1 are added to brucella agar base, autoclaved, and stored as prepared tubes of supplemented brucella agar. On day of testing, the supplemented brucella agar is melted and cooled to 48 to 50°C. The appropriate antimicrobials and the sterile laked sheep blood are added to the molten agar. Samples are then gently mixed, taking care not to introduce bubbles, and the plates are poured. Solidification of the media usually takes approximately 15 to 20 min. Plates should be placed in an incubator or laminar flow hood to be allowed to dry for 30 to 45 min after solidification. The CLSI recommends that the plates can be stored seven or fewer days in sealed plastic bags at 2 to 8°C. However, a 72-h storage limit should be applied for more stringent research or other purposes. Due to instability, plates containing imipenem (but not meropenem, ertapenem, or doripenem) or β-lactam–β-lactamase inhibitor combinations containing clavulanic acid cannot be stored and should be used on the day of preparation. If such plates are stored and used beyond the date of preparation, the antibiotic may break down and the antimicrobial MICs will appear higher than they truly are.

Inoculum Preparation

The inoculum can be prepared by suspending colonies from a 24- to 72-h brucella blood agar plate into brucella broth or another clear broth and obtaining a 0.5 McFarland standard density. Alternatively, the inoculum may be prepared by adding five or more colonies into enriched thioglycolate or other supportive broth medium and incubating them for 4 to 24 h to obtain a 0.5 McFarland standard density. Although the equivalence of a 0.5 McFarland standard may be assessed using either a photometer or by visual inspection, certain broth media may affect photometer readings. Thus, quality control (QC) should be performed in such situations, as mentioned below.

Inoculation Procedure

The organism suspensions should be applied onto the surface of the agar with an inoculum-replicating apparatus (also known as Steer's replicator) (Multiple Inoculator; CMI-Promex, Inc., Pedricktown, NJ) (11). The final inoculum on the agar is approximately 10^5 CFU per spot for *B. fragilis* and *Bacteroides thetaiotaomicron*. For species with larger cells, such as *Clostridium difficile* ATCC 700057, the inoculum is 1 $\log_{10}$ lower. These variations generally do not affect MICs and are acceptable. Agar dilution plates should be inoculated in an aerobic ambient atmosphere, since most anaerobes can tolerate short periods of exposure to oxygen. However, for certain fastidious bacteria, it may be necessary to prereduce the brucella plates and perform the inoculum preparation and the inoculation under anaerobic conditions. After the plates are stamped, the bacteria should be allowed to absorb onto the plates for 10 to 15 min. The plates should then be stacked upside down to prevent condensation from falling on the agar surface and placed in an anaerobic environment within 30 min of inoculation.

Incubation Conditions

The plates should be placed in an anaerobic jar or an alternative anaerobic environment at 35 to 37°C for 42 to 48 h. An anaerobiosis indicator should be included.

Quality Control

A QC program is designed to monitor accuracy and precision of the susceptibility testing process, including procedures, performance of reagents and equipment, and competency of persons conducting the tests. QC must be performed to demonstrate that any new medium adequately supports growth of the test organisms and that the antimicrobial agents have not deteriorated during shipping or storage. These tests must be a part of any testing program using any of the methods described above. QC strains that most closely resemble the tested organisms should be included. Recommended QC strains for the agar dilution procedure include *B. fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, *C. difficile* ATCC 700057, and *Eggerthella lenta* ATCC 43055. A chart of QC MIC ranges for anaerobes by the agar dilution method is included in CLSI document M100 (8).

Two QC strains should be used for each assessment when using the agar dilution procedure. Detailed suggestions are included in the CLSI document M11 (7). Dilution blanks of sterile, distilled water or buffer should be set up when preparing antimicrobial agents. Brucella plates with no antibiotic should be prepared when pouring the agar. An antimicrobial-free control plate should be incubated aerobically to check for aerobic contamination of the inoculation process, while another should be incubated under anaerobic conditions. If thioglycolate or another agar-containing broth medium is used for inoculum preparation, additional control plates should be inoculated with antibiotic-free broth since inoculum residue can occasionally be confused with growth of an organism on final reading of the plates. The CLSI also advises performance of colony counts on inoculum suspensions to ensure that the final inoculum is appropriate. It is also suggested that the inoculum concentration be verified for 5 to 10% of tests by performing colony counts when using broth media which may affect photometer readings. Different organisms may demonstrate variations in colony counts. For example, *Veillonella* and *Parvimonas micra*, which are composed of smaller cells than are most anaerobes, may have higher colony counts for a standard McFarland measure than do bacteria with larger cells, such as *Clostridium perfringens*.

Interpretation and Result Reporting

The MIC endpoint, as defined by the CLSI, is the minimum concentration at which a marked reduction of growth occurs on the test plate compared to the anaerobic growth control. A color pictorial guide of various MIC end points for anaerobes is included for guidance in CLSI document M11 (7). It is important to compare the antimicrobial-containing plates to the antimicrobial-free control plate when reading the tests, as different species of anaerobic bacteria can look different on the agar dilution plate. Other antimicrobial-organism combinations may be particularly difficult to interpret, such as Gram-negative organisms tested against ceftizoxime and piperacillin. Additionally, many strains of fusobacteria produce L forms (cell wall-deficient bacterial forms) which grow as transparent hazes on the surface of the agar and appear even at the highest MICs of β-lactam antibiotics.

Interpretive categories for MICs for anaerobic bacteria are listed in CLSI document M100 (8). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) publishes its breakpoints, which do not match CLSI breakpoints in all cases (EUCAST; http://www.eucast.org/clinical_breakpoints/). EUCAST also does not specify a single susceptibility testing method for anaerobes. The CLSI states

TABLE 1 Methods for susceptibility testing of anaerobic bacteria

Method	Medium	Inoculum	Incubation time (h)	Advantages	Disadvantages
Agar dilution[a]	Brucella blood agar	10^5 cells/spot	48	Reference method; multiple isolates tested per antibiotic	Labor-intensive; high expertise needed to perform; expensive
Broth microdilution[b]	Supplemented brucella broth	10^6 cells/ml (10^5/well)	48	Economical; commercial panels available; multiple antimicrobials tested per isolate	Shelf life of frozen panels may be limited; poor growth by some strains
MIC gradient diffusion	Brucella blood agar	1.0 McFarland; swab plate	24–48	Precise MIC; convenient for individual isolates; multiple drugs can be tested at one time	Expensive for surveillance purposes

[a] Media are commercially available.

[b] Frozen or lyophilized panels are available from Trek Diagnostic Systems, Thermo Fisher Scientific, Cleveland, OH.

that the intermediate category for anaerobes was established due to the difficulty in reading endpoints (8). Isolates for which the antimicrobial agent MICs are in the intermediate range may respond to therapy when maximum dosages are used alongside appropriate ancillary therapy, such as drainage or debridement. However, clinical responses should be carefully monitored. An overview of the agar dilution method, including advantages and disadvantages, is presented in Table 1.

Broth Microdilution

The broth microdilution method is easier to perform than agar dilution and offers certain advantages, such as the ability to test multiple antimicrobials at one time. With this method, small volumes of broth liquid media containing drugs in serial 2-fold dilutions are prepared on rounded or conical wells of sterile, plastic microdilution trays or plates. A standard amount of the bacteria is inoculated into each well. MIC determinations are obtained in a manner similar to that used for agar dilution. If panels are made in-house, laboratories can design their own drug panels. Panels may be prepared in advance and frozen. However, due to limitations of this technique, including poor growth of certain anaerobes which are more sensitive to oxygen, this method is recommended and validated by the CLSI only for *B. fragilis* group organisms at this time (8).

Medium

The recommended medium is brucella broth supplemented with hemin (5 μg/ml), vitamin K_1 (1 μg/ml), and 5% (vol/vol) laked (lysed) horse blood. Microdilution trays may be freshly prepared and then frozen, or they may be purchased commercially as lyophilized or frozen panels. If trays are to be frozen, antimicrobial agents should be added before freezing. Details concerning the preparation of broth microdilution plates are covered in CLSI document M11 (7). Dispensing devices or pipettes may be used to add the antimicrobials to the plates, but volumes differ based upon the device used. Each well should contain at least 0.1 ml of broth. Volumes lower than 0.1 ml are not recommended due to evaporation. Certain antimicrobials such as imipenem and clavulanic acid are more labile than other antibiotics, and panels containing these antimicrobials must be stored at lower temperatures. Trays should not be stored in a self-defrosting freezer, as repeat freeze-thaw cycles lead to antibiotic degradation.

Inoculum Preparation

Inoculum preparation is similar to that used for agar dilution. A 0.5 McFarland standard density inoculum can be prepared by suspending colonies from a 24- to 72-h brucella blood agar plate into brucella broth or another clear broth, or by incubating enriched thioglycolate or other supportive broth medium with several colonies until the desired turbidity is reached. Optimally, the inoculum suspension should be diluted in broth or saline within 15 min of obtaining the correct McFarland density, for a final approximation of 1 × 10^5 CFU in each well. Dilution techniques vary depending upon the method of tray inoculation (7).

Inoculation Procedure

Frozen trays should be brought to room temperature before use. Inoculation may be accomplished by use of a mechanical dispenser, an inoculator, or a pipette. Placement of the trays in a reducing environment for 2 to 4 h prior to inoculation may aid in the growth of oxygen-sensitive strains, but *B. fragilis* is relatively oxygen tolerant (9). Trays should be prereduced if metronidazole is included in the panel, since activity of metronidazole is dependent upon an active intermediate, which requires a reduced atmosphere. If metronidazole is not prereduced, rapidly growing or nonfastidious strains can appear falsely resistant.

Incubation Conditions

Trays should be incubated for 46 to 48 h at 35 to 37°C in an anaerobic atmosphere. Trays should not be stacked more than four high to ensure uniformity of incubation temperature of the trays. In addition, trays should not be sealed with sealing tape, since this decreases the diffusion of gas to the inoculum and may lead to poor growth or false resistance to metronidazole. However, evaporation of the broth can be problematic if the trays are not incubated in a humidified environment with a cover tray. Alternatively, the trays may be placed in a self-sealing plastic bag which is left open slightly to allow for gas exchange. It is also permissible to use perforated tape with holes for each well.

Quality Control

QC of the broth microdilution process is similar to that described for agar dilution and is described in detail in CLSI document M11 (7). Recommended QC strains for the broth microdilution procedure include *B. fragilis* ATCC 25285, *B. thetaiotaomicron* ATCC 29741, *C. difficile* ATCC 700057, and *E. lenta* ATCC 43055. A chart of QC MIC ranges for anaerobes by the broth microdilution method is included in CLSI document M100 (8). When testing an individual strain, one QC strain should be included. Each tray should include a growth control well and a sterility well that is uninoculated. It is also suggested to incubate uninoculated microdilution trays from each batch that is prepared in-house in order to confirm sterility (12). Purity checks of the inoculum suspension should be performed by incubating aliquots aerobically and anaerobically. Colony counts on inoculum suspensions should also be performed to ensure that the final inoculum is appropriate.

Interpretation and Result Reporting

Determination of the MIC may be made by viewing the plates from the bottom using a magnifying mirror viewing stand with reflective light. As for agar dilution, the MIC is the concentration at which the most significant reduction of growth is observed. A trailing effect may be seen with some antimicrobial-organism combinations, and a color pictorial guide of various MIC determinations for broth microdilution is included in CLSI document M11 (7). If there is poor growth in the drug-free positive control well, the test should not be read. Currently, CLSI breakpoints for broth microdilution and agar dilution for *B. fragilis* group organisms are the same (8). A summary of the advantages and disadvantages of the broth microdilution technique is provided in Table 1.

Commercial Test Methods

MIC Gradient Diffusion Method

The Etest (bioMérieux, Durham, NC) and M.I.C.Evaluator (M.I.C.E.) strips (Thermo Fisher Scientific, Basingstoke, United Kingdom) are convenient gradient diffusion methods and are generally easier to use in routine clinical microbiology practice than are agar dilution and broth microdilution. The Etest was introduced in 1994 and until recently was the only gradient diffusion method available for clinical laboratories. In a 2008 survey of 150 U.S. hospitals, Goldstein and colleagues reported that 65% of hospital laboratories with access to anaerobe susceptibility testing performed such testing using Etest (13). Forty percent of hospital laboratories used microbroth dilution, and none used agar dilution.

Various antimicrobial Etests are commercially available for use in the United States. Both the Etest and M.I.C.E. are thin plastic strips which are impregnated on one face with an increasing gradient concentration of an antimicrobial agent. A 1.0 McFarland is swabbed onto a plate, usually a prereduced brucella plate supplemented with hemin and vitamin K_1. The MIC is determined by the intersection of the lower part of the ellipse with the corresponding number on the test strip. The strips are generally read at 48 h; however, clostridial species and *B. fragilis* group isolates may grow well enough to be read at 24 h. The advantages of these strips are that multiple drugs can be tested on one plate, and the method is flexible in allowing laboratories to tailor antimicrobial testing according to their needs. Large overlapping inhibitory zones of certain organisms may render the test difficult to read, unless the strips are located far apart on the plate. Users should refer to the Etest package inserts for QC organism suggestions. When testing an individual strain with Etest, one QC strain should be included.

Etest strips cost approximately $2 to $3 (U.S.) apiece, and testing can be expensive if several Etests are used at one time. The complete list of current U.S. Food and Drug Administration (FDA)-approved antimicrobial agents for anaerobe testing can be found in the various package inserts for the drugs. Table 1 lists the advantages and disadvantages of the MIC gradient diffusion methodology.

Comparative studies of Etest performance against agar dilution have demonstrated high categorical agreement, approximately 98%, averaged across all anaerobes and essential agreement of 85% or higher (14–17). In one study of 197 anaerobic organisms tested by both agar dilution and Etest, intermethod correlations of all drugs were high, with penicillin showing the lowest correlation, at 91.8% (18). It has been well documented that Etest can overestimate metronidazole resistance of organisms compared to agar dilution if anaerobiosis is not adequate (19). However, this phenomenon can be avoided by prereducing the test plates in an anaerobic environment overnight prior to their use. Other studies have shown a high very major error (VME) rate (false susceptibility) of 3.2% for Etests of certain antimicrobial-microorganism combinations, such as clindamycin and ceftriaxone tested against *B. fragilis* group and *Clostridium* spp., compared to agar dilution (16).

In a comparative study of agar dilution, Etest, and M.I.C.E. using 102 clinical strains of anaerobes, Rennie et al. reported that the essential and categorical agreement between Etest and M.I.C.E. was >90% for the four drugs tested: amoxicillin-clavulanate, imipenem, metronidazole, and penicillin (20). Compared to agar dilution, a high VME rate was noted for both Etest and M.I.C.E. for penicillin (8 to 9% VME) and metronidazole (13 to 14% VME). Agar dilution MICs were generally higher for all species of anaerobes and antibiotics. The VME discrepancies were again observed among *B. fragilis* group strains and clostridia, including *C. perfringens*.

Commercially Available Broth Microdilution Panels

Two commercially available panels for anaerobic susceptibility testing are available currently, both offered by Thermo Fisher Scientific (Cleveland, OH). The first is the dried anaerobic Sensititre panel (ANO2B; Trek Diagnostic Systems, now owned by Thermo Fisher), which is available with 15 antimicrobials and is marketed for research use only (http://www.trekds.com/products/sensititre/c_anaerobe.asp). Sensititre can also design custom panels, either frozen or dried, to suit the needs of an individual laboratory. A frozen anaerobic susceptibility panel is also available through Oxoid (also now owned by Thermo Fisher) (ANA MIC panel, catalog number R8320100; http://www.thermoscientific.com/en/product/oxoid-ana-mic-panel-minimal-inhibitory-concentration-mic-susceptibility-system.html). There are limited studies on the performance of such panels for anaerobic susceptibility testing. Dowzicky and colleagues compared meropenem on the Sensititre panel to agar dilution for 47 anaerobes and demonstrated overall agreement of 93% (±1 2-fold dilution) between the two methods (21). Each drug on the panels must be FDA approved for use in anaerobic infections in order for the entire panel to be approved. Since this is not the case, FDA approval is not available for either company's panels.

β-Lactamase Testing

Anaerobes may be tested for β-lactamase production by the β-lactamase disk test using a chromogenic cephalosporin. Such disks are available through BD Diagnostics, Hardy Diagnostics, and Thermo Fisher Scientific. Hydrolysis of the β-lactam ring by β-lactamases causes the disk to change from yellow to red. Most reactions occur within 5 to 10 min. Since some β-lactamase-positive strains of *Bacteroides* spp. or other anaerobes may react more slowly (up to 30 min), it is suggested to hold the chromogenic cephalosporin disks for a full 30 min before calling the test negative. Members of the *B. fragilis* group are presumed to be resistant to ampicillin, amoxicillin, and penicillin due to β-lactamases, so β-lactamase disk testing need not be performed. Other Gram-negative anaerobes, such as *Prevotella*, *Porphyromonas*, and *Bacteroides*, may be tested by the nitrocefin disk, but it is important to realize that resistance to β-lactam drugs is not always mediated by β-lactamase production. Therefore, if an isolate is negative by the chromogenic cephalosporin disk, resistance may still be present due to other mechanisms such as alterations of penicillin-binding proteins, as with some strains of *Parabacteroides* (previously *Bacteroides*) *distasonis* and *B. fragilis* (22). Gram-positive anaerobes may also be screened for β-lactamase activity by the chromogenic cephalosporin disk test. If the isolate is β-lactamase positive, the isolate should be reported as resistant to penicillin and ampicillin, regardless of the MIC result.

Disk Diffusion

According to CLSI, disk diffusion tests are not suggested as an antimicrobial testing method for anaerobes, as the susceptibility testing results are inaccurate and do not correlate with the results of the agar dilution method (8).

RESISTANCE PATTERNS IN ANAEROBIC BACTERIA

Resistance patterns differ by geographic region and have changed significantly over time. Various authors have emphasized the screening of anaerobes for certain resistance patterns which are rising in incidence and are clinically important (23). Such patterns which deserve close attention include (i) resistance to the β-lactam–β-lactamase inhibitor combinations among the *B. fragilis* group; (ii) increasing clindamycin resistance in all anaerobes; (iii) metronidazole resistance, which is no longer confined to the *B. fragilis* group but now includes Gram-positive cocci and Gram-positive rods; and (iv) resistance of *Clostridium* to glycopeptides and lipopeptides, such as vancomycin and daptomycin. Each of these patterns is described below.

Bacteroides fragilis Group

The *B. fragilis* group consists of 24 species (including five *Parabacteroides* species). Of this group, *B. fragilis* is generally the most susceptible to antibiotics. *P. distasonis* demonstrates high MICs for β-lactams, and *B. thetaiotaomicron* also demonstrates higher rates of resistance to various antimicrobials than do other members of the *B. fragilis* group. A survey conducted by Snydman and colleagues of *B. fragilis* group isolates collected from 10 U.S. medical centers demonstrated trends in susceptibilities using agar dilution over 1997 to 2004 (4). Species distribution of 6,574 isolates included 51% *B. fragilis*, 19% *B. thetaiotaomicron*, 10% *Bacteroides ovatus*, 6% *Bacteroides vulgatus*, and 5% *P. distasonis*, with the remaining composed of a variety of species. Susceptibility rates of antibiotics within the *B. fragilis* group varied by species. Specifically, compared to other members of the *B. fragilis* group, *B. ovatus* was more resistant to the carbapenems, *B. vulgatus* was more resistant to piperacillin-tazobactam and showed the highest rates of resistance to moxifloxacin (54%), *P. distasonis* was more resistant to ampicillin-sulbactam and cefoxitin, and *B. ovatus* and *Bacteroides uniformis* showed higher rates of resistance to moxifloxacin (39% and 41%, respectively). These patterns of resistance by species have been mirrored by other studies (24–26).

Greater than 97% of all *Bacteroides* isolates are resistant to penicillin and ampicillin principally due to β-lactamase production (27). Resistance to penicillins and cephalosporins is mediated primarily by the *cepA* or *cfxA* gene. The *cepA* gene encodes a chromosomal cephalosporinase which renders the organism resistant to cephalosporins and aminopenicillins, but the isolate remains susceptible to piperacillin and β-lactam–β-lactamase inhibitor combinations. When *cepA* gene expression is enhanced by an upstream promoter, the MICs of all β-lactams increase, with the exception of carbapenems (23). However, the additional presence of a membrane modification such as a porin loss may lead to a 4-fold increase in the MICs of the β-lactams and β-lactam–β-lactamase inhibitor combinations. Currently, β-lactam–β-lactamase inhibitor combinations, such as ticarcillin-clavulanate and piperacillin-tazobactam, are active against nearly all strains of *B. fragilis* group, with >90% susceptibility in most reports (3, 28, 29). However, recent studies from various areas of the world, including the United States, Europe, Taiwan, and Kuwait, have emphasized increasing resistance to ampicillin-sulbactam or piperacillin-tazobactam of *Bacteroides* species (3, 28–32). Authors from one study characterized a *B. thetaiotaomicron* strain with a porin loss which contributed to amoxicillin-clavulanate resistance (33). This pattern of decreasing susceptibility of *B. fragilis* group isolates is important, since the number of therapeutic choices is narrowing and β-lactam–β-lactamase inhibitor combinations are not infrequently used in anaerobic infections.

The *cfxA* gene encodes a broad-spectrum β-lactamase which is responsible for high-level resistance to cefoxitin and other β-lactam drugs (26, 34). Although the principal mechanism of resistance to penicillins is β-lactamase production, penicillin-binding proteins can also lead to resistance (35). Piperacillin and other ureidopenicillins are more active than penicillin, but more than half of isolates are resistant to the former (36). Oxacillin, nafcillin, and the first-generation cephalosporins are not active against the *B. fragilis* group.

Cefoxitin and cefotetan are generally active against *B. fragilis*. However, cefotetan has lower activity against members of the *B. fragilis* group other than *B. fragilis* (32). Rates of resistance to cefoxitin and cefotetan are rising, and susceptibility testing should be performed if these antimicrobials are to be used for therapy (3). With the exception of ceftizoxime, broad-spectrum cephalosporins show poor activity against *B. fragilis* group organisms (37).

Carbapenems (imipenem, ertapenem, meropenem, and doripenem) are generally active against members of the *B. fragilis* group, and most studies report susceptibility rates of ≥95% (4, 25). Carbapenem resistance is usually mediated by a zinc metallo-β-lactamase enzyme encoded by the *cfiA* gene that confers resistance to all current β-lactams and β-lactam–β-lactamase inhibitor combination agents (38). The *cfiA* gene is present in small numbers in *Bacteroides* but is silent unless insertion sequence elements activate the gene (39). Metallo-β-lactamase enzymes can be detected phenotypically with use of a chelating compound such as

EDTA, which inactivates the enzyme and renders the isolate susceptible to the carbapenem. Alarmingly, carbapenem resistance is increasing as well but at low rates (25, 28, 31). In a susceptibility trend study of *B. fragilis* group organisms, Snydman and colleagues reported a decrease in carbapenem susceptibility from 2006 to 2009 (29). Carbapenem susceptibility rates in the study were 97.5 to 98.9%, and the susceptibility rate for the β-lactam–β-lactamase inhibitor combination agents was approximately 95%. Seifert et al. reported 95% carbapenem susceptibility of the *B. fragilis* group isolates associated with intra-abdominal infections in their multicenter German study (25). Interestingly, a report from Turkey noted only 90% susceptibility to carbapenems in a group of 66 *B. fragilis* group isolates, the majority of which were confirmed positive by PCR for the presence of the *cfiA* gene (40).

Clindamycin resistance is increasing worldwide in the *B. fragilis* group and approaches 40% (3, 4, 29, 31). Resistance is mediated by *erm* genes, which are frequently located on transferable plasmids and can be linked to transferable tetracycline resistance as well (41).

Metronidazole resistance is linked to the *nim* (nitroimidazole reductase) gene. Although rare, metronidazole-resistant strains of *B. fragilis* group isolates have been reported from numerous areas of the world (3, 28, 38). In 2011, Sherwood and colleagues published on a strain of *B. fragilis* from a leg wound which was resistant to metronidazole and numerous other drugs (38). The isolate was confirmed by PCR to possess the *nim* gene located on a potentially transferrable plasmid. Moxifloxacin and linezolid were the only antimicrobials to which the organism displayed MICs in the susceptible range. Approximately 95 to 97% of *B. fragilis* isolates in France are susceptible to metronidazole (using the EUCAST susceptible breakpoint of ≤4 mg/liter, which is one 2-fold dilution lower than the CLSI susceptibility breakpoint), and other countries have published reports of similar susceptibility (3, 23, 28). Expression of this gene leads to reduction of the nitrate residue of metronidazole into an amino derivative, thus decreasing the effect of the antibiotic on the bacterium. Of 206 *B. fragilis* isolates in one study, the *nim* gene was detected in 24%, and metronidazole MICs of the *nim*-positive isolates ranged from 1.5 to >256 mg/liter, spanning the range of susceptible to highly resistant (42). Metronidazole MICs must therefore be measured, as the *nim* gene can be found in metronidazole-susceptible strains. Although many *nim*-positive *Bacteroides* strains do not develop a high level of resistance, they can be induced by subinhibitory concentrations of the antibiotic to express high MICs (43). Thus, metronidazole treatment could potentially select for a subpopulation of *nim*-positive organisms which may express resistance and decrease clinical response to the drug. Metronidazole resistance is spreading geographically to other genus and species of anaerobes, highlighting the transmissibility of this type of plasmid-mediated resistance.

Moxifloxacin can be distinguished from levofloxacin by its added activity against anaerobes and is FDA approved for complicated intra-abdominal infections caused by anaerobes. However, rates of susceptibility to moxifloxacin are highly variable across studies, and isolates appear to be acquiring resistance rapidly. In a recent study, Snydman et al. reported resistance rates of >80% to moxifloxacin for *B. fragilis* group isolates (29). In a multicenter European surveillance study, susceptibility to moxifloxacin decreased between 2003 and 2009 from 91 to 86.4% (3). Resistance is mediated by *gyrA* mutations, efflux pumps, or modifications of topoisomerase genes.

The FDA has approved MIC breakpoints for tigecycline (susceptibility breakpoint, 4 μg/ml) for use in treatment of complicated intra-abdominal infections due to anaerobes. Although resistance at this time is relatively low in surveillance studies, there have been reports of susceptibility rates ranging from approximately 95 to 98.5% in some studies (6, 44).

In conclusion, *Bacteroides* carries various resistance mechanisms which can complicate treatment of infections. Studies have shown that susceptibility patterns of *Bacteroides* species are linked to outcome, even in the presence of mixed infections (4, 45). Therefore, *B. fragilis* group isolates from significant sites should be tested for susceptibility against a variety of antibiotics.

Prevotella and *Porphyromonas*

Fewer data are available for *Prevotella* and *Porphyromonas* susceptibility profiles than for *Bacteroides*. Overall, *Prevotella* and *Porphyromonas* are more susceptible to antimicrobials used to treat anaerobic infections than the *B. fragilis* group, while *Prevotella* is more resistant than *Porphyromonas*. Most *Prevotella* spp. are resistant to penicillin and ampicillin due to β-lactamase production. In a New Zealand surveillance study from 1999 to 2003, susceptibility rates among 45 strains of *Prevotella* spp. were as follows: 18% to penicillin, 89% to ceftriaxone, and 96% to clindamycin (5). Susceptibility rates for cefoxitin and cefotetan range from 70 to 99%, and piperacillin susceptibility rates are lower, at 55 to 80% (30, 31, 46). Liu and colleagues also reported in their study that *Prevotella* spp. demonstrated decreasing susceptibility to imipenem from 100% in 2002 to 94% in 2006 (31). Authors of a German antimicrobial surveillance study reported that 90.5% of *Prevotella* spp. tested susceptible to clindamycin. A recent Belgian study of *Prevotella* spp. and miscellaneous Gram-negative anaerobes documented decreasing clindamycin susceptibility from 91% susceptible in 1993 to 1994, 82% susceptible in 2004, and 69% susceptible in 2012 (47). Nine percent of *Prevotella* isolates showed intermediate moxifloxacin MICs (25).

Porphyromonas, on the other hand, appears more susceptible to β-lactams and other drugs than do *Prevotella* spp. However, susceptibility reports of clinically significant *Porphyromonas* spp. in surveillance studies are rare. Bahar and colleagues reported susceptibility profiles of 45 *Porphyromonas* isolates as measured by Etest (48). In their study, *Porphyromonas* demonstrated 94% susceptibility to penicillin and 97% susceptibility to cefoxitin, with 100% susceptibility to the remaining antibiotics, including ampicillin-sulbactam, imipenem, clindamycin, and metronidazole. Production of β-lactamase is rare, although clindamycin resistance has been observed in a minority of strains (49).

Both genera are almost uniformly susceptible to carbapenems, metronidazole, and tigecycline. Rare reports of metronidazole resistance in *Prevotella* spp. have been published (46). A report from France describes reduced susceptibility to metronidazole in 3 of 188 *Prevotella* spp., which also showed resistant subpopulations, similar to the findings with *B. fragilis* group organisms (50). In another study, ceftobiprole was tested against 42 strains of *Porphyromonas* and *Prevotella* (51). The ceftobiprole MICs of most *Porphyromonas* spp. were ≤0.125 μg/ml, while *Prevotella bivia* and *Prevotella melaninogenica* were less susceptible.

Other Gram-Negative Bacilli

Penicillin resistance among *Fusobacterium* spp. is relatively rare. Four of 36 (11%) *Fusobacterium* isolates in a Taiwan survey were positive for β-lactamase, as reported by Liu and colleagues (31). All *Fusobacterium* spp. were susceptible to metronidazole, piperacillin-tazobactam, cefmetazole, doripenem, and ertapenem. On the other hand, imipenem and meropenem nonsusceptibility rates were 4 to 8%, respectively, which were increased in comparison to previous years. Moxifloxacin susceptibility was shown in 81% of *Fusobacterium* isolates. Susceptibility rates vary somewhat by species. Over 90% of *Fusobacterium nucleatum* and *Fusobacterium necrophorum* isolates are susceptible to cephalosporins and cephamycins, while *Fusobacterium mortiferum* is resistant to cephalosporins (52).

Bilophila wadsworthia usually produces a β-lactamase and is therefore resistant to penicillin and ampicillin but is generally susceptible to remaining antibiotics, including clindamycin, cefoxitin, β-lactam–β-lactamase inhibitor combinations, carbapenems, and metronidazole (53, 54).

Campylobacter gracilis was previously thought to be resistant to many β-lactam agents, but studies have shown that a more resistant organism, *Sutterella wadsworthensis*, was often misidentified as *C. gracilis* in published reports (55). When properly identified and tested, *C. gracilis* is susceptible to most agents, including β-lactam–β-lactamase inhibitor combinations, cefoxitin, and clindamycin (56). *S. wadsworthensis*, on the other hand, not uncommonly displays resistance to clindamycin, piperacillin, and/or metronidazole (57).

Gram-Positive, Non-Spore-Forming Bacilli

The "*Eubacterium*" group, *Actinomyces*, *Propionibacterium*, and *Bifidobacterium* are usually susceptible to β-lactam agents, including the penicillins, cephalosporins and cephamycins, carbapenems, and β-lactam–β-lactamase inhibitor combinations (58, 59). One exception is *Eggerthella lenta* (formerly *Eubacterium lentum*), for which the MICs of third-generation cephalosporins are elevated. There are currently no CLSI clinical breakpoints for vancomycin and anaerobes; however, this agent shows *in vitro* activity against the "*Eubacterium*" group, *Propionibacterium* spp., and *Actinomyces* spp. (57). Although most lactobacilli which grow well in ambient air are vancomycin resistant (e.g., *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Lactobacillus salivarius*, and *Lactobacillus fermentum*), vancomycin shows some activity against the majority of lactobacilli which grow only anaerobically (*Lactobacillus acidophilus* group, *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus johnsonii*, and *Lactobacillus jensenii*) (60). The lactobacilli which grow aerobically should be tested for susceptibility aerobically, as outlined in CLSI document M45 guidelines for infrequently isolated or fastidious bacteria (61). On the other hand, if the lactobacilli grow only anaerobically (as is common for most vaginal lactobacilli), or if the lactobacilli are recovered from a specimen from which other anaerobes are recovered, it is currently suggested that susceptibility testing be followed based on the anaerobic guidelines in CLSI document M11 (7). This testing approach may change as more data are generated. *Lactobacillus* spp. are generally susceptible to erythromycin and clindamycin but show variable susceptibilities to cephalosporins, imipenem, vancomycin, and penicillin according to the species (62, 63). Most non-spore-forming Gram-positive anaerobes, with the exception of the "*Eubacterium*" group, are resistant to metronidazole. The newer antimicrobial agents, such as linezolid, daptomycin, dalbavancin, telavancin, oritavancin, and ramoplanin, show activity against Gram-positive aerobic organisms and also exhibit excellent *in vitro* activity against most anaerobic Gram-positive species (60, 64–66).

Gram-Positive, Spore-Forming Bacilli

C. perfringens isolates are almost universally susceptible to penicillin and clindamycin (5, 59). However, a recent Canadian population-based surveillance study of bacteremia due to *Clostridium* spp. from 2000 to 2006 demonstrated that 8/58 (14%) *C. perfringens* isolates were resistant to clindamycin (67). All *C. perfringens* isolates in the study were susceptible to penicillin and metronidazole. *C. perfringens* is typically susceptible to metronidazole, although resistant strains have been described (68). Non-*perfringens Clostridium* spp., including *C. difficile*, show variable patterns of susceptibility to a variety of glycopeptides and related antibiotics (69). Most clostridia are inhibited by 2 mg/liter of vancomycin and daptomycin and by 1 mg/liter of teicoplanin or dalbavancin (65). However, the "RIC" group of clostridia—namely, *Clostridium ramosum*, *Clostridium innocuum*, and *Clostridium clostridioforme*—is intrinsically resistant to several antibiotics. *C. clostridioforme*, which appears Gram negative in cultures, produces β-lactamase, shows low-level resistance to teicoplanin and dalbavancin, is resistant to daptomycin, but is susceptible to vancomycin (23, 70). On the other hand, *C. innocuum* is resistant to cefoxitin and cefotetan, retains high MICs (8 to 32 μg/ml) for vancomycin, but is susceptible to tigecycline and metronidazole. Finally, *C. ramosum* is resistant to vancomycin and daptomycin and shows low-level resistance to linezolid (MIC, 8 μg/ml), in contrast to other clostridial species. Goldstein and colleagues reported on the activity of ceftobiprole against clostridial species and demonstrated that most clostridia (including *C. perfringens*, *Clostridium cadaveris*, and *Clostridium subterminale*) were susceptible at ≤1 μg/ml, excluding *C. innocuum* and *C. clostridioforme* (51). The authors' findings differed from the findings of Wootton et al., who reported an MIC_{90} of 4 μg/ml for the group of 48 mixed species of clostridia (71).

C. difficile may be resistant to many β-lactam agents, including cephalosporins, fluoroquinolones, and clindamycin, but the organism usually demonstrates low MICs to metronidazole and vancomycin (72). Strains resistant to rifampin or rifaximin have been reported in laboratory surveys, and some were confirmed by clinical failures (72, 73). Metronidazole-resistant strains are rare but have been documented (74, 75). Fidaxomicin shows excellent activity against *C. difficile*, but reduced susceptibility has been documented *in vitro* and has been associated with mutations leading to target modifications (76).

Gram-Positive Cocci

The great majority of Gram-positive cocci are susceptible to β-lactams, β-lactam–β-lactamase inhibitor combinations, cephalosporins, carbapenems, and metronidazole (52). However, many Gram-positive cocci are resistant to clindamycin, including 30% of *Finegoldia magna* and *Peptoniphilus* spp. (77). *Peptococcus niger* is highly susceptible to β-lactams, β-lactam–β-lactamase inhibitor combinations, cephalosporins, carbapenems, chloramphenicol, metronidazole, and tigecycline (52, 60, 78).

TABLE 2 Indications for susceptibility testing of anaerobic bacteria[a]

Indication	Example(s)
Surveillance	
Annual monitoring of isolates at individual medical centers	*Bacteroides fragilis* group, *Prevotella* spp., *Fusobacterium* spp., *Clostridium* spp., *Bilophila wadsworthia*
Clinical	
Known resistance of a particular species	*B. fragilis* resistant to clindamycin, cephamycins, piperacillin, or fluoroquinolones; *Prevotella* spp. or *Fusobacterium* spp. resistant to penicillin or clindamycin
Failure of a usual therapeutic regimen	Any anaerobe
Pivotal role of antimicrobial agent in clinical outcome	*B. fragilis* group implicated in osteomyelitis or joint infection
Need for long-term therapy	*B. fragilis* group or *Prevotella* spp. implicated in osteomyelitis, endocarditis, brain abscess, liver abscess, or lung abscess
Infections of specific body sites	Any anaerobe implicated in a brain abscess, endocarditis, prosthetic devices or graft infection, or bacteremia

[a] The examples are suggestions only and are not intended as all-inclusive lists. See text for specific recommendations.

TABLE 3 Suggested grouping of antimicrobial agents to be considered for testing and reporting on anaerobic organisms, CLSI[a]

Agent grouping	Drugs	
	Bacteroides fragilis group and other Gram-negative anaerobes	Gram-positive anaerobes
Antimicrobials for primary testing and reporting	Amoxicillin-clavulanate Ampicillin-sulbactam Piperacillin-tazobactam Ticarcillin-clavulanate	Ampicillin Penicillin
	Clindamycin	Amoxicillin-clavulanate Ampicillin-sulbactam Piperacillin-tazobactam Ticarcillin-clavulanate
	Doripenem Ertapenem Imipenem Meropenem	Clindamycin
	Metronidazole	Doripenem Ertapenem Imipenem Meropenem
		Metronidazole
Supplemental antimicrobials for selective testing and reporting	Ampicillin Penicillin	Ceftizoxime Ceftriaxone
	Ceftizoxime Ceftriaxone	Cefotetan Cefoxitin
	Chloramphenicol	Piperacillin Ticarcillin
	Cefotetan Cefoxitin	Tetracycline
	Piperacillin Ticarcillin	Moxifloxacin
	Moxifloxacin	

[a] Adapted with permission from the Clinical and Laboratory Standards Institute from reference 8.

TABLE 4 Cumulative antimicrobial susceptibility report for *Bacteroides fragilis* group organisms for isolates collected from selected U.S. hospitals between 1 January 2007 and 31 December 2009[a]

Anaerobic organism(s)	No. of strains	Ampicillin-sulbactam		Piperacillin-tazobactam		Cefoxitin		Ertapenem		Imipenem		Meropenem		Clindamycin		Moxifloxacin		Metronidazole[b]	
		% S (≤8/4)	% R (≥32/16)	% S (≤32/4)	% R (≥128/4)	% S (≤16)	% R (≥64)	% S (≤4)	% R (≥16)	% S (≤4)	% R (≥16)	% S (≤4)	% R (≥16)	% S (≤2)	% R (≥8)	% S (≤2)	% R (≥8)	% S (≤8)	% R (≥32)
B. fragilis	872	89	4	98	1	85	6	96	2	98	2	97	2	64	28	53	38	100	0
B. thetaiotaomicron	342	86	3	92	2	32	13	96	2	99	0	99	1	27	56	44	34	100	0
B. ovatus	67	93	2	93	2	37	15	98	0	100	0	100	0	54	39	43	39	100	0
B. vulgatus	70	67	6	100	0	83	4	98	2	98	2	98	2	49	51	43	46	100	0
B. uniformis	60	87	2	93	0	42	13	97	0	100	0	98	0	35	52	35	50	100	0
B. eggerthii	58	95	0	100	0	98	2	100	0	100	0	100	0	29	55	28	55	100	0
Parabacteroides distasonis	111	69	11	91	2	41	16	97	0	100	0	99	0	30	41	54	38	100	0
B. fragilis group without *B. fragilis*	708	83	4	93	1	40	12	97	1	99	0	99	0	33	42	43	40	100	0
B. fragilis group (all 7 species listed)	1,580	86	4	95	2	65	9	97	1	98	1	98	1	50	39	49	39	100	0

[a] Reprinted with permission from the Clinical and Laboratory Standards Institute from reference 8. Data were generated from unique isolates from patient specimens submitted to three referral laboratories: Tufts New England Medical Center, Boston, MA; Loyola University Medical Center, Maywood, IL; and R. M. Alden Research Laboratory, Culver City, CA. Testing was performed by the agar dilution method. Breakpoints, in micrograms per milliliter, are in parentheses after the category (S, susceptible; R, resistant). The intermediate category is not shown but can be derived by subtraction of the percent susceptible and percent resistant for each antimicrobial agent from 100%.

[b] Resistance to metronidazole occurs infrequently.

TABLE 5 Cumulative antimicrobial susceptibility report for anaerobic organisms other than *Bacteroides fragilis* group for isolates collected from selected U.S. hospitals between 1 January 2007 and 31 December 2009[a]

Anaerobic organisms	No. of strains	Ampicillin-sulbactam		Piperacillin-tazobactam		Cefoxitin		Ertapenem		Imipenem		Meropenem	Penicillin/ ampicillin		Clindamycin		Moxifloxacin		Metronidazole	
		%S (≤8/4)	%R (≥32/16)	%S (≤32/4)	%R (≥128/4)	%S (≤16)	%R (≥64)	%S (≤4)	%R (≥16)	%S (≤4)	%R (≥16)	%R (≥16)	%S (≤0.5)	%R (≥2)	%S (≤2)	%R (≥8)	%S (≤2)	%R (≥8)	%S (≤8)	%R (≥32)
Prevotella spp.	173	98	1	99	1	99	1	100	0	100	0	1	40	49	66	30	59	24	100	0
Fusobacterium nucleatum-necrophorum	44	100	0	100	0	100	0	100	0	100	0	0	100	0	100	0	95	5	100	0
Anaerobic Gram-positive cocci[b]	168	98	1	100	0	100	0	100	0	100	0	0	96	3	78	20	82	11	98	1
Veillonella spp.[c]	28	100	0	61	7	100	0	100	0	100	0	0	57	28	89	7	79	14	86	11
P. acnes	34	100	0	100	0	100	0	100	0	100	0	0	100	0	91	3	100	0	3	97
Clostridium perfringens	73	100	0	100	0	100	0	100	0	100	0	0	100	0	96	0	99	1	100	0
C. *difficile*[d]	56	100	0	100	0	0	100	100	0	20	18	0	0	79	5	79	78	22	100	0
Other *Clostridium* spp.	43	100	0	100	0	47	26	100	0	100	0	0	79	9	56	21	74	12	100	0

[a] Reprinted with permission from the Clinical and Laboratory Standards Institute from reference 8. Data were generated from unique isolates from patient specimens submitted to three referral laboratories: Tufts New England Medical Center, Boston, MA; Loyola University Medical Center, Maywood, IL; and R.M. Alden Research Laboratory, Culver City, CA. Testing was performed by the agar dilution method. Breakpoints, in micrograms per milliliter, are in parentheses after the category (S, susceptible; R, resistant). The intermediate category is not shown but can be derived by subtraction of the percent susceptible and percent resistant for each antimicrobial agent from 100%.

[b] Anaerobic Gram-positive cocci include *Peptococcus*, *Peptostreptococcus*, *Finegoldia*, *Peptoniphilus*, and *Anaerococcus* species.

[c] Calculated from fewer than the CLSI document M39 (81) recommendation of 30 isolates.

[d] *Clostridium difficile* isolates are from intestinal sources; these results do not imply efficacy for intraluminal infections. Vancomycin MICs for all isolates were <4 μg/ml.

The accuracy of reported susceptibilities of the anaerobic Gram-positive cocci may have been limited in the past by misidentifications. For instance, *Peptoniphilus harei* (*Pt. harei*) may have been misidentified in past studies as *Peptoniphilus asaccharolyticus* (*Pt. asaccharolyticus*) (79). Veloo and colleagues from the Netherlands report differing susceptibilities among the three most common Gram-positive cocci identified by genotypic methods in their study: *P. micra*, *F. magna*, and *Pt. harei* (80).

Metronidazole resistance among Gram-positive cocci is extremely rare (80). If such resistance is encountered in an anaerobic Gram-positive isolate, the identity of the organism should be confirmed. Strains of streptococci such as the *Streptococcus anginosus* group or the microaerophilic streptococci (including *Abiotrophia* and *Granulicatella* species) may initially be identified as anaerobic Gram-positive cocci and will test resistant to metronidazole.

STRATEGIES FOR TESTING AND REPORTING OF SUSCEPTIBILITY DATA

Antimicrobial Testing Guideline

For individual patient management, susceptibility should be performed when (i) selection of an active agent is critical for disease management, (ii) long-term therapy is considered for clinical management, (iii) anaerobes are recovered from specific sterile body sites (e.g., blood, brain, bone, or joint), or (iv) the usual treatment regimen fails (Table 2). Since most anaerobic infections are polymicrobial in nature and are likely to include both β-lactamase-negative and β-lactamase-positive strains, susceptibility testing should be reported at least for the most resistant organism. Some anaerobic organisms, such as *C. perfringens*, *Clostridium septicum*, or *Clostridium sordellii*, may be the sole source of infection. In such cases, susceptibility testing should be performed, since these organisms are usually β-lactamase negative and therapy may be narrowed.

The CLSI lists certain antimicrobial agents to be considered for testing and reporting on anaerobic organisms (Table 3). The listing of antimicrobials is based upon many factors, including resistance rates and resistance mechanisms. The type of anaerobe (Gram negative versus Gram positive) guides the choice of antimicrobial testing. As noted in Table 3, certain antibiotics are listed as a cluster within one box, and only one of the agents in the box needs to be tested under usual circumstances. Antibiotics listed in the same box demonstrate similar clinical efficacy, and interpretive results (susceptible, intermediate, or resistant) are also similar. For Gram-negative anaerobes, penicillin or ampicillin may be tested and/or reported selectively for all except the *B. fragilis* group, which are almost uniformly resistant and need not be tested. For Gram-negative anaerobes other than those in the *B. fragilis* group, penicillin or ampicillin MICs may be obtained. If the β-lactamase test is positive for Gram-negative anaerobes, report the isolate as resistant to penicillin and ampicillin. However, it is important to recognize that Gram-negative anaerobes which are negative by the β-lactamase disk test may be resistant to β-lactams by another mechanism (8). Thus, a negative β-lactamase disk test should be followed by penicillin or ampicillin MIC testing if such drugs may potentially be used for treatment.

For Gram-positive anaerobes, many non-spore-forming Gram-positive rods are resistant to metronidazole, such as *Actinomyces*. Ampicillin and penicillin are recommended for primary testing for Gram-positive anaerobes, as many are β-lactamase negative. As with Gram-negative anaerobes, report the isolate as resistant to penicillin and ampicillin if the β-lactamase test is positive, regardless of the MIC result. Although CLSI MIC amoxicillin breakpoints have not been established, they are considered equivalent to ampicillin breakpoints (8). Some laboratories do not routinely perform susceptibility testing on *C. perfringens* isolates, since isolates retrieved from human infections are almost universally susceptible to penicillin and clindamycin (5, 59).

The remaining supplemental or alternative antimicrobials listed for possible testing or reporting are helpful for those patients or institutions with strains which are resistant to primary drugs. Alternative testing may also be helpful for treatment of patients who are allergic to primary drugs or for treatment of unusual isolates.

Antibiograms

Individual hospitals should perform periodic surveillance of anaerobes to monitor resistance rates in their system, if possible. Establishment of an antibiogram outlining patterns of resistance for certain anaerobes on a periodic basis would be helpful for empirical coverage of the patient with antibiotics. CLSI guideline M39 provides recommendations outlining the proper collection, analysis, and presentation of cumulative antimicrobial susceptibility test data (81). It is recommended to gather data from at least 30 isolates collected over the period of a year from each group in order to obtain a reasonable number of isolates upon which to estimate the percent susceptible. At the same time, clinically relevant organisms which are not frequently isolated can be included. However, for optimal surveillance, testing of 75 to 100 isolates is preferred, including members of the *B. fragilis* group and *Clostridium* spp. If possible, *Prevotella* spp., *Fusobacterium* spp., and other frequently isolated pathogens should be considered for inclusion. Examples of cumulative antimicrobial susceptibility reports are included for *B. fragilis* group and for anaerobic organisms other than *B. fragilis* group (Tables 4 and 5). Reported antimicrobial agents should generally be based upon the hospital's formulary, although one agent from each antimicrobial class should be tested even if not on the hospital formulary, in order to detect resistance. Such data could be important as part of the cumulative susceptibility report if and when the hospital drug formulary were changed. It is important to recognize that neither national nor even local data from other institutions within the region are sufficient to predict susceptibility of anaerobes to antimicrobial agents at one's own hospital.

CONCLUSIONS

A variety of susceptibility testing methodologies are available which allow for accurate surveillance of susceptibility trends or testing of individual isolates. As antimicrobial resistance among anaerobes has become a significant problem in recent years, there is an even greater need to perform susceptibility testing and gather data to monitor trends in changing resistance patterns.

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Susceptibility Test Methods: Mycobacteria, *Nocardia,* and Other Actinomycetes

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The World Health Organization (WHO) has described drug-resistant tuberculosis (TB) as "a major public health threat that threatens TB control" (1). Approximately 500,000 cases of multidrug-resistant (MDR) TB occur each year worldwide (2). MDR TB is defined as a case caused by tubercle bacilli that are resistant to isoniazid (INH) and rifampin (RIF), the two most effective drugs in the standard regimen. Although most of the cases of TB and drug-resistant TB occur in developing countries, a significant percentage of these diseases also occur in industrialized countries, where immigrants may make up the majority of new TB cases. Drug-resistant TB is therefore a worldwide problem. Fortunately, at the same time that the threat of drug-resistant TB is emerging, new tools are being developed for more-rapid detection of drug resistance. Among the new tools endorsed by WHO for the diagnosis of TB and detection of drug resistance are liquid culture, Cepheid GeneXpert MTB/RIF, and molecular line probe assays (1, 3). In the United States, a task force was convened to plan to combat and control drug-resistant TB. The report of this task force was published in early 2009 (4). This report emphasizes the need to develop and enhance laboratory capacities to rapidly and accurately detect drug-resistant TB. Along with the advances in new technologies, new drugs are becoming available to help meet the challenges of this changing situation.

Recently, attention has been focused on cases of TB that exhibit resistance not only to INH and RIF but also to quinolones and injectable drugs such as amikacin, kanamycin, or capreomycin. These cases, described as extensively drug-resistant TB (XDR TB), present serious challenges for diagnosis, treatment, and control of the disease (5). Successful treatment of XDR TB is possible with individualized treatment based on results of drug susceptibility testing (DST), including second-line drugs, adverse-event management, and nutritional and psychological support (6). However, most patients with XDR TB succumb to this disease because of delays in diagnosis, inability to test for susceptibility to second-line drugs, coinfection with HIV, unavailability of second-line drugs, or combinations of these factors. Again, enhancing the capacity of laboratories to quickly detect drug resistance and test for susceptibility to second-line drugs is a key component in efforts to combat this ominous new development.

This chapter includes a description of nonradioactive broth culture systems and rapid molecular systems for detection of drug resistance, as well as the standard agar proportion method. The molecular drug susceptibility testing (MDST) methods are revolutionizing the way in which drug-resistant TB is detected, creating a new standard of turnaround time—hours or days—to provide results, rather than the weeks or months previously required. We also include a method, the microscopic observation drug susceptibility (MODS) (7, 8), which does not require frequent importation of proprietary materials and represents an inexpensive technique desirable in resource-limited settings.

As described in earlier chapters, nontuberculous mycobacteria (NTM) and aerobic actinomycetes can also be causes of significant disease in humans. As goals are met for elimination of TB, NTM will constitute an increasing proportion of causative agents of human mycobacterial diseases. DST of many NTM and aerobic actinomycetes may provide useful guidance in the treatment of these infections and is also discussed in this chapter.

ANTIMICROBIAL AGENTS

Although a variety of antimicrobial agents are available for treating mycobacterial diseases, not all agents are suitable for treating all types of infections. Furthermore, in the face of antimicrobial resistance, the choice of alternative therapies can be problematic and clinical experience becomes a prevailing factor. For uncommon mycobacterial infections, the physician is not infrequently faced with a dilemma in choosing a treatment regimen because of a lack of clinical precedence or unclear efficacy. The situation is confounded further by the need to treat mycobacterial infections with a combination of agents to improve efficacy, to prevent resistance, or to overcome intrinsic resistance.

The antimicrobial agents that are used in treating mycobacterial infections are discussed below. The primary antituberculous agents are discussed first, followed by other drugs in alphabetical order. Recommended treatments for TB and the most commonly encountered NTM infections are summarized in Table 1 (9, 10). For TB, first-line drugs are less toxic, able to be given orally, and usually used to treat uncomplicated cases. The Clinical and Laboratory Standards Institute (CLSI) document on DST of mycobacteria (11) currently recommends that first-line testing include

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TABLE 1 Antimicrobial agents recommended for primary treatment of common mycobacterial infections[a]

Mycobacterium species	Site of infection	Antimicrobial agents
M. *tuberculosis* complex[b]	Any	Isoniazid + rifampin + ethambutol + pyrazinamide. Other drugs for treating MDRTB include, but are not limited to, aminoglycosides (amikacin, kanamycin, streptomycin), capreomycin, clofazamine, ethionamide, quinolones (ofloxacin, levofloxacin, moxifloxacin, etc.), cycloserine, linezolid, *p*-aminosalicylic acid, amithiozone, and newly FDA-approved bedaquiline.
M. *avium* complex	Pulmonary	Clarithromycin[c] + ethambutol + a rifamycin (rifampin or rifabutin); amikacin or streptomycin for cavitary and severe disease)
	Disseminated	Clarithromycin[c] + ethambutol ± rifabutin
M. *kansasii*	Pulmonary	Isoniazid + rifampin + ethambutol
M. *marinum*	Skin, soft tissue[d]	Clarithromycin + ethambutol, clarithromycin + rifampin, or rifampin + ethambutol
Rapidly growing mycobacteria[e]	Skin, soft tissue[d]	Clarithromycin (if susceptible) + ≥1 additional drug(s), based on susceptibility test results
M. *abscessus* subsp. *abscessus*	Pulmonary	Multidrug regimen, based on susceptibility results, that includes clarithromycin (if susceptible)[f]

[a] Recommendations for NTM are from reference 9.

[b] Almost all (>95%) M. *bovis* isolates are resistant to pyrazinamide.

[c] Azithromycin is an acceptable alternative agent.

[d] Surgical debridement may be important for successful therapy.

[e] Most common species are M. *fortuitum* group, M. *abscessus*, and M. *chelonae*.

[f] Currently there are no drug regimens of proven efficacy. Antimicrobial therapy may provide symptomatic improvement and disease regression. Surgical resection of limited disease (if possible) and multidrug therapy are optimal.

INH, RIF, ethambutol (EMB), and pyrazinamide (PZA). In the United States, streptomycin (STR) was moved to second-line testing several years ago. Second-line drugs are used when first-line therapy fails or is inappropriate. These agents include injectable drugs such as amikacin (AMK), capreomycin (CAP), kanamycin (KAN), STR, viomycin, quinolones such as ofloxacin (OFX), levofloxacin (LVX), and moxifloxacin (MXF), oral drugs such as ethionamide (ETH), *p*-aminosalicylic acid (PAS), cycloserine (CS), clofazimine, linezolid (LZD), amoxicillin-clavulanate, amithiozones, and bedaquiline (BDQ). These drugs are often accompanied by more-severe side effects, and for some of them, the efficacy for treating TB is not clear.

Drug resistance in *Mycobacterium tuberculosis* complex (MTBC) occurs randomly and at a low frequency and is usually a result of single-step mutations. Two types of drug resistance are seen: primary and acquired. Primary drug resistance occurs in an individual who is infected with a drug-resistant strain before drug treatment is initiated. Acquired resistance can emerge against any of the antituberculous agents during chemotherapy as a result of inadequate treatment.

Genes that are known to be associated with antibiotic resistance in MTBC are summarized in Table 2 (12). Although drug resistance mechanisms in MTBC are not fully understood, five categories are found in mycobacteria. These include decreased uptake of drug such as seen in dormant acid-fast bacilli, drug inactivation by constitutive β-lactamases, increased efflux as seen in fluoroquinolone resistance, alteration of the target as described below with RIF and INH, and reduced prodrug-activating enzymes as exhibited in INH and PZA resistance (12).

Isoniazid

INH, a synthetic antimicrobial agent introduced in 1952 for the treatment of TB, is remarkably specific and potently bactericidal for tubercle bacilli. INH has comparatively low toxicity and is active against virtually all wild-type strains of MTBC. While the mechanism of INH activation is not completely understood, it is likely that the process involves an oxidation reaction catalyzed by a catalase-peroxidase encoded by the *kat*G gene (13). The oxidized form of INH can then covalently bind to the nicotinamide moiety of NAD(H) to form INH-NAD(H) adducts, which in turn compete with NAD(H) for binding to an enoyl-acyl carrier protein reductase encoded by the *inhA* gene (14).

The primary effect of INH is on mycolic acid synthesis, as evidenced by the increased fragility of the mycobacterial cell, increased intracellular viscosity, decreased cellular hydrophobicity, and loss of acid fastness of INH-resistant isolates (15). The mycolic acids are produced by the fatty acid synthesis (FAS) I and II enzyme systems, the latter of which synthesizes the species-specific long-chain mycolic acids. Activated INH targets one of the FAS II enzymes, the enoyl-acyl carrier protein (ACP) reductase of InhA. INH also may interfere with NAD metabolism, energy metabolism, and macromolecular synthesis (16, 17).

The mechanism of action of INH correlates with the known mechanisms of INH resistance. Banerjee et al. (18) reported that INH and ETH resistance in MTBC correlated with a missense mutation in the *inhA* gene. Other studies showed that *kat*G mutations account for 30 to 60% of INH resistance (19) and that mutations in *inhA* promoter conferring a low level of INH resistance may not always be clinically significant (20). Mutations in the *ahp*C promoter, which regulates the expression of an alkyl hydroperoxide reductase (21, 22), are also associated with INH resistance.

INH is active only against replicating tubercle bacilli in the presence of oxygen. Resistance to INH develops rapidly when patients are given monotherapy, and the frequency of INH resistance within a population of tubercle bacilli ranges from 10^{-5} to 10^{-6}. Adverse drug reactions include infrequent, age-related hepatitis and, less frequently, peripheral neuropathy, hypersensitivity reactions such as fever and rash, and arthralgias.

Rifampin

Rifampin (RIF; also known as rifampicin), introduced in 1968, affects intracellular, slowly replicating tubercle bacilli in caseous lesions as well as the actively replicating bacilli in open cavities. RIF is also active against several other slowly growing mycobacteria but is inactive against the

TABLE 2 Genes associated with drug resistance in M. *tuberculosis*

Antimicrobial agent	Mechanism of drug action	Molecular target	Gene product	Mechanism of drug resistance
Amikacin	Inhibits protein synthesis	*rrs*	16S rRNA	16S rRNA gene mutation reduces drug activity.
Bedaquiline	Affects proton pump for the ATP synthase	*atpE*	ATP synthase	ATP synthase mutations affect bedaquiline binding; other mechanisms exist but are not fully understood.
Capreomycin	Inhibits protein synthesis	*rrs*	16S rRNA	16S rRNA gene mutation reduces drug activity.
		tlyA	rRNA methyltransferase	Inactivates *tlyA* gene, causing loss of methylation function.
Ethambutol	Inhibits cell wall arabinogalactan synthesis	*embB*	Arabinosyltransferase	Mutations affect interaction between EMB drug and arabinosyltransferase.
Ethionamide	Inhibits cell wall mycolic acid synthesis	*inhA* promoter	Regulates expression of InhA	*inhA* promoter mutations lead to overexpression of InhA, the drug target, but mutations in *inhA* promoter do not always confer ethionamide resistance.
		ethA	Flavoprotein monooxygenase (EthA)	Mutations in *ethA* prevent activation of ethionamide.
Fluoroquinolones	Inhibits action of DNA gyrase	*gyrA* *gyrB*	DNA gyrase A subunit DNA gyrase B subunit	Mutant gyrases have reduced binding affinity to quinolones.
Isoniazid	Inhibits cell wall mycolic acid synthesis. The activated INH binds to InhA reductase and blocks fatty acid elongation.	*katG*	Catalase-peroxidase	Mutations in *katG* prevent activation of INH.
		inhA	Enoyl-acyl carrier protein reductase	The reductase activity is NADH dependent. Mutations (such as ser94ala) in *inhA* gene cause mutant InhA reductase to have lowered affinity for NADH.
		inhA promoter	Regulates expression of InhA	Mutation in *inhA* promoter leads to increased expression of InhA reductase.
		ahpC promoter	Regulates expression of AhpC	Mutations in *oxyR-ahpC* intergenic region increase AhpC expression, which is associated with a compensatory role responding to loss or reduction of catalase-peroxidase activity or detoxification of the drug action by INH.
		ndh	NADH dehydrogenase	Mutations in *ndh* may lead to defect in NADH oxidation and may interfere with KatG-mediated activation of INH.
Kanamycin	Inhibits protein synthesis	*rrs*	16S rRNA	16S rRNA gene mutation reduces drug activity.
		eis promoter	Regulates expression of an aminoglycoside acetyltransferase specific to kanamycin.	Increases synthesis of the enzyme and overcomes inhibition by kanamycin
Pyrazinamide	Acidifies cytoplasm and deenergizes membrane	*pncA*	Pyrazinamidase	Mutations in *pncA* may lead to loss in pyrazinamidase activity, which is required to convert PZA to pyrazinoic acid, from a prodrug to active form.
Rifampin	Inhibits RNA transcription	*rpoB*	β subunit of RNA polymerase	Mutation in *rpoB* affects drug binding. Note: Mutations in the core *rpoB* confer various level of RIF resistance, and some do not confer in vitro RIF resistance. Some mutations may confer resistance to RIF but not to RFB.
Streptomycin	Inhibits protein synthesis	*rpsL*	Ribosomal protein S12	Mutation alters binding of drug to target.

rapidly growing mycobacteria. It easily diffuses through the mycobacterial cell membrane due to its lipophilic nature. RIF inhibits transcription by binding to the β-subunit of the DNA-dependent RNA polymerase. Drug resistance develops when mutations occur in the *rpoB* gene encoding the β-subunit of the RNA polymerase (23). Studies showed that >96% of RIF resistance could be attributed to mutations within an 81-bp core region of the *rpoB* gene (24). With increasing use of MDST for detection of TB drug resistance, several *rpoB* mutations have been found to alter susceptibility to RIF, but these may not increase the MIC sufficiently to cause the TB bacilli to test as resistant in standardized methods such as the Mycobacterial Growth Indicator Tube (MGIT) system. The clinical significance of these mutations is not clear, but some association with treatment failure has been reported (25).

Adverse drug reactions include hepatotoxicity, gastrointestinal and hypersensitivity reactions, and a red-orange discoloration of urine, tears, other body fluids, and soft contact lenses. RIF also induces increased hepatic metabolism of several other drugs, including methadone and birth control pills. Of particular concern is the interaction of RIF and, to a somewhat lesser degree, rifabutin (RFB) with protease inhibitors, which leads to enhanced hepatic metabolism and may result in subtherapeutic levels of the antiviral agents (26).

Rifabutin and Rifapentine

Rifabutin (RFB; ansamycin), a spiropiperidyl rifamycin, and rifapentine (RFP), a cyclopentyl rifamycin, have potent *in vitro* activity against MTBC. RFB also is very active against the *Mycobacterium avium* complex (MAC) (27). The mode of action and mechanism of resistance of both drugs appear to be identical to those of RIF; however, approximately 30% of RIF-resistant MTBC isolates are susceptible to RFB and RFP. The latter observation correlates with certain specific mutations in the *rpoB* gene (28). Yang et al. analyzed clinical strains of MTBC for cross-resistance to rifamycins and found that alterations at codons 513 and 531 correlate with resistance to RIF and RFB; and point mutations at codons 516 and 529, deletion at codon 518, and insertion at codon 514 influence susceptibility to RIF but not to RFB, while some alterations at codons 515, 521, and 533 did not influence susceptibility to RIF or RFB (29). As with RIF, both RFB and RFP are metabolized to the corresponding biologically active 25-desacetyl metabolite. RFB decreases the incidence of disseminated MAC disease in HIV-infected patients when used as a prophylactic agent, and it is approved for that indication (30). In addition to being more active than RIF on a weight basis, RFB has a long elimination half-life in humans and concentrates in tissues, notably lung tissues, where the levels are 10-fold higher than in serum.

Adverse drug reactions with RFB are similar to those observed with RIF, including the above-mentioned important adverse interactions with antiretroviral agents (26). Some unique RFB toxicities include leukopenia, thrombocytopenia, arthralgias, and uveitis when coadministered with clarithromycin.

Ethambutol

EMB, introduced in 1961, is a synthetic antituberculous compound that is active only against growing bacilli. Its activity against slowly growing NTM is more variable. The primary mechanism of action of EMB is bacteriostatic inhibition of cell wall synthesis, while evidence points to a specific effect on arabinogalactan synthesis (31). In MTBC, the frequency of mutation to EMB resistance is on the order of 10^{-5}, and there is evidence that some EMB resistance correlates with a specific mutation (at codon 306) in the *embB* gene, which encodes an arabinosyltransferase (32, 33). While mutations in the *embB* gene result in high-level resistance in MTBC, this mutation accounts for only 70% of resistant strains; thus, mutations in other genes are likely to play an additional role (12). Mutations at *embB* codon 306 may also affect susceptibility to other drugs used in the treatment of TB (34).

Many MAC isolates have high EMB MIC values, but it has been shown that the MIC value does not correlate with clinical response (35). It appears that EMB affects the permeability of the MAC cell wall and perhaps increases the intracellular concentration of the other, potentially more active drugs (36).

The most important adverse effect associated with EMB is decreased visual acuity due to optic neuritis, which is related to both the dose and the duration of treatment. EMB is not recommended for the treatment of children too young to be monitored for changes in vision, unless no other drug is appropriate because of resistance. The effects on vision are generally reversible when the drug is discontinued.

Pyrazinamide

PZA is a synthetic derivative (pyrazine analog) of nicotinamide and, in combination with INH, is rapidly bactericidal for replicating and nonreplicating forms of *M. tuberculosis*. PZA is inactive against other *Mycobacterium* species, including *M. bovis*, although rare PZA-susceptible *M. bovis* isolates have been described. PZA is active only at an acidic pH; therefore, the pH of the growth medium must be adjusted for accurate measurements of the *in vitro* activity of the drug. It is most likely that PZA is active only in the acidic milieu of the phagolysosome and, depending on the concentration achieved at the site of the infection, may be bacteriostatic or bactericidal. PZA is hydrolyzed in the liver to the active metabolite pyrazinoic acid, and although the mechanism of action of PZA is not fully understood, its activity depends on this conversion. *M. tuberculosis* produces a pyrazinamidase, and most strains of PZA-resistant *M. tuberculosis* lack this enzyme; however, some PZA-resistant isolates retain enzyme activity, suggesting that there are other mechanisms of resistance such as drug efflux (37).

The lack of pyrazinamidase activity and its correlation with PZA resistance have been associated with mutations in the *pncA* gene, which encodes the enzyme (38). Indeed, it now appears that 72 to 97% of PZA resistance can be attributed to mutations in the *pncA* gene (Table 2).

Adverse effects of PZA include hepatotoxicity and rarely photosensitivity and rash. Gout is an important contraindication because of the hyperuricemia associated with PZA therapy. PZA therapy is usually discontinued after the first 2 months of short-course treatment for TB, whereas INH and RIF treatment is continued for an additional 4 months.

Aminoglycosides

The aminoglycosides that are used for the treatment of mycobacterial infections include STR, KAN, and AMK. AMK is a derivative of KAN and the most potent of the aminoglycosides. Gentamicin is inactive against mycobacteria at the usual concentrations attained in serum, and tobramycin is active only against *M. chelonae*.

The primary mechanism of action of the aminoglycosides is inhibition of the post- to pretranslocation step of protein synthesis by blocking the binding of the aminoacyl-tRNA (e-type binding). Cross-resistance among the aminoglycosides often occurs when resistance mutations develop in *rrs* after treatment with amikacin, but resistance to STR or KAN may not cause cross-resistance to amikacin (39). KAN-resistant, AMK-susceptible isolates may have mutations in the *eis* promoter region (Table 2). AMK often is used in combination with one or more other agents for treatment of serious infections caused by rapidly growing mycobacteria or by MAC.

The molecular basis of STR resistance in MTBC (Table 2) results from mutations in the gene that encodes ribosomal protein S12 or from mutations in the 16S rRNA region, which is structurally linked to the S12 protein in the assembled ribosome (40, 41). Finken et al. (40) showed that mutations in the *rpsL* gene, coding for the S12 protein, were present in 20 of 38 STR-resistant strains and that there was a mutation in the *rrs* gene, encoding 16S rRNA, in 9 strains. Nair et al. (41) determined the nucleotide sequence of the *rpsL* gene and showed that STR resistance, in a small number of isolates, appeared to be a result of point mutations at codon 43 of this gene. Adverse drug reactions associated with aminoglycosides include hearing loss, tinnitis, loss of balance, and renal failure.

Amithiozone

Amithiozone (thiacetazone, tibione, or panthrone) is a thiosemicarbazole that is active against MTBC. Resistance develops quickly when monotherapy is given. Adverse drug reactions are gastrointestinal irritation and bone marrow suppression, and hepatotoxicity can occur in patients receiving concomitant INH. Additionally, there appears to be an association of Stevens-Johnson syndrome and severe epidermal necrolysis in HIV-infected patients with TB treated by regimens containing amithiozone (42). Consequently, it is recommended that amithiozone not be used to treat patients known or suspected to be infected with HIV. Amithiozone is not available in the United States and is not used in Europe because of the adverse effects, but it has been used successfully in combination with INH for the treatment of TB in some African countries, where adverse effects are believed to be less severe.

Bedaquiline

The antituberculous potential of diarylquinoline drugs was reported in 2005 (43). One of these drugs, TMC207, or bedaquiline (BDQ), appears to reduce the time to conversion to a negative sputum culture for patients with MDR TB (44). BDQ was approved by the U.S. FDA at the end of 2012 for treatment of tuberculosis (45). Diarylquinolines have a new mechanism of antituberculous action, i.e., inhibition of mycobacterial ATP synthase. Standardized protocols for laboratory testing to detect BDQ resistance have not been developed at the time of the writing of this *Manual*. Resistance has been linked to mutations in the C ring of ATP synthase, including Asp28Gly, Asp28Ala, Leu59Val, Glu61Asp, Ala63Pro, and Ile66Met mutations. These ATP synthase mutations are associated with increases in MIC, from 0.03 μg/ml in wild-type strains to 0.25 to 4 μg/ml in strains with the above-mentioned mutations, using a broth dilution susceptibility testing method (46). However, some resistant strains that do not have ATP synthase mutations have been developed in the laboratory, indicating the presence of other resistance mechanisms (47).

Capreomycin

CAP, a macrocyclic polypeptide antibiotic isolated from *Streptomyces mutabilis* subsp. *capreolus*, is an important injectable drug used for treating drug-resistant TB. The mechanism of action is similar to that of the aminoglycosides: it interferes with translation. CAP is generally considered to be a bacteriostatic agent but has been shown to be bactericidal *in vitro* against nonreplicating TB bacilli (48). Mutations in the 16S rRNA gene (*rrs*) or *tlyA* gene can confer resistance to CAP (49).

Clofazimine

Clofazimine is a substituted iminophenazine, bright red dye. It has weak bactericidal activity against M. *leprae* but is used in combination with RIF and dapsone as a conventional treatment regimen for leprosy. However, it may take up to 50 days of treatment before there is evidence of tissue antimicrobial activity, which may influence the length of time before there is a clinical response in the treatment of leprosy.

The mechanism of action of clofazimine is unknown; however, it is highly lipophilic and binds preferentially to mycobacterial DNA. The half-life is extraordinarily long (estimated to be 70 days), and the drug tends to be deposited in fatty tissues and cells of the reticuloendothelial system. Adverse drug reactions are limited primarily to a pink or red discoloration of the skin, conjunctiva, cornea, and body fluids and gastrointestinal intolerance.

Cycloserine

D-CS is an analog of D-alanine that inhibits the synthesis of D-alanyl-D-alanine, an essential component of the mycobacterial cell wall. CS is active against all mycobacteria as well as several other types of bacteria. Although it is one of the secondary drugs for treatment of TB, *in vitro* susceptibility testing is not recommended due to technical problems with the test (11). There are significant adverse drug reactions associated with CS treatment, notably, peripheral neuropathy and central nervous system dysfunction including seizures and psychotic disturbances.

Dapsone

Dapsone (diaminodiphenyl sulfone) is a synthetic compound that was shown to be active against M. *leprae* in the early 1940s. Dapsone is an antifolate that, like other inhibitors of folic acid synthesis, exerts primarily a bacteriostatic effect and is only weakly bactericidal. Common adverse drug reactions include nausea, vomiting, anorexia, and methemaglobinemia; hematuria, rash, pruritus, and fever also can occur. Traditionally, dapsone is used in combination with RIF and clofazimine for the treatment of leprosy. Acedapsone is a diacetylated form of dapsone with an extraordinarily long half-life of 46 days; as a result, this drug is administered infrequently (e.g., five injections per year), with peak concentrations in tissue occurring 20 to 35 days after administration. Acedapsone is relatively inactive against M. *leprae*, but *in vivo* it is deacetylated to the parent compound.

Ethionamide

ETH is a derivative of isonicotinic acid and, like INH, blocks mycolic acid synthesis. Isolates of MTBC that are resistant to high concentrations of INH are susceptible to ETH, suggesting that the site of action may be different from that of INH. However, mutations in the promoter of the *inhA* gene have been associated with ETH resistance (18). Side effects associated with ETH include gastrointestinal irritation and neurologic symptoms.

Macrolides

Azithromycin and clarithromycin are the most important agents in the treatment of MAC disease and are effective and approved prophylactic agents for preventing disseminated disease in HIV-infected persons (50, 51). These drugs also are useful in the treatment of disease caused by *M. marinum*, *M. haemophilum*, *M. kansasii*, *M. simiae*, and rapidly growing mycobacteria other than *M. fortuitum*. Indeed, they are viewed as potential cornerstones in the treatment of NTM infections (9, 10). Azithromycin, an azalide (a subclass of macrolides), and clarithromycin are structurally similar to erythromycin and have modifications that improve their acid stability and increase their potency, half-life, achievable concentrations in tissue, and bioavailability without causing toxicity. These macrolides are bacteriostatic agents and inhibit the growth of microorganisms by binding to the 50S subunit of the prokaryotic ribosome, blocking protein synthesis at the peptidyltransferase step. Meier et al. (52) showed that both clarithromycin- and azithromycin-resistant mutants of *M. intracellulare* have a single-base mutation at adenine 2058 in the 23S rRNA gene, a site of mutation or methylation that has been associated with macrolide resistance in other bacteria (53). The same genetic basis for macrolide resistance was found in *M. chelonae* and *M. abscessus* (54).

The ability of azithromycin to concentrate in tissues most likely accounts for its therapeutic activity in animal studies and humans (14, 55). Its level in polymorphonuclear neutrophils is nearly 1,000-fold higher than the levels in serum (56). Similarly, the concentrations of clarithromycin in tissue are 4 to 5 times greater than the concentrations in serum, and the concentration in macrophages is 20 to 30 times greater.

p-Aminosalicylic Acid

PAS is an antifolate that is active against MTBC but inactive against most other mycobacteria. There is some evidence that PAS also may affect iron transport in MTBC and salicylic acid metabolism. PAS is incompletely absorbed in the gastrointestinal tract and is associated with significant gastrointestinal side effects.

Quinolones

OFX, LVX, and MFX are fluorinated carboxyquinolones with good *in vitro* activity against both fully susceptible and drug-resistant MTBC and many NTM (57–59). LVX (the L-isomer of OFX) is approximately twice as active against MTBC as ofloxacin *in vitro* and therefore may be the preferred drug for susceptibility testing. MFX is especially useful in the treatment of multidrug-resistant TB (60). The efficacy of fluoroquinolones in the treatment of pulmonary TB may relate, in part, to the observation that these quinolones concentrate in lung tissue to levels at least four times greater than the concentration in serum.

The mechanism of action of all fluorinated quinolones is inhibition of DNA synthesis as a result of binding to the DNA gyrase (bacterial topoisomerase II) (61). Takiff et al. (62) showed that quinolone resistance in MTBC can be ascribed to mutations in the *gyrA* and *gyrB* genes, which encode the DNA gyrase subunits (Table 2). In MTBC, fluoroquinolones function by binding to the bacterial enzyme-DNA complex, with suggested mechanisms being strand breakage, SOS-mediated autolysis, and replication blockage. While there are no reports of cross-resistance between quinolones and other classes of antimycobacterial agents, there is cross-resistance within the quinolone class, such that reduced susceptibility to one quinolone may likely confer reduced efficacy to all quinolones (59). Adverse effects with fluoroquinolones may be less severe than with the other secondary agents (63).

New Antituberculous Drugs

Several new classes of drugs, including nitroimidazole and oxazolidinone drugs, have shown promise for the treatment of pansusceptible as well as drug-resistant TB. These new drugs have unique and new action mechanisms, and there is no cross-resistance between these new drugs and the existing anti-TB drugs. Some show stronger bactericidal effects than currently used drugs, which may result in shorter therapy durations. Furthermore, for treating HIV and TB coinfection, the new drugs seem to have lower interaction with protease inhibitors. These are potential advantages that remain to be proven by clinical trials. At the time of preparation of this chapter, no information was available about susceptibility testing for these drugs.

The nitroimidazole drug PA-824 is in phase II clinical testing and has been shown to be effective in enhancing bactericidal activity when combined with RIF and/or PZA in a murine model of TB (64). The oxazolidinone drug linezolid has been demonstrated to be effective in treatment of TB, including MDR TB (65, 66). However, its cost and the frequent occurrence of side effects including neuropathy and anemia may limit its usefulness. Alternative dosing regimens may prove useful in reduction of treatment durations.

Drugs Used for Susceptibility Testing

Antimicrobial agents for susceptibility testing (reference powders) can be obtained directly from the manufacturer or from commercial sources. In the United States, most antimicrobial agents are also available from U.S. Pharmacopoeial Convention, Inc., Reference Standards Order Department, 12601 Twinbrook Parkway, Rockville, MD 20852. The reference powder should be accompanied by information about its assay potency (in micrograms per milligram), expiration date, lot number, and storage condition, as well as the stability and solubility of the agent. Preparations formulated for therapeutic use in humans or animals should not be used. Unopened vials of powders should be stored as specified by the manufacturer, and opened containers should be stored in a desiccator at the recommended temperature. Stock solutions of most agents at 1,000 μg/ml or greater remain stable for at least 6 months at −20°C and for 1 year at −70 to −80°C. Directions provided by the drug manufacturer should be followed in addition to these general recommendations. Paper disks impregnated with standardized amounts of the primary and secondary anti-TB drugs are available from commercial sources for use in the disk elution modification of the proportion method. Use of these disks obviates errors in weighing and dilution, as well as errors in labeling, because the disks are coded with the drug name and concentration. This technique provides results equivalent to those obtained with solutions prepared from reference powders. Quality control (QC) testing should be performed with each new batch of antimicrobial agent.

DRUG SUSCEPTIBILITY TESTING OF *M. TUBERCULOSIS* COMPLEX

Drug Resistance

In the early 1960s, the WHO organized two meetings that led to the description of reliable criteria and techniques for testing MTBC for resistance to antituberculous drugs (67, 68). The critical proportion (percentage of resistant cells associated with treatment failure) for resistance on Löwenstein-Jensen slants varied according to the drug, e.g., 1% for INH and RIF and 10% for STR, EMB, PZA, ETH, KAN, and CS. However, based on the experience of Russel and Middlebrook with 7H10 agar (69), the Centers for Disease Control and Prevention (CDC) recommended Middlebrook 7H10 agar and 1% as the critical proportion for all drugs (70).

Resistance is fundamentally a phenomenon linked to large initial bacterial populations. In pulmonary TB, the greatest populations are those prevailing in cavities, which can contain 10^7 to 10^9 organisms, whereas the populations found in hard caseous foci, the most common type of lesion, generally do not exceed 10^2 to 10^4 organisms (71). The greater frequency of resistance during treatment of cavitary TB was shown as early as 1949 (72, 73). David at CDC (74) demonstrated the probability distribution of drug-resistant mutants and in a fluctuation test showed that M. *tuberculosis* spontaneously mutated to resistance to INH, STR, EMB, and RIF. The highest proportions of mutants observed for INH at 0.2 μg/ml, STR at 2.0 μg/ml, EMB at 5.0 μg/ml, and RIF at 1.0 μg/ml were 3.5×10^{-6}, 3.8×10^{-6}, 0.5×10^{-4}, and 3.1×10^{-8}, respectively. Thus, the proportion of mutants resistant to INH and RIF would be in the order of 10^{-13}. Implicit in all the studies of the genetic basis of antimicrobial resistance in M. *tuberculosis* is that the MDR TB phenotype is the result of accumulative mutations rather than the acquisition of an MDR transfer factor (24).

Unique Features of TB That Affect Drug Susceptibility Testing

According to the generally accepted theory, resistance appearing during drug treatment is due to selection and multiplication of the resistant mutants preexisting in the tubercle bacillus population of the host. Inasmuch as the susceptible bacilli are the predominant part of the population, initial killing involves a greater number of microorganisms. The consequence is a sharp fall in the population of bacilli during the initial period of treatment. The rise due to multiplication of the resistant mutants occurs later. This "fall-and-rise" phenomenon, as demonstrated in the patient's sputum, was described in the late 1940s (75, 76).

In 1979, Mitchison (77) suggested the "special-populations" hypothesis to explain the action of the major antituberculous drugs against the various subpopulations of tubercle bacilli. The subpopulations include (i) rapidly growing bacilli in the pulmonary lesions; (ii) bacilli that grow in short metabolic spurts and that might be susceptible to RIF but not INH; (iii) bacilli that reside in the acidic environment of the caseous lesions; and (iv) dormant, nonreplicating bacilli. The hypothesis was developed to explain in part the basis of the early bactericidal activity and the later sterilizing activity of antituberculous agents. One of the reasons for the success of the conventional multiple-drug regimen for TB is that the different drugs each make a unique contribution in eradicating tubercle bacilli within each of these special populations. Thus, the use of multiple drugs in the treatment of TB is aimed at both preventing drug resistance and achieving a maximum therapeutic effect. The existence of subpopulations with differing metabolic status also means that conventional pharmacokinetic studies do not predict the effectiveness of treatment regimens for TB (78).

Critical Concentrations

The criteria for defining drug-resistant MTBC were established on an empirical basis, i.e., that there is a certain proportion of drug-resistant mutants above which therapeutic success is less likely to be realized. The procedures used to perform drug susceptibility tests and the criteria for interpreting the results take into account two factors: (i) the critical proportion of drug-resistant mutants and (ii) the critical concentration of the drug in the test medium. On the basis of clinical and bacteriologic studies, the significant proportion of bacilli resistant to an antituberculous drug above which a clinical response is unlikely was generally set at 1%. The critical concentration of a drug is the concentration that inhibits the growth of most cells within the population of a wild-type strain of tubercle bacilli without appreciably affecting the growth of the resistant mutant cells that might be present. In other words, if the proportion of tubercle bacilli that are resistant to the critical concentration of a drug exceeds 1%, it is unlikely that the use of that drug will lead to a therapeutic success. It should be noted that this concentration may not have a direct relationship to the peak level of the drug in serum. The critical concentrations of antituberculous drugs, in different media, are given in Table 3 (11, 79–83).

Low versus High Drug Concentrations

On occasion, the agar proportion method or commercial broth systems may indicate that an isolate of MTBC is resistant to INH or EMB at the critical concentration of the drug (Table 3). When this occurs, reflexively testing the higher concentration is recommended, although there is not uniform consensus regarding the clinical relevance of the results of testing at a higher concentration when two concentrations are used (11).

For INH, low-level resistance may be suggestive of resistance to ETH (18). Additionally, when an isolate is resistant to the low INH concentration but susceptible to the high concentration, therapeutic effect may be achieved with an adjustment in INH dosage. The CLSI recommends that the following comment be appended to the results (11):

> These test results indicate low-level resistance to INH. A specialist in the treatment of drug-resistant tuberculosis should be consulted concerning the appropriate therapeutic regimen and dosages.

Service Referral

While the rate of occurrence of TB is decreasing in the United States and some other industrialized countries, drug-resistant TB is increasing in countries that are sources for immigration. Mycobacteriology laboratories may experience reduced numbers of patient cultures or specimens requiring DST at the same time that new rapid molecular technologies are becoming available. These changing conditions may warrant a reexamination of which laboratories should be performing DST. Laboratories that are unable to maintain expertise or cost-effectiveness due to low volumes of testing and laboratories that are unable to take advantage of new, more rapid technologies should consider referring specimens or cultures to reference laboratories. The time lost due to referring cultures to a reference laboratory may be more than offset by the use of rapid techniques in the reference laboratory.

When To Perform Drug Susceptibility Tests

The CDC and CLSI guidelines recommend that the first isolate of MTBC obtained from each patient be tested for

TABLE 3 Test concentrations[a] of antimycobacterial agents against M. *tuberculosis* complex

Antimicrobial agent[g]	Medium and concn(s) (μg/ml)[b]			
	Liquid systems		Agar proportion	
	MGIT 960	VersaTrek	7H10	7H11
First-line agents				
RIF[c]	1	1	1	1
INH[d]	0.1, 0.4	0.1, 0.4	0.2, 1	0.2, 1
PZA	100	300	NR[e]	NR[e]
EMB[d]	5	5, 8	5, 10	7.5
Second-line agents				
INH-high[d]	0.4	0.4	1	1
EMB-high[d]		8	10	
Amikacin	1		4	
Capreomycin	2.5		10	10
Ethionamide	5		5	10
Kanamycin	2.5		5	6
Levofloxacin	1.5		1	
Ofloxacin	2		2	
p-Aminosalicylic acid			2	8
Rifabutin[c]	0.5		0.5	0.5
Streptomycin[f]	1, 4		2, 10	2, 10

[a] Where more than one concentration for an agent is listed, the lower concentration is the "critical concentration."

[b] The concentrations shown are from reference 11.

[c] About 30% of RIF-resistant isolates are rifabutin susceptible.

[d] INH and EMB may first be tested at the critical concentration. When INH or EMB has tested resistant at the critical concentration, the higher concentration of the drugs may be tested with other second-line agents.

[e] NR, not recommended.

[f] Laboratories may choose to test only the lower concentration.

[g] RIF, rifampin; INH, isoniazid; PZA, pyrazinamide; EMB, ethambutol.

susceptibility to the primary drugs (11, 84). This ensures the most effective treatment for a patient and contributes to the surveillance database for TB control. Susceptibility testing should be repeated if cultures fail to convert to negative after 3 months of therapy or if there is clinical evidence of failure to respond to therapy. If a laboratory is unable to provide this service, then isolates should be referred to another laboratory for timely testing.

Second-line DST should be performed when resistance to RIF or any two first-line drugs is detected. In addition, when an isolate exhibits monoresistance to the critical concentration of INH, it should also be tested for susceptibility to secondary agents if the clinician is planning to include a fluoroquinolone in the treatment regimen (11).

When To Report Drug Resistance

Drug susceptibility results should be reported as soon as possible. When drug resistance is detected and it is verified that the resistance is not due to contamination or mixing with NTM, the attending physician should be notified as soon as possible in addition to sending a written report. If repeat testing is required to confirm the drug resistance, a preliminary report indicating that drug resistance has been detected and retesting is in progress should be issued as soon as possible, and the physician should also be notified. Once the initial resistance has been confirmed, a final report should be issued. When delay in DST is anticipated due to culture impurity, a preliminary report indicating so should be issued.

Methods of Drug Susceptibility Testing of *M. tuberculosis* Complex

The methods generally accepted for determining the antimicrobial susceptibility of MTBC are based on the growth of the microorganisms on solid or in liquid medium containing a specified concentration of a single drug. Four culture-based methods have been described: the agar proportion method, the modified proportion method using commercial broth systems, the absolute-concentration method, and the resistance ratio method (85, 86). Only the agar proportion and the commercial broth systems are discussed further in this chapter; the last two are described elsewhere (86). There are two FDA-cleared, automated, and nonradiometric broth systems: the MGIT 960 system (Becton Dickinson) (87, 88) and the VersaTREK Mycobacteria Detection and Susceptibility Testing (Thermo Scientific). VersaTREK also developed a microtiter plate MIC method. Based on the CLSI guidelines (11), a rapid broth susceptibility testing method should be used in conjunction with rapid methods of primary culture and identification to provide drug susceptibility results within 28 days of receipt of the specimens in the laboratory (84). Before implementing any such rapid broth system, one should validate the system by using a standardized reference method.

Agar Proportion Method

The agar proportion method for DST of MTBC was developed in the early 1960s by G. Canetti (67, 68). The method was subsequently modified and standardized and has been considered the standard method of susceptibility testing of MTBC to all drugs except PZA in the United States and many European countries for years. The agar proportion method is not suitable for testing PZA, because the medium often fails to support growth of TB bacilli when prepared at the required pH of 5.5 (79). The preferred medium for agar proportion is Middlebrook 7H10 agar. Drugs can be either prepared from reference powders (agar diffusion) or added as drug-impregnated discs (disc elution) (79).

Media

In an effort to provide uniformity in the DST of MTBC by the agar proportion method, Middlebrook 7H10 agar supplemented with oleic acid-albumin-dextrose-catalase (OADC) is the recommended standard medium (11, 79). Most clinical isolates of MTBC grow on this medium, and under a dissecting microscope, the transparency of 7H10 agar facilitates the recognition of mixed mycobacterial species or the presence of contaminants. Occasionally, the drug susceptibility test may be invalid due to insufficient growth of drug-resistant strains of MTBC on 7H10 medium. To test those isolates, 7H11 medium may be substituted for 7H10, and higher concentrations of some drugs should be used, as shown in Table 3 (89). QC of the medium, especially the OADC supplement, is critical.

Inoculum and Incubation

The source of the inoculum for a susceptibility test by agar proportion may be growth from a primary culture or a subculture of a solid medium or broth (indirect method) or a specimen that is smear positive for acid-fast bacilli (direct method). The total incubation duration is 3 weeks.

For the indirect method, the source of the inoculum is a pure culture, usually from the primary isolation media. Careful attention should be paid to the selection of colony types so that the final inoculum is representative of all types present to ensure that there is a balance of potentially resistant and susceptible bacilli. A sufficient number of colonies must be selected to make a suspension that is equivalent to a 1.0 McFarland standard. If there is insufficient growth, a subculture into Dubos Tween-albumin or 7H9 broth should be made and incubated at 35 to 37°C until the turbidity matches a 1.0 McFarland standard. The suspension should not contain clumps of organisms. The actual number of CFU per milliliter is likely to vary from suspension to suspension, so that two dilutions (usually 10^{-2} and 10^{-4}) of the suspension must be used to inoculate two sets of media. If there is scant growth, 10^{-1} and 10^{-3} dilutions should be used or the isolate should be subcultured in broth until sufficient growth is obtained. Positive broth cultures from commercial systems may also be used to prepare cell suspensions.

For the direct method, the inoculum is either a digested, decontaminated clinical specimen or an untreated, normally sterile body fluid, in which acid-fast bacilli are seen in stained smears. To ensure adequate but not excessive growth in the direct susceptibility test, specimens are diluted according to the number of organisms observed in the stained smear of the clinical specimen. A typical dilution scheme is shown in Table 4. Theoretically, this type of inoculum is more representative of the population of the tubercle bacilli in a particular lesion in the host. It is prudent to include an undiluted inoculum if the smear-positive specimen is from a patient who is receiving antimicrobial therapy, because a significant proportion of the bacilli seen on the smear may be nonviable. The direct susceptibility test has two major advantages. First, results can be reported within 3 weeks from the time of specimen receipt in the laboratory for a majority of smear-positive specimens. Second, the proportion of resistant bacteria better represents the bacterial population in the patient. Use of the direct method may be warranted when drug-resistant TB is suspected or in a region where the prevalence of drug-resistant TB is high. However, cost can become a critical factor when there is a high incidence of smear-positive specimens containing NTM. Also, it is not possible to accurately calibrate the inoculum, which may result in insufficient or excessive growth on drug-free quadrants and render the test invalid. In addition, if contaminants grow, results are not interpretable. The rate of failure for direct susceptibility testing can reach 15% or more, necessitating retesting by the indirect method.

TABLE 4 Guidelines for selection of the dilution of a specimen concentrate prior to inoculation of 7H10 medium for susceptibility testing using the direct method

Dilutions to test[a]	No. of acid-fast bacilli/field observed with:	
	Carbol fuchsin stain[b]	Fluorochrome stain
Undiluted, 10^{-2}	<1	<5
10^{-1}, 10^{-3}	1–10	5–50
10^{-2}, 10^{-4}	>10	>50

[a] Dilutions of a concentrated specimen are prepared based on the number of bacilli observed in the initial acid-fast smear. Sterile distilled water is used to prepare the dilutions; the carbol fuchsin stain is examined with the oil immersion objective (1,000×), and the fluorochrome stain is examined with the high-dry objective (450×). If the patient is receiving therapy, not all bacilli observed in the smear may be viable; therefore, the undiluted specimen should be tested as well as the appropriate dilution based on the microscopic criteria given in this table.

[b] From reference 79.

Quadrant plates are commonly used for agar proportion, and 0.1 ml (about 3 drops from a Pasteur pipette) of each dilution of the inoculum is placed onto each quadrant, using one dilution per set of plates. After inoculation, the plates are allowed to dry thoroughly in the biosafety cabinet. After the plates are dried, they may be placed in CO_2-permeable polyethylene bags (clear sandwich bags) or sealed individually with CO_2-permeable tapes. They then are incubated at 35 to 37°C in 5 to 10% CO_2 in air. The plates must be protected from light during storage and incubation to prevent the formation of formaldehyde from the medium ingredients. As a good practice, after 1 week of incubation, the drug plates should be checked for the presence of contaminants. If contaminants are present, the susceptibility test should be repeated with a pure isolate.

Reading and Interpreting Results

The criterion for resistance in MTBC is based on the fact that when the proportion of resistant mutants to a drug exceeds 1%, the drug does not have adequate therapeutic efficacy (68). In the proportion method, the percent resistance is obtained by dividing the colony count on the drug-containing quadrant by the colony count on the control quadrant. When the percent resistance is greater than 1%, the isolate is considered resistant to the drug.

For a valid test, the control quadrant of the lower dilution should have at least 50 colonies. If there are fewer than 50 colonies on the control quadrant of the lower dilution (10^{-2} or 10^{-1}) by 3 weeks, this indicates insufficient growth, and the test is invalid and should be repeated. The total incubation period is 3 weeks; however, if mature colonies appear on the control quadrant in less than 3 weeks, resistant results can be reported. Susceptible results should not be reported until week 3. If cultures are incubated beyond 3 weeks, false resistance may result due to degradation of the antimicrobial compound.

Colonial morphology and pigmentation should be carefully examined grossly and microscopically for compatibility with MTBC and the absence of NTM or other organisms. This is especially critical when the susceptibility test is performed by the direct method. Small colonies of rapid growers, as well as the rough, dry colonies of some MAC strains, may appear similar to MTBC colonies on 7H10 agar. Also, rapidly growing mycobacteria may be slow to grow on primary isolation media, and they will appear as MDR TB when tested against primary antituberculous agents. Susceptibility testing results by the direct method must never be reported without a preliminary identification of the organism.

Microcolonies may be observed when using the agar proportion method, especially when testing EMB. Several possibilities may account for the presence of microcolonies: growth of susceptible organisms in the presence of degraded drug, true resistance, or partial resistance. One study reported that most strains that had microcolonies in the EMB quadrant had EMB-susceptible results when tested with the BACTEC 460 method (90). The significance of microcolonies is unknown, and each laboratory should determine how to best report these findings, based on its own in-house experience. With advances in molecular technologies, testing for the presence of drug resistance-associated mutations may help differentiate true resistance from growth of susceptible organisms due to degraded drugs.

Quality Control

A reference strain of *Mycobacterium tuberculosis*, H37Rv (ATCC 27294), which is susceptible to all primary and secondary antituberculous drugs, is recommended for QC. Strains of M. *tuberculosis* that are resistant to INH, RIF, and/or other drugs are available from the American Type Culture Collection; however, these strains are resistant to high concentrations of the respective drugs and are not ideal for QC testing. Ideally, a strain of MTBC that shows resistance at or near the cutoff values of a particular drug should be used for QC. A number of strains used in the CDC's Model Performance Evaluation Program offer these characteristics. Also, for safety considerations, it is not advisable to use a single MTBC strain that is resistant to more than two drugs. Aliquots of suspensions of QC strains of M. *tuberculosis*, adjusted to match a 1.0 McFarland standard in 7H9 broth, can be stored at −70°C for up to 6 months. QC testing of all agar components should be performed with each new batch of medium or antimicrobial agent, and media should be checked for sterility and shown to support adequate growth.

The MGIT 960 System for Drug Susceptibility Testing

In 1995, the MGIT was introduced for the growth and detection of mycobacteria from clinical specimens. Each MGIT tube contains modified Middlebrook 7H9 broth, and in the bottom is a fluorescence-quenching-based oxygen sensor (silicon rubber impregnated with ruthenium pentahydrate). When actively growing organisms utilize the dissolved oxygen, the reduced oxygen concentration enables the generation of fluorescence, which is then measured and expressed as growth units (GU). The MGIT 960 system is a fully automated system that continuously monitors the growth of microorganisms hourly through fluorescence detection. The system uses a nonradiometric medium and does not require the use of needles for addition of growth supplement or inoculation of organisms. In 2002, the system was approved by the FDA for susceptibility testing of MTBC to STR, INH, RIF, and EMB (SIRE) and PZA. Several studies showed that the performance of the MGIT 960 system for testing primary drugs is equivalent to that of the radiometric BACTEC 460 system (no longer commercially available) and the agar proportion method and that the time to results is comparable to that for the BACTEC 460 (87, 91–95).

The MGIT SIRE drug kit containing lyophilized INH, RIF, EMB, and STR and the SIRE supplement is specifically designed for testing susceptibility to these four drugs with the MGIT 960 system. The MGIT 960 employs a two-tier test protocol; SIRE drugs and PZA are tested separately. STR is a secondary agent and not necessary to test. PZA is discussed later. The first-tier drugs and their concentrations are as follows: STR, 1.0 μg/ml; INH, 0.1 μg/ml; RIF, 1.0 μg/ml; and EMB, 5.0 μg/ml. If resistance to INH or STR is detected at the first-tier DST, the laboratory may choose to test INH at 0.4 μg/ml and STR at 4 μg/ml in addition to the initial concentrations. MGIT 960 is not FDA approved for testing second-line agents, but several studies validated the use of MGIT 960 for testing the susceptibility of MTBC, including multidrug-resistant strains, to several secondary agents (81–83, 92, 96).

Inoculum and Incubation

The inoculum for DST may be prepared from either liquid or solid media. To ensure accuracy and reproducibility of results, strict adherence to the guidelines of the manufacturer is imperative. An MGIT DST set contains a growth control tube (without drugs) and drug-containing tubes. The preinoculation preparation includes the addition of 0.8 ml of the SIRE supplement to each MGIT tube in the set and 0.1 ml of a drug solution to the tube designated for that specific drug.

A positive MGIT tube can be used as a source of inoculum. According to the manufacturer's package insert, the MGIT tube, after being detected positive by MGIT 960, needs to incubate for an additional 1 to 2 days, and DST should be set up within 1 to 2 days of incubation. The MGIT tube must be mixed well and used to inoculate the drug-containing MGIT tubes. The inoculum for the growth control is a 1:100 dilution of the inoculum used for the drug-containing MGIT. The volume of the inoculum is 0.5 ml. If DST cannot be set up within 1 to 2 days of incubation after being positive, it can be tested with a 1:5 dilution of the MGIT tube following 3 to 5 days of incubation after being positive. After 5 days, the growth in the MGIT tube is too heavy for DST. A new MGIT tube must be seeded and incubated; the seeded MGIT tube can be tested following the manufacturer's time schedule described above. The proper inoculum for seeding is 0.5 ml of a 1:100 cell suspension equivalent to 0.5 McFarland standard. If the growth of the seeded MGIT tube is detected in <4 days, the growth is too heavy or the culture is not pure.

Growth from a solid medium can also be used as a source of inoculum. The isolate must not be older than 14 days after the first appearance of colonies on the medium. To ensure obtaining diverse populations, growth should be taken from different parts of the medium. The suspension is adjusted to a 0.5 McFarland standard and then diluted 1:5 with sterile normal saline before inoculation into the drug-containing MGIT tubes. The inoculum for the growth control is a 1:100 dilution of the inoculum used for the drug-containing tube.

After inoculation, the MGIT DST set must be registered in the MGIT 960 system with a proper drug panel. It is important to select a panel that matches the DST set being inoculated. The growth in each MGIT tube is automatically monitored hourly by the MGIT 960 system.

Reading and Interpreting Results

When the GU of the growth control reaches 400 within 4 to 13 days, the MGIT 960 system flags the completion of the drug susceptibility test and interprets the DST results based on the following rules. An isolate is called resistant to a drug when the GU of the drug-containing MGIT tube is greater than 100, and it is called susceptible when the

GU of the drug-containing MGIT tube is equal to or less than 100. The test is invalid if the GU of the growth control reaches 400 in less than 4 days, indicating that the growth is too fast (the inoculum is either too heavy or contains contaminants), or in more than 13 days, indicating that the growth is too light.

When the drug susceptibility test is completed, the MGIT 960 system allows the user to print the DST report. It is important to verify the drug order of the MGIT tubes against the drug order on the report, especially when the report contains resistant results. It is pivotal to verify any resistant result by excluding apparent resistance due to the presence of NTM or other bacteria before a preliminary report is issued. The following steps are helpful for verification of resistant results. First, examine the turbidity of the MGIT tube with a resistant result. MTBC usually grows as small clumps and settles at the bottom of the tube, and the broth usually remains clear. Without disturbing the MGIT tubes, if the broth appears turbid, the presence of NTM or other bacteria may be suspected. Second, make a smear from an MGIT tube and perform acid-fast staining to confirm that the cellular morphology is compatible with that of MTBC without presence of NTM or other bacteria. MTBC usually appears as cording or tight clumps. Although antituberculous agents may alter the typical cording morphology, a dispersed distribution of acid-fast bacilli or random loose clumps throughout the entire smear suggests the presence of NTM. When, on rare occasions, differentiating MTBC from NTM is difficult, it is advisable to make a subculture from the MGIT tube showing drug resistance onto 7H10 or 7H11. Examining the microscopic colonial morphology in a few days will help to differentiate MTBC from other mycobacteria.

When any DST result obtained with the MGIT 960 system is questionable, repeat testing of the isolate by agar proportion or with MGIT 960 should be performed. If a culture contains non-acid-fast bacteria or NTM, a pure culture of MTBC must be obtained before retesting is attempted. Because reisolation of MTBC may take several weeks, molecular testing for drug resistance mutations should be considered. Each laboratory should determine its own policy with regard to the necessity of retesting to confirm initial resistant results. As a good practice of quality assurance, when a laboratory first implements the MGIT 960 system or microbiologists are newly trained to use the system, reproducibility of retesting results should be documented. In addition, retesting an isolate to confirm rarely occurring resistant results such as mono-RIF resistance or mono-EMB resistance should be considered.

Quality Control

A reference strain of *M. tuberculosis* such as H37Rv (ATCC 27294) that is susceptible to all primary drugs should be tested with each lot of SIRE drug kit and MGIT medium received. It should also be tested with each test run or once a week if multiple test runs are performed each week (11).

PZA Testing

PZA susceptibility testing by MGIT 960 became commercially available in 2002. The MGIT PZA testing uses the same platform as previously described for the SIRE drugs. Differences are as follows. (i) The broth in the MGIT PZA tube has a reduced pH. (ii) The PZA drug kit contains PZA and PZA supplement, which is not interchangeable with the SIRE supplement. (iii) The PZA growth control is inoculated with a 1:10 dilution of the inoculum to PZA-containing MGIT tubes. The preinoculation preparation includes the addition of 0.8 ml of MGIT PZA supplement to the tubes designated for the growth control and the PZA tube, and 0.1 ml of PZA solution only to the tube designated for PZA. Inoculum preparation from positive MGIT tubes or from a solid medium is the same as that for the SIRE drugs. The interpretation criteria are also the same as those for the SIRE drugs.

The procedure for verification of the instrument-determined resistance for the SIRE drugs applies to PZA resistance as well. Since mono-PZA resistance is rare for *M. tuberculosis*, retesting of PZA to confirm the initial resistance and further identification to the species level (i.e., *M. tuberculosis* versus *M. bovis*) are recommended.

The reproducibility of PZA susceptibility testing appears to be inferior to that obtained with other first-line drugs (97, 98). Inoculum preparation is critical, and it appears that a reduced inoculum of 0.25 ml may reduce false-resistant results compared with the standard inoculum of 0.5 ml (99). A combination of culture-based testing and *pncA* sequencing may provide the most reliable and accurate prediction of PZA susceptibility or resistance (100).

VersaTREK

VersaTREK is a fully automated, continuously monitoring system for growth and detection of mycobacteria, as well as susceptibility of MTBC to all primary drugs. VersaTREK technology is based on detection of pressure changes (oxygen consumption due to microbial growth) within the headspace above the broth medium in a sealed bottle. The medium consists of a Middlebrook 7H9 broth, which has been enriched with growth supplement (glycerol and Casitone), and contains cellulose sponges as a growth support matrix that increases the surface area for exposure to oxygen.

Inoculum and Incubation

The VersaTREK Myco susceptibility kit for testing INH, RIF, and EMB and the VersaTREK Myco PZA kit for testing PZA were FDA cleared for diagnostic use. They contain lyophilized drugs and the diluent. The working drug solutions are prepared by reconstituting the drugs with 25 ml of the diluent. Preparation of the bottles for DST requires the addition of 1.0 ml of growth supplement and 0.5 ml of antibiotic working solution using a syringe. The final concentrations in the Myco bottles are as follows: 0.1 μg/ml of INH, 1.0 μg/ml of RIF, and 5.0 μg/ml of EMB. INH and EMB can be tested at higher concentrations (0.4 and 8.0 μg/ml, respectively) if testing two levels of drug concentrations is desired or indicated. The final concentration for PZA is 300 μg/ml. The inoculum can be prepared from either solid media (e.g., Löwenstein-Jensen or 7H10/7H11) or liquid media (e.g., VersaTREK Myco or 7H9). The turbidity of the cell suspension is standardized to a 1.0 McFarland standard, and a 1:10 dilution of the suspension is used for inoculating the drug-containing VersaTREK Myco bottles and the control bottle. The inoculum is 0.5 ml. The inoculated Myco bottles are kept in the instrument at 35°C, and growth is automatically monitored every 24 minutes.

Reading and Interpreting Results

DST results are manually determined. A drug susceptibility test is valid for interpretation when the time to detection of growth in the control bottle (without drugs) is within 3 to 10 days of inoculation. Drug-containing bottles are monitored for another 3 days after the control bottle has turned positive. An isolate is considered resistant to a drug if the time to detection of growth in the drug-containing bottle is less than or equal to 3 days after that for the control bottle. An isolate is considered susceptible if no growth occurs in the drug-containing bottle or if the time to growth detection is more than 3 days after that for the control bottle. A small number of studies demonstrate good agreement among the VersaTREK Myco system, the BACTEC 460TB, MGIT 960, and the agar proportion method. Detection times are very comparable to those obtained by the BACTEC 460TB and MGIT 960 (101–103).

Quality Control

Each new lot of the VersaTREK Myco Susceptibility kit and VersaTREK Myco PZA kit, Myco bottles, and growth supplement must be tested with strains of MTBC appropriate for QC. A pansusceptible strain of MTBC should be included with each test run (11).

Alternative Drug Susceptibility Testing by Other Approaches

Since 2000, DST of mycobacteria has become a very dynamic field, spawning many new technologies that may prove successful in clinical laboratories. Some of these techniques are based on improved methods for measuring inhibition of growth, while others are based on molecular detection of mutations associated with resistance (104–112).

TREK Sensititre MYCOTB MIC Plate Method

The TREK Sensititre MYCOTB MIC plate method uses a 96-well plate format with drug dilutions prepared in microbroth for testing both first-line and second-line drugs. As of the time of this publication, it has not received FDA clearance for diagnostic use; thus, it is for research use only. Biosafety precautions should be followed strictly when setting up DST using the microtiter plate, as with other procedures handling live MTBC organisms. The Sensititre MYCOTB MIC microtiter plate contains lyophilized antibiotics including AMK (0.12 to 16 µg/ml), CS (2 to 256 µg/ml), EMB (0.5 to 32 µg/ml), ETH (0.3 to 40 µg/ml), INH (0.03 to 4 µg/ml), KAN (0.6 to 8 µg/ml), MFX (0.06 to 8 µg/ml), OFX (0.25 to 32 µg/ml), PAS (0.5 to 64 µg/ml), RFB (0.12 to 16 µg/ml), RIF (0.12 to 16 µg/ml), and STR (0.25 to 32 µg/ml). It tests 12 drugs on a single MIC plate, and results are available within 10 to 21 days after the test setup. The DST setup requires growth from solid media for preparation of inoculum; this requirement may cause a delay for setting up a test. In comparison with the agar proportion method, the performance of this method is considered satisfactory to excellent with the categorical agreement from 94 to 100% in one study and 80 to 99% in another study with EMB, MFX, and cycloserine at the lower end of agreement (113, 152). PZA is not included in the panel due to a requirement for acidic testing conditions; it is necessary to use another method to test PZA. CAP is also not included in the panel; unavailability of testing CAP for MDR TB may be considered a drawback. With drug resistance prevalence at 10%, it is neither necessary nor cost-effective to test second-line drug susceptibility of all isolates, 90% of which are pansusceptible. However, the system has potential advantages. The availability of MIC values may provide an explanation for issues of poor reproducibility with the agar proportion or MGIT methods, which may be associated with strains whose drug MIC values are close to the critical concentrations. Furthermore, emerging resistance may be able to be detected sooner by the increase of MIC values during the treatment course. While the Sensititre system represents a new way of performing DST for MTBC, the objectivity and reproducibility of reading MICs, the variability of agreement with the agar proportion method for various drugs, the cost-effectiveness, and the overall usefulness of the MIC approach on regimen formulation for treating TB patients are issues to be further investigated.

Microscopic Observation Drug Susceptibility

The MODS system is a culture-based DST method. Unlike the broth culture systems mentioned above, the MODS method does not require the use of proprietary culture media purchased from a single source. Middlebrook 7H9 broth medium, supplemented with OADC and a selective antibiotic/antifungal cocktail, is used in 24-well microtiter plates (7). As described by Moore et al., 12 wells are used for each specimen, including 4 control wells with no antituberculous drug and 8 drug-containing wells including low and high concentrations of INH, RIF, EMB, and STR. MODS results are read by examining each well with an inverted microscope. Growth of MTBC is indicated by the presence of cording clumps. The method enables rapid detection of growth and provides a quick indication of susceptibility or resistance to the primary drugs. Because proprietary media are not used and ingredients can be purchased from a variety of suppliers, the method is relatively inexpensive. Safety concerns regarding the use of the 24-well microtiter plate may have been addressed by the practice of enclosing the plates in sealed plastic bags and making the microscopic observations through the bags without opening them. MTBC can be tentatively identified, especially in high-prevalence settings, by the corded or clumped appearance of nonpigmented growth after more than 5 days of incubation. However, the MODS method entails some requirements that may be difficult to achieve in limited-resource settings, including acquiring and storing labile ingredients such as OADC. In addition, it requires the sophisticated facilities and equipment, including a biological safety cabinet, high-speed centrifuge, incubator, inverted microscope, etc., and safety practices of a biosafety level 3 laboratory.

Colorimetric Redox (Reduction/Oxidation) DST Methods

These methods using redox indicators for detection of growth may be performed using liquid or solid media. Use in liquid culture will usually provide the best time to result, comparable to that of the BACTEC MGIT 960 system (or the manual MGIT DST method). A colorimetric redox method may be a good alternative for first- and second-line DSTs in resource-poor settings that do not have the BACTEC MGIT 960 system or do not use the manual MGIT DST method. The media and reagents are nonproprietary, which may decrease costs (114–116).

Molecular Drug Susceptibility Testing Methods

Mutations in genes associated with resistance to antimycobacterial agents (Table 2) can be detected by a variety of methods. Real-time PCR coupled to fluorescence detection by molecular beacons or other probes has been used for detecting INH resistance in MTBC (107, 117). Various molecular methods, including pyrosequencing (118), Sanger sequencing, and next-generation sequencing, have also been developed for detecting resistance to INH, RIF, EMB, PZA, quinolones, and injectable drugs (32, 38, 107, 108, 110, 112, 119–127).

Commercial molecular testing products and well-validated laboratory-developed assays have made their way into many clinical and public health laboratories. Indeed, the use of those rapid molecular methods for detection of INH and RIF resistance has shortened the time to diagnosis of

MDR TB and enables patients with MDR TB to be started on appropriate therapy 40 to 50 days earlier than those who were diagnosed by conventional culture (128, 129). Molecular tests may similarly become widely used for detection of second-line drug resistance and XDR TB. Examples are the MDDR service provided by CDC (http://www.cdc.gov/tb/topic/Laboratory/guide.htm) (120), laboratory-developed pyrosequencing assays (110, 121, 123), and Hain MTBDR*sl* assays (106, 130, 131). Rapid identification of patients with XDR TB has been realized, although follow-up culture is recommended to achieve optimal accuracy and sensitivity. Use of a molecular technique allows XDR TB patients to be detected sooner and enables effective treatment to be initiated in a timely fashion to save lives, including those of patients who are immunocompromised.

Molecular methods can be divided into two major categories, which are probe-based methods and sequence-based methods. Cepheid's GeneXpert MTB/RIF assay using molecular beacon probes, and Hain's MTBDR*plus* and MTBDR*sl* assays using line probes are examples of probe-based methods. Sanger sequencing and pyrosequencing are examples of sequence-based methods. Probe-based methods detect the presence or absence of mutations, while sequence-based methods provide sequences of either wild-type organisms or mutants. Since mutations are not always associated with drug resistance (132), when a mutation is detected by probe-based methods without providing the identity of the mutation, confirmation by a sequence-based method is recommended (153). Revealing the identity of a mutation allows discerning the association of the mutation with drug resistance.

The GeneXpert MTB/RIF assay uses microfluidics with five molecular beacons in a simple-to-operate enclosed system for detection of MTBC and RIF resistance (133). It is FDA cleared for testing smear-positive and smear-negative sputa for diagnostic use. Detection of MTBC is established when there are at least two probes yielding valid positive signals, and detection of RIF resistance is determined when the presence of a mutation (or mutations) in the 81-bp RIF resistance-determining region of the *rpoB* gene is detected. When RIF resistance is detected, it should be confirmed by a sequencing-based method to prevent a false detection.

The Hain probe assays use reverse hybridization technology to detect mutations. These assays have not received FDA clearance for diagnostic use in the United States, but they have been widely used in many countries. Both MTBDR*plus* and MTBDR*sl* assays contain wild-type probes and several frequently encountered mutant probes. When a wild-type band is missing, it indicates the presence of a mutation. If at the same time, a mutant band is present, this informs the user of a specific mutation present in the specimen. Absence of a wild-type band without the presence of a mutant band indicates the presence of a mutation, but the mutation identity is not provided by the assay.

In contrast to probe-based methods, sequence-based methods not only detect the presence or absence of mutations but also provide sequence information. With the availability of sequence information and the knowledge of the association of a mutation with drug resistance or susceptibility, the user would be able to interpret the molecular result properly and prevent reporting false resistance for mutations that do not confer drug resistance. This is particularly important in regions with low prevalence of drug resistance, where the positive predictive value of detecting a mutation conferring resistance is likely to be low.

Furthermore, because the knowledge of mutations that contribute to drug resistance in MTBC is incomplete, molecular methods will likely not detect all drug resistance. For this reason, follow-up culture-based DST should be considered whenever resources permit. With this algorithm, the molecular testing is added to existing methods and increases the cost of TB laboratory work. Molecular detection of drug resistance may therefore be limited to cases in which drug resistance is suspected or a susceptible population has been exposed. It is also worth remembering that rapid detection of patients with drug-resistant disease can lead to improved patient therapy and reduced infectious periods (128). These can lead to cost savings.

NONTUBERCULOUS MYCOBACTERIA

Broth microdilution is the method recommended by the CLSI for susceptibility testing of NTM. The CLSI provides guidelines for testing MAC, *M. kansasii*, *M. marinum*, and the rapidly growing mycobacteria (11). These few mycobacteria, which are discussed in the following sections, were selected because sufficient data on which to base recommendations exist. For other slowly growing, nonfastidious NTM, CLSI suggests using the same method and drugs as those recommended for *M. kansasii*. However, there is little, if any, meaningful information concerning the correlation between susceptibility test results and clinical outcome. Therefore, clinicians who request that susceptibility testing be performed on NTM other than those listed above should be aware of the limitations of such testing and interpret results with caution.

Susceptibility testing of NTM should be performed only on clinically significant isolates, such as those from blood, other sterile body fluids, or tissues. Determining the clinical significance of isolates from the respiratory specimens, however, may be problematic. Because several NTM are found in the environment and may colonize the respiratory tract, their recovery from sputum, for example, does not necessarily indicate clinical disease. The American Thoracic Society (ATS) recommends the following microbiologic criteria as an aid for diagnosing NTM lung disease (9): (i) positive culture results from at least two separate expectorated sputum samples (a minimum of three sputum specimens should be collected for mycobacterial culture), or (ii) positive culture result from at least one bronchial wash or lavage specimen, or (iii) transbronchial or other lung biopsy specimen with histopathologic features consistent with a mycobacterial infection (granulomas or chronic inflammation with stain positive for acid-fast bacilli) and positive culture for NTM. One culture-positive, smear-negative sputum specimen is not likely to be clinically significant.

General recommendations regarding the broth microdilution method and QC that apply to all NTM are discussed in the following paragraphs. Specific details related to MAC, *M. kansasii*, *M. marinum*, and the rapidly growing mycobacteria are discussed in individual sections.

Test Method

The inoculum suspension may be prepared by sweeping the confluent portion of growth on a solid medium with a sterile cotton swab or directly from a broth culture. If colonies are used, growth on the swab is transferred to 4.5 ml of sterile water containing glass beads (e.g., 7 to 10 3-mm beads) until the turbidity matches the turbidity of a 0.5 McFarland standard by visual examination or by using a nephelometer. The suspension is mixed vigorously on a vortex mixer for 15 to 20 seconds and then allowed to sit so any remaining large clumps can settle; the supernatant is then used for the inoculum suspension.

If freeze-dried microtiter plates are used without the broth already added, the final inoculum (with an organism density of approximately 5 × 10^5 CFU/ml) is prepared by transferring 50 μl of the suspension to 10 ml of cation-adjusted Mueller-Hinton broth for rapidly growing mycobacteria or 10 ml of cation-adjusted Mueller-Hinton broth plus 5% OADC for slowly growing NTM. Tubes are inverted 8 to 10 times to mix the suspension thoroughly. Alternatively, if using prepared plates that contain antimicrobials in 100 μl of broth, it is necessary to calculate the volume of standardized suspension to be added to 36 ml of water diluent to obtain a final organism concentration of 1 × 10^5 to 5 × 10^5 CFU/ml (1 × 10^4 to 5 × 10^4 CFU per well in a 0.1-ml volume). The volume depends on the delivery system that is being used.

Final inoculum suspensions are mixed well (by inverting the tube several times or vortexing) and poured into plastic troughs, after which 100 μl is transferred to each well of the microdilution tray. Each inoculated tray is covered with an adhesive seal and incubated in ambient air. Simultaneously inoculating a nutrient agar plate, such as 5% sheep blood or Trypticase soy agar, with a loopful of the final inoculum to check for purity is recommended. Incubation temperature and time are discussed for each species or group in the following sections.

Quality Control

QC testing should be performed on each new batch of test plates and once each week or each time testing is performed, if done less often than weekly (11). Strains recommended for MAC, *M. kansasii*, and the rapidly growing mycobacteria are listed in the sections in which these mycobacteria are discussed. Currently, interlaboratory proficiency testing available for testing NTM is limited. Therefore, laboratories are encouraged to submit isolates to reference laboratories with extensive experience in testing these mycobacteria for confirmatory tests in lieu of a formal proficiency test program.

M. AVIUM COMPLEX

MAC is among the most frequently encountered *Mycobacterium* species in many clinical laboratories. Isolation of MAC from blood is especially important in HIV-infected patients. Although the incidence of disseminated MAC in HIV-infected patients has dramatically decreased due to effective prophylaxis and the immune system restoration associated with highly active antiretroviral therapy, it still occurs in patients who develop resistance to antiretroviral treatment or who exhibit drug intolerance or poor compliance. In many of these patients, recovery of MAC represents a relapse of MAC disease rather than a new infection. MAC is also an important cause of chronic pulmonary lung disease (9, 10) and in the United States is the leading cause of lung disease due to an NTM (134). Patients with MAC lung disease generally may be placed in one of the following groups: those who have underlying lung disease with apical fibrocavitary lesions, postmenopausal women with bronchiectasis and nodular opacities, or patients with cystic fibrosis.

Antimicrobial Agents

Correlation between *in vitro* MAC susceptibility test results and clinical response has been demonstrated in a controlled clinical trial only with the macrolides clarithromycin and azithromycin (135). Because the mechanism of acquiring mutational resistance in isolates of MAC is the same for clarithromycin and azithromycin and because there are technical difficulties associated with testing azithromycin, current CLSI guidelines (document M24-A2) state that clarithromycin is the only primary drug to which susceptibility of MAC isolates should be evaluated (11). However, recent data have shown that AMK, either intravenous or inhaled (for pulmonary disease), also is useful clinically, supporting initial testing of AMK as well as clarithromycin (136).

Wild-type (untreated) MAC isolates typically have clarithromycin MICs of ≤8 μg/ml and are considered susceptible to macrolides. MAC isolates from patients who have relapsed after treatment, on the other hand, have clarithromycin MIC values of ≥32 μg/ml and no longer respond clinically to macrolide therapy. Virtually all such isolates have a mutation in the adenine at position 2058 or 2059 of the 23S rRNA gene, the presumed macrolide binding site on the ribosomal unit (52, 53).

Clarithromycin and azithromycin are approved by the FDA for the treatment of MAC disease, but because resistance develops quickly with macrolide monotherapy, single-drug therapy with either agent is contraindicated. A common regimen for MAC disease is shown in Table 1 (9). Very importantly, although EMB, RIF, RFB, and STR are useful clinically and broth dilution susceptibility testing of MAC to these agents may be performed, the results may not reliably predict clinical outcome. Because of the lack of data establishing a correlation between MIC values and clinical response, breakpoints separating susceptible from resistant strains have not been determined. Therefore, it is strongly recommended that susceptibility results for the above antituberculosis drugs not be reported for MAC. AMK, either intravenous or inhaled (for pulmonary disease), also is useful clinically, and Brown-Elliott and colleagues have suggested AMK breakpoints (shown in Table 5), based on data from 462 isolates (136). Most MAC isolates are intrinsically resistant to INH and PZA; these antimicrobial agents play no role in the treatment of MAC infection and should not be tested.

Treatment of macrolide-resistant MAC infections is problematic, and the role of susceptibility testing in guiding therapy for these patients is not clearly defined. Although data are limited, some experts believe that moxifloxacin and linezolid, which CLSI lists as secondary agents, may be clinically useful in select patients (9).

Indications for Susceptibility Testing

The ATS recommends that clarithromycin susceptibility testing be performed on all new, previously untreated clini-

TABLE 5 Antimycobacterial agents, interpretative criteria, and quality control recommendations for testing *M. avium* complex by broth microdilution

Antimycobacterial agent	MIC (μg/ml) for category[a]			QC range of MICs (μg/ml) for *M. avium* ATCC 700898
	S	I	R	
Primary				
Clarithromycin[b]	≤8	16	≥32	0.5–2
Amikacin[c]	≤16	32	≥64	4–16
Secondary				
Moxifloxacin	≤1	2	≥4	0.25–2
Linezolid	≤16	32	≥64	4–16

[a] S, susceptible; I, intermediate; R, resistant.

[b] Clarithromycin is the class drug for macrolides and is the only drug that need be tested.

[c] Amikacin data are from reference 136.

cally significant MAC isolates and on MAC isolates from patients who relapse while on macrolide therapy or prophylaxis (9). Susceptibility testing should be repeated after 3 months for patients with disseminated disease and after 6 months for patients with chronic pulmonary disease if the patient does not improve clinically and remains culture positive.

Test Methods

As mentioned above, broth microdilution is recommended for susceptibility testing of all NTM. Agar-based methods should not be used. The CLSI currently recommends cation-adjusted Mueller-Hinton broth with 5% OADC or OAD (pH 7.2 to 7.4) for broth microdilution testing, although some isolates grow poorly in this medium.

As previously mentioned, when broth microdilution is performed, the inoculum may be prepared directly from an agar plate or a broth culture and should be approximately 5×10^5 CFU/ml. Although it often is not practical, only transparent colony types should be tested, if possible, because this variant generally is more virulent and more resistant to antimicrobial agents than the opaque variant. Tween 80 or other surfactants should not be used to disperse clumps of bacilli because of the potential synergistic effect of the surfactant activity of Tween 80 and antimicrobial agents.

Incubation is at 35 to 37°C in ambient air. Trays are first examined at 7 days. If growth is insufficient, trays are reincubated and read again at days 10 to 14 of incubation. The endpoint for broth microdilution assay is visible turbidity.

Reporting Results

Both the MIC value and an interpretation should be reported (Table 5) (11). Because untreated wild-type strains of MAC rarely, if ever, are intermediate or resistant to clarithromycin, the CLSI subcommittee recommends that laboratories confirm such results by repeat testing (11). A confirmed intermediate result may indicate emerging resistance; therefore, patients with such an isolate should be carefully monitored, and susceptibility testing should be performed on subsequent MAC isolates. Based on data from a recent study, Brown-Elliott et al. (136) recommend testing MAC isolates against AMK in addition to clarithromycin; suggested breakpoints are shown in Table 5. Reporting secondary agents (i.e., moxifloxacin and linezolid) should be restricted to situations in which the clinician has extensive experience in the use of these drugs in the treatment of MAC disease or for research purposes.

Quality Control

M. *avium* ATCC 700898 is recommended for QC when testing clarithromycin and AMK. The range of acceptable results for clarithromycin is 0.5 to 2 µg/ml at pH 7.3 to 7.4 and 4 to 16 µg/ml for AMK (136). M. *marinum* ATCC 927 is an acceptable alternative QC organism for clarithromycin; the acceptable range is 0.25 to 1 µg/ml. When secondary drugs are tested, acceptable ranges for moxifloxacin and linezolid for M. *avium* ATCC 700898 are 0.25 to 2.0 µg/ml and 4 to 16 µg/ml, respectively. When testing M. *marinum* ATCC 927, acceptable ranges are 1 to 4 µg/ml for both drugs. Another option for QC of moxifloxacin or AMK is *Pseudomonas aeruginosa* ATCC 27853 (acceptable ranges can be found in the most recent CLSI M100 document [137]).

M. KANSASII

M. *kansasii* is the second most common cause of pulmonary disease due to NTM in the United States (9), second only to MAC. The therapeutic regimen currently recommended by the ATS for treatment of M. *kansasii* pulmonary disease consists of RIF, INH, and EMB (9), of which RIF is the component critical for treatment success. An alternative regimen that also is effective includes RIF, EMB, and clarithromycin (138). In patients with HIV infection receiving protease inhibitors, RFB is used in place of RIF (9). Isolates of M. *kansasii* from previously untreated patients are predictably susceptible to RIF. However, resistance to RIF can develop during therapy, and a patient's history of RIF therapy may be unknown or unclear. Therefore, CLSI recommends that all initial isolates of M. *kansasii* be tested for susceptibility to RIF only or, if short-course or intermittent therapy with RIF, EMB, and clarithromycin is planned, to both RIF and clarithromycin (11). Susceptibility testing should be repeated if cultures remain positive after 3 months of appropriate therapy.

Untreated wild-type strains of M. *kansasii* are susceptible to the critical concentrations of RIF and EMB used to test M. *tuberculosis* (discussed earlier in the chapter) but are resistant to the critical concentration of INH (0.2 µg/ml by agar proportion) and show variable susceptibility to the higher concentration (1.0 µg/ml). Although INH is used empirically, there currently are no laboratory or clinical data to support its use or nonuse. Therefore, routine susceptibility testing of M. *kansasii* to INH is not recommended and should be discouraged.

Susceptibility testing is done by broth microdilution using cation-adjusted Mueller-Hinton broth with 5% OADC or OAD and incubating at 35 to 37°C for 7 to 14 days in CO_2 or ambient air, although CO_2 should be avoided when testing macrolides. Acceptable QC strains and expected MIC results for RIF are as follows: M. *kansasii* ATCC 12478, ≤1 µg/ml; M. *marinum* ATCC 927, ≤0.25 to 1 µg/ml; and *Enterococcus fMPaecalis* ATCC 29212, 0.5 to 4 µg/ml.

Isolates of M. *kansasii* that are resistant to RIF (MIC ≥1 µg/ml) should be tested for susceptibility to the following drugs: RFB, EMB, STR, INH (only if a provider insists), clarithromycin (unless initially tested as a primary drug), AMK, ciprofloxacin (as the class representative for the older fluoroquinolones, which are less active *in vitro* than moxifloxacin), trimethoprim-sulfamethoxazole, linezolid, and moxifloxacin. MIC values for resistance to these agents are shown in Table 6 (11). Although INH and STR may be useful clinically, breakpoints to establish susceptibility and resistance for NTM have not been established; therefore, only the MIC (with no interpretation) should be reported for these drugs.

M. MARINUM

M. *marinum* causes chronic granulomatous lesions of the skin and soft tissues (called "swimming pool granuloma" or "fish tank granuloma") and occasionally bone. Routine susceptibility testing of M. *marinum* is not recommended and should be discouraged. Isolates are consistently susceptible to several clinically useful antimicrobial agents, including RIF, EMB, doxycycline (or minocycline), trimethoprim-sulfamethoxazole, and clarithromycin (11). Additionally, the risk of acquired mutational resistance to one or more of these agents is extremely low. Successful treatment may require surgical excision or debridement as well as antimicrobial

TABLE 6 Secondary antimycobacterial agents and MIC values indicating resistance for testing *M. kansasii* and other slowly growing nontuberculous mycobacteria (excluding *M. avium* complex)[a]

Antimycobacterial agent	MIC indicating resistance (μg/ml)
Amikacin	>32
Ciprofloxacin[b]	>2
Clarithromycin[c]	>16
Ethambutol hydrochloride	>4
Isoniazid	—[d]
Linezolid	>16
Moxifloxacin	>2
Rifabutin	>2
Streptomycin	—[e]
Trimethoprim-sulfamethoxazole	>2/38

[a] Table and footnotes are adapted from reference 11.

[b] Ciprofloxacin and levofloxacin are interchangeable, but both are less active *in vitro* than moxifloxacin.

[c] Clarithromycin is considered a primary drug in patients with *M. kansasii* infection receiving the short-course and/or intermittent therapeutic regimens consisting of rifampin, ethambutol, and clarithromycin. For patients with *M. kansasii* infection receiving the classic regimen of rifampin, ethambutol, and isoniazid, clarithromycin is a secondary agent. Clarithromycin is the class representative for the "newer" macrolides (clarithromycin, azithromycin, roxithromycin).

[d] Breakpoints to establish susceptibility and resistance for NTM have not been established. Testing susceptibility of *M. kansasii* to isoniazid should be discouraged because *in vitro* results do not correlate with clinical outcome. If testing is performed at the provider's insistence, report the MIC value only, with no interpretation.

[e] Breakpoints to establish susceptibility and resistance for NTM have not been established. Report the MIC value only, with no interpretation.

therapy. However, if a patient fails to respond clinically after several months of appropriate therapy and remains culture positive, susceptibility testing of *M. marinum* should be considered. As for MAC and *M. kansasii*, broth microdilution using cation-adjusted Mueller-Hinton broth supplemented with OADC or OAD is recommended. Incubation is at 28 to 30°C for 7 days. The drugs suggested by CLSI to test and the MIC values (in μg/ml) indicating resistance are as follows: RIF (>2), clarithromycin (>16, the class agent for newer macrolides), AMK (>32), doxycycline or minocycline (>4), ciprofloxacin (>2), moxifloxacin (>2), and trimethoprim-sulfamethoxazole (>2/38) (11).

OTHER SLOWLY GROWING NTM

Many other slowly growing NTM may cause human disease, and susceptibility testing of these species may be requested. Although data are limited, CLSI suggests broth dilution using cation-adjusted Mueller-Hinton broth with OADC for nonfastidious species (11). The same primary and secondary agents listed for *M. kansasii* should be tested and reported with the same interpretive criteria (31). In general, testing *M. gordonae* is inappropriate because isolates almost always represent contaminants and only rarely are the cause of actual disease. For fastidious species, such as *Mycobacterium haemophilum*, susceptibility testing has been performed (139); however, there is insufficient information to recommend a standard method of testing.

RAPIDLY GROWING MYCOBACTERIA

Over 30 species of rapidly growing mycobacteria have been identified, but most human disease is due to *M. abscessus* subsp. *abscessus*, *M. chelonae*, and *M. fortuitum* group, which are important causes of skin and soft tissue infections, especially following penetrating trauma with possible soil or water contamination (140). CLSI recommendations for susceptibility testing of rapidly growing mycobacteria are based predominantly on results of studies involving these three species/groups, but they apply to other clinically significant rapid growers as well. For some drugs (e.g., clarithromycin, imipenem, and tobramycin), susceptibility test results apply to certain species or groups of rapidly growing mycobacteria; therefore, identification of isolates to the species level is important. If cultures (from any site except respiratory) are positive after 6 months of appropriate antimicrobial therapy, susceptibility testing should be repeated, and the species identity should be confirmed.

Test Method

As for all NTM, broth microdilution is recommended for susceptibility testing of the rapidly growing mycobacteria (11, 141). Antimicrobial agents that should be tested are AMK, cefoxitin (up to 256 μg/ml), ciprofloxacin, clarithromycin, doxycycline (or minocycline), imipenem, linezolid, moxifloxacin, trimethoprim-sulfamethoxazole, and tobramycin. Tobramycin is used predominantly for treatment of *M. chelonae* infections and should not be used to treat infections due to other rapid growers, because MIC values generally are in the resistant range.

The inoculum is prepared as described above, and trays are incubated at 28 to 30°C in air and examined at 72 h. If growth in the growth control well is sufficient (at least 2+, or definite turbidity and "clumpy" growth [11]) at that time, the MIC can be recorded. If not, trays are reincubated and read again on days 4 and, if needed, 5. Day 5 is the final reading for all drugs except clarithromycin, which should be read at 7 to 10 days and, if the isolate is susceptible at that reading, again at 14 days (11). The final day 14 reading is recommended to ensure detection of inducible macrolide resistance; however, if clarithromycin resistance (MIC, ≥8 μg/ml) is recognized at an earlier reading, the result can be finalized at that time.

The MIC is the lowest concentration of antimicrobial agent that completely inhibits visible growth, with one exception. As with other types of bacteria, "trailing" is common when trimethoprim-sulfamethoxazole is tested, and the MIC values for this agent should be read at approximately 80% inhibition of growth. Trailing also is occasionally seen with isolates of *M. fortuitum* group and clarithromycin; when this occurs, the endpoint is read at the end of the trailing (i.e., complete inhibition).

Reporting Results

MIC values and an interpretation, based on the breakpoints for the rapidly growing mycobacteria listed in Table 7 (11), are reported for each drug. A few species/groups of rapidly growing mycobacteria are almost always susceptible to certain drugs. This is true for *M. abscessus* and AMK; for *M. fortuitum* group, *M. smegmatis* group, and *M. mucogenicum* and imipenem; and for *M. chelonae* and tobramycin. Therefore, if the MIC result for one of these isolate-drug combinations indicates resistance, susceptibility testing should be repeated, and the identification should be confirmed. If the repeat result indicates resistance, the isolate should be sent to a qualified reference laboratory for confirmation of the results if the drug in question is being considered for therapy.

Quality Control

The QC strain recommended for monitoring test performance and for verifying the concentration of antimicrobial

TABLE 7 Broth microdilution interpretive criteria for rapidly growing mycobacteria[a]

Antimicrobial agent	MIC (μg/ml) for category		
	Susceptible	Intermediate	Resistant
Amikacin[b]	≤16	32	≥64
Cefoxitin	≤16	32–64	≥128
Ciprofloxacin[c]	≤1	2	≥4
Clarithromycin[d]	≤2	4	≥8
Doxycycline/minocycline	≤1	2–4	≥8
Imipenem[e]	≤4	8–16	≥32
Linezolid	≤8	16	≥32
Moxifloxacin	≤1	2	≥4
Trimethoprim-sulfamethoxazole	≤2/38		≥4/76
Tobramycin[f]	≤2	4	≥8

[a] Table and footnotes are adapted from reference 11.

[b] Isolates of *M. abscessus* with an MIC of ≥64 μg/ml should be retested. If the repeat result is ≥64 μg/ml, the MIC should be reported with the following comment: "The MIC is greater than expected for this species; if the drug is being considered for therapy, the laboratory should be notified so the isolate can be sent to a reference laboratory for confirmation of resistance."

[c] Ciprofloxacin and levofloxacin are interchangeable. Both are less active than the newer 8-methoxyfluoroquinolones.

[d] The final reading for nonpigmented rapidly growing mycobacteria should be at 14 days to ensure detection of inducible macrolide resistance, unless the isolate is resistant at an earlier reading. Clarithromycin is the class representative for newer macrolides (i.e., azithromycin and roxithromycin).

[e] If the MIC is >8 μg/ml for *M. fortuitum* group, *M. smegmatis* group, and *M. mucogenicum* group, the test should be repeated with an incubation period of no more than 3 days. If the repeat result is >8 μg/ml, the MIC should be reported with the following comment: "The MIC is greater than expected for this species; if the drug is being considered for therapy, the laboratory should be notified so the isolates can be sent to a reference laboratory for confirmation of resistance. Imipenem results do not predict results for meropenem or ertapenem. Activity against rapidly growing mycobacteria is greater for imipenem than for meropenem or ertapenem."

[f] Tobramycin is used predominantly for treatment of *M. chelonae* infections. If the MIC to tobramycin is >4 μg/ml for an isolate of *M. chelonae*, the test should be repeated. If the repeat result is >4 μg/ml, the MIC should be reported with the following comment: "The MIC is greater than expected for this species; if the drug is being considered for therapy, the laboratory should be notified so the isolate can be sent to a reference laboratory for confirmation of resistance."

agents is *M. peregrinum* ATCC 700686. *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 17853, and/or *E. faecalis* ATCC 29212 are acceptable alternatives. Acceptable QC ranges of MIC values are found in CLSI document M24-A2 (11).

NOCARDIA AND OTHER AEROBIC ACTINOMYCETES

Clinical Significance

Nocardia spp. and other aerobic actinomycetes (*Actinomadura*, *Rhodococcus equi*, *Gordonia*, *Tsukamurella*, and rarely *Streptomyces* spp.) can cause serious disease in immunocompromised and occasionally even healthy hosts (142–149). *In vitro* susceptibility testing should be performed on all clinically significant isolates to serve as a guide for therapy and to monitor for resistance.

Testing Method

The recommended method for testing *Nocardia* and other aerobic actinomycetes is broth microdilution (11), although breakpoints for interpretation of MIC values have been established only for *Nocardia* species. Additionally, based on data from a recent multisite reproducibility study, use of a disk diffusion test for sulfisoxazole (250-μg disk) should be considered when testing *Nocardia* species as a check of the inoculum adequacy and to confirm sulfonamide MIC results (150). Because *Rhodococcus equi* grows within 24 h in most susceptibility panels for Gram-positive bacteria, it can be tested following the guidelines for staphylococci described in the most recent edition of CLSI documents M07 (151) and M100 (137).

The primary and secondary antimicrobial agents recommended for susceptibility testing are listed in Table 8. For *R. equi* only, there are two additional primary drugs: vancomycin and rifampin. Although broth dilution is preferred, for a few species-drug combinations, the accuracy of the results may be questionable. For example, ceftriaxone results may be falsely resistant when testing *Nocardia brasiliensis*, and when testing *Nocardia farcinica*, imipenem results may be falsely resistant. In addition, there appears to be a problem with reproducibility of broth microdilution testing of ceftriaxone with *Nocardia cyriacigeorgica* and *Nocardia wallacei* and sulfonamides with *N. farcinica* and *N. wallacei* (150). Studies to further investigate these potential problems are needed.

Inoculum preparation is described in detail in CLSI document M24 (11). First, a heavy suspension is prepared in

TABLE 8 Broth microdilution breakpoints for *Nocardia*[a]

Antimicrobial agent	MIC (μg/ml) for:		
	Susceptible strains	Intermediate strains	Resistant strains
Primary			
Amikacin	≤8		≥16
Amoxicillin-clavulanic acid	≤8/4	16/8	≥32/16
Ceftriaxone	≤8	16–32	≥64
Ciprofloxacin[b]	≤1	2	≥4
Clarithromycin[c]	≤2	4	≥8
Imipenem	≤4	8	≥16
Linezolid	≤8		
Minocycline	≤1	2–4	≥8
Moxifloxacin	≤1	2	≥4
Trimethoprim-sulfamethoxazole	≤2/38		≥4/76
Tobramycin	≤4	8	≥16
Secondary			
Cefepime	≤8	16	≥32
Cefotaxime	≤8	16–32	≥64
Doxycycline	≤1	2–4	≥8

[a] Table and footnotes are adapted from reference 11.

[b] Ciprofloxacin and levofloxacin are interchangeable. Both are less active than the newer 8-methoxyfluoroquinolones.

[c] Class representative for newer macrolides.

cation-supplemented Mueller-Hinton broth or sterile, deionized water or saline, using colonies on a blood or Trypticase soy agar plate that was incubated at 35 ± 2°C in air until growth is sufficient (usually 3 to 7 days). Large clumps of organisms are broken up by using a micropestle or glass beads and mixing vigorously on a vortex mixer. The suspension is allowed to sit for approximately 15 minutes so that the clumps settle. Several drops of the supernatant are added to 2 ml of water or saline in a tube compatible with a nephelometer available in the laboratory. The turbidity of the suspension is adjusted to equal the density of a 0.5 McFarland standard.

Panels and Mueller-Hinton agar plates are inoculated with the appropriately prepared suspension. Panels are covered with an adhesive seal, placed in a plastic bag, and incubated at 35 ± 2°C in ambient air for 72 h. If growth in the growth control well is sufficient at that time, MIC values are recorded; if not, panels are reincubated and read daily until growth is adequate, for up to a total of 5 days. To assess the adequacy of the inoculum, the quantity of organism growth on the sulfisoxazole disk diffusion test should be evaluated after incubation in ambient air at 35 ± 2°C for 3 to 5 days. Growth should appear as obvious streak marks with clear areas between the streaks; it should not be confluent.

Reporting of Results

For broth microdilution, both an MIC value and an interpretation (as listed in Table 8) should be reported for *Nocardia* species. Sulfisoxazole disk diffusion is interpreted as follows: a zone of inhibition of ≥35 mm indicates susceptibility to sulfonamides, and a zone of ≤15 mm indicates resistance (150). A zone size of 16 to 34 mm is uninterpretable because currently there are insufficient data to determine intermediate susceptibility. If the results of broth dilution and disk diffusion testing are discrepant, testing should be repeated and/or sent to a reference laboratory. Because most isolates of *Nocardia* are susceptible to sulfonamides, a result of resistant should be confirmed before it is reported. For all other aerobic actinomycetes, including *R. equi*, an MIC value only should be reported, because breakpoints for these drugs have not been determined for genera other than *Nocardia*.

Quality Control

Recommended reference strains for QC are *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, and for amoxicillin-clavulanic acid only, *Escherichia coli* ATCC 35218. Acceptable ranges for these strains are found in the current edition of CLSI document M100 (137). In addition to these organisms, Conville and colleagues recommend including *Nocardia nova* ATCC BAA-2227 in the QC battery to show the attributes of *Nocardia* strains grown in the presence of antimicrobial agents (150).

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Molecular Detection of Antibacterial Drug Resistance

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The detection of antibiotic resistance is one of the most important responsibilities of the clinical microbiology laboratory. This is traditionally achieved by phenotypic testing (chapters 71 to 73). However, a limitation of the phenotypic approach is that delays in appropriate therapy can adversely affect clinical outcomes (1–4). As most clinically important antibiotic resistance has a genetic basis, molecular methods to directly detect resistance are theoretically attractive because they are rapid and automated and do not require bacterial growth. However, the molecular detection of resistance determinants also poses significant challenges, perhaps foremost of which is the genetic complexity of antibiotic resistance. Resistance can arise due to the acquisition of resistance determinants (e.g., β-lactamase genes) but can also reflect changes in gene expression (e.g., efflux pumps, porins) or the acquisition of mutations (e.g., fluoroquinolone target mutations). The detection of a resistance gene may not necessarily imply phenotypic resistance if the gene is expressed at low levels or is nonfunctional. On the other hand, methods based on genotype may actually be superior to those based on phenotype in some instances, such as when heteroresistance is present or if a resistance phenotype is only weakly expressed under *in vitro* testing conditions (e.g., some methicillin-resistant *Staphylococcus aureus* [MRSA], *Klebsiella pneumoniae* carbapenemases [KPCs], or New Delhi metallo-β-lactamase-1 [NDM-1]). An ever-expanding diversity of resistance determinants is likely to result in continuing problems of sensitivity for molecular assays. The presence of PCR inhibitors, inefficient extraction of nucleic acids, and other technical issues may also interfere with detection, so it is essential for assays to include internal controls. Another limiting factor for most clinical laboratories potentially interested in using molecular assays to detect antibiotic resistance is the considerable cost. Therefore, at present, molecular methods to detect antibiotic resistance are limited to a few common and well-defined resistance mechanisms. A number of investigational approaches appear promising, and the application of molecular diagnostic tools to detect antibiotic resistance is likely to become more widespread in the future. This chapter provides an overview of current FDA-cleared molecular assays for the detection of antibiotic resistance. In addition, some commercial assays in development are briefly discussed. Analyte-specific reagents and "home-brew" assays continue to play an important research role but are impractical for the typical clinical laboratory; the interested reader is referred to other sources (5, 6).

TECHNOLOGY

Although the use of molecular assays in the clinical microbiology laboratory is not new, recent technological advances have made it possible for laboratories to implement user-friendly instrumentation that requires little to no training in advanced molecular techniques. Testing can be performed directly on clinical specimens (primarily for surveillance purposes) or on cultured isolates (e.g., positive blood cultures). Reference strains for assay validation may be readily obtained from sources such as the American Type Culture Collection (7). Current FDA-cleared molecular technologies for the detection of resistant determinants are based on two methods: PCR and solid-phase microarrays. When comparing these methods for molecular resistance detection, a key distinction should be recognized. Nucleic acid amplification in real-time PCR may allow an assay to be performed directly from a specimen but limits the number of fluorophores that can be used for simultaneous target detection. One solution is to perform independent target amplification and detection steps. In the FilmArray (BioFire) system, the initial targeted multiplex PCR is followed by distribution of the products into individual wells for a second round of PCR and detection. In contrast to PCR-based technologies, solid-phase or liquid arrays can simultaneously detect hundreds of targets, but they require a high target copy number, limiting their ability to be used for the direct analysis of clinical specimens in which low numbers of organisms are present. Unlike PCR, there are only a few instances in which direct-from-specimen microarray technologies have been applied to detect antimicrobial resistance in the clinical laboratory (8). However, combined amplification and array technologies are routinely found in research settings and are likely to become increasingly employed in clinical settings. One example is the FDA-cleared Verigene Respiratory Virus Plus assay (Nanosphere), which incorporates an amplification step prior to array-based detection. In a panel marketed outside the United States, this assay also detects oseltamivir resistance resulting from the H275Y mutation. It is likely that such combinations will find future clinical applications.

To our knowledge, seven manufacturers (some with multiple assays) have obtained FDA clearance for the molecular detection of antimicrobial resistance markers, as of July 2013. BD GeneOhm, bioMérieux NucliSENS EasyQ, ELITech ELITe MGB, and Roche Lightcycler are PCR-based platforms with high-complexity CLIA (Clinical Laboratory Improvement Amendments of 1988) designations. Cepheid

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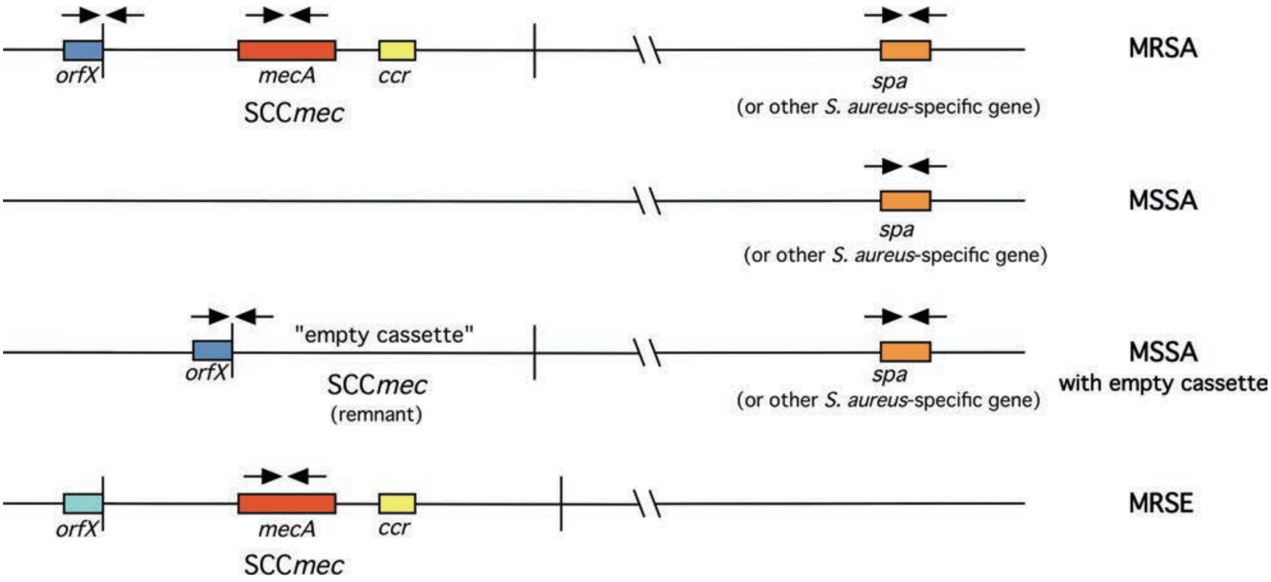

FIGURE 1 Targets of PCR-based assays to detect MRSA. *orfX*, open reading frame of unknown function and the site of SCC*mec* insertion in methicillin-resistant *Staphylococcus aureus*; *mecA*, the gene encoding PBP2′, responsible for methicillin resistance; *ccr*, cassette chromosome recombinase genes; *spa*, gene encoding protein A, an example of an *S. aureus*-specific target; MRSE, methicillin-resistant *Staphylococcus epidermidis*. doi:10.1128/9781555817381.ch77.f1

GeneXpert is a self-contained, PCR-based system with a moderate-complexity designation. The moderate-complexity BioFire FilmArray blood culture identification (BCID) panel uses PCR to detect 20 Gram-positive or Gram-negative bacteria and yeasts, as well as the *mecA*, *vanA*, *vanB*, and KPC resistance determinants. Nanosphere Verigene is a moderate-complexity slide array assay that encompasses extraction, hybridization of target DNA to gold nanoparticle probes, and detection within a closed system.

Broad-range PCR with detection by electrospray ionization mass spectrometry (PCR/ESI-MS) has been used in a commercial system that combines organism identification with the detection of resistance determinants (PLEX-ID; Abbott) (9, 10), but the considerable cost of this system has limited its usefulness in most clinical settings. Matrix-associated laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) has permitted rapid identification of microbial isolates in the clinical laboratory but lacks sufficient sensitivity to detect the proteins responsible for drug resistance (e.g., β-lactamases). Indirect methods that use MALDI-TOF MS to detect the products of antibiotic hydrolysis by carbapenemases have been developed (11–13), but the practical utility of these assays remains to be determined. Other technologies with possible future applicability to antimicrobial resistance detection include denaturing high-performance liquid chromatography (HPLC) (14, 15) and various deep-sequencing modalities (16, 17). A number of additional assays currently under development or in use outside the United States are mentioned in the corresponding resistance target section.

COMMONLY USED RESISTANCE TARGETS

Methicillin-Resistant *Staphylococcus aureus*

Staphylococcus aureus is presently the most common cause of bacterial infections in industrialized countries (18). Particular effort has been exerted to develop assays for the rapid detection of MRSA, which carries an increased risk of symptomatic infection and has fewer effective treatment options (19, 20). However, methicillin-susceptible *S. aureus* (MSSA) also continues to be an important community-acquired pathogen and appears to be playing an increasingly important role in hospital infections as well (21). The rapid detection of MRSA is important for guiding infection control measures and antimicrobial treatment. MSSA detection is also clinically important because different antimicrobial agents are required for optimal MSSA treatment (3, 22–24). The rapid identification of MSSA/MRSA in blood cultures has been shown to result in decreased length of hospital stay and health care costs (25).

Molecular assays that recognize MRSA on the basis of a single target detect the junction between the SCC*mec* (staphylococcal cassette chromosome *mec* element) cassette (which carries the *mecA* resistance determinant along with additional genes) and the flanking *orfX* gene (Fig. 1). It is important to note that such assays may yield false-negative results when MRSA strains carry SCC*mec* variants, as 11 types of SCC*mec* cassettes have now been described (26–28). SCC*mec* XI has been recently described in MRSA strains from bovine and human sources in Europe; this unusual SCC*mec* variant carries the *mecC* gene, which is only 70% identical to *mecA* and poses a new challenge to molecular diagnosis (26). It is advisable to validate a molecular MRSA assay using strains of local origin, as the prevalence of variant strains not detected by a molecular assay may be quite high at some geographic locations (29, 30). Another potential pitfall of single-target MRSA assays is the existence of MSSA strains that carry SCC*mec* remnants lacking an intact *mecA* gene, sometimes referred to as “empty cassettes” (31–35). Such strains are phenotypically methicillin susceptible but can be responsible for false-positive results when single-target MRSA assays are employed. A less common cause of a false-positive molecular MRSA assay is a strain carrying a mutant *mecA* allele that is no longer functional (36).

An alternative approach to the molecular detection of MRSA combines the *mecA* target and a second locus specific for *S. aureus*, such as *sa442*, *nuc*, *femA-femB*, *spa*, or *ldh1* (37–42) (Fig. 1). This approach can be used to detect both MSSA and MRSA. However, mixed specimens may provide ambiguous results. In particular, the concomitant presence of MSSA and methicillin-resistant coagulase-negative staphylococci (CoNS) may be falsely interpreted as MRSA. In one study, cocolonization with methicillin-resistant CoNS and MSSA was found in 3.4% of specimens from anterior nares (43). Rare *S. aureus* strains lacking *nuc* or *spa* have been reported (44, 45).

Molecular assays for the detection of methicillin resistance in *S. aureus* are available for both surveillance testing and to assist in diagnosis of infection (Table 1). Assays approved for MRSA surveillance from nasal swabs include the BD GeneOhm MRSA ACP and BD MAX MRSA assays (Becton Dickinson), Xpert MRSA (Cepheid), LightCycler MRSA advanced test (Roche), and NucliSENS EasyQ MRSA test (bioMérieux) (32, 33, 46, 47). Each of these tests is a PCR-based assay to detect the SCC*mec-orfX* junction. The MRSA/SA ELITe MGB assay (Elitech) is designed for nasal swab specimens; discrimination between MRSA and a mixture of MSSA and methicillin-resistant CoNS is achieved by quantitative comparison of *mecA* and *S. aureus* target abundance.

Assays for the combined detection of MRSA and MSSA to assist in the diagnosis of infection include the BD GeneOhm StaphSR assay (Becton Dickinson), Xpert MRSA/SA BC and Xpert MRSA/SA SSTI (Cepheid), and the Verigene Gram-Positive blood culture test (Nanosphere). The BD GeneOhm Staph SR, Xpert MRSA/SA BC, and Verigene Gram-Positive blood culture tests are used to rapidly identify MRSA and MSSA once Gram-positive cocci are identified in blood cultures (48, 49). In addition, the Verigene assay also detects other Gram-positive pathogens (50) and has targets to differentiate *S. aureus*, *S. epidermidis*, and *S. lugdunensis* from other *Staphylococcus* species. For methicillin resistance to be reported, the assay must detect the presence of a particular staphylococcal species. In polymicrobial cultures, methicillin susceptibility cannot be inferred, as detection of *mecA* may be problematic (51). The Xpert MRSA/SA SSTI assay is performed on swabs obtained from infected skin or soft tissue lesions (49). Although any molecular assay may detect DNA from nonviable organisms, this feature may be particularly problematic for the MRSA/SA SSTI assay. Patients with severe soft tissue infections have frequently received antibiotics prior to the obtaining of diagnostic specimens. The false-positive rate (relative to culture) for patients receiving antibiotics within 3 weeks of testing is reported to be 13.8% and 9.5% for MSSA and MRSA, respectively (package insert). In addition, the MRSA/SA SSTI assay may yield false results in the presence of mixed staphylococcal infections.

Commercial multipathogen detection assays currently in development that can distinguish MRSA and MSSA include the FilmArray BCID Panel (BioFire), VYOO Pathogen Detection assay (Analytik Jena), Gram Positive 12 Easy-Plex PCR Panel (AusDiagnostics) (52), SepsiTest (Molzym), and the next generation of the Plex-ID System (Abbott). The VYOO and SepsiTest assays are intriguing because they are able to detect pathogens in whole blood without requiring culture, but initial studies have shown poor concordance with conventional culture results (53, 54). In addition, PNA-FISH (AdvanDx), a rapid method for pathogen identification from blood culture, is evaluating a probe to target *mecA* mRNA (55).

Vancomycin-Resistant Enterococci

Enterococci are commensal residents of the gastrointestinal tract and female genital tract that account for approximately 10% of nosocomial infections (56, 57). The vast majority of enterococcal infections are caused by *Enterococcus faecalis* and *Enterococcus faecium* and predominately occur in patients requiring long-term care (e.g., intensive care unit, transplant, cancer, hemodialysis). The emergence of vancomycin-resistant enterococci (VRE) in the inpatient setting is of particular concern, given that vancomycin is commonly administered as empirical therapy for suspected bloodstream infections (58). Thus, infection with VRE is associated with increased morbidity and mortality, primarily due to the propensity of VRE to infect patients already at high risk from comorbidity (59).

In the United States, approximately 30% of enterococcal isolates are resistant to vancomycin (57). High-level vancomycin resistance occurs in enterococci via acquisition of mobile transposable genetic elements carrying the *vanA* or *vanB* resistance determinants. *E. faecium* is more frequently vancomycin resistant than *E. faecalis*, with *vanA* more commonly encountered than *vanB*. For this reason, currently marketed molecular assays identify VRE by the presence of *vanA*. Although the detection of *vanA* has been shown to be highly specific (aside from rare reports of *vanA*-carrying *S. aureus* and streptococcal species) (60, 61), the concomitant use of culture-based methods is required to definitively link the detection of the *vanA* gene to VRE carriage, to perform susceptibility testing, and to perform strain typing in an outbreak setting. Additionally, the regional prevalence of *vanB*-carrying VRE is an important consideration when deciding on a molecular assay, as not all commercial assays detect these strains.

As with MRSA, the rapid detection of VRE colonization to prevent health care-associated infections is widely recommended. Currently, two FDA-cleared assays are marketed in the United States for the detection of VRE from perianal and rectal swabs (Table 1). Both of these assays, GeneOhm VanR (BD) and Xpert VanA (Cepheid), target the *vanA* gene, the most prevalent vancomycin resistance determinant in enterococci in North America (62). In addition, the VanR assay also detects *vanB*. However, a number of studies have illustrated the presence of this gene in fecal or rectal specimens without culturable enterococci, questioning the specificity of this target. The *vanB* gene has been found in commensal nonenterococcal bacteria (e.g., *Streptococcus mitis*, *Streptococcus gallolyticus*, *Eggerthella lenta*, *Clostridium* spp., and *Ruminococcus lactaris*) (8, 63), necessitating culture confirmation of a *vanB*-positive result from a molecular assay.

The Verigene Gram-Positive blood culture nucleic acid test (Nanosphere) is a multiplexed nucleic acid test with the capability to identify 12 Gram-positive organisms as well as the presence of *mecA*, *vanA*, and *vanB* resistance determinants (Table 1). This assay is FDA cleared for use on positive blood cultures that have undergone a Gram stain assay to confirm the presence of Gram-positive organisms. With regard to enterococci, the test targets *hsp60* for detection of *E. faecalis* and *E. faecium*, but not other *Enterococcus* species. This allows the accurate identification of most VRE responsible for bloodstream infections that carry either *vanA* or *vanB* (64).

TABLE 1 FDA-cleared molecular assays for the detection of methicillin resistance in *Staphylococcus aureus* and vancomycin resistance in *Enterococcus* species[a]

Manufacturer	Organism(s) detected	Assay name	Target	Purpose	Specimen type	Run time (min)	Hands-on time (min)[b]	Process[c]	LOD[d]
BD GeneOhm	MSSA, MRSA	MRSA ACP	SCC*mec-orfX* junction	Surveillance	Nasal swab	60	15[e]	Batch	130–576 CFU/swab
		StaphSR	SCC*mec-orfX* junction	Infection	Positive blood culture	60	15[e]	Batch	10 CFU/rxn
	VRE[f]	VanR	*vanA* and *vanB*	Surveillance	Perianal and rectal swabs	60	15[e]	Batch	1–14 CFU/rxn
Cepheid Xpert	MSSA, MRSA	MRSA	SCC*mec-orfX* junction	Surveillance	Nasal swab	66	5	Random access	10–100 CFU/swab
		MRSA/SA BC	*spa*, *mecA*, SCC*mec*	Infection	Positive blood culture	60	5	Random access	100–400 CFU/rxn
		MRSA/SA SSTI	*spa*, *mecA*, SCC*mec*	Infection	Skin and soft tissue swabs	60	5	Random access	51–242 CFU/swab
	VRE[f]	vanA	*vanA*	Surveillance	Rectal swab	45	5	Random access	37 CFU/swab
Elitech ELITe MGB	MRSA, MSSA	MRSA/SA ELITe MGB	*mecA*/*S. aureus* target	Surveillance	Nasal swab	120	15	Batch	165 CFU/ml swab
Roche LightCycler	MRSA	MRSA advanced test	SCC*mec*	Surveillance	Nasal swab	75	15	Batch	240 CFU/swab
NucliSENS EasyQ	MRSA	MRSA	*mecA*, SCC*mec*	Surveillance	Nasal swab	90	15	Batch	182–493 CFU/swab
BioFire FilmArray	MRSA, MSSA, MSSE, MRSE, VRE	BCID	Staphylococcal and enterococcal targets, *mecA*, *vanA*, *vanB*	Infection	Positive blood culture	60	5	Random access	6.12×10^7–9.50×10^8 CFU/ml
Nanosphere Verigene	MRSA, MSSA, MSSE, MRSE, VRE	BC-GP	Staphylococcal and enterococcal targets, *mecA*, *vanA*, *vanB*	Infection	Positive blood culture	150	5	Random access	1.9×10^5–1.2×10^8 CFU/rxn

[a] Abbreviations: FDA, Food and Drug Administration; MSSA, methicillin-susceptible *S. aureus*; MRSA, methicillin-resistant *S. aureus*; VRE, vancomycin-resistant *Enterococcus*; LOD, limit of detection; min, minutes; CFU, colony forming unit; rxn, reaction; BCID, blood culture identification; BC-GP, Gram-positive blood culture.

[b] The approximate hands-on time is variable and would be scalable by the number of samples tested.

[c] Assays requiring substantial hands-on time and/or inclusion of external controls per run to validate results were considered best suited for batched processing.

[d] For assays with multiple targets, LOD differs depending upon target; ranges are given.

[e] When performed using the Cepheid Lightcycler, not BD MAX automation.

[f] Assay does not detect *Enterococcus*, only the resistance determinant.

Beta-Lactamases in Gram-Negative Bacteria

One of the greatest threats to the antimicrobial armamentarium has been the emergence of beta-lactamases in Gram-negative bacteria with the capability of hydrolyzing broad-spectrum penicillins, cephalosporins, and carbapenems. These enzymes include the extended-spectrum beta-lactamases (ESBLs), AmpCs, and carbapenemases (65–69). The accurate detection of broad-spectrum beta-lactamases is important for both infection control and treatment, and detection on the basis of the MIC or other phenotypic testing is imperfect (68, 70). A rapid and affordable molecular assay able to detect broad-spectrum beta-lactamases would be clinically useful but at present represents an unmet need. The greatest challenge to molecular detection is the enormous diversity of beta-lactamases, with more than 200 described ESBLs and numerous classes of carbapenemases, including the carbapenemases KPC (*K. pneumoniae* carbapenemase), NDM-1 (New Delhi metallo-beta-lactamase), VIM (Verona integron-encoded metallo-beta-lactamase), IMP (imipenem metallo-beta-lactamase), and OXA (oxacillinase). An additional consideration is that the detection of beta-lactamase genes does not provide information about copy number and expression, which are important determinants of phenotypic beta-lactam resistance.

A number of commercial assays for the molecular detection of broad-spectrum beta-lactamases are currently undergoing evaluation (Table 2). Check-Points (Wageningen, Netherlands) offers Check-MDR assays in both real-time PCR and microarray formats for the detection of ESBLs (TEM, SHV, CTX-M), AmpCs (CMY, ACC, DHA, ACT/MIR), and carbapenemases (KPC, NDM, VIM, IMP, OXA-48), in various configurations (71–73). The BD MAX platform (Becton-Dickinson) offers a CRE (carbapenem-resistant *Enterobacteriaceae*) assay for research use only (RUO) that detects KPC, NDM, and OXA-48 genes. The Hyplex (Amplex Diagnostics GmbH) system for detection of ESBLs (TEM, SHV, CTX-M) or carbapenemases (VIM, IMP, KPC, OXA-48, NDM) detects products from a multiplexed PCR using enzyme-linked immunosorbent assay (ELISA)-based principles (74). The NucliSENS EasyQ platform (bioMérieux) now offers a KPC assay (75), and the FilmArray (BioFire), PLEX-ID (Abbott), and VYOO (Analytik Jena) panels include a limited number of beta-lactamase targets. The Verigene Gram-Negative blood culture test (Nanosphere) is designed to identify common Gram-negative pathogens directly from positive blood cultures as well as to detect CTX-M, KPC, NDM, VIM, IMP, and OXA targets.

OTHER RESISTANCE TARGETS

At present, there appears to be limited interest in the commercial development of molecular assays for other resistance determinants. Some possible candidates might be considered.

Aminoglycosides

Resistance to aminoglycosides most often results from the actions of modifying enzymes: acetyltransferases (AAC), nucleotidyltransferases (ANT), phosphotransferases (APH), and 16S rRNA methylases. These genes may be detected by PCR, but sequence diversity among the resistance genes is considerable. Multiplex PCR assays to detect high-level aminoglycoside resistance in enterococci have been developed (76). Since aminoglycosides are principally used in combination with other agents, the need for rapid detection of aminoglycoside resistance genes may not be as great as with other agents.

Anaerobic Bacteria

The prevalence of resistance of anaerobic bacteria to antimicrobial agents appears to be increasing, including resistance to clindamycin (*erm*), metronidazole (*nimA*), and carbapenems (*cfiA*) (77). PCR assays to detect these determinants have been developed (78–82). However, the value of routine susceptibility testing of anaerobic bacteria is questionable (83).

Beta-Lactam-Resistant Pneumococci

Beta-lactam resistance in *Streptococcus pneumoniae* has become an increasing concern since first reported in 1967 (84). This resistance problem has posed a difficult challenge for molecular diagnosis. Beta-lactam resistance results from mosaic variants of low-affinity penicillin-binding proteins (PBP1a, PBP2b, and PBP2x), which vary in their individual contributions to penicillin and cephalosporin resistance. PCR-based assays have been developed to detect some of the most common variants associated with resistance (85–87). As beta-lactam antibiotics can still be used to treat nonmeningeal infections with *S. pneumoniae* strains exhibiting intermediate levels of resistance (88), the utility of a rapid resistance assay may be limited, with the exception of the setting of meningitis.

Ceftriaxone-Resistant *Neisseria gonorrhoeae*

The emergence of ceftriaxone resistance in *N. gonorrhoeae* is of particular concern given the reliance on non-culture-based methods for gonococcal detection in the clinical setting. Incorporating resistance detection into currently available nucleic acid amplification tests would be highly desirable, but this is currently unavailable. One approach used in the research setting is the molecular detection of mosaic variants of *penA*, encoding penicillin-binding protein 2, that confer reduced beta-lactam susceptibility (89). The future development of molecular *penA* assays will need to account for the presence of mosaic PBP2 in commensal *Neisseria* species, which is likely to be of particular importance in pharyngeal specimens (90). Combined detection of cephalosporin resistance and mutations conferring quinolone resistance (see "Fluoroquinolones") would allow the use of a quinolone for isolates determined to be susceptible. However, at present, no molecular assay is commercially available to detect antimicrobial-resistant gonorrhea.

Fluoroquinolones

Fluoroquinolone agents are widely used in clinical medicine. Therefore, a rapid assay to detect resistance is theoretically attractive. However, resistance most frequently results from mutations in the *gyrA* and *parC* genes, encoding subunits of DNA gyrase and topoisomerase IV that are targeted by these antibiotics. Detection of these point mutations would require techniques such as sequencing, microarray, mismatch amplification mutation assay (MAMA), or allele-specific PCR (91–94). Alternatively, an assay might target *Escherichia coli* strain ST131, which appears to be responsible for more than one-half of the infections caused by fluoroquinolone-resistant *E. coli* at diverse geographic locations in the United States (95). Although alterations of the target site account for the predominance of high-level resistance, low-level resistance has been associated with additional mechanisms, making molecular detection of low-level resistance difficult.

TABLE 2 FDA-cleared and select RUO molecular assays for the detection of beta-lactamase resistance in Gram-negative bacteria[a]

Manufacturer	Target of detection	Assay name	Designation	Purpose	Specimen type	Run time	Hands-on time[b]	Process[b]	LOD[c]
Biofire FilmArray	KPC	BCID	FDA cleared	Infection	Positive blood culture	60 min	5 min	Random access	0.5×10^8–1.0×10^8 CFU/ml
Nanosphere Verigene	KPC, NDM, VIM, IMP, OXA-48, CTX-M	BC-GN	FDA cleared	Infection	Positive blood culture	2 h	5 min	Random access	NA
BD MAX	KPC, OXA-48, NDM	CRE	RUO	Infection or surveillance	Colonies	2 h	10 min	Random access	NA
NucliSENS EasyQ	KPC	KPC	RUO	Infection or surveillance	Colonies, rectal swabs, stools	1 h 40 min	15 min	Batch	1–4 CFU/rxn
Abbott PLEX-ID	KPC	Broad bacterial assay	RUO	Infection	Positive blood culture	6 h	>1 h	Batch	NA
Analytik Jena VYOO	SHV, CTX-M	VYOO 5	RUO	Infection	Blood	6 h	>1 h	Batch	3–10 CFU/ml
Amplex Hyplex	VIM, IMP, KPC, OXA-48, NDM	Superbug ID	RUO	Infection or surveillance	Colonies or direct from specimen	5 h	30–60 min	Batch	NA
	TEM, SHV, CTX-M, OXA	ESBL ID	RUO	Infection or surveillance	Colonies or direct from specimen	5 h	30–60 min	Batch	NA
Check-Points	KPC, NDM, VIM, IMP, OXA-48, ESBL,[d] AmpC[d]	Check-MDR CT103[e]	RUO	Infection or surveillance	Colonies, rectal and perirectal swabs, stools	6 h	>1 h	Batch	2–100 CFU/rxn
	KPC, OXA-48, VIM, NDM	Check-Direct CPE	RUO	Surveillance	Rectal and perirectal swabs	1 h 40 min	40–60 min	Batch	<5 CFU/rxn

[a] Abbreviations: FDA, Food and Drug Administration; RUO, research use only; KPC, *Klebsiella pneumoniae* carbapenemase; NDM, New Dehli metallo-beta-lactamase; OXA, carbapenem-hydrolyzing oxacillinase; VIM, Verona integron-encoded metallo-beta-lactamase; IMP, imipenemase; CTX-M, cefotaximase; SHV, sulfhydryl variable beta-lactamase; BCID, blood culture identification; BC-GN, Gram-negative blood culture; CRE, carbapenem-resistant *Enterobacteriaceae*; LOD, limit of detection; rxn, reaction; NA, not available.

[b] Approximate hands-on time is variable and heavily dependent upon extraction and amplification methods as well as the number of samples tested.

[c] For assays with multiple targets, LOD differs depending upon target; ranges are given.

[d] ESBL targets: CTX-M (CTX-M-1 group, CTX-M-1 like, CTX-M-15 like, CTX-M-3 like, CTX-M-32 like, CTX-M-2 group, CTX-M-8 & -25 group, CTX-M-9 group); TEM (TEM wt, TEM E104K, TEM R164S, TEM R164C, TEM R164H, TEM G238S); SHV (SHV wt, SHV G238S, SHV G238A, SHV E240K); AmpC targets: CMY I/MOX, CMY II/FOX, ACC, DHA, ACT/MIR.

[e] Other Check-MDR assays (CT101 and CT102) are configured to include fewer targets; MDR Carba and MDR ESBL assays are also available for beta-lactamase detection using real-time PCR instrumentation.

Linezolid

The oxazolidinone linezolid is an important last-line agent for the treatment of resistant Gram-positive cocci. Although resistance is infrequent, mutations that affect the linezolid-binding site have been described. In both staphylococci and enterococci, the most commonly reported mechanism of linezolid resistance is a G2576T mutation in domain V of 23S rRNA, making this region an attractive target for the molecular detection of resistance (96). A confounding issue in the development of an assay detecting the G2576T mutation is that the phenotypic level of resistance correlates with the number of rRNA operons carrying a mutation; strains that are phenotypically linezolid susceptible despite the presence of the G2576T mutation have been described. A number of additional mutations in 23S rRNA or the ribosomal proteins L3 and L4 have been described in linezolid-resistant strains, and the relationship between resistance and these mutations is currently under investigation. Linezolid resistance following acquisition of the *cfr* gene, encoding a 23S rRNA methyltransferase, has also been reported. This plasmid-borne mechanism of resistance has been implicated in outbreaks of infections caused by linezolid-resistant staphylococci and has recently been detected in a clinical isolate of *E. faecalis* (97–99). If linezolid resistance becomes more prevalent, a molecular assay to detect the 23S G2576T mutation and the presence of *cfr* may become useful.

Mupirocin

Mupirocin is an isoleucyl tRNA synthase inhibitor used for MRSA decolonization. Resistance to mupirocin is conferred by the *mupA* gene, which encodes a mupirocin-resistant isoleucyl tRNA synthase. Molecular assays for *mupA* detection have been developed (100) and may become increasingly useful if the prophylactic use of mupirocin becomes more widespread (101).

Mycobacteria

Assays for the rapid detection of mutations associated with resistance to isoniazid, rifampin, ethambutol, pyrazinamide, and streptomycin have been developed, and some are commercially available for research use. Additional information may be found in chapter 76.

Trimethoprim

Variant *dfr* genes, encoding dihydrofolate reductase, the target of trimethoprim, have been increasingly observed in *E. coli* isolates causing urinary infections, sometimes inserted into integrons (102). A rapid assay to identify the common *dfr* variants might help to identify patients unlikely to respond to empiric treatment with trimethoprim-sulfamethoxazole.

CONCLUSIONS

At present, FDA-cleared molecular assays for the detection of antibiotic-resistant pathogens are limited to those identifying MRSA and VRE, employing PCR and solid-phase array platforms. These assays must be employed with careful attention to the specific targets used for detection, as potential false-positive and false-negative results may be obtained. Selection of a specific assay must take specimen type, test volume, hands-on and turnaround time, on-demand capability, and cost into consideration. Although genetic diversity and cost pose significant obstacles, new assays for the detection of broad-spectrum beta-lactamases of Gram-negative bacteria appear promising. With a continuing urgent clinical need for the rapid detection of antimicrobial resistance, and with many novel technologies in development, the molecular diagnosis of antibacterial drug resistance is likely to see expanded application in coming years.

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VIROLOGY

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Taxonomy and Classification of Viruses

ELLIOT J. LEFKOWITZ

78

INTRODUCTION

Taxonomy

Taxonomy at its most basic level involves the classification and naming of objects. Living objects have been grouped for hundreds of years according to the Linnaean system (1), a classification scheme that places living things hierarchically into groups of species followed by groupings into higher-level taxa (from genera to families, orders, classes, phyla, kingdoms, and domains, including a number of now-recognized intervening ranks) dependent on common shared characteristics. Taxonomic assignments were originally based on visible structural similarities between organisms. Assignments now also include molecular and genetic information.

Taxonomy functions beyond mere categorization. By having information about, and an understanding of, a few of the organisms in a group of closely related taxa, it is often possible to extend that knowledge to other organisms in related taxa for which much less biological information may be available. For viruses, this process of comparative analysis plays a critical role in increasing our overall knowledge of the molecular biology, pathogenesis, epidemiology, and evolution of poorly understood or newly isolated viruses. This knowledge enhances our ability to respond to new threats by supporting the development of diagnostics, vaccines, and other antiviral therapies.

Viruses

Viruses are not easily placed on the evolutionary tree of life (2–4). They are not accurately represented by side branches sprouting off from the main branches or by their own single branch growing out from the base of the tree. In fact, it is likely that viruses have multiple independent evolutionary origins (5, 6) that cannot be easily or completely separated from the evolution of their hosts, as they cannot reproduce or evolve separately from their hosts (7, 8). Indeed, the host represents one of the most important characteristics of a virus that must be considered when making taxonomic assignments. Therefore, viruses might be better represented as individual twigs arising from branches spread throughout the rest of the tree.

In addition to distinct evolutionary histories, viruses differ from other domains of life in the variety of possible coding molecules they utilize to store their genetic programs (9, 10). Every other domain of life has, as the basic reservoir of its genetic program, double-stranded DNA (dsDNA). Virus genomes may be composed of dsDNA, single-stranded DNA (ssDNA), dsRNA, or ssRNA (which may be positive or negative sense with respect to the mRNA coding strand). In addition, reverse transcription may be a part of their molecular programs. Genome topology (linear, circular, single segment, or multiple segments) also varies among different viruses. All of these unique features, as well as numerous other criteria, are taken into account when classifying viruses and making taxonomic assignments.

VIRAL TAXONOMY: ICTV CLASSIFICATION OF VIRUSES

The process of making taxonomic assignments is the responsibility of a number of different organizations that have the internationally recognized authority to oversee or contribute to the process by defining the rules, methods, and nomenclature to be used in making assignments for particular domains or subdomains of life (11–13). For viruses, the Virology Division of the International Union of Microbiological Societies has charged the International Committee on Taxonomy of Viruses (ICTV) with the task of developing, refining, and maintaining a universal viral taxonomy (14, 15).

The ICTV currently recognizes five hierarchical ranks that are used to define the universal viral taxonomy: the order, family, subfamily, genus, and species. The 2013 ICTV viral taxonomy (16) comprises 7 orders, 103 families, 22 subfamilies, 455 genera, and 2,827 species. The official viral taxonomy is published on the ICTV website (http://www.ictvonline.org) and in the ICTV reports (14) (although the ICTV ninth report, compiled in 2010 and 2011, contains the 2009 ICTV taxonomy [17] and therefore lags behind the most recently published ICTV taxonomy published in 2014 [16]). The first report of the ICTV was based on deliberations made at and after the 1968 (Helsinki) International Congress of Virology (18). Subsequent reports have been published at regular intervals, authored by numerous and collaborating virologists, with the most recent report, the ninth, published in 2012. These reports provide a history of the efforts and the logic used in forming taxa, term definitions, the official taxonomy, and a description of higher-level taxa.

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Initially, the ICTV only recognized and made assignments to ranks at the genus level and above. In 1991, the rank of species was added (19). A virus species is defined as "the lowest taxonomic level in the hierarchy approved by the ICTV. A species is a monophyletic group of viruses whose properties can be distinguished from those of other species by multiple criteria." This represents the new definition as redefined by the ICTV in 2013 (20). Note that, in general, every species is a member of a genus, which in turn is a member of a family. A few families are also members of one of the seven orders that are currently recognized, but the majority of viral families do not belong to an order. Some species are not yet assigned to a genus (but may be assigned to a family), and a few genera are not yet assigned to a family; additional biological information must become available before these assignments can be made. Subfamily and order assignments are optional and therefore not necessary to complete a taxonomic hierarchy.

The ICTV is governed by a series of statutes and follows rules and definitions provided by the International Code of Virus Classification and Nomenclature (20, 21) to make taxonomic assignments and name virus taxa. Any modifications or additions to the official ICTV taxonomic classification and names, as well as changes to the statutes or code, must be approved by the voting membership of the ICTV. Any individual can submit proposals to the ICTV for the modification of existing taxa or the creation of new taxa.

In addition to defining the process and rules for virus classification, the ICTV Code also defines the rules to be followed for name assignment. Higher-level taxon names are composed of a single word ending with a suffix that is dependent on taxon rank. The suffix "*-virales*" identifies an order. Families are identified by the suffix "*-viridae*"; subfamilies are identified by the suffix "*-virinae*"; and genera are identified by the suffix "*-virus*." Species names should be as concise as possible but normally comprise two or more words. It is common for a species name to end with the word "*virus*" or have "*-virus*" as a suffix, but this is not required. When written, names of taxa are italicized, and the first letter of the name is capitalized. (As a part of a species name, the first letter of any proper noun, such as a geographic location, is also capitalized.) It is often difficult to determine in a particular context if a species name should be written in the formal italicized manner, since species names often coincide with the common names used to refer to a virus. Taxa are abstract concepts that do not physically exist. As an example, when referring to the species *Variola virus* that belongs to the genus *Orthopoxvirus* and the family *Poxviridae*, all names refer to abstract taxa and are therefore written in the formal italicized manner. However, when referring to variola virus, the physical entity that causes smallpox, the name is neither capitalized nor italicized.

It is important to note that the ICTV is only concerned with making taxonomic assignments at the species level and higher, although many viral isolates assigned to one particular species can be further subdivided into categories based on sequence phylogeny, immune reactivity, or other properties. In many cases, these subspecies-level assignments are made on an *ad hoc* basis by an individual investigator and reported in a journal article. In other instances, a more organized effort may have been made to subdivide a species into a series of "types" based on a defined set of demarcation criteria. Examples of viruses for which subspecies-level assignments have been made include hepatitis C virus (22), dengue virus (23), and HIV (24).

CRITERIA FOR TAXONOMIC CLASSIFICATION

Character-Based Descriptors

Taxonomic classification is accomplished by comparing and contrasting sets of characters that can be used to define the properties of any particular taxon. Any aspect of viral biology can be defined by a set of characters. These characters may have values represented by quantitative measures, such as the triangulation (T) number used to categorize icosahedral virion capsid structure, or they may be purely qualitative descriptors, such as the presence or absence of a host-derived lipid envelope.

Viruses are described by choosing a set of appropriate characters and then assigning values to these characters as necessary. The ICTVdb species and isolate database in the past provided one example of a comprehensive character list for describing viruses (25, 26). This list is summarized in Table 1. More than 2,500 different characters are avail-

TABLE 1 ICTVdb character list

Major category(ies)	No. of available characters	Character examples
Classification	31	Name; NCBI taxon ID; ICTVdb decimal code; synonyms
Taxonomic structure	13	Order; family; subfamily; genus; species
Isolate information	77	Location; date; host; tissues; method of isolation
Virion properties	1,050	Morphology (size, envelope, capsid); physicochemical and physical properties; nucleic acid (genome type and configuration); proteins
Genome organization and replication	462	Attachment; penetration; transcription; translation; protein processing; genome replication; assembly
Biological properties	954	Host range; transmission; disease; pathology
Antigenicity	75	Antigenic determinants; epitopes; serological relationships; immunity; variation; diagnostics; vaccines
Comments, references, and contributors	35	Publication; database; website; contact

able for use, and many of these have multiple values that might be associated with any one viral taxon or isolate. For example, a particular isolate may be described by the location and date of its isolation, each of which would be associated with the appropriate value (e.g., country and year). Characters describing a particular species may include its host range and a list of diseases associated with viruses belonging to that species. More than 40,000 different descriptors or values can be associated with the character set of the ICTVdb.

There is no one master character list utilized by the ICTV for classifying viruses. This is because every taxon is unique and characters useful for describing one taxon may be entirely inappropriate for describing another. The ICTVdb character list may be useful in providing a list of potential characters that might be used for any particular classification, but significant research still needs to be performed to determine the most appropriate set of choices. Character selection and character value assignment can only be performed by investigators with relevant expertise. Therefore, the research scientists who comprise the ICTV study groups that are responsible for making taxonomic assignments for each viral family set the rules of classification for the viruses that fall under their areas of expertise (14, 27, 28).

The rules for taxonomic classification are defined by a set of demarcation criteria specific to each rank of every taxonomic hierarchy. These demarcation criteria can then be used by research scientists either to determine that a newly isolated virus belongs to an existing species (and therefore there is no need for it to be further classified) or to submit a proposal to the appropriate study group for the creation of a new species (or higher-level taxon) if the isolate, based on the demarcation criteria, is sufficiently different from other viruses that it warrants the creation of a new taxon. Demarcation criteria are provided in each chapter of the ICTV reports describing any particular taxon. Table 2 provides examples of some of the specific criteria utilized to define a species and its upper-level taxa for the species *Human enterovirus* C (polioviruses were recently reclassified as belonging to this species) (14, 28). Different sets of characters are utilized at each level in the taxonomic hierarchy to describe the viruses that would be assigned to that level and below. The order-, family-, and genus-level characters are universal and must be present in all viruses assigned to that taxon, while the species-level characters do not necessarily need to be present in all members of the species but are frequently shared by the viruses belonging to the species.

Sequence-Based Characters

Characters that describe virus morphology and structure have always been important classification criteria. With the advent of genome sequencing, sequence-based comparison has assumed an increasingly important role in classification. Table 2 provides a number of different sequence-based criteria at each rank that are used to define the important characteristics of that rank. There are conserved functional amino acid domains present in all members of the order *Picornavirales*. Conserved protease and polymerase protein sequences help define the family. A specific level of amino acid conservation across the whole polyprotein is required to place a virus within the *Enterovirus* genus. An even higher level of sequence identity is required for members of a particular species. Classification based on sequence comparison is now one of the major defining characteristics of all viral taxa. How these comparisons are made, how the relationships are measured, and the extent to which they are included in the taxon demarcation criteria vary significantly from taxon to taxon. This is understandable given the inherent differences in mutability between viruses, especially between viruses of different genomic composition. For example, viruses with dsDNA genomes show much less variability at the sequence level than RNA viruses. This difference is a direct consequence of the error rates of their DNA and RNA polymerases (5). Therefore, the measures used (as defined by each ICTV study group) to define specific sequence-based demarcation criteria will also vary from one taxon to another.

Sequence-based comparisons can be measured using a variety of different techniques. The most basic involve pairwise comparisons in which two sequences are aligned and the number of nucleotide or amino acid differences between each aligned position is counted. When comparing multiple sequences, a table of distances is compiled that provides the percent similarity between every possible pairwise comparison in the set. Depending on the particular taxon and hierarchical rank under study, nucleotide or amino acid sequences can be compared and alignments of complete viral genomes, a portion of the genome, or individual genes can be utilized. By examining these sequence distance tables, a study group can set specific similarity levels that define the demarcation criteria for classification of viruses into different taxa.

More-sophisticated analyses based on pairwise sequence comparisons can be utilized to provide alternative methods for visualizing differences, choosing cutoffs, and making assignments. One method used in recent years is the pairwise analysis of sequence conservation (PASC) (29). PASC utilizes pairwise sequence alignments of either whole genomic nucleotide sequences or individual gene nucleotide or amino acid sequences. A pairwise alignment is constructed between every possible pair of available sequences. Once all pairwise alignments have been constructed, the percent identity is calculated for each aligned pair and then plotted versus the number of aligned pairs producing similar identities. Figure 1A provides an example of a PASC plot using amino acid alignments of the DNA polymerase gene of all virus isolates that belong to the family *Poxviridae* (30). As can be seen, several distinct peaks are produced, each of which corresponds to comparisons between viruses classified into particular ranks of the *Poxviridae* taxonomic hierarchy. The lowest percent identity (20 to 30%) corresponds to comparisons between viruses from different subfamilies. Genus-level comparisons produce multiple peaks from 45 to 75% identity. Peaks for interspecies comparisons vary between 80 and 98% identity, with the most prominent interspecies peaks occurring at 97 and 98% identity. Intraspecies comparisons (comparisons between strains of the same species) show very high levels of identity (99% and greater). Figure 1B shows how these taxa are arrayed on the *Poxviridae* phylogenetic tree. By utilizing PASC, a new virus isolate can be compared to all existing isolates, and by plotting its similarity to isolates already assigned to a known taxon, a determination can be made as to whether the new isolate can be assigned to an existing taxon or if a proposal for creation of a new taxon should be considered. It is interesting that, from a biological point of view, the distinct patterns of conservation that are exhibited by the peaks present in the PASC graph suggest that the evolutionary history of the protein being analyzed (in this case, poxvirus DNA polymerase) has selected for protein sequences that exhibit distinct peaks of fitness. PASC is now utilized by a number

(Text continues on page 1402)

TABLE 2 Criteria for taxonomic classification

- Order: *Picornavirales*
  - Virion
    - Nonenveloped, icosahedral particles, ~30 nm in diameter
    - Capsid proteins composed of three distantly related jelly roll domains forming particles with pseudo-T=3 symmetry
  - Genome
    - Positive-sense ssRNA
    - One or two monocistronic genome segments
    - 5′-bound VPg protein
    - Genome serves as the mRNA
    - Genome typically contains a 3′ poly(A) tail
  - Protein
    - Primary polyprotein translation product proteolytically cleaved into mature proteins by one or more virus-encoded proteinases
    - Functional domains include a superfamily III helicase (Hel); chymotrypsin-like proteinase (Pro); and superfamily I RNA-dependent RNA polymerase (Pol)
    - Nonstructural proteins are arranged as Hel-VPg-Pro-Pol

- Family: *Picornaviridae*
  - Genome
    - Single monocistronic genome segment
  - Protein
    - Conserved genome organization
    - Conserved set of functional mature proteins
    - Protein sequence conservation (protease-polymerase region)

- Genus: *Enterovirus*
  - Protein
    - At least 50% amino acid identity over the length of the polyprotein
    - VPg sequence conservation
    - Lacks an L protein
    - Possesses a type 1 internal ribosomal entry site
  - Host
    - Virus replication primarily in (but not limited to) the gastrointestinal tract

- Species: *Human enterovirus C*
  - Host
    - Share a limited range of host cell receptors
    - Share a limited natural host range (e.g., human)
  - Genome
    - Conserved genome map (organization of protein functional domains)
    - Common polyprotein proteolytic processing program
    - Share a significant degree of compatibility in proteolytic processing, replication, encapsidation, and genetic recombination
  - Sequence similarity
    - Amino acid identity: 70% in the polyprotein
    - Amino acid identity: 60% in the P1 structural proteins
    - Amino acid identity: 70% in the 2C + 3CD nonstructural proteins
    - Similar base G+C composition (within 2.5%)
  - Phylogeny
    - Monophyletic

FIGURE 1 Taxonomic demarcation via sequence similarity. (A) PASC was carried out on the viral DNA-dependent DNA polymerase gene (the vaccinia virus E9L gene homolog) for every completely sequenced poxvirus genome. Each protein was aligned to every other protein, and the percent identity of each pairwise comparison was then included in a histogram plot of all possible comparisons. Peaks are identified across the top of the figure according to the taxa represented by particular pairwise sequence comparisons. (B) Phylogenetic reconstruction of the *Poxviridae* family of viruses based on their DNA polymerase protein sequences. Subfamily and genera demarcations are identified. Terminal nodes are labeled according to genus. Sequences belonging to one of the genera labeled either group A or B coincide with the A and B comparison peaks at the top of panel A. (C) Phylogenetic prediction based on the multiple nucleic acid sequence alignment of the core genomic region of each representative orthopoxvirus species or strain. BR, strain Brighton Red; GRI, strain GRI-90. Reprinted with modification with permission of Elsevier from reference 30. doi:10.1128/9781555817381.ch78.f1

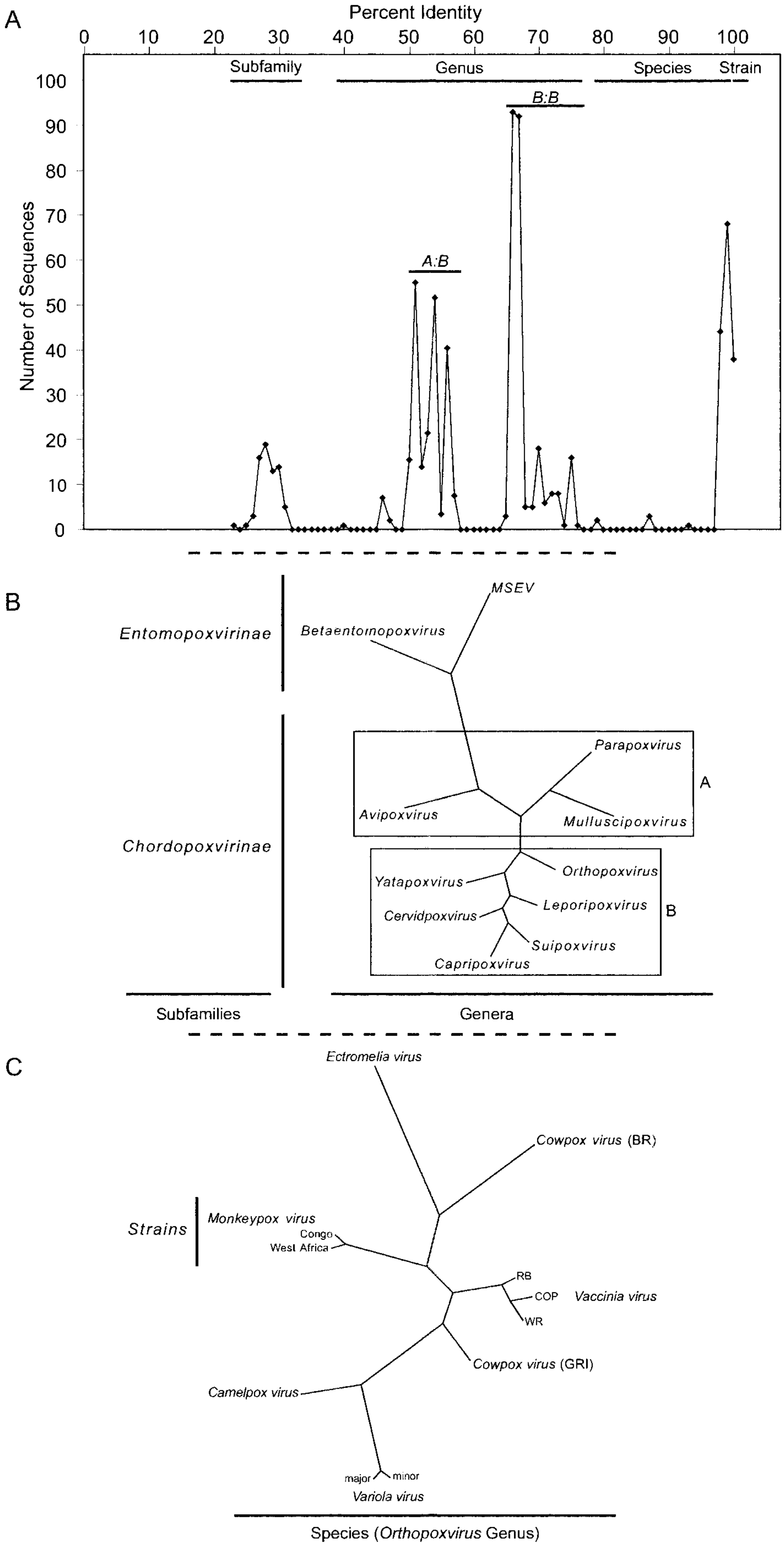
A
Percent Identity
0
10
20
30
40
50
60
70
80
90
100
Subfamily
Genus
Species
Strain
B:B
A:B
Number of Sequences
100
90
80
70
60
50
40
30
20
10
0
B
MSEV
Entomopoxvirinae
Betaentomopoxvirus
Parapoxvirus
A
Avipoxvirus
Mulluscipoxvirus
Chordopoxvirinae
Yatapoxvirus
Orthopoxvirus
Leporipoxvirus
B
Cervidpoxvirus
Suipoxvirus
Capripoxvirus
Subfamilies
Genera
C
Ectromelia virus
Cowpox virus (BR)
Strains
Monkeypox virus
Congo
West Africa
RB
COP
Vaccinia virus
WR
Cowpox virus (GRI)
Camelpox virus
major
minor
Variola virus
Species (Orthopoxvirus Genus)

TABLE 3 Taxonomic classification of viruses infecting humans

Genome composition and order	Family	Subfamily	Genus	Species (ICTV type species or common examples)
dsDNA, linear				
Herpesvirales	*Herpesviridae*	*Alphaherpesvirinae*	*Simplexvirus*	*Human herpesvirus 1* (herpes simplex virus type 1); *Human herpesvirus 2* (herpes simplex virus type 2); *Macacine herpesvirus 1* (B virus)
			Varicellovirus	*Human herpesvirus 3* (varicella-zoster virus)
		Betaherpesvirinae	*Cytomegalovirus*	*Human herpesvirus 5* (human cytomegalovirus)
			Roseolovirus	*Human herpesvirus 6A, 6B*, and *7*
		Gammaherpesvirinae	*Lymphocryptovirus*	*Human herpesvirus 4* (Epstein-Barr virus)
			Rhadinovirus	*Human herpesvirus 8* (Kaposi's sarcoma-associated herpesvirus)
Unassigned	*Adenoviridae*		*Mastadenovirus*	*Human mastadenovirus A* to *G*
Unassigned	*Poxviridae*	*Chordopoxvirinae*	*Molluscipoxvirus*	*Molluscum contagiosum virus*
			Orthopoxvirus	*Cowpox virus*; *Monkeypox virus*; *Vaccinia virus*; *Variola virus*
			Parapoxvirus	*Orf virus*
			Yatapoxvirus	*Yaba monkey tumor virus*
dsDNA, circular				
Unassigned	*Papillomaviridae*		*Alphapapillomavirus*	*Alphapapillomavirus 1* (human papillomavirus 32)
			Betapapillomavirus	*Betapapillomavirus 1* (human papillomavirus 5)
			Gammapapillomavirus	*Gammapapillomavirus 1* (human papillomavirus 4)
			Mupapillomavirus	*Mupapillomavirus 1* (human papillomavirus 1)
			Nupapillomavirus	*Nupapillomavirus 1* (human papillomavirus 41)
Unassigned	*Polyomaviridae*		*Polyomavirus*	*BK polyomavirus*; *JC polyomavirus*
ssDNA, linear				
Unassigned	*Parvoviridae*	*Parvovirinae*	*Bocaparvovirus*	*Primate bocaparvovirus 1* and *2*
			Dependoparvovirus	*Adeno-associated dependoparvovirus A* and *B*
			Erythroparvovirus	*Primate erythroparvovirus 1* (human parvovirus B19)
ssDNA, circular				
Unassigned	*Anelloviridae*		*Alphatorquevirus*	*Torque teno virus 1* (torque tenovirus)
dsDNA, reverse transcribing circular				
Unassigned	*Hepadnaviridae*		*Orthohepadnavirus*	*Hepatitis B virus*
ssRNA, linear, negative sense				
Mononegavirales	*Bornaviridae*		*Bornavirus*	*Borna disease virus*
	Filoviridae		*Ebolavirus*	*Bundibugyo ebolavirus*; *Sudan ebolavirus*; *Tai Forest ebolavirus*; *Zaire ebolavirus*
			Marburgvirus	*Marburg marburgvirus*
	Paramyxoviridae	*Paramyxovirinae*	*Henipavirus*	*Hendra virus*; *Nipah virus*
			Morbillivirus	*Measles virus*
			Respirovirus	*Human parainfluenza virus 1* and *3*
			Rubulavirus	*Human parainfluenza virus 2* and *4*; *Mumps virus*
		Pneumovirinae	*Metapneumovirus*	*Human metapneumovirus*
			Pneumovirus	*Human respiratory syncytial virus*
	Rhabdoviridae		*Lyssavirus*	*Rabies virus*
ssRNA, linear, negative sense, segmented				
Unassigned	*Orthomyxoviridae*		*Influenzavirus A*	*Influenza A virus*
			Influenzavirus B	*Influenza B virus*
			Influenzavirus C	*Influenza C virus*
ssRNA, linear, positive sense				
Nidovirales	*Coronaviridae*	*Coronavirinae*	*Alphacoronavirus*	*Human coronavirus 229E*; *Human coronavirus NL63*
			Betacoronavirus	*Betacoronavirus 1*; *Human coronavirus HKU1*; *Severe acute respiratory syndrome-related coronavirus*
		Torovirinae	*Torovirus*	*Human torovirus*

(Continued on next page)

TABLE 3 *(Continued)*

Genome composition and order	Family	Subfamily	Genus	Species (ICTV type species or common examples)
Picornavirales	*Picornaviridae*		*Aphthovirus*	*Foot-and-mouth disease virus*
			Enterovirus	*Enterovirus* A (human coxsackievirus A2; human enterovirus 71); *Enterovirus B* (human coxsackievirus B1; human echovirus); *Enterovirus* C (human poliovirus 1 to 3; human coxsackievirus A1); *Enterovirus D* (human enterovirus 68, 70, and 94); *Rhinovirus* A, B, and C
			Hepatovirus	*Hepatitis A virus*
			Parechovirus	*Human parechovirus*
Unassigned	*Astroviridae*		*Mamastrovirus*	*Mamastrovirus* 1, 6, 8, and 9 (human astrovirus)
Unassigned	*Caliciviridae*		*Norovirus*	*Norwalk virus*
			Sapovirus	*Sapporo virus*
Unassigned	*Flaviviridae*		*Flavivirus*	*Dengue virus*; *Japanese encephalitis virus*; *Kyasanur Forest disease virus*; *Langat virus*; *Louping ill virus*; *Murray Valley encephalitis virus*; *Omsk hemorrhagic fever virus*; *Powassan virus*; *St. Louis encephalitis virus*; *Tick-borne encephalitis virus*; *Wesselsbron virus*; *West Nile virus*; *Yellow fever virus*; *Zika virus*
			Hepacivirus	*Hepatitis C virus*
Unassigned	*Hepeviridae*		*Hepevirus*	*Hepatitis E virus*
Unassigned	*Togaviridae*		*Alphavirus*	*Barmah Forest virus*; *Chikungunya virus*; *Eastern equine encephalitis virus*; *Mayaro virus*; *O'nyong-nyong virus*; *Ross River virus*; *Semliki Forest virus*; *Sindbis virus*; *Venezuelan equine encephalitis virus*; *Western equine encephalitis virus*
			Rubivirus	*Rubella virus*
ssRNA, linear, ambisense, segmented				
Unassigned	*Arenaviridae*		*Arenavirus*	*Chapare virus*; *Guanarito virus*; *Junín virus*; *Lassa virus*; *Lujo virus*; *Lymphocytic choriomeningitis virus*; *Machupo virus*; *Sabiá virus*
Unassigned	*Bunyaviridae*		*Hantavirus*	*Hantaan virus*; *Puumala virus*; *Sin Nombre virus*; *Thottapalayam virus*; multiple other species
			Nairovirus	*Crimean-Congo hemorrhagic fever virus*; *Dugbe virus*
			Orthobunyavirus	*Bunyamwera virus*; *Bwamba virus*; *California encephalitis virus*; *Guama virus*; *Madrid virus*; *Nyando virus*; *Oropouche virus*; *Tacaiuma virus*
			Phlebovirus	*Punta Toro virus*; *Rift Valley fever virus*; *Sandfly fever Naples virus*
dsRNA, linear, segmented				
Unassigned	*Picobirnaviridae*		*Picobirnavirus*	*Human picobirnavirus*
Unassigned	*Reoviridae*	*Sedoreovirinae*	*Orbivirus*	*Changuinola virus*; *Corriparta virus*; *Great Island virus*; *Lebombo virus*; *Orungo virus*
			Rotavirus	*Rotavirus* A, B, and C
			Seadornavirus	*Banna virus*
		Spinareovirinae	*Coltivirus*	*Colorado tick fever virus*
			Orthoreovirus	*Mammalian orthoreovirus*
ssRNA, linear, dimer, reverse transcribing				
Unassigned	*Retroviridae*	*Orthoretrovirinae*	*Deltaretrovirus*	*Primate T-lymphotropic virus 1* and 2
			Lentivirus	*Human immunodeficiency virus 1* and 2
		Spumaretrovirinae	*Spumavirus*	*Simian foamy virus*
ssRNA, circular, negative sense				
Unassigned	Unassigned		*Deltavirus*	*Hepatitis delta virus*

TABLE 4 Summary of important characteristics used to differentiate families of viruses infecting humans

Classification (family)	Virus properties						
	Virion					Genome	
	Envelope	Shape	Size (nm)	Nucleocapsid symmetry	Molecule[a]	Structure	Size (kb or kbp)
Adenoviridae	–	Isometric	70–90	Icosahedral	dsDNA, linear	Monopartite, inverted terminal repeats, 5′ ends covalently linked to terminal protein	26–48
Anelloviridae	–	Isometric	30	Icosahedral	ssDNA (–), circular	Monopartite	2–4
Arenaviridae	+	Spherical, pleomorphic	50–300	Helical	ssRNA, linear	2 ambisense (±) segments	11 (total)
Astroviridae	–	Isometric	28–30	Icosahedral	ssRNA (+), linear	Monopartite	6–8
Bornaviridae	+	Spherical	90–130	Helical	ssRNA (–), linear	Monopartite	9
Bunyaviridae	+	Spherical, pleomorphic	80–120	Helical	ssRNA, linear	3 negative or ambisense segments	11–19 (total)
Caliciviridae	–	Isometric	35–40	Icosahedral	ssRNA (+), linear	Monopartite, 5′-end covalently linked protein (VPg)	7–8
Coronaviridae	+	Spherical, pleomorphic	120–160	Helical	ssRNA (+), linear	Monopartite	26–32
Deltavirus[b]	+	Spherical	36–43	Helical	ssRNA (–), circular	Monopartite, requires hepatitis B virus for replication	1.7
Filoviridae	+	Filamentous pleomorphic	80 × 660–800	Helical	ssRNA (–), linear	Monopartite	19
Flaviviridae	+	Spherical	40–60	Icosahedral	ssRNA (+), linear	Monopartite	9–13
Hepadnaviridae	+	Spherical	42–50	Icosahedral	dsDNA, circular	Monopartite, reverse transcribing	3–4
Hepeviridae	–	Isometric	27–34	Icosahedral	ssRNA (+), linear	Monopartite	7
Herpesviridae	+	Spherical	160–300	Icosahedral	dsDNA, linear	Monopartite, terminal and internal repeats, multiple isomeric forms	125–240
Orthomyxoviridae	+	Pleomorphic	80–120	Helical	ssRNA (–), linear	6–8 segments, depending on genus	10–15 (total)
Papillomaviridae	–	Isometric	55	Icosahedral	dsDNA, circular	Monopartite, supercoiled	7–8
Paramyxoviridae	+	Pleomorphic	150–300	Helical	ssRNA (–), linear	Monopartite	13–20
Parvoviridae	–	Isometric	21–26	Icosahedral	ssDNA, linear	Monopartite	4–6
Picobirnaviridae	–	Isometric	33–37	Icosahedral	dsRNA, linear	2 segments	4.2 (total)
Picornaviridae	–	Isometric	30	Icosahedral	ssRNA (+), linear	Monopartite, 5′-end covalently linked protein (VPg)	7–9
Polyomaviridae	–	Isometric	40–45	Icosahedral	dsDNA, circular	Monopartite, supercoiled	5
Poxviridae	+	Brick shaped or oval	140–260 × 220–450	Complex	dsDNA, linear	Monopartite, inverted terminal repeats, both ends covalently closed	130–375
Reoviridae	–	Isometric	60–85	Icosahedral	dsRNA, linear	10–12 segments, depending on genus	19–32 (total)
Retroviridae	+	Spherical	80–100	Icosahedral	ssRNA (+), linear	Monopartite, diploid, reverse transcribing	7–13
Rhabdoviridae	+	Bullet shaped	75 × 180	Helical	ssRNA (–), linear	Monopartite	11–15
Togaviridae	+	Spherical	60–70	Icosahedral	ssRNA (+), linear	Monopartite	10–12
Prions[c]	NA[d]	Rods	Protein PrP^{SC}	NA	NA	No nucleic acid; self-replicating infectious prion protein (PrP) with a mol wt of 33,000–35,000	NA

[a] +, positive sense; –, negative sense.
[b] Genus (unassigned family).
[c] Not classified by the ICTV.
[d] NA, not applicable.

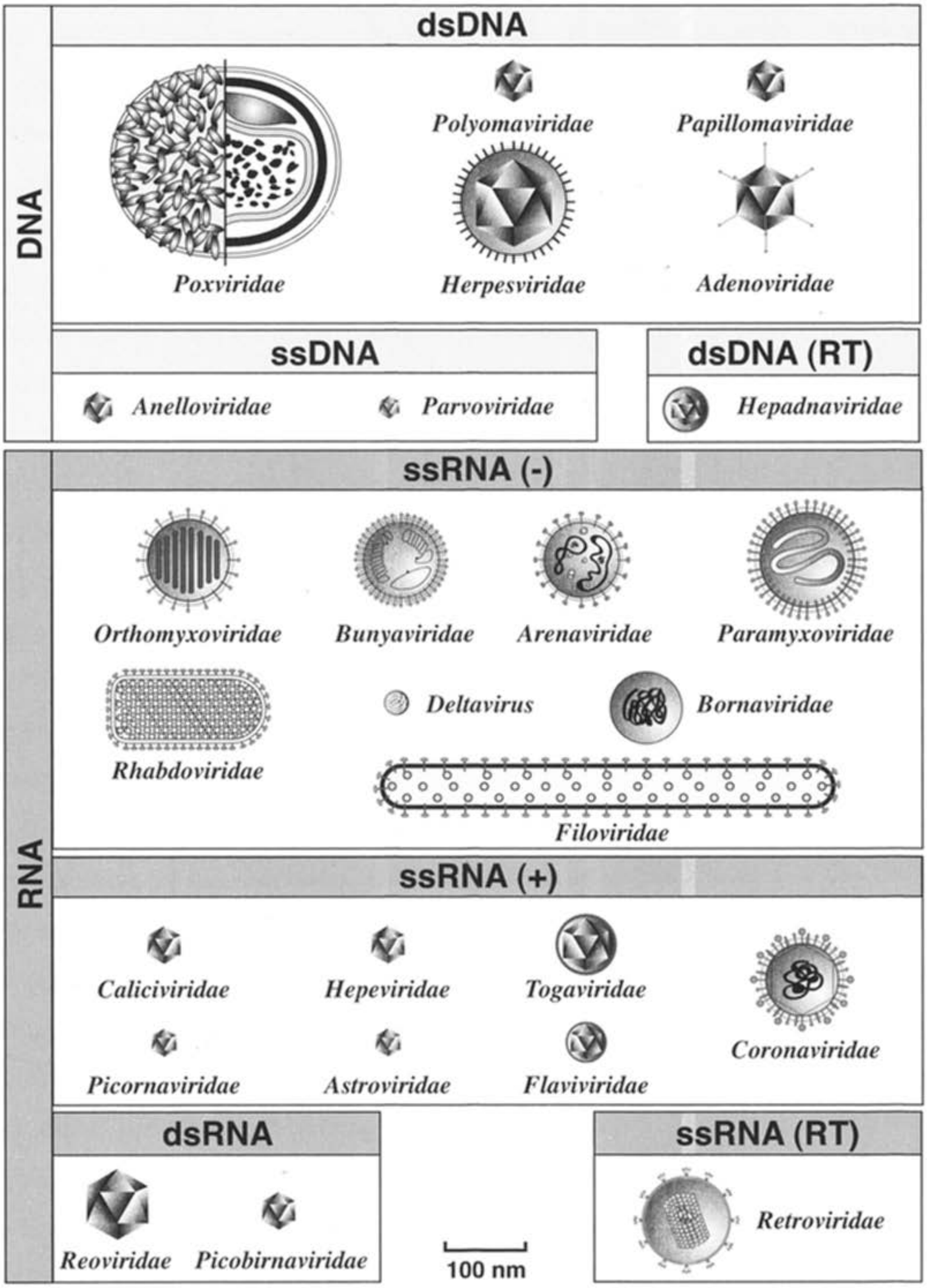
dsDNA
DNA
Poxviridae
Polyomaviridae
Papillomaviridae
Herpesviridae
Adenoviridae
ssDNA
Anelloviridae
Parvoviridae
dsDNA (RT)
Hepadnaviridae
ssRNA (-)
RNA
Orthomyxoviridae
Bunyaviridae
Arenaviridae
Paramyxoviridae
Rhabdoviridae
Deltavirus
Bornaviridae
Filoviridae
ssRNA (+)
Caliciviridae
Hepeviridae
Togaviridae
Coronaviridae
Picornaviridae
Astroviridae
Flaviviridae
dsRNA
Reoviridae
Picobirnaviridae
100 nm
ssRNA (RT)
Retroviridae

FIGURE 2 Virion morphology. Depiction of the shapes and sizes of viruses of families that include animal, zoonotic, and human pathogens. The virions are drawn to scale, but artistic license has been used in representing their structure. In some, the cross-sectional structure of capsid and envelope are shown, with a representation of the genome; for small virions, only their size and symmetry are depicted. RT, reverse transcribing; +, positive-sense genome; –, negative-sense genome. Reprinted with modification with permission of Academic Press from reference 14.
doi:10.1128/9781555817381.ch78.f2

of ICTV study groups for making taxonomic assignments (31, 32).

One final sequence-based analysis that is extensively utilized as a demarcation criterion for making taxonomic assignments is phylogenetic analysis (28, 33). Phylogenetic analysis utilizes a multiple-sequence alignment constructed from the nucleotide or amino acid sequence of a whole or partial genome or whole or partial protein sequence (with the exact parameters set for any particular taxon by the appropriate study group). This alignment is then used as the basis for phylogenetic reconstruction in which the evolutionary history of the virus isolates is inferred by applying one of a variety of possible phylogenetic prediction algorithms. The result is a phylogenetic tree showing branching patterns that reflect the evolutionary history of the individual isolates, with branch lengths that are proportional to the number of evolutionary changes that have occurred between each node (both internal and terminal) of the tree. Figure 1B displays a tree that shows the branching topology and distances for subfamilies and genera of the family *Poxviridae*. At this level, it is not possible to discern the species topology for any of the genera, but species-level arrangements can be visualized if, for example, individual species for the genus *Orthopoxvirus* are compared separately (Fig. 1C) (30). The phylogenetic position of a newly described virus can be compared to that of any existing isolate on these trees to determine taxonomic assignment in a manner similar to PASC. Close inspection of Fig. 1C also reveals that the differentiation between species (e.g., *Monkeypox virus* and *Ectromelia virus*) is clearly distinguishable from the differentiation between isolates of the same species (e.g., the Congo and West African monkeypox virus clades).

TAXONOMY OF HUMAN PATHOGENS

As categorized according to the 2013 ICTV taxonomy (16), viruses that infect humans fall into four orders: the *Herpesvirales*, *Mononegavirales*, *Nidovirales*, and *Picornavirales*. (The other existing orders, the *Caudovirales*, *Ligamenvirales*, and *Tymovirales*, contain only bacteriophages, archaea viruses, and plant viruses, respectively.) Human pathogens are further subdivided into 25 families (not all of which belong to an order), 13 subfamilies, and 65 genera (with each genus usually comprising multiple species). Table 3 provides an overview of the taxa that contain human pathogens along with representative species for each genus. Table 4 provides a few of the structural features that define each viral family. Finally, Fig. 2 displays stylized representations of virion morphology for each family.

TAXONOMY DATABASES

The ICTV

The ICTV produces an extensive amount of information during the process of classifying and naming viruses that is published regularly in the ICTV reports. The ICTV website provides a database of the most recent officially approved viral taxonomy since publication of the last reports (which occurs, on average, every 4 to 5 years). Additional features of the website include a searchable hierarchical list of the current taxonomy; a downloadable spreadsheet, the "Master Species List," that contains a listing of all taxa; access to past and present taxonomy proposals submitted for review to the ICTV Executive Committee; and a forum for discussion of ICTV-related issues. The ICTV also publicizes news and information regarding its efforts in the Virology Division News section of *Archives of Virology* (34). In addition, following approval of any updates to the taxonomy by the voting membership of the ICTV, an article that reviews all of the changes and additions is published in the *Archives of Virology* (16).

The NCBI

The National Center for Biotechnology Information (NCBI) provides access to a wide variety of databases containing various types of biological information (35). This includes GenBank, the primary repository of sequence data, including viral sequences (36). NCBI also provides RefSeq, a database of reference sequences derived from GenBank records (37). RefSeq contains one representative genomic sequence for each viral species. Viral RefSeq records have been extensively annotated by NCBI curators, and in many cases, they are also reviewed by investigators with expertise on individual viral species. Each viral sequence is linked to the NCBI taxonomic database using a taxonomy identification that is assigned to each taxon at every rank of the viral taxonomic hierarchy. The ICTV and NCBI taxonomies should be completely congruent; however, this has not always been the case. The ICTV and NCBI have therefore worked extensively in the past few years to update the NCBI viral taxonomy so that it reflects the official ICTV taxonomy, and this effort is mostly complete. Unfortunately, NCBI taxonomy is not automatically updated when new ICTV taxonomy is approved. A lag period of several months, therefore, exists before any officially approved ICTV taxonomy is fully represented in the NCBI taxonomy that is linked to GenBank and RefSeq sequence records.

FUTURE CHALLENGES

A number of challenges will affect viral taxonomic assignment now and in the future. These challenges include discovery of novel, previously unknown viruses (38, 39); consideration of the full complement of genetic mechanisms and machinery that viruses use to evolve, including recombination and horizontal gene transfer (40–43); management of vast increases in the amount of available information, such as data derived from metagenomic sequencing projects (44–46); determination of new data types (characters) for describing viruses; availability of only a limited set of characters for classification (such as solely sequence information) (47, 48); and creation of additional higher-level taxonomic ranks based on an increase in our knowledge of viral evolution (27, 28). Luckily, new analytical methods, new approaches to classification, and the dedication of virologists worldwide will allow us to handle these challenges and deal with future challenges as they arise.

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Specimen Collection, Transport, and Processing: Virology*

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Given that laboratory data significantly influence medical diagnoses, it is critical that all phases of testing (pre-, intra-, and postanalytical) be approached systematically to ensure the accuracy of results and to detect and correct potential errors. The preanalytical phase is often the most vulnerable part of the testing process and accounts for the majority of errors in laboratory diagnostics (1). Preanalytical steps for viral diagnostics involving specimen selection, collection, transport, and processing are described in this chapter, as well as details for collection of specific sample types. While these recommendations are general guidelines, laboratories must also follow manufacturers' instructions for testing of samples with commercially available kits and reagents.

Preanalytical variables should be controlled to maintain specimen integrity. Therefore, it is important for the laboratory to make available to all specimen-collecting areas within the institution, or other locations submitting samples, a comprehensive manual of tests available, indications for appropriate use, and methods of specimen collection. The manual should detail the appropriate (i) specimen types for specific clinical syndromes (Table 1); (ii) collection devices/containers and transport media, when indicated; (iii) collection techniques; (iv) volume of specimen required; (v) specimen labeling procedures; (vi) transport times and storage conditions; and (vii) clinical data, if available (2).

SPECIMEN SELECTION

Viruses infecting humans cause a wide range of diseases. Signs and symptoms of infection largely influence which specimens are collected for virus testing. Additional factors to be considered are epidemiologic factors (e.g. travel, exposures, and season) and the patient's immune status. It is important to recognize that viruses differ in their pathogenic mechanisms, their ability to disseminate from the primary site of infection, and which organ systems they infect. Generally, specimen selection is guided by the test best suited to establish a particular diagnosis (synopsized in chapter 81). The diagnosis of certain illnesses requires a basic understanding of viral pathogenesis, organ involvement, and viral epidemiology to determine the appropriate specimen(s) and tests. Specimen selection based on suspicion of a particular virus may be complicated by the fact that similar clinical syndromes can be caused by many different viruses (Table 1). By collecting only specimens needed to detect a specific virus, other important etiologies may be missed.

Depending on the anatomic site and means of collection, specimens are either nonsterile (i.e., contaminated with bacteria and/or fungi) or sterile. This determines the extent of specimen processing that occurs prior to viral culture, since bacteria and fungi grow rapidly in nutrient-rich cell culture systems. Non-culture-based test methods are less affected by this distinction. Sterile specimens are obtained from sites that are free of microorganisms in the absence of infection (e.g., blood, cerebrospinal fluid [CSF], and tissue) and avoid contact with nonsterile sites during the collection process. Identification of a virus in these sites is associated with a high likelihood of causality for disease. Nonsterile specimens are obtained from sites that contain normal biota (e.g., upper respiratory tract, skin, lower genital tract, and stool) or from sterile sites for which contact with nonsterile sites is unavoidable during specimen collection (e.g., sputum and voided urine).

SPECIMEN COLLECTION

Since no single diagnostic approach may be optimal for detecting every possible virus in a clinical situation, it may be necessary to utilize a combination of test methods to collect a number of different sample types and/or longitudinal samples for testing to yield the most medically useful information (2). Specimens should be collected as soon as possible after the onset of symptoms. For many viral infections in otherwise healthy and immunocompetent individuals, the likelihood of obtaining positive results is generally greatest within the first 3 days after onset of symptoms and diminishes rapidly as the course of infection proceeds (3). In cases of disseminated disease or in immunocompromised patients, viruses may be identified in clinical samples for prolonged periods (4). Generally, the level and duration of virus shedding depend on the virus, infected organ or organ system, and host factors such as age and immune status (5–7). Specimens with high concentrations of viral particles, viral antigens (Ags), or viral nucleic acids (NAs) improve the laboratory's ability to make an accurate diagnosis. Specimen collection techniques can greatly affect specimen quality and, therefore, test results. Collecting an adequate specimen is straightforward when fluids (e.g., CSF, blood, or urine) are sampled, as standard collection procedures are used and are not subject to great variability. However,

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TABLE 1 Specimens and methods for detection of viruses

Source and syndrome	Virus(es)[a]	Specimen(s)[b]	Test method(s)[c]
Cardiac			
Myocarditis/pericarditis	Adenoviruses	Blood, respiratory, tissue	Culture, histology, IA, NA
	CMV	Blood, tissue, urine	Culture, histology, NA
	Enteroviruses	Blood, respiratory, stool, tissue	Culture, NA
	Influenza viruses	Respiratory, tissue	Culture, IA, NA, serology
Cutaneous			
Exanthematous rash	Adenoviruses	Blood, respiratory	Culture, IA, NA
	EBV	Blood	NA, serology
	Enteroviruses	Blood, respiratory, stool	Culture, NA
	HHV-6	Blood	NA, serology
	Measles virus	Blood, respiratory, urine	Culture, serology
	Parvovirus B19	Blood	NA, serology
	Rubella virus	Blood, respiratory, urine	Culture, serology
Papillomas/papules	Molluscum contagiosum	Tissue	Histology
	Papillomaviruses	Tissue/cells	Histology, NA
Vesicular rash	Enteroviruses	Blood, respiratory, stool, vesicle	Culture, NA
	HSV	Lesion	Culture, IA, NA
	Poxviruses[d]	Blood, respiratory, tissue, vesicle	Culture, EM, histology, NA
	VZV	Respiratory, vesicle	Culture, IA, NA
Gastrointestinal			
Diarrhea	Adenoviruses 40 and 41	Stool	IA, NA
	Astroviruses	Stool	NA
	Caliciviruses	Stool	NA
	CMV	Blood, stool, tissue	Culture, histology, NA
	Human bocavirus	Stool	NA
	Parechoviruses	Stool	Culture, NA
	Rotavirus	Stool	IA, NA
Hepatitis	Adenoviruses	Blood, tissue	Culture, histology, IA, NA
	CMV	Blood, tissue	Culture, histology, NA
	EBV	Blood	NA, serology
	HAV	Blood	Serology
	HBV	Blood	IA, NA, serology
	HCV	Blood	NA, serology
	HDV	Blood	IA, serology
	HEV	Blood	Serology
	HSV	Blood, tissue	Culture, histology, IA, NA
Parotitis	Adenoviruses	Respiratory	Culture, IA, NA
	CMV	Blood, respiratory	Culture, NA
	EBV	Blood	NA, serology
	Enteroviruses	Blood, respiratory, stool	Culture, NA
	HIV-1	Blood	Serology
	Mumps virus	Blood, respiratory, urine	Culture, NA, serology
Hematologic			
Bone marrow suppression	CMV	Blood, bone marrow	Culture, IA, NA
	Dengue virus	Blood, bone marrow	NA, serology
	EBV	Blood, bone marrow	Histology, NA
	HAV	Blood	NA, serology
	HBV	Blood	NA, serology
	HCV	Blood	NA, serology
	HHV-6	Blood, bone marrow	NA
	HHV-8	Blood, bone marrow	Histology, NA
	HIV-1	Blood	NA, serology
	Parvovirus B19	Blood, bone marrow	NA, serology
Hemolytic anemia	CMV	Blood, bone marrow	Culture, IA, NA
	EBV	Blood, bone marrow	Histology, NA
	HBV	Blood	NA, serology
	HCV	Blood	NA, serology
Lymphoid disorders	EBV	Blood, bone marrow	Histology, NA
	CMV	Blood, bone marrow	Culture, IA, NA
	HIV-1	Blood	NA, serology
	HHV-8	Blood, bone marrow	IA, NA
	HTLV-1	Blood, bone marrow	NA, serology

(Continued on next page)

TABLE 1 *(Continued)*

Source and syndrome	Virus(es)[a]	Specimen(s)[b]	Test method(s)[c]
Neonatal/fetal	CMV	Blood, urine, amniotic fluid	Culture, NA
	Enteroviruses	Blood, CSF, urine	Culture, NA
	HBV	Blood	Serology
	HIV	Blood	NA, serology
	HSV	Blood, CSF, vesicle	Culture, IA, NA
	Parechoviruses	Blood, CSF, respiratory, urine	Culture, NA
	Parvovirus B19	Blood, amniotic fluid	NA, serology
	Rubella virus	Blood, urine	Culture, NA
	VZV	Amniotic fluid, blood, CSF, vesicle	Culture, IA, NA, serology
Neurologic			
Encephalitis	Arboviruses[d]	Blood, CSF	EM, NA, serology
	BK virus	CSF, tissue	NA
	CMV	CSF	NA
	EBV	CSF	NA
	Enteroviruses	CSF	Culture, NA
	HHV-6	CSF	NA
	HSV	CSF	NA
	HIV	CSF	NA
	Measles virus	CSF	NA, serology
	Mumps virus	CSF	Culture, NA, serology
	Parechoviruses	CSF	Culture, NA
	Rabies	Blood, CSF, saliva, tissue (nuchal)	IA, NA, serology
	VZV	CSF	NA
Meningitis	Arboviruses	Blood, CSF	NA, serology
	Enteroviruses	CSF	Culture, NA
	HSV	CSF	NA
	LCMV	CSF	Serology
	Mumps virus	CSF	Culture, NA, serology
	Parechoviruses	CSF	Culture, NA
	VZV	CSF	NA
PML[e]	JC virus	CSF, tissue	Histology, NA
Ocular			
Conjunctivitis	Adenoviruses	Eye, respiratory	Culture, IA, NA
	Enteroviruses	Eye, respiratory	Culture, NA
	HSV	Eye	Culture, IA, NA
Keratitis	Adenoviruses	Eye, respiratory	Culture, IA, NA
	Enteroviruses	Eye, respiratory	Culture, NA
	HSV	Eye	Culture, IA, NA
	VZV	Eye	Culture, IA, NA
Uveitis/retinitis	CMV	Eye	NA
	HSV	Eye	NA
	VZV	Eye	NA
Respiratory			
Bronchiolitis	Adenoviruses	Respiratory	Culture, IA, NA
	Enteroviruses	Respiratory	Culture, NA
	Human bocavirus	Respiratory	NA
	Human coronaviruses	Blood, respiratory	EM, NA, serology
	Human metapneumovirus	Respiratory	NA
	Influenza viruses	Respiratory	Culture, IA, NA
	Parainfluenza viruses	Respiratory	Culture, IA, NA
	RSV	Respiratory	Culture, IA, NA
	Rhinoviruses	Respiratory	Culture, NA
Croup	Human bocavirus	Respiratory	NA
	Human metapneumovirus	Respiratory	NA
	Influenza viruses	Respiratory	Culture, IA, NA
	Parainfluenza viruses	Respiratory	Culture, IA, NA
	Rhinoviruses	Respiratory	Culture, NA
	RSV	Respiratory	Culture, IA, NA

(Continued on next page)

TABLE 1 Specimens and methods for detection of viruses *(Continued)*

Source and syndrome	Virus(es)[a]	Specimen(s)[b]	Test method(s)[c]
Pharyngitis/upper respiratory infection	Adenoviruses	Respiratory	Culture, IA, NA
	EBV	Blood	Serology
	Enteroviruses	Respiratory	Culture, NA
	HSV	Respiratory	Culture, NA
	Human coronaviruses	Respiratory	NA
	Influenza viruses	Respiratory	Culture, IA, NA
	Parainfluenza viruses	Respiratory	Culture, IA, NA
	Rhinoviruses	Respiratory	Culture, NA
	RSV	Respiratory	Culture, IA, NA
Pleurodynia	Enteroviruses	Respiratory	Culture, NA
Pneumonia	Adenoviruses	Respiratory	Culture, IA, NA
	CMV	Blood, respiratory, tissue	Culture, histology, IA, NA
	Hantaviruses	Blood, tissue	NA, serology
	HSV	Respiratory, tissue	Culture, histology, NA
	Human coronaviruses	Blood, respiratory	NA, serology
	Human metapneumovirus	Respiratory	NA
	Influenza viruses	Respiratory	Culture, IA, NA
	Measles virus	Blood, respiratory, urine	Culture, serology
	Parainfluenza viruses	Respiratory	Culture, IA, NA
	Rhinoviruses	Respiratory	Culture, NA
	RSV	Respiratory	Culture, IA, NA
	VZV	Respiratory, tissue	Culture, histology, NA
Rhinitis/coryza	Adenoviruses	Respiratory	Culture, IA, NA
	Enteroviruses	Respiratory	Culture, NA
	Human coronaviruses	Respiratory	NA
	Human metapneumovirus	Respiratory	NA
	Influenza viruses	Respiratory	Culture, IA, NA
	Parainfluenza viruses	Respiratory	Culture, IA, NA
	Rhinoviruses	Respiratory	Culture, NA
	RSV	Respiratory	Culture, IA, NA
Urogenital			
Cervicitis/urethritis	Adenoviruses	Genital	Culture
	CMV	Genital, tissue	Culture, histology, NA
	HSV	Genital	Culture, NA
Genital warts, carcinoma	HPV	Genital, tissue	Histology, NA
Hemorrhagic cystitis	Adenoviruses	Urine	Culture, NA
	BK virus	Urine	NA
	CMV	Urine	Culture, NA
Herpetic lesions	HSV	Lesion	Culture, IA, NA
	VZV	Lesion	Culture, IA, NA
Orchitis/epididymitis	Mumps virus	Blood	Serology
Papules	Molluscum contagiosum	Tissue	Histology
Proctitis	HSV	Rectal	Culture, NA

[a] Refer to specific chapters for individual viruses; only common viral pathogens are listed. HAV, hepatitis A virus; HDV, hepatitis D virus; HEV, hepatitis E virus; HHV, human herpesvirus; HTLV-1, human T-cell lymphotropic virus 1; LCMV, lymphocytic choriomeningitis virus.

[b] Common specimens for viral diagnostics; refer to specific chapters for individual viruses. "Respiratory" includes throat and nasal swabs, nasal wash, nasopharyngeal aspirate, tracheal, and BAL specimens. "Eye" includes conjunctiva, cornea, and aqueous and vitreous fluids.

[c] Common detection methods used. EM, electron microscopy; IA, immunoassay such as immunofluorescence-antibody assay, enzyme-linked immunosorbent assay, or immunochromatographic test; NA, nucleic acid detection.

[d] Hemorrhagic fever viruses and smallpox virus processing in BSL4 facilities.

[e] PML, progressive multifocal leukoencephalopathy.

collection of swab specimens is prone to variability, which can affect the amount of live virus, viral NA, or viral Ag present on the swab.

For serologic diagnosis, the timing of serum collection and type of antibody detected vary for specific viruses. An acute-phase serum specimen should be obtained early in the course of illness for virus-specific IgM testing. Convalescent-phase serum for IgG testing should be obtained 2 to 4 weeks after the acute-phase specimen or symptom resolution, and both acute- and convalescent-phase specimens should be tested concurrently to identify any significant changes in antibody concentration. However, due to the delay in collecting a convalescent-phase specimen, the final result will generally not affect patient treatment. For some viruses (e.g., severe acute respiratory syndrome coronavirus), timing of the collection of the convalescent-phase specimen may extend beyond 4 weeks after the onset of symptoms to reliably rule out infection (8). Additionally, in disease due to reactivation of latent or persistent viruses, there may or may not be a detectable rise in IgM and/or IgG levels. Generally, IgM is more sensitive to freezing and thawing than is IgG, so repetitive freeze-thaw cycles should be avoided.

For serologic diagnosis of HIV, since the newer generation of immunoassays can detect acute infection prior to the detection of HIV-1/2-specific antibodies, a repeatedly reactive result should be confirmed by an HIV-1/HIV-2 antibody differentiation assay (e.g., Multispot HIV-1/HIV-2 Rapid Test; Bio-Rad Laboratories, Redmond, WA) and/or HIV-1 RNA assay (e.g., Aptima HIV-1 RNA Qualitative Assay; Gen-Probe, San Diego, CA) (9, 10). Hepatitis C virus (HCV) antibody testing cannot distinguish between persons whose past HCV infection has resolved and those who are currently HCV infected. Therefore, persons with reactive results after HCV antibody testing should be evaluated for the presence of HCV RNA in their blood (11). To distinguish between true positivity and biologic false positivity for HCV antibody, a second assay that is different from the first may be performed. Biologic false positivity is unlikely to be exhibited by more than one test when multiple assays are used on a single specimen (12).

Health care workers who collect specimens from patients should wear personal protective equipment as appropriate for standard, contact, airborne, or droplet precautions (13, 14). The laboratory should always be notified in advance if rare agents representing a danger to laboratory workers (e.g., hemorrhagic fever viruses or novel influenza viruses) are suspected. Samples in which such viruses are suspected should not be manipulated in laboratories lacking the ability to undertake such handling under the appropriate biosafety level (BSL) conditions. Some specimens can be handled under enhanced BSL2 conditions, or BSL3 practices for certain viruses (e.g., severe acute respiratory syndrome coronavirus). Likewise, laboratories should provide instructions to those collecting and submitting specimens from individuals suspected of harboring a non-BSL2 pathogen. These should generally not be cultured in routine clinical laboratories, but rather referred to the Centers for Disease Control and Prevention (CDC; Atlanta, GA). Manipulation of these specimens and tissues, including sera obtained from convalescent patients, may pose a serious biohazard and should be minimized outside of a BSL4 laboratory. Use of vacuum tube collection systems is considered safer than use of syringes and needles, which must be disassembled before their contents are transferred to another tube. Procedures that generate aerosols should be minimized. NA extraction procedures, conducted in a biosafety cabinet, have the advantage of inactivating viruses prior to analysis.

TRANSPORT MEDIUM

Depending on the specimen source and type of testing requested, it may be appropriate to place the sample in viral transport medium (VTM). Liquid specimens such as amniotic fluid, blood, CSF, urine, and bronchoalveolar lavage (BAL) fluid do not generally require VTM (15). Specimens that are susceptible to drying (e.g., swabs and tissue) must be kept moist in VTM or other buffered solutions designed to maintain the titer of infectious virions for culture and the stability of viral Ags for direct Ag tests or viral NAs for NA tests (NATs). However, molecular testing can be an exception, as certain NAs have been found to be quite stable in a desiccated state. Dried samples for NATs have the advantages of (i) relatively long storage capability at room temperature; (ii) reduced biohazard risk to laboratory personnel, with no spill risk and reduced infectivity; and (iii) simple and inexpensive ambient temperature shipping of specimens where maintenance of a cold chain is limited. However, the versatility of a liquid specimen offers obvious advantages (i.e., it can be split and tested by multiple methods). When a complete microbiologic workup is requested on a specimen, including viral, bacterial, mycobacterial, and fungal testing, VTM should not be added to the specimen. Rather, samples should be collected independently or aliquoted into the appropriate transport media.

The most useful types of transport systems should allow for simultaneous detection of viruses by any number of methodologies, including culture, Ag detection, and NA testing. Although some common viral agents can withstand extended storage in suitable transport media at room temperature, it is generally the case that virus viability decreases over time, and at higher temperatures, this rate of decay is often accelerated. To stabilize the rate of decay, viral transport systems are usually kept on ice until they can be processed for testing. The optimal transport medium should further stabilize virus viability with minimal loss of virus titer as well as contain components to control potential microbial contamination, have a long shelf life, and be readily available at a reasonable cost (15, 16). Formulations of VTM typically consist of a buffered salt solution such as Hanks' balanced salt solution buffered with HEPES to maintain a neutral pH, protein-stabilizing agents such as bovine serum albumin or gelatin, and antimicrobials to prevent bacterial and/or fungal overgrowth. Some laboratories have reported that media designed for collection and transport of bacteriology specimens are also acceptable for collection and transport of viral specimens. In infants with pneumonia, the sensitivity of NA testing for adenovirus, influenza viruses, and respiratory syncytial virus (RSV) in nasopharyngeal (NP) swabs stored and frozen in skim milk-tryptone-glucose-glycerol media ranged from 65.2 to 100% compared to results with NP aspirates in VTM (17). Under experimental conditions, bacteriologic swab transport systems allowed for NA detection of influenza A, echovirus 30, herpes simplex virus 2 (HSV-2), and adenovirus up to 5 days after storage at 4°C or room temperature, albeit with lower sensitivity than swabs in VTM (18).

VTM can be prepared in the laboratory or purchased (Table 2). An advantage of commercially prepared media is that the burden of quality control is shifted from the laboratory to the manufacturer (19). Some transport media also sustain the viability of chlamydiae, mycoplasmas, and ureaplasmas. The inclusion of sucrose in the media serves as a cryoprotectant to maintain the integrity of viruses if specimens are frozen (−70°C or lower) for prolonged periods. Laboratories should validate the suitability of their selected media for their particular applications, as performances can vary (19, 20).

A sample collection and transport device that contains an absorbent matrix for drying of specimens, ViveST (ViveBio, Norcross, GA), has been used primarily for preservation of NA at ambient temperature, although some viruses remain viable for up to 7 days with the addition of fetal bovine serum (21, 22). Influenza RNA was detectable in clinical specimens after 3 weeks of room temperature storage in ViveST devices with minimal loss in RNA recovery compared with frozen samples (23). CyMol (Copan Italia, Brescia, Italy), an alcohol-based transport medium for collection and preservation of cells for cytologic and NA testing, compared favorably with VTM for maintenance of influenza A viral RNA for up to 14 days at 4°C or colder (24). However, direct fluorescent-antibody (DFA) testing from swabs in VTM was more sensitive than for those in CyMol.

Manufacturers of commercial assays for NA testing or Ag detection either supply transport media or make recommendations for transport systems that are compatible with their test methods. The manufacturer's package insert should therefore be consulted for information on appropriate

TABLE 2 Commercial sources of transport media for viral diagnostics

Medium	Composition	Vol (ml)	Storage temp (°C)
BD Universal Viral Transport Medium[a] (BD Diagnostics)	Basal constituents,[b] gelatin, L-cysteine, sucrose, colistin, vancomycin	1 or 3	2–25
Copan Universal Transport Medium[a] (Copan Diagnostics)	Basal constituents, gelatin, L-cysteine, sucrose, colistin, vancomycin	1, 1.5, 3, 10	2–25
Copan Viral Transystem (Copan Diagnostics)	Balanced salt solution, sodium thioglycolate	1.2	2–25
CVM Transport Medium[a] (Hardy Diagnostics)	Basal constituents, gelatin, sucrose, colistin, vancomycin	1 or 2	2–8
FlexTrans Viral Transport Medium (Trinity Biotech)	Basal constituents, sucrose, gentamicin, streptomycin	2	2–8
Meridian Viral Transport[a] (Meridian Bioscience)	Tryptose phosphate broth, gelatin, gentamicin	1.5	5–25
MicroTest M4[a] (Remel)	Basal constituents, gelatin, sucrose, colistin, vancomycin	3	2–8
MicroTest M4RT[a] (Remel)	Basal constituents, gelatin, sucrose, gentamicin	3	2–30
MicroTest M5[a] (Remel)	Basal constituents, protein stabilizers, sucrose, colistin, vancomycin	3	2–8
MicroTest M6[a] (Remel)	Basal constituents, gelatin, sucrose, colistin, vancomycin	1.5	2–30
Multitrans Medium[a] (Starplex Scientific)	Basal constituents, gelatin, sucrose, sodium bicarbonate, colistin, vancomycin	3	2–25
Puritan Universal Transport Medium[a] (Puritan Diagnostics)	Basal constituents, gelatin, sucrose, colistin, vancomycin	1 or 3	2–25
Sigma VCM medium[a] (Medical Wire & Equipment)	Balanced salt solution, HEPES, disodium hydrogen orthophosphate, sucrose, lactalbumin hydrolysate, colistin, vancomycin, amphotericin B	1, 1.5, 3	5–25
Sigma Virocult medium[a] (Medical Wire & Equipment)	Balanced salt solution, disodium hydrogen orthophosphate, lactalbumin hydrolysate, chloramphenicol, amphotericin B	1 or 2	5–25
ViraTrans Medium (Trinity Biotech)	Basal constituents, gentamicin, penicillin, streptomycin	2	2–8
Virocult medium[a] (Medical Wire & Equipment)	Balanced salt solution, disodium hydrogen orthophosphate, lactalbumin hydrolysate, chloramphenicol, amphotericin B	1.2	5–25

[a] Product available as swab-transport tube combination.

[b] Basal constituents: Hanks' balanced salt solution, bovine serum albumin, L-glutamic acid, HEPES buffer (pH 7.3), phenol red, and amphotericin B.

collection and transport systems. Most VTM products are compatible with NA and Ag detection tests (15, 25).

TRANSPORT CONDITIONS

Once a clinical specimen has been collected, it should be transported to the laboratory as soon as possible, since virus viability decreases with time (i.e., some clinically important viruses are more labile than others). The optimal time and temperature for transport, processing, and storage (discussed below) of specimens should be adhered to in order to generate accurate and meaningful results. Typically, specimens for viral culture should be transported to the laboratory promptly, ideally within 2 to 4 h of collection, since overall diagnostic yield improves when specimens are expeditiously processed for viral culture. The preferred transport temperature is 2 to 8°C, except for blood specimens, which can be transported at ambient temperature. Enzymes present in specimens and capable of inactivating viruses or degrading proteins and NAs are less active at refrigeration temperatures. For longer transport times or submission to reference laboratories, specimens should be stored and shipped under conditions that would preserve the integrity of the sample, preferably −70°C or lower. Viability is not a requirement for Ag or NA detection methods; therefore, transport time may be less significant, unless degradation of intact cells or viral NA is a consideration. However, for timely diagnosis, delay should be avoided during transport of these specimens to the laboratory.

SPECIMEN STORAGE AND PROCESSING

Accredited laboratories are required to specify rejection criteria for specimens that are collected, transported, or stored under improper conditions. Specimens that are unacceptable for testing include those that are (i) unlabeled or improperly labeled, (ii) received in improper or leaking containers, (iii) not appropriate for a particular test, (iv) transported under improper conditions, or (v) received beyond the acceptable transport time limit. At a minimum, the specimen container should be labeled with the patient's full name, medical record number or unique identifier, date and time of collection, specific source, and the name of the collector. Each specimen should be accompanied by a requisition containing the same information as on the specimen container as well as the patient's location, test(s) requested, source of specimen, requesting clinician with contact information, and suspected clinical diagnosis (26). Specimens that are deemed unacceptable but cannot be re-collected (e.g., tissue collected during surgery) may be processed and tested according to the laboratory's clearly defined procedure allowing such exceptions.

Specimens submitted for virus isolation can be held at refrigeration temperature (2 to 8°C) for up to 2 days prior to inoculation onto cell culture. Many viruses lose infectivity at ambient or even refrigeration temperature. For example, 90% of RSV infectivity is lost after 4 days at 4°C and 24 h at 37°C (27). Freezing of specimens (−70°C or lower) is not recommended for virus isolation unless specimen processing will not occur within 2 days. The temperature in standard freezers (−20°C) is not low enough to maintain virus infectivity. Certain viruses may also lose infectivity with repeated freeze-thaw cycles, particularly enveloped viruses. Detailed specimen processing protocols for viral culture have been described (28, 29). Specimens should be processed using all appropriate biosafety guidelines (30), and a biosafety cabinet should be used when manipulating specimens suspected to contain virus(es) considered to be contagious by airborne routes.

Molecular assays are particularly sensitive to substandard preanalytical conditions. For RNA targets such as HIV or HCV, the Clinical and Laboratory Standards Institute recommends that EDTA whole-blood samples be centrifuged and the plasma removed to a secondary tube within 4 h of phlebotomy, a conservative time frame that may vary depending on the assay manufacturer's requirements (31). However, studies have shown that for HIV-1 viral load testing, whole blood with EDTA and cell-free EDTA plasma can be stored at room temperature for up to 30 h, at 4°C for up to 14 days, and at −70°C for extended periods of time without significant decreases in viral load signal (32, 33), and HCV RNA is stable in EDTA whole blood for at least 24 h at room temperature (34). HIV proviral DNA can be detected in acid citrate dextrose (ACD)- or EDTA-treated whole blood for up to 10 days at both 4°C and ambient temperature (35). The preanalytical steps for selected Food and Drug Administration (FDA)-cleared/approved NATs are listed in Table 3.

Using RNAlater (Qiagen, Gaithersburg, MD), an RNA stabilization buffer, HCV and HIV plasma viral loads were stable for up to 28 days at 37°C and HCV viral loads were essentially equivalent after 5 days when transported either frozen or in RNAlater at ambient temperature (36). Under experimental conditions, nonenveloped and enveloped viruses, including HIV-1, retained infectivity after more than 72 h of storage at room temperature in RNAlater (37). Arboviral RNA was stable for at least 35 days when virus-containing samples were stored at 4 or −20°C in AVL buffer (Qiagen), a lysis buffer for NA purification that contains guanidine isothiocyanate and RNase inhibitors (38). AVL buffer has the added property of inactivating viral infectivity. Enterovirus RNA was shown to be stable in CSF specimens stored at 4 and −80°C for up to 2 weeks, but the half-life when stored at room temperature was calculated to be 9 days. In NP wash specimens, there was a >65% reduction in influenza A RNA concentration when samples were maintained at room temperature, 4°C, or −80°C for 2 weeks (39). If no specific RNase inhibitors are used, long-term storage of diagnostic RNA samples should be done preferably at −70°C or lower to inhibit RNase activity, as RNase may limit the stability of RNA even in frozen samples at −20°C (40).

For DNA targets such as cytomegalovirus (CMV), the data on DNA quantification after storage are conflicting, with some studies reporting no decline in CMV DNA levels after 24 h (41), 72 h (42), and 2 weeks (43), while another

TABLE 3 Processing and storage conditions for blood specimens with selected FDA-cleared/approved NATs

Test (manufacturer)	Specimen types	Preseparation conditions	Short-term storage of plasma/ serum	Max no. of freeze-thaw cycles
Aptima HCV RNA Qualitative (Gen-Probe)	EDTA, ACD, citrate, heparin plasma, PPT, or serum	≤24 h, 15–30°C	≤48 h, 2–8°C; or ≤−20°C	3
Aptima HIV-1 RNA Qualitative (Gen-Probe)	EDTA, ACD, citrate plasma, PPT, or serum	≤3 days, ≤25°C	≤5 days, 2–8°C; or ≤−20°C	3
COBAS Amplicor HCV (Roche)	EDTA plasma or serum	≤6 h, 2–25°C	≤3 days, 2–8°C; or −70°C	3
COBAS Amplicor HIV-1 (Roche)	EDTA or ACD plasma	≤6 h, 2–25°C	≤24 h, 15–30°C; ≤5 days, 2–8°C; or −70°C	3
COBAS AmpliPrep/COBAS TaqMan CMV (Roche)	EDTA plasma	≤6 h, 2–25°C	≤7 days, 2–8°C; or ≤6 wk, ≤−20°C	3
COBAS AmpliPrep/COBAS TaqMan HBV (Roche)	EDTA plasma or serum	≤24 h, 2–25°C	≤3 days, 15–30°C; ≤7 days, 2–8°C; or ≤6 wk, −20 to −80°C	5
COBAS AmpliPrep/COBAS TaqMan HCV (Roche)	EDTA plasma or serum	≤6 h, 2–25°C	≤3 days, 2–8°C; or ≤6 wk, ≤−70°C	5
COBAS AmpliPrep/COBAS TaqMan HIV-1 (Roche)	EDTA plasma	≤6 h, 2–25°C	≤24 h, 15–30°C; ≤5 days, 2–8°C; or −20 to −80°C	5
RealTime HBV (Abbott)	EDTA plasma or serum	≤6 h, 2–30°C	≤24 h, 15–30°C; ≤3 days, 2–8°C; or ≤−70°C	1[a]
RealTime HCV (Abbott)	EDTA plasma or serum	≤6 h, 2–30°C	≤24 h, 15–30°C; ≤3 days, 2–8°C; or ≤−70°C	3[a]
RealTime HIV-1 (Abbott)	EDTA or ACD plasma	≤6 h, 15–30°C; or ≤24 h, 2–8°C	≤24 h, 15–30°C; ≤5 days, 2–8°C; or ≤−70°C	3[a]
Versant HCV RNA 3.0 (Siemens)	EDTA or ACD plasma, serum	≤4 h, 15–30°C	≤48 h, 2–8°C; ≤3 days, −20°C; or ≤−60°C	4
Versant HIV-1 RNA 3.0 (Siemens)	EDTA or ACD plasma or PPT	≤4 h, EDTA or ACD; ≤2 h, PPT	≤48 h, 2–8°C; ≤24 h, 15–30°C (PPT only); or ≤−60°C	3

[a] Thaw specimens at 15–30 or 2–8°C; once thawed, specimen may be stored at 2–8°C for ≤6 h.

showed increased levels in blood stored at room temperature or at 4°C prior to plasma separation (44). The increased levels of CMV DNA in plasma separated beyond 2 h after collection may be falsely positive in latently infected patients due to the lysis of leukocytes and release of CMV into plasma during storage. In another study, CMV DNA in EDTA blood was stable for 2 weeks when stored at room temperature, 4°C, and −80°C, whereas CMV DNA in serum had a half-life of <1, 2, and 3 days when stored at room temperature, 4°C, and −80°C, respectively (39). At both 5 and 25°C storage conditions, there was no statistically significant decline in hepatitis B virus (HBV) DNA concentration in plasma for up to 28 days (33). For HSV, the quantity in oral and genital specimens stored in VTM for 16 months at −20°C was within 1 log of the original concentration for >90% of specimens (45). In CSF specimens, HSV DNA was detectable by PCR for up to 30 days when stored at temperatures ranging from 23 to −70°C (46). Human papillomavirus (HPV) DNA stored in two commercial, liquid-based cytology media at 2 to 8°C was stable for >2 years. However, the reproducibility of results after storage may be affected by the methods of NA extraction and detection (47). Generally, DNA in tissue is stable for up to 24 h at 4°C, for at least 2 weeks at −20°C, and for at least 2 years at −70°C or lower (31).

No exhaustive studies exist that evaluate the stability of NAs in stool specimens, although rotavirus RNA has been shown to be stable for up to 2.5 months at ambient temperature (48). Adenovirus DNA in fresh stool collected on SDS-EDTA-treated chromatography paper strips was detected after 4 months of storage at −20 to 37°C (49), and under experimental conditions, strips containing various concentrations of norovirus were stable for reverse transcription-PCR detection for up to 2 months at temperatures ranging from −80 to 37°C (50).

COLLECTION METHODS AND PROCESSING OF SELECTED SPECIMENS

For commercially available, FDA-cleared/approved assays, laboratories must follow manufacturers' instructions for collection and processing of specimens. Assay performance must be verified by the individual laboratory prior to implementing any change in the preanalytical steps described in the manufacturer's package insert (51, 52). For laboratory-developed tests, each test site must establish and validate appropriate processing and storage conditions, the extent of which may rely on studies performed by the laboratory or those in published sources.

Amniotic Fluid

Although rare, viral infections during pregnancy may be detrimental to the fetus or newborn. Testing amniotic fluid for viral pathogens is generally based on maternal history or ultrasound-guided indications. Fluid is collected by amniocentesis, and 2 to 5 ml should be submitted to the laboratory in a sterile container. NA detection methods are the most commonly used. NAs from samples should be extracted as soon as possible and stored at 2 to 8°C for up to 48 h or frozen at −70°C or lower if testing is delayed beyond 48 h (31). Virus can be isolated in culture, but sensitivity is generally lower than with NAT.

For the most accurate prenatal diagnosis of congenital CMV infection, amniotic fluid should be collected at between 21 and 23 weeks of fetal gestation (53, 54). If maternal varicella-zoster virus (VZV) infection occurs during the first or second trimester, VZV DNA may be detected in amniotic fluid, but its presence is not necessarily indicative of the development of congenital varicella syndrome. The presence of parvovirus B19 in amniotic fluid should be correlated with other clinical and prenatal diagnostics since up to 25% of asymptomatic fetuses may have detectable virus (54).

Blood

Serum, plasma, purified peripheral blood leukocytes, and whole blood have all been used to detect and quantify viral pathogens. The most suitable blood compartment for testing depends on the virus targeted. Quantitative molecular methods performed on blood samples are useful in assessing patient prognosis, treatment response, and antiviral resistance. For viral load monitoring over time, it is particularly important that the sample matrix is not varied. Which blood compartment to test depends on the underlying condition of the patient and whether the viral target dictates that a cellular or cell-free portion be utilized (55–58).

Approximately 3 to 5 ml of blood should be collected in the appropriate anticoagulant vacuum tube. For pediatric specimens, smaller volumes are acceptable, but <1 or 2 ml may be inadequate for testing. The most commonly used anticoagulants are EDTA, heparin, and ACD. Anticoagulated blood may be fractionated to allow recovery of leukocytes (see below). EDTA and ACD are the preferred anticoagulants for obtaining plasma for NA testing, as heparin is inhibitory to many NA amplification chemistries (31). In addition, frozen EDTA plasma specimens have improved NA stability compared with heparin (32).

Whole blood used for NA amplification must be processed to remove inhibitors of DNA and RNA polymerases such as heme and metabolic precursors of heme. Many different extraction protocols are used in both commercial and laboratory-developed amplification tests to remove inhibitors and purify NAs (31). Plasma is obtained by centrifuging blood collected in tubes containing spray-dried anticoagulant (EDTA) at 1,500 × *g* for 20 min at 25°C. Plasma Preparation Tubes (PPTs; BD, Franklin Lakes, NJ) have spray-dried EDTA and a gel barrier that results in physical separation of plasma and cellular constituents, eliminating the need for decanting plasma. The use of PPTs for HIV-1 RNA quantitation have shown falsely elevated viral loads from centrifuged tubes that were stored frozen or for prolonged periods at 2 to 8°C (59–61), likely due to the presence of cells containing proviral DNA (62, 63). This limitation can be overcome by an additional centrifugation step prior to testing or use of an extraction method that recovers only RNA (64, 65). No decline in HCV RNA levels was observed in blood stored in PPT at 4°C for 72 h (66).

When serum is required for serologic or molecular testing, 4 to 8 ml of blood is collected in a serum separator tube (e.g., SST [BD Diagnostics], Corvac [Covidien, Dublin, Ireland], or Vacuette [Greiner Bio-One, Monroe, NC]) or a tube without anticoagulant. After allowing blood to coagulate for 30 min, tubes are centrifuged at 1,000 to 1,300 × *g* for 10 min. The serum fraction, removed to a sterile tube, can be stored at 2 to 8°C for up to 48 h or frozen at −20°C or lower for longer periods. Whole blood drawn for antibody determination should not be frozen.

For virus isolation, 5 to 10 ml of anticoagulated blood (EDTA or heparin) should be collected by venipuncture and transported to the laboratory at room temperature. Processing of the specimen for leukocyte fractionation should occur within 8 h. Whole blood should not be used to inoculate cell cultures because of toxicity caused by red blood cells. The buffy coat fraction may also contain erythrocytes.

To adequately remove red blood cells and recover both polynuclear and mononuclear cells for inoculation, density gradient methods should be used, a number of which are commercially available (28). Isolated leukocytes can be cocultured with human diploid fibroblasts such as MRC-5 cells (tube or shell vial culture) or H&V-Mix FreshCells (Diagnostic Hybrids, Athens, OH) (shell vial culture) (67), directly stained with fluorescence-labeled monoclonal antibodies (e.g., CMV pp65 antigenemia assay) (68), or extracted to detect viral genomes by molecular techniques. Quantifying leukocyte input is essential for optimizing assay performance (69, 70). For recovery of CMV in culture, the shell vial technique is more rapid and sensitive than conventional tube culture (71).

Bone Marrow

Bone marrow can be used to identify viral etiologies of hematologic disorders, including hemophagocytic lymphohistiocytosis, aplastic anemia, and chronic pure red cell aplasia (72, 73) (Table 1). At least 2 ml of bone marrow aspirate should be collected in the appropriate anticoagulant tube. The recommended anticoagulants for NA detection tests include EDTA and ACD (31). Although the diagnostic yield may be low, bone marrow aspirates for culture should be collected in a syringe or vacuum tube with heparin or EDTA and processed to fractionate the leukocytes as soon as possible after collection (74, 75). Bone marrow specimens should be stored at 2 to 8°C prior to NA extraction. Freezing and thawing of bone marrow lyses red cells, causing release of heme, a known inhibitor of PCR (76).

Cerebrospinal Fluid

CSF is an important specimen for diagnosis of viral central nervous system infections (Table 1). CSF is collected using sterile technique, by inserting a spinal needle into the L4-L5 interspace, located in the midline between the left and right iliac crest. Preferably, 2 to 5 ml should be collected in a sterile, leak-proof container. VTM is not added to sterile body fluids such as CSF, because they do not require antimicrobial treatment and dilution in transport media may cause false-negative results. Transport and store for up to 48 h at 2 to 8°C, and freeze at −70°C or lower for longer-term storage. NAs should typically be extracted prior to testing since CSF contains globulins, cell-derived proteins, and other uncharacterized substances that inhibit the activity of thermostable polymerases used in PCR (77, 78). However, some studies have demonstrated the suitability of simple methods such as exposure to high temperature or repetitive freeze-thawing to release NAs (79, 80). For virus isolation, specimens with visible blood may affect recovery of viruses since antibodies may be present in sufficient concentration to inhibit viral growth. No treatment of CSF is needed before inoculation of cell cultures.

Dried Blood Spots

Use of dried blood spot (DBS) specimens eliminates the need for venipuncture and allows for greater flexibility in storage temperature and transport time. DBS samples have been used in NATs for diagnosis of and monitoring of therapeutic response in HIV infection, HBV quantification and genotyping, diagnosis of neonatal HSV infection, and detection of congenital CMV infection. Antibodies to viruses such as hepatitis A virus, HBV, HCV, Epstein-Barr virus (EBV), measles virus, and rubella virus can be detected in DBSs, although the performance characteristics vary compared with serum testing depending on the virus. DBS card samples can be prepared from a few drops of blood, typically 15 to 200 µl, obtained from a capillary blood stab, i.e., a finger prick (81). A minimum of 2 h is needed for air drying of the blood spot. Once dried, DBSs should be placed in hermetically sealed bags or containers with sufficient desiccant to minimize moisture exposure that could allow for growth of microorganisms (31). DBSs may be transported and stored at room temperature for relatively long periods (depending on the virus) or frozen at −20°C or lower for longer-term storage. Punched-out disks from DBS cards normally contain the equivalent of 3.1 to 12.4 µl of blood depending on the punch size (3 to 6 mm) (81). Once dried, HIV-1 RNA in a DBS is stable at ambient temperature or −70°C for at least 1 year and the virus is typically inactivated (82). Compared with plasma viral loads, >90% of finger-stick DBS samples from solid-organ transplant recipients showed <1 $\log_{10}$ deviation in CMV viral load and the kinetics of CMV viral load during antiviral therapy were comparable (83). In this study, the 95% limit of detection of CMV in DBSs was estimated at 2,700 copies/ml (675 IU/ml).

Eye

Eye specimens include swabs or scrapings of the conjunctiva, corneal scrapings, and vitreous and aqueous fluids. The conjunctiva should be cleared of any exudate present with a swab before a specimen is collected. Using a second flexible, fine-shafted Dacron or rayon swab premoistened with sterile saline, infected cells from the clean conjunctiva can be obtained and transferred to a vial of VTM or used to prepare a slide for immunofluorescent staining. The AdenoPlus test (Rapid Pathogen Screening, Inc., Sarasota, FL), a lateral-flow immunochromatographic cartridge test for the detection of ocular adenovirus infection, uses a built-in sampling pad to touch the eye and collect fluid from the conjunctiva. Test sensitivity for the AdenoPlus has been reported to be 85%, with a specificity of 98% compared to PCR (84). Corneal scrapings and swabs of the cornea are best collected and placed in VTM by a specially trained clinician. Thin smears may be made directly from scrapings or swab specimens for use in immunofluorescent staining. Retinal pathogens are detected in the aqueous and vitreous fluids by NA testing, as they are not easily cultivated from these samples. Direct testing of these surgically obtained fluids has resulted in PCR inhibition (85); therefore, the original specimen must be extracted.

Genital

The most common viral causes of genital lesions are HSV-1 and -2, which are easily detected by direct Ag (DFA) methods, NATs, or culture. Cells and fluid from genital ulcers are collected and processed as lesions from skin (discussed below).

Testing of HPV genotypes associated with a high risk of developing cervical cancer (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) is performed using molecular methods on cervical specimens collected with swabs or brushes (detailed in chapter 104). Specimens may be collected in manufacturer-specific systems or FDA-approved/cleared liquid-based cytology solutions used concomitantly for Pap smear preparation. Freshly collected cervical biopsy specimens, at least 2 to 5 mm in cross section, may be analyzed in some commercially available NATs. All specimens should be placed immediately into the specimen transport medium provided by the assay manufacturer and stored, transported, and processed according to their directions. Patient-collected cervicovaginal swab or lavage specimens (86–89) and swabs of penile lesions (90, 91) have also proven reliable for detection of high-risk HPV infection. Anal specimens for HPV testing can be collected using a saline-moistened Dacron swab and transported in liquid-based cytology media (92, 93).

Cervical brush specimens in standard transport medium for the hc2 High-Risk HPV DNA Test (Qiagen) may be stored for up to 2 weeks at room temperature, after which specimens can be stored for an additional week at 2 to 8°C or for up to 3 months at −20°C. Specimens in PreservCyt liquid-based cytology medium may be held at 2 to 30°C for up to 3 months for hc2 testing, 20 to 30°C for up to 18 weeks for Cervista (Hologic, Bedford, MA) testing, and 2 to 30°C for up to 6 months for COBAS HPV (Roche Diagnostics, Indianapolis, IN) testing. For the Aptima HPV Assay (Gen-Probe), cervical specimens in ThinPrep Pap Test vials containing PreservCyt solution should be transported and stored at 2 to 30°C, with no more than 30 days at temperatures above 8°C, and, for longer storage, at −20°C for up to 24 months. PreservCyt specimens cannot be frozen prior to testing for high-risk HPV.

Oral

An oral specimen provides a noninvasive means of detecting infection, and for some viruses, the onset of salivary shedding can predict recent acquisition. The different types of oral specimens include oral mucosal cells (including lesions), whole saliva, glandular duct saliva (from parotid, sublingual, and submandibular glands), and oral mucosal transudates (OMTs). OMTs can be used for both culture and NAT methods (94). Oral mucosal cells are dislodged with a swab or plastic spatula; the collection device is then placed in VTM for transport to the laboratory. Saliva is collected by initially tilting the head forward and catching fluid from the lower lip into a collection container and then by catching residual, expectorated fluid after 5 min (94). Parotid gland saliva, useful for diagnosing infectious parotitis, is collected with a swab ~30 s after massaging the area between the cheek and teeth at the level of the ear (95).

Salivary gland fluid consists primarily of secretory IgA, whereas OMTs contain a mixture of secretory IgA, IgG, and IgM. OMTs (also called gingival crevicular fluid, crevicular fluid saliva, or crevicular fluid) arise from the capillaries in the buccal mucosa and the base of the pocket between the teeth and gums (gingival crevice) and can be collected with commercially available devices and transported to the laboratory at 2 to 8°C (96). OMTs have been used for detection of both IgM and IgG antibodies to measles, mumps, rubella, HIV, and hepatitis viruses (97–99). The performance characteristics of oral fluid for the determination of antibodies to HIV and HCV approach those of blood-based specimens (100, 101). However, HIV antibody testing with oral fluid alone is not recommended for patients at high risk of infection, particularly in low-prevalence settings, and should be followed up by testing of a blood specimen (101).

Respiratory

Viruses causing upper and lower respiratory tract infections can be detected using Ag detection, culture, and NATs (Table 1). Ideally, diagnostic testing should be completed within a time frame (generally 24 h) that can affect patient management (102).

The primary site of replication for many respiratory viruses is the ciliated epithelial cells of the posterior nasopharynx and, to a lesser extent, the anterior nares and oropharynx. Therefore, nasal and throat specimens are traditionally not acceptable for virus detection because many viruses are present at low levels in these sites. However, these specimens may be suitable for use in highly sensitive NATs or when immunocompromised patients may be at risk of bleeding with harsher collection methods.

An NP aspirate is collected by inserting a narrow catheter or tube through the nostril into the posterior nasopharynx. A mucus trap or syringe is connected to the other end, and suction is applied while slowly withdrawing the tube back through the nostril. Any secretions remaining in the tubing should be flushed into the trap or syringe by aspirating VTM or sterile saline (28, 29). A nasal wash is collected by instilling several milliliters of sterile saline into the nasal cavity using a bulb or syringe with catheter tubing attached. The contents are immediately aspirated by releasing the bulb or pulling the syringe plunger and placed into a tube containing VTM or sterile container. Nasal washes can yield high rates of respiratory virus detection by NAT with minimal patient discomfort compared with swab, aspirate, and brush sampling (103).

Swabs for respiratory virus testing should be polyester, Dacron, or rayon with plastic or aluminum shafts. Wooden-shaft swabs may contain substances that are toxic to cultured cells. Calcium alginate swabs should not be used since they may impair recovery of enveloped viruses, may interfere with fluorescent-antibody tests, and are inhibitory to some NATs. Flocked swabs, made from nylon fiber using a proprietary spray-on technology, are designed for optimum specimen absorption and release and have been shown to collect more respiratory epithelial cells than conventional rayon swabs for DFA testing of respiratory viruses (104, 105). Midturbinate flocked swabs are designed with a tapered cone shape, a sampling depth indication gauge, and a greater length and diameter of flocked nylon in order to sample a larger surface area of respiratory mucosa, including the inferior and middle turbinate bones. These can be self-collected and yield comparable numbers of respiratory epithelial cells to nasal and NP swabs; in children, they compared favorably with NP aspirates for DFA detection of common respiratory viruses (106, 107). Polyurethane foam-tipped swabs provide an alternative to nylon or Dacron swabs for sampling of the anterior nares in patients who might be at risk for bleeding. Self-collected foam nasal swabs have been shown to be more sensitive than nasal washes for detection of several respiratory viruses by NAT in immunocompromised patients, and they performed better than nylon flocked swabs for rapid influenza Ag testing in children (108, 109).

A swab of the posterior nasopharynx typically yields more virus than a swab of the anterior nares or throat. Here, the flexible, fine-tipped swab is inserted through the nostril along the floor of the nose into the nasopharynx until resistance is felt, then rotated several times, removed, and placed in VTM (28). The swab should be pointed toward the ear rather than toward the top of the head and traverse half the distance between the angles of the nares and pinna. For DFA or NA testing, the sensitivity of flocked NP swabs for detection of respiratory viruses compares favorably to nasal wash or NP aspirate specimens (110, 111). Throat swabs alone are generally inferior specimens for diagnosis of upper respiratory tract infections. However, combined testing of throat swabs with either NP swabs or aspirates can improve the yield of diagnostic etiologies of respiratory infection, as can the testing or pooling of other types of respiratory specimens (111–113). Throat swabs are collected by depressing the tongue and swabbing the tonsillar area and posterior pharynx thoroughly.

Although the oropharynx and nasopharynx are common portals for the introduction of viruses into the respiratory tract, the presence or absence of a virus in the upper respiratory tract may not be sufficient evidence of lower respiratory tract disease, as evidenced by cases of severe influenza infection in which upper respiratory tract samples tested negative for the presence of virus while those from the lower respiratory tract had detectable virus (114, 115). BAL specimens are collected by inserting a fiber-optic bronchoscope into the involved segment of the lung, instilling saline, and applying suction to remove the lavage specimen. BAL specimens for NA testing should be transported and tested within 24 h of collection, stored at 4°C for up to 72 h, or frozen at −70°C or lower for future testing (31). Although sputum is not a particularly worthwhile specimen for virus isolation, it is often submitted because of the ease of collection and need for other microbiologic analyses. Sputum can be a suitable specimen for respiratory virus detection using NATs (reviewed in reference 116) and has been shown to increase the diagnostic yield over combined nasal and throat specimens in adults with respiratory illness (117). However, viruses identified in sputum do not necessarily represent lower respiratory tract infection since they may originate in the oropharynx. Sputum for NATs should be collected in a sterile container and transported to the laboratory at room temperature within 30 min or, if longer, transported at 4°C (31).

Respiratory fluids (e.g., NP aspirates and nasal washes) or swabs in VTM for culture should be vortexed to release cell-associated virus and centrifuged at 500 to 1,000 × *g* for 10 min unless there is a minimal amount of cellular debris. The supernatant can be used directly as inoculum or further clarified by filtration (0.45-μm pore size) to remove any additional bacterial, fungal, and/or cellular debris. The pelleted cells (washed two or three times in phosphate-buffered saline) may be used for immunofluorescent staining (28, 29). Application of the concentrated cell suspension to a slide by cytospin preparation can increase the sensitivity of immunofluorescent staining and reduce the number of inadequate specimens (118). Centrifuged or filtered specimens are not suitable for Ag or NA detection since these assays should not be affected by microbial contamination. The loss of host cells may compromise Ag test sensitivity, and NATs have not been approved for use with acellular specimens.

Mucus in respiratory specimens can significantly affect Ag detection. In fluorescent-antibody tests, mucus can inhibit adherence of cells to slides and can cause nonspecific fluorescence. It also prevents penetration of the sample into filtration devices. To prevent these complications, mucus threads can be broken by repeated aspiration through a small-bore pipette.

Skin

Viruses can cause rashes with many different appearances, including maculopapular, petechial, and vesicular. Recovery of viruses from maculopapular and petechial rashes requires biopsy of skin, which is not routinely performed. Viral causes of disseminated diseases manifested by maculopapular and petechial rashes are usually identified by their clinical features and laboratory testing of specimen types other than skin; often blood is used for serologic or NA testing (Table 1) because the virions are not typically found in those lesions, resulting from the host immune response to infection (119).

Vesicular lesions are commonly caused by HSV, VZV, and enteroviruses. Cells and fluid from fresh vesicles are preferred over other lesion types such as pustules, ulcers, and crusts, which may not contain sufficient virus for Ag detection, NA testing, or culture. Vesicular fluid can be collected by use of capillary pipettes, syringes, or on swabs placed in VTM. Vesicles that have not been opened may have fluid contents withdrawn by means of a sterile tuberculin syringe fitted with a 26- or 27-gauge needle. Prior preparation of the area with disinfectants such as iodophors or alcohol may inactivate the viruses. Therefore, use local disinfection after the specimen has been collected. Promptly rinse fluids collected by syringe aspiration into VTM. Alternatively, vesicles may be uncapped using a sterile needle or scalpel and the vesicle fluids absorbed onto the tip of a sterile swab and placed in VTM. The margins and base of the lesion should be swabbed briskly to obtain infected epithelial cells (29). Cells from ulcers or crusted lesions (after removal of the crust) should be collected by rolling a swab over the same area. Specimens should be collected without causing bleeding, since the presence of neutralizing antibody in blood can impair recovery. Smears for immunofluorescent staining (e.g., HSV and VZV) can be prepared at bedside by spreading the material thinly onto a small area in the center of a clean slide. After air drying, slides should be fixed in acetone for 5 to 10 min prior to staining. Swabs or vesicle fluid in VTM can be tested by NATs, Ag detection, and/or culture. Specimens for viral culture can be inoculated directly after vortexing or first clarified by centrifugation and filtration if debris is present.

Stool and Rectal Swabs

Stool is the optimal specimen for identification of viruses causing gastroenteritis. Many gastroenteritis viruses (e.g., rotavirus) are noncultivable and require Ag or NA tests for detection. Ideally, specimens should be collected within the first 2 to 4 days of illness since detection rates are reduced in later stages of infection unless the patient's underlying condition allows for prolonged shedding. However, most enteroviruses can be recovered in stool for several weeks after onset of symptomatic infection.

Approximately 2 to 5 ml of liquid stool or 2 to 5 g of formed stool should be collected in a clean, dry, leak-proof container or in VTM, depending on the type of testing to be performed. Fresh stool specimens can be stored at 4°C for 2 to 3 days if they are not tested immediately after collection. For prolonged storage, specimens should be kept frozen, preferably at −70°C or lower. Specially formulated paper strips have also been used for collection, transport, and storage of stool samples for NA testing of rotavirus, adenovirus types 40 and 41, and norovirus (49, 50, 120). The rectal swab is generally considered an inferior specimen to stool, as it usually collects an insufficient amount of specimen; the utility is directly related to the amount of visible stool collected. For collection, the swab is inserted several centimeters past the anal sphincter, rotated for several seconds, removed, and placed in VTM.

As bacteria comprise a significant proportion of the mass of stool, centrifugation, filtration, or both are often necessary to prevent microbial contamination of cell cultures. For virus isolation, prepare a 10 to 20% (wt/vol) stool suspension in VTM with antimicrobials and glass beads, vortex for 1 min, and centrifuge the suspension at 3,000 × *g* for 15 to 30 min. The clarified supernatant can be filtered through a 0.2- to 0.45-μm-pore-size filter prior to inoculation of cell cultures (28, 29).

Tissue (Biopsy or Autopsy)

Tissue specimens obtained during surgery or at autopsy can be assessed by several diagnostic methods. Lung, liver, lymph node, kidney, spleen, cardiac, and brain tissue can be used to identify the viral etiology of many clinical syndromes (Table 1). Due to the invasive nature of specimen collection, the laboratory should ensure that a sufficient quantity of tissue is available for all testing requested; otherwise, testing can be prioritized by the clinician. The tissue can be divided in the laboratory in consultation with the pathologist to determine which portions should be submitted for viral diagnostics (121). A small piece of tissue from the leading edge of the affected area should be excised and kept moist in a sterile container, typically with sterile saline if comprehensive microbiologic and histologic testing is requested. Formalin-fixed tissue is unsuitable for viral isolation and may affect the performance of NATs (31). Touch preparations of cells can be made by pressing tissue against the clean surface of a glass slide multiple times. Once air dried, the slide is fixed in acetone prior to application of staining reagents.

If NA is to be extracted, biopsies or large tissue specimens should be kept moist with sterile normal saline or placed in a suitable NA preservative. While the stability of NAs in tissue specimens varies with tissue type, it is recommended to transport tissues to the laboratory on wet ice or frozen (31). Fresh tissue specimens for NA detection are minced, treated with proteolytic enzymes, and extracted. Formalin-fixed, paraffin-embedded tissues for NA testing should be stored and transported at ambient temperatures, deparaffinized, and extracted. However, fresh tissue is superior for NA recovery since formalin treatment induces considerable NA degradation (31). For viral isolation from tissue, it is recommended to prepare a 10 to 20% (wt/vol) homogenate using VTM as a diluent from small or minced tissue fragments that have been aseptically ground in a tissue grinder (28, 29). The homogenate can be centrifuged (600 to 900 × *g* for 10 min) or allowed to settle by gravity and the supernatant used as inoculum.

Urine

Collect urine specimens as soon as possible after the onset of illness or when congenital or perinatal infection is first suspected. A volume of 5 to 10 ml of urine (midstream or catheter obtained) is collected in a sterile container; no VTM is required. The nature and timing of the collection is not known to have any effect on the reliability of testing methods. For NATs, ambient storage of fresh, unprocessed urine should be minimized since the low pH and high urea content rapidly denature DNA and RNA (31). NAs should be extracted before testing since urine contains substances that can inhibit PCR (122–124). NAs may be extracted from urine obtained on filter paper disks, or the disks may be used directly in the NAT as a template without additional purification or elution steps (125–127).

Prior to inoculating cell culture, urine can be filtered (0.45-μm pore size) or centrifuged (1,000 × *g* for 10 min) to remove bacteria and debris; the pH can be neutralized with sodium bicarbonate (7.5% solution) to reduce toxicity (28). Alternatively, antibiotics can be added directly to the urine or it can be diluted with VTM to neutralize pH and introduce antimicrobials (29). Allow the mixture to stand at room temperature for 15 min prior to inoculation of cell cultures.

TRANSPORTATION REGULATIONS

Shipment of specimens to reference or public health laboratories is common practice. All packages that contain infectious substances must meet the shipping regulations of various organizations or agencies. In the United States, the main source for regulations governing specimen and biological shipments is the U.S. Department of Transportation. The International Air Transport Association regulates all shipments on air carriers and provides its own *Dangerous Goods Regulations* (128). Since there is some harmonization between the national and international regulatory bodies, the International Air Transport Association requirements are usually acceptable under all regulations.

The *Dangerous Goods Regulations* include specific instructions for packaging and labeling of shipments containing biological or infectious substances such as patient specimens or cultured microorganisms (128). In the clinical laboratory, materials being shipped generally fall into one of three categories: exempt human specimen, category A, or category B. Category A infectious substances (UN2814 infectious substance, affecting humans) are those transported in a form that, when exposure to it occurs, can cause potentially life-threatening illness (e.g., Ebola virus). A list of category A microorganisms is available at www.phmsa.dot.gov. An infectious substance that does not meet the criteria for inclusion in category A is assigned to category B (UN3373 biological substance, category B) unless it is a patient specimen for which there is minimal likelihood that pathogens are present, in which case it would be labeled an exempt human specimen.

For transportation of any category A infectious substance, packaging must include an inner container, an itemized list of contents, and an outer packaging. The inner packaging must comprise one or more leak-proof primary receptacles and leak-proof secondary packaging capable of withstanding an internal pressure differential of at least 95 kPa. For liquid specimens, an absorbent material in sufficient quantity to absorb the entire contents must be placed between the primary and secondary packaging. Category B packing instructions are essentially identical to those for category A, although no packing list need be included. Exempt patient specimens are packaged with leak-proof primary and secondary receptacles within an outer container of adequate strength. Training of personnel for packaging and shipping dangerous goods must be documented, and recertification is required every 2 years. The Department of Transportation may inspect any shipper or receiver of dangerous goods unannounced at any time.

SUMMARY

The importance of appropriate specimen collection and handling to ensure accurate laboratory results cannot be overstated, and the laboratory must serve as a resource for clinicians. In some instances, proper collection, transport, and processing of specimens is determined by the manufacturer, whereas those laboratories that utilize in-house-developed tests need to verify these preanalytical factors themselves prior to routine, clinical testing (51).

The performance characteristics of viral diagnostic tests (sensitivity, specificity, positive predictive value, and negative predictive value) are, to a large extent, dependent on the integrity of viral or host components present in the specimen. For molecular methods, successful testing is based largely on the quality of the NA purified from the clinical specimen. That quality is directly related to how the specimen is stored and transported to the laboratory after it has been collected from the patient. Specimens for detection of viral Ags require that adequate amounts of intact cellular material from the site of infection be collected and maintained to prevent degradation. Serologic testing is affected by the timing of collection relative to disease progression.

Successful recovery of virus in culture requires maintenance of viability, which can be enhanced by (i) the timing and method of collection to incorporate high titers of virus, (ii) protection from thermal inactivation or drying, and (iii) use of an effective transport system.

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Reagents, Stains, Media, and Cell Cultures: Virology

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80

INTRODUCTION

In 1915, vaccinia virus was first propagated in cell culture for the purpose of vaccine production (1). However, the potential role of cell culture for clinical diagnostics was not highlighted until 1949, when Enders et al. (2) first described the use of cultivated mammalian cells and the observation of cytopathic effect (CPE) for the detection of polioviruses. Today, living cells are used to support the growth of a number of cell-dependent organisms, including viruses and certain bacteria such as *Chlamydia* spp. and, more rarely, *Mycoplasma* spps. In addition, cultured cells can be used to demonstrate the effects of bacterial toxins excreted from pathogens such as *Shigella* spp., toxigenic *Escherichia coli*, including O157:H7, and toxigenic *Clostridium difficile*, among others. Traditional tube cell culture and rapid cell culture methods (e.g., shell vial) are dependent on the interactions of viruses with a variety of animal, human, and/or insect cells and utilized within the laboratory setting as substrates for growth, identification, and enumeration of pathogenic viruses (3, 4).

In the era of molecular detection and quantification of viral pathogens, the applicability of viral isolation has been questioned. For many viruses, it is well documented that molecular detection methods are preferred (i) for their greater sensitivity; (ii) for their faster potential for detection and reporting (hours versus overnight or days to weeks); (iii) for their ability to quantify more accurately the amount of virus present in the sample; and (iv) in instances where viral culture may place the laboratory at risk due to the highly pathogenic nature of the virus (e.g., variola virus, Ebola virus, and avian influenza virus). However, cell culture methods have useful applications (i) when the potential viral agent is not known; (ii) when the cost of other methods of testing is greater than that of cell culture; (iii) for documentation of active infection; (iv) to perform antiviral susceptibility testing; (v) to assess response to antiviral treatment by the detection of viable virus; (vi) for serologic strain typing; (vii) for vaccine and therapeutic clinical trials; and (viii) for laboratories that do not have the ability to perform molecular detection methods. Some molecular assays, for practical reporting purposes, may take as long as overnight cell culture and may not lend themselves to single-specimen testing as well as cell culture does. For these reasons, cell cultures are still an indispensible research and clinical laboratory tool, particularly when combined with the use of highly specific monoclonal antibodies (MAbs) for the detection of common viruses and *Chlamydia* spp. or when cell lines are engineered to produce virus-induced enzymes (5), such as β-galactosidase for the detection of herpes simplex viruses (HSV) (6).

This chapter describes the cell lines, reagents, stains, and media used in association with traditional tube and rapid viral culture techniques. Included are examples of both well-characterized and emerging infectious viral agents that may be encountered while working with viral culture. Sample collection, specimen processing, and culture requirements for individual or classes of viruses are discussed in the appropriate chapters. The reader is referred to a comprehensive review of cell culture (7) and reference document M41-A from the Clinical and Laboratory Standards Institute (Wayne, PA), which provides guidance for viral culture methods, including the applicable biosafety measures required (8). The fourth edition of the *Clinical Virology Manual* lists the virology services offered by federal reference laboratories (9) and state public health laboratories (10). This chapter's appendix lists the manufacturers and suppliers of cell lines, media, and reagents referred to throughout the chapter.

REAGENTS

Balanced salt solutions (Hanks' and Earle's)

Hanks' balanced salt solution and Earle's balanced salt solution (EBSS) are the two most commonly used formulations. However, Hanks' balanced salt solution has a better buffering capacity with CO_2 and EBSS has a better buffering capacity with air.

Density gradient media

Density gradient media or cell preparation tubes (BD, Franklin Lakes, NJ) are used for the isolation of peripheral blood mononuclear and polymorphonuclear lymphocytes. Separated cells can be used for the direct detection of viruses, such as cytomegalovirus (CMV), using immunostaining methods. Detailed descriptions of the specific uses of the gradient media or tubes and commercial sources are listed in chapter 79.

Dulbecco's PBS

Dulbecco's phosphate-buffered saline (PBS) is a maintenance-type medium containing sodium pyruvate and glucose.

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HEPES

HEPES is an organic chemical-buffering agent that maintains physiological pH despite changes in carbon dioxide concentration, in contrast to bicarbonate buffers. HEPES is used in culture media.

Formalin for cell culture preservation

Formalin can be used to preserve viral CPE in cell culture for both educational and research purposes.

Earle's minimal essential medium........................	81 ml
Formaldehyde (37 to 40% concentration)..........	30 ml

Fill CPE-positive culture tubes with the solution, seal, and store at room temperature.

HAD test

Hemadsorption (HAD) refers to the attachment of red blood cells (RBCs) to infected cell culture monolayers. Influenza A and B; parainfluenza 1, 2, 3, and 4; and mumps viruses possess a surface hemagglutinin protein that is expressed on the cell surface of infected cells (11). The hemagglutinin protein binds RBCs and adsorbs them to the infected cell membrane. HAD may be performed when there is no visual CPE in culture or as a rapid screen for the presence of an orthomyxovirus or paramyxovirus in cell culture with a suspicious CPE. HAD testing is usually performed at 3 to 7 days of incubation or at the end of the incubation period (generally 10 to 14 days). MAb staining of the cell monolayer for infectious agents such as influenza and parainfluenza has largely replaced HAD procedures in most clinical laboratories because staining tends to be more specific and provide more rapid results, which can be critical to patient care.

Test procedure

Variations in HAD testing may occur from laboratory to laboratory. Positive- and negative-control tubes must be included when the test is performed.

1. Wash fresh guinea pig (GP) RBCs two or three times in PBS weekly, discarding the supernatant after each centrifugation. Prepare a final 4 to 10% GP-RBC stock suspension in PBS and store at 4°C. The GP-RBC suspension should be used within 7 days.
2. Using the 10% GP-RBC stock suspension, prepare a 0.4% suspension prior to testing (0.4 ml of the 10% suspension plus 9.6 ml of PBS).
3. Remove the cell culture tube media and replace with 1 ml of cold PBS. Add 0.2 ml of the 0.4% GP-RBC suspension to the tube cultures and incubate at 4°C for 30 min.
4. Shake each tube and examine the tubes at 40× and 100× lens objectives for HAD to the monolayer or hemagglutination in the supernatant. Place HAD-positive tubes in the incubator at 37°C for 1 h to release the adsorbed RBCs.
5. To detect parainfluenza virus type 4, place HAD-negative tubes at room temperature for 30 min and reexamine for HAD.

Saline

Normal or physiological saline (0.85%) is commonly used as a diluent.

10× Gentamicin-amphotericin B solution

Several different combinations of antibiotics and amphotericin B are added to transport media, particularly for pretreatment of specimens such as stool prior to culture inoculation, or are used in refeed medium to reduce both bacterial and fungal contamination. Commercial media containing the appropriate strength of antibiotics and amphotericin B can be purchased. Alternatively, a 10× gentamicin-amphotericin B solution can be added by the laboratory at a ratio of 1:10 (0.1 ml of 10× gentamicin-amphotericin B to every 1.0 ml of specimen). Following receipt by the laboratory of the transport medium containing a specimen, centrifugation may be helpful to reduce cellular artifact overlayers or bacterial or fungal contaminants; however, this is not always required. The specimen supernatant is inoculated onto the appropriate cell line(s).

Eagle's minimal essential medium (EMEM)	89 ml
Gentamicin (50 mg/ml)	1 ml
Amphotericin B (250 µg/ml)	10 ml

Combine all ingredients and store frozen at −20 to −70°C in working-size aliquots.

Trypsin solutions

In lieu of scraping cells from tubes or wells, trypsin solutions are used to disburse cells from the monolayer for repassage and for immunostaining of cell-associated viruses such as adenovirus, CMV, and varicella-zoster virus (VZV). Trypsin solutions made with 2.5% PBS or Versene EDTA solution are commercially available.

Tween 20-PBS

Tween 20-PBS may be used to wash cell monolayers prior to staining with fluorescent MAbs, but is not always required. Follow manufacturers' directions for specific staining protocols if using a commercially available product. Tween 20-PBS is stored at room temperature and should be discarded if the solution is turbid or a precipitate develops.

VIROLOGY STAINS

Direct examination of clinical specimens using several methods, such as slide touch preps from unfixed tissue from an excision or biopsy, cytologic examination of tissue scrapings (e.g., Tzanck assay for HSV), smears from mucous membrane scrapings (e.g., HSV or VZV), or sample concentration by cytospin or centrifugation (e.g., respiratory viruses), can provide relatively rapid results. Slides can be prepared at the bedside (e.g., skin scrapings for HSV or VZV) or in the laboratory (e.g., respiratory swabs in transport media) and fixed with 80 to 100% reagent-grade acetone, 95% alcohol, or a cytological fixative, depending on the method. Traditional staining with hematoxylin and eosin and Wright-Giemsa stains can demonstrate characteristic cell morphologies such as the "owl's eye" nuclear inclusions indicative of CMV or the "smudge cells" that contain large basophilic inclusions consistent with adenovirus. Today, the definitive identification of certain viruses or *Chlamydia* spp. directly in clinical samples is mainly done using MAbs labeled with fluorescein isothiocyanate (FITC), methylrhodamine isothiocyanate, or phycoerythrin (PE) with an Evans blue and/or propidium iodide counterstain. Results are available within 15 to 60 min. Additionally, direct fluorescent-antibody (DFA) or indirect fluorescent-antibody (IFA) testing is used for confirmation of viruses isolated in cell culture and for blind staining when using centrifugation-enhanced virus isolation methods (discussed in subsequent sections). Procedures require minimal equipment (incubator, fluorescent microscope, pipettes, and centrifuge) and moderate technical expertise to perform and interpret the results (reviewed in reference 7). Commercial reagents, commonly provided at working strength and quality tested to ensure sensitive and specific reactions, cleared by the Food and Drug Administration for *in vitro* diagnostic testing are listed in Table 1. Reagents may be cleared for use with direct specimen testing or for culture confirmation, and in some instances for both applications. Various formats of MAb reagents are available for immunofluorescence testing methods that (i) detect *Chlamydia* spp. (Fig. 1) and *Chlamydophila* spp. but do not differentiate; (ii) target a single virus (Fig. 2); (iii) detect and differentiate two or more viruses (Fig. 3); (iv) target a single virus and contain a pool of MAbs that detect multiple additional viruses but do not differentiate (Fig. 4); and (v) detect a family of viruses or multiple viruses using a pool of MAbs but do not differentiate (Fig. 5). Both the location of the fluorescent staining (e.g., cytoplasmic versus nuclear) and the staining pattern (e.g., speckled versus homogenous) can aid in virus differentiation (Fig. 3).

CELL CULTURES

Good manufacturing practices-regulated commercial vendors (see the appendix) provide tissue culture cells that are sterile, stabilized at the proper pH, thoroughly tested for susceptibility to common pathogens, and carefully screened to be free of potentially harmful endogenous agents, such as foamy viruses and mycoplasmas that will interfere with the detection of the intended pathogens. Monolayered, ready-to-use cells can be produced at the vendor facility within a day or two of order, allowing the laboratory flexibility in the quantity and delivery date. Culture cells are provided in a number of ready-to-use formats including traditional 16- by 125-mm glass round-bottom screw-cap tubes, 1-dram vials (shell vials), flasks, or cluster trays. Ready-to-use cell cultures have a shelf life that is defined by the manufacturer in days or weeks, depending on the cell line or intended pathogen to be recovered. Some cells are also supplied frozen in shell vials and may be stored for months or years at −70°C or below, ready to use with a simple thaw step and change of medium. Other frozen cells are supplied in cryovials and other containers at a stated density and require the laboratory to subculture them to obtain monolayers in a flask, multiwell plate, shell vial, or tube, depending on the end application (12). Frozen cells can be used as needed and for unexpected situations such as unanticipated increases in the volume of samples, sudden viral outbreaks, or delays in cell shipments.

For laboratories that require special cells not readily available from commercial sources, specific cell types can sometimes be obtained from research laboratories or the American Type Culture Collection (Manassas, VA) and then propagated within the user laboratory. Once the cells are received, the laboratory must confirm sterility, absence of mycoplasma contamination, and the appropriate passage or cell duplication number necessary to ensure sensitivity for viral or chlamydial isolation. Cell cultures must be maintained in an environment that allows appropriate cellular replication and proper utility for the tests desired (13). A number of references offer detailed procedures for growing cells from frozen or fresh flasks (4, 8); such procedures are not addressed in this chapter.

To ensure the safety of the technical staff and to prevent cell culture contamination, the laboratory must follow strict procedures for the handling of biohazardous materials throughout the testing process (8, 13). These include (i) the use of class II or higher biological safety cabinets with HEPA filters and, if possible, external venting, certified at least annually; (ii) facilities and procedures that are appropriate to the biohazard level of the viruses tested as defined by the Centers for Disease Control and Prevention (Atlanta, GA) (14); and (iii) training and annual competency assessment of the laboratory staff. Virology benches and safety cabinets should be disinfected at least daily with a high-level disinfectant, such as 10% sodium hypochlorite (bleach).

Traditional cell culture

The common culturable human viral pathogens causing significant infections are readily detected by using a variety of cell types, including the established cell lines listed in Table 2. Cell lines may be primary (e.g., rhesus monkey kidney and rabbit kidney), used for one or two passages; diploid (e.g., human embryonic lung), used for 20 to 50 passages; or heteroploid (e.g., human epidermoid lung carcinoma), which can be passaged indefinitely. The laboratory must maintain sufficient cell types and incubate cell cultures for an optimal length of time and under the appropriate conditions that permit the recovery of the potential range of detectable viruses for all specimen types processed by the laboratory (8, 13, 15). Cell culture systems can be variable and are susceptible to conditions that can adversely affect results, including cell culture source and lineage, age and condition of the monolayer, number of passages, shipping conditions, and the presence of contaminating agents. For example, in 2012, cultures of rhesus monkey kidney cells distributed to clinical laboratories throughout the United States were found to be contaminated with *Coccidioides immitis* that was endogenous to the harvested monkey kidney (16, 17). As a result of rapid communication of this information and adherence to laboratory safety procedures, there were no adverse effects in terms of personnel infection with this highly infectious fungus (16). Therefore, quality control procedures and specific testing guidelines must be followed (8, 13, 18). Shipments of cell culture material should be observed microscopically to confirm that the confluency of the monolayer is appropriate (75 to 90%); the cells are attached to the substratum; cell appearance is typical; and no evidence of contaminating viruses, bacteria, or fungi is present, usually signified by cytopathic appearance of the cells before use or turbidity of the cell medium. Cell culture media that will be added to newly shipped cells should also be free of contamination (clear) and near a neutral pH (salmon pink in color). If the laboratory introduces additives (e.g., L-glutamine or antibiotics) to commercial media, the final solution must be checked for sterility, pH, growth promotion, and the absence of toxicity to cells. The lot number and date of use for all media, buffers, reagents, and additives should also be recorded. Tubes of cell cultures should be stored in a slanted position with the cell monolayer covered by the medium. Tissue culture cells should ideally be inoculated within 7 days of receipt (8 to 10 days of seeding) for optimal propagation of cell-dependent organisms or demonstration of cytotoxicity. The laboratory should retain all documentation provided by the manufacturer, including cell culture records with cell types, source, passage number, and age of cells. Uninoculated lot-matched tubes, cluster plates, or shell vials that are incubated, maintained, and observed in the same manner as inoculated tissue cells serve as negative controls for CPE, toxicity, exogenous contamination, and procedures such as DFA, HAD, and hemagglutination. Daily inoculation of positive controls to monitor traditional tube culture performance

TABLE 1 Commercially available DFA and IFA reagents for the detection of chlamydiae and viruses[a]

Target[b]	Use as per manufacturer	Manufacturer and test name
Chlamydia spp.	DSD	Trinity Bartels Chlamydia DFA kit
Chlamydia spp.	CC	Trinity Bartels Chlamydia CC FA kit
C. trachomatis	CC	Trinity Biotech MicroTrak *C. trachomatis* Culture Confirmation
C. trachomatis	DSD	Trinity Biotech MicroTrak *C. trachomatis* Direct Specimen Kit
Chlamydia spp.	CC	Bio-Rad Pathfinder Chlamydia
C. trachomatis	DSD	Bio-Rad Pathfinder *C. trachomatis* Direct Specimen
Chlamydia spp.	CC	Quidel/Diagnostic Hybrids D^3 DFA Chlamydia
Chlamydia spp.	CC	Meridian Merifluor Chlamydia
Chlamydia spp.	DSD and CC	Remel Imagen Chlamydia
CMV	DSD	Argene CMV CINA Antigenemia Kit
CMV	DSD	Millipore Light Diagnostics CMV pp65 Antigenemia IFA Kit
CMV	CC	Millipore Light Diagnostics CMV DFA Kit
CMV	CC	Millipore Light Diagnostics CMV IFA Kit
CMV	CC	Quidel/Diagnostic Hybrids D^3 DFA CMV IE Kit
CMV	CC	Trinity Bartels CMV IEA Indirect Antibody Test
CMV	DSD and CC	Trinity Bartels CMV Fluorescent Monoclonal Antibody Test
CMV	CC	Trinity Biotech MicroTrak CMV Culture Identification Test
Enterovirus	CC	Millipore Light Diagnostics Pan-Enterovirus IFA
Enterovirus groups	CC	Millipore Light Diagnostics Enterovirus Blends IFA
Enterovirus	CC	Quidel/Diagnostic Hybrids D^3 IFA Kit
Enterovirus	CC	Remel Imagen Enterovirus
HSV-1/2 detection	DSD and CC	Millipore Light Diagnostics SimulFluor HSV 1/2 Kit
HSV-1/2 and VZV	DSD and CC	Millipore Light Diagnostics SimulFluor HSV/VZV
HSV species	CC	Quidel/Diagnostic Hybrids D^3 HSV ID
HSV-1/2 typing	CC	Quidel/Diagnostic Hybrids D^3 HSV Typing
HSV-1/2 species	CC	Quidel/Diagnostic Hybrids ELVIS HSV Identification
HSV-1/2 typing	CC	Quidel/Diagnostic Hybrids ELVIS HSV ID and Typing
HSV-1/2 detection and typing	CC	Remel Imagen Herpes Simplex Virus
HSV detection	CC	Trinity Bartels HSV Fluorescent Monoclonal Antibody Test
HSV-1/2 detection and typing	CC	Trinity Bartels HSV ID and Typing Kit
HSV detection	CC	Trinity Biotech MicroTrak HSV Culture ID Test
HSV-1/2 detection and typing	CC	Trinity Biotech MicroTrak HSV1/HSV2 Culture ID/Typing Test
HSV-1/2 detection and typing	DSD	Trinity MicroTrak HSV1/HSV2 Direct Specimen ID/Typing
VZV	DSD	Meridian Merifluor VZV
VZV	DSD and CC	Millipore Light Diagnostics VZV DFA Kit
VZV	CC	Quidel/Diagnostic Hybrids VZV Detection
Respiratory panel B pool	DSD and CC	Bartels Respiratory Viral Pool
Respiratory panel B/identification	CC	Bartels Viral Respiratory Screening and Identification Kit
RSV	DSD	Bartels RSV Direct Fluorescent Antibody Test
Respiratory panel B	CC	Millipore Light Diagnostics SimulFluor Respiratory Screen
Respiratory panel B	CC	Millipore Light Diagnostics Respiratory Screen DFA Kit
Respiratory panel B	CC	Millipore Light Diagnostics Respiratory Screen/ID DFA Kit
Influenza viruses A and B	DSD and CC	Millipore Light Diagnostics SimulFluor Flu A/B
Parainfluenza viruses 1, 2/3	CC	Millipore Light Diagnostics SimulFluor Para 1, 2/3
Parainfluenza viruses 1, 2, 3, adenovirus (dual)	CC	Millipore Light Diagnostics SimulFluor Para 1, 2, 3, /Adeno
Parainfluenza virus 4	DSD	Chemicon International
RSV/influenza virus A (dual)	DSD and CC	Millipore Light Diagnostics SimulFluor RSV/Flu A
RSV/parainfluenza virus 3 (dual)	CC	Millipore Light Diagnostics SimulFluor RSV/Para 3
Respiratory panel A/identification	DSD and CC	Quidel/Diagnostic Hybrids D^3 FastPoint L-DFA Respiratory Virus ID
Influenza viruses A and B	DSD and CC	Quidel/Diagnostic Hybrids D^3 FastPoint L-DFA Influenza A/B ID
RSV and HMPV	DSD and CC	Quidel/Diagnostic Hybrids D^3 FastPoint L-DFA RSV/MPV ID
Respiratory panel B/identification	DSD and CC	Quidel/Diagnostic Hybrids D^3 Ultra Screening and Typing Kit
Respiratory panel C (dual)	DSD and CC	Quidel/Diagnostic Hybrids D^3 Duet FluA/respiratory Screening and Identification Kit
Respiratory panel D (dual)	DSD and CC	Quidel/Diagnostic Hybrids D^3 Duet RSV/respiratory Screening and Identification Kit
2009 FluA H1N1	DSD and CC	Quidel/Diagnostic Hybrids D^3 Ultra 2009 H1N1
HMPV	DSD and CC	Quidel/Diagnostic Hybrids D^3 MPV
Respiratory panel B pool	DSD and CC	Remel Imagen Respiratory Screen
Influenza viruses A and B	DSD and CC	Remel Imagen Influenza A and B
Parainfluenza virus group/typing	DSD and CC	Remel Imagen Parainfluenza Group and Typing
RSV	DSD	Remel Imagen RSV
RSV	DSD and CC	Quidel/Diagnostic Hybrids RSV

[a] Refer to manufacturers' websites for Food and Drug Administration and *in vitro* diagnostic status of reagents. Although uses for the reagents may be suggested on manufacturers' websites, all suggested applications may not have been validated by the manufacturer. Abbreviations: adeno, adenovirus; CC, culture confirmation; DSD, direct specimen detection; dual, a 2-fluorophore stain; FluA, influenza virus A; ID, identification; IF, immunofluorescence; Para, parainfluenza virus.

[b] Respiratory panel A: adenovirus, influenza viruses A and B, parainfluenza viruses 1, 2, and 3, RSV, HMPV; respiratory panel B: adenovirus, influenza viruses A and B, parainfluenza viruses 1, 2, and 3, RSV; respiratory panel C: adenovirus, influenza viruses A and B, parainfluenza viruses 1, 2, and 3, and differential identification of RSV; respiratory panel D: adenovirus, influenza virus B, parainfluenza viruses 1, 2, and 3, RSV, and differential detection of influenza virus A.

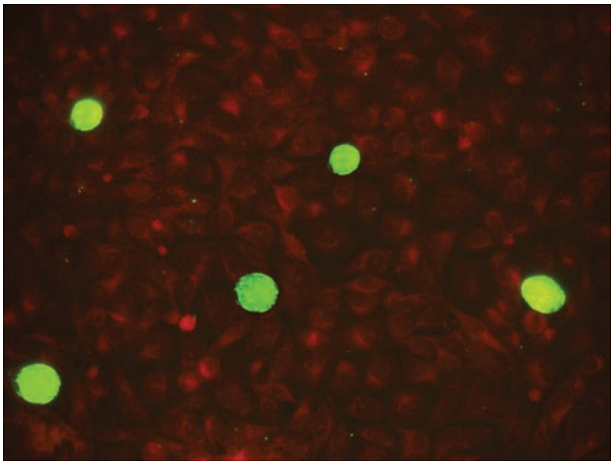

FIGURE 1 Immunofluorescence detection of *Chlamydia trachomatis* in McCoy cells. Magnification, ×200. Courtesy of Quidel/Diagnostic Hybrids.
doi:10.1128/9781555817381.ch80.f1

is not routinely performed. However, commonly isolated viruses may be used to perform quality control on cell lines when new shipments are received in the laboratory, and they are a source of positive-control material for detection and confirmation methods such as HAD and DFA.

With the exception of a few slower-growing viruses such as CMV, or when viruses are present at very low titers, the time to detection by traditional tube culture methods is generally between 1 and 7 days of inoculation (4). The standard approach for detecting viral proliferation is the microscopic examination of the unstained cell culture monolayer for the presence of CPE. The presence of a virus is indicated by degenerative changes in monolayer cells, including shrinking, swelling, rounding of cells, clustering, and formation of syncytia, or by complete destruction of the monolayer. Identification of the virus is then based on the CPE characteristics, the cell line involved, the time to detection, specimen type, and confirmation, generally by staining with virus-specific MAbs. Alternatively, for the identification of viruses for which MAbs may not be available, molecular methods or ancillary traditional testing (e.g., acid resistance for rhinoviruses) must be performed. In addition, for certain viruses (influenza, parainfluenza, and mumps viruses) that do not always demonstrate CPE, HAD testing may be done (11).

Centrifugation-enhanced rapid cell culture

Centrifugation-enhanced inoculation using cells grown on coverslips in 1-dram shell vials and pre-CPE detection of viral antigen in the monolayer cells by use of MAbs was first described for the detection of *Chlamydia trachomatis* (19). This technique was adapted for the routine detection of CMV using MRC-5 shell vials and staining with MAbs directed against early CMV proteins (20). This pioneering method has reduced the time for virus detection from as long as 10 to 30 days to 16 to 72 h. The important factor in reducing the time to detection is the stressing of the monolayer during centrifugation (21). This process has been shown to increase cell proliferation, decrease cell generation times, alter cell metabolism, increase cell longevity, and activate specific genes.

Rapid cell culture is now commonly used for the detection of *Chlamydia* spp., CMV, enteroviruses, VZV, HSV, mumps viruses, and the main respiratory viruses (adenovirus; human metapneumovirus [HMPV]; influenza A and B viruses; parainfluenza viruses 1, 2, and 3; and respiratory syncytial virus [RSV]) (reviewed in reference 7). Centrifugation-enhanced rapid cell culture can be used with standard cell lines (Table 2; Fig. 1) and has been adapted for use with cocultivated cells (Table 3) for the detection of respiratory viruses (R-Mix and R-Mix Too; Quidel Corp./Diagnostic Hybrids, Inc., Athens, OH) (Fig. 2), for the detection of HSV and VZV (H&V-Mix; Quidel/Diagnostic Hybrids) (Fig. 6) (22–24), and with genetically engineered cells such as ELVIS (enzyme-linked virus-inducible system; Quidel/Diagnostic Hybrids) (Table 3) for the detection of HSV (Fig. 3) and Super E-Mix (Quidel/Diagnostic Hybrids) for the detection of enteroviruses (Fig. 5) (25, 26). Identifica-

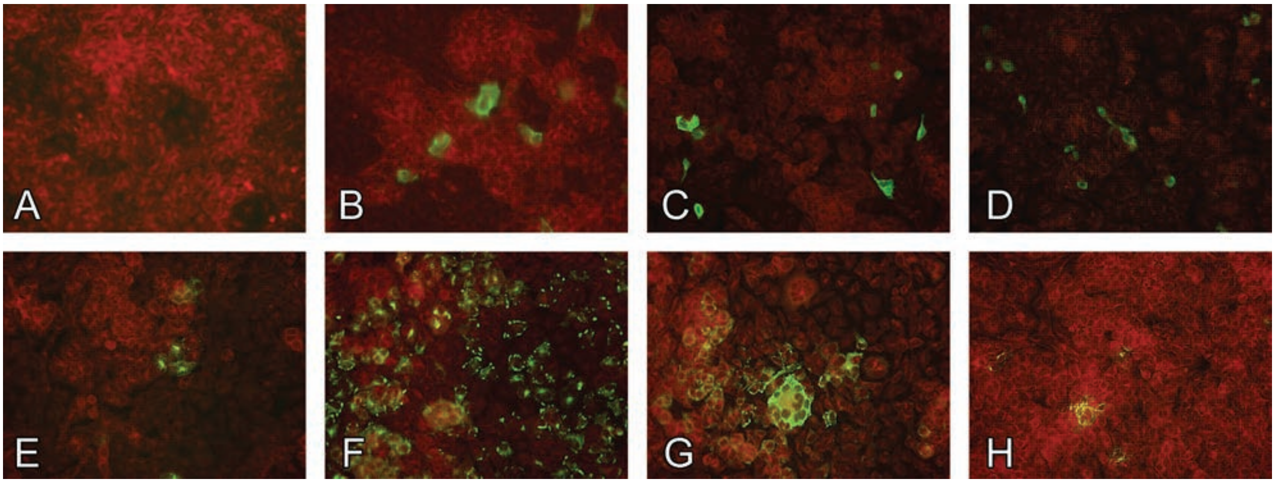

FIGURE 2 Immunofluorescence detection of respiratory pathogens in R-Mix cells using D3 Ultra Kit reagents (Quidel/Diagnostic Hybrids). (A) Uninoculated R-Mix cells; (B) adenovirus; (C) influenza A virus; (D) influenza B virus; (E) parainfluenza virus type 1; (F) parainfluenza virus type 2; (G): parainfluenza virus type 3; (H) RSV. Magnification, ×170. Courtesy of Quidel/Diagnostic Hybrids. doi:10.1128/9781555817381.ch80.f2

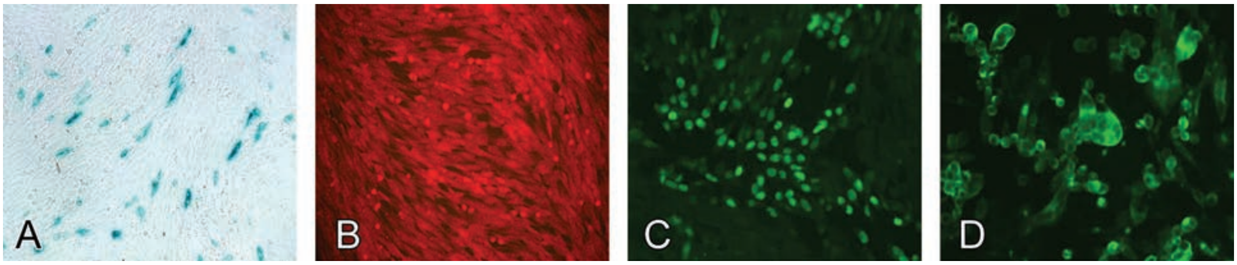

FIGURE 3 Detection of HSV-1 and HSV-2 in ELVIS cells (Quidel/Diagnostic Hybrids). (A) Blue cells positive for HSV with X-Gal stain; (B) immunofluorescence of uninoculated ELVIS cells; (C) HSV-1-positive ELVIS immunofluorescence (note nuclear pattern); (D) HSV-2-positive ELVIS immunofluorescence (note cytoplasmic pattern). Magnification, ×170. Courtesy of Quidel/Diagnostic Hybrids. doi:10.1128/9781555817381.ch80.f3

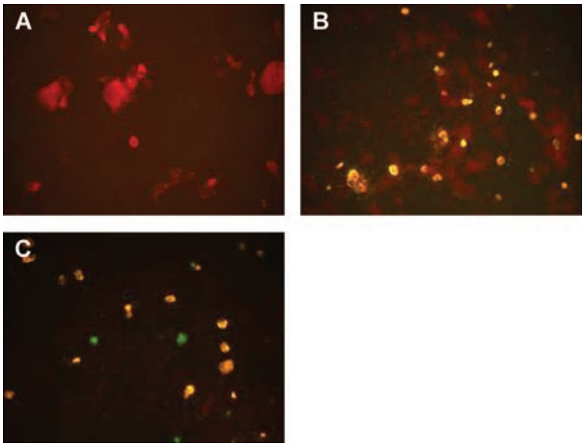

FIGURE 4 Immunofluorescence detection of respiratory pathogens from clinical specimens using Duet stains (Quidel/Diagnostic Hybrids). (A and B) Influenza A Duet stain (influenza A [gold] and respiratory pool [green] for adenovirus; influenza B; parainfluenza virus types 1, 2, and 3; and RSV): negative cells (A); influenza A-positive cells (B). (C) RSV Duet stain (RSV [gold] and respiratory pool [green] for adenovirus; influenza A; influenza B; and parainfluenza viruses 1, 2, and 3): RSV-positive and influenza A-positive cells. Magnification, ×170. Courtesy of Quidel/Diagnostic Hybrids. doi:10.1128/9781555817381.ch80.f4

tion of the viral pathogen when using centrifugation-enhanced rapid cell culture is not dependent on the visualization of CPE, allowing technologists not skilled in CPE recognition to perform viral testing. Instead, the virus is detected either by "blind staining" or staining "pre-CPE" with PE-, peroxidase-, or FITC-labeled virus-specific MAbs or a combination of PE- and FITC-labeled antibodies or by virus-specific induction of enzymes that are detected by the ELVIS HSV system using substrates such as β-galactosidase (6, 25, 26). A significant benefit of using cocultivated cells such as R-Mix, R-Mix Too, H&V-Mix, and Super E-Mix is that they allow the identification of multiple viruses from a single shell vial or cluster tray well, rather than having to use multiple shell vials to cover the range of viruses that the laboratory may wish to detect in a particular sample type. One shell vial can be substituted for up to four different tube cell cultures that require much longer (10 to 14 days) to identify fewer subspecies of the group.

Since rapid detection formats are not based on the detection of CPE but use blind staining for either single viruses or multiple viruses, positive- and negative-control slides are required for each day of patient testing. When a single-culture system detects multiple viruses, the detection reagents must be validated for all targets (including any pooled MAb reagents) upon receipt in the laboratory (13, 15). Virus isolate controls can be tested daily and virus types rotated so that during the course of 1 week, the lots of cells and reagents have been tested against all the routinely isolated viruses.

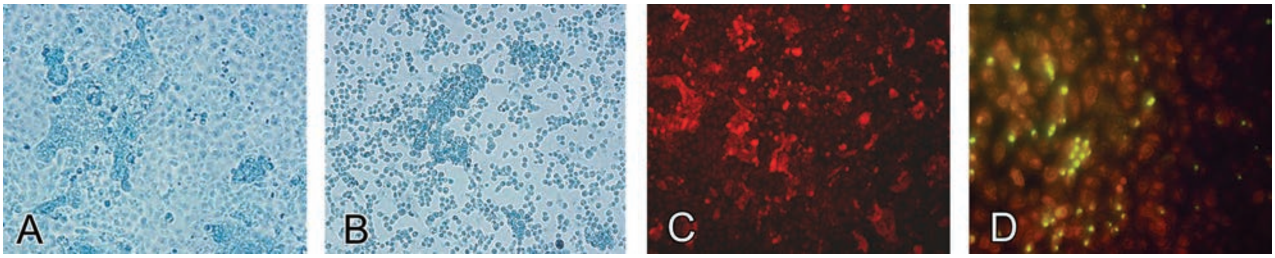

FIGURE 5 Detection of coxsackie B virus in Super E-Mix cells (Quidel/Diagnostic Hybrids). (A) Unstained, uninoculated cells; (B) unstained coxsackie B virus CPE; (C and D) immunofluorescence staining with pan-enterovirus antibody pool of uninoculated Super E-Mix cells (C) and coxsackie B virus-infected cells (D). Magnification, ×170. Courtesy of Quidel/Diagnostic Hybrids. doi:10.1128/9781555817381.ch80.f5

TABLE 2 List of cell lines and virus susceptibility profiles

Cell line	Origin	Virus(es)[a]
A-549	Human lung carcinoma	Adenovirus, HSV, influenza virus, measles virus, mumps virus, parainfluenza virus, poliovirus, RSV, rotavirus, VZV
AGMK[b]	African green monkey kidney	Enteroviruses, influenza virus, parainfluenza virus
AP61	Mosquito	Arboviruses
B95 or B95a	Epstein-Barr virus-transformed lymphoblastoid	Measles virus, mumps virus
BGMK	Buffalo green monkey kidney	*Chlamydia* spp., coxsackie B virus, HSV, poliovirus
C6/36	Mosquito	Arboviruses
Caco-2	Human epithelial colorectal adenocarcinoma	Astrovirus, HCoV (NL63)
CV-1	African green monkey kidney	Encephalitis viruses (some), HSV, measles virus, mumps virus, rotavirus, SV40, VZV
Graham 293	Human embryonic kidney transformed with adenovirus type 5	Enteric adenoviruses
H292		Adenovirus, coxsackie B virus, echovirus, HSV, mumps virus, parainfluenza virus, poliovirus, RSV, rubella virus
HeLa	Human cervix adenocarcinoma	Adenovirus, CMV, coxsackie B virus, echovirus, HSV, poliovirus, rhinovirus, vesicular stomatitis virus (Indian strain), VZV
HeLa 229	Human cervix adenocarcinoma	Adenovirus, *Chlamydia* spp., CMV, echovirus, HSV, poliovirus, rhinovirus, vesicular stomatitis virus (Indian strain), VZV
HEL	Human embryonic lung	Adenovirus, CMV, echovirus, HSV, poliovirus, rhinovirus, vesicular stomatitis virus (Indian strain), VZV
HEK	Human embryonic kidney	Adenovirus, BK virus, enterovirus, HSV, measles virus, mumps virus, rhinovirus
HEK 293	Human embryonic kidney transformed with adenovirus type 5	Enteric adenoviruses
HEp-2	Human epidermoid carcinoma	Adenovirus, *Chlamydia* spp., coxsackie B virus, HSV, measles virus, parainfluenza virus, poliovirus, RSV
HNK	Human neonatal kidney	Adenovirus, HSV, VZV
Hs27 (HFF[c]; MRHF)	Human foreskin fibroblast	Adenovirus, CMV, echovirus, HSV, mumps virus, poliovirus, rhinovirus, VZV
HuH-7	Human hepatocyte	HCoVs (OC43, 229E)
LLC-MK2	Original, rhesus monkey kidney	Arboviruses (some), enteroviruses (including coxsackie A and B viruses, echoviruses, polioviruses), HMPV (NL-63), influenza virus, MERS-CoV, mumps virus, parainfluenza virus, poxvirus groups, rhinovirus
Mv1Lu	Mink lung	CMV, HSV, influenza virus
McCoy[c]	Mouse fibroblast	*Chlamydia* spp., HSV
MDCK	Madin-Darby canine kidney	Adenovirus (some types), coxsackievirus, influenza virus, reovirus
MNA	Mouse neuroblastoma	Rabies virus
MRC-5	Human fetal lung	Adenovirus, CMV, coxsackie A virus, echovirus, HSV, influenza virus, mumps virus, poliovirus, rhinovirus, RSV, VZV, cytotoxicity for *C. difficile*
NCI-H292	Human pulmonary mucoepidermoid	Adenovirus, BK polyomavirus, enteroviruses (most), HSV, measles virus, reoviruses, rhinoviruses (most), RSV, vaccinia virus
RD	Human rhabdomyosarcoma	Adenovirus, coxsackie A virus, echovirus, HSV, poliovirus
RK[b]	Rabbit kidney	HSV, paramyxoviruses
RhMK[b]	Rhesus monkey kidney	Arboviruses, coxsackie A and B viruses, echoviruses, influenza virus, parainfluenza virus, measles virus, mumps virus, polioviruses
SF	Human foreskin	Adenovirus, CMV, coxsackie A virus, echovirus, HSV, poliovirus, VZV
Vero	African green monkey kidney	Adenovirus, arboviruses (some), *Chlamydia* spp., coxsackie B virus, HMPV, HSV, measles virus, MERS-CoV, mumps virus, poliovirus type 3, rotavirus, rubella virus
Vero E6	African green monkey kidney	Adenovirus, coxsackie B virus, HSV, measles virus, mumps virus, poliovirus type 3, rotavirus, rubella virus, SARS-CoV
Vero 76	African green monkey kidney	Adenovirus, coxsackie B virus, HSV, measles virus, mumps virus, poliovirus type 3, rotavirus, rubella virus, West Nile virus
WI-38	Human lung	Adenovirus, CMV, coxsackie A virus, echovirus, HSV, influenza virus, mumps virus, poliovirus, rhinovirus, RSV, VZV

[a] Abbreviations: HCoV, human coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus; SARS-CoV, severe acute respiratory syndrome coronavirus.

[b] Primary cell cultures.

[c] Available as fresh and frozen ReadyCells (Quidel/Diagnostic Hybrids).

TABLE 3 List of cocultured cell lines and virus susceptibility profiles[a]

Cell mixture[b]	Cell type	Virus(es)
R-Mix[c]	Mv1Lu (mink lung)	CMV, HSV, influenza virus, SARS-CoV
	A549 (human lung carcinoma)	Adenovirus, HSV, influenza virus, measles virus, mumps virus, parainfluenza virus, poliovirus, rotavirus, RSV, VZV
R-Mix Too[c]	MDCK (Madin-Darby canine kidney)	Influenza virus
	A549 (human lung carcinoma)	Adenovirus, HSV, influenza virus, measles virus, mumps virus, parainfluenza virus, poliovirus, rotavirus, RSV, VZV
H&V-Mix	CV-1 (African green monkey kidney)	Encephalitis viruses (some), HSV, measles virus, mumps virus, rotavirus, SV40, VZV
	MRC-5 (human fetal lung)	Adenovirus, CMV, echovirus, HSV, influenza virus, mumps virus, poliovirus, rhinovirus, RSV, VZV
Super E-Mix[c]	sBGMK (Buffalo green monkey kidney) (with degradation activating factor)	*Chlamydia*, coxsackie A and B viruses, echovirus, HSV, poliovirus
	A549 (human lung carcinoma)	Adenovirus, HSV, influenza virus, measles virus, mumps virus, parainfluenza virus, poliovirus, RSV, rotavirus, VZV
ELVIS	Transfected baby hamster kidney HSV UL97 promoter/*E. coli lacZ* gene	HSV-1, HSV-2

[a] Abbreviations: SARS-CoV, severe acute respiratory syndrome coronavirus.

[b] Available from Quidel/Diagnostic Hybrids.

[c] Available as both fresh and frozen cells that do not require propagation but are ready to use.

ELVIS

ELVIS uses a genetically engineered cell line (BHKICP6LacZ) that was first described by Stabell and Olivo (6). The promoter sequence of the HSV UL97 gene and the *Escherichia coli lacZ* gene were used to stably transform a baby hamster kidney cell line. When the cell line is infected with HSV, the virion-associated transactivator protein VP16 and other transactivating factors such as ICP0 strongly transactivate the UL97 promoter, which in turn activates the *lacZ* gene, resulting in high levels of β-galactosidase activity. Addition of 4-chloro-3-indol-β-D-galactopyranoside (X-Gal) turns a colorless substrate to blue, indicating the presence of HSV-infected cells (Fig. 3A). If HSV typing is required, a fluorescence-labeled MAb that specifically detects HSV-2 (Fig. 3D) and a nonlabeled MAb that specifically binds to HSV-1 are incorporated in the staining procedure. If the infected (blue) cells are not detected with the HSV-2 MAb, then the monolayers are stained with anti-mouse fluorescence-labeled antibody to detect the HSV-1 MAb (Fig. 3C). ELVIS is completed within 16 to 24 h for both positive and negative results (25, 26).

Cell cytotoxicity assays

Tissue culture assays using cell types such as human foreskin fibroblasts, MRC-5 cells, and Vero epithelioid cells are commonly used for the detection of toxin-producing strains of *Clostridium difficile* (27–29). Cell culture testing for the presence of *C. difficile* cytotoxin has demonstrated improved sensitivity compared to *C. difficile* toxin A/B enzyme immunoassays. Enteroviruses can cause a CPE similar to that caused by *C. difficile* toxin; therefore, the procedure includes the use of a *C. difficile* antitoxin to *C. difficile* toxin, which creates a more specific assay. Fecal filtrate is diluted, bacteria are removed by membrane filtration, and the tissue culture cells are exposed to the patient material, with and without *C. difficile* antitoxin. Test wells without *C. difficile* antitoxin demonstrating CPE within 12 to 48 h are presumed positive for *C. difficile* toxin if the control well with *C. difficile* antitoxin shows no evidence of CPE. As little as 1 pg of toxin can cause a visible change in cells over a period of hours up to 1 to 2 days.

CELL CULTURE MEDIA

Transport Media and Collection Swabs

An important consideration in using cell culture is ensuring collection of cellular material and maintaining the viability of the organisms from the time of sample collection to inoculation in cell culture. Therefore, time to processing, transport temperature, and the use of transport media need to be evaluated for each sample type. Cell culture media with additives, such as antibiotics and fungicidal agents to inhibit microbial growth, are used to maintain the viability of viruses during sample transport. Transport media may be specific for virus isolation (viral transport media) or also allow the isolation of *Chlamydia*, *Mycoplasma*, and *Ure-*

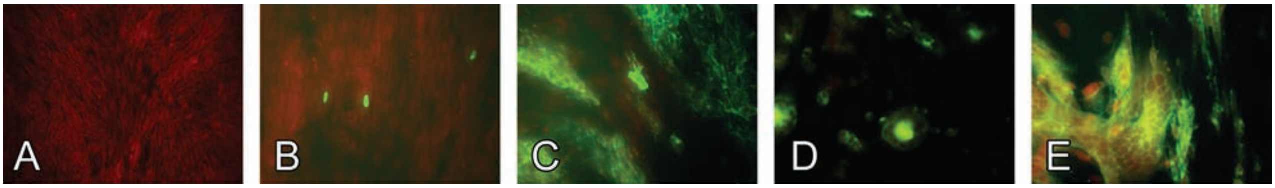

FIGURE 6 Immunofluorescence detection of *Herpesviridae* family viruses in H&V-Mix cells (Quidel/Diagnostic Hybrids). (A) Uninoculated H&V-Mix cells; (B) CMV; (C) VZV; (D) HSV-1; (E) HSV-2. Magnification, ×170. Courtesy of Quidel/Diagnostic Hybrids.
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aplasma (universal transport media). The types of transport media, uses and components, collection procedures, and swabs are detailed in chapter 79.

Growth medium with 10% fetal bovine serum

Cell culture media are an important part of the production and maintenance of cells. Cell culture stocks are generally grown in a richer medium than that which is used for the maintenance of the cells. The higher level of serum or protein in growth medium rapidly enhances cell growth and is used for a day or more before it is replaced by maintenance medium. Standard growth medium consists of EMEM with EBSS and supplemented with 10% heat-inactivated fetal bovine serum (FBS), one or more antimicrobials (gentamicin, penicillin, streptomycin, vancomycin, and/or amphotericin B), and HEPES buffer. Since simian viruses are endogenous to primary monkey kidney cell cultures, media that contain simian virus 5 (SV5) and SV40 antisera are available (Quidel/Diagnostic Hybrids).

Maintenance medium

Maintenance medium contains nutrients and buffers that protect the cells during the stage when rapid growth is not desired but healthy monolayers must be controlled for inoculation of specimens and recovery of the etiologic agent. Seeded flasks, tubes, trays, or shell vials may be fed with medium multiple times prior to release from the vendor to ensure that the cells are growing according to specifications and arrive at the user site ready to be used. At the user site, most cells remain stable for a period of a few days without needing changing of the medium with which they were shipped. However, before use, it is common to remove the shipping medium and add a maintenance medium such as 2% FBS EMEM or 0% FBS Hanks', mimimal essential medium, or, for those viruses that would be inhibited by serum proteins (myxoviruses and paramyxoviruses), R-Mix refeed medium. Trypsin and antimicrobial agents can be added if indicated. Other refeeding media for viruses include E-Mix refeed medium (Quidel/Diagnostic Hybrids) and 5 or 10% FBS growth medium. Tube cell cultures may require refeeding on a weekly or twice-weekly basis; however, the use of shell vial or cluster plate technology usually reduces refeeding to one time just prior to inoculation. Commercial media with and without enrichments are available, such as PBS, L-glutamine, trypsin, and bactericidal and fungicidal agents.

Chlamydia isolation medium

Some cell-dependent bacteria such as *C. trachomatis* require the cell host to restrict protein synthesis, thus allowing the infecting microbe to replicate more easily. For this reason, media used for propagation of *Chlamydia* spp. in cell culture commonly contain cycloheximide, a protein synthesis inhibitor. Isolation media for *Chlamydia* spp. detection using shell vials generally contain EMEM with EBSS, supplemented with 10% FBS, HEPES, glucose, nonessential amino acids, antibiotics (cycloheximide, gentamicin, or streptomycin), and amphotericin B.

EMEM pH 2–3

Rhinoviruses require a lower temperature of incubation than many other pathogenic human viruses, and their characteristic CPE can be confused with enterovirus CPE, which has a similar appearance. At this time, MAbs are not available to identify rhinoviruses. For this reason, the ability of acid solutions to neutralize rhinoviruses, thus reducing viral titers, can be used to differentiate rhinoviruses from enteroviruses, which are not sensitive to low pH. EMEM pH 2–3 can be used to test for acid sensitivity.

RPMI 1640 medium

RPMI medium consists of glucose, essential amino acids, other amino acids, vitamins, HEPES buffer, antibiotics (such as penicillin and streptomycin), and phenol red as an indicator. This enriched medium is used for lymphocyte cell cultivation and culture of HIV.

Proprietary Media

CMV TurboTreat medium

CMV TurboTreat pretreatment medium (Quidel/Diagnostic Hybrids) consists of EMEM with EBSS without phenol red, 10% FBS, HEPES, and gentamicin. Pretreatment of Mv1Lu, R-Mix, and MRC-5 cultures overnight, prior to specimen addition, enhances the recovery of CMV (30).

ELVIS replacement medium

ELVIS replacement medium is used with the ELVIS test system and comprises EMEM, FBS, streptomycin, and amphotericin B.

R-Mix refeed and rinse medium

R-Mix refeed and rinse medium is used with R-Mix or R-Mix Too fresh and R-Mix or R-Mix Too ReadyCells (frozen shell vial) culture systems (Quidel/Diagnostic Hybrids) and is a defined serum-free medium with trypsin, penicillin, and streptomycin.

APPENDIX

Sources of Virology Reagents, Stains, Media, and Cell Lines

American Type Culture Collection
Manassas, VA 20110
http://www.atcc.org

BD Biosciences
Franklin Lakes, NJ 07417
http://www.bdbiosciences.com

Bio-Rad Laboratories
Hercules, CA 94547
http://www.bio-rad.com

Cambrex Corporation (BioWhittaker Cell Products)
East Rutherford, NJ 07073
http://www.cambrex.com

Chemicon International (Millipore)
Temecula, CA 92590
http://www.millipore.com

Lonza, Inc.
Allendale, NJ 07401
http:/www.lonza.com

Meridian Bioscience
Cincinnati, OH 45244
http://www.meridianbioscience.com

Millipore (Chemicon)
Billerica, MA 01821
http://www.millipore.com

Quidel/Diagnostic Hybrids
Athens, OH 45701
http://www.quidel.com or http:/www.dhiusa.com

Remel
Lenexa, KS 66215
http://www.remelinc.com

Trinity Biotech (Bartels, MicroTrak)
Carlsbad, CA 92008
http://www.trinitybiotech.com

Trinity Biotech (Bartels)
Bray, County Wicklow, Ireland
http://www.trinitybiotech.com

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Algorithms for Detection and Identification of Viruses

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81

Virology is a dynamic field that in recent decades has moved from the periphery to the mainstream of clinical laboratory practice. When the first edition of the *Manual of Clinical Microbiology* was published in 1970, diagnostic virology was practiced in a limited number of laboratories, primarily in public health, research, and academic settings. Methods focused on conventional cell cultures, classical serologic techniques, and light and electron microscopy. Time to result was slow, and it was often said the patient was dead or better by the time the result was received.

Over the intervening years, diagnostic advances have transformed the field by allowing accurate results in a clinically useful time frame. These technology improvements include enzyme, chemiluminescence, and chromatographic immunoassays for antigen and antibody detection, IgM class capture assays, monoclonal antibodies for rapid identification of culture isolates, shell vial centrifugation cultures, and direct detection of viral proteins in clinical specimens by immunofluorescence. The most transformative, however, have been nucleic acid amplification techniques (NAATs), which are both rapid and sensitive; can be automated, high-throughput, or random access; and can detect and quantify viruses not amenable to routine culture.

Since the last edition of this *Manual*, the dramatic growth in the development and implementation of clinically useful molecular methods has accelerated. This trend has been facilitated by the increasing availability of commercial reagents and Food and Drug Administration (FDA)-cleared kits, some requiring minimal molecular expertise and no extraction step, and the greater variety of instruments for nucleic acid extraction and real-time amplification, including some integrated systems. Novel detection methods have permitted the diagnosis of multiple respiratory viruses in a single multiplex PCR test. One FDA-approved test, although low-throughput, is simple to perform, detects up to 21 respiratory pathogens, and provides “on demand” results within 1 h. A high-throughput but more complex test was also recently approved by the FDA for detection of common gastrointestinal viral, bacterial, and parasitic pathogens. Quantitative monitoring of viral load in blood has become more widely applied due to implementation of real-time PCR techniques and the availability of FDA-approved kits. Substantial effort has been and continues to be invested in the development of international quantitative standards (e.g., cytomegalovirus and Epstein-Barr virus) that will permit cross-institutional comparisons and interpretive guidelines. Thus, molecular assays have become the standard of care for the diagnosis of many viral infections, for predicting patient outcomes, and for monitoring response to antiviral therapies. Laboratories without classical virology expertise can now implement state-of-the-art molecular testing. The main obstacle for offering molecular testing in-house is no longer lack of technical expertise but cost, for both equipment and reagents.

In addition to the advantages of molecular testing, some pitfalls are also becoming apparent as the tests are more widely used. For example, the sensitivities and specificities of assays to detect the same virus can vary significantly between laboratories, especially for laboratory-developed tests. In addition, despite targeting conserved regions of the genome, strain variability and mutations can lead to underquantification of viral load, or even falsely negative results. Furthermore, as tests become more sensitive, low levels of clinically irrelevant or nonviable viruses may be detected and can be misleading to clinicians. Similarly, interpreting the clinical relevance of multiple viral pathogens in the same sample when relative quantification is not available is problematic. Requirements for staff training, test validation, and quality control can also be challenging.

At the other end of the spectrum are rapid tests requiring minimal or no equipment or reagent additions. These tests often employ immunochromatographic detection for viral antigen or antibody and can be used at the point of care for immediate impact on clinical decisions. However, the 2009 novel H1N1 influenza pandemic revealed the need to improve the sensitivity of point-of-care rapid tests. While newer tests with fluorescent labels and walk-away readers have improved accuracy, much work remains. Several integrated, rapid, and simple NAAT devices suitable for point-of-care testing are in the pipeline and may fill this niche.

Thus, with progress have come new challenges. Laboratories need to choose which tests to offer. Selecting the appropriate test will depend on the virus(es) sought, sample site, clinical presentation, clinical purpose (e.g., screening, confirmation, diagnosis, or monitoring), patient characteristics, and disease prevalence. Performance characteristics, staff expertise, and cost will also influence that choice. Laboratories must recognize the uses and also the limitations of each test in order to guide clinicians in test selection and in interpretation of results. This *Manual* should serve

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TABLE 1 Methods for detection and identification of viruses[a]

Virus(es)	Applicability[b] of: Nucleic acid detection	Antigen detection	Virus isolation	Antibody detection	Electron microscopy	Comments
Adenoviruses	A	A	A	C	C	NAAT, culture, and IFA widely used for respiratory specimens. NAAT used to monitor viral load in compromised hosts. Rapid antigen assays used for ocular and enteric adenoviruses.
Arboviruses	B	D	C	A	C	Serology is primary diagnostic method. Most arboviruses are readily cultured; isolation of some agents may require BSL3 or -4 facilities.
Bocaviruses	A	D	D	C	D	NAAT is the only test available for diagnosis. Clinical relevance awaits further investigation.
Coronaviruses	A	C	C	B	C	NAAT becoming more widely used for respiratory CoVs as part of multiplex panels. NAAT and antibody tests for SARS- and MERS-CoV available only in public health or research laboratories.
Cytomegalovirus	A	A	A	B	C	Shell vial culture useful for nonblood specimens. Quantitative NAAT and pp65 antigenemia used to assess risk of disease and response to therapy. Serology primarily used to determine prior infection.
Enteroviruses and parechoviruses	A	D	A	D	D	Enterovirus NAAT preferred for CNS infection. Parechovirus NAAT required for optimal detection of infection.
Epstein-Barr virus (EBV)	A	B	D	A	D	Serology is test of choice for diagnosis of mononucleosis. Quantitative NAAT useful for monitoring viral load in blood posttransplant or in EBV-related proliferative and neoplastic diseases. IHC or ISH can be used on tissue biopsies.
Filoviruses and arenaviruses	A	A	A	B	C	Testing confined to specialized laboratories. Antigen and NAAT key to rapid diagnosis. BSL4 facility needed for culture, except for LCMV. Patients with severe filovirus disease may die without developing antibody. LCMV diagnosed primarily by serology.
Hantaviruses	A	B	C	A	C	Testing confined to specialized laboratories. Serology and NAAT equally useful for diagnosis. IHC used in fatal cases. BSL4 facility needed for culture. Isolation difficult.
Hepatitis A virus	C	C	D	A	D	Serology is standard diagnostic test. False-positive IgM problematic in low-prevalence areas.
Hepatitis B virus	A	A	D	A	C	Detection of specific viral antigens and antibodies allows for diagnosis and for monitoring of the course of infection. NAAT used to monitor therapy and determine genotype.
Hepatitis C virus	A	D	D	A	D	Serology used for diagnosis. NAAT used to confirm active infection. Quantitative NAAT used to monitor response to therapy. Genotyping helps determine drug regimen and duration of therapy.
Hepatitis D virus	B	B	D	A	D	Testing confined to reference laboratories. Diagnosis is relevant only in the presence of hepatitis B virus infection. IHC of biopsy tissue useful for diagnosis.
Hepatitis E virus	B	D	D	A	D	Serology is standard diagnostic test, but tests vary tremendously in sensitivity and specificity. False-positive IgM problematic in low-prevalence areas. NAAT required for accurate diagnosis in transplant patients but is not commercially available.

(Continued on next page)

TABLE 1 Methods for detection and identification of viruses[a] *(Continued)*

Virus(es)	Applicability[b] of:					Comments
	Nucleic acid detection	Antigen detection	Virus isolation	Antibody detection	Electron microscopy	
Herpes simplex virus (HSV)	A	A	A	B	C	NAAT is test of choice for CNS infection and used for other sample types as well. IFA used for rapid detection in skin/mucous membrane lesions or tissue specimens. Serology, including HSV-2-specific serology, used to determine prior infection.
Human herpesviruses (HHV) 6 and 7	A	C	C	B	C	NAAT is test of choice for diagnosis. Serology can document primary infection in children. Interpretation of HHV-6 NAAT can be complicated due to possible chromosomal integration by virus.
Human herpesvirus 8	B	B	D	A	C	Serology used to identify infected persons; sensitivity and specificity hampered by difficulty in setting cutoff values. NAAT of blood may be useful for monitoring KS risk. IHC more specific than NAAT for KS.
Human immunodeficiency virus	A	A	C	A	D	Serology is primary diagnostic method. Antigen/antibody combination tests reduce seronegative window in acute infection. Quantitative RNA tests used to guide therapy and monitor response. Proviral DNA tests useful for diagnosis of neonatal infection.
Human metapneumovirus	A	A	B	D	C	NAAT is test of choice for diagnosis. IFA and shell vial culture also useful. Conventional culture difficult.
Human T-cell lymphotropic virus (HTLV)	B	D	C	A	D	Serology is primary diagnostic method. NAAT useful for virus identification in HTLV Western blot-positive but untypeable specimens.
Influenza viruses	A	A	A	B	D	NAAT most sensitive and can provide subtype. Antigen tests rapid and simple but suboptimal in sensitivity and specificity. IFA and rapid culture more accurate. Serology useful for epidemiological studies or retrospective diagnosis.
Measles viruses	C	B	B	A	C	Serology used most commonly for diagnosis and determination of immunity. NAAT available in public health laboratories. Isolation useful if attempted early (prodromal period to 4 days postrash).
Mumps virus	B	C	A	A	C	Serology used most commonly for diagnosis and determination of immunity. NAAT useful for diagnosing outbreaks among vaccinated individuals.
Noroviruses	A	C	D	D	B	NAAT is test of choice but challenging due to strain variability. Reagents for antigen detection not commercially available.
Papillomaviruses	A	D	D	D	C	NAAT is test of choice for detection and genotype differentiation. Cytopathology useful for diagnosis. Serologic diagnosis of exposure not available.
Parainfluenza viruses	A	A	A	C	D	NAAT more sensitive than isolation. IFA also used for rapid detection.
Parvoviruses	A	C	D	A	C	Serology used to diagnose B19 infection in immunocompetent individuals. NAAT is test of choice for B19-exposed fetuses and for immunocompromised hosts. Bocavirus NAAT included in some multiplex respiratory virus panels.

(Continued on next page)

TABLE 1 Methods for detection and identification of viruses[a] *(Continued)*

Virus(es)	Applicability[b] of: Nucleic acid detection	Antigen detection	Virus isolation	Antibody detection	Electron microscopy	Comments
Polyomaviruses	A	B	C	C	B	NAAT is test of choice, but genetic variability can lead to falsely low or negative results. JC virus DNA detection in CSF useful for presumptive diagnosis of PML. BK DNA quantification in plasma or urine used for preemptive diagnosis of PVAN. IHC and EM useful for biopsy tissues.
Poxviruses	A	C	A	A	A	NAAT allows virus inactivation and rapid detection. EM is very useful for rapid diagnosis but has limited availability. Smallpox virus isolation requires BSL3 or -4 and should be attempted only in WHO Collaborating Centres. Vaccinia virus requires BSL2 and grows readily in cell culture.
Rabies virus	A	A	A	A	C	For human rabies, testing done at CDC. NAAT and culture used for saliva, CSF, and tissue; IFA for skin biopsy; serology for CSF and serum. Serology available at commercial laboratories only used to monitor antibody titers in vaccinated professionals.
Respiratory syncytial virus	A	A	A	C	C	NAAT most sensitive. Rapid antigen tests, especially IFA, more sensitive than culture. Serology useful for epidemiological studies.
Rhinoviruses	A	C	A	D	D	NAAT much more sensitive than culture; cross-reaction with enteroviruses can occur.
Rotaviruses	A	A	D	D	C	Direct antigen detection is standard test for diagnosis. New NAAT gastroenteritis panels include rotavirus. EM useful if available.
Rubella virus	B	D	A	A	D	Serology used most commonly for diagnosis and determination of immunity. NAAT available in public health laboratories. Isolation useful if attempted early (prodromal period to 4 days postrash). In CRS, virus can be isolated for weeks to months after birth.
Transmissible spongiform encephalopathy agents	B	C	D	D	C	Histology most useful diagnostic test. Surrogate markers popular but lack specificity. Western blot for PrP performed in specialized laboratories. Human genome sequencing useful for diagnosis of genetic disorders.
Varicella-zoster virus	A	A	A	B	C	NAAT most sensitive and increasingly used. IFA more sensitive than culture. Serology most useful for determination of immunity.

[a] Viral nucleic acids (DNA or RNA) can be detected and quantified by direct hybridization or by the performance of amplification methods such as PCR. Viral antigens can be detected by a variety of immunoassays, such as enzyme-linked immunosorbent assays, agglutination assays, immunofluorescence or immunoperoxidase techniques, and immunochromatography. Virus isolation includes conventional cell culture with detection of viral growth by cytopathic effects or hemadsorption, and shell vial centrifugation culture with detection of viral antigens by immunostaining. Antibody detection involves measurement of total or class-specific immunoglobulins directed at specific viral antigens. Electron microscopy involves the visualization of viral particles by negative staining or immunoelectron microscopy, or by thin-section techniques. Abbreviations: BSL, biosafety level; CNS, central nervous system; CoV, coronavirus; CRS, congenital rubella syndrome; EM, electron microscopy; IFA, immunofluorescence assay; IHC, immunohistochemistry; ISH, *in situ* hybridization; KS, Kaposi's sarcoma; LCMV, lymphocytic choriomeningitis virus; MERS, Middle East respiratory syndrome; PML, progressive multifocal leukoencephalopathy; PrP, prion protein; PVAN, polyomavirus-associated nephropathy; SARS, severe acute respiratory syndrome.

[b] A, test is generally useful for the indicated diagnosis, preferred method may vary with sample type; B, test is useful under certain circumstances or for the diagnosis of specific forms of infection, as delineated in the right-hand column and in the text of the individual chapters; C, test is seldom useful for general diagnostic purposes but may be useful for epidemiological studies or for the diagnosis of unusual conditions; D, test is not available or not used for laboratory diagnosis of infection.

as a key resource for accomplishing these tasks. The choices available for each virus differ and continue to evolve. Table 1 provides a concise overview for each virus group; however, the reader is referred to the specific chapters for more detailed discussions.

While it can be daunting for laboratories to acquire and maintain expertise in the variety of test methods and platforms now available, it is extremely gratifying to witness the impact of state-of-the-art testing on patient care. As we move forward, it is critical that laboratorians communicate with one another to address problems, including the optimization and standardization of methods, and, in addition, encourage input and feedback from clinicians. Due to the speed of methodological change and the continuing discovery of new viruses and new therapies, keeping abreast of the most recent literature is strongly recommended.

Human Immunodeficiency Viruses*

BERNARD M. BRANSON AND S. MICHELE OWEN

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TAXONOMY

Historical Perspective and Origin

The human immunodeficiency virus (HIV) is the etiologic agent of acquired immune deficiency syndrome (AIDS). The clinical manifestations of AIDS were first recognized in 1981 (1). Search for the cause of this severe cellular immune dysfunction led to isolation of lymphadenopathy-associated virus in 1983 (2). The following year, additional researchers isolated cytopathic retroviruses from persons with AIDS, which they termed human T-lymphotrophic virus type III (3, 4). These viruses were soon confirmed to be identical, and in 1986 the International Committee on the Taxonomy of Viruses designated human immunodeficiency virus (HIV) as the name for the virus causative of AIDS (5).

HIV exists as two major viral species. Both are members of the genus *Lentivirus* within the family *Retroviridae*. HIV type 1 (HIV-1), identified first, is the more virulent of the two and is responsible for the majority of AIDS cases worldwide. HIV-2, first isolated in 1986, has biological and morphological properties similar to those of HIV-1, but differs in some of its antigenic components (6). HIV-2 is less pathogenic and has a more limited geographic distribution than HIV-1. Both HIV-1 and HIV-2 are related to simian immunodeficiency viruses (SIV), which are found in 26 species of African primates but do not cause disease in their native hosts (7). Phylogenetic evidence indicates that HIV-1 arose as a consequence of at least three separate zoonotic transmissions of SIV from chimpanzees to humans; four interspecies SIV transmissions from sooty mangabeys are the origins of HIV-2 (7, 8). Genetic sequences of HIV have been identified retrospectively in human plasma specimens from as early as 1959 (9).

HIV Classification

HIVs are classified based on the phylogenetic relatedness of their nucleotide sequences. The current classification is hierarchical and consists of types, groups, subtypes, sub-subtypes, and recombinant forms (10). Subtypes are often referred to as clades. The most distantly related HIVs are categorized as types: HIV type 1 and HIV type 2. HIV-1 is further characterized into groups: the major (M) group; the more divergent outlier (O) group; the non-M, non-O (N) group; and group P, a new lineage closely related to a gorilla SIV (10–12). Most HIV infections occur with HIV-1 group M, which is differentiated into nine subtypes (A, B, C, D, F, G, H, J, and K). Subtypes A and F are further classified into sub-subtypes A1, A2, A3, F1, and F2. The sequences within any one subtype are more similar to each other than to sequences from other subtypes throughout their genomes and represent different lineages of HIV. Eight HIV-2 groups (A through H) have been defined; only two, A and B, have been recovered from more than a single individual. When viruses from two or more HIV-1 lineages infect one individual and exchange their genetic material, they are termed recombinant viruses (13). If transmission of the recombinant virus to three or more persons has been documented by whole-genome sequencing, it is referred to as a circulating recombinant form (CRF), and the CRF is given a numeric designation (13). As of December 2013, CRF01 to CRF55 are recognized (Los Alamos Laboratory National HIV Sequence Database, http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html). Recombinant viruses that have been identified but not documented to have been further transmitted are referred to as unique recombinant forms (URF). The variation in nucleotide sequences can have implications for the biology and transmission of the virus and for patient survival, and also helps to identify the geographic distribution and epidemiology of HIV infection. From the diagnostic perspective, sequence variation can have significant implications for the reactivity and cross-reactivity of diagnostic tests designed to detect specific viral proteins or peptides.

DESCRIPTION OF THE AGENTS

Structure and Genomic Organization

HIVs are enveloped single (positive)-stranded RNA viruses. The HIV genome is organized similarly to other retroviruses. It contains the *gag*, *pol*, and *env* genes, which encode structural proteins, viral enzymes, and envelope glycoproteins, respectively. These are flanked by long terminal repeats. In addition, the genome comprises open reading frames for *trans*-acting transcriptional activator (Tat), the regulator of viral expression (Rev), and several other proteins such as Vif, Vpr, and Nef.

Mature viral particles measure 100 to 150 nm in diameter and have a conical core surrounded by a lipid envelope

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*This chapter contains information presented by Brigitte P. Griffith, Sheldon Campbell, and Angela M. Caliendo in chapter 79 of the 10th edition of this *Manual*.

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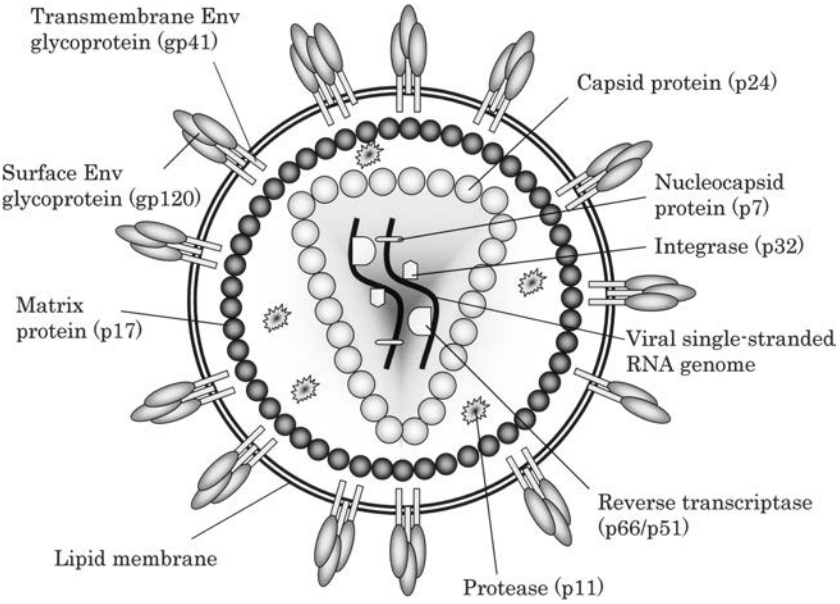
Transmembrane Env glycoprotein (gp41)
Capsid protein (p24)
Surface Env glycoprotein (gp120)
Nucleocapsid protein (p7)
Integrase (p32)
Matrix protein (p17)
Viral single-stranded RNA genome
Reverse transcriptase (p66/p51)
Lipid membrane
Protease (p11)

FIGURE 1 Schematic representation of a mature HIV virion showing the localization of viral proteins. doi:10.1128/9781555817381.ch82.f1

(Fig. 1). The core contains two copies of single-stranded RNA, approximately 10 kb in length, which are surrounded by structural proteins that form the nucleocapsid and the matrix shell as well as by-products of the *pol* gene. The lipid envelope is acquired as the virus buds from infected cells. The HIV-1 glycoprotein gp120/41 forms spikes that protrude from this envelope. The glycoprotein, a product of the *env* gene, is synthesized as a precursor, gp160, which is cleaved into the heavily glycosylated gp120, forming the majority of the external portion, and gp41, which contains the membrane-spanning domain. The major HIV-1 structural proteins encoded by the *gag* gene include p17, p24, p7, and p9. Products of the *pol* gene include protease, reverse transcriptase, RNase, and integrase.

HIV-2 is structurally analogous to HIV-1, but some of its protein components differ, most notably the outer envelope and transmembrane glycoproteins gp125 and gp36, and the core proteins p16 and p26. The major gene products that are of significance for the diagnosis of HIV-1 and HIV-2 are listed in Table 1.

TABLE 1 Major HIV proteins of diagnostic significance

HIV genes and products	Viral protein/glycoprotein mol wt	
	HIV-1	HIV-2
env		
Precursor	gp160	gp125
External glycoprotein	gp120	gp105
Transmembrane glycoprotein	gp41	gp36
pol		
Reverse transcriptase	p66	p68
Reverse transcriptase	p51	p53
Endonuclease	p31	p31/34
gag		
Precursor	p55	p56
Core	p24	p27
Matrix	p17	p16

Replication Cycle

The replication cycle of HIV-1 is accomplished *in vivo* in approximately 24 hours (14). Replication begins with the attachment of virus to the target cell via the interaction of gp120 and the cellular receptor CD4. This binding results in gp120 conformational changes that allow the virus to interact with other cellular coreceptor sites, CXCR4 or CCR5. The interaction with CXCR4 occurs primarily with T-cell-tropic, syncytium-inducing viruses (15). In contrast, the β-chemokine receptor CCR5 is involved in attachment of macrophage-tropic non-syncytium-inducing HIVs and is thought to be the predominant mode of new infections (16).

After fusion with the host cells, HIV enters the cell and RNA is released. HIV particles contain reverse transcriptase (RT), an enzyme that plays a crucial role in the replication process. RT possesses three distinct functions: RNA-dependent DNA polymerase, which serves to synthesize complementary DNA (cDNA); ribonuclease H (RNase H), which degrades RNA from the cDNA–RNA complex; and a DNA-dependent DNA polymerase, which synthesizes a DNA strand complementary to the cDNA. The reverse-transcribed genome is associated with several viral proteins and is transported into the nucleus. The double-stranded DNA copy becomes integrated into the genome of the infected cell by the virally encoded integrase; this integrated retroviral DNA genome is called the provirus. The cDNA then serves as a template for viral RNA. Activation of HIV transcription and gene expression is modulated by cellular transcription factors and by viral regulatory proteins, including Tat, Rev, Nef, and Vpu. The regulatory genes *tat* and *rev* greatly influence the rate of viral replication. The Tat protein increases transcription from the long terminal repeat, and the Rev protein facilitates export of unspliced or partially spliced RNAs encoding the viral structural proteins. At the end of the replication cycle, the virion assembles and buds through the plasma membrane.

EPIDEMIOLOGY AND TRANSMISSION

Epidemiology

Approximately 75 million people worldwide have become infected with HIV since the start of the pandemic. In 2012, 2.3 million people became newly infected, and an estimated 35 million people were living with HIV (UNAIDS 2013 Global Fact sheet). HIV-1 has been reported in virtually every country on earth. Group M viruses are responsible for most HIV-1 infections, with a predominance of subtype C. Subtype B is most prevalent in the United States, Europe, and Australia but is rarely found in Africa, where subtypes A, C, and D predominate. HIV-2 is found predominantly in West African nations, where it has infected an estimated 1 to 2 million people. In recent years, HIV-2 infections have been reported in countries with historical and socioeconomic ties to West Africa, including Brazil, France, Portugal, and India, and in other countries among immigrants from countries where HIV-2 is endemic (17). In 2012, approximately 9.7 million people living with HIV had access to antiretroviral therapy in low- and middle-income countries; updates from 2013 indicate that globally, new HIV infections have fallen by 33% since 2001, and AIDS-related deaths have fallen by 30% since the peak in 2005 (18).

The CDC estimated that 1.1 million persons in the United States were living with HIV at the end of 2010, of whom 181,000 (15.8%) were unaware of their diagnosis (19). The demographic characteristics of HIV infection continue to evolve. The CDC now generates annual estimates of the number of new HIV infections, including both those that are diagnosed and undiagnosed. In contrast to new HIV diagnoses (which can occur at any point after HIV infection), HIV incidence (the number of new HIV infections in a single year) reflects the leading edge of HIV transmission and HIV infection trends. Approximately 50,000 persons acquired HIV-1 each year from 2007 to 2010 (20). From 2008 to 2010, the number of new HIV infections among adult females decreased 21%, from 12,000 in 2008 to 9,500 in 2010. In contrast, the number of new infections in males increased 7%, from 35,500 in 2008 to 38,000 in 2010. In 2010, blacks/African-Americans accounted for 44% of new HIV infections, followed by whites (31%) and Hispanics/Latinos (21%). Men who have sex with men continue to bear the heaviest burden of HIV: in 2010, 68% of new HIV infections overall, and 78% of new infections among males, were attributed to male-to-male sexual contact. Among females, 84% of new HIV infections were attributed to heterosexual contact (20). After rapidly increasing through the 1980s, deaths due to HIV infection peaked in 1994-1995, decreased rapidly through 1997 with the introduction of combination antiretroviral therapy, and continued to decrease steadily through 2010. However, HIV infection remains one of the leading causes of death among persons 25 to 44 years old. The age-adjusted death rate due to HIV infection has been highest among blacks/African-Americans and second highest among Hispanics/Latinos.

Transmission

HIV-1 and HIV-2 have the same modes of transmission. The most common mode of infection is sexual transmission at the genital mucosa through direct contact with infected body fluids, including blood, semen, and vaginal secretions (21). Infection may also occur through inoculation of infected blood, via transfusion of infected blood products, transplantation of infected tissues, from an infected mother to her infant during pregnancy, or by reuse of contaminated needles. The risk of HIV-1 infection from occupational percutaneous exposure to HIV-1-infected blood has been estimated to be 0.3% (22). Postexposure prophylaxis may reduce this risk by 81% (23). The U.S. Public Health Service currently recommends 4 weeks of postexposure prophylaxis with three or more antiretroviral drugs after an occupational exposure (24).

The majority of HIV transmissions from mother to child occur in resource-poor countries. The level of maternal HIV-1 RNA or DNA in blood and genital fluids has been documented to correlate strongly with mother-to-child transmission (25). HIV transmission can occur *in utero*, during labor and delivery, and during breast-feeding. In the absence of therapeutic intervention, the risk of mother-to-child transmission can range from 15 to 30% and is further increased with breast-feeding. However, the risk can be reduced to less than 2% if antiretroviral therapy is administered to women during pregnancy and labor (26). Antiretroviral treatment of the infant immediately after birth can also significantly decrease the risk of HIV-1 infection in the newborn.

CLINICAL SIGNIFICANCE

Virological Parameters during the Course of HIV Infection

The natural history of HIV-1 infection can be divided into three phases: a transient acute retroviral syndrome associated with primary infection, an asymptomatic period during which active viral replication continues and disease progresses, and finally, advanced disease resulting in severe immune dysfunction and AIDS. Each of these stages is associated with specific changes in virological and immunological parameters. After HIV-1 infection, HIV-1-specific markers appear in the blood in the following chronological order: HIV-1 RNA, p24 antigen, HIV-1 IgM antibody, and HIV-1 IgG antibody (Fig. 2). The exact time at which each of these markers can be detected depends on a number of factors including characteristics of the infecting virus, the type of test used, and individual host immune responses. Immediately after exposure and transmission, HIV-1 replicates in the mucosa, submucosa, and lymphoreticular tissues, and the virus cannot be detected in plasma. This so-called eclipse phase generally lasts 10 days (range, 7 to 21 days) (27, 28). Once HIV-1 RNA reaches a concentration of 1 to 5 copies/ml, it can be detected by sensitive qualitative methods of nucleic acid amplification; at concentrations of 50 copies/ml, it can be detected by quantitative assays used clinically to monitor viral load. HIV-1 p24 antigen is then expressed and quantities rise to detectable levels within 4 to 10 days after the initial detection of HIV-1 RNA (29). However, p24 antigen detection is transient, because as antibodies begin to develop, they bind to the p24 antigen and form immune complexes that interfere with p24 assay detection unless the assay includes steps to disrupt the antigen–antibody complexes. Next, immunoglobulin (Ig) M-class antibodies are expressed, which can be detected by 3 to 5 days after p24 antigen is first detectable and 10 to 13 days after the appearance of viral RNA (29). Finally, IgG-class antibodies emerge and persist throughout the course of HIV infection. The time period after infection when HIV antibody is not detectable is referred to as the window period. In most infected persons, HIV antibody becomes detectable within 1 to 2 months after infection.

Acute Retroviral Syndrome

An estimated 50 to 70% of individuals with HIV infection experience an acute clinical syndrome 3 to 6 weeks after

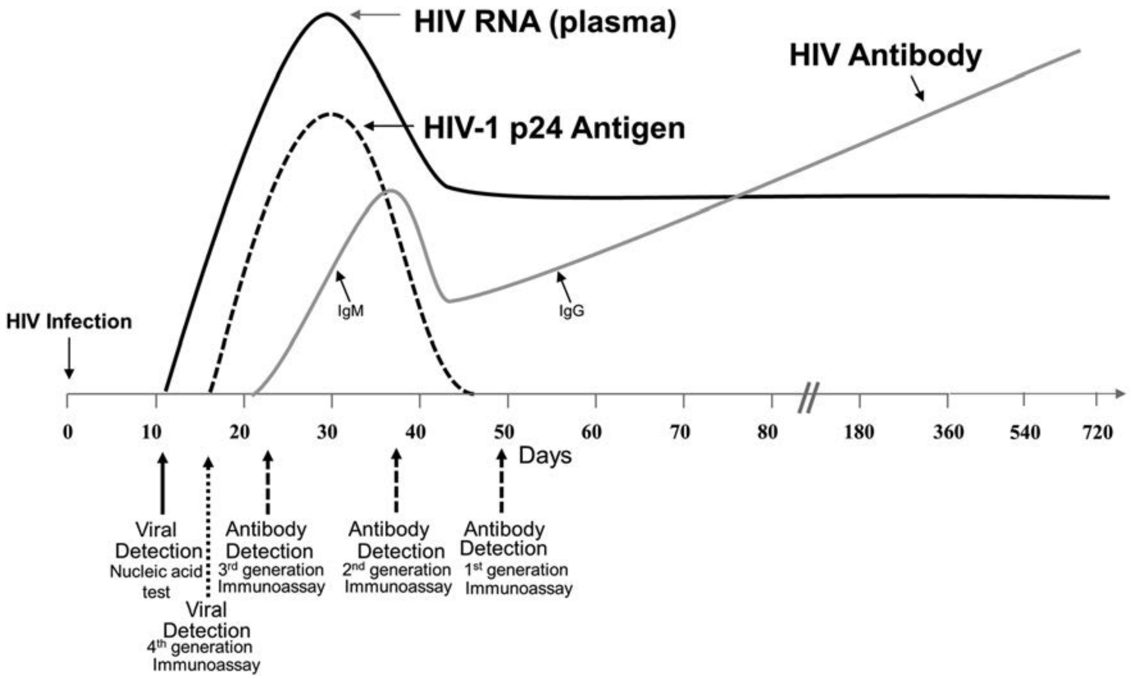

FIGURE 2 Time course of appearance of laboratory markers for HIV-1 infection. Units for vertical axis not noted because their magnitude differs for RNA, p24 antigen, and antibody. Adapted data from reference 90 and updated with data from references 29, 97, and 107.
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primary infection during which antibodies are often not detectable. Acute HIV-1 infection is a transient symptomatic illness that usually lasts 7 to 14 days and is associated with high levels of HIV-1 replication and a developing virus-specific immune response. Acute HIV-1 infection has been described as a mononucleosis-like syndrome. Clinical symptoms include fever, maculopapular rash, oral ulcers, lymphadenopathy, malaise, weight loss, arthralgia, pharyngitis, and night sweats (30–32). During acute HIV-1 infection, plasma viremia rises, reaching levels of up to 100 million copies of HIV-1 RNA per ml of plasma (33). Destruction of HIV-1-specific CD4+ T lymphocytes and widespread dissemination of the virus, with seeding of lymphoid organs and other tissue reservoirs, occurs. During resolution of primary infection, CD4+ T-cell counts rebound and viremia declines before reaching a steady level. This viral set point reflects ongoing viral replication and immune system damage and is an important prognostic indicator (34, 35). In prenatally infected infants, the HIV-1 RNA pattern differs from that in infected adults: high HIV-1 RNA levels may persist in HIV-infected children for prolonged time periods.

Clinical Latency

In most patients, primary infection with or without the acute syndrome is followed by a prolonged period of clinical latency or smoldering low disease activity. In untreated patients, the length of time from initial infection to the development of clinical immunodeficiency disease varies greatly; the median interval is 10 years. HIV disease with active virus replication is ongoing and progressive during this asymptomatic period. The number of CD4+ lymphocytes declines slowly and the virus continues to replicate. During this asymptomatic period, high titers of virus can be found in lymphoid and other tissue compartments (36). In rapid progressors, AIDS can develop within 1 to 2 years. In contrast, 5 to 10% of HIV-1- infected individuals are long-term nonprogressors and remain symptom-free for longer than 20 years (37). Approximately 0.5% of persons with HIV infection, termed elite controllers, are able to spontaneously maintain undetectable levels of virus without antiretroviral therapy.

Disease Progression to AIDS

In the absence of therapy, the continuous replication of HIV-1 in productively infected cells, together with the elimination of host cells and chronic immune activation, results in deterioration of the immune system. Studies of HIV-1 replication dynamics have shown that, in productively infected lymphocytes, the interval between infection, virus production, and cell death is very short. The half-life of HIV-1 in plasma is thought to be 6 hours, the length of the replication cycle in CD4+ lymphocytes has been estimated at 2.6 days, and 35 million CD4+ cells are thought to be lost daily (38). As the ability of the host to eliminate productively infected cells declines, CD4+ lymphocytes with integrated provirus accumulate. These resting memory CD4+ lymphocytes serve as a long-lived reservoir for HIV-1. This reservoir decays slowly (mean half-life of 44 months) even in treated patients who have had no detectable viremia for as long as 7 or more years (39). Clinical manifestations of HIV disease can affect nearly every organ system. Destruction of the immune system is clinically manifested by the occurrence of opportunistic infections and tumors. Central nervous system involvement also occurs, most commonly HIV-associated dementia (40). A decline of the CD4+ lymphocyte count to below 200 cells/μl marks the onset of immunologic AIDS and predisposition to opportunistic infections caused by viruses, bacteria, fungi, and protozoa, and to neoplastic disease, HIV encephalopathy, wasting syndrome, and progressive multifocal leukoencephalopathy, which are often the cause of death.

The course of HIV-1 infection and the clinical characteristics of AIDS in children differ from those in adults. The disease progresses rapidly in infants with vertically acquired HIV-1 infection. The most common AIDS-defining conditions in children include *Pneumocystis jirovecii* pneumonia and recurrent bacterial infections.

Public health organizations, including the CDC and the WHO, have published HIV disease classification systems for public health surveillance purposes. All case definitions of HIV infection now require laboratory-confirmed evidence of HIV infection. The current CDC classification system for adults and children categorizes HIV-1-infection into five stages (0, 1, 2, 3, and unknown). Stage 0, indicative of early infection, is defined by a negative or indeterminate HIV test result obtained within 180 days of a positive HIV test result. Stage 0 can be established either by testing history (a positive test result obtained after a previous negative or indeterminate test result) or by a laboratory testing algorithm that demonstrates the presence of HIV-specific viral markers, such as p24 antigen or nucleic acid (RNA or DNA), in conjunction with a negative or indeterminate antibody test result. Stages 1, 2, and 3 are categorized on the basis of age-specific CD4+ T-lymphocyte counts or percentages indicative of increasing severity (Table 2). Cases with no information on CD4+ T-lymphocyte count or percentage are classified as stage unknown (64).

Therapy and Vaccination

Significant scientific advances in the development of effective antiretroviral therapy have occurred in the past 25 years. The first effective drug against HIV-1, zidovudine, was approved by the FDA in March 1987. Currently, over 30 antiretroviral drugs or combinations of antiviral drugs have been approved by the FDA for the treatment of HIV infections (see www.aidsinfo.nih.gov for a list of FDA-approved drugs). Approved antiviral agents used to treat HIV-1 fall into five classes: nucleoside RT inhibitors, nonnucleoside RT inhibitors, protease inhibitors, integrase inhibitors, and entry and fusion inhibitors. Guidelines for the use of antiretroviral agents in adults and in children evolve rapidly. These are updated on a regular basis, and the most recent guidelines can be obtained from the AIDSinfo website (http://aidsinfo.nih.gov).

Several obstacles have hindered the development of effective vaccines against HIV-1, including the inability of HIV-1 to induce neutralizing antibodies to contemporaneous virus particles, the lack of understanding of correlates of protective immunity, the genetic diversity of the virus, and the limitation of animal models (41, 42).

COLLECTION, STORAGE, AND TRANSPORT OF SPECIMENS

Serum and plasma are the most common specimen types used for routine HIV antibody and antigen determinations in the laboratory. The most common tubes for plasma collection are potassium EDTA, sodium citrate, sodium and lithium heparin, and plasma preparation tubes. Prompt separation of serum and plasma from the clot/cellular elements is important to decrease hemolysis and maintain the most viable specimen for testing. Serum or plasma specimens can be transported either at room temperature, refrigerated, or frozen (after removal from cellular components) in screw-cap plastic vials. For antibody immunoassays, serum or plasma specimens may be shipped refrigerated (2 to 8°C) or at ambient temperature (≤ 37°C) for up to 7 days. For HIV antigen/antibody combination assays, the time that specimens can remain at room temperature is more restrictive. It is necessary to minimize room-temperature storage for optimum preservation of p24 antigen. Specimens for the antigen/antibody combination assays available in the U.S. as of December 2013 can be stored at room temperature for a maximum of 48 to 72 hours, or refrigerated at 2 to 8°C for up 7 days. It is important to include specimen transport times as part of the maximum allowable storage times at any given temperature. For long-term storage, serum or plasma specimens should be frozen at −20°C or lower.

Some HIV antibody assays are approved for use with dried blood spots, oral fluid, and urine; and some rapid point-of-care HIV assays can be performed with finger-stick whole blood, venipuncture whole blood, or oral fluids. Collection devices, procedures, and storage requirements are specified by the test manufacturer for use with these alternative specimen types.

Quantitative HIV-1 RNA viral load assays are most commonly performed on plasma specimens. Plasma for viral load determination is generally collected in potassium EDTA or acid citrate dextrose (ACD) tubes. Heparin anticoagulants inhibit PCR and should not be used for any nucleic acid assay that involves PCR amplification. To ensure accurate HIV-1 RNA quantification in plasma, proper collection, processing, storage, and transport of plasma specimens are essential. For all assays, plasma must be separated from blood cells in a timely manner to prevent RNA degradation. Assay manufacturers specify that whole blood can be maintained at room temperature for 6 to 24 hours or stored at 2 to 8°C for up to 24 hours before centrifugation, depending on the specific assay used. HIV-1 RNA is generally stable in cell-free plasma refrigerated at 2 to 8°C for 5 to 7 days. For long-term storage, plasma samples should be frozen at −20 to −70°C. HIV-1 RNA remains stable for at least three freeze-thaw cycles (43).

TABLE 2 HIV infection stage, based on age-specific CD4+ T-lymphocyte count or CD4+ T-lymphocyte percentage of total lymphocytes

Stage[a]	Age on date of CD4 T-lymphocyte test					
	<1 year		1–5 years		6 years through adult	
	Cells/µl	%	Cells/µl	%	Cells/µl	%
1	≥1,500	≥34	≥1,000	≥30	≥500	≥26
2	750–1,499	26–33	500–999	22–29	200–499	14–25
3	<750	<26	<500	<22	<200	<14

[a] The stage is based primarily on the CD4+ T-lymphocyte count; the percentage is considered only if the count is missing. Stage 0 is defined by a negative or indeterminate HIV test result obtained within 180 days of a positive HIV test result; cases with no information on CD4+ T-lymphocyte count or percentage are classified as stage unknown.

To simplify the process of collecting and transporting plasma, plasma preparation tubes (PPT) (BD Vacutainer PPT; Becton Dickinson), which contain dried EDTA and a gel separator that forms a barrier between the plasma and cellular elements after centrifugation, are available. PPT provide a closed collection system for the preparation and transport of plasma specimens. Plasma samples do not need to be aspirated from a PPT after centrifugation, eliminating plasma transfer and relabeling steps. This improves convenience, decreases risks of error, and improves safety. Initial manufacturer recommendations allowed for either refrigerated or frozen transport of centrifuged PPT. However, discrepancies in viral loads have been reported to occur in plasma specimens that were frozen *in situ* (44, 45). The differences were noted only for samples with viral loads close to the assay limit of detection; a large proportion of samples with undetectable viral load in a standard EDTA aliquot were found to have detectable HIV-1 RNA in the corresponding PPT. As a result, the tube manufacturer has recommended that plasma in PPT be stored without freezing; plasma from PPT should be transferred to a secondary tube if plasma specimens need to be stored frozen. Each of the FDA-approved viral load kits has specific volume, collection tube, and storage requirements.

The same collection system and procedures should be used for subsequent patient monitoring because different systems may produce discrepant values. For example, specimens collected in ACD tubes have also been shown to yield results that are approximately 15% lower than results obtained with EDTA plasma (46, 47). If a change in collection system or technology is planned, new baseline HIV-1 viral load values should be determined.

Laboratories that test specimens from pediatric patients should investigate flexibility of specimen volume because it may be difficult to obtain the 1 ml of plasma needed for several of the assays. Viral load assays have also been used to measure HIV-1 RNA in specimens other than plasma, including serum, dried blood spots, cerebrospinal fluid, cervical secretions, and seminal plasma (43). The RNA quantity in serum has been found to be lower than that in paired plasma samples; applications with specimens other than plasma must be validated because they are not included as approved specimen types for the current FDA-approved viral load assays.

Qualitative HIV-1 RNA assays can be conducted with serum and plasma. For the FDA-approved qualitative diagnostic assay, APTIMA (Hologic Gen-Probe Incorporated), blood specimens can be collected in glass or plastic tubes. EDTA, ACD, sodium citrate, EDTA PPT, or serum tubes can be used. For this assay, whole blood can be stored at ≤ 25°C for up to 72 hours; temperatures not to exceed 30°C are acceptable for not more than 24 hours. Specimens can be stored for an additional five days at 2 to 8°C after centrifugation. Plasma separated from cells can be stored for longer periods of time at ≤−20°C.

For HIV-1 antiretroviral drug resistance assays (genotypic and phenotypic), plasma collected from EDTA, ACD, or PPT EDTA tubes can be used. Plasma must be separated from the cellular elements within 6 hours of collection and frozen to prevent degradation of RNA. Higher integrity samples are achieved by minimizing the time before centrifugation and freezing of processed samples. Plasma specimens should be transported frozen and stored at −70°C for optimal results. While there are no FDA-approved methods for HIV-1 DNA amplification or viral culture, there are instances where these procedures are used for clinical purposes after validations are completed that meet clinical regulatory guidelines or for research purposes. Whole blood is commonly used in these applications. Blood should be collected in EDTA, ACD, or cell preparation tubes. For preparation of the peripheral blood mononuclear cells required for HIV DNA amplification, the blood specimen should not be refrigerated or frozen but instead should be kept at ambient temperature for no longer than 4 days.

DIRECT DETECTION

p24 Antigen Assays

In the early days of the AIDS epidemic, p24 antigen testing played an important role as a tool for the diagnosis, prognosis, and evaluation of antiretroviral activity and for monitoring of HIV-1-infected cultures. In 1996, the FDA approved the Coulter HIV-1 p24 Ag assay (Coulter Co., Miami, FL) for screening blood products. This test was used for screening blood and plasma donors in the United States for a few years until it was replaced with more sensitive nucleic acid tests in 1999 (48). At present, use of individual p24 antigen assays is very limited in the United States. The presence of HIV p24 antigen can be detected in plasma or serum by antigen capture enzyme immunosorbent assay (EIA). Because p24 antigen tests can produce false-positive reactions due to interfering substances and immune complexes, positive samples need to be confirmed by a neutralization procedure (49). p24 antigen is usually not detectable during the first 2 weeks after infection, but during acute infection, it becomes detectable before HIV-1 antibody (Fig. 2). However, detection of p24 antigen is transient because as HIV-1 antibodies begin to develop, they bind to the p24 antigen and form immune complexes that interfere with p24 assay detection unless the assay includes steps to disrupt the antigen–antibody complexes (50, 51). Modifications to the HIV-1 antigen test have been made to boost the sensitivity of the assay, including acid or heat treatment for dissociation of antigen–antibody complexes (51–53), and increased sensitivity has been achieved by the addition of signal amplification (54). Heat denaturation combined with tyramide signal amplification has been reported to increase the sensitivity of p24 antigen detection to levels comparable to detection of viral RNA by PCR; this method can be effective for monitoring responses to antiretroviral therapy (55–57) and for pediatric diagnosis (58, 59) in resource-limited settings when nucleic acid testing is not feasible.

HIV RNA and DNA Qualitative Assays

The Aptima HIV-1 qualitative RNA assay has clinical utility for identification of acute HIV-1 infection and neonatal HIV-1 infection, and for confirmation of HIV-1 infection in an individual whose specimen is repeatedly reactive for HIV antibodies. The test targets both the long terminal repeat and the *pol* gene of the HIV-1 genome, which allows detection of all HIV-1 group M, N, and O viruses. There are three general steps to the assay: target-specific capture of HIV-1 RNA from the clinical specimen, transcription-mediated amplification, and detection using a hybridization protection assay. The assay has a limit of detection of 30 copies/ml of plasma with a specificity of 99.8%.

Detection of HIV-1 DNA can also be used for HIV diagnosis in special situations such as during the acute phase of infection prior to appearance of antibody, in newborns of infected mothers, or in individuals with suppressed viral replication due to immunologic control or antiretroviral therapy. HIV-1 DNA qualitative assays use PCR to amplify conserved regions of the HIV-1 genome to detect proviral

HIV-1 DNA in peripheral blood mononuclear cells. Only one qualitative HIV-1 DNA PCR assay is commercially available (Roche Amplicor HIV DNA assay, version 1.5), but it is not approved by the FDA and may be phased out by the manufacturer (60). Laboratory-developed tests based on nested and real-time PCR procedures are also used. The limit of detection and ability to detect non-B subtypes vary among these assays. The sensitivity and specificity of standard HIV-1 DNA PCR assays for the diagnosis of neonatal HIV-1 infection have been reported to be 96% and 99%, respectively, at 1 month of age for HIV-1 subtype B (61). In the absence of antiretroviral therapy, comparisons of RNA versus DNA testing for infant diagnosis have shown little difference (62). It remains to be determined whether the more intensive combination antiretroviral regimens that women may receive during pregnancy for treatment of their own HIV infection will affect the sensitivity of RNA detection in their infants. The potential utility of qualitative DNA PCR assays in the diagnosis of vertical HIV-1 transmission has been greatly increased with the development of simple and sensitive procedures for dried blood spots. False-negative HIV-1 DNA results have been reported in infants with non-B subtypes (63), but the latest version 1.5 of the Roche Amplicor assay appears to have improved sensitivity for most HIV-1 subtypes.

HIV RNA Viral Load Assays

Viral load assays, which measure the quantity of HIV-1 RNA present in plasma, are used as prognostic indicators, to monitor response to therapy, and to determine infectiousness (65). HIV-1 viral load assays have been used most commonly to guide treatment decisions, but because of the relationship between viral load and potential for virus transmission, they are also used as a tool for guiding treatment as prevention interventions (66). In the United States, five commercial assays are FDA-approved for the quantification of HIV-1 RNA in plasma. The two most recently approved assays utilize real-time RT-PCR technology. A comparison of the specimen requirements and characteristics of the assays is shown in Table 3. The RealTime HIV-1 assay (Abbott Molecular, Des Plaines, IL) and the Cobas TaqMan HIV-1 version 2.0 assay (Roche Diagnostics, Indianapolis, IN) have been optimized to detect all group M subtypes, including all circulating recombinant forms and group O. The other viral load assays have not been optimized for group O viruses and will likely underquantify HIV-1 RNA levels. None of the viral load assays detect HIV-2.

The two real-time RT-PCR assays offer several advantages over the conventional viral load assays, including a very broad linear range (40 to 10,000,000 copies/ml for the RealTime HIV-1 assay and 20 to 10,000,000 copies per/ml for Cobas TaqMan HIV-1 version 2.0 assay), extensive automation, and decreased risk of carryover contamination. The RealTime HIV-1 assay uses the automated m2000 system, which has two components: the m2000sp for nucleic acid extraction and loading of sample and master mix into the 96-well optical reaction plate, and the m2000rt for amplification and detection. The assay contains an internal control, which is an unrelated RNA sequence that is added to the sample lysis buffer prior to extraction. RNA is captured by magnetic particles, washed to remove unbound material, and eluted. After the master mix and sample are combined into the reaction plate, the reaction plate is covered and loaded into the m2000rt; these are the only manual steps of the assay. The amplification and detection utilize TaqMan technology. The HIV-1 oligonucleotide probe is a partially double-stranded complex; the long strand is complementary to the HIV-1 target (integrase) and is labeled at the 5′ end with a fluorophore. The shorter strand is complementary to the 5′ end of the long strand and is labeled with a quencher moiety at its 3′ end. When HIV-1 target is present, the HIV-1-specific strand preferentially hybridizes to the target, allowing emission of fluorescence (67, 68). For calculations of viral load values, two assay calibrators are run in replicates of three to generate a calibration curve; the slope and intercept of the curve are stored on the instrument and used to calculate viral load values. The limit of detection of the assay is 40 copies/ml for a 1.0-ml sample volume, 75 copies/ml for a 0.5-ml sample volume, and 150 copies/ml for a 0.2-ml sample volume. The RealTime assay has been designed to quantify all group M, N, and O viruses and CRFs (67, 69).

The Cobas AmpliPrep/Cobas TaqMan HIV-1 test version 2.0 is also based on real-time TaqMan technology, and targets two HIV-1 gene regions, LTR and *gag*. The extraction process is automated on the Cobas AmpliPrep instrument using a generic magnetic silica-based capture method and includes a quantitation standard (QS) that is

TABLE 3 FDA-approved viral load assays and specimen requirements

Test and manufacturer	Amplification method; target	Anticoagulant	Plasma volume[a]	Range (copies/ml)	Standards and controls
Amplicor HIV-1 Monitor version 1.5 (Roche Diagnostics, Indianapolis, IN)	RT-PCR, *gag* gene	EDTA, ACD			1 internal QS, 3 external controls
Standard			200 µl	400–750,000	
Ultrasensitive			500 µl	50–100,000	
Cobas AmpliPrep/Cobas TaqMan HIV-1 Version 2.0 (Roche Diagnostics, Indianapolis, IN)	Real-time RT-PCR, *LTR*, *gag* gene	EDTA	1 ml	20–10,000,000	1 internal QS, 3 external controls
RealTime HIV-1 (Abbott Molecular, Des Plaines, IL)	Real-time RT-PCR, integrase gene	EDTA, ACD	1 ml	40–10,000,000	1 internal control, 3 external controls, calibration curve each new lot or every 6 months
Versant HIV-1 RNA 3.0 (bDNA) (Siemens Healthcare Diagnostics, Tarrytown, NY)	bDNA, *pol* gene	EDTA, ACD	1 ml	75–500,000	6 external standards, 3 external controls

[a] For tests that use an automated extraction instrument (AmpliPrep and RealTime), the listed specimen volume is the volume of sample that is loaded on the instrument, which is greater than the actual volume of specimen used for the extraction.

added to each specimen at a known concentration along with the lysis buffer. The extracted sample and the reaction mix are added to amplification tubes on the AmpliPrep instrument, and the amplification and detection are completed on either the Cobas TaqMan analyzer or the Cobas TaqMan 48 analyzer. Docking the Cobas TaqMan analyzer to the AmpliPrep instrument creates a fully automated system. Alternatively, the amplification tubes can be manually loaded into the Cobas TaqMan 48 analyzer. For calculation of viral load values, the fluorescent readings for the QS and target are checked by the instrument software to ensure they are valid, crossing threshold values are determined, and the viral load value is calculated from lot-specific calibration constants provided by the manufacturer. The amount of QS added to each sample is constant, so if the critical threshold for the QS has been affected, the HIV-1 target concentration is adjusted accordingly. The Cobas AmpliPrep/Cobas TaqMan HIV-1 test has been designed to quantify all group M viruses including CRFs and can also detect group O and N viruses (69–71).

The Amplicor HIV-1 Monitor assay (Roche Diagnostics, Indianapolis, IN) is based on reverse transcription of the target HIV-1 RNA and on PCR amplification of the resulting cDNA. The HIV-1 RNA copy number is calculated based on the input copy number of the QS RNA. One advantage of the assay is that there is minimal risk of amplicon contamination because the kit includes UTP and uracil-N-glycosylase. One disadvantage of the Amplicor HIV-1 Monitor assay is that it has a limited dynamic range, requiring both the standard and ultrasensitive procedures to be performed for specimens that fall outside the dynamic range. The Cobas Amplicor HIV-1 Monitor assay captures amplicons on magnetic beads, using the Cobas analyzer to automate the amplification and detection steps. The Cobas AmpliPrep/Cobas Amplicor assay automates the nucleic acid extraction, followed by amplification and detection on the Cobas instrument. Because of the popularity of the recently introduced real-time PCR format, the Amplicor assays are currently being phased out in the United States.

The Versant HIV-1 RNA 3.0 assay (Siemens Healthcare Diagnostics, Tarrytown, NY) is a branched DNA (bDNA) method that quantifies HIV-1 RNA by signal amplification instead of target (nucleic acid) amplification (72). The quantity of HIV-1 RNA is determined from an external standard curve run on the same plate; six kit standards of known viral concentration are used. Compared with other viral load assays, the reproducibility of the bDNA assay is superior, particularly at the low end of the dynamic range (73). The Versant HIV-1 RNA 3.0 bDNA assay offers the advantage of high throughput: in the current version, 84 specimens can be run on one plate. In addition, the assay can reliably quantify all subtypes of HIV-1. Disadvantages of the bDNA assay include the requirement for a large volume of plasma, the absence of an internal QS for each sample tested, and lower specificity compared to target amplification methods (32).

The high cost and complex technical requirements of nucleic acid-based testing strategies have resulted in the development of other types of assays for measuring virus quantity in specimens collected in resource-limited settings. Two such assays are the heat-denatured signal-boosted p24 antigen assays (Perkin Elmer, Wellesley, MA) and RT assay (ExaVir Load assay; Cavidi AB, Uppsala, Sweden) (74, 75). Although both methods are less sensitive and reproducible than the nucleic acid-based assays, they are affordable alternatives to HIV RNA quantification. Numerous efforts are also underway to develop point-of-care quantitative assays for HIV-1 nucleic acids (76).

ISOLATION PROCEDURES

Because HIV can be isolated from the blood of the majority of HIV-infected individuals, HIV culture was frequently utilized in the early years of the epidemic as a diagnostic or prognostic marker or for assessing the efficacy of antiviral therapy (35, 77). The procedure for HIV culture is elaborate and time-consuming (78). Although a positive culture provides direct evidence of HIV infection, HIV culture is utilized primarily in research laboratories and not for routine diagnosis. To isolate HIV, the patient specimen is first cultured by mixing patient cells with cells from healthy donors stimulated with phytohemagglutinin and interleukin-2; fresh stimulated donor cells must be added weekly because HIV-1 produces cell death. In the second step, the presence of RT or p24 antigen released in the culture supernatants is assayed periodically, generally every 3 to 7 days, for approximately 1 month of culture.

Viral culture assays have been used in phenotypic resistance assays and to determine the viral fitness of HIV-1 (79). However, phenotypic resistance assays based on recombinant DNA technology and amplification of plasma viral RNA have obviated the need for viral isolates (80). A commercially available fitness assay, also referred to as viral replication capacity (RC) assay, has been developed by Monogram BioSciences (South San Francisco, CA). The RC assay measures the ability of HIV-1 from a patient undergoing antiretroviral treatment to replicate *in vitro* compared to a wild-type reference virus. The patient RC value is expressed as a percentage of the RC of the wild-type reference standard. The assay uses a retroviral vector constructed from an infectious clone of HIV-1. The vector contains a luciferase expression cassette inserted within a deleted region of the envelope gene (52). HIV-1 protease and RT sequences are amplified from the patient plasma samples and inserted into the vector. The amount of luciferase produced by patient-derived viruses is then compared to the amount of luciferase produced by well-characterized wild-type reference virus. RC has been suggested as an additional parameter for making decisions regarding antiretroviral therapy. Patients who do not experience increases in viral loads despite the accumulation of multiple resistance mutations have been shown to harbor virus with decreased RC (81). In addition, certain drug resistance mutations have been shown to reduce RC (83, 84); lower RC has also been observed in persons who become HIV controllers (85).

SEROLOGIC TESTS

Diagnosis of HIV infection usually has been accomplished via detection of HIV antibody using a sensitive initial immunoassay validated by a subsequent supplemental test (86). Because HIV infection typically persists for the lifetime of the individual, and the initial infection may be minimally symptomatic, most patients who are identified by antibody detection are in the clinically latent or late phases of the illness. Immunoassays for HIV antibody are rapid and economical, but they have an important limitation because of the window period, the time between initial infection and the expression of detectable antibody (29). During the window period, active viral replication and high levels of viremia occur. Different types of antibody immunoassays have different window periods, but all tests based on detection of antibody miss patients during early infection. Recognition that the risk of transmission from persons with acute infection is much higher than that from persons with established infection (87) and indications of the clinical benefits from antiretroviral treatment during acute HIV infection (88) have served as the impetus for adoption of techniques that detect HIV earlier after initial infection. Antigen-antibody combination immunoassays and an HIV-1 nucleic acid test (NAT) that allow earlier detection of HIV have received FDA approval and play an increasingly important role in HIV diagnosis.

Initial Screening Tests

Considerable progress in the development of HIV immunoassays has been made since the discovery of the virus in 1983. Current methods are summarized in Table 4. HIV immunoassays based on different design principles are generally grouped into generations. The earliest (first-generation) immunoassays are indirect EIAs that used coated (or immobilized) viral lysate antigens derived from cell culture on a solid phase for antibody capture and an indirect format that detected antibody using an anti-human IgG conjugate. To increase specificity, significant specimen dilution is required to overcome cross-reactivity with cellular protein contaminants. Second-generation immunoassays use synthetic peptide or recombinant protein antigens alone or combined with viral lysates to bind HIV antibodies, and they use an indirect immunoassay format that employs labeled antihuman IgG or protein A, which binds to IgG with high affinity (89), to detect IgG antibodies. Design of the specific antigenic epitopes in the second-generation assays improves sensitivity for HIV-1, HIV-1 group O, and HIV-2, allowing earlier detection of IgG antibodies (90). Eliminating cellular antigens that contaminate viral lysates improves specificity by eliminating cross reactivity with cellular proteins. Third-generation immunoassays also use synthetic peptide or recombinant antigens to bind HIV antibodies, but in an immunometric antigen sandwich format: HIV antibodies in the specimen bind to HIV antigens on the assay substrate and to antigens conjugated to indicator molecules. This allows detection of both IgM and IgG antibodies. Lower sample dilutions and the ability to detect IgM antibodies (which are expressed before IgG antibodies) further reduce the window period during early seroconversion (91, 92). Fourth-generation immunoassays use synthetic peptide or recombinant protein antigens in the same antigen sandwich format as third-generation assays to detect IgM and IgG antibodies, and also include monoclonal antibodies to detect p24 antigen. Inclusion of p24 antigen capture allows detection of HIV-1 infection before seroconversion (93–96). Most fourth-generation antigen/antibody immunoassays (termed "combo" assays") do not distinguish antibody reactivity from antigen reactivity. Analyses of specimens from seroconversion panels have established the approximate time of detection by the different generations of immunoassays. Estimates derived from several data sources are outlined schematically in Fig. 2 (29, 90, 94, 97).

Conventional Immunoassays

Conventional immunoassays for laboratory use consist of EIA and chemiluminescent immunoassay (CIA) methods. Most of the commonly used assays incorporate specific antigens for the detection of HIV-1 groups M and O and HIV-2. HIV antigens (and anti-p24 antibodies in fourth-generation assays) are adsorbed to a solid phase (usually plates, beads, or tubes), which binds HIV antibodies (or p24 antigen) in the specimens. Antibodies (and p24 antigen with fourth-generation assays) are conjugated to enzymes (alkaline phosphatase or horseradish peroxidase) or to acridinium esters. The indirect EIA format uses an enzyme-labeled antiglobulin conjugate; the antigen-sandwich EIA and CIA formats use a conjugate with enzyme-labeled HIV antigens (or anti-p24 monoclonal antibodies). With an EIA, the end result is a color change, measured as optical density by a spectrophotometer, that is proportional to the amount of antibody (or antigen) in the specimen. CIA results are expressed in relative light units, also proportional to the amount of antibody (or antigen) in the specimen. Results are compared to those of a calibrator; a result with a signal-to-cutoff ratio of >1.0 is considered to be reactive. Both EIAs and CIAs are suitable for automation. CIAs, characterized by a shorter processing time and wider dynamic range than EIAs, have been developed for random-access immunochemistry platforms with the potential to make laboratory-based HIV testing simpler and faster.

Rapid Immunoassays

The logistics of conventional laboratory HIV immunoassays require phlebotomy and, typically, a follow-up visit for test results after the specimen has been processed. This complicates HIV testing for many hard-to-reach populations for which phlebotomy is impractical; many persons may also fail to return for their test results (98). In addition, an immediate HIV test result is medically desirable in certain circumstances when antiretroviral prophylaxis should be initiated promptly, for example, in assessment of the source patient after an occupational blood or body fluid exposure, and for pregnant women in labor whose HIV status is

TABLE 4 FDA-approved conventional laboratory HIV serologic tests

Test	Markers used for detection	Analytes detected	Generation
Conventional EIAs			
Avioq HIV-1 Microelisa System	Viral lysate, native gp160	IgG antibodies	Second
Bio-Rad GS HIV-1/2 *PLUS* O	Recombinant p24, gp160, HIV-2 gp36, synthetic group O peptide	IgG and IgM antibodies	Third
Bio-Rad GS HIV Combo Ag/Ab EIA	Synthetic gp41, recombinant gp160, HIV-2 gp36, synthetic group O peptide, p24 monoclonal antibodies	IgG and IgM antibodies p24 antigen	Fourth
Chemiluminescent assays			
Abbott Architect HIV Ag/Ab Combo	Synthetic and recombinant gp41 and HIV-2 gp36, group O peptide, p24 monoclonal antibodies	IgG and IgM antibodies P24 antigen	Fourth
Ortho Vitros Anti-HIV 1+2	Recombinant p24, gp41, gp41/120, HIV-2 gp36	IgG and IgM antibodies	Third
Siemens Advia Centaur HIV 1/O/2	Recombinant gp41/120, p24, HIV-2 gp36, synthetic group O peptide	IgG and IgM antibodies	Third

unknown (24, 99). To meet these needs, rapid HIV tests have been developed that are suitable for use at the point of care as well as in clinical laboratories.

Rapid HIV immunoassays are single-use devices that use either immunoconcentration (flow-through) or immunochromatographic (lateral-flow) principles (100). Flow-through assays require the sequential addition of specimen, conjugate reagent, and a clarifying buffer through a membrane. Lateral-flow assays contain all necessary reagents and are extremely simple to perform because they require the addition of only specimen or specimen and buffer. Rapid tests differ in their required specimen volumes (range 3 µl to 50 µl for whole blood, serum, or plasma) and processing times (range 1 to 20 minutes). Rapid tests that can use direct, unprocessed specimens (whole blood or oral fluid) have been waived under the Clinical Laboratory Improvement Amendments (CLIA) and are especially well suited for testing outside of traditional laboratory settings. Nine rapid HIV antibody tests were FDA-approved in the United States as of December 2013 (Table 5). Disadvantages of rapid assays include their subjective interpretation, possible errors if the reader has vision problems such as color blindness, and potential for procedural errors when performed by less skilled personnel (101, 102). Accurate specimen volumes must be dispensed and tests read within specified time limits, control lines must be observed, and instructions must be followed carefully. Rapid tests are useful primarily for small-volume testing. Accurate timing of steps can be adversely affected when multiple specimens are tested simultaneously. Nevertheless, rapid HIV tests have become valuable tools for clinical situations in which rapid results are essential, and for outreach settings (103).

Published studies suggest that rapid HIV antibody immunoassays perform similarly to laboratory-based conventional immunoassays in established HIV infection (104). However, most use colloidal gold bound to protein A for detection of IgG antibodies; sensitivity with plasma seroconversion panels is similar to that of second-generation conventional immunoassays (94, 97). Comparative studies of rapid tests performed on oral fluid, whole blood, and serum demonstrate that rapid tests identified fewer HIV infections than conventional laboratory immunoassays, and sensitivity with oral fluid specimens is lower than that with whole blood or serum in persons from populations with increased prevalence of early HIV infection (105, 106). One rapid HIV test that detects and distinguishes HIV antibodies and p24 antigen, the Alere Determine HIV Combo, has received FDA approval (Table 5). Its overall sensitivity is similar to that of third-generation assays; sensitivity of the antigen component is lower than that of laboratory-based fourth-generation assays (106–108).

Alternative Specimens for Antibody Testing

Alternative specimens to blood, serum, or plasma for HIV antibody testing include oral fluid, urine, and dried blood spots. These may be useful in testing of patients who are reluctant to undergo phlebotomy or who have poor vascular access, in mass-screening settings, in locations in which phlebotomy is impossible, in infants, and for seroprevalence studies. It is essential to employ only specimen collection systems and testing methods designed and validated for the specific specimen type. Oral fluid is a complex mixture of secretions from several different sets of glands, as well as transudated plasma from the capillaries of the gum and mucosa. Glandular secretions of saliva primarily contain secretory IgA, which is not a reliable target for diagnostic testing. Most IgG in the oral cavity derives from the crevicular space between the gums and the teeth, and not from salivary glands, or can be obtained by inducing an oral mucosal transudate. The OraSure specimen collection device is designed to collect oral mucosal transudate for conventional testing (109) with an FDA-approved oral fluid-based HIV-1 EIA and Western blot. Two rapid tests are FDA approved for use with oral fluid as well as whole blood and plasma specimens. However, false-negative and false-positive immunoassay results and indeterminate Western blot results occur more frequently with oral fluid than with serum specimens (105, 110–112). Dried blood spots, after

TABLE 5 FDA-approved rapid and point-of-care HIV tests

Test	Manufacturer	Specimen types	CLIA category[a]	Antigens represented	FDA approval
OraQuick Advance Rapid HIV-1/2 Antibody Test	Orasure Technologies, Inc.	Oral fluid, whole blood, plasma	Waived; moderate complexity	gp41, gp36	2002
Reveal G3 Rapid HIV-1 Antibody Test	MedMira, Inc.	Serum, plasma	Moderate complexity	gp41, gp120	2003
Uni-Gold Recombigen HIV-1/2	Trinity BioTech	Whole blood, serum, plasma	Waived; moderate complexity	gp41, gp120, gp36	2003
Multispot HIV-1/HIV-2 Rapid Test	Bio-Rad Laboratories	Serum, plasma	Moderate complexity	gp41, gp36	2004
Alere Clearview HIV 1/2 Stat Pak	Chembio Diagnostics	Whole blood, serum, plasma	Waived; moderate complexity	gp41, gp120, gp36	2006
Alere Clearview Complete HIV 1/2	Chembio Diagnostics	Whole blood, serum, plasma	Waived; moderate complexity	gp41, gp120, gp36	2006
INSTI HIV-1 Antibody Test Kit	Biolytical Laboratories	Whole blood, serum, plasma	Waived; moderate complexity	gp41, gp36	2010
Chembio DPP HIV 1/2 Assay	Chembio Diagnostics	Oral fluid, whole blood, serum, plasma	Waived; moderate complexity	gp 41, gp120, gp36	2012
Alere Determine HIV 1/2 Ag/Ab Combo	Alere	Whole blood, serum, plasma	Waived; moderate complexity	gp41, gp120, gp36; p24 antibodies	2013
Geenius HIV 1/2 Supplemental Assay	Bio-Rad Laboratories	Whole blood, serum, plasma	Moderate complexity	p24, p31, gp41, gp160, gp36, gp140	2014

[a] Waived status applies only when tests are used with direct, unprocessed specimens (whole blood or oral fluid).

appropriate elution, can be tested for HIV antibody, p24 antigen, and HIV RNA. Results from HIV antibody and p24 antigen tests are comparable to those from matched serum or plasma specimens (58, 113, 114). Because of the lower specimen volume, sensitivity for RNA in dried blood spots is 78 to 100% compared with plasma at viral loads of <1,000 copies/ml, but increases to 100% at a threshold of 500 copies/ml (115, 116). Depending on the technology and the viral load distribution, the percentage of dried blood-spot samples that are within 0.5 $\log_{10}$ of viral load in plasma ranges from 52 to 100%. HIV viral load tests performed on dried blood spots also measure cell-associated RNA and proviral DNA, potentially leading to false-positive results or elevated values compared with plasma, especially at low copy numbers (116). In urine, IgG is found in small quantities relative to serum. Sensitivity and specificity of the FDA-approved EIA and Western blot for urine specimens was lower than with matched serum specimens tested with conventional EIAs (117).

Screening for Atypical and HIV-2 Infections

The prevalence of HIV-1 group M subtypes and CRFs varies geographically. All subtypes and most CRFs are found in sub-Saharan Africa. Subtype B is the predominant strain in the United States, Europe, Canada, and Australia. However, the prevalence of non-B subtypes in these countries is increasing. In the United States, non-B infections account for approximately 3 to 5% of HIV infections (118, 119). Current HIV immunoassays reliably detect the overwhelming majority of HIV-1 group M, B, and non-B subtype infections (120, 121). Only three group O infections have been documented in the United States, all in individuals with a link to west central Africa. HIV-1 group O infections can be missed by immunoassays that do not contain specific reagents for the detection of antibody to group O (122).

Immunoassays specific for detection of HIV-1 antibodies can detect HIV-2 infections because of cross-reactivity to HIV-1 antigen present in the assay. However, detection of HIV-2 infection by HIV-1–only immunoassays is highly variable: different assays detect 51 to 100% of HIV-2 infections (97). The capability of HIV-1/2 and HIV-2 assays to detect HIV-2 infections must be demonstrated for the assays to obtain approval by regulatory agencies, and most currently available FDA-approved HIV-1/2 assays incorporate gp36 antigen for reliable detection of HIV-2 (Tables 4 and 5).

Supplemental Assays for HIV

Initial screening immunoassays for HIV are optimized to provide very high sensitivity, often at the expense of specificity. HIV diagnostic testing therefore relies on a sequence of tests used in combination to improve the accuracy of HIV laboratory diagnosis. Specimens that are nonreactive on the initial immunoassay are generally considered HIV negative. If the initial immunoassay result is reactive, it must be followed by one or more supplemental tests. Four assay methods are FDA approved as supplemental tests: HIV-1 Western blot, indirect immunofluorescent assay (IFA), qualitative HIV-1 RNA assay, and HIV-1/HIV-2 antibody differentiation assay. Both the Western blot and IFA are highly specific, but because they rely on viral lysate antigens and anti-human IgG conjugates, they detect HIV-1 later during seroconversion than most currently available conventional initial immunoassays and thus may produce false-negative or indeterminate results (123, 124). In addition, because of cross-reactivity, the HIV-1 Western blot has been interpreted as positive for HIV-1 in 46 to 85% of specimens from persons infected with HIV-2 (125, 126). An HIV-1 qualitative RNA assay can be used for the diagnosis of acute HIV-1 infection in serum or plasma from patients without antibodies to HIV-1, and as a supplemental test, when it is reactive, for HIV-1 with specimens that are repeatedly reactive for HIV antibodies. However, HIV-1 RNA is undetectable in 2 to 5% of HIV-1 Western blot-positive specimens from infected persons. Therefore, a specimen with a nonreactive HIV-1 RNA qualitative assay result after repeatedly reactive HIV-1/HIV-2 antibody immunoassay results must undergo supplemental antibody testing to confirm whether HIV-1 or HIV-2 antibodies are present (92, 97). Two HIV-1/HIV-2 antibody differentiation assays have been approved by the FDA for use as supplemental tests and for differentiation of HIV-1 and HIV-2 antibodies. Because the differentiation assays use an indirect EIA format with an anti-human IgG conjugate, they also detect HIV antibodies later during seroconversion than most currently available conventional initial immunoassays (123, 124).

Historically, the HIV-1 Western blot has been the gold standard for HIV diagnosis (86). The Western blot owes its specificity to separation and concentration of viral components (127). A viral lysate of HIV is applied in a gel under an electric field; the mixture of viral components is separated by their molecular weights into specific "bands." Each viral component becomes relatively pure as it is separated. Components are blotted separately onto a membrane, which is cut into strips. The testing laboratory incubates the strips with patient serum, plasma, or dried blood-spot eluates and then develops the reaction with an enzyme-labeled antihuman antibody. Antibodies to the following HIV-1-associated antigens can be detected: gp160, gp120, p66, p55, p51, gp41, p31, p24, p17, and p15. Additional viral bands may be described by some manufacturers, and the molecular weight of some antigens might vary slightly between assays produced by different manufacturers. Because Western blot antigens are prepared from HIV grown in cell culture, nonviral cellular proteins may be present on the nitrocellulose strip and lead to nonspecific reactions. The interpretation of HIV-1 Western blots predominantly follows CDC guidelines. A positive result requires detection of at least two of three antigens: p24, gp41, or gp120/160 (86). The absence of all bands is a negative result. The presence of HIV-associated bands not meeting the criteria for positivity, or the presence of nonviral bands, is interpreted as an indeterminate result. After a reactive initial HIV-1/HIV-2 immunoassay, negative or indeterminate HIV-1 Western blot results should also be followed with additional testing specific for HIV-2 antibodies (128). Western blots that include HIV-2 antigens (e.g., gp36 or gp105) are available outside the United States, but none are FDA-approved. Line immunoassays employ a principle similar to that of the Western blot, but recombinant or synthetic antigens are placed on the strip instead of viral lysate antigens from an electrophoretic gel. This approach has the advantage of using only viral antigens in the reaction, eliminating the background from cross-reactivity with nonspecific cellular proteins. The manufacturer also has control over the quantity and type of antigens represented, and can include HIV-2 and group O antigens to confirm these infections with a single assay (129). The Inno-LIA HIV I/II Score test (Innogenetics, Ghent, Belgium) is a line immunoassay widely available outside the United States.

The Fluorognost HIV-1 IFA (Sanochemia) has been used as both an initial screening test and as a supplemental test for HIV-1 infection with serum, plasma, and dried blood-spot eluates. HIV-infected and uninfected lymphocytes are fixed on a slide. The slide is incubated first with patient serum and then with a fluorescent-labeled antihuman antibody (130). For interpretation, patterns of fluorescence in infected and uninfected cells are compared for each patient specimen; fluorescence on the infected lymphocytes that exceeds that from nonspecific antibody binding on the uninfected lymphocytes is interpreted as a positive result. Considerable skill is required, and indeterminate results can be produced in patients with autoantibodies and other conditions. In addition to its use as an independent supplemental test, IFA can be used to resolve indeterminate HIV-1 Western blots. An HIV-2-specific IFA has been described but it is not FDA approved.

The APTIMA HIV-1 Qualitative Assay is FDA approved for detection of HIV-1. HIV-1 RNA in the test specimen is hybridized to capture nucleotides homologous to highly conserved regions of HIV-1. The hybridized target is then captured onto magnetic microparticles and separated from plasma in a magnetic field. Target amplification occurs via a transcription-based nucleic acid amplification. Detection is achieved by chemiluminescent-labeled nucleic acid probes that hybridize specifically to the amplicon. The chemiluminescent signal, measured in a luminometer, is reported as relative light units. Analytical sensitivity is 30 copies/ml of HIV-1 RNA. Quantitative viral HIV-1 and HIV-2 RNA and DNA NAT are also available but are not FDA approved for HIV diagnosis.

The Multispot HIV-1/HIV-2 Rapid Test (Bio-Rad Laboratories, Redmond, WA) is a flow-through rapid EIA that differentiates HIV-1 and HIV-2 antibodies in a single-use cartridge. Microscopic particles are coated separately with a synthetic gp41 peptide and recombinant gp41 antigen for HIV-1, and with a synthetic gp36 peptide for HIV-2. The microparticles are immobilized separately on the reaction membrane in three separate test spots. Antibodies against HIV-1 or HIV-2 in the test specimen bind to the antigens in the spots on the membrane. Alkaline phosphatase-labeled goat anti-human IgG is added to the cartridge and binds to the antibody–antigen complexes immobilized in the spots. When development reagent is added to the cartridge, a purple color develops at the spots in proportion to the amount of antibodies present. The membrane is examined visually for the presence of purple color on the test spots. Reactivity at both gp41 spots is required for defining a positive supplemental HIV-1 test result; reactivity at only one HIV-1 spot is interpreted as indeterminate. Sensitivity of the Multispot antibody differentiation assay for established HIV infection is comparable to that of the HIV-1 Western blot, but it produces fewer indeterminate results and accurately identifies HIV-2 antibodies, including those in specimens misclassified as HIV-1 by the HIV-1 Western blot (123, 124, 131, 132). Because of cross-reactivity, approximately 0.4% of reactive specimens remain dually reactive at the HIV-1 and HIV-2 spots after recommended dilution procedures (124). Although most dually reactive specimens represent HIV-1 infections with cross-reactivity to HIV-2 (133), one U.S. study of five dually reactive specimens found detectable HIV-2 RNA in the one specimen with strong HIV-2 reactivity, suggesting that strong reactivity at the HIV-2 spot indicated the need for further investigation with HIV-2 NAT (132).

The Geenius HIV-1/2 Supplemental Assay (Bio-Rad Laboratories, Redmond, WA) received FDA approval as a supplemental test in October 2014. The assay employs the dual path platform principle for rapid differentiation of HIV-1 from HIV-2 in serum, plasma, and whole blood specimens. The dual path principle appears to be more useful for multiplex testing for different antibodies on the same strip. Specimen is added to the cartridge device and flows from one direction across the test strip, onto which four separate HIV-1 antigens (p24, p31, gp41, and gp160) and two HIV-2 antigens (gp36 and gp140) have been adsorbed. The detection agent (colloidal gold conjugated to protein A) is added to flow from a perpendicular direction. The test uses an automated reader and software that provides interpretation of results based on the presence and intensity of the bands (163, 172).

ANTIVIRAL SUSCEPTIBILITIES

Resistance testing is an essential element in the management of antiretroviral therapy. It is important for the selection of initial regimens because of the prevalence of transmitted drug resistance in therapy-naïve patients and for the selection of antiretroviral treatment for patients who are failing their current regimen due to the development of antiviral resistance (http://aidsinfo.nih.gov).

The clinical utility of HIV resistance testing has been evaluated in a number of prospective randomized clinical trials (79, 134, 135). Patients whose antiretroviral treatment was based on the results of resistance testing had greater decreases in viral load than did patients in whom the antiretroviral regimen was based on prior antiretroviral usage, and the use of resistance testing to guide treatment is cost-effective (136, 137). Two types of methods are available to assay for HIV resistance. Genotyping tests examine the population of viral genomes in the patient sample for the presence of mutations known to confer decreased susceptibility to antiretroviral drugs. Phenotypic assays measure viral replication of the patient's virus in the presence of antiretroviral drugs. In addition, HIV tropism assays have become important for evaluating susceptibility to HIV entry inhibitors that target viruses that use CCR5 for cell entry. Table 6 provides a list of the currently available commercial HIV-1 resistance and tropism assays.

Genotyping Assays

The initial steps of genotypic assays include extraction of viral RNA from plasma and RT-PCR to amplify viral RNA sequences that code for portions of the viral genome that are targeted by antiretroviral drugs. These sequences include genes in the pol region of the virus for reverse transcriptase, protease, and integrase as well as envelope regions related to the entry and fusion inhibitors. The nucleotide sequence is determined and examined for the presence of known resistance mutations. This is accomplished most commonly by using automated sequencing technology. The nucleotide sequence of the gene of interest is obtained and compared to the sequence of wild-type virus to identify resistance mutations. The process requires alignment and editing of the sequence, comparison to the wild-type sequence, and final interpretation to identify mutations associated with resistance to specific antiretroviral drugs. FDA-approved kits containing sequencing reagents and the software programs required for sequence alignment and interpretation are commercially available. The Trugene HIV-1 genotyping kit and OpenGene DNA sequencing system (Siemens Healthcare Diagnostics), and the ViroSeq HIV-1 genotyping system (Abbott Molecular) perform in an equivalent manner (138). These tests detect mutations in the reverse transcriptase and protease genes but do not detect mutations associated with resistance to the integrase or entry inhibitors. Additional genotypic tests that detect mutations associated with other classes of drugs, such as integrase inhibitors (raltegravir, elvitegravir, dolutegravir) and entry inhibitors (enfuvirtide, maraviroc), are available in commercial laboratories but they are not sold as standalone kits. The databases used for interpretation of resistance mutations require regular updating as the number of new antiretroviral drugs continues to expand (173).

One limitation of genotypic assays is that they are only able to detect mutants that comprise major fractions of the

TABLE 6 HIV-1 resistance and tropism assays

Test	Format	Description
Trugene HIV-1 genotyping kit and Open Gene DNA sequencing system (Siemens Healthcare Diagnostics, Tarrytown, NY)	Genotypic resistance	Detects mutations known to be associated with drug resistance to protease inhibitors and reverse transcriptase inhibitors
Viroseq HIV-1 genotyping system (Abbott Molecular, Des Plaines, IL)	Genotypic resistance	Detects mutations known to be associated with drug resistance to protease inhibitors and reverse transcriptase inhibitors
Integrase and Envelope Genotypes Various commercial labs	Genotypic resistance	Sequence analysis of integrase or envelope genes to identify mutations associated with resistance to integrase inhibitors[a] or fusion inhibitor[b]
PhenoSense HIV (RT and protease inhibitors) (Monogram Biosciences, South San Francisco, CA)	Phenotypic resistance	Measures decrease in virus replication in presence of reverse transcriptase and protease inhibitors
PhenoSense for entry inhibitor susceptibility (Monogram Biosciences, South San Francisco, CA)	Phenotypic resistance	Measures decrease in virus replication in presence of entry inhibitors[b,c]
PhenoSense integrase (Monogram Biosciences, South San Francisco, CA)	Phenotypic resistance	Measures decrease in virus replication in presence of integrase inhibitors[a]
SensiTrop II HIV coreceptor tropism (Pathway Diagnostics, Malibu, CA)	Genotypic cell tropism	Virus gene sequences used to predict CCR5 cell tropism before initiation of CCR5 antagonist[c]
Trofile Co-Receptor Tropism (Monogram Biosciences, Inc. South San Francisco, CA)	Phenotypic cell tropism	Single round virus replication assay that uses envelope sequences from patient viruses to determine the ability to replicate in cells with CCR5, CXCR4, or both coreceptors before initiation of CCR5 antagonist[c]

[a] Raltegravir, elvitegravir, dolutegravir.

[b] Enfuvirtide.

[c] Maraviroc.

patient's virus: resistant variants must constitute at least 25 to 30% of the virus population (139). Although the clinical significance of resistant mutations that are present at low levels remains to be fully elucidated, there is evidence that minor mutations that are missed by standard genotyping assays can lead to failure of subsequent treatments (140). Because standard genotyping assays lack sensitivity for low-frequency drug resistance mutations, efforts are underway to increase sensitivity by various methods including PCR-based assays (141), single-genome analysis (142), and deep sequencing (143, 144). These techniques are not FDA approved and the single-genome sequencing approach is not yet a practical clinical tool because the process is expensive and labor-intensive. However, next-generation deep-sequencing technologies offer the promise of reducing the cost of sequencing, and evaluations of this approach for clinical laboratories are underway (143).

Phenotyping Assays

Phenotyping assays measure the ability of HIV-1 to grow in the presence of various concentrations of an antiretroviral agent. The amount of drug required to inhibit virus replication by 50% or by 90% is determined and given as a 50% or 90% inhibitory concentration (IC_{50} or IC_{90}). The IC_{50} or IC_{90} obtained with the patient sample is compared to a control wild-type virus, and the result is reported as a relative difference. Early phenotypic resistance assays were labor-intensive because they necessitated the isolation and culture of HIV from the patient's specimen. Currently, commercially available methods use HIV-1 RNA amplified from plasma and are based on recombinant DNA technology. These phenotypic assays are automated but remain labor-intensive and technically complex. They have not been developed into a kit format and are only performed at commercial laboratories such as Monogram Biosciences (South San Francisco, CA). The first step involves extraction of HIV-1 RNA from plasma. Reverse transcription and PCR amplification of the protease and RT genes follow. The amplified genes from the patient's specimen are then inserted into vectors, and recombinants are used in culture to examine resistance. The PhenoSense assay (Monogram BioSciences) uses an HIV-1 vector with a luciferase reporter gene that replaces the viral envelope gene, allowing viral replication to be quantified by measuring luciferase activity. PhenoSense tests are also available for measuring resistance to the entry inhibitor and integrase inhibitors. As with genotypic testing, the phenotypic assays can only detect mutant variants that comprise at least 25% of the viral population.

Tropism Assays

An entry inhibitor, maraviroc, has brought about a need for viral tropism assays because the drug is only effective against virus that uses CCR5 as a coreceptor for entry. The drug is not active against CXCR4-tropic virus or dual/mixed tropic virus. The tropism assay must be performed prior to initiating maraviroc to determine whether the virus is CCR5 tropic. Like drug resistance assays, there are two general approaches in use, and two tropism assays are commercially available. The Trofile (Monogram Biosciences) uses the phenotypic approach, and SensiTrop II HIV coreceptor tropism assay (Pathway Diagnostics, Malibu, CA) uses the genotypic approach. For the Trofile assay, the *env* gene from the patient is amplified and used to construct pseudoviruses. Coreceptor tropism is then determined by measuring the ability of the pseudoviral population to infect CD41/U87 cells that express either CXCR4 or CCR5. Depending on which cells they infect, the viruses are then designated CXCR4 tropic, CCR5 tropic, or dual/mixed tropic (145). The SensiTrop assay uses a heteroduplex tracking assay combined with sequence analysis to identify minor viral populations that may be CXCR4 tropic. Only patients that are solely CCR5 tropic are candidates for a CCR5 inhibitor. Resistance to maraviroc has been reported, as have results from the development of mutations that allow the virus to use CXCR4 coreceptors or mutations that lead to structural changes in the envelope that prevent the drug from being effective (146, 147). In addition to the commercially available tropism assays, laboratories are developing additional research tools for the prediction of virus tropism, including prediction of affinity for CCR5 binding (148) and next generation sequencing to predict viral quasi-species binding to CXCR4 (149).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Use and Interpretation of Serologic Tests

Currently available conventional immunoassays are exquisitely sensitive and specific. False-negative results may occur during early infection before the appearance of antigen or antibodies (105, 106). Delayed seroconversion has also been reported in persons taking antiretroviral therapy for pre- and postexposure prophylaxis (150, 151). False-positive results are rare, but have been reported to occur transiently after recent immunizations and may also occur in recipients of experimental HIV vaccines (152–154). As with any screening test, positive predictive value may be low in populations with low prevalence. The currently FDA-approved rapid methods have comparable sensitivity to conventional immunoassays for established HIV infection, but they produce more false-negative results during early infection (155). False-negative and false-positive results occur more frequently with oral fluid than with blood or serum specimens (105, 112).

No single supplemental assay is adequate to confirm the presence of HIV infection in all specimens after a reactive initial immunoassay. The Western blot and IFA are less sensitive than either third- or fourth-generation immunoassays during early infection and may give false-negative or indeterminate results (91, 123). HIV-1 RNA may be undetectable in 2 to 5% of infected persons who are antibody-positive (97, 156). Therefore, in Septermber 2014 the CDC and Association of Public Health Laboratories recommended a new diagnostic algorithm for use with serum or plasma specimens that no longer relies on the Western blot (Fig. 3) (96). This algorithm maximizes the ability to detect acute HIV-1 infections and to correctly classify HIV-2 infections. Testing begins with an initial HIV-1/2 antigen/antibody combo immunoassay. If reactive, this is followed by an HIV-1/HIV-2 antibody differentiation assay. Reactive results on the initial immunoassay and the antibody differentiation assay confirm the presence of HIV-1 or HIV-2 antibodies, and no further testing is then necessary. Specimens that are nonreactive or indeterminate on the antibody differentiation assay undergo testing with HIV-1 NAT. A reactive fourth-generation immunoassay result, negative antibody differentiation assay result, and reactive HIV-1 NAT result is consistent with the presence of acute HIV-1 infection; a nonreactive NAT indicates false-positive results from the initial immunoassay. The same sequence of supplemental tests should be performed if a third-generation assay is used as the initial test. Reports of HIV test results should specify all assays that were used, the results of each assay, and an interpretation of the test results. Suggestions for reporting language are available from the New York State Health Department website (https://www.health.ny.gov/diseases/aids/providers/regulations/testing/docs/guidelines_diagnostic_testing.pdf) and the Clinical Laboratory Standards Institute (157).

Positive results from the testing algorithm indicate the need for HIV medical care and an initial evaluation that includes additional laboratory tests (such as HIV-1 viral load, CD4+ lymphocyte determination, and an antiretroviral resistance assay) to stage HIV disease and to assist in the selection of an initial antiretroviral drug regimen. Up-to-date recommendations for the initial evaluation are maintained at http://aidsinfo.nih.gov/guidelines. No diagnostic test or algorithm can be completely accurate in all cases of HIV infection. Inconsistent or conflicting test results obtained during the clinical evaluation, or results inconsistent with clinical findings, warrant additional testing of follow-up specimens with different assays.

Specimens submitted for testing after a reactive rapid HIV test result proceed through this same algorithm, beginning with an antigen/antibody combo immunoassay. Assays used in this algorithm are not suitable for use with oral fluid, dried blood spots, or urine. Testing of these alternative specimens is conducted with the specific immunoassays and HIV-1 Western blots approved for these types of specimens.

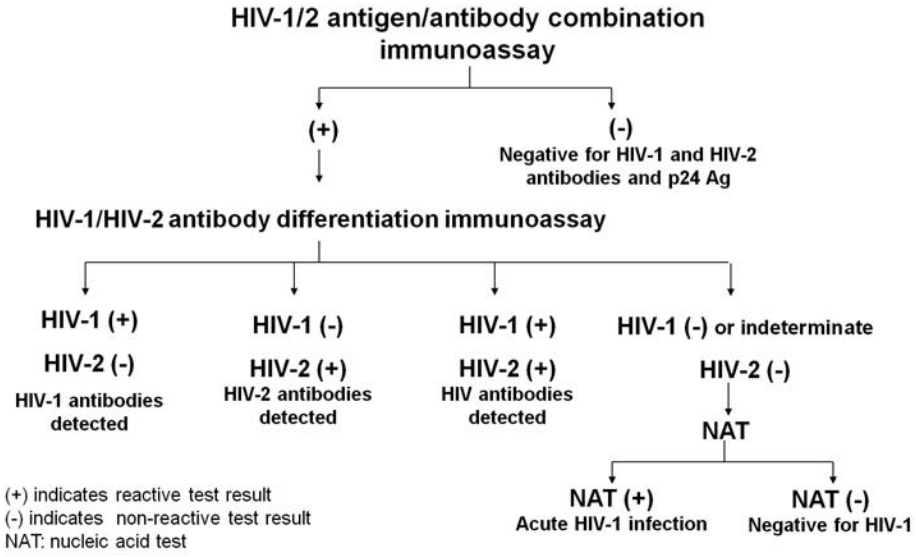
HIV-1/2 antigen/antibody combination immunoassay
(+)
(-)
Negative for HIV-1 and HIV-2 antibodies and p24 Ag
HIV-1/HIV-2 antibody differentiation immunoassay
HIV-1 (+)
HIV-2 (-)
HIV-1 antibodies detected
HIV-1 (-)
HIV-2 (+)
HIV-2 antibodies detected
HIV-1 (+)
HIV-2 (+)
HIV antibodies detected
HIV-1 (-) or indeterminate
HIV-2 (-)
NAT
NAT (+)
Acute HIV-1 infection
NAT (-)
Negative for HIV-1
(+) indicates reactive test result
(-) indicates non-reactive test result
NAT: nucleic acid test

FIGURE 3 Diagnostic HIV testing algorithm showing sequence of follow-up testing. doi:10.1128/9781555817381.ch82.f3

Use and Interpretation of Qualitative HIV RNA and DNA Assays

In adults, HIV-1 RNA tests are primarily used to diagnose acute infection, either as part of the diagnostic algorithm or as part of strategies that conduct pooled testing of specimens that are immunoassay-negative (158, 159). Nucleic acid tests are needed for infant diagnosis because serological tests are not useful for the detection of HIV infection in infants; maternal antibodies can persist in uninfected, HIV-exposed infants until 18 months of age. Both HIV-1 RNA and DNA tests can be used for the diagnosis of neonatal HIV-1 infection. DNA has often been the preferred method for determining an exposed infant's HIV-1 infection status, but recent studies suggest that HIV-1 RNA is more sensitive at birth and at 4 weeks of age (160). The DNA or RNA assay can be performed on whole blood or dried blood spots, but the specimen collected at birth must be a neonatal and not a cord blood sample: cord blood samples yield a high rate of false-positive results (161). Current recommendations are to test HIV-exposed infants within 14 days of birth, at 1 to 2 months, and at 4 to 6 months after birth; some experts also recommend testing at birth for improved treatment outcomes (162, 164). HIV-1 infection in neonates is diagnosed by two positive RNA or DNA tests performed on separate blood samples regardless of age. Qualitative test characteristics vary, and the population tested may influence the optimal test to be used. The Aptima test may be more sensitive than some HIV-1 DNA tests for the detection of non-B subtypes, CRFs, and group O virus. An advantage of proviral DNA tests is that they generally remain positive even in individuals receiving effective antiretroviral therapy and individuals that naturally suppress virus replication. However, no proviral DNA assays are currently FDA approved.

Use and Interpretation of Viral Load Assays

The clinical utility of HIV-1 viral load testing has been well established. Viral load assays are used widely to monitor changes in plasma viremia during antiretroviral therapy, because they are useful for predicting time to progression to AIDS and for monitoring responses to therapy. The magnitude of the decrease in viral load is dependent on the effectiveness of the antiretroviral therapy. The goal of optimum therapy is to suppress viral loads below the detection limit of the assay. Baseline testing of HIV-1 RNA viral load should be obtained before initiating therapy, and determinations should be repeated before changing antiretroviral therapy regimens. After initiation of therapy, patients should be tested within 2 to 8 weeks to assess drug efficacy and then every 3 to 4 months to assess durability of response. Updated guidelines on using viral load to guide antiretroviral treatment are maintained at http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html.

Biological variation of HIV-1 RNA levels among clinically stable patients has been estimated to be approximately 0.3 $\log_{10}$ (165). Reproducibility of commercial assays ranges from 0.1 to 0.3 $\log_{10}$ depending on the region of the assay's dynamic range. Changes in viral load values must exceed 0.5 $\log_{10}$ copies/ml to represent significant changes in viral replication. The clinical implications of HIV RNA levels near the lower limit of quantitation in patients on antiretroviral therapy are controversial. Persistent HIV RNA levels that are >200 copies/ml are often associated with evidence of viral evolution and accumulation of drug resistance mutations (166); persistent plasma HIV RNA levels in the 200 to 1,000 copies/ml range should therefore be considered as virologic failure. "Blips" of viremia (e.g., viral suppression followed by a detectable HIV RNA level and then subsequent return to undetectable levels) are usually not associated with subsequent virologic failure (167).

Numerous studies comparing the performance of the conventional FDA-approved viral load assays show high correlations between assays in terms of net changes in plasma RNA levels after antiretroviral therapy (168–170). Early assays showed larger variations in absolute values between assays with the same sample, but current-generation assays have been standardized so that the differences in values between assays are narrowing. The two real-time RT-PCR assays show improved correlation and agreement between viral load values. Although there is improved overall agreement between the different viral load assays, it is still considered optimal for patient care to monitor viral load values over time with the same assay. Specifically, some variability exists among platforms in immunologically stable patients with very low viral loads near the limits of detection (170).

HIV-1 RNA load testing is sometimes requested to resolve equivocal serological findings, to facilitate the diagnosis of HIV-1 infection during the acute phase of infection, or for pediatric diagnosis. Viral load tests can be performed for these purposes with a physician's order, but they are not approved by the FDA for the diagnosis of HIV-1 infection and they should be validated within a laboratory prior to routine use for these purposes. False-positive results can be obtained because of contamination during specimen processing, carryover of amplified products, or by selecting incorrect thresholds for defining positivity.

Use and Interpretation of Resistance Assays

Clinical guidelines for the use of HIV-1 resistance testing in adults are published and updated on a regular basis (171). In general, resistance testing is recommended for patients entering care even if therapy is not immediately initiated, when initiating antiretroviral therapy, for patients failing therapy, for pregnant women, and for patients with acute infection. Genotyping assays are generally more widely available, technically easier to perform, faster, and less expensive than phenotyping assays. In some cases, a resistance mutation may be detectable before a change in the phenotype has occurred, and therefore, genotypic and phenotypic results are not always correlated. One limitation of genotyping assays originates from the complexity of data that these assays generate. In the face of the rapid development of new drugs and new information on HIV-1 resistance, it remains challenging to keep databases updated on which mutations are associated with specific drug combinations. Updated lists of drug-resistant mutations for all classes of drugs are available at the International AIDS Society-USA website (http://www.iasusa.org) and the Stanford University website http://hivdb.stanford.edu/.

Interpretation of HIV-1 genotyping results is complex; it requires knowledge of the identity of mutations associated with each drug, the interactions of resistance mutations, and the genetics of cross-resistance. Most systems use a rule-based approach; a group of experts establishes interpretation algorithms based on the types or combinations of mutations that are associated with resistance to specific drugs. In the Trugene and ViroSeq assays, these algorithms are used to generate automated HIV-1 genotyping reports. Depending on the mutations detected, the report will indicate, for each drug in each of the antiviral categories, whether HIV in the patient sample shows no evidence of resistance, resistance, or possible resistance, or if evidence is insufficient to categorize the virus in one of the three other categories. These rule-based systems provide easy-to-interpret information for clinicians, but the databases require regular updating. The manufacturer's database update may lag behind the published literature, so clinicians may find it necessary to refer to one of the online databases for the most up-to-date information. For this reason, it is very helpful for laboratories to report both the specific mutations and the interpretation to clinicians so they can easily use online databases.

Phenotyping assays provide results in a format that is more familiar to clinicians. Results are reported as a relative change in IC_{50} compared to wild-type virus. In addition, there is less need for expert interpretation because susceptibility is measured directly. One problem with phenotypic resistance testing is that drugs are used—but are not tested—in combination. Thus, synergistic effects are not detectable. Cutoffs for a significant change in IC_{50} can also vary greatly depending on the drug. Initially, biological cutoffs were established based on the reproducibility of the assays; however, over time, clinical cutoffs correlated with outcome have been established for most drugs. A second disadvantage to phenotypic testing is a longer turnaround time for a result.

Both genotypic and phenotypic assays generally only yield results if the plasma used for testing contains at least 500 to 1000 HIV-1 RNA copies/ml. Depending on the extraction method used, it may be possible to obtain results for specimens with a lower viral load. Concentration of the virions in plasma by high-speed centrifugation may allow sequencing of specimens with viral loads of <500 copies/ml, but this process may also concentrate inhibitors and interfering substances. Because of the labor and expense involved with genotyping assays, laboratories should establish the lower viral load limit for reliable sequencing results. Cross contamination can occur with both genotypic and phenotypic resistance assays because both procedures rely on an RT-PCR step to amplify HIV-1 gene sequences. Both methods have clinical utility in managing patients and are widely used in clinical practice. However, the commercially available genotypic assays listed in Table 6 rely on bulk sequencing methods. To be detected, the resistant viral strain must constitute at least 20 to 30% of the viral quasispecies. Because of this limitation, minority resistant strains might be present that could be missed, and new methods such as allele-specific PCR and next-generation sequencing techniques have been developed for detecting drug resistance mutations that exist at low levels (141, 143, 144). Given their wider availability, faster turnaround time, and lower cost, most clinicians use genotyping for the initial evaluation of resistance. Phenotypic assays, on the other hand, can help in defining the significance of newly recognized resistance mutations and in elucidating the effects of complex mutation interactions, and may be very helpful in determining salvage regimens.

The findings and conclusions in this chapter are those of the authors and do not necessarily represent the views of the CDC. The use of product names in this manuscript does not imply their endorsement by the U.S. Department of Health and Human Services.

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Human T-Cell Lymphotropic Viruses

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TAXONOMY

Human T-cell lymphotropic virus types 1 and 2 (HTLV-1 and HTLV-2) are members of the *Deltaretrovirus* genus in the *Retroviridae* family (1). HTLVs likely originated from cross-species transmission of simian T-cell lymphotropic viruses (STLVs); combined, this group of viruses is also referred to as primate T-lymphotropic viruses (PTLVs) (2, 3).While the close phylogenetic relationships of HTLV-1 and STLV-1 indicate a simian origin for HTLV-1, HTLV-2 and STLV-2 are only distantly related, so the exact simian origin of HTLV-2 is unknown. Recently, two novel HTLVs were identified in hunters in Cameroon and were called HTLV-3 and HTLV-4 (2, 4–6). HTLV-3 likely originated from monkeys infected with highly related STLV-3 viruses (2, 4, 5). HTLV-4 is equidistant from all other HTLVs and was recently found to have a gorilla reservoir in Cameroon (7, 8). HTLVs and STLVs are distinct from the *Lentivirus* genus that includes human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) and simian immunodeficiency viruses (SIVs) (1). Classification of HTLV groups using Roman numerals was replaced over a decade ago with Arabic numerals following the guidelines of the International Committee on Taxonomy of Viruses (1).

DESCRIPTION OF THE AGENT

HTLV-1 and HTLV-2 are enveloped viruses about 80 to 100 nm in diameter; they contain an electron-dense, centrally located nuclear core with less prominent envelope spikes and bud from the cell surface. Electron micrograph studies have not been performed on HTLV-3 and -4 but likely possess morphologies comparable to HTLV-1 and -2. Within the core are two positive-sense single-stranded RNA genomes. Once a cell is infected, the RNA genome is converted by reverse transcriptase to DNA and integrates into the host genome.

HTLVs are complex retroviruses with regulatory genes in addition to the structural and enzymatic *gag*, *pol*, and *env* genes found in all classical retroviruses. HTLV-1, HTLV-2, HTLV-3, and HTLV-4 all have similar genomic organization: group specific antigen (*gag*), protease/polymerase (*pro/pol*), envelope (*env*), accessory gene region (*pX*), flanked by long terminal repeats (LTRs) (Fig. 1). Each HTLV group is highly divergent, sharing about 60% nucleotide identity (8–10). The *gag* gene encodes the structural proteins, matrix (p19), capsid (p24), and nucleocapsid (p15) (Fig. 2). The *pro* and *pol* genes encode the protease and the reverse transcriptase enzymes, respectively. The *env* gene encodes the transmembrane (TM) and external or surface (SU) envelope glycoproteins, gp21 and gp46, respectively.

HTLVs use alternative splicing and internal initiation codons to produce several regulatory and accessory proteins encoded by at least four open reading frames (ORFs) located in the *pX* region of the viral genome between *env* and the 3′ LTR (11, 12). The *pX* region encodes the spliced, regulatory proteins Tax and Rex and several additional ORFs, some of which are also believed to be involved in viral replication and transport (Fig. 1). Unlike other retroviruses, HTLVs also encode a protein on the antisense strand, called antisense protein of HTLV or APH, that is a repressor of Tax-mediated viral transcription (Fig. 1) (13). APH was first discovered in HTLV-1; it contains a basic leucine zipper (bZIP) motif and was thus originally called HBZ (HTLV-1 bZIP protein) (14, 15). HBZ is also believed to control cellular replication and promote T-lymphocyte proliferation associated with leukemia as seen in some HTLV-1-infected persons (13, 16, 17). APH proteins have now been identified in all PTLV groups, suggesting the evolutionary and biological importance of these proteins (8, 10, 13).

HTLV-1 and HTLV-2 use a receptor complex with three different molecules, including glucose transporter type 1 (GLUT-1), VEGF-165 receptor Neuropilin 1 (NPR-1), and heparin sulfate proteoglycans (HSPG), for cellular entry (18–23). HTLV-1 preferentially infects CD4$^+$ T cells, whereas CD8$^+$ T cells are the primary target for HTLV-2 (18). *In vitro* studies have shown that HTLV-3 can infect both CD4$^+$ and CD8$^+$ T cells and also uses GLUT-1, but does need HSPGs or NRP-1 for cellular entry, suggesting it may use a different complex of receptor molecules (19). Cell receptor studies for HTLV-4 have not been reported yet.

EPIDEMIOLOGY AND TRANSMISSION

Recently, meta-analyses of reliable epidemiologic data from about 1,100 published papers since the discovery of HTLV in 1981 showed that 5 to 10 million people worldwide are estimated to be infected with HTLV-1, with endemic foci in southwestern Japan, the Caribbean and surrounding islands, large parts of sub-Saharan Africa and Central and South America, some rare areas in Melanesia, the Middle East (northern part of Iran), and Romania (24). The seroprevalence rate in adults ranges from 2 to 17% in the Caribbean

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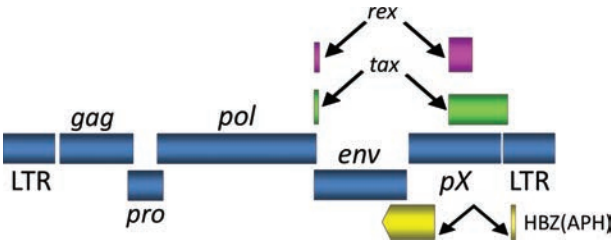

FIGURE 1 Genomic organization of HTLV. LTR, long terminal repeat; Gag, group-specific antigen; *env*, envelope; *pol*, polymerase (including reverse transcriptase and integrase); *pro*, protease; *rex*, regulator of viral expression; *tax*, transcriptional activator; HBZ, HTLV-1 basic leucine zipper-like protein also known as the antisense protein of HTLV (APH). Miscellaneous accessory genes (not shown) are located in the pX region, located between the *env* gene and the 3′ LTR region of HTLV. The Gag and Env proteins are the most immunogenic, and antibodies to these proteins are commonly detected by serological tests (EIA and Western blotting). PCR assays are typically designed to detect regions within the LTR region and *gag*, *pol*, *env*, and/or *tax* genes. doi:10.1128/9781555817381.ch83.f1

islands and the highest rates (1.7 to 17.4%) are observed in southern Japan (24, 25). While similar meta-analysis estimates of global numbers of HTLV-2 infections have not been reported, it is endemic in Amerindian tribes throughout the Americas, as well as in a few Pygmy tribes in Central Africa with seroprevalences of about 3% in Pygmies, 1 to 5.8% in South America, 8 to 10% in Central America, and 2 to 13% in North America (26–29). HTLV-3 and HTLV-4 have only been found in a few primate hunters in Cameroon and do not appear to have spread locally or globally, though screening has been limited to only certain non-African populations (2, 4, 5, 30–32). In all areas of endemicity, HTLV-1 and -2 seroprevalence increases with age, especially in women (24, 25, 33). In the United States and Europe, the seroprevalence for both HTLV-1 and HTLV-2 among low-risk populations is less than 1% (34, 35). However, high-risk populations such as intravenous drug users (IDUs) in the U.S. and Europe, in whom HTLV-2 infection predominates over HTLV-1 infection, are reported to have seroprevalences from 0.4 to 20% (24, 25). African-American IDU populations, especially in New Orleans, are at increased risk for HTLV-2 infection (25). IDUs in South Vietnam showed a very high prevalence (>60%) of HTLV-2 infection, believed to have been introduced and spread by American military personnel during the Vietnam war (36).

HTLV-1 and HTLV-2 infections are transmitted sexually (mainly male to female), vertically (mother to child, mostly by prolonged breast-feeding), and parenterally (drug use and blood transfusion) (24, 25, 33). In nonendemic populations, intravenous drug use and sex with IDUs are the most important risk factors for HTLV-2 transmission (24, 25, 33). Both cross-sectional and prospective studies support sexual transmission, and there was a strong concordance of seropositivity between spouses in an area where HTLV-2 is endemic (24, 25, 33). One study showed that transmission mode can affect viral evolution rates, with HTLV-2 strains transmitted between IDUs evolving 150 to 350 times faster than in mother-to-infant transmission (37). The increase in viral transmission rate between IDUs, which can be many transmissions per year, was proposed to account for the increase in the HTLV-2 evolutionary rate, whereas vertical transmission would be expected to occur just once every 14 to 30 years for a given viral lineage and thus would evolve more slowly (51, 70). This increased rate of transmission and evolution among IDUs may increase the risk for the emergence of HTLV-2 strains with a higher virulence.

Over the past 20 years, highly effective screening programs for volunteer blood donors have been implemented in Japan, Australia, the U.S., Canada, and several Caribbean and European countries to reduce the risk of transfusion-related HTLV-1 and HTLV-2 transmission (25, 33–35). For example, in the U.S. between 1991 to 1996, the incidence rate of seroconversion associated with HTLV in blood donors was estimated to be 1.59 per 100,000 persons per year (34), and the residual risk of transmitting HTLV infection by transfusing screened blood was estimated to be 1 in 641,000 (34). From 1998 to 2001, the estimated incidence of new infections among repeat blood donors to the American Red Cross (ARC) was 0.239 per 100,000 person years and the estimated risk of collecting blood during the infectious window period was 1:2,993,000 (38). More recently, the ARC found that the HTLV incidence and residual risk of transfusion-transmissible HTLV infection among repeat donors during 2007–2008 decreased to 0.21 per 100,000 person years and 1:3,394,086, demonstrating further the success of universal blood screening (39).

Many molecular epidemiologic studies have shown HTLV proviruses to be remarkably stable genetically, which has allowed strong resolution of HTLV phylogenies and molecular dating of these viruses (8, 10, 40–42). The differing evolution rates for HTLV-1 and HIV-1 are due to clonal expansion of HTLV-infected cells versus active replication of HIV-1 (43, 44). While the overall genome of HTLV is highly conserved, nucleotide divergence in the LTR, *tax*, *pol*, and *gp21 env* genes has been exploited to genotype HTLV-1, HTLV-2, and more recently HTLV-3 and -4 (30, 45–47). The impact of diversity and emerging variants continues to challenge HTLV serology. An understanding of phylogenetic relationships is therefore useful. There are at least seven major geographic HTLV-1 subtypes which evolved from STLV-1 through several independent interspecies transmissions between simian and human hosts at different geographic locations (2, 3, 48): subtype A (cosmopolitan), subtype B (Central African), subtype C (Australo-Melanesian), subtype D (Central African, mainly among Pygmy tribes), subtype E found in an Efe Pygmy from the Congo, subtype F from persons in the Democratic Republic of Congo (DRC), and subtype G from primate hunters in Central and West Africa (2, 3, 24, 48). HTLV-2 has three subtypes named 2a, 2b, and 2d (26, 29) whose primate origin has been linked to a single primate, since STLV-2 has only been found in bonobos (3, 49). Subtype 2a is commonly found among IDUs worldwide, whereas subtype 2b is found primarily among Amerindians and in some Pygmies from Cameroon. Subtype 2d has been found in only one Pygmy from DRC. Earlier studies proposed subtype 2c in one Amerindian tribe from Brazil; however, subsequent analysis of complete genomes confirmed this to be a subtype 2a variant (50). HTLV-3 most likely originated from recent cross-species transmission in persons exposed to nonhuman primates in Cameroon (2, 4, 5, 28, 51). Phylogenetic analysis has identified HTLV-3 infection originating from at least four highly divergent STLV-3 subtypes such that there are also four HTLV-3 subtypes (2, 4, 5, 28, 51, 52). Subtypes A and C include the original STLV-3 identified in a baboon from East Africa and STLV-3 in spot-nosed monkeys (*Cercopithecus nictitans*) from Cameroon, but human infection has not been reported yet with either

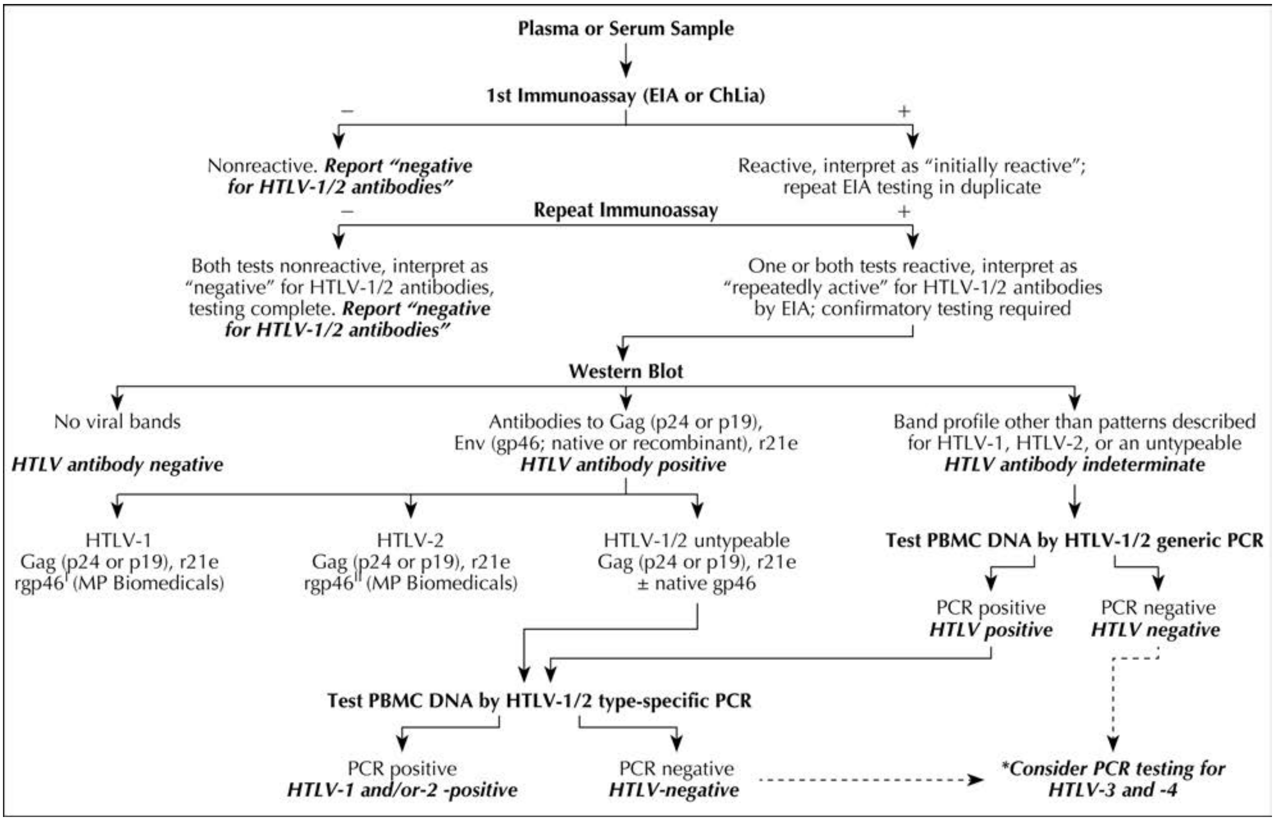
Plasma or Serum Sample
1st Immunoassay (EIA or ChLia)
−
+
Nonreactive. Report "negative for HTLV-1/2 antibodies"
Reactive, interpret as "initially reactive"; repeat EIA testing in duplicate
Repeat Immunoassay
−
+
Both tests nonreactive, interpret as "negative" for HTLV-1/2 antibodies, testing complete. Report "negative for HTLV-1/2 antibodies"
One or both tests reactive, interpret as "repeatedly active" for HTLV-1/2 antibodies by EIA; confirmatory testing required
Western Blot
No viral bands
HTLV antibody negative
Antibodies to Gag (p24 or p19), Env (gp46; native or recombinant), r21e
HTLV antibody positive
Band profile other than patterns described for HTLV-1, HTLV-2, or an untypeable
HTLV antibody indeterminate
HTLV-1
Gag (p24 or p19), r21e
rgp46I (MP Biomedicals)
HTLV-2
Gag (p24 or p19), r21e
rgp46II (MP Biomedicals)
HTLV-1/2 untypeable
Gag (p24 or p19), r21e
± native gp46
Test PBMC DNA by HTLV-1/2 generic PCR
PCR positive
HTLV positive
PCR negative
HTLV negative
Test PBMC DNA by HTLV-1/2 type-specific PCR
PCR positive
HTLV-1 and/or-2 -positive
PCR negative
HTLV-negative
*Consider PCR testing for HTLV-3 and -4

FIGURE 2 Serologic testing algorithm for the detection and confirmation of HTLV-1 and -2 infections. If the initial screening immunoassay (EIA or ChLIA) is reactive, a repeat assay with the same specimen is performed in duplicate. If one or both of the repeat tests are reactive, the specimen is classified as repeatedly reactive and supplemental testing is done for confirmation. WB criteria shown are those used by the manufacturer and not the U.S. Public Health Service working group. In some cases, further follow-up is done using HTLV generic and/or type-specific PCR. *, HTLV-3 and -4 PCR is suggested if patient is linked to Cameroon or West Central Africa. r21e, recombinant p21 envelope (Env) protein; rgp46^{I} and rgp46^{II}, recombinant glycoprotein in Env specific for HTLV-1 or HTLV-2, respectively; Gag, group specific antigen; PBMC, peripheral blood mononuclear cells. doi:10.1128/9781555817381.ch83.f2

subtype (53–55). Subtype B includes both STLV-3 and HTLV-3 from West-Central Africa, while subtype D occurs in both monkeys and a human from Cameroon (2–5, 28, 49, 51, 55). HTLV-4 has only been identified in a single hunter from Cameroon (2). These studies suggest that cross-species transmission of a wide range of STLV lineages to humans is ongoing and not limited to rare historical events. It is currently unknown whether HTLV-3 and -4 represent dead-end infections in humans or have the potential to spread regionally or globally. A new STLV group, STLV-5, has been proposed recently for a macaque STLV originally classified as STLV-1 since detailed phylogenetic analysis showed it is highly divergent from STLV-1 (56, 57). A human counterpart of STLV-5 has not yet been found. The genetic heterogeneity within HTLV has provided valuable information on geographic clustering, movement of ancient populations, and viral transmission (3, 8, 10, 24, 40, 41, 48, 58, 59).

CLINICAL SIGNIFICANCE

HTLV-1 has been associated with two major diseases: adult T-cell leukemia/lymphoma (ATLL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Disease occurs in less than 5% of infected individuals (24, 25, 33). ATLL exists in four forms: acute, chronic, smoldering, and T-cell non-Hodgkin's lymphoma. The acute form comprises about 75% of all ATLLs and can be rapidly fatal. ATLL is characterized by severe malignant proliferation of CD4^{+} CD25^{+} T-lymphocytes diagnosed biologically by seropositivity for HTLV-1, the presence of morphologically distinct CD3^{+}/4^{+}/25^{+} lymphocytes with cleaved nuclei (flower cells), and clonal integration of HTLV-1 proviruses in the tumor cells, as detected by Southern blotting, inverse PCR, or newer technologies such as ultra-deep sequence analysis (17, 60, 61). Both Tax and HBZ are believed to be involved in ATLL development (13, 17, 62).

The clinical features of the chronic neuromyelopathy HAM/TSP are muscle weakness in the legs, hyperreflexia, clonus, extensor plantar responses, sensory disturbances, various urinary manifestations, impotence, and low-back pain (24, 25, 33, 62, 63). Spasticity of the lower legs is the main disability of HAM/TSP, with patients often requiring wheelchairs. The main biological diagnosis includes the presence of high titers of HTLV-specific antibodies in the serum and cerebrospinal fluid (CSF). Levels of HTLV-1-infected cells in the CSF are over twice those found in the peripheral blood for HAM/TSP patients compared to levels in HTLV-1-infected persons with other neurological disorders, and PCR determination of proviral loads in these compartments thus has diagnostic value (62). The estimated lifetime risk of developing HAM/TSP among HTLV-1-infected persons is 0.2 to 4% but can vary according to the geographical area, and the disease occurs more frequently in persons over 40 years old (24, 25). Other uncommon inflammatory disease associations include infective dermatitis, uveitis, polymyositis, thyroiditis, and pneumonitis and HTLV-associated arthropathy (24, 25, 33, 62, 63). There have also been case reports of increased opportunistic infections in HTLV-1-infected persons such as cytomegalovirus (CMV), *Pneumocystis jirovecii*, herpes zoster, *Cryptococcus*, and *Mycobacterium avium* (25). Increased viral load has been identified in both ATLL and HAM/TSP patients, compared to asymptomatic infected persons, and is one of the best predictors for disease development, though patients with high proviral loads can remain asymptomatic for decades (24, 25, 33, 62).

HTLV-2 has not been definitively associated with malignancy; however, it has shown rare association with a neurological disease resembling HAM/TSP that progresses more slowly and with milder symptoms (25, 64). The estimated lifetime risk of disease development for HTLV-2-infected persons is unknown but appears to be less than that estimated for persons with HTLV-1 infection (25, 26). While the majority of HTLV-2-infected persons remain asymptomatic (>95%), recent studies report an increased incidence of infectious diseases (such as bronchitis, pneumonia, tuberculosis, and kidney and/or bladder infections) in HTLV-2-infected persons (25, 64). A recent study also found a higher association of all-cause cancer and cancer mortality in a cohort of HTLV-2-infected persons in the U.S., suggesting that more research is needed to understand the pathogenic potential of HTLV-2 (65).

HTLV coinfection with HIV can occur in high-risk groups, such as persons from endemic HTLV areas and IDUs. To date, a few studies evaluating the impact of HIV-1 coinfection with HTLV have shown that there is an increased frequency of HTLV-1-associated clinical outcomes (lymphoma and neurological disease), especially in persons with high CD4$^+$ T cell counts. In contrast, a delayed progression to AIDS is seen in some HTLV-2/HIV-1-coinfected persons (66–68). Moreover, HIV therapy that includes highly active antiretroviral therapy in HTLV-2/HIV-1-coinfected persons does not affect HTLV-2 proviral burden but has been reported to provide a protective effect from AIDS progression (67, 69). These limited numbers of studies suggest that HIV-1 coinfection may increase the morbidity of the HTLV-1-infected patients. A recent study also showed that the Tax proteins of both HTLV-1 and HTLV-2 inhibited HIV-1 replication *in vitro*, suggesting a protective effect of HTLV in HIV-1 infection (66). However, additional studies are needed to determine if coinfection affects the mortality of HTLV-infected patients. The pathogenic potential of HTLV-3 and HTLV-4 is not known and is limited by the small number of infected persons identified in surveys of apparently healthy populations (2, 4, 5, 28). In addition, limited studies of persons receiving care at outpatient clinics in Europe or individuals with some cancers in the U.S. have not identified evidence of HTLV-3 and HTLV-4 infection (30–32, 70).

Treatment options for HTLV-associated diseases are limited (17, 25). Chemotherapy has not proven effective against aggressive ATLL forms (71–73). Although zidovudine (AZT) and alpha interferon (IFN-α) yield some responses and improve ATLL prognosis, alternative therapies are critically needed (71–73). New drugs such as arsenic trioxide, proteasome inhibitors, retinoids, and angiogenesis inhibitors, as well as cellular immunotherapy, are under investigation (71–73). Antiretroviral treatments such as lamivudine and high-dose IFN-α and IFN-β have been evaluated with HAM/TSP patients and resulted in decreases in proviral loads, alterations in T-cell phenotypes, and anti-*tax* cytotoxic T-cell frequency changes (74). However, results of further follow-up on immunomodulatory therapies have been disappointing, and combination antiretroviral therapies (zidovudine and lamivudine) have been discouraging because of only partial or temporary success in decreasing proviral loads (75, 76). One study using STLV-1-infected baboons treated with zidovudine and sodium valproate showed significant viral load reduction, but clinical trials in HTLV-1-infected humans using both therapeutic agents have not been reported (77). Symptomatic or targeted treatment is still the foundation of HAM/TSP therapy (74, 75). Recently, the integrase inhibitor raltegravir was shown to have potent *in vitro* anti-HTLV-1 activity (78). However, the clinical effects of raltegravir treatment are not clear. In a small study of two persons with HAM/TSP and three asymptomatic HTLV-1-infected persons, treatment with raltegravir for 12 months did not show any sustained reduction in proviral load (79). For uveitis, topical and systemic corticosteroids appear to improve sight, whereas infective dermatitis responds well to antibiotic treatment (25).

Although animal model studies of various HTLV vaccines have induced neutralizing antibodies (nAb) and strong cytotoxic T-lymphocyte responses using peptides, and synthetic inhibitory peptides and nAb have blocked envelope-mediated viral entry, these vaccines have not yet been used in clinical trials (25). Recently, a subunit vaccine composed of recombinant SU proteins was successful in robustly activating cell-mediated cytotoxic responses and neutralized viral infectivity in mouse models (80). While these studies are promising for development of potential HTLV vaccines, more research is needed to determine their antiviral and prophylactic efficacy.

COLLECTION AND STORAGE OF SPECIMENS

Serum or plasma is suitable for use in serologic assays for HTLV detection and can be drawn at the time of presentation. Samples should be redrawn at 3 months from seronegative patients who are suspected of having HTLV infection, or those with seroindeterminate Western blot patterns, and should be retested to capture potentially infected persons during the seroconversion window period (81). Alternatively, these persons can also be tested by PCR to investigate possible infection, though these assays are not yet FDA approved. Serum and plasma specimens can be stored at 4°C or frozen for later use. Package inserts should be consulted for storage limitations and limitations on the number of freeze-thaw cycles permitted. Fresh whole blood for the preparation of peripheral blood mononuclear cells (PBMCs), and not plasma or serum, is an appropriate specimen for nucleic acid testing (NAT) and virus isolation since HTLV-1 and HTLV-2 are cell-associated viruses. PBMCs are typically isolated from fresh whole blood on a Ficoll-Hypaque gradient (discussed in chapter 80). Heparin as a blood anticoagulant is not recommended for NAT as it interferes with the enzyme used in PCR. DNA is prepared using standard methods and can be stored at −20°C until needed. For virus culture, whole blood is collected in sodium citrate, EDTA, or heparin tubes and PBMCs are isolated on a Ficoll-Hypaque gradient.

DIRECT EXAMINATION

PCR Detection of Nucleic Acids

Amplification of proviral DNA by PCR is the preferred method for determining infection status, testing the validity of serologic assay results, distinguishing among the four HTLV groups, and studying tissue distribution *in vivo* (81). Qualitative PCR procedures utilizing primers in the *pol*, *env*, or *tax* genes and the LTR have been used to confirm and differentiate between HTLV-1 and -2 (and more recently HTLV-3 and -4) infections (Table 1) (2, 45, 46, 70, 82). These assays utilize HTLV consensus primers that allow amplification of all four viruses, with typing achieved either by hybridizing the product to an HTLV-specific probe or by sequence analysis. A second approach employs type-specific primers and probes in separate amplifications. The PCR products can be detected with labeled internal probes by Southern blot hybridization and with sequencing (45, 82). Most of these assays are performed for research use only, but some are now offered by commercial laboratories.

Viral detection and quantification of proviral DNA can be performed simultaneously using real-time PCR; RNA viral load estimation from plasma is not feasible in HTLV infection since these viruses are cell-associated (30, 47). Noncommercial real-time PCR assays have excellent sensitivity and specificity and a broad dynamic range, from 10 to 10^6 copies/reaction, and have been used to define the relationship between proviral load and both pathogenesis and person-to-person transmission risks (25, 83). For example, proviral load is higher among HAM/TSP and ATLL patients than among asymptomatic carriers, suggesting that proviral load may be a prognostic indicator for future disease development (25, 33, 62, 68, 84). Both sexual and mother-to-child transmission are associated with higher HTLV proviral load in the index case (25, 33, 62, 68, 84, 85). Thus, quantification of HTLV proviral load has provided a better understanding of correlates of transmission and disease progression (25, 33, 62, 68, 84).

VIRUS ISOLATION AND IDENTIFICATION

Isolation of HTLV has been difficult since these viruses are cell-associated (25, 86). Nevertheless, cocultivation of HTLV-infected PBMCs with activated, allogeneic HTLV-

TABLE 1 Summary of serological and supplemental confirmatory tests for HTLV infection

Detection method	Description	Reference(s)	Availability in U.S.[a]
Serological screening tests			
EIA	HTLV-1- and HTLV-2-infected cell lysates and/or recombinant antigens used to detect HTLV antibodies by colorimetric readout. Excellent sensitivity and specificity for HTLV-1 and -2. Sensitivity for HTLV-3 and -4 detection is unknown.	25, 46, 81, 89	One FDA-licensed test
ChLIA	Similar to EIA but with chemiluminescent detection	90	One FDA-licensed test
Particle agglutination	Viral lysate coats gelatin or latex particles. Antigen-specific antibodies bind, which results in agglutination of particles. Visual readout by operator.		Research use only
Serological supplementary tests			
WB	HTLV-1 viral lysates ± recombinant antigens used to detect HTLV-1- and -2-specific antibodies. May contain recombinant antigens to differentiate antibodies to HTLV-1 and HTLV-2. HTLV-3 and -4 can give variable WB profiles.	46, 89, 95	Research use only
LIA	Recombinant or peptide HTLV-1 and HTLV-2 antigens printed onto membrane. Antibodies to individual viral proteins are visualized. HTLV-3 and -4 can give variable results.	46, 89, 96	Research use only
IFA	Antibodies bind to HTLV-1-, HTLV-2-, and STLV-3-infected cells. Detected by secondary anti-human antibody with fluorescent label. Differentiates between antibodies to HTLV-1, HTLV-2, and HTLV-3.	25, 46	Research use only
Nucleic acid detection tests			
Qualitative PCR	Distinguishes between HTLV groups either by using HTLV consensus primers with typing done by HTLV-specific primers and probes in separate tests, or by sequence analysis	45, 82	Research use only
Quantitative PCR	Determines proviral DNA copy number by limiting dilution, quantitative competitive PCR, or real-time PCR using HTLV degenerate and/or type-specific primers	25, 30, 47, 83	Research use only

[a] FDA licensed; may be used for blood donor screening and as an aid in clinical diagnosis of HTLV-1 or HTLV-2 infection and related diseases. Research use only (not for clinical use).

negative PBMCs or cord blood cells is used to obtain viral isolates. Tissue culture supernatants are collected weekly for up to 4 to 6 weeks, and the presence of HTLV-1 or HTLV-2 p19 Gag antigen in the supernatant is tested using a research use only antigen capture assay (Zeptometrix Corp., Buffalo, NY). Alternatively, PCR methods can be used to monitor for infection in tissue culture cellular DNA. Because of the time required and labor-intensive nature of this method, virus isolation and tissue culture detection are generally not done for HTLV diagnosis but rather serve as research tools to further characterize the viruses. HTLV-3 and HTLV-4 have not yet been isolated *in vitro*.

TYPING SYSTEMS AND SEROLOGIC TESTS

Testing for antibodies to HTLV-1 and HTLV-2 should be performed for all blood donors and patients presenting with relevant clinical signs and symptoms. In the U.S., since 1988 all blood donors have been screened for antibodies to both HTLV-1 and HTLV-2 using assays that include both HTLV-1 and HTLV-2 antigens (34, 81, 87). Testing for HTLV-1 and HTLV-2 should also be offered to persons who are from areas where HTLV is endemic, who engage in high-risk behaviors such as needle sharing, and who have had sexual contact with persons from either group. Currently, HTLV-1 and HTLV-2 testing is not routinely performed for fertility or pregnancy testing in the U.S. This is different from some European countries, as well as some islands in the West Indies, where recommendations have been made to test pregnant and breast-feeding women originating from areas of HTLV-1 endemicity like Japan (24, 25, 35). In addition, in the U.S. all donors of viable, leukocyte-rich cells or tissue (e.g., hematopoietic stem/progenitor cells and semen) are screened for HTLV-1 and -2 (88). However, since favorable outcomes for recipients of HTLV-1/2-positive organs in the U.S. have been reported, discussion is ongoing to eliminate pretransplant HTLV-1/2 screening if a proposed retrospective testing study shows a very low incidence of HTLV infection and an absence of clinical sequelae in donors and recipients (88).

The most common screening assays detect antibodies to HTLV in serum or plasma (25, 34, 81). The immunodominant regions of structural and regulatory proteins are well characterized and are used in diagnostic assays that detect and differentiate HTLV-1 and HTLV-2. The major tests for HTLV-1 and HTLV-2 are described in Table 1. The serologic testing algorithm consists of a primary screening assay followed by testing for confirmation and identification of HTLV type (Fig. 2). Assays for the specific detection and discrimination of HTLV-3 and -4 are not currently available (89).

Primary screening assays include enzyme immunoassays (EIA), chemiluminescent immunoassays (ChLIA), particle agglutination (Fujirebio America, Fairfield, NJ), and immunofluorescence assays (IFAs) (Table 1). EIAs are sensitive and simple colorimetric tests that use purified HTLV-infected cell lysates and/or recombinant antigens or synthetic peptides. ChLIA is similar to EIA, but uses chemiluminescence for the detection step. The addition of HTLV-2 antigens to screening tests significantly improved the detection of antibodies to HTLV-2, compared with results using tests that contained only HTLV-1 antigens (25, 90). The EIA and ChLIA can be automated and performed on a large scale (90). However, neither EIAs nor ChLIAs can differentiate between HTLV-1 and HTLV-2 infection because of significant sequence identity of the structural proteins between the two viruses; therefore, these screening assays are referred to as tests for HTLV-1/2.

Comparative analysis of various commercial screening assays containing both HTLV-1 and HTLV-2 antigens indicates that sensitivity ranges from 98.9 to 100% for confirmed HTLV-1-positive specimens and 91.5 to 100% for confirmed HTLV-2-positive specimens. Specificity ranges from 90.2 to 100% (25, 91, 92). Currently, one HTLV-1/2 EIA (Avioq HTLV-I/II Microelisa System, Avioq, Inc., Research Triangle Park, NC) and one HTLV-1/2 ChLIA (Abbott PRISM HTLV-I/HTLV-II, Abbott Laboratories, Abbott Park, IL) are licensed by the FDA and are available for use in the U.S. The Avioq assay was previously called the Vironostika HTLV-I/II Microelisa System and was manufactured by bioMérieux but was not commercially available in the U.S. for a period of time. Both FDA-approved assays have reported overall sensitivity point estimates of 100% (95% confidence interval [CI] of 99.97 to 100% for Avioq and 99.89 to 99.96% for Abbott). Specificities of 99.93% (95% CI, 99.89 to 99.96%) for Abbott PRISM HTLV-I/HTLV-II and 99.95% for Avioq (95% CI, 99.89 to 99.98%) were reported from clinical trial data that supported the licensure of these tests. Both assays can be used for blood donor screening and as an aid in clinical diagnosis of HTLV-1 and HTLV-2 infection and related diseases. Further attempts have been made to develop a dual EIA algorithm to increase the predictive values of HTLV tests (Fig. 2) (34, 93). Pooling of samples for seroepidemiologic studies has been proposed; however, pooled testing is not recommended for blood donor testing (94).

While most screening assays are highly sensitive, specimens containing low titers of antibodies to HTLV-1 and HTLV-2 from certain areas of HTLV endemicity, or specimens from early seroconverters, may be missed by EIA screening. NAT can be used to detect infected individuals either with low antibody titers or within their window period prior to the development of an antibody response (median window period, 51 days; range, 36 to 72 days) (34). However, while NAT assays may be commercially available, they are not licensed by the FDA, are used mainly for research, and are not in routine clinical use.

Supplemental confirmatory or differentiation tests for HTLV-1 and HTLV-2 infection include Western blots (WB) containing viral lysates supplemented with recombinant proteins, line immunoassays (LIAs; Innogenetics, Ghent, Belgium), and IFAs (Table 1). Although WB assays using purified viral lysates are highly sensitive for detecting p24 and p19 Gag antibodies, they do not always detect antibodies to native Env glycoproteins. An alternative approach used in a second-generation confirmatory assay is a modified WB that contains type-specific gp46 Env recombinant proteins from HTLV-1 and -2 and a truncated form of recombinant p21 Env (r21e) that reduces nonspecific reactivity, improves performance (specificity and sensitivity), and allows differentiation between HTLV-1 and HTLV-2 (MP Diagnostics WB 2.4; MP Biomedicals, Science Park, Singapore) (Fig. 3) (95).

LIA is another supplementary test consisting of recombinant and synthetic HTLV-1- and HTLV-2-specific antigens printed onto strips (INNO-LIA, Innogenetics) (96); this assay can differentiate between HTLV-1 and HTLV-2 infection. IFAs that detect binding of antibodies from a specimen to HTLV-1- or HTLV-2-infected cells can also be used to discriminate HTLV-1/2 infections and to determine antibody titer (24, 25). No confirmatory or supplementary assay has been approved or licensed for clinical use by the FDA; however, WB and LIA have been certified for use in Europe

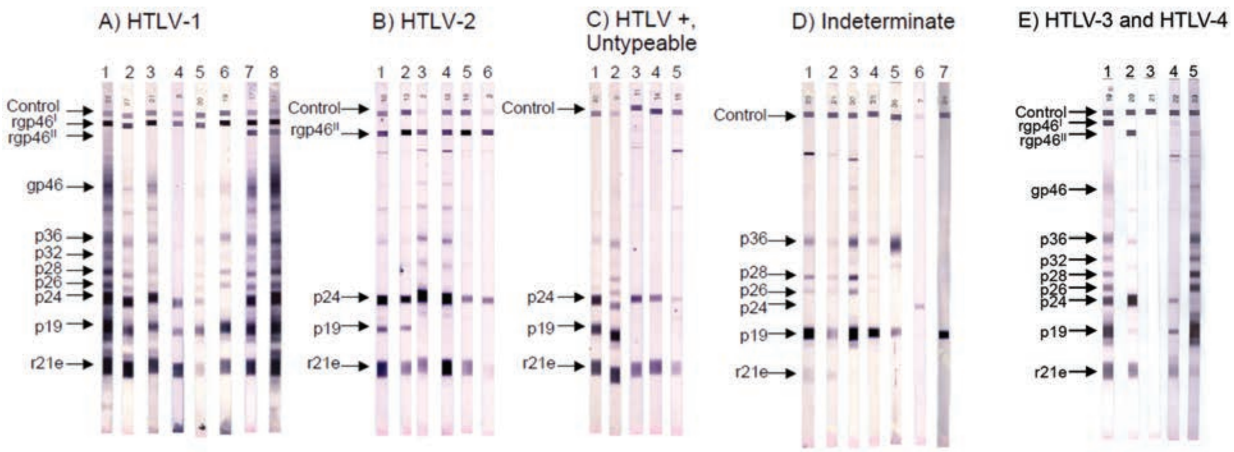

FIGURE 3 Western blot (WB) analysis of representative plasma or serum specimens from persons infected with (A) HTLV-1, (B) HTLV-2, (C) HTLV-1/2 untypeable, (D) indeterminate, and (E) HTLV-3 and HTLV-4. Representative seroreactivity patterns are shown for WBs from MP Biomedical (HTLV-2.4 version), which contain HTLV-1 antigens spiked with recombinant r21e (common to HTLV-1 and HTLV-2) and two external envelope recombinant proteins specific for HTLV-1 (rgp46^{I}) and HTLV-2 (rgp46^{II}). (A) Typical patterns for HTLV-1 reactivity (lanes 1 to 5), atypical reactivity lacking p24 Gag response (lane 6), and specimens with high antibody titers showing dual reactivity to both rgp46 proteins (lanes 7 and 8; titration of sera results in reactivity only to rgp46^{I}). (B) Typical patterns for HTLV-2 reactivity (lanes 1 to 6; note that reactivity to the p24 band is stronger than to the p19 band, which is usually absent from sera from HTLV-2-infected persons). (C) HTLV-1/2-positive but untypeable specimens, with reactivity to Gag (p24, with or without p19) and r21e but not gp46^{I} or gp46^{II}. Lanes 1 and 2: characteristic patterns of specimens that are usually found to contain HTLV-1 after additional testing; lanes 3 to 5: characteristic patterns of specimens that are usually found to contain HTLV-2 after additional testing. (D) Typical patterns from HTLV indeterminate specimens. Shown are typical HTLV Gag indeterminate profiles frequently found in plasma or sera from individuals originating from tropical regions (Central Africa, Papua New Guinea, etc.) (lanes 1 to 4) and those from low-risk populations (lanes 5 to 7). In the great majority of cases, neither HTLV-1 nor HTLV-2 infection could be demonstrated in samples with such seroreactivity using PCR testing. (E) Seroreactivity observed in HTLV-3-infected (lane 4) and HTLV-4-infected (lane 5) persons from Cameroon. Plasma from an HTLV-3-infected person (Cam2026ND) was weakly reactive to p24, p19, r21e, and rgp46^{I}. Plasma from the HTLV-4-infected person (Cam1863LE) was weakly reactive to p24, r21e, and rgp46^{II}, but was strongly reactive to p19, similar to that seen in HTLV-1-infected specimens. Lanes 1 and 2 are from HTLV-1- and -2-infected persons, respectively, while lane 3 is reactivity of negative control plasma. doi:10.1128/9781555817381.ch83.f3

and other areas of endemicity (Brazil, Argentina, West Indies, Iran, and Japan) to confirm any repeatedly EIA-reactive sample (35).

HTLV-3 and HTLV-4 were detected in research studies using commercially available EIAs and WB assays or research-based IFAs that use HTLV-1 and -2 antigens (2, 46, 89). The sensitivity for detecting these new HTLVs with these assays is unknown, and a broad range of WB or LIA profiles is observed which can be confused with those of HTLV-1 or -2. Thus, PCR and sequence analysis are critical for resolving infection with the specific HTLV group.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

A typical algorithm for HTLV testing for diagnostic purposes is outlined in Fig. 2. If the initial screening immunoassay (EIA or ChLIA) is reactive, a repeat assay on the same specimen is performed in duplicate. If one or both of the repeat tests are reactive, the specimen is classified as repeatedly reactive (RR). The RR specimens are subjected to confirmatory supplemental testing that is typically done by WB when available (Fig. 3 and Table 1). Based on the data available in 1992, the U.S. Public Health Service (PHS) recommended that the diagnostic criteria for confirmation of HTLV-1 or HTLV-2 seropositivity by supplemental tests include demonstration of antibodies to p24 Gag and to native gp46 Env and/or r21e (81). Subsequent data with more sensitive assays suggest that alternative patterns of WB reactivity may be considered. The second-generation modified WB is the most commonly used research-use WB assay worldwide (MP Diagnostics WB 2.4). Specimens with reactivity to p19 Gag (with or without p24 Gag), r21e, and rgp46^{I} are referred to as HTLV-1 positive (Fig. 3A). Sera with reactivity to p24 Gag (with or without p19 Gag), r21e, and rgp46^{II} are referred to as HTLV-2 positive (Fig. 3B). Specimens without immunoreactivity to any WB bands are considered negative for antibodies to HTLV-1 and HTLV-2 (false-positive EIA specimens). Specimens reacting with p24 Gag (with or without p19 Gag) and r21e, but with no reactivity to either rgp46^{I} or rgp46^{II}, are considered HTLV positive but untypeable (Fig. 3C) since PCR analysis of untypeable specimens has identified the presence of HTLV-specific sequences in some cases (2, 45, 82). This type of reactivity could indicate the presence of divergent HTLV such as HTLV-3 and HTLV-4. However, to date HTLV-3 and -4 have only been found in Cameroon (2, 12–14). Similar interpretive criteria are used for LIA (see manufacturer's instructions).

Both the WB assays and LIA can give indeterminate results (immunoreactivity to a single HTLV gene product or multiple bands not defined as positive above, conditions that do not meet the criteria for seropositivity [Fig. 3D]). Antibody to only Gag proteins (p24, p19) is the most common indeterminate pattern that is observed in EIA-reactive specimens and is an area of intense investigation (97–99). Extensive PCR analyses using primers to detect multiple gene regions have failed to detect HTLV-1 or HTLV-2 proviral sequences in low-risk seroindeterminate persons, thus indicating that these individuals are not likely to be infected with HTLV-1 or HTLV-2 (45, 82, 97–99). The possibility that such indeterminate WB results may represent a novel retrovirus with partial homology to HTLV has been explored; however, no DNA amplification was observed using generic PCR primers that would detect HTLV-related viruses (45, 82, 99), with the exception of HTLV-3 and HTLV-4 (2, 4–6, 51). Limited studies have established that individuals with indeterminate WB profiles generally do not have risk factors for HTLV infection (97–100). Indeterminate WB results among low-risk persons may represent antibodies to different viral, microbial, and cellular antigens that cross-react with HTLV proteins (97–99) (Fig. 3E). The predictive value of positive WB results is increased in patients with a history of any potential HTLV risk factor, including residence in endemic areas, history of a blood transfusion before 1988 or IDU, having multiple sexual partners without condom protection, or having sexual partners or parents from an endemic region (25, 81).

In rare instances, specimens with confirmed HTLV infection have an indeterminate WB pattern of reactivity to p19 Gag (in the absence of p24 Gag) and r21e (25, 100). Likewise, in some instances, antibody to r21e may represent an early antibody response during seroconversion (25, 81). Individuals with such reactivity should be retested in 3 months by EIA and/or PCR (81).

Recent data suggest that a very small number of U.S. patients with chronic progressive neurological disease with HTLV-indeterminate WB patterns may be infected with a defective HTLV or have HTLV-1 in low copy numbers (97). Persons who have clinical neurological symptoms or are from high-prevalence areas (e.g., African, Asian, South American, and Caribbean countries) with HTLV-1/2-indeterminate reactivity should be further investigated to exclude HTLV-3 and HTLV-4.

In the U.S., decisions to accept blood donations and defer blood donors are currently based only on screening test (ChLIA) results because of the lack of FDA-licensed supplemental tests. Persons who are ultimately confirmed to be antibody-positive to HTLV-1 or HTLV-2 (i.e., when licensed supplemental testing becomes available) will be permanently deferred from donating blood (34, 39). Blood donors with plasma specimens that are RR on screening but not confirmed as seropositive for HTLV-1 or HTLV-2 (including false EIA-reactive and supplemental test indeterminate specimens and specimens for which supplemental testing was not performed) should be notified and deferred if the same test result is obtained on two separate donations (34, 39, 81). Screening tests are weighted toward better sensitivity than specificity because of the public health implications of false-negative results (101). Therefore, in the absence of known risk factors for a given blood donor, repeatedly reactive results from a single-test kit most likely represent false-positive reactions. However, in the absence of an FDA-approved confirmatory test it is difficult to understand the true specificity of a screening assay in finding RR results. The positive predictive value (PPV) of a test result is always a function of the prevalence of disease in the screened population, which for HTLV is extremely low in blood donors following implementation of screening in 1988 (48). The lower the prevalence, the lower the PPV, and the lower the specificity of a test. Thus, blood specimens are routinely tested using the dual-screening test algorithm (Fig. 2) to increase the probability of detecting actual infection (34, 39, 93). Since sensitivities are similar in each individual screening test and nonspecific reactivity is not, specimens reactive in both tests are likely true positives (34, 39). Nonetheless, WB-indeterminate results are seen in RR blood donors but have been shown by PCR to be HTLV negative in nearly all cases (82). Thus, additional testing by PCR or from specimens collected at a later time point is required to resolve infection in these persons. Many studies have reported high concordance between WB and PCR results of infected and uninfected U.S. blood donors (101–103).

Persons with HTLV-1- or HTLV-2-positive or indeterminate test results are counseled according to the guidelines established by the USPHS Working Group at CDC (81). These guidelines state that persons should be informed that HTLV is not HIV and that their risk of developing HTLV-related diseases is low. HTLV-1- or HTLV-2-infected persons are asked not to donate blood, semen, organs, or other tissues and not to share needles or syringes. To prevent transmission of HTLV, the infected person is counseled to use protective measures during sexual activity and women are counseled to refrain from breast-feeding. Persons who have indeterminate results on two separate occasions at least 3 months apart should be advised that their specimens were reactive in screening for HTLV-1/2, but that these results could not be confirmed by more specific tests. Further, they should be reassured that indeterminate results are very rarely caused by HTLV-1 or HTLV-2 infection. Repeat serologic and/or PCR testing should be offered to persons with indeterminate test results.

Use of trade names is for identification only and does not imply endorsement by the U.S. Department of Health and Human Services, the Public Health Service, or the Centers for Disease Control and Prevention. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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Influenza Viruses

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TAXONOMY

The influenza viruses are members of the family *Orthomyxoviridae*. Antigenic differences in two major structural proteins, the matrix protein (M) and the nucleoprotein (NP), are used to separate the influenza viruses into three genera within the family: *Influenzavirus A*, *Influenzavirus B*, and *Influenzavirus* C. Members of these three genera are also referred to as influenza type A, B, and C viruses, respectively. The influenza A viruses are further classified into subtypes based on characteristics of the two major surface glycoproteins, the hemagglutinin (HA) and neuraminidase (NA). Subtypes are recognized by the lack of cross-reactivity in double immunodiffusion assays with animal hyperimmune sera corresponding to each antigen (1). Eighteen HA subtypes and 11 NA subtypes are now recognized (2). Within a subtype, strains may be further subclassified into lineages, or clades, based on phylogenetic analysis of gene sequences. An example is the classification of the Eurasian lineage of highly pathogenic H5N1 strains into clades and the further subdivision of circulating strains into second-, third-, and fourth-order clades (3).

The following information is used in the naming of individual virus strains: type, species of origin (if nonhuman), geographic location of isolation strain, laboratory identification number, year of isolation, and subtype (influenza A viruses only). Thus, an example of a human strain of influenza is A/Texas/50/2012 (H3N2), while A/quail/Vietnam/36/2004 (H5N1) is an example of an avian strain isolated in an epizootic in Asia.

DESCRIPTION OF THE AGENTS

Orthomyxoviruses are enveloped, single-stranded, RNA viruses with segmented genomes of negative sense. Influenza A and B viruses have eight RNA segments, and influenza C viruses have only seven segments. Gene segments range from ~800 to ~2,500 nucleotides in length, and the entire genome ranges from 10 to 14.6 kb. The segmented genome of influenza viruses allows the exchange of one or more gene segments between two viruses when both infect a single cell. This exchange, called *genetic reassortment*, results in the generation of new strains containing a mix of genes from both parental viruses. Genetic reassortment between human and avian influenza virus strains led to the generation of the 1957 A(H2N2) and 1968 A(H3N2) pandemic strains, and it also played a role in the emergence of the pandemic A(H1N1pdm09) virus in 2009 and in A(H7N9) avian strains from China that have caused infections in people (4).

Influenza viruses are spherical and pleomorphic, with diameters of 80 to 120 nm after serial passage in culture. Filamentous forms also occur and may be as large as several micrometers. The lipid envelope is derived from the host cell membrane, through which maturing virus particles bud, and HA and NA form characteristic rod-like spikes (HA) and spikes with globular heads (NA) on the virus surface. As its name implies, the HA can agglutinate red blood cells from both mammalian (e.g., human [type O], guinea pig, horse) and avian (e.g., chicken, turkey) species by binding to sialic acid residues. The HA protein, the major antigenic determinant, is used to identify viruses using immune sera. The lipid envelope surrounds the nucleocapsid, which has helical symmetry and consists of the genomic RNA segments, several copies of the polymerase proteins, and the NP. The matrix-1 (M1) protein is located between the nucleocapsid and the envelope, and the matrix-2 (M2) protein forms an ion channel across the envelope in influenza A viruses.

EPIDEMIOLOGY AND TRANSMISSION

Influenza viruses cause annual epidemics in areas with temperate climates, while in tropical climates, seasonality is less apparent and influenza viruses can be isolated throughout the year. In the temperate regions of the Northern Hemisphere, epidemics generally occur between December and March, and in the Southern Hemisphere, the epidemic period is usually between May and August. Epidemics are characterized by a sudden increase in febrile respiratory illnesses and absenteeism from school and work, and within a community, the epidemic period usually lasts 3 to 8 weeks. A single subtype (A) or type (B) of influenza virus usually predominates, but epidemics have occurred in which both A and B viruses or two influenza A virus subtypes were isolated. Global epidemics, or pandemics, occur less frequently and are seen only with influenza A viruses. Pandemics occur following the emergence of an influenza A virus that carries a novel HA and that can be readily transmitted from person to person. The pandemic strain may develop due to genetic reassortment following coinfection of a susceptible host with human and animal influenza viruses or through gradual adaptation of an avian strain to mammalian hosts.

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Influenza viruses are transmitted from person to person primarily via droplets generated by sneezing, coughing, and speaking. Direct or indirect (fomite) contact with contaminated secretions and small-particle aerosols is another potential route of transmission. The relative importance of these different routes has not been determined for influenza viruses (5). As for human infections caused by avian strains of influenza virus, direct contact with infected birds has been the most common factor of transmission, and direct inoculation into the pharynx or gastrointestinal tract may lead to infection (6).

There has been concern about the pandemic potential of avian strains of influenza since 1997, when several human cases of infection with A(H5N1) viruses occurred in Hong Kong in association with a large poultry outbreak. The outbreak was controlled by slaughtering all poultry in Hong Kong, but A(H5N1) viruses again caused outbreaks in poultry in China in 2003. By late 2005, the virus had spread to other parts of Asia, as well as to parts of Europe, Africa, and the Middle East. Human cases of A(H5N1) have been directly associated with outbreaks in poultry, and as of 2013, more than 600 human infections have been documented. Most cases have occurred in southeastern Asia, but several cases have also been documented in the Middle East and northern Africa. Most human cases have been due to direct contact with infected birds, but limited human-to-human transmission has also occurred (6). Several mutations in influenza virus genes are required for avian influenza viruses to replicate efficiently in mammalian cells and to transmit by droplet aerosol between ferrets, an animal model of human infection (7–9). A(H5N1) viruses continue to evolve and increase diversity, raising the possibility that they may acquire the ability to spread efficiently among humans.

Other avian influenza A virus subtypes are also of concern. An outbreak of A(H7N7) virus in commercial poultry farms in the Netherlands in 2003 was associated with respiratory illness in more than 400 persons, although only a single person died (10). In 2013, an A(H7N9) virus emerged in poultry markets in China, and more than 100 persons were infected (11). The greatest risk of infection has been exposure to infected poultry, similar to what has been observed with human cases of A(H5N1).

Swine are another source of novel influenza virus strains that can infect people. In 2009, a novel influenza A(H1N1)pdm09 was initially identified as a cause of significant febrile respiratory illnesses in Mexico and the United States, and it rapidly spread to many countries around the world, prompting the World Health Organization (WHO) to declare an influenza pandemic. The new strain subsequently replaced previously circulating seasonal A(H1N1) strains. In recent years, a number of infections with another swine virus with an antigenically distinct HA (variant H3N2) were identified in several states (12). Fortunately, most cases are associated with direct or indirect contact with swine, and the A(H3N2)v strain has not spread among the population like the A(H1N1)pdm09 strain. The transmission of influenza viruses to people from avian and swine species highlights the need for vigilant surveillance for such events.

CLINICAL SIGNIFICANCE

Influenza A and B virus infections typically cause a febrile respiratory illness characterized by fever, cough, upper respiratory tract symptoms (e.g., sore throat, rhinorrhea, nasal congestion), and systemic symptoms (e.g., headache, myalgia, malaise). This constellation of symptoms is called influenza, although other clinical presentations, ranging from asymptomatic infection to viral pneumonia, also occur. Illness begins abruptly after a 1- to 5-day incubation period (average, 2 days). Fever generally lasts for 3 to 5 days, but dry cough and malaise may persist for several weeks. Complications include otitis media in children, sinusitis, viral pneumonia, secondary bacterial pneumonia, exacerbation of underlying cardiac or pulmonary disease, myositis (including rhabdomyolysis), neurologic problems (seizures, acute encephalitis, and postinfectious encephalopathy), Reye's syndrome (associated with aspirin use), myopericarditis, and death (13, 14). In contrast, influenza C viruses cause mild respiratory illnesses that are clinically indistinguishable from common colds.

Influenza A(H5N1) viruses also cause a febrile respiratory illness, although lower respiratory tract illness has been more prevalent. Upper respiratory tract symptoms may be absent, and gastrointestinal symptoms (watery diarrhea, vomiting, and abdominal pain) occur in some patients (6). Acute encephalitis may occur. Infection is associated with a high mortality rate (~60%), with most patients dying of progressive pneumonia. Viral replication is prolonged, and levels of several inflammatory mediators (e.g., interleukin-6, interleukin-8, interleukin-1β) in plasma have been higher in fatal than in nonfatal cases. Surviving patients develop measurable serum antibody responses 10 to 14 days after symptom onset (6). Influenza A(H7N9) virus infections also can cause severe respiratory illness and mortality, although overall, the severity has been less (~30% mortality) than that observed with A(H5N1) virus (15).

Influenza A and B virus infections spread rapidly through the community, with documented clinical attack rates as high as 70% after a common source exposure in an enclosed space. Epidemic disease is associated with an increase in hospitalization rates, especially in young children and elderly persons, and an increase in mortality rates in elderly persons. Mortality rates have been higher in epidemics caused by influenza A(H3N2) viruses than in those caused by A(H1N1) or B viruses in the past 20 years. Additional information on the clinical presentation, manifestations, and complications of the diseases can be found in clinical textbooks (13, 14).

Four licensed antiviral medications are available for the treatment of influenza virus infection. Amantadine and rimantadine are adamantanes that block the M2 ion channel. The adamantanes have no activity against influenza B viruses, and unfortunately, the currently circulating influenza A viruses have developed resistance so that the adamantanes are not clinically useful as monotherapy for these viruses either. Zanamavir and oseltamivir are NA inhibitors and are active against both influenza A and B viruses. Clinically significant resistance can occur following treatment of children or immunocompromised patients with oseltamivir. Treatment of outpatients with any of these medications should be initiated within 2 days of symptom onset to have demonstrable clinical benefit, although treatment of virus-positive hospitalized patients can be started at any time during the illness (16). These drugs have also been used for prophylaxis, but annual immunization with a trivalent influenza vaccine is the primary means of prevention of influenza.

Inactivated influenza vaccines (IIVs), live attenuated influenza virus (LAIV) vaccine, and a recombinant hemagglutinin vaccine (RIV) are licensed in the United States (17). The IIVs are derived from viruses grown in cell culture (ccIIV) or embryonated chicken eggs that are harvested and then inactivated. Viral proteins are partially purified and standardized to contain 15 μg of HA per dose. The IIVs may be trivalent (IIV3), containing influenza A(H1N1), A(H3N2), and B virus strains, or quadrivalent (IIV4), containing influenza B virus strains from two lineages (B/Victoria and B/Yamagata), as well as A(H1N1) and A(H3N2) strains. A high-dose IIV3 containing 60 μg of each HA is also licensed for adults 65 years and older. The RIV3 vaccine contains 45 μg of baculovirus-expressed, recombinant hemagglutinin for an A(H1N1) and A(H3N2), and B strain. The LAIV4 vaccine is quadrivalent and contains the same strains recommended for IIV4. A reassortant vaccine virus for each strain to be included is derived to contain six internal genes from a parental attenuated influenza (A or B) virus and the HA and NA from the WHO-recommended vaccine strain. The vaccine is licensed in the United States for use in persons 2 to 49 years old. It is given topically into the nose, and virus replicates in the upper respiratory tract.

Due to constant virus evolution causing gradual antigenic changes in the HA protein, viruses included in the influenza vaccines must be updated periodically. The strains to be included in the vaccine are selected twice annually by WHO. Vaccine strains for northern hemisphere countries are selected in January and February to make vaccine for use in September. New vaccine alternatives, including those given by other routes and in combination with adjuvants, are undergoing clinical studies.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Influenza viruses infect the respiratory epithelium and can be found in respiratory secretions of all types. The level of virus shedding parallels the severity of clinical symptoms in uncomplicated influenza and is maximal in the first several days of illness. Samples should be collected during this time (first 2 or 3 days) to maximize the likelihood of virus detection. A variety of upper respiratory tract samples, alone or in combination, is routinely used for virus identification, including nasal aspirates, nasal wash fluids, nasal or nasopharyngeal swabs, throat swabs, and throat wash fluids. Virus titers tend to be lower in samples collected from the throat, so assays of these samples alone tend to be less sensitive (18, 19). However, reports of human infection caused by A(H5N1) strains suggest that throat samples and lower respiratory tract samples may have better diagnostic yields than samples collected from the nose (6). Lower respiratory tract samples, including sputa, tracheal aspirates, and bronchoalveolar lavage fluids, may yield virus and can be assayed when indicated. Virus can occasionally be identified in nonrespiratory clinical samples (6).

Once collected, the clinical samples should be placed in viral transport medium. A number of transport media are suitable for influenza viruses, including veal infusion broth, Hanks' balanced salt solution, tryptose phosphate broth, sucrose phosphate buffer, and commercially available cell culture medium. All of these media are supplemented with 0.5% bovine serum albumin or 0.1% gelatin to stabilize the virus and antimicrobials (antibiotics and antifungals) to inhibit the growth of other respiratory flora. However, the use of transport medium may interfere with the test performance for certain commercially available virus detection assays; the package inserts of these assays should be consulted if they are to be used for diagnosis (Tables 1 and 2). Influenza virus infectivity is maintained for up to 5 days when samples are placed in transport media and maintained at 4°C (20). Clinical samples should be transported to the diagnostic laboratory as rapidly as possible after collection under these conditions. If a sample cannot be cultured during this time frame, it should be stored immediately at −70°C; storage at higher temperatures (e.g., −20°C) leads to the loss of virus viability. Immediate transport and processing of samples after collection are necessary for immunofluorescence detection of virus antigen in exfoliated epithelial cells.

DIRECT DETECTION

Microscopy

Influenza viruses have been detected in clinical specimens by direct and indirect visualization of their typical morphological appearance by electron microscopy (EM). Immune EM has been the most sensitive EM method and allows differentiation of virus type and subtype when specific hyperimmune sera are used in the assay (21). However, large numbers of viruses ($>10^5$ to 10^6/ml) must be present in the clinical sample for successful detection using this diagnostic approach. Because of the need for an experienced microscopist and access to an electron microscope, the relatively high costs of assay performance, and the greater sensitivity of other diagnostic approaches, EM is not routinely used for diagnosis of influenza virus infection.

Antigen Detection

Antigen detection assays are used in a variety of formats to rapidly detect influenza viruses in clinical specimens and to confirm the identity of isolates grown in culture. These assays are based on detecting the interaction of viral proteins with specific antibodies. A variety of different formats are used, including direct and indirect fluorescent-antibody (FA) staining, radioimmunoassay, enzyme immunoassay, immunochromatographic assay, and fluoroimmunoassay.

FA assays identify viral antigens present on or in infected, exfoliated epithelial cells in respiratory secretions. Cells are collected on swabs or in aspirates or wash fluids and are washed in cold buffer to remove mucus before being applied and fixed to a microscope slide. Use of cytocentrifugation for application of the cells to slides can improve the number and morphology of cells for evaluation and enhance the accuracy of interpretation. Virus-specific antibodies are applied to the fixed cells; monoclonal antibodies directed against viral proteins that are conserved and expressed in large quantities (e.g., M, NP) are used because of their greater specificity compared with polyclonal sera and are available from a number of manufacturers. A fluorochrome is conjugated to the virus-specific antibody in direct FA (DFA) assays, and it is conjugated to a second antibody that reacts with the virus-specific antibody in indirect FA (IFA) assays. Antibody staining of cells is detected with a fluorescent microscope. Contaminating mucus can cause nonspecific fluorescence that can be reduced by treating the samples with *N*-acetylcysteine or dithiothreitol and by centrifuging cells through Percoll. DFA and IFA assays take 2 to 4 hours to perform, although some diagnostic laboratories batch samples and do not perform tests as soon as the sample is received, delaying the availability of results. In theory, IFA assays should be more sensitive and less specific than DFA assays, but there is significant overlap in the sensitivities (50% to 90%) and specificities (generally >90%) of these assays noted in published reports (22). An advantage of FA assays is that sample quality can be determined by observing whether an adequate number of epithelial cells are present. In addition, kits are available to screen for other respiratory viruses (e.g., respiratory syncytial virus, parainfluenza viruses, adenovirus), as well as for influenza

TABLE 1 Commercially available kits for detection of influenza A or B viruses by fluorescent antibody staining[a]

Assay format	Kit name (manufacturer)	Acceptable clinical samples for direct detection; cell culture confirmation	Comments	Influenza virus types detected	Assay sensitivity/ specificity for direct detection per manufacturing brochure	Assay sensitivity/ specificity for isolate identification per manufacturing brochure	Other viruses detected
IFA	Bartels Respiratory Viral Detection Kit (Trinity Biotech)	NA, NPA, NPS, NW, TX; cell culture	Also available as individual influenza A or B components	A and B	A: 86%/99% B: 65%/98%	A: 100%/ 99.9% B: 100%/100%	Adeno, P1, P2, P3, RSV
DFA	D^3 DFA Respiratory Virus Screening & ID kit (Diagnostic Hybrids, Inc.)	NA, NPA, NW; cell culture	Also available as individual influenza A or B components	A and B	A: 96.6–100%/ 100% B: 100%/100%	A: 100%/99.5–100% B: 100%/99.5–100%	Adeno, P1, P2, P3, RSV
DFA	Imagen Influenza Virus A and B (Remel, Inc.)	NPA; cell culture		A and B	A: 96.2%/ 100% B: 86.7%/ 99.5%	A: 100%/100% B: 100%/100%	None
DFA	PathoDx Respiratory Virus Panel (Oxoid)	Cell culture only	Not approved for direct use on clinical specimens	A and B	N/A	A: 100%/100% B: 100%/100%	Adeno, P1, P2, P3, RSV
DFA	Light Diagnostics Simulfluor Viral Diagnostic Screen (Millipore)	NPA, NPS, NW, TS; cell culture	Also available as Flu A/ Flu B kit	A and B	A: 80%/98.6% B: 50%/100%	A: 97.8%/ 100% B: 100%/100%	Adeno, P1, P2, P3, RSV

[a] IFA, indirect fluorescent antibody; DFA, direct fluorescent antibody; NA, nasal aspirate; NPA, nasopharyngeal aspirate; NPS, nasopharyngeal swab; NW, nasal wash; TS, throat swab; Adeno, adenovirus; P1, P2, P3, parainfluenza virus types 1, 2, 3; RSV, respiratory syncytial virus.

A and B viruses (Table 1). These multiplex assays allow efficient screening for other viral causes of febrile respiratory disease. Disadvantages include the need for specialized equipment (a fluorescent microscope) and the impact of technician expertise on assay performance characteristics (i.e., sensitivity and specificity). Each laboratory should establish its own performance characteristics compared to those of cell culture.

A number of immunoassays that use different reporter formats (isotopic, colorimetric, fluorometric, and chromatographic) have been developed for the detection of influenza virus antigen in clinical specimens. Many of these assays take at least 2 hours to perform and have 50 to 80% sensitivity compared with culture methods (23). Rapid influenza diagnostic test (RIDT) kits use the immunoassay formats for rapid (<30 min) detection of influenza A and B viruses in clinical specimens and are used much more commonly than other antigen detection immunoassay formats (Table 2). The kits use monoclonal antibodies to detect the presence of the influenza A or B nucleoprotein by enzyme immunoassay or chromatographic immunoassay. All of the kits provide results within 30 minutes, and some of them can be used as point-of-care tests (i.e., those classified by the Clinical Laboratory Improvement Amendments [CLIA] as waived). The types of specimens that are appropriate for testing vary among the kits, and specific instructions for sample collection and processing must be followed for optimal results. Assay performance characteristics in clinical settings are affected by the age of the patient (generally lower sensitivity in adults), the amount of virus in the clinical sample, and the type of specimen analyzed (16, 22). The sensitivity of RIDTs for identification of infection with influenza A virus strains containing novel hemagglutinins (e.g., H1pdm09, H5, H7) has frequently been lower than for previously circulating seasonal influenza A strains, so such tests should not be relied on when such infections are suspected (24, 25). In fact, a negative RIDT result should not prevent prescription of antiviral treatment of a patient with suspected influenza, especially when influenza is prevalent

TABLE 2 Commercially available rapid influenza diagnostic test kits for rapid (≤30 min) detection of influenza A or B virus[a]

Assay format	Kit name (manufacturer)	Acceptable clinical samples	Sample collection restrictions	Virus type(s) detected (differentiation of A and B)	Assay sensitivity(ies) (%)/specificity(ies) (%)[b]	Assay performance time (min)	Assay complexity[c] (510K number)	References
Dipstick chromatographic immunoassay	Clearview Exact Influenza A & B (Alere)	NS	Do not use calcium alginate swabs	A and B (yes)	A, 19–59%/95–100%	15	CLIA waived (K092349)	71, 72
Dipstick chromatographic immunoassay	OSOM Influenza A& B (Sekisui Diagnostics)	NS	Use only swabs supplied with the kit	A and B (yes)	No published studies	10	CLIA moderate (K061508)	No published studies
Dipstick chromatographic immunoassay	TRU FLU (Meridian Bioscience, Inc.)	NA, NPS, NS, NW	Do not use calcium alginate swabs	A and B (yes)	No published studies	15	CLIA moderate (K071657)	No published studies
Flow through enzyme immunoassay	Directigen Flu A+B (Becton-Dickinson)	BAL, NA, NPS, NS, NW, TS	For swabs, use polyester or rayon-tipped with aluminum wire; do not use calcium alginate	A and B (yes)	A, 41–87%/98–100% B, 29–88%/97–100%	15	CLIA moderate (K001364)	73–76
Lateral flow chromatographic immunoassay	BD Veritor (Becton Dickinson)[d]	NPS, NS		A and B (yes)	No published studies	10	CLIA waived (K112277)	No published studies
Lateral flow chromatographic immunoassay	BD Veritor (Becton Dickinson)[d]	NA, NPS, NW		A and B (yes)	No published studies	10	CLIA moderate (K121797)	No published studies
Lateral flow chromatographic immunoassay	BinaxNOW Influenza A&B (Alere)	NA, NPS, NW, NS	For swab samples, use cotton, rayon, foam, or polyester swabs; do not use calcium alginate	A and B (yes)	A, 10–78%/96–100% B, 15–100%/90–100%	15	CLIA waived (K062109)	24, 73, 77–79
Lateral flow chromatographic immunoassay	Biosign Flu A&B (Princeton BioMeditech)	NA, NPS, NS, NW		A and B (yes)	No published studies	15	CLIA moderate (K083746)	No published studies
Lateral flow chromatographic immunoassay	Directigen EZ Flu A+B (Becton-Dickinson)	NA, NPS, NW, TS	For swabs, use polyurethrane foam swabs	A and B (yes)	A, 41–82%/97–100% B, 30–53%/100%	15	CLIA moderate (K042472)	73, 80–82
Lateral flow chromatographic immunoassay	QuickVue Influenza Test (Quidel)	NA, NS, NW	Do not use any kind of transport media to store or transport sample	A and B (no)	Combined, 37–95%/76–99%	10	CLIA waived (K991633)	83–85
Lateral flow chromatographic immunoassay	QuickVue Influenza A+B Test (Quidel)	NA, NPS, NS, NW	Limited transport media supported	A and B (yes)	A, 18–93%/92–100% B, 0–41%/90–100%	10	CLIA waived (K031899)	80, 86–89

Lateral flow chromatographic immunoassay	SAS FluAlert Influenza A&B Test (SA Scientific, Inc.)	NA, NW		A and B (yes)	No published studies	15	CLIA moderate (K080380)	No published studies
Lateral flow chromatographic immunoassay	SAS FluAlert Influenza A Test (SA Scientific, Inc.)	NA, NW		A only	No published studies	15	CLIA waived (K041439)	No published studies
Lateral flow chromatographic EIA	SAS FluAlert Influenza B Test (SA Scientific, Inc.)	NA, NW		B only	No published studies	15	CLIA waived (K041441)	No published studies
Lateral flow chromatographic EIA	Xpect Flu A&B (Remel)	NS, NW, TS,	For swab samples, use rayon or Dacron-tipped swabs with aluminum or plastic shafts; do not use calcium alginate	A and B (yes)	A, 40–92%/99–100% B, 20–98%/99–100%	15	CLIA moderate (K031565)	77, 90, 91
Lateral flow fluorescent immunoassay	RAMP Flu A+B (Response Biomedical)[d]	NA, NPS, NW	For swab, use sterile foam, polyester, nylon or rayon; do not use calcium alginate	A and B (yes)	A, 41–75%/80–99.8% B, 31–87%/99–100%	15	CLIA moderate (K071591)	24, 78, 91
Lateral flow fluorescent immunoassay	Sofia Influenza A+B FIA (Quidel)[d]	NA, NPS, NS	Use nylon-flocked swab for NPS and kit swab for NS	A and B (yes)	A, 73–82%/96–100% B, 59–86%/98–100%	15	CLIA waived[e] (K112177)	89, 92

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[a] Additional information on rapid tests can be found at the following website: http://www.cdc.gov/flu/professionals/diagnosis/rapidlab.htm. BAL, bronchoalveolar lavage; NA, nasal aspirate; NPS, nasopharyngeal swab; NS, nasal swab; TS, throat swab; NP, nucleoprotein.

[b] Test characteristics compiled from published literature on clinical samples.

[c] CLIA (Clinical Laboratory Improvement Amendments) waived laboratory assays employ methodologies that are so simple and accurate as to render the likelihood of erroneous results negligible. CLIA moderate-complexity assays require some knowledge, training, reagent preparation, processing, proficiency, ability to troubleshoot or interpret, and judgment in the performance of the test.

[d] Requires a reader for assay interpretation.

[e] Analysis of nasal aspirate/wash samples is classified as moderate complexity.

in the community, and follow-up testing with culture or RT-PCR should be considered (16, 26). The lower sensitivity of the rapid antigen tests for these viruses is not just due to using nucleic acid tests as a gold standard (24), and it varies depending on the kit used (27). The lower sensitivity associated with many of the RIDTs is leading to a reassessment of their status by the U.S. Food and Drug Administration (FDA) with a proposal to establish minimum sample sensitivity requirements using appropriate culture or molecular methods as the gold standard to monitor device performance over time to evaluate its ability to identify contemporary strains, and to have provisions for evaluating an RIDT's ability to detect a novel reassortant influenza virus when it emerges in the setting of a public health emergency (28).

A novel lateral flow immunochromatographic assay (AVantage; Arbor Vita, Sunnyvale, CA) has been cleared by the FDA for the identification in nasal or throat swabs of some influenza A(H5N1) strains. The NS1 protein of many clade 1 and 2 influenza A(H5N1) virus strains, but not clade 0 or seasonal influenza virus strains, contains a unique sequence that binds to PDZ domains (29). The test uses a recombinant protein containing a PDZ-binding domain and gold-labeled monoclonal antibody that recognizes a wide range of influenza A virus strains in a sandwich immunoassay. However, the performance of this assay with clinical samples has not yet been determined, and variability in the PDZ domain among H5N1 viruses has been reported that may affect the ability of this test to detect some A(H5N1) viruses.

Nucleic Acid Analyses

Molecular methods are increasingly being used both for the detection and for the characterization (see below) of influenza viruses. The most commonly used molecular method is RT-PCR. Viral nucleic acids are first extracted from clinical samples. The use of guanidinium thiocyanate with silica particles or commercial kits based on this approach reliably removes inhibitors of the enzymatic amplification that are often present in clinical specimens. Automated extraction instruments can be used in place of manual methods, decreasing the amount of time personnel must spend in sample preparation while increasing the reproducibility of the procedure. Reverse transcriptase is used to synthesize cDNA from viral RNA using random hexamers or virus gene-specific oligonucleotides. The cDNA is then amplified by using virus gene-specific oligonucleotides as primers and a heat-stable DNA polymerase. Resulting amplicons are identified as virus-specific by using a variety of different methods (e.g., identification by size, hybridization, restriction enzyme mapping, sequencing).

A large number of different RT-PCR assays have been developed since the initial description in 1991 of an RT-PCR method to detect and distinguish influenza A, B, and C viruses (30). Assays that identify and distinguish different influenza virus types have targeted conserved genes, such as the matrix gene, and subtype-specific assays have amplified a portion of the HA gene. Nested PCR assays have been developed to improve assay sensitivity, but the inherent problem of carryover contamination associated with the use of this assay format limits its utility for most diagnostic laboratories. Real-time RT-PCR assays, which are less vulnerable to cross-contamination, can directly and rapidly detect influenza viruses in clinical specimens with a sensitivity approaching or exceeding that of culture (31). Multiplexed assays able to identify influenza viruses and other respiratory viruses have been developed and have performance characteristics that meet or exceed those of cell culture (32, 33). A variety of methodologies are used to detect amplified products, and different equipment is needed based on each assay's characteristics. Multiplexed respiratory virus panels may be less sensitive than monoplex molecular assays that target a single virus (34, 35). The availability and FDA clearance of such assays (Table 3) are leading many diagnostic laboratories to use these assays for respiratory virus diagnosis in place of the more time-consuming cell culture methods, and the improved sensitivity of molecular methods is replacing culture methods as the gold standard for influenza virus detection (34, 35).

A number of isothermal molecular amplification assays are undergoing evaluation for direct detection of influenza viruses in clinical samples. These include nucleic acid sequence-based amplification (NASBA), reverse transcription-loop mediated amplification (RT-LAMP), RT-helicase–dependent amplification (HDA), and RT-nicking enzyme amplification reaction (RT-NEAR) (36). Nucleic acid amplification occurs at a single temperature without requiring the cycling associated with PCR. All of these assays require initial synthesis of complementary DNA using a reverse transcriptase. NASBA uses T7 RNA polymerase to generate RNA amplicons, while the other listed methods use a DNA polymerase to produce DNA amplicons. For the DNA-based methods, separation of double-stranded DNA occurs enzymatically rather than as a result of the heat denaturation used in PCR reactions. Successful amplification is detected using a variety of different methods, including molecular beacon probes, turbidity assays (RT-LAMP), and probe hybridization using electrochemical readouts. As with RT-PCR assays, these isothermal amplification methods are more sensitive than culture or immunofluorescent-antibody staining for the diagnosis of influenza virus infection, and they hold the promise for future use as potential point-of-care tests (37, 38).

ISOLATION PROCEDURES

Influenza virus isolation procedures should be performed under biosafety level 2 (BSL-2) conditions. When the clinical sample comes from a patient suspected to be infected with a highly pathogenic avian influenza (HPAI) virus strain, attempts at virus isolation should be performed under BSL-3 or higher conditions (39). Human clinical samples should be processed in separate laboratories and by staff members other than those handling clinical material from swine or birds (40).

Cell Culture

Influenza viruses can be grown in a number of different cell lines, including primary monkey kidney cells, Vero cells, human diploid lung fibroblasts, and Madin-Darby canine kidney (MDCK) cells (41, 42). Although some variability can be seen from season to season, MDCK and primary monkey kidney cell lines have similar isolation frequencies (41), and MDCK cells are more sensitive than Vero or diploid lung fibroblast cells (42). Thus, MDCK cells (CCL 34; American Type Culture Collection, Manassas, VA), a continuous polarized cell line, are the most common cell line used for isolation of influenza viruses and will support the growth of type A, B, and C strains. Continuous cell lines do not produce proteases that will cleave the viral HA, a step necessary to produce infectious viral progeny, so exogenous protease must be added to the maintenance medium. L-(Tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin at a concentration of 1 to 2 μg/ml provides the necessary proteolytic activity and is the

TABLE 3 Commercially available and selected other molecular detection assays for influenza viruses[a]

Assay format	Kit name (manufacturer)	Acceptable clinical samples	Virus type(s), (subtypes) detected	FDA approval (510K number)	Instrumentation	Other viruses detected	References
Real-time RT-PCR	CDC Human Influenza Virus Real-time RT-PCR Detection and Characterization Panel (CDC)	BAL, BW, NA, NPS, NS, NW, TA, TS	A (H1, H3, 2009 H1, H5), B (Yamagata lineage, Victoria lineage)	Yes (K08570, K130551, K101564)	ABI 7500 Fast DX Real-Time PCR instrument	None	93
Real-time RT-PCR	IMDx Flu A/B and RSV for Abbott m2000 (Abbott Molecular)	NPS	A, B	Yes (K131584)	Abbott m2000	RSV	None
Multiplex real-time RT-PCR	*artus* Influenza A/B RG Kit (Qiagen GmbH)	NPS	A, B	Yes (K113323)	Rotor-Gene Q MDx instrument	None	94
Multiplex real-time RT-PCR	Cepheid Xpert Flu Assay	NA, NPS, NW	A (2009 H1), B	Yes (K120911)	GeneXpert Infinity-48 or Infinity-80 systems	None	95
Multiplex RT-PCR with probe detection using voltammetry	eSensor Respiratory Virus Panel (GenMark Diagnostics)	NPS	A (H1, H3), B	Yes (K113731)	eSensor XT-8 System	RSVA, RSVB, PIV1, PIV2, PIV3, hMPV, Ad, HRV	96
Multiplex RT-PCR with endpoint melt curve analysis	FilmArray Respiratory Panel (BioFire Diagnostics)	NPS	A (H1, H3)	Yes (K110764)	FilmArray Instrument	RSV, PIV1, PIV2, PIV3, PIV4, hMPV, HRV/Ent, Ad, OC43, HKU1, 229E, NL63	96
Multiplex real-time RT-PCR	Liat Influenza A/B Assay (IQuum, Inc.)	NPS	A, B	Yes (K111387)	Liat Analyzer	None	None
Multiplex RT-PCR with electrospray ionization-mass spectrometry (ESI-MS)	PLEX-ID Flu (Abbott Laboratories)	NPS	A (H1, H3), B	Yes (K121003)	PLEX-ID system	None	97
Multiplex real-time RT-PCR	ProFast+ Assay (Gen-Probe Prodesse, Inc.)	NPS	A (2009 H1, seasonal H1, H3)	Yes (K101855)	Cepheid SmartCycler II	No	97
Multiplex real-time RT-PCR	ProFlu+ Assay (Gen-Probe Prodesse, Inc.)	NPS	A, B	Yes (K110968)	Cepheid SmartCycler II	RSV	32
Multiplex real-time RT-PCR	Quidel Molecular Influenza A+B (Quidel)	NS, NPS	A, B	Yes (K112172, K113777)	ABI 7500 Fast Dx, Cepheid SmartCycler II	No	None
Multiplex real-time RT-PCR	Simplexa Flu A/B & RSV (Focus Diagnostics)	NPS	A, B	Yes (K102170)	3M Integrated Cycler	RSV	98, 99

(*Continued on next page*)

TABLE 3 Commercially available and selected other molecular detection assays for influenza viruses[a].

Assay format	Kit name (manufacturer)	Acceptable clinical samples	Virus type(s), (subtypes) detected	FDA approval (510K number)	Instrumentation	Other viruses detected	References
Multiplex RT-PCR, target-specific primer extension, fluidic microbead microarray	xTAG Respiratory Virus Panel Fast (Luminex Molecular Diagnostics)	NPS	A (H1, H3), B	Yes (K103776)	Thermal cycler plus Luminex 100 or 200 system	RSV, hMPV, Ad, HRV	96
Multiplex RT-PCR, target-specific primer extension, fluidic microbead microarray	xTAG Respiratory Virus Panel (Luminex Molecular Diagnostics)	NPS	A (seasonal H1, H3), B	Yes (K112119)	Thermal cycler plus Luminex 100 or 200 system	RSV A, RSV B, PIV1, PIV2, PIV3, hMPV, HRV, Ad	34, 49, 96, 100
Multiplex RT-PCR, target-specific primer extension, microarray chip	Infiniti RVP Plus (AutoGenomics, Inc.)	NPA	A, B	No	Thermal cycler plus Infiniti Analyzer	RSV A, RSV B, PIV1, PIV2, PIV3, PIV4, hMPV, HRV, Ent, Ad, OC43, HKU1, 229E, NL63	35
Multiplex RT-PCR with tagged oligonucleotides cleavage extension	AnyplexTM RV16 (Seegene, Inc.)	BAL, NPA, NPS	A, B	No	Multiple platforms for amplification, Seegene software for melting curve analysis	RSV A, RSV B, PIV1, PIV2, PIV3, PIV4, hMPV, HRV, Ad, OC43, 229E/NL63, Boca, Ent	100
Multiplex RT-PCR with auto-capillary electrophoresis	Seegene RV12 or RV15[b] ACE Detection Assay (Seegene, Inc.)	BAL, NPA, NPS	A, B	No	ScreenTape (Lab901), MCE-202 MultiNA	RSV A, RSV B, PIV1, PIV2, PIV3, PIV4[b], hMPV, HRV, Ad, OC43, 229E/NL63, Boca[b], Ent[b]	100, 101
Multiplex RT-PCR	Verigene Respiratory Virus Nucleic Acid Test (RV+) (Nanosphere, Inc.)	NPS	A (H1, H3, 2009 H1), B	Yes (K083088)	Verigene Processor and Verigene Reader	RSV A, RSV B	99

[a] Additional information on rapid tests can be found at the following website: http://www.cdc.gov/flu/professionals/diagnosis/molecular-assays.htm. BAL, bronchoalveolar lavage; NA, nasal aspirate; NPS, nasopharyngeal swab; NS, nasal swab; TS, throat swab; NP, nucleoprotein; RSV, respiratory syncytial virus; PIV, parainfluenza virus; hMPV, human metapneumovirus; HRV, human rhinovirus; Ent, enterovirus; Ad, adenovirus; Boca, bocavirus; OC43, 229E, NL63, HKU1, human coronavirus variants.

[b] Additional virus strains identified in RV 15 panel.

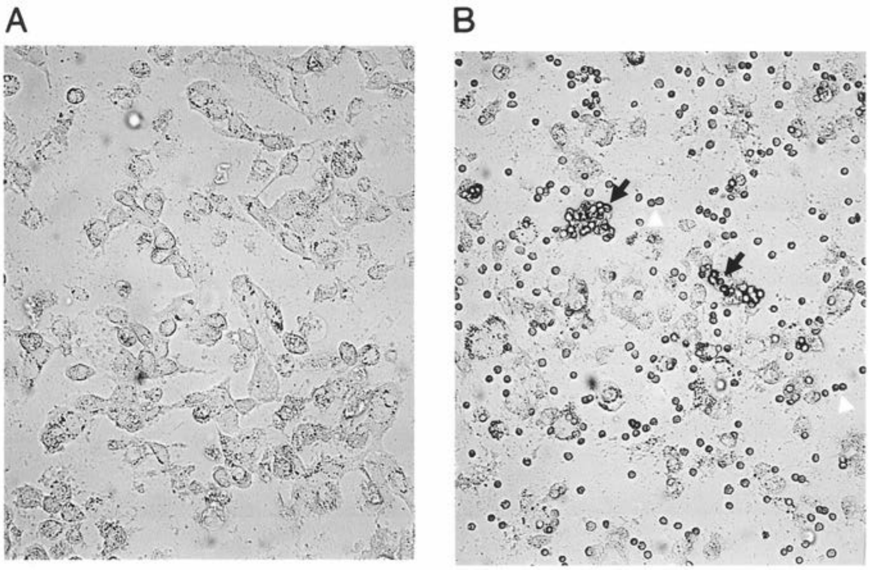

FIGURE 1 Influenza virus-infected MDCK cells. (A) Cytopathic changes. (B) Hemadsorption with guinea pig red blood cells. Red blood cells adsorb to both infected cells (black arrows) and the plastic previously occupied by infected cells and where residual hemagglutinin protein is still present (white arrowheads). doi:10.1128/9781555817381.ch84.f1

recommended protease for virus isolation. Chymotrypsin cleavage of the HA prevents the trypsin-mediated enhancement of viral infectivity, and TPCK treatment inactivates chymotrypsin activity, which may contaminate pancreatic extracts of trypsin.

MDCK cells are propagated in growth medium that contains 5 to 10% fetal calf serum (FCS). FCS contains inhibitors that prevent the production of infectious virus, so the FCS must be removed before inoculation of the clinical sample (43, 44). The inhibitory effects of FCS can be prevented by washing the cell sheet with Hanks' buffer or serum-free medium sufficiently to remove the protein-containing growth medium and then adding serum-free medium to cover the cell sheet. The clinical sample is then inoculated into the medium. After a 2-hour incubation, the inoculum-medium mixture is removed and replaced with serum-free medium supplemented with TPCK-treated trypsin. Alternatively, the sample can be inoculated directly onto cells with serum-free medium supplemented with TPCK-treated trypsin and incubated overnight before changing of the medium the next day. The cultures are maintained at 33 to 34°C and monitored for virus growth.

The replication of influenza viruses typically leads to cytopathic effects (CPE) and destruction of the cell sheet within a week following inoculation. CPE may be inapparent or absent in the presence of viral replication, but viral replication can be identified by the ability of the viral HA to bind to sialic residues on the erythrocytes of different animal species. Cultures should be screened every 2 or 3 days by hemadsorption (binding of erythrocytes to the viral HA of infected cells) or hemagglutination (cross-linking of erythrocytes by virus in the culture medium) for evidence of viral replication. To evaluate hemadsorption of cells grown in a tissue culture tube, the monolayer is first examined for CPE (Fig. 1A), and the medium is removed and stored. The cell sheet is rinsed three times with 1 ml of 0.05% guinea pig red blood cells. One milliliter of 0.5% guinea pig red blood cells is then added, and the tube is stored at 4°C for 20 min, with the red blood cell suspension covering the cells. The tube is then shaken, and adherence of red blood cells to the cell sheet is determined microscopically (Fig. 1B). If cytopathic changes are scored as less than 4+ (i.e., less than 75% of cell sheet with CPE), the tissue culture tubes are rinsed with phosphate-buffered saline and re-fed with culture medium. The media collected initially from tubes with 4+ cytopathic changes can be used for further characterization. All procedures are performed in a BSL-2 safety cabinet, and care must be taken to prevent cross-contamination between cultures. Guinea pig red blood cells are more sensitive for detection of influenza virus than are avian cells, but influenza C virus does not agglutinate guinea pig red blood cells. Chicken red blood cells can be used in agglutination assays to identify influenza C viruses. Although most isolates will demonstrate growth within 1 week after inoculation, virus from samples with low infectious titers may require extended culture incubation for 10 to 14 days and additional blind passaging of negative cultures. Presumptive isolates are characterized further, as outlined below.

A disadvantage of traditional cell culture methods is the time needed to obtain a positive result (average, 4 to 5 days). More rapid methods have been developed by inoculating samples onto cell culture monolayers maintained in shell vials or multiwell plates. This approach can use either cell lines employed in traditional cell culture for identification of influenza virus (e.g., MDCK cells) or mixed-cell cultures (e.g., A549 cells plus mink lung cells) to screen for multiple respiratory viruses (R-Mix FreshCells; Diagnostic Hybrids, Athens, OH) that are reported to detect seasonal influenza virus strains as well as strains with novel hemagglutinins (24, 45). The cells are fixed after 24 to 72 hours, and type-specific monoclonal antibodies are used to detect viral antigen. Sensitivity can be lower than that achieved by using standard isolation methods, although R-mix cells have been reported to have 82 to 100% sensitivity for detection of influenza A and B viruses (24, 45). Shell vial assays have the disadvantage of not producing virus for additional studies (e.g., antigenic characterization). Screening for viral

TABLE 4 Methods to identify and characterize influenza virus isolates

Assay	Advantages	Limitations
Assays using type or subtype-specific antisera		
ELISA	Standard assay with known performance characteristics; most labs experienced with assay format	For subtyping of influenza A strains, need to update sera periodically to detect circulating strain
Hemagglutination-inhibition	Standard assay with known performance characteristics; no special equipment needed; gold standard for antigenic characterization	For subtyping of influenza A strains, need to update sera periodically to detect circulating strain; many clinical labs not experienced with this method
Immunofluorescence or immunoperoxidase staining of infected cells	Standard assay with known performance characteristics; many labs experienced with assay format; monoclonal antibodies commercially available	For subtyping of influenza A strains, need to update monoclonal antibodies periodically to detect circulating strain
Molecular methods		
RT-PCR	Very sensitive assays	Potential for carryover contamination; need for stringent laboratory controls
Amplicon size	Ease of performance	Potential for false positives results due to nonspecific amplification
Probe hybridization	Most commonly used approach for confirmation of PCR results; real-time formats eliminate need for post-amplification processes	Depending on hybridization format used, may add time to performance of assay
Restriction analysis	Ease of performance	Need to know specific sequence; requires specific nuclease site; increased handling of post-PCR samples
Genetic sequence	Highest level of identity; sequence data that may be used in other studies	Need for specialized equipment; technically complex; increased cost
Microarray analysis	Potential to analyze multiple genetic sequences simultaneously	Investigational; limited experience

antigen by immunofluorescence also can be used at the end of the 10- to 14-day incubation period for standard culture before discarding of cells (46). This step is usually not necessary if screening by hemadsorption or hemagglutination is being performed, but it may detect virus in the absence of cytopathic changes if other strategies for virus detection are not used.

Isolation from Embryonated Chicken Eggs

The amniotic and allantoic cavities of 10- to 11-day-old embryonated chicken eggs are inoculated with the clinical sample for isolation of influenza A and B viruses. Seven- to 8-day-old eggs are used for isolation of influenza C viruses, although these viruses are also isolated using 10- to 11-day-old eggs. Embryonated eggs have endogenous proteases that are able to cleave the viral HA to yield infectious virus, so exogenous administration of proteases is not necessary. Inoculated eggs are incubated at 33 to 34°C for 2 or 3 days (5 days for influenza C viruses), then both amniotic and allantoic fluids are collected and assayed for hemagglutination activity. Influenza A and B viruses can grow both in cells lining the allantoic cavities and in those lining the amniotic cavities, whereas influenza C virus grows only in cells lining the amniotic cavities of embryonated eggs. If no hemagglutination activity is detected, influenza viruses may still be recovered by performing one or two blind passages. A pool containing equal volumes of the amniotic and allantoic fluids is inoculated into eggs as described earlier (43).

IDENTIFICATION AND TYPING SYSTEMS

A variety of methods are used to identify and characterize influenza virus isolates. The most common are based on immunologic or molecular approaches (Table 4). The initial step is to identify the isolate as an influenza virus and to distinguish it from other respiratory viruses that can agglutinate or adsorb red blood cells (e.g., parainfluenza viruses, mumps virus). In many instances, it is sufficient to identify the virus by type by using immunofluorescent or immunoperoxidase stains or an enzyme-linked immunosorbent assay (ELISA) using commercially available, type-specific antibodies targeting the viral NP or M proteins. These assays are particularly useful for working with cell culture isolates. The rapid immunochromatographic assays described in Table 2 may be able to identify isolates and type them, but data on the use of these assays for this purpose are limited, and these assays are not approved for this use. Importantly, the immunochromatographic assays may also give false-negative results when the quantity of virus in a cell culture harvest is low.

Hemagglutination inhibition (HAI) assays have been performed for more than 70 years and are still used for identification (40, 47). HAI assays can be type-, subtype-, or strain-specific, and they are particularly useful for examining antigenic relationships among strains of the same subtype. HAI is the WHO gold standard for antigenic characterization of influenza isolates and vaccine strain selection. Immune sera are usually produced in ferrets, sheep, or chickens. The hemagglutination activity of the virus is quantitated, and a standard amount of viral HA (4 HA units) is mixed with serial 2-fold dilutions of the immune serum and turkey or guinea pig red blood cells. A 4-fold or greater difference in HAI activities between the isolate and the reference strain is an indication that the isolate may be an antigenic variant. Because the HA undergoes antigenic change over time, subtype-specific antisera for interpandemic strains must be prepared and standardized periodically. Thus, subtype identification by HAI is usually performed only as part of surveillance activities or investigation of a case in which there is a strong epidemiologic suspicion of infection with a nonhuman strain.

Molecular assays can be used for virus identification and characterization. The same RT-PCR assays used for detection of viruses in clinical samples also can be used to identify clinical isolates. An advantage of molecular assays over immunology-based assays is that the molecular assays can identify influenza A virus subtypes even after significant antigenic variation has occurred because there are well-conserved regions of the HA gene that serve as targets for the primers and probes used for identification. Multiplex assays can also be used to distinguish influenza A and B viruses or to identify HA and NA subtypes (48, 49). Results are determined by identification of amplicon size, by hybridization to type- or subtype-specific probes, and by direct sequencing of the amplicons. If the sequences of different variants are known, it may be possible to identify unique differences by digesting amplified DNA with restriction endonucleases that generate restriction fragment-length polymorphisms (RFLP) unique to each strain. For example, this method was used to distinguish two A(H3N2) variants that cocirculated during a single season (50). Influenza A/Wuhan/359/95 (H3N2) virus-like variants generated amplicons that could be digested with the BstF5I restriction enzyme, whereas amplicons from influenza A/Sydney/05/97 (H3N2) virus-like variants could be digested by HindIII. Given the difficulty to design and perform RFLP analysis, together with the reduced cost and time required to perform DNA sequencing, direct sequencing of amplicons, or the entire HA gene, has become a more common way to track and characterize specific strains. Electrospray ionization/mass spectrometry is another method that can be used to analyze virus-specific PCR amplicons and identify novel variants and reassortants when the viral genomic sequence is unknown, as was done with the initial identification of the A(H1N1)pdm09 virus as a likely swine-origin virus (51).

DNA microarrays are being used increasingly in diagnostics for identification of specific pathogens. Oligonucleotide probes are arrayed on a chip or membrane, and hybridization of virus-specific sequences is then detected. The viral sequences can be generated by cDNA synthesis from viral genomic RNA or by amplification of fragments of genomic RNA by RT-PCR. Microarray analysis strategies have been developed that distinguish influenza virus types (A versus B) and subtypes (H1, H3, H5, N1, and N2) (52–54) but currently are too costly for most individual laboratories to develop. Nevertheless, this technology has the promise of being able to more fully characterize strains in surveillance studies and to provide rapid and accurate identification of influenza virus strains.

SEROLOGIC TESTS

Influenza virus infections are also identified by using serologic methods. Most persons have been infected previously with influenza viruses, so detection of virus-specific immunoglobulin M or other immunoglobulin subclasses has not been particularly useful (55). An exception may be detection of immunoglobulin M responses to novel HAs from avian strains (56). Instead, paired acute- and convalescent-phase serum samples collected at least 10 days apart are needed to detect a significant (4-fold or greater) increase in serum antibody levels. The requirement for paired sera to identify infection makes serology an impractical method for identification of influenza virus infection in the acutely ill individual. Instead, serology is used primarily in surveillance and in epidemiologic studies. The most widely used assay formats include complement fixation, HAI, neutralization, and enzyme immunoassay. Complement fixation identifies type-specific antibodies to the NP, but it is not as sensitive as the other commonly used serologic assays in detecting significant rises in antibody levels. HAI and neutralization antibodies in serum are functionally significant in that higher serum antibody levels correlate with protection from infection and illness, and these antibody levels are used to measure responses to vaccination as well as to identify infection. HAI antibodies block the binding of the viral HA to sialic acid residues on red blood cells and thus inhibit hemagglutination. Each of the components in the HAI assay may affect the outcome of the test.

Human and animal sera may contain nonspecific inhibitors of hemagglutination, but methods to remove these inhibitors have been developed (43). The source of the viral antigen can affect results in that virus initially isolated in cell culture may detect a greater frequency of antibody rises than egg-grown virus (57). The species from which the red blood cells are derived can affect assay results. Chicken and turkey red blood cells are commonly used to measure HAI antibody to human strains of influenza viruses, but they may fail to detect HAI antibodies to avian strains such as A(H5N1). Substitution of horse red blood cells can improve HAI assay sensitivity for detection of antibodies to avian influenza virus strains (58). Neutralizing antibodies block viral infectivity and provide a more sensitive assay for detection of antibodies to influenza A and B viruses (59). Although neutralization assays have been available for several decades, they are less standardized than HAI antibody assays (40, 60). Nevertheless, neutralization assays are the preferred method for detection of antibody to highly pathogenic avian influenza (HPAI) virus strains (60). Because these assays require the use of live virus, their use with HPAI virus strains is restricted to laboratories with BSL-3 or higher facilities. Enzyme immunoassays are also used for detection of antibody responses to whole-virus antigen or specific viral proteins. The conjugate and antigen used in the assay are factors that affect the performance characteristics (sensitivity and specificity) of these assays. Enzyme immunoassays are used to measure specific immunoglobulin responses in a variety of clinical specimens (serum samples and respiratory secretions).

ANTIVIRAL SUSCEPTIBILITIES

Plaque inhibition assays are the gold standard for measuring susceptibility to amantadine and rimantadine, but the assays are cumbersome and time-consuming to perform. ELISA methods have also been used to measure decreases in the expression of viral antigens in the presence of these drugs. These assays can be used in combination with genotypic characterization of the M2 gene since *in vitro* and *in vivo* resistance to these drugs is associated with specific M2 gene mutations (61). RT-PCR amplification followed by RFLP analysis or direct sequencing of amplicons is a genotypic method used to identify resistant viruses (62). Amplification of the influenza A M2 gene followed by pyrosequencing is a rapid, high-throughput method that allows the rapid and reliable identification of adamantane (amantadine and rimantadine) mutations (63).

Cell culture assays do not reliably identify antiviral susceptibility to the NA inhibitors, zanamivir and oseltamivir. Instead, NA enzyme inhibition assays with chemiluminescent or fluorescent substrates are used to identify resistance (64). A commercially available diagnostic assay (NA-Star, Applied Biosystems, Foster City, CA) is available for *in vitro* screening of influenza virus isolates (65). The results of these assays also correlate with mutations in the NA gene that can be identified by sequencing (65). Molecular approaches can be used to identify known NA gene mutations associated with NAI resistance (e.g., E119V and R292K in A(H3N2), H274Y in A(H1N1), R152K in influenza B) (64, 66). Both traditional terminal deoxynucleotide (Sanger) sequencing and pyrosequencing of the NA gene have been used successfully to identify these mutations (67, 68). Another strategy to quickly screen a large number of isolates is application of a real-time RT-PCR assay that uses a probe that recognizes wild-type (susceptible) NA sequence. This approach identified all A(H1N1) strains with a H274Y NA gene mutation (67).

Mutations in the HA gene may also lead to a resistance phenotype through decreased binding affinity of HA to cell surface receptors and decreased reliance on NA function to release budding viruses from infected cells. No reliable cell culture system currently exists for identifying HA resistance mutations, so identification relies on sequencing of the receptor binding site of the HA gene.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The results of a diagnostic test must be considered in the context of the overall setting in which the test is ordered. Clinicians play a critical role in assessing the plausibility of a test result, but the laboratory also can contribute to this appraisal. Seasonal, epidemiologic, and clinical factors are elements that must be evaluated in addition to the type of assay used. Unexpected laboratory results can be recognized by the laboratory as well as by the clinician. For example, a positive influenza test result when influenza is not recognized to be circulating in the community should prompt an assessment as to whether epidemiologic (e.g., travel history) or clinical (e.g., immunocompromised host) factors support the diagnosis of influenza virus infection. Similarly, a negative result, especially with a less sensitive assay (e.g., an RIDT), should not preclude prescription of antiviral treatment to a patient with signs and symptoms of influenza. Close interactions between the laboratory and clinician are a vital component of a quality-control program.

No diagnostic assay has 100% sensitivity and specificity, so false-negative and false-positive results can be expected to occur. Many factors that contribute to lowered sensitivity and specificity are known and can be addressed in ongoing quality control programs. False-negative results may be due to poor quality or inappropriate clinical sample collection, delays in sample transportation or processing, inadequate sample storage (e.g., wrong temperature or transport medium), the time of sample collection during the clinical illness (e.g., later in the illness than recommended, when viral shedding has decreased), and the performance characteristics of the diagnostic assay (i.e., lower sensitivity). False-positive results may also be due to other characteristics of the diagnostic assay (i.e., nonspecific reactions), as well as to cross-contamination within the laboratory, mislabeling of specimens, and microbial contamination. Standard operating procedures in the collection, transportation, and processing of clinical samples should be established and followed to minimize the occurrence of the inaccurate test results. Reagents should be standardized, and periodic assessments of assay performance should be performed with known positive and negative controls. The timing of these assessments will be based on the type and number of tests being performed and the sources of reagents.

Each laboratory must decide on the goals of its influenza virus diagnostic program in selecting the diagnostic assays to be performed. Rapid and sensitive assays can favorably impact patient management by allowing the prescription of targeted antiviral therapy and the institution of appropriate infection-control isolation procedures. Positive test results may form the basis for offering prophylactic therapy to close contacts of infected patients, especially those with high-risk medical conditions. Early and rapid laboratory diagnosis also can be important for evaluating influenza-like illnesses in the setting of a nosocomial outbreak, at the beginning of the influenza season (before influenza is recognized to be circulating in the community), and in persons with a history of contact with pigs or birds or travel to an area where influenza virus is circulating. The laboratory's expertise, staffing, and available equipment also will influence test selection. For example, a fluorescent microscope and an experienced technician are necessary for the performance of immunofluorescence assays, and a thermal cycler along with other equipment is needed for RT-PCR assays. If the clinical specimen being tested comes from a patient who may be infected with an HPAI virus strain (e.g., H5N1), non-culture-based assays are currently recommended for laboratories that do not meet the BSL-3 or higher conditions recommended for growth of these strains (39). Commercially available antigen-detection assays or the more sensitive H5-specific RT-PCR assays may be performed using BSL-2 work practices. In the United States, influenza A virus-positive samples from patients meeting the clinical (febrile [>38°C] respiratory illness [cough, sore throat, or dyspnea]) and epidemiologic [contact with poultry or domestic birds or with a patient with known or suspected A(H5N1) virus infection in an A(H5N1)-affected country] parameters for suspected A(H5N1) virus infection are referred to the Centers for Disease Control and Prevention (CDC) for further evaluation. Selected negative samples may also be sent to the CDC for analysis in consultation with the local public health department (39).

As new strains of influenza virus emerge, the sensitivities of established methods to detect these strains may change. For example, cell lines may have diminished sensitivity to new strains, or the ability to detect influenza virus antigen in infected tissue culture cells (e.g., by hemadsorption) may decrease (46). Thus, it is prudent to reevaluate periodically the performance characteristics of established methods, especially if results do not correlate with those expected based on clinical and epidemiologic criteria.

Influenza diagnosis is also performed for reasons other than patient management. On the local level, knowledge that influenza is circulating in a community allows diagnosis of influenza based on clinical symptoms (febrile respiratory illness with cough) with a sensitivity (60 to 80%) similar to that of many rapid antigen tests (69). Influenza viruses isolated in national and global surveillance systems are characterized antigenically and genetically to identify variants. Information gained from these surveillance activities is used in the annual selection of strains for inclusion in updated trivalent influenza vaccines. Surveillance and characterization of isolates also allow the identification of infection with novel subtypes, as has occurred with influenza A(H5N1) viruses in southeast Asia and A(H7N7) strains in the Netherlands (70).

The findings and conclusions in this chapter are those of the authors and do not necessarily represent the views of the CDC. The use of *product names in this manuscript does not imply their endorsement by the U.S. Department of Health and Human Services.*

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Parainfluenza and Mumps Viruses

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TAXONOMY

The human parainfluenza viruses (HPIVs) and mumps virus are members of the *Paramyxoviridae* family. This family includes the *Paramyxovirinae* subfamily, to which both HPIV and mumps virus belong. However, within this subfamily, the *Respirovirus* genus is home for HPIV-1 and HPIV-3, and the *Rubulavirus* genus is home for HPIV-2, HPIV-4, and mumps virus. The *Paramyxovirinae* subfamily includes two additional genera: *Morbillivirus*, to which measles virus belongs, and *Megamyxovirus*, to which Hendra and Nipah viruses belong. The *Pneumovirinae* subfamily completes the *Paramyxoviridae* family. Within this subfamily are the *Pneumovirus* genus, which includes respiratory syncytial virus (RSV), and the *Metapneumovirus* genus, which includes human metapneumovirus. The *Paramyxoviridae* family of viruses produces significant human and veterinary diseases, with its effects noted among virus families as "one of the most costly in terms of disease burden and economic impact to our planet" (1).

DESCRIPTION OF THE AGENTS

Although some of the HPIV types and mumps virus are classified into different genera, they are all pleomorphic, enveloped, medium-sized helical viruses, usually ranging from 150 to 250 nm in diameter. All have single-stranded, nonsegmented RNA with negative polarity. The genome encodes six common structural proteins. The largest, L, is a polymerase. L, along with the phosphoprotein P and the nucleocapsid protein N (or NP), is associated with the viral RNA to make up the viral nucleocapsid. The surface glycoproteins, hemagglutinin-neuraminidase (HN) and fusion protein (F), project from the viral envelope and can be seen with an electron microscope. The sixth structural protein is a membrane (M) protein. In viral replication, N protein binds to viral RNA, L and P function in transcription and replication, and the HN and F surface glycoproteins interact with the M protein, which attracts completed nucleocapsids to areas of infected membrane that will become the envelopes of the new virions during budding. The HN surface glycoproteins also function in virus-host cell attachment via sialic acid receptors, and the F proteins function in virus-host cell membrane fusion that allows the viral nucleocapsid to enter and infect a host cell.

Four serotypes of HPIV, types 1, 2, 3, and 4, have been identified. HPIV-4 can be further subdivided into HPIV-4A and HPIV-4B; because these are so closely related, they are considered HPIV-4 for the remainder of this discussion. Only one antigenic type of mumps virus has been identified, although strains show differences in expression of the small hydrophobic (SH) gene; 12 genotypes have been identified and are designated A to L. HPIV and mumps virus are inactivated by temperatures above 50°C, organic solvents, UV irradiation, formalin treatment, low pH (3.0 to 3.4), drying, and desiccation. Preservation by freezing at −70°C or colder is effective, and the addition of 0.5% bovine serum albumin, skim milk, 5% dimethyl sulfoxide, or 2% chicken serum prior to freezing prolongs survival.

Although they are similar in structure and antigenic composition, HPIVs and mumps virus currently present very different pictures of disease production in the United States. The HPIVs are common agents of respiratory infections in children and adults in the United States, and virology laboratories routinely test for HPIV by direct antigen testing, virus isolation, and molecular methods. In contrast, mumps virus, which was considered one of the common diseases of childhood prior to the introduction of an effective vaccine in 1968, is now relatively uncommon. Although sporadic mumps outbreaks occur, most virology laboratories no longer focus on the isolation and identification of mumps virus. Because of these differences, the HPIVs and mumps virus are discussed separately below.

PARAINFLUENZA VIRUSES

Epidemiology and Transmission

HPIVs are thought to be transmitted by large-droplet aerosols and by contact with contaminated surfaces. The viruses have been shown to survive for up to 10 h on porous surfaces; however, HPIV-3 experimentally placed on fingers was shown to lose more than 90% of its infectivity in the first 10 min (2). Currently, HPIV-1, -2, -3, and -4 represent approximately 5% of all viruses isolated in routine hospital diagnostic laboratories (3) and 13% of viruses isolated from respiratory specimens (4). HPIV-1 occurs most often in the fall of the year and biennially. The incidence of HPIV-2 is generally lower than that of HPIV-1 and HPIV-3, occurring biennially with HPIV-1, in alternate years from HPIV-1, or yearly. HPIV-3 occurs yearly, in spring and summer, but can circulate year-long in temperate climates. HPIV-4 is seldom isolated, but diagnostic methods specifically targeting this virus are not as widely available.

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Clinical Significance

HPIV-1, -2, and -3 are associated with upper respiratory tract infections in infants, children, and adults. The mean incubation period is estimated to be between 2 and 6 days for HPIV infection (5). These are typically fairly mild and self-limited (6) and mortality is rare in developed countries (7). Reinfection is common because natural infection does not induce lifelong immunity. HPIV accounts for the majority of cases of viral croup, which is the most common cause of upper airway obstruction in children of 6 months to 6 years of age. Croup is characterized by inspiratory stridor, barking cough, and hoarseness (8).

HPIV-1 is associated with up to 50% of cases of croup reported in the United States, with the majority of cases occurring in children of 7 to 36 months of age. HPIV-2 is associated with croup in immunocompromised or chronically ill children. HPIV-2 also causes typical lower respiratory tract syndromes in otherwise healthy children, with about 60% of infections occurring in children younger than 5 years of age; peak incidence is in children between 1 and 2 years of age. HPIV-3 is more frequent in infants younger than 6 months of age, with 40% of infections occurring during the first year of life. Only RSV causes more lower respiratory tract infections in neonates and young infants than HPIV-3. Although HPIV-4 is less frequently isolated, it can cause all of the different respiratory syndromes (9). As many as 7% of all hospitalizations for viral acute respiratory illness in children younger than 5 years of age have been shown to be due to HPIV infection (10). HPIV-1, -2, and -3 have also been found in as many as one-third of lower respiratory tract infections in children younger than 5 years of age in the United States and are second only to RSV as a cause of hospitalization for viral lower respiratory tract infections (9).

Although associated with infections in children, HPIVs have the potential for serious pulmonary infections in adults, with HPIV-1 and HPIV-3 being among the four most commonly identified pathogens in adults requiring hospitalization for community-acquired pneumonia (11). HPIVs also cause lower respiratory tract infections in the elderly. Of nine common infections, HPIVs were significantly associated with mortality in those aged ≥75 years; this association was of lower magnitude than that of influenza A and RSV—which were significantly associated with mortality in all age groups—but of higher magnitude than that of the other pathogens (12).

HPIV is increasingly recognized as a source of severe morbidity and mortality in immunocompromised patients, especially in those with congenital immunodeficiencies, and is capable of infecting tissues in the gastrointestinal and urinary systems in these individuals (6). HPIVs replicate productively in respiratory epithelium and generally do not spread systemically unless the host is severely immunocompromised (13). HPIVs cause more than 50% of the respiratory infections in pediatric bone marrow transplant recipients and 19% in pediatric solid organ transplant recipients (14). HPIV infection, most commonly HPIV-3, was identified in 3.3% of 5178 pediatric and adult hematopoietic stem cell transplant recipients, causing both upper and lower respiratory tract infection and resulting in poor outcomes for those with lower respiratory infections (15). HPIVs were detected in 3% of pediatric patients with cystic fibrosis, second only to rhinovirus (16), and, similarly, in 5% of very low birth weight infants, next in frequency behind rhinovirus and RSV (17). In Korea, HPIVs were the most commonly isolated pathogen among 137 pediatric cancer patients with respiratory viral infections, with 80% of these acquired nosocomially (18).

There are currently no U.S. Food and Drug Administration (FDA)-cleared antivirals for treatment of HPIV infection. Ribavirin has been used to treat HPIV infections in immunocompromised patients, but results have varied. Elizaga et al. (19) reported no efficacy for this therapy. In contrast, successful HPIV treatment was reported for intravenous ribavirin therapy (20), oral ribavirin along with methylprednisolone (21), and high ribavirin doses used with early intervention (22). Management of symptoms through administration of corticosteroids has been recommended (8).

There are currently no FDA-cleared vaccines for the HPIVs. The search continues for an effective HPIV vaccine using live-attenuated recombinant HPIVs carrying various mutations and utilizing a bovine PIV-3 strain. However, these remain in clinical trials, and success has been only moderate (7).

The diagnosis of HPIV infection may be based primarily on clinical signs and symptoms, and laboratory diagnostic studies may not be needed for all patients. However, laboratory assays to confirm HPIV infection, thus differentiating it from the many other viral infections that present with similar respiratory tract signs and symptoms, are widely available, and confirmation of infection may improve patient management and decrease costs (1, 23).

Collection, Transport, and Storage of Specimens

Testing to confirm HPIV infection may include virus isolation, antigen or nucleic acid detection, or antibody detection (Table 1). Viral specimen collection, transport, and storage guidelines are provided in chapter 79 in this *Manual*. Because detection of HPIV often involves immunofluorescence assays performed directly on the clinical material, care should be taken to include cellular material in samples collected from respiratory sites. The nasopharynx and oropharynx are primary locations of initial HPIV replication, and children shed virus from 3 to 4 days prior to the onset of clinical symptoms until approximately 10 days past onset. Virus recovery from adults is much more difficult than that from children, although immunocompromised patients and adults with chronic diseases, especially lung disease, have been shown to persistently shed HPIV for many months (9). Throat swabs, nasopharyngeal swabs, nasal washes, and nasal aspirates have all been used successfully to recover HPIV, but specimens from the nasopharynx—which is the primary location of initial HPIV replication—are best. Specimens should be collected, placed in viral transport medium, and kept at 4°C until cell culture inoculation. Inoculation within 24 h of collection is recommended (9).

Peripheral blood samples for use in HPIV antibody assays should be collected in tubes without anticoagulant, and serum should be separated from the clot as soon as possible to ensure sample integrity. Serum samples may be stored at 4°C if testing will be performed within 24 to 48 h but should be frozen at −20°C or lower if testing is delayed.

Direct Examination

Microscopy

Electron microscopy can easily demonstrate the presence of HPIV, but HPIV and the other paramyxoviruses appear the same. Cellular material from the lung, other bronchial tissues, pancreas, kidney, and bladder exhibit the typical appearance of giant-cell formation under a light microscope when they are infected with HPIV (6).

TABLE 1 Diagnostic methods for parainfluenza and mumps virus detection

Virus	Virus isolation	Antigen detection	Molecular methods	Antibody detection
HPIV-1, -2, and -3	Widely available; replicate in PMK cells in 4 to 8 days, little to no CPE; HAD positive.	IF[a] only (for direct specimen detection and culture confirmation); widely available as single tests or as part of respiratory virus panel; sensitivity, 70 to 83% compared to culture.	Most sensitive method; available at many laboratories as HPIV-1, -2, -3 panel or as part of 12–15 target respiratory viral panel.	May be available at reference laboratories; cross-reactivity with other *Paramyxoviridae*.
HPIV-4	Replicates in PMK cells in 4 to 8 days; HAD stronger at room temperature.	IF only (for culture confirmation); testing not widely available.	Available at many laboratories as part of 12–15 target respiratory viral panel.	Not available at most laboratories.
Mumps virus	Widely available; replicates in PMK cells in 6 to 8 days, with little to no CPE; HAD positive.	IF only (for culture confirmation); testing not widely available.	May be available at reference laboratories.	Immune status (IgG) testing widely available. IgM testing: some newer methods more effective, not widely available. Cross-reactivity with other *Paramyxoviridae* limits usefulness in confirming acute infection.

[a] IF, immunofluorescence.

Antigen Detection

HPIV-1, -2, and -3 antigens are routinely detected in clinical specimens through the use of immunofluorescence techniques that employ monoclonal antibodies (MAbs). Cells from nasopharyngeal washes, aspirates, and swabs are fixed on the microscope slide, usually in several cell spots or dots or by cytocentrifugation (24). At least 20 columnar epithelial cells must be present if the assay is to be valid. Many laboratories use pooled MAbs containing antibodies against seven common respiratory pathogens; these are adenovirus, influenza A and B viruses, HPIV-1, -2, and -3, and RSV. These MAbs are applied in either a direct (DFA) or indirect (IFA) fluorescent-antibody staining protocol that detects antigens of all seven viruses. When a positive result is seen, further testing must be done to determine which virus is present. The sensitivity of HPIV antigen detection compared to virus isolation in cell culture varies from laboratory to laboratory and with the various DFA and IFA staining methods and reagents but has been reported to range from 70% (3) to 83% (24). Specificity is very high.

Pooled and individual MAbs in DFA or IFA formats are commercially marketed in the United States and are FDA-cleared for use in detecting HPIV-1 to -3 antigens directly in clinical specimens and for culture confirmation. Distributors of these reagents include but are not limited to the following: DakoCytomation USA, Carpinteria, CA; Diagnostic Hybrids/Quidel (DHI), Athens, OH; Millipore Corporation Light Diagnostics, Temecula, CA; and Trinity Biotech, Carlsbad, CA. FDA-cleared MAbs in the DFA format are available from Millipore Corp. for HPIV-4 culture confirmation. HPIV-infected cells demonstrate bright fluorescence that is primarily cytoplasmic and often punctate, with irregular inclusions. A brief overview of the protocol for one HPIV DFA method can be found in reference 62. The principles of DFA and IFA are reviewed in chapter 7 of this *Manual*.

MAb pools that screen for common respiratory viruses are marketed in two DFA formats. In one format, a pool of labeled MAbs is used in initial staining to screen for seven viruses (influenza A, influenza B, RSV, adenovirus, and HPIV-1 through -3); fresh smears are then prepared and stained with individual MAbs to identify the infecting virus. A second DFA format allows definitive identification of more than one virus simultaneously through the use of two different fluorescent dyes with overlapping spectra (Light Diagnostics SimulFluor reagents, Millipore Corp.; Duet reagents, DHI). The reagents are cleared by the FDA for direct specimen testing and for culture confirmation. When stained preparations are examined with a fluorescence microscope with a fluorescein isothiocyanate (FITC) filter set, one antibody will produce apple-green fluorescence, and the second will appear gold or golden orange. The SimulFluor reagents have shown excellent sensitivities and specificities, comparable to those of individual stains, for the respiratory viruses (24).

A rapid format for staining cells in solution is also available (D^3 FastPoint; DHI). This system features three dual-labeled (R-phycoerythrin versus FITC) MAb preparations also containing propidium iodide. HPIV-1, -2, and -3 are detected but not differentiated in this system. In FastPoint testing, after a short incubation of specimen material with the three MAb preparations, the samples are placed on a microscope slide and examined in the wet state with a fluorescence microscope with an FITC filter set. Premarket trials by the manufacturer showed 85 to 100% sensitivity and 98% to 100% specificity for HPIV-1, -2, and -3 detection by the FastPoint method compared to other HPIV antigen detection methods.

HPIV-4 is the least frequently encountered of the HPIV types. It is not detected by most respiratory virus immunofluorescence screening reagent pools, which screen for only HPIV-1, -2, and -3. Although MAbs are available for immunofluorescence staining of HPIV-4 cell culture isolates, these are not FDA cleared for direct HPIV-4 antigen detection in clinical specimens.

Nucleic Acid Detection Techniques

In the last decade a number of molecular assays have been used for HPIV detection. Initially these were lab-developed assays that detected individual HPIVs. More recently, multiplexed assays that detect HPIVs, either alone (Prodesse Proflu +, Hologic GenProbe, San Diego, CA) or in combination with other respiratory viruses (Luminex xTAG RVPv1, Luminex Molecular Diagnostics, Austin TX; eSensor RVP, GenMark DX, Carlsbad, CA; FilmArray RP, Biofire Diagnostics, Salt Lake City, UT), have received FDA clearance for *in vitro* diagnostic use. These involve various applications of molecular technology and have unique performance characteristics (Table 2). Of the four, only the FilmArray includes HPIV-4; the others detect only HPIV-1 through -3. These assays have been compared to antigen detection, virus isolation, and other individual and multiplexed molecular methods, and have shown excellent sensitivity and specificity (25–33).

Most method comparisons have included fewer than 20 samples positive for HPIV types 1 and 3 and fewer than 5 positive HPIV-2s. HPIV-4 was often not evaluated because it was not present in the samples tested or not detected by one or more of the comparator methods. Evaluations of HPIV detection consistently show improved sensitivity over traditional cell culture and antigen detection by immunofluorescence for molecular multiplex methods (25, 27, 31). Although individual lab-developed PCRs were shown to detect slightly more HPIVs than the GenMark eSensor (28) and Biofire (30), all of the FDA-cleared multiplex methods showed sensitivities of 92 to 100% and specificities of nearly 100% for HPIV detection compared to each other and to individual PCRs (25–28, 30, 33). The Luminex xTag RVPv1 showed elevated HPIV-1 signal in the presence of a strongly positive HPIV-3 result, but this was not a substantial problem because the HPIV-3 signal was remarkably stronger (33).

The enhanced sensitivity of molecular methods over antigen detection and virus isolation is especially important for immunocompromised patients and older patients in whom the viral titer may not be high. Most large virology laboratories employ molecular technologies for detection of all of the common viral respiratory pathogens. As this technology is further refined and made more "user-friendly,"

TABLE 2 FDA-cleared molecular viral respiratory panels that detect HPIVs

Name (manufacturer)	Viruses detected	Method, turnaround, complexity
FilmArray RP (BioFire Diagnostics–formerly Idaho Technologies, Salt Lake City, UT)	Parainfluenza 1–4 (also adenovirus, coronaviruses [HKU1, NL63, OC43, and 229E], metapneumovirus, influenza A [H1, H3, and H1 2009] and B, RSV, rhino/entero). Includes bacterial targets of *Chlamydophila pneumoniae*, *Mycoplasma pneumonia*, *Bordetella pertussis*	Fully automated closed system with extraction, amplification, and readout in self-contained plastic pouch. Pouch is placed in instrument-loading station, rehydrated, and injected with sample. The instrument mixes/moves reagents and heats/cools to perform cell lysis, magnetic bead-based nucleic acid isolation, reverse transcription, and multiple PCRs (1st stage multiplex and 2nd stage nested PCRs). Uses endpoint DNA melting-curve analysis to automatically generate a result for each target. Takes 3–5 min of hands-on time and 1.25 h for complete assay. One sample at a time, moderately complex
eSensor RVP (GenMark DX, Carlsbad, CA)	Parainfluenza 1–3 (also adenovirus [B/E, C], metapneumovirus, influenza A [H1, H3, and H1 2009] and B, RSV [A and B], rhinovirus).	Open system requires separate extraction (with addition of bacteriophage MS2 as internal control) and conventional endpoint PCR amplification. After amplification, ferrocene-labeled signal probes specific for the various targets are added, and target DNA hybridizes to the corresponding probe. Mixture is added to a detection cartridge that passes the solution across a single-file microarray of gold-plated electrodes coated with single-stranded oligonucleotide capture probes specific for individual targets. Hybridization brings ferrocene label close to the gold electrode. Electrical current is applied to electrodes, facilitating electrochemical detection using voltammetry. Batch testing, 6–7 h turnaround. Highly complex
Luminex xTAG RVPv1 (Luminex Molecular Diagnostics, Austin, TX)	Parainfluenza 1–3 (also adenovirus, metapneumovirus, influenza A [H1 and H3] and B, RSV [A and B], rhino/entero)	Open system requires separate extraction, reverse transcriptase PCR, and steps to attach PCR product to Luminex beads. Amplicons are hybridized to target-specific primers possessing unique DNA tags. DNA polymerase extends perfectly formed complements, at the same time incorporating biotin-dCTP into the extension product. The extension products are added to microwells containing polystyrene microbeads, each of which contains an anti-tag sequence unique to a specific viral target and all of which are treated with colored dyes to distinguish each bead set. Each tagged primer hybridizes onto its unique anti-tag complement. Biotinylated extension products hybridizing onto the bead surface are detected with a streptavidin-phycoerythrin reporter molecule. Dual lasers identify bead sets and amount of bound target. Most hands-on time of methods shown, 6–8 h turnaround. Batch testing. Highly complex
Prodesse ProParaflu + (Hologic Gen-Probe, San Diego, CA)	Parainfluenza 1–3	Open system requires separate extraction. Real-time RT-PCR using multiplex HPIV reagents from Prodesse, 1–2 h turnaround time. Highly complex

(i.e., less technical expertise required, faster turnaround time, less sophisticated specimen processing, instrumentation, and facilities due to closed systems), smaller laboratories will also be able to take advantage of this superior diagnostic approach. Experts in viral molecular diagnostics caution users that molecular panels are expensive and should be selected carefully to best fit the needs of the individual laboratory and the patient population it serves. Large multiplexed viral respiratory panels may not be the preferred assay for diagnosis when clinical symptoms are consistent with a particular viral infection already known to be circulating in the community (34).

Isolation and Identification

Many commonly used cell lines support the growth of HPIV, but the best growth is seen in primary monkey kidney (PMK) cells, including rhesus, cynomolgus, and African green monkey cells (9). LLC-MK2 cells are also acceptable for primary isolation when 2 to 3 µg/ml of trypsin is added to the cell culture medium (9). Madin-Darby canine kidney (MDCK), HeLa, Vero, and HEp-2 cells may be used for transferring isolates but are not recommended for primary isolation (35). At this writing, commonly used cell lines are available commercially from DHI.

In preparing for cell culture inoculation of specimens collected on swabs and transported in a viral transport medium, the transport medium tube is mixed extensively on a vortex mixer, and excess fluid is expressed from the swab by pressing the swab against the side of the tube; the swab is then discarded. The transport medium is then centrifuged at 1,500 × *g* for 10 min. The medium is decanted from cell culture tubes that are to be inoculated, and the supernatant from the centrifuged transport medium, usually 0.2 ml for each tube, is applied to each cell culture monolayer. The inoculated tubes are incubated in a horizontal position in a 35 to 37°C incubator for 1 h before excess inoculum is discarded and fresh cell culture medium is added. Inoculated cell culture tubes are incubated in rotating racks at 35 to 37°C and examined microscopically on alternate days for 14 days.

In PMK cells, some HPIVs produce a cytopathic effect (CPE) of rounded cells and syncytium formation in 4 to 8 days. However, many HPIVs will produce little, if any, CPE in traditional cell culture tubes. Fortunately, HPIVs produce hemagglutinin proteins that are inserted into the membranes of infected cells. These proteins have an affinity for erythrocytes, a phenomenon called hemadsorption (HAD), and this property can be used as another approach for detecting HPIVs in cell cultures. For HAD testing, culture medium is replaced with a dilute suspension of guinea pig erythrocytes, and the cell culture tubes are refrigerated at 4°C for 30 min; the tubes are then examined microscopically (3). When a hemadsorbing virus is present, erythrocytes adhere to the infected cell monolayer. If hemadsorbing virus is absent, erythrocytes will not adhere and will float free when the tube is tilted or tapped. Uninfected and infected control tubes should be included in HAD testing. HAD testing may be performed at the end of the typical incubation period of 14 days, or earlier, after 3 to 7 days of incubation (36). HPIV-4 hemadsorbs weakly compared to HPIV-1, -2, and -3 at 4°C and more strongly at room temperature. Other members of the *Paramyxoviridae* family, including mumps virus, and other viruses, such as influenza virus, also give positive HAD test results. Whether the presence of HPIV is suspected based on the appearance of typical CPE or by a positive HAD test result, confirmatory testing must be done to definitively identify the virus.

Confirmatory testing is routinely completed by immunofluorescence techniques involving the use of HPIV MAbs in DFA or IFA assays, as described for HPIV antigen detection in clinical samples. MAbs against HPIV-1, -2, and -3 are readily available in the various respiratory virus antibody testing kits. However, HPIV-4 antibodies are not included in most testing kits and must be purchased separately. Infected cells are scraped from the cell culture monolayer, applied to a microscope slide, fixed, and stained. The presence of apple-green fluorescence when the smear is viewed with a fluorescence microscope confirms the identification of HPIV. Confirmatory testing may also be accomplished by many other methods, including hemagglutination inhibition, complement fixation, and neutralization assays. These techniques are laborious and time-consuming compared to immunofluorescence methods and are seldom used.

Cell cultures grown on coverslips in shell vials or in microwell plates provide an alternative to traditional tube cell cultures for isolation of HPIVs. Centrifugation of the inoculated vials and plates, usually at 700 × *g* for 1 h, is an important feature of the inoculation process. Inoculated vials or plates are incubated for 24 to 48 h or as long as 5 days. Detection of viral proliferation depends on pre-CPE detection of viral antigens by application of HPIV MAbs to the monolayers in a typical DFA or IFA staining assay. Use of shell vial cultures with centrifugation-enhanced inoculation and pre-CPE detection by immunofluorescence staining has been shown to be very useful in HPIV detection.

Cocultivated mink lung (Mv1Lu) and A549 cells in shell vials, marketed as R-Mix (DHI), are used with centrifugation-enhanced inoculation and pre-CPE detection by a DFA staining technique. Staining involves pooled MAbs to seven respiratory viruses (adenovirus, influenza A and B viruses, HPIV-1, -2, and -3, and RSV). In a recent comparison of respiratory virus detection in 3,800 clinical samples in R-Mix and in shell vials of PMK, A459, and MRC-5 cells, 33 of 38 (87%) HPIVs were detected after overnight incubation in the R-Mix cells (37). This included 26 of 30 HPIV-1 isolates, 2 of 2 HPIV-2 isolates, and 5 of 6 HPIV-3 isolates. Cocultivated MDCK cells and A549 cells comprise R-Mix Too (DHI). This combination, which does not support the replication of severe acute respiratory syndrome coronavirus, was prepared as an alternative to R-Mix. The HPIVs proliferate very well in R-Mix Too (unpublished data) and are easily detected (Fig. 1).

An overview of the recommended protocol from the manufacturer of R-Mix and R-Mix Too (DHI) is shown here; however, this may be adjusted to meet the needs of the laboratory. Briefly, the processed clinical specimen for respiratory virus detection is inoculated into three vials containing R-Mix or R-Mix Too cells, and the vials are spun in a centrifuge (700 × *g* for 1 h). At 16 to 24 h, one vial is stained with respiratory virus screening reagent (tests for seven respiratory viruses). If fluorescence is observed, the cells are scraped from one of the remaining shell vials, rinsed in phosphate-buffered saline, and spotted onto an eight-well slide; seven of the wells are stained with individual MAbs against the seven respiratory viruses, and the eighth well is used as a control well. If no fluorescence is observed when the first vial is stained, the second vial is stained after an additional 16 to 24 h of incubation. If this vial is positive, the cells are scraped from the remaining vial and stained as described above. If the second vial is negative, the culture may be considered negative for the seven respiratory viruses (i.e., the remaining vial is discarded and the culture is terminated), or the third vial may be observed for CPE for up to 7 days.

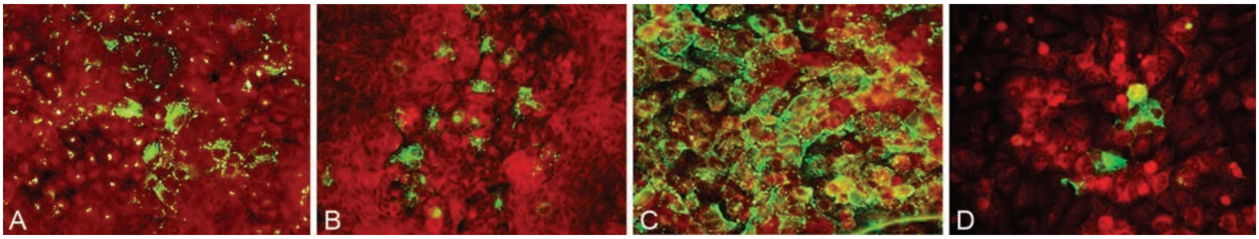

FIGURE 1 Immunofluorescence staining of HPIV-infected R-Mix Too and mumps virus-infected R-Mix cell cultures. R-Mix Too cells were infected with HPIV-1 (A), HPIV-2 (B), or HPIV-3 (C). (D) R-Mix cells infected with mumps virus. Magnification, ×200 (all). (A to C) Courtesy of Indiana Pathology Images; (D) courtesy of Diagnostic Hybrids, Inc., Athens, OH. doi:10.1128/9781555817381.ch85.f1

Serologic Tests

Most children are born with HPIV-neutralizing antibodies of maternal origin, but these diminish by 6 months of age, allowing more than two-thirds of children to be infected by HPIV during the first year of life. Most children have early serologic evidence of HPIV-3 infection, with HPIV-1 and -2 antibodies developing later, at 2 to 3 years of age. Antibodies to HPIV-4 peak in school-age children (9). Immunoglobulin M (IgM) is produced in most primary HPIV infections of children, so IgM detection in a single sample supports diagnosis of current infection. Likewise, measurement of a significant increase in the IgG antibody level, which is an increase of 4-fold or more in assays that test serial 2-fold dilutions of serum samples collected 2 weeks apart, supports diagnosis of current infection. Cross-reactivity among the various HPIV types and other paramyxoviruses, such as mumps virus, makes serologic diagnosis more difficult. Serologic differentiation of the HPIV types is unreliable by all methods; this determination can be made only through virus isolation and antigen typing with MAbs or identification of the viral RNAs.

Antibodies to the HN protein may appear early, but both HN and F protein antibodies must be present for protection. Immune status determinations are not useful for HPIV. Serology in general is seldom used as a diagnostic approach for HPIV infection because of the wide availability and improved reliability of virus isolation and viral antigen and nucleic acid detection methods.

Principles of various immunoassays are described in detail in chapter 7 of this *Manual*. HPIV antibodies can be detected by many types of assays, including hemagglutination inhibition, complement fixation, and neutralization assays; all of these detect total antibody (IgG and IgM), and most laboratories do not offer these methods due to their cumbersome nature. A detailed procedure for hemagglutination inhibition testing for HPIV antibodies was published previously (35). HPIV antibody detection can be accomplished by enzyme immunoassay (EIA), but commercial kits are not available. Complement fixation is the least sensitive method for antibody detection, and EIA is the most sensitive.

Evaluation, Interpretation, and Reporting of Results

Isolation of HPIV-1, -2, -3, and -4 in traditional or shell vial/microwell cell cultures and detection of HPIV RNA in clinical samples are the best evidence for current or very recent infection because HPIV is seldom present in the absence of infection. Rapid reporting of positive results is important (35). As molecular panels that include the HPIVs become more widely available in formats that produce rapid results, they will become the diagnostic method of choice due to their excellent sensitivity and specificity. Although the sensitivity of HPIV antigen detection by immunofluorescence is lower than that of virus isolation in cell culture or molecular detection, the rapid availability of results and high specificity make such testing useful in patient management. Confirmation of HPIV infection via the serologic route and HPIV immune status determinations are seldom employed due to the difficulties with cross-reactions among the paramyxoviruses.

MUMPS VIRUS

Epidemiology and Transmission

More than 150,000 cases of mumps were expected each year in the United States before the aggressive vaccination programs implemented in the late 1960s. The mumps vaccine was combined with the measles and rubella vaccines to make a trivalent vaccine (MMR). In the United States, the initial dose of MMR is administered to children between 12 and 15 months of age. Vaccination produced a dramatic decline in the incidence of mumps, resulting in fewer than 3,000 cases in the United States by 1985. There was a brief resurgence of mumps in 1986 and 1987 that peaked at 8,000 to 12,000 cases. Following the resurgence, a requirement for a second dose of the MMR vaccine was implemented. This dose is administered to children between ages 4 and 6 or 11 and 13 years (38) and has been expected to confer life-long immunity. The number of mumps cases continued to decline in the United States until the largest U.S. outbreak in 2 decades occurred in the Midwestern states in 2006 (39). More than 6,500 cases were involved in this outbreak. The highest incidence of mumps was in persons aged 18 to 24 years; 83% of these were college students, and 84% of this group, as well as 63% of all those infected in the outbreak, had received two doses of mumps vaccine. The cause of this outbreak is unclear. Potential explanations include waning immunity and incomplete vaccine-induced immunity to circulating wild-type virus (39). In addition, the overall estimate of mumps antibody seroprevalence in the U.S. in 1999–2004 was 90% (40). This is the lower end of the level of immunity (90–92%) needed to achieve herd immunity.

During 2009–2010, another mumps outbreak occurred in the U.S. The outbreak started at a New York summer camp for Orthodox Jewish boys and resulted in 3,502 cases of mumps, 97% of which were in Orthodox Jewish persons, most of whom lived in three neighborhoods in New York (41). As with the 2006 outbreaks, a high percentage of infected individuals had received either one (14%) or two

(76%) doses of the MMR vaccine, so waning of vaccine-induced protection against mumps was again suggested as a contributing factor. In addition, it was concluded that the level of immunity required to protect against clinical mumps may depend on the size of the inoculum of virus involved in the exposure, indicating that a particular antibody titer versus protection from infection is not absolute (41). However, in a study of antibody in students prior to the 2006 Kansas outbreak, it was shown that pre-outbreak neutralizing titers were significantly lower among patients who developed mumps than in individuals neither exposed nor infected and in individuals exposed but not infected during the outbreak. However, although those who developed mumps had lower pre-outbreak mumps antibody levels than non-infected individuals, antibody titers overlapped, and no cutoff points clearly separated the groups (42).

At this writing, the incidence of mumps has returned to its pre-2006 outbreak levels. It remains unclear whether a change in the vaccine itself or in vaccine administration schedules will be needed to prevent future outbreaks in the United States. Mumps remains common throughout much of the world, usually infecting 6- to 10-year-old children in the spring in unvaccinated populations.

The virus is transmitted from person to person through respiratory droplets or contaminated fomites and is highly contagious, with approximately 85% of susceptible contacts becoming infected when first exposed. Humans are the only known host and reservoir for the virus. The virus replicates initially in epithelial cells in the upper respiratory tract and in regional lymph nodes. Initial replication is followed by viremia, which results in infection of the salivary glands and other sites. Mumps virus can be isolated from 7 days before through 8 days after the onset of parotitis, but the isolation is more likely and the highest virus loads occur closest to parotitis onset and decrease rapidly thereafter. Disease transmission likely occurs before and within 5 days of parotitis onset. Transmission can also occur during the prodromal phase and with subclinical infection (43). The virus is excreted in the urine for as long as 14 days after the onset of illness.

Clinical Significance

The average incubation period for mumps is 16 to 18 days. Mumps virus infection is asymptomatic in 25 to 30% of cases and is associated with nonspecific or respiratory symptoms in 50% of cases. Symptoms are typically mild and characterized by slightly elevated temperature and enlargement of one or both parotid glands in 30 to 40% of cases. Complications of mumps include meningoencephalitis (up to 15% of cases) and, in postpubertal individuals, orchitis in males (20 to 30%) and oophoritis in females (7%). Polyarthritis and pancreatitis have also been associated with mumps.

When mumps was a common disease of childhood, the diagnosis was made largely on clinical grounds alone. With the decrease in incidence of mumps, many physicians no longer readily recognize the symptoms. In addition, typical clinical signs and symptoms may be absent in underimmunized or immunocompromised individuals. Parotitis, the hallmark of clinical diagnosis, is now known to be present in other viral and nonviral diseases or conditions. Mumps-like symptoms in acutely ill children who previously received the MMR vaccine have been associated with Epstein-Barr virus, HPIV, adenovirus, and human herpesvirus type 6 (44). Therefore, laboratory confirmation of mumps virus infection is now more important in making the diagnosis.

Collection, Transport, and Storage of Specimens

The accepted laboratory criteria for the diagnosis of mumps virus infection are isolation of the virus from clinical specimens, detection of mumps virus RNA via molecular methods, a significant rise between acute- and convalescent-phase antibody titers in serum, or a positive IgM result for mumps. Guidelines for specimen collection, transport, and storage of specimens are presented in chapter 79 in this *Manual*. Specimen collection guidelines differ for diagnosing mumps in unvaccinated and previously vaccinated individuals. Information on sample procurement in both hosts is provided below.

Specimens for mumps virus isolation in cell culture include the following: saliva, blood, urine, and cerebrospinal fluid (CSF). Virus can be isolated from saliva 7 days before and up to 8 days after the onset of parotitis, recovered from the urine for up to 2 weeks after onset of symptoms, isolated from CSF during meningitis, and detected rarely in peripheral blood. Throat swabs and urine samples have been shown to have similar efficacies as sources of mumps virus (45). It is always advisable to collect samples for virus isolation early in the course of the infection, when the viral titer is the highest. Samples for virus isolation and molecular testing should be collected even sooner in previously vaccinated individuals. In these individuals neither virus isolation nor molecular methods are likely to yield positive results unless samples are collected within 3 days after onset of parotitis (46). The virus is stable for several days at 4°C, although inoculation of susceptible cell cultures within a few hours of specimen collection is recommended for optimal virus isolation. The virus may survive for months or longer when frozen at −70°C or lower. Information regarding materials and methods for specimen collection (samples for virus detection as well as blood samples for antibody testing), storage, and shipment is available online from the Centers for Disease Control and Prevention (CDC) at http://www.cdc.gov/mumps/lab/specimen-collect.html.

An alternative to serum collected by venipuncture is whole blood obtained by finger stick, heel prick, or venipuncture and then spotted on filter paper and dried. Following elution and dilution of the dried blood samples, commercial antibody assays may be used to test the sample for mumps virus antibody. Results of EIA in testing for mumps virus IgG and IgM by use of samples collected as blood spots have shown excellent correlation with those obtained in testing of fresh serum. The dried blood spots may be stored for 6 to 24 months without a significant change in antibody testing results (47).

In order to compare IgG or total antibody levels, two samples should be collected, the first (acute phase) as soon as possible after onset of symptoms and the second (convalescent phase) within 2 to 3 weeks following the acute-phase sample. A single serum sample collected within 4 to 10 days of onset is all that is required for IgM-specific antibody testing. If the purpose of the serologic testing is simply to determine the patient's mumps immune status, a single serum sample collected at random is sufficient. Neither comparison of IgG levels in sequential samples nor detection of IgM in a single sample collected early in the infection has been shown to be an effective diagnostic approach for confirming mumps virus infection in previously vaccinated individuals.

Direct Examination

Microscopy

Diagnosis of mumps does not typically involve examination of biopsy samples. However, microscopic examination of affected salivary glands reveals an edematous interstitium diffusely infiltrated by macrophages, lymphocytes, and plasma cells, which compress acini and ducts. Neutrophils and necrotic debris may fill the ductal lumen, causing focal damage to the ductal epithelium (48).

Antigen Detection

Mumps virus antigen detection by immunofluorescence in cells from CSF and salivary glands was described in the early 1970s (49, 50). More recent studies of immunofluorescence staining of throat swab specimens for mumps virus antigen have shown sensitivities as high as 98 to 100% compared to mumps virus isolation in cell culture (45). Although mumps virus MAbs are available commercially, most are not FDA cleared for use in direct detection of mumps virus antigens in tissues and other clinical samples, and most virology laboratories do not offer direct antigen analysis for mumps virus.

Nucleic Acid Detection

Various molecular approaches have been developed to aid in mumps diagnosis. Mumps virus RNA has been detected by reverse transcriptase (RT)-PCR in oral fluid, CSF (51, 52), saliva or throat, and urine (52) specimens and in mumps virus isolates from cell culture (51).

Although there are currently no commercially available FDA-cleared molecular assays for mumps detection, two of these assays have been developed and standardized by the CDC, with information available online. Step-by-step procedures are provided and commercial sources of reagents are identified for one standard RT-PCR to detect the SH gene of mumps virus (http://www.cdc.gov/mumps/downloads/lab-rt-pcr.doc) and for one real-time RT-PCR assay (http://www.cdc.gov/mumps/downloads/lab-rt-pcr-assay-detect.doc). Molecular assays are more sensitive than virus isolation in culture and can provide sequence information for the coding region of the SH gene that is needed to determine the viral genotype. Laboratories with advanced molecular assay capabilities may be able to offer mumps detection by RT-PCR, but most routine virology laboratories rely on mumps virus isolation as the standard method for diagnosing mumps virus infection.

Isolation and Identification

Mumps virus proliferates in traditional cell cultures of several cell lines commonly used in viral diagnostic laboratories. These include PMK, human neonatal kidney, HeLa, and Vero cells. Recently, a marmoset lymphoblastic cell line, B95a, was shown to be as sensitive for mumps virus isolation as PMK cells (53). A hybridized Vero cell line transfected with a plasmid carrying the gene for human CDw150—a signaling-lymphocytic activation molecule (SLAM)—and called Vero/SLAM has been used for mumps virus isolation (54). Although used originally for isolation of measles virus, these cells have been shown to be effective for mumps virus isolation as well (46, 55). Directions for cell culture inoculation for swab samples in viral transport medium are the same as those described above in "Parainfluenza Viruses" under "Isolation and Identification."

In cells infected with mumps virus, CPE characterized by rounded cells and multinucleated giant cells is typical, usually appearing after 6 to 8 days of incubation at 35 to 37°C. However, this characteristic CPE may be very subtle, may not appear at all, or may be confused with a similar CPE produced by endogenous contaminant viruses that sometimes infect PMK cells.

Mumps virus, like the HPIVs, inserts hemagglutinin proteins into the membranes of infected cells, so mumps virus-infected cells can be demonstrated through HAD testing. The HAD testing protocol is described above in "Parainfluenza Viruses" under "Isolation and Identification." Whether mumps virus is detected by CPE production or by a positive HAD result, confirmatory testing must be performed. Confirmatory testing for mumps virus is routinely performed by IFA staining. FDA-cleared mumps MAbs in an IFA format for culture confirmation are available from Millipore Corp.

Many laboratories currently use centrifugation-enhanced inoculation and pre-CPE detection with MAbs to detect mumps virus within 24 to 48 h after inoculation. Various cell lines can be used in either shell vial or microwell plate formats for this purpose. The recommended centrifugation speeds and times may vary for inoculation, ranging from 700 × *g* for 45 min (45) to 3,000 × *g* for 20 min (37). Up to 66% of mumps virus-positive samples were identified within 2 days of inoculation in the shell vial system; after 5 days of incubation, the shell vial system was 96% sensitive compared to mumps virus isolation in traditional cell cultures (56). When various cell lines were compared for efficacy of mumps virus isolation in shell vials, Vero and LLC-MK2 cells were the most sensitive (100%), followed by MDCK (78%), MRC-5 (44%), and HEp-2 (22%) cells (57). Mumps virus can also be isolated in R-Mix cells (Fig. 1). An overview of the recommended protocol from the manufacturer of R-Mix (DHI) is shown above in "Parainfluenza Viruses" under "Isolation and Identification."

Because mumps virus infections in previously vaccinated individuals result in decreased levels of virus shedding into the buccal cavity, virus isolation may be difficult (58). Specimens collected and cultured within 3 days of onset of parotitis may aid in confirming the presence of mumps virus, but negative results do not rule out the infection (39).

Serologic Tests

In mumps virus infection, IgM is detectable initially within 3 to 4 days of appearance of clinical symptoms and persists for 8 to 12 weeks. IgG is detectable within 7 to 10 days of the onset of symptoms, is maintained at high levels for years, and remains detectable for life. Traditional serologic diagnosis of mumps is based on the detection of virus-specific IgM in a single sample or measurement of a significant increase in the titer of IgG or total antibody, i.e., 4-fold or greater increase for methods that use serial 2-fold dilutions, between two specimens collected 2 weeks apart. Antibodies produced in mumps virus infection often cross-react with related viruses, which complicates the interpretation of results. Assays for mumps immune status indicate whether mumps virus IgG is present at detectable levels or is absent or undetectable. These qualitative results are sufficient, and reporting of titers is unnecessary given that there is no particular antibody level that correlates with protection (41, 42). With the rarity of mumps cases in the United States at present, most laboratories focus mumps virus serology on immune status testing.

In previously vaccinated persons, serologic diagnosis has very limited use. IgM may be produced weakly or not at all in a secondary immune response. In a recent outbreak, mumps virus IgM antibodies were detected in fewer than 15% of mumps virus-infected persons who were previously immunized, and 95% of these patients were positive for mumps virus IgG (46).

Basic principles of immunoassays are described in chapter 7 in this *Manual*. Mumps virus antibodies can be detected by many types of assays, including hemagglutination inhibition, complement fixation, and neutralization assays; all of these detect total antibody (IgG and IgM). These methods are usually laborious and are available only at reference or specialty laboratories. A detailed procedure for mumps virus antibody hemagglutination inhibition testing was published previously (59). The complement fixation technique can be used with two different mumps virus antigens, V and S. Antibodies against the S antigen appear earlier and rise quickly, in contrast to the antibodies to the V antigen, which appear later. The presence of both V and S antibodies is thought to signal a recent past infection, while V antibodies alone signal a long-past infection (35). An IgM-capture EIA was used recently for detection of mumps virus IgM (46).

Most diagnostic laboratories use commercially supplied mumps virus antibody EIA kits, in either manual or automated formats, or IFA testing systems for mumps virus antibody determinations. Most of these measure only mumps virus IgG. Antibodies are produced against various mumps virus proteins, but protection is most closely correlated with antibodies to the mumps virus HN protein. Most assays use whole virus or viral extract antigens that detect HN antibody effectively. Written protocols, along with the proper reagents and controls, are included with each commercial product, and each manufacturer's guidelines must be followed if assays are to yield high-quality results. The procedural steps for performance of antibody testing are not published here due to the need for strict adherence to manufacturers' guidelines. None of these mumps virus antibody assays has been shown to be superior in regard to sensitivity or specificity.

Standard diagnostic tests that detect virus or virus-specific antibody perform inconsistently for individuals with prior immune exposure via either immunization or natural infection. Detection of activated mumps-specific antibody-secreting memory B cells (ASCs/plasmablasts) by EIA has been shown to be a more reliable test (60). ASCs are detectable in circulation only following recent activation by antigen. In testing for ASCs, peripheral blood mononuclear cells are cultured in the presence of polyclonal mitogens, and the ASCs are detected by exposure to viral antigen. Trials with this method detected mumps ASCs in recently MMR-vaccinated individuals and in those with clinical mumps during a mumps outbreak. Detection of ASCs appears to be more sensitive for longer periods of time that RT-PCR or IgM EIA and may be useful for diagnosing mumps cases that cannot be confirmed with standard methods and for testing asymptomatic case contacts in an outbreak. Interestingly, mumps ASCs appear to be produced in lower numbers than measles or rubella ASCs, suggesting that mumps infection may not generate robust B-cell memory (60).

Evaluation, Interpretation, and Reporting of Results

Isolation of mumps virus in traditional or shell vial/microwell cell cultures is evidence of current or very recent infection. The same can be said for the detection of mumps virus RNA in clinical samples. Rapid antigen detection may also confirm the presence of mumps virus. However, neither molecular methods nor rapid antigen assays are routinely available in U.S. laboratories, so mumps virus isolation in culture remains the most sensitive approach. Confirmation of mumps virus infection via the serologic route is less straightforward. Mumps virus IgM may be detectable, along with significant increases in IgG, in patients infected with related viruses, including the other paramyxoviruses, such as HPIV-1, -2, -3, and -4. In general, cross-reactivity with related viruses can be ruled out by testing for antibodies to the related viruses in parallel with mumps virus antibody testing. The greatest increase in antibody level should identify the true infection. Given the potential lack of serologic test specificity, virus isolation and RNA detection in clinical samples (preferably buccal swabs) remain the most effective ways to confirm infection in unvaccinated individuals.

Virus isolation and RNA detection have also been recommended for mumps diagnosis in individuals with a documented immunization history. Buccal swabs should be procured early (within 3 days of parotitis onset), as the duration of viral replication is likely to be shorter in these hosts. Despite these recommendations, it should be recognized that none of the traditional diagnostic approaches is highly effective for diagnosis of mumps in previously vaccinated individuals. IgM is not consistently observed, so is not a reliable indicator of recent infection. Virus detection can also be variable, presumably due to low viral loads. Alternative tests may have better diagnostic efficacy. Recent data from the 2009 outbreak suggest that the IgM capture assay used at the CDC has improved sensitivity compared to commercially available tests in previously vaccinated individuals (~50% for CDC capture versus 9–24% for commercial assays) (61). Additionally, use of nucleoprotein RNA as a target enhanced the sensitivity of real-time PCR compared to the historically used SH-based test. The CDC provides guidance online for mumps testing at http://www.cdc.gov/mumps/lab/qa-lab-test-infect.html.

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Respiratory Syncytial Virus and Human Metapneumovirus*

N. ESTHER BABADY AND YI-WEI TANG

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RESPIRATORY SYNCYTIAL VIRUS

Taxonomy

Respiratory syncytial virus (RSV) belongs to the *Paramyxoviridae* family, subfamily *Pneumovirinae*, genus *Pneumovirus* (1). Other members of the *Pneumovirus* genus include morphologically and biologically similar animal viruses, such as the pneumonia virus of mice, bovine RSV, ovine RSV, and caprine RSV (1). RSV and other *Pneumoviruses* differ from *Paramyxoviridae* viruses in the number and order of genes and the lack of hemagglutinin and neuraminidase activity (2). RSV was first isolated in chimpanzees with coryza in 1956 and subsequently recovered from children with pulmonary disease in 1957 by cell culture (3, 4). RSV derives its name from the characteristic formation of multinucleated giant cells (syncytia) in monolayer cell cultures of nonpolarized epithelial cells (5, 6). Only one serotype of RSV has been identified and further subdivided into two major antigenic groups designated RSV A and RSV B (1, 7). Additional variability, both genetic and antigenic, exists within each subgroup, with the RSV A subgroup being significantly more diverse strains than the RSV B subgroup (8). The prototypes RSV A and RSV B strains are the Long and A2 viruses and the CH-18537 and 8/60 viruses respectively (7).

Description of the Agent

Although mostly spherical, RSV virions are pleomorphic with spherical, filamentous, or both forms observed by electron cryotomography (Fig. 1) (9). The enveloped RSV virion consists of a single-stranded, negative-sense, nonsegmented RNA genome with 10 genes that are transcribed into 11 monocistronic polyadenylated mRNAs, each of which encodes a major polypeptide chain (1, 7). The virus lipid envelope is derived from the host plasma membrane and contains three structural proteins: the F (fusion) protein, the G (glycosylated) protein, and the SH (short hydrophobic) protein. The F protein initiates viral penetration by fusing viral and cellular membranes and promotes viral spread by fusing the infected cells to adjacent uninfected cells. The G protein mediates attachment of the virus to the host cells and the SH protein accumulates within lipid-raft structures of the Golgi complex during RSV infection (5, 6). The six other structural proteins include the nucleoprotein (N), the phosphoprotein (P), the polymerase or large protein (L), a matrix protein (M), and two proteins M2-1 and M2-2 (3, 8). The N protein serves as the major structural protein for the nucleocapsid, while the P and L (polymerase or large) are involved in transcription and replication (5, 6). The M protein is present in detergent-solubilized cores and inhibits viral replication in preparation for budding. The M2-1 protein is a transcription regulator that promotes the association of the M and N proteins while the function of the M2-2 protein is unclear (10). Two nonstructural proteins, NS1 and NS2, are unique to *Pneumovirus* and some studies have suggested that these proteins can inhibit apoptosis (11) and the host interferon-α/β response to viral infection (12).

Differences between RSV A and RSV B subgroups are primarily due to variability in the G protein with only one of six epitopes shared between the two subgroups (13). The variability in the G protein also accounts for the diversity of RSV strains within each subgroup. In contrast, the F protein is highly conserved, and antibodies against F cross-react with viruses from both subgroups A and B (5, 6). Studies have suggested that differences in the clinical presentation of RSV infections might be due to antigenic differences with RSV A being more virulent than subgroup B, resulting in greater disease severity among hospitalized infants (14, 15).

RSV is highly vulnerable to environmental changes. Only 10% of RSV remained infectious after exposure to 55°C for 5 minutes. At room temperature, 10% infectivity was present after 48 hours, and at 4°C, 1% of the infectivity remained after 7 days. The RSV infectivity titer fell by approximately 90% after each freezing and thawing cycle (6, 16). The virus is inactivated quickly by ether, chloroform, and a variety of detergents such as 0.1% sodium deoxycholate, sodium dodecyl sulfate, and Triton X-100. The survival of RSV in the environment depends in part on drying time as well as humidity (16). Long-term storage of RSV can be enhanced by flash freezing in an alcohol and dry ice bath and by adding stabilizing agents such as glycerin or sucrose.

Epidemiology and Transmission

Transmission of RSV occurs via droplets and direct or indirect contact with infected patients and/or contaminated

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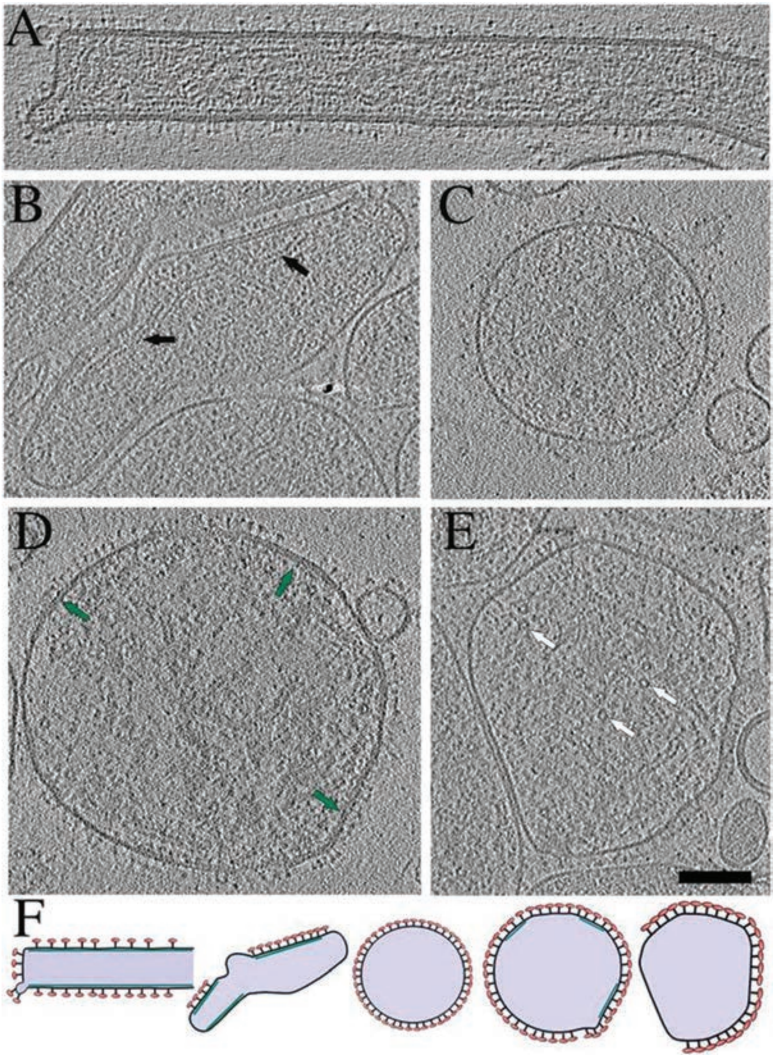

FIGURE 1 Tomography of A2 HRSV virions. Virion morphology ranges from completely filamentous (A) to completely spherical (C) with intermediate forms (B and D) that have some tubularly curved parts but are otherwise spherically curved. Spherical particles are highly deformable when in the proximity of other particles and the membrane proximal to the neighboring particle is free of glycoprotein spikes (E). (F) Virions in panels A through E are illustrated schematically in alphabetical order. Black arrows: side views of the RNP; white arrows: top views of the RNP; green arrows: secondary density layer under the membrane in a spherical particle (bar, 100 nm). Tomographic slices are 3.8 nm thick. Reprinted from reference 9 with permission.
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surfaces (17, 18). Environmental factors including temperature, humidity, and precipitation can significantly impact the rate of transmission of RSV (19). Studies have shown that a combination of low temperature and high humidity can result in increased RSV activity in temperate climates while a high number of rainfall days correlates with an increase in activity in tropical climates.

RSV infections occur year round worldwide. In the temperate northern hemisphere, RSV infections occur in the winter with a peak between December and February (Fig. 2A). In the temperate southern hemisphere, RSV infections peak between May and July (Fig. 2C), and in a few tropical locations, including Taiwan, Hong Kong, Singapore, Malaysia, and Colombia, semiannual RSV peaks have been identified (Fig. 2B and 2D) (20). Epidemiology data have established that both RSV A and RSV B can circulate in the same region in a given respiratory season with one subtype, commonly RSV A, dominating. The pattern of dominance, however, can shift between seasons with RSV genotype strains evolving and new strains emerging (21–23).

A meta-analysis review of studies published between 1995 and 2009 estimated that in 2005, 33.8 million new episodes of acute lower respiratory tract infections were caused by RSV worldwide in children less than 5 years old with 66,000 to 199,000 associated deaths, mostly in developing countries (24). In the United States, early reports estimated that 100,000 hospitalizations and 4,500 deaths were related to RSV infection with excess expenses from $300 to $600 million for hospitalized infants with RSV in the United States (16).

RSV is associated with 20% of hospitalizations, 18% of emergency department visits, and 15% of office visits during

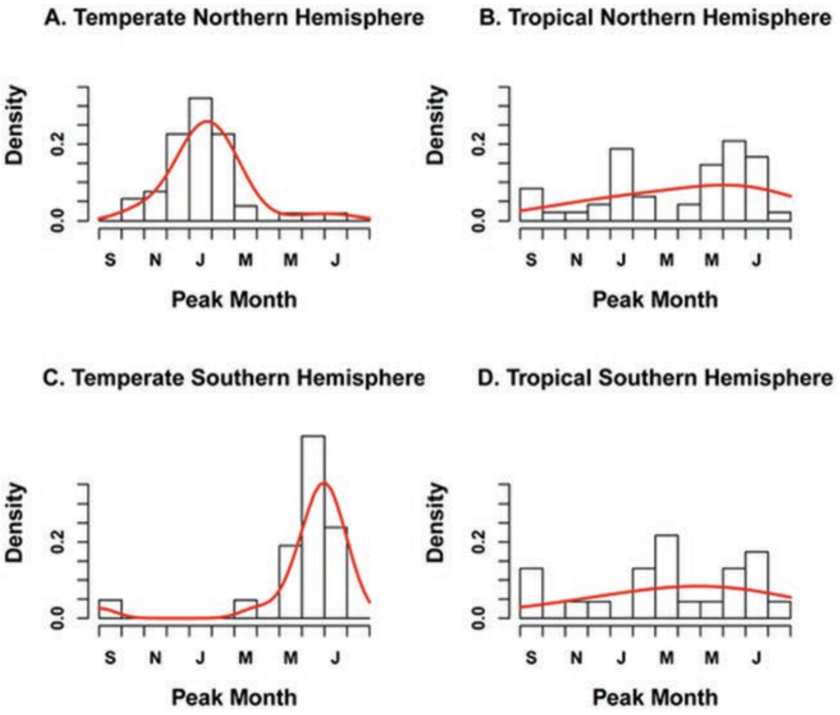

FIGURE 2 Distribution of RSV peak month by geographic zone (n = 96 locations). The black histogram represents observations while the red curve illustrates the fit of a Gaussian density kernel. Reprinted from reference 20 with permission. doi:10.1128/9781555817381.ch86.f2

the peak season. Hospitalization rates from RSV were three times higher than those of influenza (25). The overall increased prevalence of RSV subgroup A over B viruses may be due to a more transient nature of subgroup A-specific immune protection (26). Virtually all children are infected by the time they reach 3 years of age and repeated infections with RSV are common throughout life. No age group appears completely protected against reinfection due to prior exposure (16).

RSV causes hazardous nosocomial infection and produces outbreaks each year with widespread infection in both children and adults, including medical personnel, who may have a mild enough illness as to not cause absences from work (27). In a classic experiment, volunteers in close physical contact with RSV-infected infants were more readily infected than volunteers who remained 6 feet away from the infected infant, suggesting that small-particle aerosol is less important in the spread of RSV than direct contact with infectious secretions via fomites or large-particle aerosols (6, 16). A targeted infection control intervention has been demonstrated to be cost-effective in reducing the rate of RSV nosocomial infections (27, 28). Contact isolation procedures are recommended for RSV-infected patients when they are hospitalized (16, 27).

Clinical Significance

Children

RSV is recognized as the most serious cause of severe acute lower respiratory tract illnesses in young children and infants (29). Clinical syndromes caused by RSV include upper respiratory tract infections characterized by cough, low-grade fever, and rhinorrhea (29). The incubation period ranges from 2 to 8 days with a median of 4.4 days based on a systematic review (30). Common lower respiratory tract infections include bronchiolitis, tracheobronchitis, and pneumonia (29). One study estimates that, each year in the United States, 2.1 million children younger than age 5 years required medical attention for RSV, which is far higher than previously thought (25). Crowding increases the attack rate of RSV. Schools or day-care centers provide ideal settings for the spread of RSV to susceptible individuals. Infants who have not breast-fed appear to be at greater risk for developing severe RSV infections in some studies, but a strong effect has not always been noted (28). Breastfeeding appears to have the strongest protective effect in female infants (31). Most children with RSV infection are previously healthy children with no underlying conditions and experience recovery from illness after 8 to 15 days (25). Risk factors for acquiring RSV include being younger than 2 years of age and residential crowding (25, 32). Development of severe disease has been associated with the following risk factors: male gender, premature birth, lack of breastfeeding, bronchopulmonary dysplasia, chronic and congenital heart disease, smoke exposure, immunodeficiency, sickle cell anemia, and high viral loads (33). While hospitalization is rarely required in healthy children (0.5 to 2%), up to 25% of high-risk children require hospitalization (16).

Adults

RSV infection in adults was first reported in the 1960s. Immunity to RSV is incomplete and adults are subject to reinfection (34). Adult populations at increased risk for severe RSV infections include nursing home or long-term care residents; adults with underlying heart and lung diseases, such as chronic obstructive pulmonary diseases, chronic cardiac diseases, and asthma; and immune-compromised patients, including cancer and transplant patients (35). In adults older than 50 years, RSV can cause significant morbidity resulting in hospitalization rates of 6.1 to 11% (36, 37).

The incidence of RSV infection following hematopoietic stem cell transplantation (HSCT) and solid organ transplantation (SOT) ranges from 1 to 12% with 18 to 55% progressing to lower respiratory tract infection and death due to RSV in 7 to 33% of patients (38). Risk factors associated with acquisition of RSV infection in HSCT recipients include male gender, cytomegalovirus seropositivity, allogeneic transplant, and pre-engraftment status (39, 40). Due to the potential complications associated with RSV infections in HSCT recipients, a delay in transplantation of patients with upper respiratory tract infection due to RSV has been suggested (41).

Data on RSV disease in SOT recipients are limited. The incidence and risk of RSV infections in SOT recipients are lower than in the HSCT recipient groups but higher than in immunocompetent individuals. The most common symptoms reported for RSV infections in SOT are fever, cough, and dyspnea, and complications, such as bronchiolitis obliterans, may occur in lung transplant recipients (42–45).

Treatment and Prevention

Treatment of RSV infections can be challenging. Current options that have been cleared by the FDA include ribavirin and palivizumab (PVZ) for RSV treatment and prophylaxis respectively (46). PVZ is FDA cleared for RSV prophylaxis of high-risk children. PVZ is a monoclonal antibody that functions by preventing RSV entry into host cells and is administered as a monthly injection in high-risk children during the respiratory season (46). PVZ prophylaxis reduces rate of hospitalization due to RSV in young children with chronic lung disease, extremely premature birth, and significant congenital heart disease (16, 47). A second-generation monoclonal antibody, motavizumab, has also been evaluated for prophylaxis in high-risk children including preterm infants and children with congenital heart disease (48, 49).

Ribavirin is a broad-spectrum nucleoside analogue that targets RNA viruses and can be administered orally, intravenously, or as an aerosol, with aerosolized ribavirin being the preferred method (46). Data from a large retrospective study in allo-HSCT recipients, including both children and adults, supported the early initiation of ribavirin therapy for RSV upper respiratory tract infections to prevent progression to lower respiratory tract infection and subsequent mortality from RSV lower respiratory tract infections (50). Ribavirin has been used alone or in combination with PVZ, total intravenous Ig, or corticosteroids in both SOT and HSCT recipients (46). Although there is a worldwide need for a preventative vaccine in the pediatric population, an effective and safe RSV vaccine is not yet licensed. Experimental live attenuated, vectored, and subunit vaccines are in development.

Collection, Transport, and Storage of Specimens

Most general viral specimen collection principles also apply to specimen collection for RSV detection and culture, including collecting specimens during the acute phase of illness, maintaining recovered cells in intact form, delivering specimens promptly to the diagnostic laboratory, refrigerating specimens if stored temporarily, and sealing well in an O-ring sealed cryovial if stored on dry ice. Heating or freezing the specimen will result in a decreased number of infectious virions in the sample. If a delay of longer than 2 hours is expected between collection and receipt of the sample, specimen containers should be kept at 4°C, including during transport to the laboratory, to effectively isolate RSV. RSV is labile and should ideally be transported to the laboratory and processed for culture within 4 hours of collection as viral titers can significantly decrease with prolonged storage at 4°C. Alternatively, specimens may be snapped frozen to prevent viral degradation (51). However, differences in recovery of RSV from various specimen types and transport or storage temperatures are less pronounced with the use of sensitive nucleic acid testing (52–54).

Appropriate respiratory specimens for RSV detection should reflect the site of infection. RSV infects respiratory epithelial cells and can spread to the lower respiratory tract following 1 to 3 days of incubation (29). For upper respiratory tract infections, the ideal specimens include nasal or nasopharyngeal washes, aspirates, and swabs (55). Nasopharyngeal aspirates have traditionally been considered the best specimen for recovery of respiratory viruses, but insertion of the catheter through the nostril and aspiration are invasive and painful. Hence, swab samples are commonly obtained, as they provide adequate sensitivity without the discomfort associated with collection of washes or aspirates or the requirement for suction devices (56). Similarly, nasopharyngeal swabs collect a greater number of epithelial cells, but nasal swabs are associated with the least discomfort (57, 58). Studies comparing nasopharyngeal aspirates to nasal swabs for the detection of RSV by culture and/or immunofluorescence showed a lower recovery rate for nasal swabs (97% versus 67–79%) (58).

Washes and aspirates should be collected in sterile containers and transported directly to the laboratory. Swabs should be placed in tubes containing viral transport medium. Any synthetic (Dacron or rayon) tipped swabs without calcium alginate are acceptable (59), although nylon flocked swabs collected significantly more epithelial cells than rayon swabs as observed by direct fluorescent antibody (DFA) assays and therefore would provide the greatest number of cell-associated RSV (56, 57). Throat swab and saliva specimens are inferior but might be acceptable when it is impractical to obtain nasal or nasopharyngeal specimens (60).

For lower respiratory tract infections, bronchoalveolar lavage (BAL) fluids, bronchial washings, sputa and endotracheal aspirates can be used, with BAL having the best recovery when compared to nasal washes or nose and throat swabs (51, 53). Endotracheal tube aspirate or bronchoalveolar lavage fluid collection is preferred in immune-compromised adults who are being mechanically ventilated (51).

The likelihood of identifying RSV as the etiology of a patient's infection is greatest when a specimen is obtained within the first several days of illness. Viral cultures or antigens usually become negative 1 week after onset of illness in about 50% of patients. However, shedding of live virus in immune-compromised infants has been documented up to 3 weeks after onset of illness (16, 61, 62). The quantity of virus in the upper respiratory tract (nasal aspirates) is nearly equivalent to that found in the lower respiratory tract when tested by quantitative culture or real-time PCR methods (63, 64). In healthy adult volunteers experimentally inoculated with RSV, the mean duration of RSV shedding was 7.4 (±2.5) days while naturally infected older adults could shed the virus on average for 10 days to 13 days but for as long as ≥20 days (65, 66).

Direct Examination

Microscopy

Although RSV can be visualized by electron microscopy, its application has been limited to research laboratories.

However, characteristic syncytial cytopathic effects (CPE) in lung biopsy facilitate the diagnosis.

Antigen Detection

Rapid Antigen Detection Tests

Rapid RSV antigen tests are commercially available in a variety of platforms or test formats including immunofluorescent assay (IFA) (Table 1), enzyme immunoassay (EIA), dipstick immunoassay (DIA) and chromatographic immunoassay (CIA) (Table 2). Rapid antigen tests can be performed in less than 30 minutes and do not require experienced technologists to perform and interpret test results. Results are visualized as a color change or the appearance of a line or sign on a solid membrane as a result of antigen-antibody complex binding. Most rapid antigen tests have waived status and can be performed as point-of-care tests (67). However, the sensitivity of these assays is highly variable (44 to 95%) and highly dependent on the prevalence of RSV in the community (68–70). Hence, false-positive and false-negative results are a concern depending on the time of the year and the performance of these tests is much reduced during the RSV off season (71, 72). In addition, the accuracy of these rapid tests can be influenced by the type of specimen obtained and the age of the patient. Rapid antigen tests tend to perform better in children than in adults because children generally have higher RSV titers. In contrast, these tests have limited value in the diagnosis of RSV respiratory illness in adults (36, 72). Several studies performed on RSV-infected older adults showed antigen detection in nasal specimens to be very insensitive (69). The extreme low sensitivity in adults is due to low magnitude and short duration of RSV shedding in adult respiratory secretions and the problems associated with obtaining nasal washes in frail older adults (6, 73).

It is recommended that rapid antigen tests be reflexed to culture or nucleic acid amplification tests for confirmation (70, 74). In comparison to a reverse transcription (RT)-PCR assay, the BD Directigen RSV assay had a 23% false-negative rate (68). The sensitivity and specificity of CIA kits were 83% and 93% when DFA and molecular assays were used as the gold standard (75–77). The two DIA assays, Respi-Strip (Coris BioConcept) and QuickVue RSV (Quidel), can provide results within 15 minutes (78). Automated readers are available for interpretation of some of the rapid antigen tests, including the 3M RSV and the Sofia RSV FIA, with the goal of reducing errors that could arise from subjective interpretation of test results (79) (Table 2).

Several novel direct antigen-targeting techniques have been described and have potential diagnostic applications in RSV rapid antigen detection (80, 81) (Table 2). Key factors that influence choice include test accuracy, turnaround time, acceptable specimen types, compatible transport media, and a reliable supply of reagents or kits.

Direct Fluorescent Antibody Tests

DFA tests are based on the detection of RSV antigens in respiratory specimens using fluorophore-labeled monoclonal mouse antibodies (82). The use of monoclonal antibodies in these assays has greatly improved the sensitivity and specificity of the test (Fig. 3). DFA for RSV has reported sensitivities and specificities ranging from 84 to 100% and 86 to 100% when compared to culture or nucleic acid amplification tests (NAATs) (71, 83). RSV DFA reagents are commercially available from a number of companies (Table 1). "Multiplex" DFA reagents to simultaneously detect a panel of common respiratory viruses, including RSV, are also available commercially (84).

A multianalyte point-of-care antigen detection test system (mariPOC; ArcDia International Oy Ltd., Turku, Finland) was developed to detect eight respiratory viruses, including RSV, by a fully automated, random-access immunoassay method. The sensitivity and specificity (95% confidence intervals) of the mariPOC test were 89% (78 to 99%) and 100% for RSV detection, respectively (85). Sensitivity and specificity of DFA detection are comparable to that of cell culture (84); however, test turnaround time is longer than for other rapid antigen tests. DFA tests offer the advantage of evaluating the specimen quality by visualizing cells on the slide, but they are fairly labor intensive and require experienced technologists (67, 86).

Nucleic Acid Detection

Compared with conventional diagnostic tests, NAATs have increased diagnostic yields for RSV detection with sensitivities ranging from 93.5 to 100% and specificity approaching 100% (Table 3). The RSV N gene has been used as the principal target for developing NAA-based assays because (i) nucleotide sequences in the N gene are highly conserved and (ii) the N gene is preferentially transcribed because it is located nearer the 3′ end of the genome where transcription and replication initiate. Different technical platforms have been described to detect RSV nucleic acids. PCR-based procedures incorporating one-step, nested, random-access, monoplex, or multiplex RT-PCR have been developed and are used for RSV nucleic acid amplification (68, 87–93). Other non-PCR amplification procedures have been described, including nucleic acid sequence-based amplification (NASBA) (94, 95), loop-mediated isothermal amplification (LAMP) (77, 96), and multiplex ligation-dependent probe amplification (MLPA) (97).

Several diagnostic commercial multiplex molecular diagnostic assays have been cleared by the U.S. FDA for detection of RSV nucleic acids. Although the only specimen type that is currently cleared by the FDA for most of these NAATs is nasopharyngeal swabs, most published reports have included analysis of other respiratory specimen types, including nasal swabs and bronchoalveolar lavage fluids, with sensitivity and specificity similar to that of nasopharyngeal swabs (98–100). In addition, a wide range of research-use-only tests and analyte-specific reagents (ASR) are available. These assays range from moderate to high complexity with turnaround time as short as 1 hour, allowing implementation of sensitive and rapid molecular assays in a wide range of diagnostic settings (101, 102).

Isolation Procedures

Isolation of the virus from respiratory secretions by cell culture remains the gold standard method for RSV diagnosis due to its excellent specificity. Furthermore, unlike NAAT, viral culture is not affected by mutations in amplification targets, which may result in false-negative results (103), and most specimen types are amenable to culture (74). However, due to the labile nature of RSV, viral culture is no longer the most sensitive and reliable method for diagnosis of RSV infections. Diagnostic samples for culture should be kept cold and inoculated as quickly as possible. Inadequate specimen collection or delays in processing may result in lower sensitivity by culture than antigen tests.

For primary isolation, human heteroploid cells, such as HEp-2, HeLa, and A549 cells, are usually preferred. Other cell lines that may be used but are usually less sensitive include human kidney, amnion, and diploid fibroblastic cells

TABLE 1 Commercial RSV and hMPV DFA reagents and their performance

Virus	Products	Company	Manufacturer claimed performance[a]	Application	Remarks (references)
RSV	Bartels RSV DFA	Trinity Biotech, Jameston, NY	Sensitivity, 88–100%; specificity, 100%	Direct specimen detection and culture confirmation	Single- or dual-reagent DFA available. Acquired recently by Trinity Biotech (71).
	Light Diagnostics Respiratory Viral Panel DFA	Millipore, Temecula, CA	Sensitivity, 100%; specificity, 86%	Direct specimen detection and culture confirmation	In either single RSV or panel format. The panel covers RSV, influenza A/B, adenovirus, and parainfluenza viruses 1, 2, and 3 (173).
	SimulFluor Respiratory Screen, RSV/flu A or RSV/Para 3	Millipore, Temecula, CA	Sensitivity, 83.3–100%; specificity, 83.8–100%	For screening in clinical specimens and culture	Three formats available. Covers RSV, influenza A/B, adenovirus, and parainfluenza 1, 2, and 3 (78, 84).
	D^3*Ultra* DFA Respiratory Kit	DHI/Quidel, Athens, OH	Sensitivity, 93%; specificity, 99%	Direct specimen detection and culture confirmation	Covers influenza A and B, RSV, parainfluenza 1–3, and adenovirus.
	Imagen RSV	Oxoid Ltd., Hampshire, UK	Sensitivity, 93%; specificity, 98%	Detection of identification in human clinical specimens	Mixture of 3 monoclonal antibodies covering RSV, influenza A and influenza B (106)
	PathoDx RSV and Respiratory Virus Panel	Remel Inc., Lenexa, KS	Sensitivity, 77.6–100%; specificity, 98–100%	For clinical specimen detection and culture confirmation	The respiratory panel covers RSV, influenza A/B, adenovirus, and parainfluenza 1, 2, and 3.
HMPV	D^3*Ultra* DFA HMPV Kit	DHI/Quidel, Athens, OH	Sensitivity, 62.5%; specificity, 99.8%;	Direct specimen detection and culture confirmation	Reference was a combination of culture and NNA assays (71, 147).
	Imagen hMPV DFA test	Thermo Fisher Scientific, Ely, United Kingdom	Sensitivity, 63.2%; specificity, 100.0%	For clinical specimen detection	Sensitivity and specificity were calculated by using culture and NAAT as standards (71).
	Light Diagnostics HMPV Reagent	Millipore, Temecula, CA	ND	Direct specimen detection and culture confirmation	An ASR kit carried by Millipore (149)
RSV and HMPV	mariPOC test	ArcDia International Oy Ltd., Turku, Finland	Sensitivity, 89% (RSV), 50% (MPHV); specificity, 100.0% (both)	For clinical specimen detection	A multianalyte point-of-care antigen detection test system; reference was multiplex RT-PCR (85).

[a]Compared to cell culture unless specifically notified. In many cases, the culture used for comparison and validation was suboptimal, making the rapid tests look artificially better. It should be recognized that performance in each laboratory may be different. ND, not done.

TABLE 2 Main commercially available RSV rapid antigen products and their performance characteristics

Technique platform	Products	Company	Compatible specimens	Assay time (min)	Claimed sensitivity/ specificity[a]	Validated sensitivity/ specificity[a]	Comments	Reference(s)
EIA, flow-through	Directigen RSV	BD Diagnostic Systems, Sparks, MD	NPW, NPA, NPS, TA	15	93–97%/90–97%	61–86%/69–95%	Instant, clear-cut readings. Mucous specimen hard to pass through. Two antibodies utilized to detect two RSV antigens.	69
CIA, lateral flow	Directigen EZ RSV	BD Diagnostic Systems, Sparks, MD	NPW, NPA, NPS	15	89%/93%	59–86.5%/ 92.3–98%	Specific antibody used to avoid possible interference with the immunoglobulin therapy.	68, 69, 77
	BinaxNOW RSV	Inverness Medical, Princeton, NJ	NW or NPS	15	89–93%/93–100%	89–94.6%/ 88.5–100%	CLIA waived test	69
	SAS RSV	SA Scientific, San Antonio, TX	NPW, NPA, NPS	15	95.6%/94.1%	57–97%/73–100%	The original equipment manufacturer for SAS, Sure-Vue, ImmunoCard, Xpect and Clearview	76
	Sure-Vue RSV	Quidel Corp., San Diego, CA	NPW, NPA, NPS	15	95.6%/94.1%	NA	18-month shelf life	
	Immuno-Card STAT! RSV Plus	Meridian Bioscience, Inc., Cincinnati, OH	NPW, NPA, NPS	15	Wash or aspirate, 77.8–84.2%; swab, 89.5–94.4%/ 100.0%	91%/80%	Analytical sensitivity from 10 to 10,000 virions/ ml for both subgroups A and B	174
	Clearview RSV	Inverness Medical, Princeton, NJ	NPW, NPA NPS	15	93.7%/97.7%	NA	CLIA waived test; formerly QuickLab RSV	
	Xpect RSV	Remel Inc., Kansas City, KS	NPW, NPS	15	95.6%	67–78%/96–98%	Low rate of uninterpretable results. Even mildly mucoid specimens can fail to migrate.	75
	3M Rapid Detection RSV test	3M Health Care, Saint Paul, MN	NW, NPA, NPS	15		60–87%/96–99%	Automated reader available	79
	Sofia RSV	Quidel Corp., San Diego, CA	NW, NPA, NPS		86–89%/97–98%	NA	Automated reader available	
DIA	RSV Respi-Strip	Coris BioConcept, Gembloux, Belgium	NPW and/or culture supernatant	10	92%/98%	NA	Two reagents (extraction buffer and the immunostrips) and results are available within 25 min.	78
	QuickVue RSV	Quidel Corp., San Diego, CA	NW, NPA, NPS	15	83–99%/90–92%	NA	Negative results do not preclude RSV infection and should be confirmed by culture or NAAT.	

[a] Compared to cell culture unless specifically noted. NA, not applicable or not available; EIA, enzyme immunoassay; CIA, chromatographic immunoassay; DIA, dipstick immunoassay; NW, nasal wash; NPS, nasopharyngeal swab.

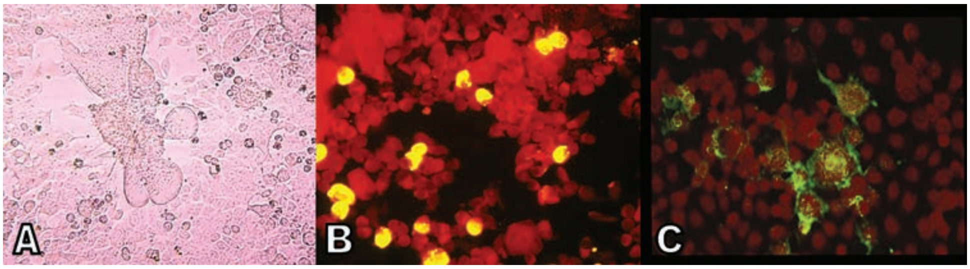

FIGURE 3 Microscopic detection of RSV and hMPV. (A) RSV-infected HEp-2 cells have fused, forming large syncytia. (B) Direct immunofluorescence of RSV on a nasopharyngeal swab specimen. (C) Indirect immunofluorescence of HMPV-infected LLC-MK2 centrifugation culture stained with monoclonal antibody MAb-8 (142). Images courtesy of http://www.virology.org/hpphoto3.html (A) and David Ferguson and Marie Landry (B and C). doi:10.1128/9781555817381.ch86.f3

and monkey kidney cells (5, 6, 16). Cell line cultures show characteristic syncytial CPE after 3 to 7 days of incubation (Fig. 3). However, the degree of syncytia formation depends on the type of cell culture, the confluence of the cell monolayer, the medium, the strain of virus, the multiplicity of infection, and whether the RSV strain is laboratory adapted. The reported sensitivities of conventional tube cell culture have ranged from 57 to 90%, in part due to compromise of virus viability, the technical expertise required, and the cell lines used in the laboratory (74).

The shell vial assay has significantly shortened the length of time needed for detection of the virus to about 16 hours. This method combines centrifugation and immunofluorescence to detect expression of viral antigens on infected cells before development of CPE (74). The use of R-Mix cultures (Diagnostic Hybrids/Quidel Corp, Inc., Athens, OH), which contain a mixture of human lung carcinoma A549 and mink lung Mv1Lu cells, has been shown to be a rapid and sensitive method for the detection and identification of respiratory viruses (104, 105). With significantly decreased costs compared to conventional culture, the use of R-Mix was slightly more sensitive than RMK, HEp-2 and MRC-5 cell lines used in conventional cultures, and it was several days faster (104). Screening of R-Mix cells after overnight incubation was more sensitive and produced more timely results for RSV and other respiratory viruses, and was more reliable than direct antigen testing (105, 106). In addition, R-Mix cell cultures have the major advantage of identifying viruses not detected by direct staining. A cryopreserved R-Mix ReadyCells preparation can be stored frozen, thawed, and used as needed with minimal addition of re-feeding media (107). A second cell line, R-Mix Too (Diagnostic Hybrids, Inc./Quidel Corp., Athens, OH), which contains a mixture of human lung carcinoma A549 and Madin-Darby canine kidney cells, is also used for rapid growth of respiratory viruses including RSV and shows comparable detection rate to R-Mix cells (74).

Identification

The appearance of characteristic syncytial CPE in cell culture, together with a negative hemadsorption test, may be adequate to establish the presence of RSV during the epidemic season. However, since other respiratory viruses (e.g., parainfluenza virus 3 and measles virus) can produce similar CPE in certain cell lines, clinical virology laboratories confirm RSV detection by performing subgroup-specific IFA on infected cells from cell culture vials as a standard practice (74).

Typing Systems

Infections with RSV of subgroup A or B may result in differing clinical manifestations and outcomes; therefore, RSV subtype information may become useful in clinical patient management if type-specific vaccines or treatments are developed (16). More commonly, RSV subgroup information has been used widely to facilitate epidemiological investigations (26). Several of the molecular commercial assays include concomitant detection and subtyping of RSV into RSV A and RSV B (Table 3).

Methods commonly used for RSV genotyping include restriction fragment length polymorphisms (RFLP), DNA sequencing, and heteroduplex mobility assays (HMA) of the G protein (108, 109). More than 100 genotypes of RSV group A (i.e. GA2, GA5, GA7) and group B (i.e., GB3 and GB4) have been identified (15, 110–112). Genotyping studies have shown an association between RSV subgroups and clinical severity, with RSV subtype A generally associated with more severe disease (14, 15). Furthermore, molecular epidemiology studies confirmed that multiple subgroups co-circulate each year and that the predominant genotype may shift with the season (15). These highly discriminatory typing techniques have been useful in monitoring possible nosocomial RSV infections and facilitating the interruption of RSV outbreaks (26).

Serologic Tests

Serology is generally not used for clinical diagnosis of RSV infections. Most children have serologic evidence of RSV infection by 2 years of age. A significant proportion (10% to 30%) of young patients with documented RSV infections remain serologically negative, probably due to the immaturity of the infant immune response (16). Secretory antibodies to RSV in nasopharyngeal secretions may also be detected (113, 114), and low RSV-specific nasal IgA is an independently significant risk factor for RSV infection (114). Repeated infections with viruses of the same subgroup of RSV occur frequently; therefore, it is usually necessary to test acute and convalescent sera and observe an increase in titers to confirm the diagnosis. Furthermore, assays that detect IgM antibody to RSV show low sensitivity, particularly for infants younger than 6 months of age, precluding a definitive diagnosis in a clinically relevant time frame (16, 115).

Serologic diagnosis of RSV infection has been more useful for analysis of vaccine take, defined by 4-fold rise in titers, and for epidemiologic monitoring of infections in the community (116). Current means of serologic diagnosis for RSV infections include IFA, EIA, complement fixation

TABLE 3 Commercial NAATs for detection and identification of RSV and hMPV[a]

Virus	Test	Manufacturer	Method	Additional targets	Regulatory status	Sensitivity (%)	Specificity (%)	Reference(s)
RSV	NucliSENS EasyQ RSV A+B	bioMérieux Durham, NC	RNA extraction Real-time NASBA	None	ASR CE	95–99	87–99	94, 95
	Cepheid RSV	Cepheid Sunnyvale, CA	RNA extraction Real-time PCR	None	ASR CE	98–100	100	68
	ProFlu+	Gen-Probe San Diego, CA	RNA extraction Real-time PCR	Flu A, Flu B	FDA CE	95–100	97–99	90
	Simplexa Flu A/B & RSV	Focus Diagnostics Cypress, CA	RNA extraction Real-time PCR	Flu A, Flu B	FDA CE	95	100	175
	Simplexa Flu A/B & RSV Direct	Focus Diagnostics Cypress, CA	Sample heating Real-time PCR	Flu A, Flu B	FDA CE	91	100	176
	Verigene RV+	Nanosphere Northbrook, IL	Integrated extraction and nanoparticle technology	Flu A, Flu B	FDA CE	100	99	175
	MGB Alert Influenza A&B/RSV	Nanogen San Diego, CA	RNA extraction Real-time PCR	Flu A, Flu B	RUO	ND	ND	89
hMPV	NucliSENS EasyQ hMPV	bioMérieux Durham, NC	RNA extraction Real-time NASBA	None	ASR CE	ND	ND	
	MGB Alert hMPV	Nanogen San Diego, CA	RNA extraction Real-time PCR	Flu A, Flu B	RUO			
	Pro hMPV	Gen-Probe San Diego, CA	RNA extraction Real-time PCR	Flu A, Flu B	FDA CE			
RSV & hMPV	RSV+hMPV Molecular assay	Quidel Molecular San Diego, CA	RNA extraction Real-time PCR	None	FDA CE	ND	N.D	
	xTAG Respiratory Viral Panel[b]	Luminex Molecular Diagnostics Austin, TX	RNA extraction PCR and RT-PCR xMAP suspension array	Flu A, Flu B, PIV1–4, AdV, EnV/RhV, CoV	FDA CE	85–100	99–100	92, 177
	xTAG Respiratory Viral Panel Fast[b]	Luminex Molecular Diagnostics Austin, TX	RNA extraction PCR and RT-PCR xMAP suspension array	Flu A, Flu B, PIV1–4, AdV, EnV/RhV, CoV, Boca	FDA CE	60–100	99–100	98, 99
	FilmArray Respiratory Panel[b]	BioFire Diagnostics Salt Lake City, UT	Integrated extraction and nested PCR Melt curve analysis	Flu A, Flu B, PIV1–4, AdV, EnV/RhV, CoV, Boca, *S. pneumo*, *B. pertussis*, *M. pneumo*	FDA CE	86–100	99–100	100, 154
	eSensor XT-8 Respiratory Viral Panel	GenMark Diagnostics Pasadena, CA	RNA extraction PCR and RT-PCR Electrochemical detection	Flu A, Flu B, PIV1–3, AdV, RhV	FDA	99–100	99	157

RespiFinder-19	PathoFinder Maastricht, Netherlands	Pre-extraction Multiplex ligation-dependent probe amplification Capillary electrophoresis	Flu A, Flu B, PIV1–4, AdV, RhV, CoV, *S. pneumo*, *B. pertussis*, *M. pneumo*, *L. pneumo*	CE	95–96	100	97
RespiFinder SMART 22	PathoFinder Maastricht, Netherlands	Pre-extraction Multiplex ligation-dependent probe amplification Melt curve analysis	Flu A, Flu B, PIV1–4, AdV, RhV, CoV, Boca, *S. pneumo*, *B. pertussis*, *M. pneumo*, *L. pneumo*	CE	ND	ND	178
Resplex II Panel v2.0	Qiagen Valencia, CA	Pre-extraction PCR and RT-PCR xMAP suspension array	Flu A, Flu B, PIV1–4, AdV, RhV, SARS CoV	CE RUO	95–96	100	88, 91
MultiCode PLx Respiratory Viral Panel	Eragen Biosciences Madison, WI	Pre-extraction PCR and RT-PCR xMAP suspension array	Flu A, Flu B, PIV1–4, AdV, RhV, CoV	RUO	97.4–100	99.4–100	179
Seeplex Respiratory Virus 15 ACE	Seegen Seoul, South Korea	Pre-extraction PCR and RT-PCR Gel electrophoresis	Flu A, Flu B, PIV1–4, AdV, RhV, CoV	CE	89.5 96	99.5 100	155
Infiniti Respiratory Viral Panel	AutoGenomics, Inc. Carlsbad, CA	Pre-extraction real-time PCR and microarray detection	Flu A, Flu B, PIV1–4, AdV, EnV, RhV, CoV	RUO	90–100	100	93
Plex-ID Respiratory Viral Panel	Abbott Molecular DesPlaines, IL	Pre-extraction Broad range, multilocus PCR Electrospray ionization mass spectrometry	Flu A, Flu B, PIV1–4, AdV, RhV, CoV, Boca	RUO	ND	ND	156

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[a] Abbreviations: Flu, influenza virus; ASR, analyte-specific reagent; CE, Communauté Européenne; RUO, research use only; PIV, parainfluenza virus; AdV, adenovirus; EnV, enterovirus; Flu, influenza; RhV, rhinovirus; CoV, coronavirus; Boca, bocavirus; *S. pneumo*, *Streptococcus pneumoniae*; *B. pertussis*, *Bordetella pertussis*; *M. pneumo*, *Mycoplasma pneumoniae*; *L. pneumo*, *Legionella pneumophila*; ND, not done; SARS, severe acute respiratory syndrome.

[b] Not all targets are FDA cleared.

(CF), and neutralization assays (16, 113, 115). EIA is analytically more sensitive than CF and has the feature of eliminating false IgM-positive results (115). Oral-fluid samples have been used in the place of serum for RSV IgG and IgA antibody detection and surveillance by EIA (113). Virus neutralization tests have been developed only for research and epidemiologic studies as plaque reduction assays or in a microneutralization format.

Antiviral Susceptibilities

Resistance to ribavirin and palivizumab has not been recognized in the clinical setting. Several RSV escape mutants derived in the laboratory have been shown to be resistant to palivizumab prophylaxis in cell culture and cotton rats (117). A total of 458 RSV isolates from treated subjects were recovered during 1998 to 2002 and were tested for resistance. The evidence to date suggests that development of palivizumab-resistant mutants does not appear to play a significant role in hospitalizations for breakthrough RSV disease occurring in infants receiving palivizumab prophylaxis. The nucleotide and amino acid sequence of the F protein epitope to which palivizumab binds appears to be highly conserved among clinical isolates, even in children receiving palivizumab prophylaxis (118).

Evaluation, Interpretation, and Reporting of Results

Test results should be interpreted with knowledge of the natural history of RSV infections in the context of the patient's clinical presentation and medical history. Serologic results have little clinical value, and rapid antigen, DFA, and NAAT-based molecular assays are the tests of choice for laboratory diagnosis. Culture for RSV detection has lower sensitivity than DFA or NAAT and is mainly used as a backup test, especially for specimens yielding negative antigen results, or when further characterization has been requested.

The availability of rapid antigen assays with a turnaround time as short as 15 minutes makes it possible for clinicians to receive results in a timely fashion. However, due to their relatively low sensitivity, false-negative results can be expected and should not be interpreted as excluding the possibility of RSV infection. To improve detection, DFA or RT-PCR assays should be used as backup tests. Additionally, several of the rapid antigen assays are only cleared and/or recommended for use in infants and children and negative results in other age groups should be interpreted with caution.

Cell culture is considered to have the highest specificity, followed by antigen tests or NAAT. NAATs, which are the most sensitive tests for RSV detection, are particularly useful for testing of respiratory specimens collected from adults and in the off-season. With the increased number of multiplexed molecular FDA-approved assays, simultaneous detection and identification of common viral pathogens, including RSV, is becoming the main tool for detection and subtyping of RSV. Special attention should be paid to potential carryover contamination and cross-reaction between tested viruses when evaluating and implementing these tests. Whereas multiplex NAATs are currently qualitative, real-time NAATs can provide an estimate of viral load. Additionally, when more than one virus is detected, the viral load can be helpful in identifying the predominant pathogen. Viral load estimates might also be helpful in determining the rate of RSV clearance, as the increased sensitivity of NAAT often results in persistent detection of RSV RNA even after symptoms have resolved. This is especially crucial in immunocompromised patients or pretransplant patients, where a positive RSV test result could result in further therapy or a delay in transplant procedures, respectively. Nevertheless, the clinical relevance of quantitative respiratory virus PCR awaits further investigation and standardization.

The positive and negative predictive values of these tests vary according to the prevalence of the disease in a particular population during certain seasons. The diagnosis of RSV infection is often made with reasonable accuracy in combination with clinical and epidemiologic findings in infants with lower respiratory tract disease during the epidemic season. However, due to the significant overlap in symptoms and seasonality, diagnostic testing is recommended to accurately identify the infecting virus. Effective exchanges of relevant information between clinicians and the laboratory are essential to good patient care. RSV is extremely labile and improper specimen collection, transport and processing, even when sensitive methods such as RT-PCR are used, can lead to false-negative results. The importance of correct specimen collection and prompt specimen transport cannot be overemphasized. Prompt reporting of test results may assist clinicians in discontinuing unnecessary forms of therapy or in implementing infection control precautions, thereby preventing nosocomial spread of infection.

HUMAN METAPNEUMOVIRUS

Taxonomy

Human metapneumovirus (HMPV) virus was not discovered until 2001, a half century later than its closest cousin RSV (119). HMPV was classified as the first human member of the *Metapneumovirus* genus, in the subfamily *Pneumovirinae*, the family *Paramyxoviridae*, and the order *Mononegavirales*. The avian pneumoviruses (APV) are highly related to HMPV. APV and HMPV were separated by taxonomists into the separate genus because they can be distinguished from members of the *Pneumovirus* genus by their lack of genes encoding the nonstructural proteins NS1 and NS2, and by a different gene order in the RNA genome (120).

Description of the Agent

HMPV is an enveloped respiratory virus with a single-stranded nonsegmented negative-sense RNA genome revealing pleomorphic particles measuring 150 to 600 nm (120). Although APV and HMPV are related to RSV viruses, they differ in that the gene order in the nonsegmented genome is slightly altered and APV/HMPV are lacking the two nonstructural proteins NS1 and NS2 that are located at the 3′ end of RSV genomes. The HMPV genome, ranging in length from 13,280 to 13,278 nucleotides, encodes nine proteins in the order 3′-N-P-M-F-M2-SH-G-L-5′ (the M2 gene is predicted to encode two proteins M2-1 and M2-2 using overlapping open reading frames, as in RSV). HMPV nucleoprotein (N) and phosphoprotein (P) proteins interact and are recruited to cytoplasmic viral inclusion bodies in HMPV-infected cells that can be detected easily by immunofluorescent staining. Similar to RSV, nucleotide sequences in the N gene are highly conserved. HMPV fusion protein (F) induces virus-cell fusion by binding to heparin sulfate and uses multiple Arg-Gly-Asp-binding integrins as attachment and entry receptors (121). The glycosylated protein (G), which is the most variable protein among HMPV isolates, binds to the host cell via cellular glycosaminoglycans, including heparin sulfate-like molecules (122).

Epidemiology and Transmission

Nearly all children have evidence of prior infection by the age of 5 years, when they are tested by serology (119). A prospective, population-based surveillance for acute respiratory illness or fever among inpatient and outpatient children in three U.S. counties from 2003 through 2009 revealed that HMPV infection is associated with a substantial burden of hospitalizations and outpatient visits among children throughout the first 5 years of life, especially during the first year (123). The burden of HMPV illness on the intensive care unit in terms of resource utilization may be considerable (124). Approximately 12% of outpatient lower respiratory tract illness was associated with HMPV infection, and 3.9% of hospitalizations in children due to acute respiratory illness or fever were associated with HMPV. HMPV has been found in association with respiratory tract disease in every continent; dozens of country-specific case series have been reported (120).

HMPV has a seasonal distribution in temperate countries with most cases occurring in winter and spring (Fig. 4), often overlapping in part or in whole with the annual RSV epidemic (123). Long-term studies have shown that sporadic infection does occur year round. HMPV strains vary genetically and antigenically and have been classified into two broad groups: group A and group B, with each group divided into genetic subgroups 1 and 2 (119, 120). The A2 sublineage shows the greatest diversity; phylogenetic analysis showed a further bipartition of A2 into two new genetic clusters designated A2a and A2b (125–127). Both antigenic group A and group B were noted to cocirculate in the same location during the epidemic periods and had various patterns of predominance. Antigenic variability is thought to contribute to reinfection throughout the life of the patient and may pose a challenge to vaccine development (125–127).

The incubation period is thought to be about 3 to 5 days. Humans are the only source of infection. The usual period of viral shedding has not been defined, but is likely to be weeks following primary infection in infants. Formal transmission studies have not been reported, but experts believe that transmission occurs by close or direct contact with contaminated secretions involving large particle aerosols, droplets or contaminated surfaces (120). HMPV, as a nosocomial pathogen, has been underestimated until recently. Several health care-associated HMPV infections have been reported in hospitalized children and adults, and contact isolation with excellent hand washing for health care providers is necessary to prevent spread (122, 128–131). There should be increased awareness of HMPV infection within health care settings, particularly when the population at risk has a high prevalence of underlying co-morbidities (129).

Clinical Significance

HMPV is associated with the common cold and with lower respiratory tract illnesses such as bronchiolitis, pneumonia, croup, and exacerbation of reactive airways disease. The signs and symptoms caused by HMPV are very similar to those caused by RSV; providers cannot distinguish between the two infections based on clinical assessment alone. Like RSV, HMPV infection leads to significant morbidity in infants and other special populations, including patients who are immunocompromised, high-risk, or elderly. Most children with HMPV infection were previously healthy. Female gender, hospital acquisition of HMPV infection, and presence of chronic medical conditions each independently increase mortality (123). The virus causes upper and lower respiratory tract illnesses. About half of the cases of lower respiratory tract illness in children occur in the first 6 months of life, suggesting that young age is a major risk factor for severe disease (123). Both young adults and the elderly can suffer HMPV infection that leads to medically attended illnesses including hospitalization, but severe disease occurs at lower frequencies in adults than in young children (132). RSV and HMPV coinfections have been reported, which is not surprising given the overlapping seasons. Coinfection with bacteria is not common, except for the complication of otitis media, which occurs frequently (123).

Immunocompromised patients are particularly susceptible with resultant morbidity and mortality. A large proportion of respiratory tract samples submitted to diagnostic virology laboratories are obtained from immunocompromised patients. HMPV infection is associated with wheezing more commonly in patients with underlying pulmonary disease, especially asthma (123). HMPV causes severe lower respiratory tract disease in pre- and post-HSCT recipients, with mortality rates at 100 days of 43%. Steroid therapy, oxygen requirement of >2 liters or mechanical ventilation, and bone marrow as cell source were significant risk factors for overall and virus-related mortality in multivariable models, whereas the virus type was not (133).

The majority of immunocompetent children infected with HMPV can be managed with supportive care. Both ribavirin and polyclonal human immunoglobulin have been used for HMPV infections in immunocompromised patients. In a case report of a 2-year-old girl undergoing intensive chemotherapy for Burkitt lymphoma who developed severe HMPV pneumonia, rapid and complete recovery was observed after treatment with oral ribavirin and intravenous Ig (134). Ribavirin has been used in conjunction with Ig in severely immunocompromised patients with some evidence of efficacy (135). In a series of nine immunocompromised patients who were diagnosed with pneumonia secondary to HMPV, two treated with aerosolized and oral ribavirin along with intravenous immunoglobulin recovered (136). Oral ribavirin therapy alone did not improve clinical outcomes in hematologic disease patients infected with HMPV (137). HMPV vaccine is not available. Recently, it was reported that a HMPV-like particle vaccine induced cross-protection against HMPV infections in mice (138).

Collection, Transport, and Storage of Specimens

Reports in the literature suggest that HMPV can be recovered or detected by antigen detection, culture, and RT-PCR from nasal washes, nasopharyngeal aspirates, nasopharyngeal swabs, and bronchoalveolar lavage specimens. Typically, nasopharyngeal aspirates are collected from infants by using a 10F catheter. Washing the catheter after dispensing, in addition to collection of material in the collection trap, may enhance yield over use of the trap material alone (139). Nasopharyngeal flocked swabs have been gradually used to replace aspirate specimens without significantly decreasing diagnostic yield (140). Transport is generally performed using standard viral transport media. Specimens should be processed as soon as possible after collection and stored at 4°C for less than 72 hours before processing. Incubation at 37°C has been shown to lead to a rapid decrease in HMPV titer (141). For long-term storage of specimens or laboratory stocks, a temperature of −70°C is preferred with specimens in O-ring sealed cryovials.

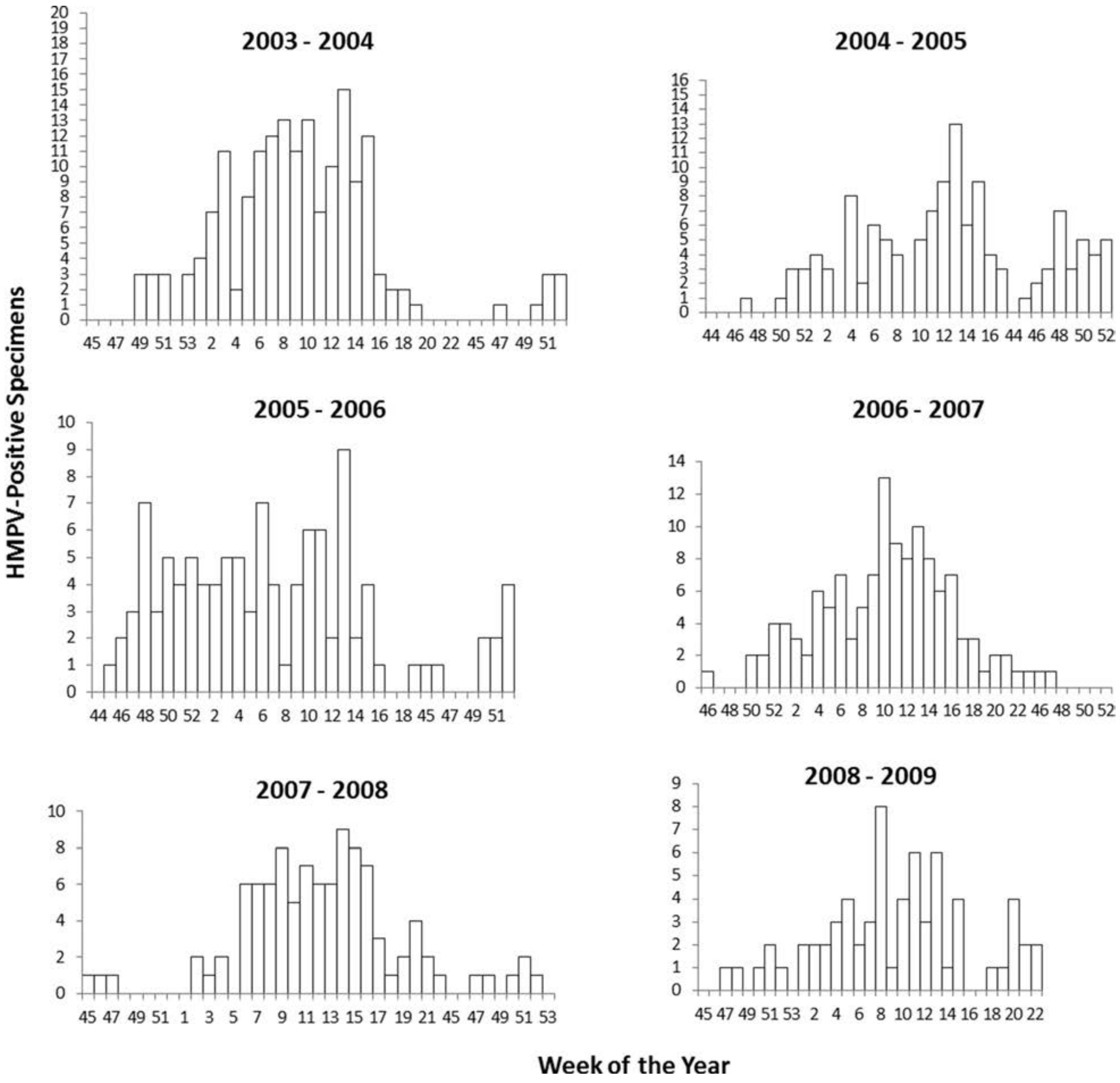

FIGURE 4 Seasonal distribution of HMPV infections. HMPV-positive specimens by week in Cincinnati Children's Hospital, University of Rochester Medical Center, and Vanderbilt University Medical Center from 2003 through 2009. Reprinted from reference 123 with permission. doi:10.1128/9781555817381.ch86.f4

Direct Examination

Microscopy

The virus can be seen by electron microscopy (119). This method was used to study host binding domain to the HMPV F protein; however, it is not sufficiently sensitive or reproducible for clinical diagnosis use.

Antigen Detection

Several rapid antigen tests using different platforms have been reported for use on nasopharyngeal aspirate and swab samples using HMPV-specific monoclonal antibodies (120, 142, 143). An antigen detection EIA kit is commercially available from Biotrin (Dublin, Ireland). The initial studies reported a sensitivity of 81% and a specificity of 100% in comparison to viral culture and RT-PCR (144) and an agreement of 94% with the Pro HMPV Real Time Assay from Prodesse (Gen-Probe) (145). Another antigen detection kit using immunochromatography (the Check HMPV assay previously known as the SAS HMPV test; SA Scientific, San Antonio, TX) was evaluated on nasal swab specimens with overall sensitivity and specificity of 82.3% and 93.8%, respectively, when RT-PCR was used as standard (146). This assay was used to rapidly identify index patients during an HMPV outbreak in a long-term-care facility (130).

In addition to rapid EIA format, DFA staining has been widely implemented for HMPV detection in the clinical setting (Table 1). Mouse monoclonal antibodies specific for HMPV have been developed and used for DFA staining of nasopharyngeal samples. Several DFA reagents are commercially available including the D^3*Ultra* DFA HMPV (Diagnostic Hybrids Inc./Quidel Corp, Athens, OH) (71, 147), the Imagen HMPV DFA (71, 148) and the Light Diagnostics HMPV DFA Reagent (Millipore, Temecula, CA) (149). Recently, a multianalyte point-of-care antigen detection test system (mariPOC) was reported to detect eight respiratory viruses including HMPV. Among the 158 nasopharyngeal swabs collected from children with respiratory symptoms and/or fever, the mariPOC detected eight HMPV, resulting in a sensitivity of 50% and specificity of 100% in comparison multiplex RT-PCR (85).

Nucleic Acid Detection

The most sensitive test for identification of HMPV in clinical samples to date is RT-PCR. Numerous real-time RT-PCR tests targeting the N and F genes have been reported to detect HMPV from the four known genetic lineages (71, 91, 150–152). Although the copy number of the N gene transcript should be higher than that of the F gene because of its more proximal position relative to the virus 3′ leader region where transcription begins, both F and N primer sets have been shown to be sensitive in RT-PCR tests. Quantitative RT-PCR tests have been employed to define viral load in patient samples (152). A NASBA test also has been described; however, the sensitivity of this assay (limit of detection of 100 copies) appears to be lower than that of quantitative RT-PCR (153). Several molecular devices, including NucliSENS EasyQ HMPV (bioMérieux, Durham, NC), MGB Alert HMPV Detection Reagent ASR (Nanogen, San Diego, CA), Quidel Molecular RSV+HMPV Assay (Quidel Corp., San Diego, CA), and Pro HMPV Assay (GenProbe Inc, San Diego, CA), are commercially available specifically for detection of HMPV; their performance characteristics are contrasted in Table 3.

Recent advances in nucleic acid amplification and identification techniques have allowed for the development of molecular systems that can simultaneously detect and differentiate multiple respiratory viruses, including HMPV in clinical laboratories with varied sizes, resources, or capacities. Several commercial devices, with different amplification and detection platforms, have been reported including RespiFinder (PathoFinder BV, Maastricht, The Netherlands) (97), ResPlex II assay (QIAGEN, Valencia, CA) (91), FilmArray Respiratory Pathogen Panel (BioFire Inc., Salt Lake City, UT) (100, 154), Infiniti Respiratory Viral Panel (AutoGenomics, Inc., Carlsbad, CA) (93), Seeplex Respiratory Virus Detection Assay (Seegene, Inc., Seoul, Korea) (155), PLEX-ID RVP Panel (Abbott Molecular Inc., Des Plaines, IL) (156), xTAG Respiratory Viral Panel (Luminex Molecular Diagnostics, Toronto, Canada) (92, 177), and eSensor XT-8 Respiratory Viral Panel (GenMark Diagnostics, Inc., Pasadena, CA) (157) (Table 3). Among them, the xTAG RVP, the FilmArray Respiratory Pathogen Panel, and the eSensor XT-8 Respiratory Viral Panel have received FDA-approval for HMPV to date (92, 157). Most of these devices targeted highly conserved HMPV N or F genes.

Some of these systems have potential for high-throughput testing, and others allow rapid near-patient testing (92, 158, 159). The multiplex testing reveals positive tests for more than one agent in a significant proportion of cases (88, 160); however, the potential coinfections should be interpreted with caution for several reasons. First, PCR is susceptible to false-positive tests due to cross-contamination in some laboratories that perform frequent amplifications. Second, viral genome can be detected by RT-PCR in respiratory secretions for several weeks or more, even after live virus shedding has ceased. It is difficult to know in this situation if the presence of positive virus-specific nucleic acids signifies active infection or simply a recent acute infection. Potential future applications of quantitative molecular tests would be to differentiate acute and resolved infections as well as to determine the significance of positive findings (152, 160–162).

Isolation Procedures

HMPV was first isolated in 2001 in the Netherlands using tertiary monkey kidney cells (119). HMPV replicates poorly in most conventional cell cultures used for respiratory virus diagnosis such as HEK, HEp-2, and Madin-Darby canine kidney cells. Primary isolation is facilitated by the presence of a low concentration of trypsin, which is not used routinely in diagnostic virology cultures in many laboratories. A recent study indicated that the most permissive monkey kidney epithelial cells were LLC-MK2 and Vero, while the most permissive human airway epithelial cell line was BEAS-2B. LLC-MK2 cells were tolerant of trypsin and thus remain an ideal cell line for HMPV cultivation (141). Centrifugation cultures significantly increased the infectivity of HMPV for cells in monolayer culture (141, 142). HMPV grown in LLC-MK2 cell or Vero cell monolayer cultures is slow and often requires several blind passages before CPE appears, especially following primary isolation. The CPE in cell lines is not particularly striking, often appearing only as focal areas of rounding of cells and minor patches of cell-cell fusion (119, 163). A shell vial culture method using R-Mix cells with mixed monolayer of human adenocarcinoma cells (A549 cells) and mink lung cells (Mv1Lu cells) (Diagnostic Hybrids Inc./Quidel Corp), and an HMPV-specific monoclonal antibody had a sensitivity of 100% compared with tube culture in one study (164).

Identification

The characteristics of the CPE are not distinct enough that the virus could be identified on this basis, even by trained observers (119, 163). HMPV is suspected when a virus is isolated during the epidemic season in Vero or LLC-MK2 cells and is not identified by specific immunofluorescence tests for related viruses (Fig. 3). Specific identification must be made using immunofluorescence with a commercial monoclonal antibody, or by a licensed RT-PCR test on RNA extracted from cell lysates.

Typing Systems

The typing of strains of HMPV is mainly of interest to epidemiologists at this time and does not currently provide guidance in clinical care or therapy. The nomenclature and classification of genotypes, subgroups, strains, variants, and isolates of HMPV are still being developed. The HMPV G glycoprotein genes exhibit the greatest diversity with nucleotide and amino acid sequence identities ranging from 52 to 58% and 31 to 35%, respectively, between subgroups A and B (120, 165). Within the A subgroup, the A2 sublineage shows the greatest diversity and has been further divided into two new genetic clusters designated A2a and A2b (125–127). Studies using experimental infection of animals and reciprocal cross-neutralization studies suggest for the most part that the viruses of varying subgroups all fall within one serotype (120).

Serologic Tests

Serology is generally not used for clinical diagnosis of acute infections, because diagnosis requires a comparison of acute- and convalescent-phase titers. Several serology methods using both virus-infected cell lysates and recombinant viral proteins in enzyme-linked immunosorbent assay (ELISA) have been reported (119, 166–168). Serum neutralizing antibody assays have been used to demonstrate the induction of functional antibodies in both humans and experimentally infected animals. Most of these tests have used reduction of virus plaques or CPE as the readout; however, a more objective microneutralization test using a recombinant HMPV expressing green fluorescent protein has been reported (169). The use of molecular diagnostics may have exaggerated the HMPV infection prevalence determined by serology methods (170). In contrast, additional HMPV diagnostic yield by adding serology to PCR was revealed in Kenyan patients (171).

Antiviral Susceptibilities

Both ribavirin and polyclonal human Ig have been used for HMPV infections in immunocompromised patients (134–137, 172). No controlled trials have been conducted and antiviral effect remains determined. Resistance to ribavirin and immunoglobulin has not been recognized in the clinical setting.

Evaluation, Interpretation, and Reporting of Results

Several FDA-approved rapid antigen (DFA) and molecular tests are commercially available for detection of HMPV. Therefore, the laboratory diagnosis of HMPV should be based on the detection of HMPV genomes by NAAT or antigen tests by DFA in nasopharyngeal aspirates or swabs. Currently, the diagnosis is best made when an HMPV-specific RT-PCR test is found to be positive. Multiplex PCR assays are available to detect and differentiate a panel of respiratory viruses, which helps distinguish the infection from RSV and other viral respiratory tract infections. Although CLIA-waived, point-of-care tests are not commercially available for HMPV, some of the molecular assays allow rapid near-patient testing that helps prompt patient care and control nosocomial transmission. Serology and typing are mostly of utility in studies of epidemiology at this time.

Because the average duration of shedding during infection is not defined, and the role of coinfection in disease expression is not well understood, the clinical interpretation of a positive HMPV molecular test must be made with caution, especially when RNA from another virus is detected simultaneously in the same sample. In general, the diagnosis of HMPV infection is most likely when a positive nucleic acid test for HMPV infection occurs when testing a respiratory secretion during late winter or early spring in temperate climates from a patient with acute respiratory illness and negative tests for other respiratory viruses. Real-time PCR-based quantitative molecular test results including threshold cycle (Ct) values may be useful to determine the significance of positive findings as well as to differentiate acute and resolved infections. Inversely correlated to the HMPV loads in clinical specimens, higher Ct values would be more consistent with a resolved infection.

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Measles and Rubella Viruses

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MEASLES VIRUS

Taxonomy

Measles is the prototypic member of the genus *Morbillivirus* in the family *Paramyxoviridae*, and it is the only member of the genus that causes human disease (1). With rare exception, the members of this genus have restricted host ranges, indicative of long-term association with, and adaptation to, their respective zoonotic hosts. Other genus members include rinderpest virus, canine distemper virus, peste des petits ruminants virus, and cetacean and phocine distemper viruses.

Description of the Agent

Measles virus is an enveloped, nonsegmented, single-stranded, negative-sense RNA virus with a diameter of 120 to 250 nm. The measles virus genome is 15,894 nucleotides in length and contains six structural genes organized on the single strand of RNA in a gene order consistent with most of the paramyxoviruses, i.e., 3′-N, P, M, F, H, and L-5′. The genome encodes at least eight proteins; three gene products are coded for by the phosphoprotein (P) cistron. The nucleoprotein (N) gene encodes the N protein, which encapsulates both full-length minus-strand (genome) and full-length plus-strand (antigenome) RNAs. The protein products of the P and the polymerase (L) genes interact with the full-length viral RNA and form the ribonucleoprotein complex. Viral nucleocapsid structures are surrounded by a membrane, derived from the plasma membrane, which includes the gene products from the M, F, and H genes (matrix, fusion, and hemagglutinin proteins, respectively). The F and H envelope proteins are N-linked transmembrane glycoproteins, and are responsible for fusion of virus with host cell membranes (1). The H glycoprotein is the major target for neutralizing antibody, and interacts with the two cellular receptors for measles virus, CD46 (2) and human signaling lymphocyte activation marker (hSLAM) (3). Recently, an epithelial cell protein, nectin-4, was identified as a third measles receptor. This receptor also interacts with the H glycoprotein and is present in adherans junctions of tracheal airway epithelial cells (4, 5).

Although measles virus has only a single serotype (monotypic), antigenic and genetic variability has been detected between and among wild-type viruses and vaccine viruses. The nucleotide sequence variability among wild-type viruses is most evident in the genes encoding the N and H proteins (7 to 10%), and the maximum sequence variability has been determined to reside in the last 450 nucleotides of the coding region of the N gene (~12%) (6). Based on this sequence region, a standard nomenclature has been established. For molecular epidemiologic purposes, the genotype designations are considered the operational taxonomic unit, while related genotypes are grouped by clades. The World Health Organization (WHO) currently recognizes 8 clades designated A, B, C, D, E, F, G, and H. Within these clades, there are 24 recognized genotypes (7). A recent report by Rota and others (8) provides an excellent overview of the current status of the molecular epidemiology of measles and the global distribution of the various genotypes.

Epidemiology and Transmission

Measles virus infections are transmitted via aerosols, droplets, or contaminated fomites. Measles is a highly contagious disease, and susceptible individuals who come into close contact with measles patients have a 99% probability of acquiring the disease. In the prevaccine era, greater than 90% of individuals would acquire measles infections before 10 years of age.

In unvaccinated populations, measles causes periodic epidemics, with interepidemic periods of 2 to 5 years. These periods decrease as population size and density increase and are directly related to the availability of susceptible individuals for sustained disease transmission.

In vaccinated populations, the interval between measles outbreaks increases, and sufficiently high levels of vaccination can interrupt endemic transmission. With vaccination, the age distribution of cases is determined by which groups are likely to lack vaccine- or measles-induced immunity. In the United States, an extensive effort to achieve high levels of first-dose measles vaccination at 12 to 15 months of age and the addition of a recommended second dose of vaccine in school-age children has resulted in a decrease in reported measles cases from 400,000 to 500,000 per year in the 1960s to record lows of approximately 63 cases from 2000 to 2007, and increasing somewhat due to importations in recent years (9–11). Endemic transmission of measles in the United States was interrupted in 1994 (12), and measles was declared eliminated from the U.S. in 2000 (13). Recently, a panel of experts was convened to certify the maintenance of elimination of measles and of rubella (14).

The decade or more absence of circulating measles virus and, therefore, natural "boosting" of the antibody response,

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has led some investigators to question whether endemic measles transmission might be reestablished if population antibody immunity were to wane below theoretical protective levels (15). Investigation of outbreaks associated with importations of measles into the U.S. or similar measles-eliminated settings have resulted in the majority of spread cases occurring among unvaccinated individuals. However, limited spread was occasionally observed among one- and two-dose recipients of measles-containing vaccine. These suspect measles cases often presented with modified symptoms, such as reduced fever and modified rash illness in terms of appearance and duration, and were reminiscent of cases referred to as "secondary vaccine failure" cases. In fact, laboratory testing indicated that these individuals had a heightened memory response, manifested as a high-avidity IgG and an unusually high neutralizing-antibody response (>50,000 mIU) within the first few days of rash (16, 17). Confirmed measles infections in health care workers with numerous pediatric contacts resulted in no additional cases, indicating that these modified infections pose a low transmission risk (18) and little threat to the eradication effort. Nevertheless, good public health practice would dictate that these modified measles cases be monitored to assure that contacts remain uninfected, particularly unvaccinated contacts.

Although measles remains a formidable disease of children in many areas of the world, tremendous strides have been made in global measles control and mortality reduction between 2000 and 2010, with recent estimates suggesting infections and deaths declined by approximately 75% during this period (18). Effective use of available live-attenuated measles vaccines and a variety of multidose vaccination strategies (second opportunities for vaccination) have combined to eliminate measles in many large geographic regions, including the Americas, Australia, the Scandinavian countries, and the United Kingdom (19, 20). Unfortunately, large measles outbreaks in Africa and delays in implementing vaccination campaigns and other control measures in India have slowed the global march toward the mortality-reduction goal. Successful achievement of this goal will require redoubling of political and especially financial commitments at a time when such resources are very difficult to secure. Nevertheless, the World Health Assembly recommended proceeding to the eventual global eradication of measles, if measurable progress towards reaching the regional measles elimination goals can be achieved (21).

Clinical Significance

Uncomplicated Clinical Course

Approximately 1 week to 10 days following exposure, the clinical presentation begins with cough, coryza, conjunctivitis, and fever. The prodromal stage then progresses over the next 3 to 4 days, with all symptoms intensifying and the associated fever reaching as high as 105°F.

Koplik's spots, pathognomonic for measles, appear on the buccal mucosa in 50 to 90% of cases 2 to 3 days before rash onset, and may persist for 1 to 2 days following rash onset. These lesions are small, irregular red spots with a bluish white speck in the center. The erythematous rash appears approximately 2 weeks following exposure and is first evident on the forehead or behind the ears. The rash presents as red macules 1 to 2 mm in diameter, becoming maculopapules over the next 3 days. The exanthem is usually most confluent on the face and upper body and initially blanches on pressure. By the end of the second day the trunk and upper extremities are covered with rash, and by the third day the lower extremities are affected. The rash resolves in the same sequence, first disappearing from the face and neck. The lesions turn brown and persist for 7 to 10 days and then are followed by a fine desquamation. In most cases, recovery is rapid and complete.

Death resulting from respiratory and neurological causes (see below) occurs in 1 of every 1,000 measles cases, but estimates during the 1989 to 1991 outbreaks were three to four times higher (22). The risk of death is greater for infants and adults than for children and adolescents. However, rates of acute measles virus infections of infants and children in developing countries, particularly malnourished populations, can approach 10%, with rates of morbidity being much higher.

Complications

The most common complications associated with measles virus infection are otitis media (7 to 9%), pneumonia (1 to 6%), and diarrhea (6%). Pneumonia may occur as a primary viral pneumonia (Hecht pneumonia) or as a bacterial superinfection. Measles commonly involves the central nervous system (CNS), with as many as 50% of cases reported to have electroencephalogram abnormalities during the acute or convalescent phase of the illness (23).

Notable CNS complications include acute disseminated encephalomyelitis and subacute sclerosing panencephalitis (SSPE). Acute disseminated encephalomyelitis occurs approximately 1 week after rash onset in 1 per 1,000 cases and is manifested clinically by seizures, lethargy, irritability, and/or coma. It results in the death of 5 to 30% of patients, causes residual deficits in about 30% of survivors (24, 25), and is presently considered an autoimmune disease since an immune response to myelin basic protein has been reported and measles virus has not been isolated from these patients (26).

SSPE is a progressive, inevitably fatal, late neurological complication caused by persistent measles virus infection of the CNS that occurs in approximately 1 per 100,000 measles cases (27), although recent studies estimate the rate to be 1 per 11,000 cases (28). Only wild-type measles virus nucleic acid sequences have been found in association with this disease. The average time from natural measles virus infection to manifestations of SSPE is 7 years. The disease characteristically involves personality changes, decreased motor and intellectual capabilities, involuntary movements, and muscular rigidity and ultimately leads to death. The virus is difficult to recover from brain specimens, requiring cocultivation or brain tissue explant techniques, yet measles virus proteins and RNA can readily be detected in brain tissue (29, 30). Patients with SSPE usually have high titers of measles virus-specific IgG antibodies in their sera and cerebrospinal fluid.

Infection during pregnancy is associated with an increased risk of miscarriage and prematurity, although there is no convincing evidence that maternal infection with measles virus is associated with congenital malformations. Clinical illness in the newborn after intrauterine exposure follows a shortened incubation period and may vary from mild to severe.

Atypical measles syndrome occurred in children who had been vaccinated with multiple doses of formalin-killed measles vaccine and subsequently exposed to wild-type measles virus. The syndrome was believed to occur as a result of immunopathological responses due to a combination of the Arthus reaction and delayed hypersensitivity (31). The syndrome has little relevance today, but the experience serves to emphasize the safety concerns that should be addressed by those wishing to develop alternative measles vaccines or to explore alternate routes of vaccine delivery.

The use of combined, live-attenuated measles-mumps-rubella (MMR) vaccines has been the subject of controversy particularly as their use might be associated with immune-mediated bowel disorders, ileal lymphonodular hyperplasia, and a regressive form of autism (32, 33). Reviews conducted by expert panels assembled by the Institute of Medicine, as well as numerous carefully controlled epidemiological and laboratory studies in many countries including the United States and United Kingdom, have failed to confirm those reports (34, 35). Most recently, the Office of Special Masters of the US Court of Federal Claims rejected test cases claiming compensation for injuries due, in part, to MMR playing a causal role in autism-spectral disorders. A portion of the ruling was based on exhaustive review of the current literature regarding any causal relationship between MMR vaccine and autism spectral disorders. On January 28, 2010, a full retraction from the published record of the original paper by Wakefield and coworkers (33) was made by *The Lancet* (published online February 2, 2010).

Other described complications include thrombocytopenia, stomatitis, laryngotracheobronchitis, hepatitis, appendicitis and ileocolitis, pericarditis and myocarditis, glomerulonephritis, hypocalcemia, and Stevens-Johnson syndrome.

In immunocompromised patients, syndromes such as giant-cell pneumonia and measles inclusion-body encephalitis (MIBE) have been observed (36, 37). Measles-induced giant-cell pneumonia is usually unrecognized due to the absence of rash. It usually occurs in patients with deficits in cell-mediated immunity, and several cases have been diagnosed following vaccination of children with severe combined-immunodeficiency syndrome (38). MIBE is generally fatal and is best described as the unchecked replication of measles virus in the CNS in the face of an impaired or absent cell-mediated immune response. There are anecdotal reports of more severe and even fatal measles in patients with AIDS (39). However, vaccination is considered safe unless patients are severely immunocompromised.

Collection, Transport, and Storage of Specimens

In general, specimens for successful virus isolation should be collected early in the acute phase of the infection, when the virus is present in high concentrations, and transported to a laboratory under conditions that maintain the infectivity of labile viruses. Suitable samples for isolation of measles virus or for detection of viral antigen can be whole blood, serum, throat and nasopharyngeal secretions, urine, and, in special circumstances, brain and skin biopsy samples. Specimens should be processed as soon as possible after collection and are best kept at 4°C rather than frozen, since freezing causes significant loss of recoverable virus. Specimens should be frozen and shipped with dry ice only if the time between specimen collection and delivery to the laboratory is expected to exceed 48 h.

Transport medium should be used to maintain measles virus until isolation of virus from clinical specimens can be performed. Samples requiring viral transport medium and commercial sources of viral transport medium are reviewed in chapter 79. Measles virus is lymphotropic, and macrophages are a known source of infectious virus during natural infection (40). Thus, peripheral blood mononuclear cells (PBMCs) are an excellent source for the isolation of measles virus. PBMCs are obtained from heparinized blood (diluted 1:3 in saline) by sedimentation through density gradients.

Serum specimens from patients with suspected measles should be assayed in an IgM enzyme-linked immunosorbent assay (ELISA) within 7 to 10 days of rash onset, if possible. A single serum specimen is sufficient in most cases (41). To assess seroconversion following measles vaccination, serum specimens can be tested with IgG enzyme immunoassays (EIAs) or plaque reduction neutralization (PRN) tests (see below). Ideally, paired specimens should be obtained: the first prior to vaccination and the second approximately 3 to 4 weeks later, so that a rise in measles virus-specific IgG can be measured. Serum samples should ideally be stored at −20°C, but antibody is stable for extended periods at 4°C. Samples for IgM determinations should not be frozen and thawed more than five times. Spinal fluid samples should also be obtained if neurological complications are present or suspected.

Respiratory specimens are appropriate for testing. Nasal aspirates or bronchial lavage samples yield virus more frequently than throat swabs, because of the greater likelihood of obtaining infected cells. For immunofluorescence assays (IFAs), slides of respiratory specimens should be fixed in cold acetone for 2 min and stored at 4°C.

For isolation of virus from urine, a large volume of midstream (clean-catch), preferably morning urine should be collected into a sterile container. Measles virus is very cell associated; thus, the urine is centrifuged at 800 × *g* for 30 min. The pelleted cells and sediment are resuspended in 1 to 2 ml of Hanks' balanced salt solution in preparation for cell culture, PCR, or other diagnostic methods (42).

Two approaches show promise in terms of alternatives to serum specimen collection for the diagnosis of measles: the use of oral mucosal transudates (OMT) (see chapter 79) and the use of blood spots on filter paper. OMT samples have been used both for detection of measles virus-specific IgM and IgG and for detection of hepatitis, rubella, mumps, and other infections (43–45). This specimen collection method has been used successfully for many years for routine measles surveillance in the United Kingdom (46). The use of OMT samples has appeal because the collection method is noninvasive. The specimens can be used for rubella testing, do not require processing in the field, and offer the opportunity to detect measles virus-specific IgM for case confirmation and nucleic acids for molecular characterization (47, 48). Commercial serological assays performed with OMT samples can result in heightened background reactivity and decreased sensitivity; cold chain issues remain a problem with OMT specimens in warmer climates. Devices (such as OraSure) containing compounds that stabilize IgM should be avoided if molecular testing is to be performed as these additives inhibit nucleic acid amplification enzymes.

Like OMT samples, blood spots collected onto filter paper do not require processing in the field, but a cold chain does not appear necessary. They can be used to test for rubella as well as for measles (49–52) and they can be used for molecular characterization of the measles virus genome (53), although virus isolation would not be possible. In addition, the eluted serum from blood spots can likely be tested in commercially available measles ELISAs without the loss of sensitivity and specificity (49, 53, 54), making this technology very attractive for widespread use. Although still considered an invasive technique, fingerstick as a method for sample collection is often more acceptable to parents than phlebotomy (55).

In June 2007, WHO convened a special meeting of the Laboratory Network collaborators to review the available data on use of OMT and dried blood spots (DBS). Among the recommendations that emerged, the committee endorsed the use of both methods as viable options for measles and rubella surveillance in all regions, especially where patients might resist venipuncture, or where special challenges exist with specimen storage or transportation (47).

Direct Examination

Cytologic Examination

Characteristic cytopathic effects (CPE) of measles virus infection include multinucleated cells and cellular inclusions (intracytoplasmic and intranuclear). Cytologic examination of various lymphoid tissue specimens and secretions frequently reveals the presence of giant cells with multiple overlapping nuclei, Warthin-Finkeldey giant cells. Slides can be stained with either Wright stain or hematoxylin and eosin. Tissue samples may be fixed in 10% formalin, embedded in paraffin, sectioned, and then stained with hematoxylin and eosin (56). Staining of tissue specimens with monoclonal antibodies (see below) to the measles virus N protein has been used for the diagnosis of giant-cell pneumonia and MIBE (57).

Immunofluorescence Assay

Detection of measles virus can be achieved using an immunofluorescent antibody (IFA) to examine clinical specimens as well as cell cultures infected with clinical material. The standard assay uses a commercially available monoclonal antibody to the N protein of measles virus and fluorescein-conjugated goat anti-mouse antiserum (see "Confirmation of Measles Virus Isolation" below). Nasopharyngeal aspirates or swabs from the posterior nasopharynx are the specimens of choice and can be diluted in sterile saline solution and centrifuged at 800 × *g* to pellet the cells. The cell pellets are then washed several times with sterile saline before being applied to a glass slide and fixed in cold 80% acetone for 10 min at −20°C (58, 59). Using the N monoclonal antibody, specific granular or punctate fluorescent staining restricted to the cytoplasm is often observed in multinucleated giant cells.

Nucleic Acid Detection Techniques

Standard and real-time reverse-transcriptase quantitative PCR (RT-PCR and RT-qPCR) assays have been used in research settings to detect measles virus RNA in a variety of clinical specimens and infected cells (60–63). While measles IgM serology remains the recommended routine diagnostic test for acute measles infections, molecular detection methods can be more advantageous in certain circumstances. Recently real-time RT-qPCR was included as an acceptable method for measles case confirmation along with IgM serology, IgG seroconversion, and 4-fold rises in antibody titer using a quantitative IgG method (64). The measles RT-qPCR assay is used in conjunction with IgM serology in elimination settings to provide the best opportunity of confirming suspect measles cases. Collection of both blood and respiratory specimens when the patient first presents with symptoms is now strongly recommended.

RT-PCR should be considered for measles case confirmation where IgM testing is compromised by the concurrent or recent use of measles-containing vaccine as part of outbreak response or in settings of recent vaccine distribution, such as supplemental immunization activities (65). Likewise, molecular detection methods can be used when cell culture isolation is not a practical alternative, and/or when genetic characterization of the virus is required. RT-PCR amplification and nucleotide sequencing of the last 450 nucleotides of the coding region of the N protein gene permit genotype analysis and delineation between vaccine and wild virus (see "Genotyping" below). The use of molecular techniques to detect and track diseases like measles and rubella has expanded tremendously, particularly within the WHO LabNet. However, these tests are performed primarily in Regional Reference Laboratories (RRL) and in some cases National Laboratories where measles has been largely controlled or is near elimination. In contrast, molecular methods have not been implemented beyond the RRL level in places where case-based surveillance is less of a priority, such as areas challenged by uncontrolled measles or large measles outbreaks (8, 66).

Isolation and Identification of Measles Virus

Measles virus can be isolated from the conjunctiva, nasopharynx, and blood during the latter part of the prodromal period and during the early stages of rash development. Although virus has been isolated from the urine as late as 4 to 7 days after rash onset, viremia generally clears 2 to 3 days after rash onset in parallel with the appearance of antibody. Thus, virus can be most readily isolated within a period from 2 to 4 days prior to rash onset to about 4 days after rash onset.

Though other cell cultures and lines, such as primary monkey kidney and Vero cells, have traditionally been used, an Epstein-Barr virus-transformed B lymphoblastoid cell line derived from marmoset lymphocytes until recently has been the preferred cell line for primary isolation of measles virus (67). These cells (B95a cells) were found to be as much as 10,000 times more sensitive for isolation of measles virus from clinical specimens than other cell lines, such as Vero cells and primary monkey kidney cells. Laboratorians should note that this cell line does produce Epstein-Barr virus and should be handled as infectious material at all times. B95a cells can no longer be obtained from commercial sources, thus dramatically restricting the widespread use of this cell line.

Recently, a Vero cell line transfected with a plasmid encoding the protein for the hSLAM molecule has been constructed (68). The hSLAM molecule has been shown to be a cell surface receptor for both wild-type and laboratory-adapted strains of measles. Testing conducted to date indicates that the sensitivity of Vero/hSLAM cells for isolation of measles virus is equivalent to that of B95a cells. Vero/hSLAM cells also express the simian CD46 molecule. The cell line has been recommended for use in the WHO Laboratory Network as a replacement for the B95a cell line. The CPE that results following measles infection is essentially that observed in measles-infected Vero cells (Fig. 1). The advantage to the Vero/hSLAM cells is that they are not persistently infected with Epstein-Barr virus, and therefore, are not considered as hazardous material like B95a cells. This provides a significant safety advantage for laboratorians and greatly facilitates international shipments. The disadvantage of the Vero/hSLAM cells is that they must be cultured in medium containing Geneticin to retain hSLAM expression. This increases the cost of the cell culture medium. Laboratories (U.S. public health and WHO LabNet laboratories) should only accept Vero/hSLAM cells from a WHO-approved source (RRL or Global Specialized Laboratory). Upon receipt, the cells should be passaged in medium containing 400 µg of Geneticin/ml as described in the protocol distributed with the cell line. Laboratories should passage the cells two to four times in the presence of Geneticin to expand the number of cell culture vessels sufficient for preparation of 20 to 50 vials of seed stock for liquid-nitrogen storage.

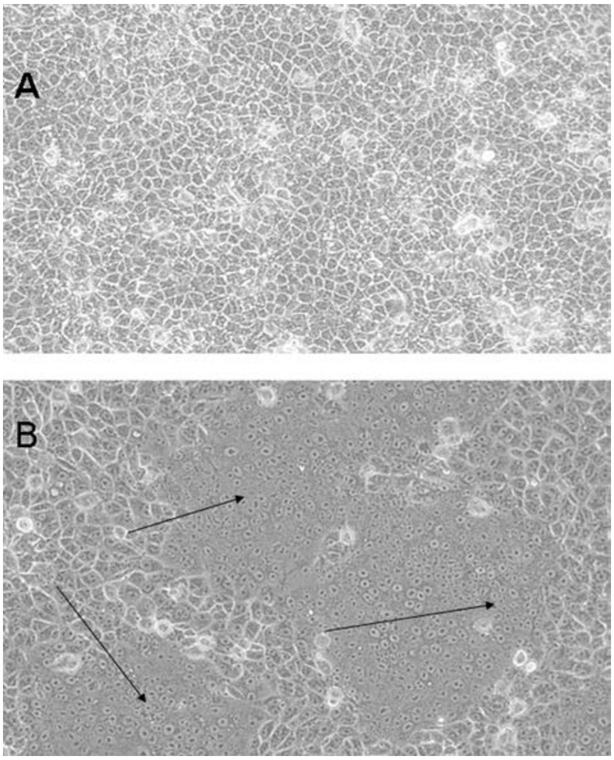

FIGURE 1 CPE of measles virus when propagated in Vero/hSLAM cells, a cell line that is transfected with a gene encoding the wild-type virus receptor (CDw150) for measles virus. The cell line naturally expresses the simian form of CD46 on the cell surface. (A) Uninfected monolayer of Vero/hSLAM cells. (B) Measles virus-infected monolayer of Vero/hSLAM cells with apparent syncytium formation and multinucleated giant cells (arrows). doi:10.1128/9781555817381.ch87.f1

To prepare cells for virus isolation procedures, Vero/hSLAM cells can be recovered from liquid nitrogen and passaged up to 15 times in medium without the addition of Geneticin. These cells should be used for virus isolation attempts only and should be discarded after 15 passages. Even with addition of Geneticin to the medium, the cells should not be passaged forward beyond about 20 to 30 passages, as with the standard Vero cell line. Cells that have been passaged without Geneticin in the medium should never be used to prepare cell stocks for liquid-nitrogen storage or shipped to another network laboratory for use in virus isolation.

Confirmation of Measles Virus Isolation

Confirmation of isolation is most often achieved by IFA detection and uses a monoclonal antibody to detect the N protein or other internal antigen of measles virus in infected cells. The infected cells are fixed onto a microscope slide. Binding of the measles virus-specific antibody is detected using a goat anti-mouse antibody that is conjugated to fluorescein isothiocyanate. IFA test kits are available from Millipore Corporation, Billerica, MA (Light Diagnostics, catalog no. 3187). It is also possible to configure an indirect IFA test without using a commercial kit. Most monoclonal antibodies to the N protein (Light Diagnostics catalog no. 5030; available from Millipore Corporation) or other internal antigens (catalog no. 20-902-170205; GenWay BioTech, Inc., San Diego, CA) perform well in the IFA procedure described above. Monoclonal antibodies directed against other viral proteins such as the hemagglutinin and fusion proteins may recognize conformational epitopes that are not stable after acetone fixation. When configuring an in-house IFA, the appropriate working dilutions of monoclonal antibody and fluorescein isothiocyanate-labeled conjugate have to be determined by experimental titration.

Genotyping

Amplicon sequencing and phylogenetic analysis (genotyping) of the last 450 nucleotides of the measles N gene has proven useful in suggesting the possible source of virus involved in outbreaks, tracking transmission pathways during outbreaks, and differentiating between vaccine and wild-type strains of measles (8, 66, 69). The latter application is the most pertinent to measles diagnostics, but until recently, rarely performed at the state or local public health laboratory level. In general, these laboratories are asked to collect appropriate viral specimens from suspected cases, and send them to the Centers for Disease Control and Prevention (CDC) if the results of IgM serology are positive.

In 2012, the CDC, in collaboration with the Association of Public Health Laboratories (APHL), established four Vaccine Preventable Disease Reference Centers (VPD RCs) at four state public health laboratories. The VPD RCs were established to provide an efficient means of measles (rubella and other viral and bacterial VPDs) testing based on CDC-developed and -evaluated protocols to state and local health departments that have limited budgets for testing for diseases of low incidence or low-volume testing. To date, 32 state and nine local health departments submit specimens for testing to the VPD RCs. The RCs have provided surge capacity for three major measles outbreaks in 2013–2014. In addition, the RCs provide serological and molecular confirmatory testing and genetic characterization for other VPDs, surveillance support, and confirmation of vaccine adverse events in a shared-service model to 32 state and 9 local-submitter public health laboratories. Results are contemporaneously transmitted to the submitting public health departments and CDC (70).

Genetic characterization of wild-type measles viruses provides a means to study the transmission pathways of the virus; molecular epidemiology in conjunction with conventional case investigation and epidemiology has permitted the linking of imported cases to their foreign sources. Genotyping is also an essential indicator for establishing and maintaining the elimination status of the U.S. and the region of the Americas (14). Laboratory-based surveillance for measles and rubella, including genetic characterization of wild-type viruses, is performed throughout the world by the WHO LabNet, which serves 160 of 193 member countries in all WHO regions. In particular, the genetic data can help confirm the sources of virus or suggest a source for unknown-source cases as well as to establish links, or lack thereof, between various cases and outbreaks. Virologic surveillance has helped to document the interruption of transmission of endemic measles in some regions. Thus, molecular characterization of measles viruses has provided a valuable tool for measuring the effectiveness of measles control programs, and virologic surveillance needs to be expanded in all areas of the world and conducted during all phases of measles control (6, 14, 71, 72). It must be emphasized, however, that conventional epidemiology and case investigation must be done hand in hand with the molecular studies to achieve the optimal outcome of this approach. Drawing conclusions from genotype determinations without proper epidemiologic investigation can lead to false assumptions regarding geographic origin or source of infections.

Serologic Diagnosis

The recommended laboratory method for the confirmation of clinically diagnosed measles is a serum-based IgM ELISA. Several of these tests are commercially available and are used worldwide by public health, clinical, and commercial laboratories. These assays have been used as the primary confirmatory test by the laboratory network of the Pan American Health Organization throughout the measles control and elimination phases throughout the Americas. The WHO LabNet has also recommended use of the IgM ELISA for laboratory confirmation of measles (73). As regions enter the elimination phase, measles cases become more difficult to diagnose and ELISA results become less reliable. As mentioned above, many RRLs have already implemented additional testing such as molecular methods, as well as IgG, and in some cases IgG avidity testing to supplement surveillance and case classification (66, 72, 74). However, the ELISA still remains the mainstay of confirmatory measles testing. The ELISAs can be done using a single serum specimen, are relatively rapid (2 to 6 h), are simple to perform, and can be used to diagnose acute measles virus infection from the time of rash onset until at least 4 weeks after rash onset. Thus, the IgM ELISAs fulfill all of the basic criteria for the accurate, effective, and efficient diagnosis of measles. Both indirect and IgM capture formats have been used (75–77). Though the IgM capture assay format is often regarded as more sensitive than the indirect format, comparative studies of some commercial indirect formatted ELISA kits have demonstrated that the two formats have equivalent sensitivities and specificities (78, 79).

Traditional antibody tests such as hemagglutination inhibition (HI), PRN, and ELISA have been used extensively in the serologic confirmation of the clinical diagnosis of measles. However, because of the availability of sensitive and specific commercial kits, ELISA has become the most widely used test format. Commercial ELISAs also have the ability to measure measles virus-specific IgM as well as IgG responses and, therefore, have particular importance in confirming clinical diagnosis as well as monitoring measles-control programs. Although limited in number, a few available IgG assay kits were found to have sensitivities and specificities that compared favorably with PRN (80–82). In general, IgG measurements by ELISA or similar assay have been used for surveillance (antibody prevalence) studies, while PRN has been reserved for those studies interested in questions regarding immunity. Most laboratories have trained personnel and are already equipped to run ELISAs.

Commercial laboratories and some large clinical centers performing large-volume testing of measles (and rubella) specimens require high throughput automated platforms in conjunction with ELISA, chemiluminescent, and Luminex immunoassays. Although the formats for some of the assays may differ from the traditional ELISAs, their performance characteristics and rapid turnaround times make them viable alternatives for large surveillance, seroprevalence, and seroconversion studies (83, 84).

Standard ELISAs

Currently, only one commercial assay includes a measles virus antigen produced by a yeast recombinant DNA expression system rather than antigen produced by infected cell cultures. ELISAs using recombinant-expressed N protein in both capture and indirect formats have high sensitivity and specificity compared to those of other commercial ELISAs (76, 77).

Though the configurations of the commercial measles virus IgM kits vary, the tests are simple and straightforward to perform by following the manufacturers' protocols. Indirect ELISA has been successful in the detection of IgG (76). For this indirect test, either whole virus antigen diluted in 0.05 M bicarbonate buffer (pH 9.5) or recombinant antigen diluted in phosphate-buffered saline is distributed into polystyrene microtiter plates. Serum specimens are diluted in phosphate-buffered saline containing 4% normal goat serum (plus 4% Sf9 cell lysate for recombinant antigen) and 0.05% EDTA and then added to the washed plates. Bound antibody can be detected with standard commercial reagents such as goat anti-human IgG conjugated to either alkaline phosphatase or biotin. Assays are developed with the appropriate substrate, and the plates can be read either by eye or spectrophotometer.

Plaque-Reduction Neutralization (PRN)

The PRN assay, which measures neutralizing antibodies that are directed against the surface glycoproteins of measles virus, is more sensitive and specific than hemagglutination inhibition (HI) or ELISA (85). Since functional antibodies are detected, the PRN provides the best serologic correlate for the assessment of immune protection (86, 87). However, the PRN test is not practical for routine serologic diagnosis because it is very labor-intensive, requires paired serum samples, and takes 5 to 7 days to complete. Recently, a fluorescent-based plaque-reduction microneutralization assay for measles virus immunity (88) has been developed that permits higher-throughput processing of small quantities of serum specimens and is more amenable for use in large serosurveys for assessing immune status. This assay has been standardized against the conventional PRN assay (80) using the WHO 2nd International Standard for antimeasles serum and awaits further validation.

In the PRN assay, measles virus-specific antibody in serum combines with and neutralizes measles virus, preventing it from infecting a cell monolayer and forming a plaque under the overlay. The endpoint for the test is the highest dilution of serum that reduces the number of plaques by 50%. Serum dilutions are made in 96-well microtiter trays, making either 2- or 4-fold dilutions (depending on the expected titer of the serum). Once the dilutions of serum samples are made, an equal volume (120 μl) of a dilution of virus containing 25 to 35 plaque-forming units (PFU) is added to each well and incubated for 2.5 h at 36°C. After incubation, 100 μl of the serum-virus mixture is added to each of two 16-mm-diameter wells on a tissue culture plate(s) containing Vero cell monolayers. These plates are then incubated for 1 h at 36°C. Following incubation, the inoculum is removed by aspiration, and the monolayers are covered with overlay medium consisting of either 2% carboxymethyl cellulose in Leibovitz-15 medium or 1% agarose in Eagle's medium. The plates are then incubated for 4 days at 36°C, and the monolayers are stained with a solution of neutral red in cell culture medium. On day 5, the overlay is removed and the plaques are counted. The numbers of PFU in duplicate wells representing a given serum dilution are averaged.

New ELISAs

A new and potentially promising development has been the use of ELISAs to measure the avidity of IgG antibodies to measles virus (89, 90). As the immune response matures, low-avidity antibodies are replaced with high-avidity antibodies. These avidity differences can be detected by using protein denaturants, 6 to 8 M urea or diethylamine (91) in the washing step of the indirect ELISA for measles virus

IgG. An avidity index is then calculated by comparing the optical densities obtained with and without the denaturing agent in the wash buffer. These tests show promise in differentiating between primary and secondary responses to vaccination and to natural infection (92). Measles avidity assays are limited in their use and can only be used to rule in a case of measles. Nevertheless, this can be very useful in elimination settings where IgM assays yield indeterminate or questionable results that appear to conflict with clinical presentation and patient history. Although there are several commercial products available, these assays remain investigational tools due to the lack of appropriate guidelines for use and the availability of well-characterized standardized controls.

Evaluation, Interpretation, and Reporting of Results

The interpretation of negative culture results should be made with caution, since many factors influence the outcome. Some of the most important considerations include the timing of sample collection, transportation to the laboratory, preparation for culture, and finally, and probably most importantly, the cell culture system used for virus isolation. In a recent survey of public health laboratories that performed cell culture for measles virus, most were not using engineered cell lines that are required for optimal isolation of wild-type measles (93). This report was the basis for the establishment of VPD RCs that in turn has led to greater availability of RT-qPCR among state public health laboratories. Proper interpretation of results is therefore increasingly important given the improved availability of molecular tests. Negative RT-qPCR results cannot be used to rule out a case of measles, for many of the same reasons that negative culture results may not be definitive. Moreover, genomic regions selected for amplification for measles virus RT-qPCR assays do not distinguish between wild-type and vaccine viruses, and therefore, would not be useful in many outbreak settings where vaccine is in use to control the outbreak. The utilization of conventional RT-PCR coupled with nucleotide sequencing and genotyping as confirmatory testing is strongly advised when considering implementing RT-qPCR. Alternatively, access to these methods should be arranged.

The serum-based IgM assay is the recommended test for the confirmation of acute measles infection. False-negative results can occur when serum specimens taken too early with respect to rash onset are tested. For example, the CDC capture IgM assay, which has a sensitivity and specificity equivalent to those of commercial ELISAs, has been shown to detect IgM in only 77% of true measles cases within the first day of rash onset (94). In the case of indirect IgM assays, false-negative values can be the result of insufficient removal of high levels of measles virus-specific IgG from a test specimen. The residual IgG competes with IgM for viral antigen placed on the solid-phase support, thereby blocking IgM binding to the antigen and interfering with IgM detection. Present-day immune-absorbent reagents are much improved over earlier reagents; thus, fewer problems of this nature occur.

False-positive tests due to the presence of rheumatoid factor (RF) appear to be more frequently encountered when using the indirect IgM assay. RF is an IgM class immunoglobulin that reacts with IgG and is produced as a result of some viral and autoimmune diseases. Immune complexes may form that contain test antigen-specific IgG and RF IgM. By virtue of the IgG binding to the viral antigen, the IgM component of the RF immune complex is recognized by the detector system, thus rendering a falsely positive result. Similar false-positive results can occur in capture assays but appear to be enhanced by the presence of high levels of both antigen-specific antibody and RF (75).

The inherent sensitivities and specificities of the ELISAs in times of low disease prevalence become a factor in interpretation. This situation occurs in countries that have eliminated endemic measles but remain vigilant in performing case-based surveillance of rash illnesses. In these geographic regions, both measles IgM and IgG ELISAs are performed on serum specimens from suspected cases due to high vaccine-induced seroprevalence. In this setting, if the IgG test is positive and the IgM test is negative, the case is usually discarded (depending upon the timing of serum collection). Recall as well the rare instances of "modified measles," although many of these do have an IgM response (16, 17).

Unfortunately, ELISA specificity is not 100%. Serum specimens from patients with parvovirus B19 and rubella have inherent rates of false-positive reactions (overall rate of about 4%) when tested in measles IgM ELISAs and vice versa (95, 96). The reason(s) for these false-positive reactions is not understood, and attempts to remove immune complexes using anti-RF have proven unsuccessful.

There is also evidence that case contacts that have a resident IgG response, due either to a history of natural infection or to vaccination, may develop a secondary IgG or an IgM response to currently circulating virus (97, 98). The IgM is generally fleeting and weakly positive and, except for rare instances, should not be a source of diagnostic confusion (65).

Table 1 summarizes the possible interpretations of ELISA results. Despite the vagaries associated with the ELISAs, they are the best assays available for laboratory confirmation of clinically diagnosed measles. It should be emphasized that the vast majority of serum specimens submitted for serology will yield a test result that will be easily interpretable. However, as mentioned earlier, the current CDC Surveillance Manual for Vaccine Preventable Diseases (64) strongly encourages the collection of both blood and respiratory specimens when suspect measles cases first visit the health care provider. Additionally, laboratorians should be provided with as much clinical and epidemiological information as possible to aid in the final interpretation of the test result(s).

RUBELLA VIRUS

Taxonomy

A number of small, enveloped viruses having the same overall genetic organization and replication strategy as rubella virus exist, and they are grouped into the *Togaviridae* family. The *Togaviridae* family consists of the *Rubivirus* genus, containing only rubella virus, and the *Alphavirus* genus, containing about 25 other viruses, all of which are transmitted by arthropods (e.g., western equine encephalitis virus). Rubella virus has a restricted host range and humans appear to be the only species in which rubella circulates.

Description of the Agent

Rubella virus virions are particles about 70 nm in diameter that are composed of a core surrounded by a lipid envelope. The core consists of the positive-strand RNA genome (~9,760 nucleotides) and the virus protein C. The viral envelope contains two viral glycoproteins, E1 and E2 (99). The viral RNA replicates in the cytoplasm of infected cells, with nonstructural proteins being translated from the 5′ two-thirds of the genomic RNA and the structural proteins being translated from a subgenomic RNA that is a copy of

TABLE 1 Interpretation of measles ELISA results

IgM result	IgG result	Infection history	Current infection	Comment(s)
+	+ or –	Not previously vaccinated, no history of measles	Recent first MCV[a]	Seroconversion,[b] post-vaccination, low avidity IgG, if present
+	+ or –	Not vaccinated, no history of measles	Wild-type measles virus	Seroconversion,[b] classic measles, low avidity IgG, if present
+	+ or –	Previously vaccinated, primary vaccine failure	Recent second MCV vaccination	Seroconversion,[b] post-vaccination, low avidity IgG confirms primary failure, if present
–	+	Previously vaccinated, IgG positive	Recent second MCV vaccination	IgG level may stay the same or increase, high avidity IgG
+	+	Previously vaccinated, IgG positive	Wild-type measles virus	May have few or no symptoms[c]
+	+	Recently vaccinated	Exposed to wild-type measles virus	Cannot distinguish if vaccine or wild-type virus infection; evaluate on epidemiological grounds[d]
+ or –	+	Distant history of measles	Wild-type measles virus	May have few or no symptoms[d]; if clinically compatible, may have been misdiagnosed initially

[a] MCV, measles virus-containing vaccine.

[b] IgG level depends on timing of specimen collection.

[c] Rare occurrence; do not consider contagious unless clinical presentation is consistent with measles.

[d] If result is IgM negative, it is helpful to rule out wild-type measles virus infection.

the 3′ one-third of the genomic RNA. New virions are produced when genomic RNA, the E1 glycoprotein, the E2 glycoprotein, and the C protein assemble at cellular membranes (100, 101).

Rubella viruses currently circulating in the world contain RNAs that differ sufficiently that two clades of rubella viruses, differing by about 10% in the nucleotide sequence, have so far been identified (102, 103). Groups of related viruses within the clades have been classified as genotypes. At present, 12 genotypes and one provisional genotype of rubella viruses have been recognized (104). Only minor immunologic differences exist among circulating viruses. Immunity to one rubella virus is sufficient to protect against clinical disease caused by other known rubella viruses.

Epidemiology and Transmission

Rubella virus was not isolated until 1962, largely because infected cells are difficult to identify in tissue culture (105, 106). Introduction of rubella vaccine in the United States (licensed in 1969), mostly through childhood immunization, immediately broke the 6- to 9-year epidemic cycle of rubella. The last major U.S. rubella epidemic was in 1964 to 1965, when approximately 20,000 congenital rubella syndrome (CRS) cases occurred (107). The combined MMR vaccine was recommended for the United States in 1972. Rubella and CRS have been eliminated in the United States; from 2004–2012, only 79 cases of rubella and six cases of CRS were reported in the U.S. (14, 108). Most mothers of CRS children were born in countries without rubella-immunization programs or with recently organized programs (108). Occasional outbreaks of rubella in some U.S. populations have not spread to the undervaccinated populations, suggesting that herd immunity has been protective (109). Indigenous rubella and CRS have not only been eliminated from the United States, but also from the Americas; rubella is targeted for elimination in Europe by 2015 (110–112). Despite the possibility of worldwide rubella eradication, rubella remains endemic in many countries and about 100,000 to 200,000 CRS cases occur annually in the world (113–115). Postnatal transmission of rubella virus is often by close contact with an infected individual, such as occurs in correctional institutions or day-care centers.

The safety of the live rubella virus vaccine strains most widely used (RA 27/3) is well documented (111, 116). A recent summary indicated only 1 of 833 infants born to rubella-susceptible mothers who were inadvertently vaccinated after conception was born with abnormalities consistent with CRS (117). However, a small theoretical risk remains. Thus, the Advisory Committee on Immunization Practices recommends avoiding pregnancy after receipt of rubella-containing vaccine for 28 days (118).

Clinical Significance

Rubella (German measles or 3-day measles) was first described in the 18th century and was accepted as a disease independent of measles and scarlet fever in 1881 (119). Postnatal rubella is characterized by an acute onset and generalized maculopapular rash with mild fever (greater than 99°F) and may include arthritis or arthralgia (mostly in postpubertal females), lymphadenopathy (specifically postauricular and suboccipital nodes), and conjunctivitis. Because disease caused by rubella virus is mild, about 50% of postnatal rubella cases are not diagnosed clinically. Although rubella had been largely ignored for 60 years, in 1941 N. McAlister Gregg first recognized that cataracts in children followed maternal rubella during gestation (120). The association between congenital rubella and a spectrum of significant birth defects including sensorineural hearing loss, cardiovascular abnormalities, cataracts, congenital glaucoma, and meningoencephalitis is now accepted. Rubella virus is now recognized as the most potent infectious teratogenic agent yet identified (121, 122).

When rubella occurs in a pregnant woman in the first 11 weeks of gestation, there is a high likelihood of defect(s) in the infant (about 90%). After 18 weeks, the likelihood of birth defect is much lower, although the infant may still be born infected with rubella virus. Congenital rubella infection (CRI) refers to infants born with rubella virus infection with or without birth defects. The pathogenesis of CRI leading to congenital rubella syndrome is not well understood, but rubella virus infection early in gestation results in an altered immune response to the virus and altered organogenesis. The significant effect on the fetus of rubella virus infection during the first trimester is likely related to the facts that the fetus cannot synthesize IgM until about 20 weeks of gestation and cell-mediated immunity does not develop until late in gestation. Congenitally infected infants shed virus for long periods, have a slowly developing immune response to rubella virus, and respond to particular rubella virus proteins differently than individuals with postnatal rubella. These three characteristics of congenital infection are all consistent with selective immune tolerance to rubella virus proteins in such infants (100). Infection during the first 11 weeks of gestation often results in multiple organ involvement and necrosis in many tissues (121). Organogenesis is affected in CRS cases, since specific organs are abnormal and other apparently normal organs have a reduced number of cells. Reinfection with rubella virus can occur, but viremia is rare; reinfection of a pregnant woman poses low risk to the fetus (121, 123).

CRI results in both shedding of virus and IgM antibodies in the neonate. Diagnosis is based on detection of rubella virus (RT-PCR or virus culture) or rubella virus-specific IgM in such patients. If congenital defects characteristic of CRS are not present, the infant is diagnosed as having CRI only. The clinical definition of CRS is standardized, but laboratory confirmation of rubella virus infection in the newborn by either serologic or virus detection techniques is critical, especially when only a single defect presents, since the defects characteristic of CRS can occur for other reasons (124).

Collection, Transport, and Storage of Specimens

Clinical specimens for culture of rubella virus are usually throat swabs or nasopharyngeal secretions diluted into transport medium (e.g., Culturette collection and transport devices). The virus can also be isolated from a number of other specimens, including cataract tissue and urine (provided that pH is controlled) (113). Urine is often a source of infectious virus from CRS patients. Specimens for virus detection should be stored at 4°C for short periods (days) or at −70°C for longer periods (weeks); virions lose infectivity at higher temperatures (e.g., 37°C). Virions are rapidly inactivated by mild heat (56°C), detergents, or lipid solvents. Rubella virus-specific IgG can be detected in urine (125). Specimens for serology or culture can be transported by standard methods (e.g., overnight carrier) at 4°C since virions are relatively stable at that temperature. Alternative specimens such as DBS and OMT (see chapter 79) have been shown to be adequate for surveillance of rubella using IgM detection (DBS and OMT) and virus detection (OMT) (47, 49, 126–129). Two caveats should be considered if these alternative specimens are used. First, diagnostic kits are usually not approved by FDA for use with DBS; second, low IgM levels in OMT necessitate the use of highly sensitive detection assays.

The timing of specimen collection is especially important in postnatal rubella. Rubella virus-specific IgM is the laboratory diagnostic criterion typically used for rubella, but about 50% of rubella cases are IgM negative on the day of rash (see Table 2 and related text below). Since postnatal rubella is a mild disease of short duration, special effort may be required to obtain a serum sample 5 to 7 days after rash, when most rubella patients are strongly IgM positive. Patients with CRS are IgM and virus positive for months; therefore, timing is less critical for these patients.

Direct Examination

Nucleic Acid Detection Techniques

Amplification of rubella virus RNA directly from a clinical specimen using RT-PCR can be used to determine if a patient is infected with rubella virus. Not all RT-PCR protocols are sufficiently sensitive to be used directly with clinical specimens. Assays that can reliably detect 3 to 10 copies of rubella virus RNA are sufficiently sensitive. Real-time and nested RT-PCR protocols, although difficult to maintain, usually meet this criterion (126, 131, 132).

Many postnatal rubella cases are IgM negative before 4 to 5 days post-rash and direct detection of viral RNA is the most sensitive test during this time period (126). For example, on the day of rash onset, direct detection of rubella virus RNA by real-time assay will confirm about twice as many suspected rubella cases as commercial IgM ELISAs. No standard real-time or RT-PCR protocol for detection of viral RNA has been established and there are currently a number of such tests being used. At present, real-time and RT-PCR are mostly used in national or reference laboratories.

Isolation and Identification of the Virus

Growth of rubella virus from clinical specimens can be used to diagnose postnatal rubella, CRS, and CRI (Table 2). Throat swabs taken on the day of rash, typically a convenient time for sample collection, are usually positive for rubella virus, even though a slightly higher percentage of cases are positive 2 days before rash onset. Virus shedding in the throat declines rapidly, and by 4 days after rash onset, only about 50% of cases

TABLE 2 Timing of biological markers of rubella virus infection[a]

Diagnostic criterion	Convenient time when many cases are positive (%)	An example of a time when >90% of cases are positive	Approx time for 50% decline[b]
Postnatal rubella			
Virus in throat[c]	Day of rash (90%)	2 days **before** rash	4 days after rash
IgM in serum by ELISA[c]	Day of rash (50%)	5 days after rash	6 wk after rash
IgG in serum by ELISA	3 days after rash (50%)	8 days after rash	Lifetime
Virus in blood by culture[d]	Day of rash (50%)	5 days **before** rash	1 day after rash
CRS[e]			
Virus in throat[c]	At birth (almost all)	2 wk after birth	3 mo of age
IgM in serum by ELISA	At birth (80%)	1 mo of age	6 mo of age
IgG in serum by ELISA[f]			

[a] Times and percentages given are approximate and are meant to guide typical specimen collection. Percentages vary depending on the sensitivity of the assay used. Note that the times listed in the third column were chosen to help guide specimen collection and may not be the earliest time when >90% of cases are positive.

[b] After maximum number of cases are positive for a given criterion, the approximate time for 50% of cases to become negative.

[c] "Alternative" specimens, oral mucosal transudate (OMT) and dried blood spot (DBS), have been evaluated for detection of virus (OMT) and IgM (OMT, DBS). See references 47 and 126–128.

[d] Data taken from reference 130.

[e] Information given is for fetal infection in the first trimester.

[f] Declining maternal IgG and developing IgG response in a CRS patient lead to high (steady) or increasing IgG levels in the CRS patient through the first year of life.

are positive. In addition, viral culture is used to monitor virus in CRS and CRI patients for the purpose of determining when isolation of these patients from susceptible contacts can be stopped. The virus will grow in a variety of cell types, including Vero, BHK21, AGMK, and RK-13 cells. The primary problem encountered with tissue culture is the lack of a cell type that produces CPE in a single passage of wild-type viruses. Historically, this problem was overcome by clever assays exploiting the fact that rubella virus growth interferes with the replication of lytic enteroviruses such as coxsackievirus A9. However, interference assays are quite difficult to maintain (133). Virus growth can now be identified in the absence of CPE using methods such as RT-PCR, IFA, and immunocolorimetric assays (ICA) to detect viral RNA or proteins (132, 134).

The sensitivity of the RT-PCR system used to detect viral RNA from infected tissue culture cells is not critical, since the amount of rubella viral RNA has been amplified by passage in tissue culture (about 10^6-fold) (102, 132). Detection of rubella virus-infected cell monolayers can also be accomplished by IFA or ICA. It is crucial that IFA and ICA reagents have low background, since rubella virus culture does not produce high levels of progeny virus (about 10^7 PFU/ml for laboratory strains such as f-Therien). Infected cells are easily identified when stained with high-quality reagents (Fig. 2). Dilution of specimens may be desirable since it is useful to have both infected and uninfected cells in the same field. Monoclonal antibodies to the E1, E2, and C proteins, which react with both reduced and non-reduced antigens on Western blots, often work well in the IFA and ICA.

Utility of Sequences Derived from Viral RNA

Sequencing of the nucleic acid amplified directly from specimens or from tissue culture material can provide useful information. Vaccine virus can be differentiated from wild-type viruses (135). Useful information on the origin of imported cases of rubella and CRS can be obtained (108, 136). Documentation of the elimination of rubella can be supported by analysis of sequences obtained over time (137).

Serologic Tests

ELISA

Detection of rubella virus-specific IgM by either IgM-capture ELISA or indirect-IgM ELISA is the fastest and most cost-effective diagnostic test for recent postnatal infection. Unfortunately, only about 50% of postnatal rubella cases are IgM positive on the day of symptom onset (Table 2) (126). Most postnatal rubella cases have virus-specific IgM detectable by capture ELISA from 5 until 40 days after symptom onset and IgG by indirect ELISA ≥8 days after symptom onset (Fig. 3) (138). A negative IgM serologic result 4 to 5 days after onset should be followed with testing of a serum sample taken as soon as possible thereafter to avoid false-negative results (110). If acute- and convalescent-phase sera are available, a 4-

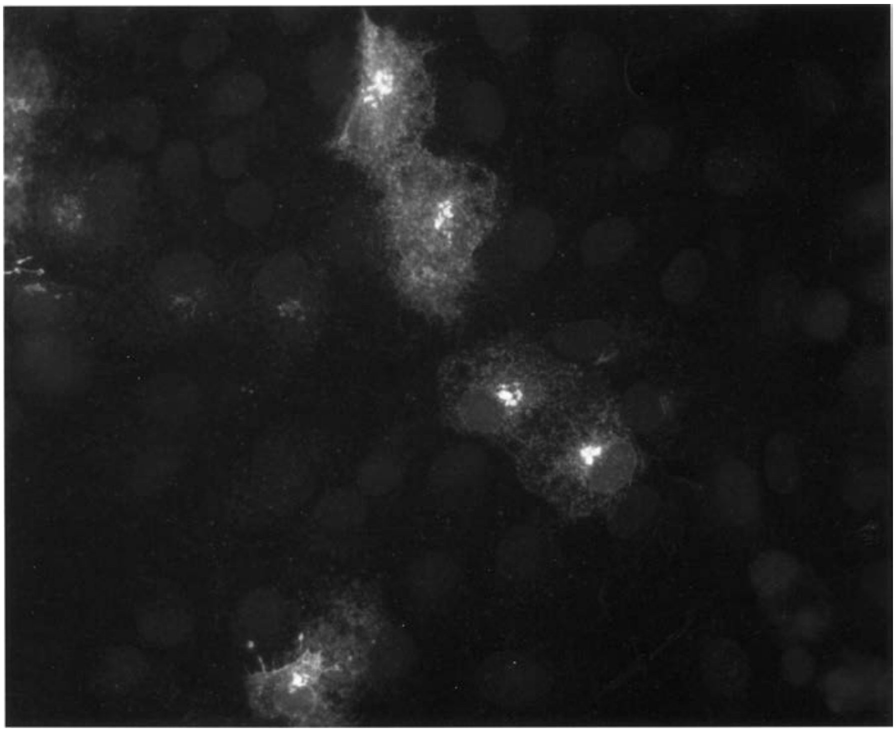

FIGURE 2 IFA of rubella virus-infected cells using monoclonal antibody to the E2 protein. Vero cells were infected with wild-type rubella virus and 3 days after infection were fixed with cold paraformaldehyde followed by permeabilization with −20°C methanol. Reaction of infected cells with monoclonal antibody to the E2 protein (monoclonal antibody 24–26; Meridian Life Science, Saco, ME) was followed by detection of bound monoclonal antibody with Alexa Fluor 488-conjugated, highly cross-absorbed goat anti-mouse IgG (Molecular Probes Inc., Eugene, OR). Cell nuclei were visualized by staining with propidium iodide. About 25% of the cells which stained with propidium iodide were positive for E2 protein. doi:10.1128/9781555817381.ch87.f2

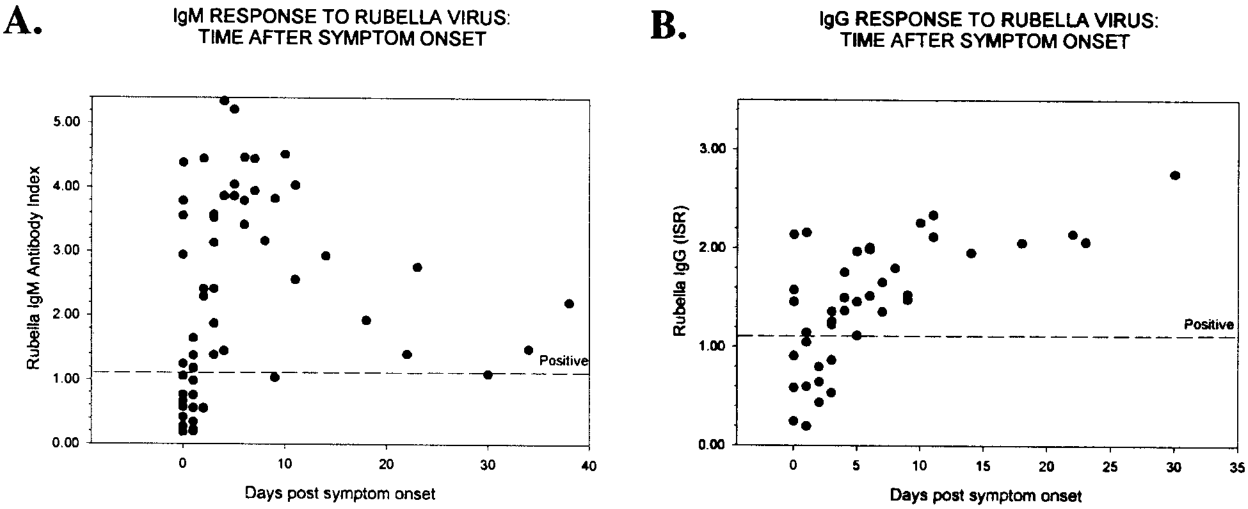

FIGURE 3 Time course of rubella virus-specific IgM and IgG detection by ELISA in sera of rubella patients. Commercial IgM capture ELISA (A) and IgG indirect ELISA (B) were used to detect rubella virus-specific antibodies at the indicated number of days after onset of symptoms (usually rash); antibody index and ISR are the commercial test designations for the ratio of the optical density obtained for the test serum to the optical density obtained for a standard (cutoff) serum. The minimum signal considered positive in each test is indicated by a dashed line. Only results from patients who tested positive for IgM to rubella virus at some time after the onset of symptoms are shown. doi:10.1128/9781555817381.ch87.f3

fold rise in rubella virus-specific IgG is diagnostic for postnatal rubella infection; such sera should be taken as early as possible after disease onset and about 2 to 3 weeks after disease onset. When IgG titers are used for diagnostic purposes, a dilution series of each serum sample should be made and ELISA results for each dilution series compared, since optical density values at a single dilution are an unreliable measure of the amount of IgG.

The same ELISAs may be used to confirm CRS. Most congenitally infected infants have IgM detectable from birth to 1 month of age (Table 2). The percentage of infants who are IgM positive declines over the first year of life. At 1 year, most infants are negative. In CRS patients, the IgG response increases gradually over the first 9 months, while maternal IgG declines. Thus, high or increasing IgG levels in the first year of life, in the absence of vaccination or significant risk of postnatal rubella, are consistent with CRS. Recommendations for the best times for collection of samples from postnatal rubella and CRS cases have been published (110).

Latex Agglutination

Commercial rapid latex particle agglutination tests consist of latex spheres coated with rubella virus antigen. These particles aggregate in the presence of either rubella virus-specific IgG or IgM. Because high throughput is possible with these assays, they are often used in immunity-screening programs (e.g., prenatal screening).

Hemagglutination Inhibition

The HI test was once the standard test for antibodies to rubella virus, and many current tests are calibrated using HI assays. However, indicator erythrocytes are not readily available and the assay is difficult to perform as a diagnostic test since removal of nonspecific inhibitors and internal standardization are required.

Immunofluorescence Assay

IFA has been used for detection of IgG and IgM antibodies to rubella virus. Typically cells expressing rubella-virus proteins and control cells are reacted with patient serum and any rubella virus-specific antibodies are then detected with fluorescent dye-labeled goat anti-human IgG (or IgM) and fluorescence microscopy. Negative human sera and uninfected control cells are useful for detecting nonspecific signal. Fluorescence should be cell associated. Staining restricted to the periphery of the cell monolayer is artifactual and not indicative of a true-positive result.

Plaque-Reduction Neutralization

PRN is performed when a quantitative assessment of the neutralizing capacity of an antiserum is necessary. A laboratory strain (e.g., f-Therien) should be used since viruses from clinical specimens do not exhibit CPE. The assay follows a format common to many viruses. The initial step is incubation of 2- or 10-fold dilutions of antiserum and a standard amount of rubella virus (usually about 100 to 200 PFU) in medium- or buffer-containing protein to inhibit losses on surfaces (e.g., 0.1% bovine serum albumin) for 1 h at 35 to 37°C followed by overnight in a refrigerator. A control consisting of virus alone must be included in the assay, since some reduction in the number of plaques is observed during the 1 h incubation. Virus-antiserum specimens are then allowed to attach to confluent Vero cell monolayers for 1 h at 35°C and then overlaid with diethylaminoethyl (DEAE) dextran (100 μg/ml)-containing medium with 0.5% agar (e.g., Oxoid). Medium in the agar overlay is typically Dulbecco's modified Eagle's medium; 1% fetal calf serum may also be included to maintain the monolayer. After 6 days, agar is removed and plates are stained with neutral red. Crystal violet can also be used; however, a wash step to remove dead cells should be used prior to crystal violet staining. The ICA for rubella virus can also be used for virus detection (134). The neutralizing capacity of the antiserum is typically reported as the inverse of the antiserum dilution giving a standard reduction in plaques, typically 90% reduction. In both vaccination of non-immune individuals and postnatal rubella cases the neutralizing capacity rises at least 100-fold, allowing the easy use of PRN data to confirm past exposure to rubella. However, information on the precise assay used is required to quantitatively compare neutralization capacities of antisera when assays are done in different laboratories (e.g., rubella virus-plaquing efficiency may vary between laboratories).

Other Serologic Tests

Avidity tests have been used when IgM detection does not reliably indicate recent infection (e.g., first serum sample was collected months after clinical symptoms). Low-avidity anti-rubella IgG suggests recent infection (96, 139, 140). This test compares the ability of detergents or chaotropic agents to dissociate case IgG and control IgGs from rubella virus proteins. Both high- and low-avidity control sera should be used in each assay. Avidity tests are not widely available and vary in performance (141).

Since the clinical symptoms of postnatal rubella and CRS are dramatically different, it is not surprising that there are significant differences in the immune responses of patients with these diseases. These differences can be observed on Western blots, in which antibodies in sera from CRS patients often demonstrate different reactivity to rubella glycoproteins than those from postnatal rubella patients (142). These tests are not widely used, but have been developed in some diagnostic laboratories (109).

Prenatal Screening

The present description of laboratory testing for rubella emphasizes identification of postnatal and congenital rubella cases. However, in the United States much of the testing is for immunity to rubella, since health care providers should test for immunity by a serum IgG test in all pregnant women at the earliest prenatal visit. There are slightly different criteria for rubella immunity that are recommended by various groups (range is about 10 to 15 IU/ml) (116). Commonly used tests, e.g., ELISA, are standardized to give positive results for 10 IU/ml, the breakpoint defining immunity to rubella in the United States (143). Much of the screening for immunity to rubella virus in the United States is done by automated random-access systems using microparticle immunoassays.

Evaluation, Interpretation, and Reporting of Results

IgM and IgG testing should be done with most sera for both suspected postnatal rubella and CRS cases, since results from both immunoglobulin classes often provide additional information useful for diagnosis. For example, results from a serum sample taken at 8 days post-rash which are positive for IgM but negative for IgG to rubella virus would be inconsistent with the immune response to rubella; usually the IgM result would be most suspect in this situation (e.g., cross-reaction with antibodies to parvovirus). A serum that tests rubella IgG antibody positive and IgM negative is inconsistent with recent postnatal rubella infection in the patient, since the IgM response should precede the IgG response and persist for weeks after rash onset.

A positive result for rubella virus culture is obtained when a positive real-time assay or RT-PCR result is obtained from the culture or at least one cluster of cells are infected as determined by IFA or ICA. Control cultures must be negative. When an IFA or ICA is used, the expected distribution of viral proteins should be obtained (e.g., E1 glycoprotein distribution when using an E1 monoclonal) (134).

Direct detection of rubella virus RNA by PCR-based protocols requires the laboratory to evaluate the significance of results from such tests. Multiple negative controls and amplified product in more than one specimen from a given patient will increase confidence of a positive diagnosis based on direct RT-PCR. The significance of negative results is usually difficult to determine since false-negativity rates are usually not available. Sequence variation in wild-type viruses, which can lead to poor primer binding and poor amplification, must be considered when evaluating the significance of negative results. Nevertheless, when serum from a patient cannot be obtained, or when confirmation of serologic results is desired, direct detection of rubella virus RNA by RT-PCR may be necessary since it is more rapid than viral culture.

There is often a considerable burden on the laboratory in the diagnosis of rubella. For example, when primary rubella virus infection is suspected for a pregnant woman, false positives and false negatives may lead to incorrect clinical decisions (144). Thus, the laboratorian may be asked to go beyond just communicating false-positivity and false-negativity rates. Testing for recent infection with other viruses that cause clinically similar diseases (e.g., human parvovirus B19) is often prudent. Positive rubella results may be more believable if no other infection is found. Specimen retesting and testing of different specimen types with alternative methods (e.g., serology and viral culture [Table 2]) often yield consistent results and reduce the likelihood of false-positive results. False positives can occur even with IgM-capture ELISA. For example, in one study, 1 of 87 sera testing positive for rubella virus-specific IgM by IgM capture ELISA was from a patient whose final diagnosis was primary human parvovirus B19 (96). False negatives can often be identified by testing multiple specimens from a patient (e.g., sera taken 1 week apart). If only a single specimen is available, it may be tested by multiple assays. For example, IgG avidity may resolve the diagnosis from a single serum sample that is IgM positive for both rubella virus and human parvovirus B19 (96).

Postnatal rubella can be clinically similar to other diseases, or it can be asymptomatic. Additionally, birth defects characteristic of CRS occur for other reasons. Thus, correct classification of suspected postnatal rubella and CRS is based on laboratory results rather than clinical presentation. Classification of a postnatal rubella case results in its categorization as suspected, probable, confirmed, or asymptomatic confirmed; for a case of congenital rubella, categories are suspected CRS, probable CRS, confirmed CRS, or infection only. Positive laboratory results are required to correctly classify asymptomatic confirmed or confirmed cases of postnatal rubella and CRS. Clinical, laboratory, and epidemiological information (e.g., international travel) all may enter into the final clinical decision(s). A full description of classification criteria and recommendations should be consulted (110). One specific diagnostic situation should be noted. A series of tests including a rubella virus IgM test should not be used to determine immunity in a pregnant woman because of the possibility of a false-positive result; immunity should be determined by IgG testing alone. Since standard TORCH (toxoplasmosis, other, rubella, cytomegalovirus, and herpes simplex virus) panels include testing for rubella virus IgM, they should not be used to determine immunity.

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Enteroviruses and Parechoviruses

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TAXONOMY

Enteroviruses (EV) are members of the *Picornaviridae* family, with "pico" meaning very small, "rna" indicating an RNA genome, and "viridae" signifying viruses. Traditional criteria for taxonomy and identification of EV to subgroups were based on patterns of replication in cell cultures, clinical syndromes, or disease, and on disease manifestations in suckling mice. The subgroups were poliovirus (PV), coxsackievirus A (CVA), coxsackievirus B (CVB), and echovirus (E). Echoviruses were initially named as the acronym "echo," meaning enteric cytopathic human orphan, since they were first isolated from the stool of asymptomatic children and caused a cytopathic effect in cell culture. The criteria classified 67 different human EV serotypes until the designation was dropped in the 1960s, with all subsequent serotypes designated enterovirus (EV) followed by a number beginning with 68 (1).

With the development of molecular sequencing and the limited availability of antisera for characterizing newly identified strains and variant strains, the traditional methods of taxonomy have become challenging. The EV taxonomy was effectively redefined through phylogenetic analysis (2). Currently, human EVs are divided into seven species: EV A through D and rhinoviruses (RVs) A to C (Table 1)(3). Several EVs that were originally thought to be distinct serotypes are likely variants of the same strain (4). Some strains have been reclassified into different groups, new EVs have been identified (EV73 to EV120) (http://www.picornaviridae.com/enterovirus/enterovirus.htm), and host names have been removed from the EV genus (5).

The genus *Parechovirus* (HPeV) comprises 16 different types (http://www.Picornaviridae.com/parechovirus/hpev/hpev.htm). Originally identified in 1956 as members of the EV genus based on growth characteristics, echoviruses 22 and 23 are genetically distinct from other EVs and were renamed HPeV1 and HPeV2 (6–8). HPeV3 was identified decades later by molecular methods (9). Analogous to the genotyping of EVs, the use of the *VP1* gene sequence to determine the HPeV genotype is standard (10, 11). As with EVs, molecular techniques have led to the identification of a number of additional types based on the *VP1* gene sequence, designated HPeV3 to 16. With new HPeV genotypes being identified by sequence analysis of the *VP1* gene, whole-genome sequence analysis will elucidate the genetic relatedness to other HPeVs. As more data become available, a better understanding of the new types, and of any evolutionary relationship with the origin, will be established.

DESCRIPTION OF THE AGENTS

Similar to other members of the family *Picornaviridae*, EVs are small (30-nm diameter in the hydrated state), nonenveloped viruses that possess a single-stranded positive (message)-sense RNA genome. Their buoyant density in cesium chloride is 1.30 to 1.34 g/cm^3 (12). The majority of the EVs, from group A to group D, are stable in acid, ether, and chloroform and are insensitive to non-ionic detergent. They are inactivated by heat (>56°C), UV light, chlorination, and formaldehyde. In cell culture, most EVs replicate optimally at 36 to 37°C, with the exception of RVs A and B, which replicate optimally at 33°C. RVs are covered more extensively in chapter 89.

The EVs are stable in liquid environments and can survive for many weeks in water, body fluids, and sewage. This is due to a number of viral properties, including thermostability in the presence of divalent cations, acid stability, and the absence of a lipid envelope.

The EV RNA genome serves as a template for both viral protein translation and RNA replication (Fig. 1) (13). The genome contains a long (approximately 750 bases) 5′ nontranslated region (5′ NTR), which immediately precedes a single open reading frame (ORF) (Fig. 1). The ORF is translated into a single polyprotein that is posttranslationally cleaved into several functional intermediates and the final 11 proteins by virus-encoded proteases. Immediately downstream of the ORF is a short non-coding region of approximately 70 to 100 bases (3′ NTR) and a terminal polyadenylated tail.

The EV 5′ and 3′ NTRs play critical roles in the EV life cycle. The 5′ NTR contains multiple regions of predicted higher-order structure (i.e., stem loops or domains). A domain located at the extreme 5′ terminus of the 5′ NTR is essential for viral RNA replication. Cap-independent translation of the EV genome is regulated by the internal ribosome entry site (IRES), which spans a discontinuous region within the 5′ NTR. For PV and several non-polio enteroviruses (NPEVs), the 5′ NTR has been documented to be a determinant of virulence phenotype and cell type specificity (14, 15). Given the crucial nature of the functions controlled by the 5′ NTR, it is not surprising that nucleotide sequences, with near absolute conservation among the EVs, exist within this region. These regions of high nucleotide identity have been exploited for the design of primers and probes used for the detection of the EVs. The 3′ NTR has also been demonstrated to play a role in viral RNA replication.

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TABLE 1 HEVs, HRVs, and HPeVs

Enterovirus species	Serotypes (3, 5)
EV A (24 serotypes, 20 infecting humans)	
Coxsackieviruses (CV)	CV-A2-A8, A10, A12, A14, A16
Enteroviruses (EV)	EV-A71, A76, A89, A92, A114, A119, A120
EV B (61 serotypes, 59 infecting humans)	
Coxsackieviruses (CV)	CV-A9, B1-6
Echoviruses (E)	E1-7, 9, 11-21, 24-27, 29-33
Enteroviruses (EV)	EV-B69, B73-B75, B77-B88, B93, B97, B98, B100, B101, B106, B107
EV C (23 serotypes)	
Coxsackievirus (CV)	CV-A1, A11, A13, A17, A19-A22, A24
Poliovirus (PV)	PV1-3
Enterovirus (EV)	EV-C95, C96, C99, C102, C104, C105, C109, C113, C116, C117, C118
EV D (5 serotypes, 4 infecting humans)	
Enterovirus (EV)	EV-D68, D70, D94, D111
Rhinovirus (RV) A (77 serotypes)	RV-A1, A2, A7-A13, A15, A16, A18-A25, A28-A34, A36, A38-A41, A43-A47, A49-A51, A53-A68, A71, A73-A78, A80-A82, A88-A90, A94-A96, A98, A100-A106
Rhinovirus (RV) B (30 serotypes)	RV-B3-6, B14, B17, B26, B27, B35, B37, B42, B48, B52, B69-70, B72, B79, B83-84, B86, B91-93, B97, B99-104
Rhinovirus (RV) C (51 serotypes)	RV-C1-51
Human parechovirus (HPeV)	Serotypes (3)
HPeV (16 serotypes)	HPeV1–16

The EV capsid is arranged in 60 repeating protomeric units that confer an icosahedral shape to the virion (Fig. 2) (13). VP1, VP2, and VP3 comprise the surface of the virion and possess an eight-stranded "beta barrel" core structure. The external loops that connect the beta strands are responsible for the differences in surface topography and antigenic diversity among the EVs. Neutralization sites, typically three or four per protomer, are most densely clustered on VP1. VP4 is located internally within the capsid and has no surface-exposed regions.

The resolution of the near-atomic structures of multiple human picornaviruses revealed that the EVs (and RVs) share a number of conserved structural motifs. Surrounding a conserved protrusion at the 5-fold axis of each pentameric unit (i.e., five protomers) is a narrow deep cleft (25 Å) or canyon. It is into this site that the specific receptors for the EVs bind when the virus encounters a susceptible host cell.

The genomic organization of HPeVs is similar to that of EVs. However, unlike the EVs, HPeVs possess only three capsid proteins, VP0, VP3, and VP1, as a result of the lack of cleavage of VP0. In addition, the HPeV IRES is more similar to those of the cardioviruses and aphthoviruses than those of the enteroviruses (16). Recently published images, obtained using cryo-electron microscopy, revealed a 28-nm-diameter HPeV particle similar to other known picornaviruses but with a somewhat smoother surface (17). Sequence similarities between HPeVs and the other picornaviruses suggest that the major capsid proteins share the typical beta-barrel core structure. Relative to the EVs, the HPeVs are predicted to have flatter surface topography (as shown with cryo-electron microscopy), with antigenic variants determined by the external loops, as for EVs.

EPIDEMIOLOGY AND TRANSMISSION

The EVs and HPeVs are ubiquitous agents found worldwide. In regions with temperate climates, the majority of EV infections (70 to 80%) occur in summer and fall. In tropical

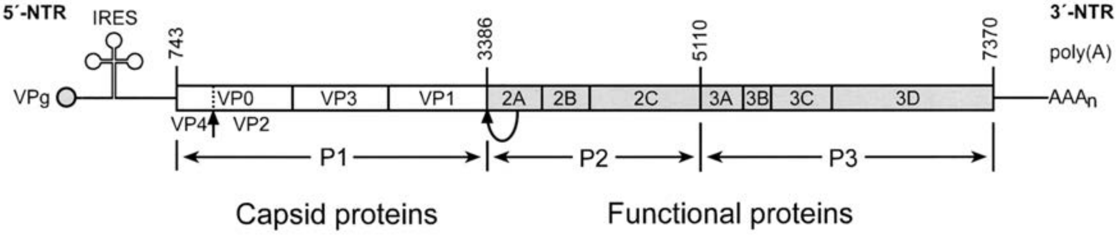

FIGURE 1 Genome organization of PV type 1. The PV genome is a single-stranded positive-sense RNA of approximately 7,500 nucleotides. Nucleotides 743 to 7370 encode, in a single open reading frame, the capsid proteins (white boxes in coding region P1) and functional proteins (gray boxes in coding regions P2 and P3). The 5′ and 3′ NTRs are shown as lines. The IRES is shown schematically with two-dimensional structure. The virus protein VPg is covalently linked to the terminal uracil of the 5′ NTR. Reprinted from reference 13. doi:10.1128/9781555817381.ch88.f1

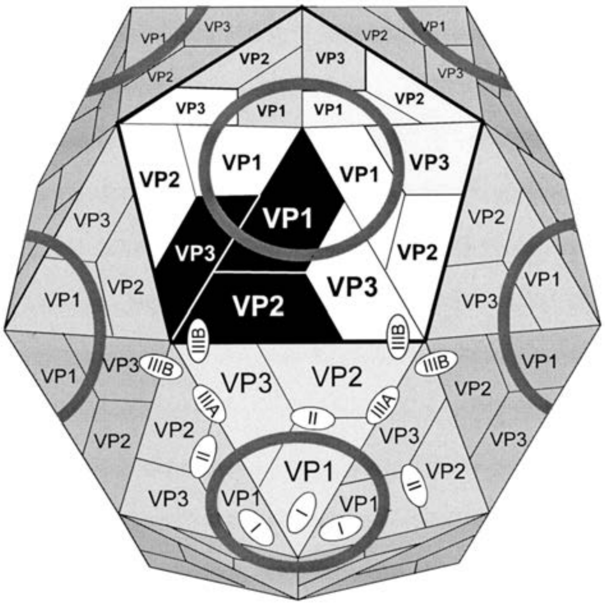

FIGURE 2 Schematic representation of the three-dimensional structure of a PV particle and the four neutralizing antigenetic (N-Ag) sites. The icosahedral capsid structure typical of EVs is composed of 60 protomers, each consisting of the capsid proteins VP1, VP2, and VP3 (black areas). Each of the 12 5-fold symmetry axes is surrounded by five protomers, forming a pentamer (surrounded by a bold black line). The attachment site for the virus-specific receptor is a depression around the 5-fold symmetry axis, also called the canyon (dark gray circles). Each of the three surface-exposed capsid proteins contains immunodominant antigenic sites at which neutralizing antibodies bind. Four N-Ag sites (white ellipses) have been mapped to surface loop extensions. Reprinted from reference 13.
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climates, EV infections occur year-round or with increased incidence during the rainy season. Seasonal differences in incidence have been seen among serotypes of HPeV. HPeV3 infections occur mainly during spring and summer, whereas HPeV1 circulates throughout the year, with a nadir in summer months (16, 18, 19).

It is estimated that the EVs infect a billion or more individuals worldwide each year, with 30 to 50 million infections in the United States (20). With the exception of strictly respiratory strains such as EV-D68 (21), EVs and HPeV are spread mainly by the fecal-oral route and less by respiratory droplets and fomites. Fecal-oral transmission predominates in areas with poor sanitary conditions. Transplacental (i.e., congenital) transmission has been documented (22). These viruses can survive on environmental surfaces for periods long enough to allow transmission from fomites. Hospital nursery and other institutional outbreaks have occurred.

The highest incidence of EV infection is observed in young infants and children of 5 to 10 years of age, with children aged <1 year accounting for almost half of the reported cases in the U.S. (23). Generally, the incubation period is 3 to 6 days, except for acute hemorrhagic conjunctivitis, which is 24 to 72 hours (24), and secondary infections are observed in >50% of susceptible household members (25). The incidence of HPeV infection is highest in children <3 years of age, again with the majority of infections occurring in infants of <1 year of age (10, 18). Furthermore, variations in the mean age at which infants had infections with various HPeV types have been documented, with HPeV3 infection occurring at a younger age (16).

Two major patterns of circulating enteroviruses have been observed between 1970 and 2005: epidemic (e.g., E9, E13, E30, and CVB5) and endemic (e.g., CVA9, CVB2, CVB4, and EV71) (23). Epidemic serotypes were characterized by substantial fluctuations in numbers over time with occasional increased incidence. Endemic serotypes had stable and usually low levels of circulation with few changes in incidence. The five most commonly reported serotypes (E9, E11, E30, and E6, and CVB5) accounted for 48.1% of the cases. Interestingly, from 2006 to 2008, CVB1 was the most commonly detected enterovirus, accounting for 17% of cases tested (26). In the 35 years prior, CVB1 accounted for a small proportion (2%) of all EVs in the United States (23). In the United States, HPeV1 accounts for 1.8% of all typed EV isolates reported to the Centers for Disease Control and Prevention, while HPeV2 accounted for only 0.1% (16).

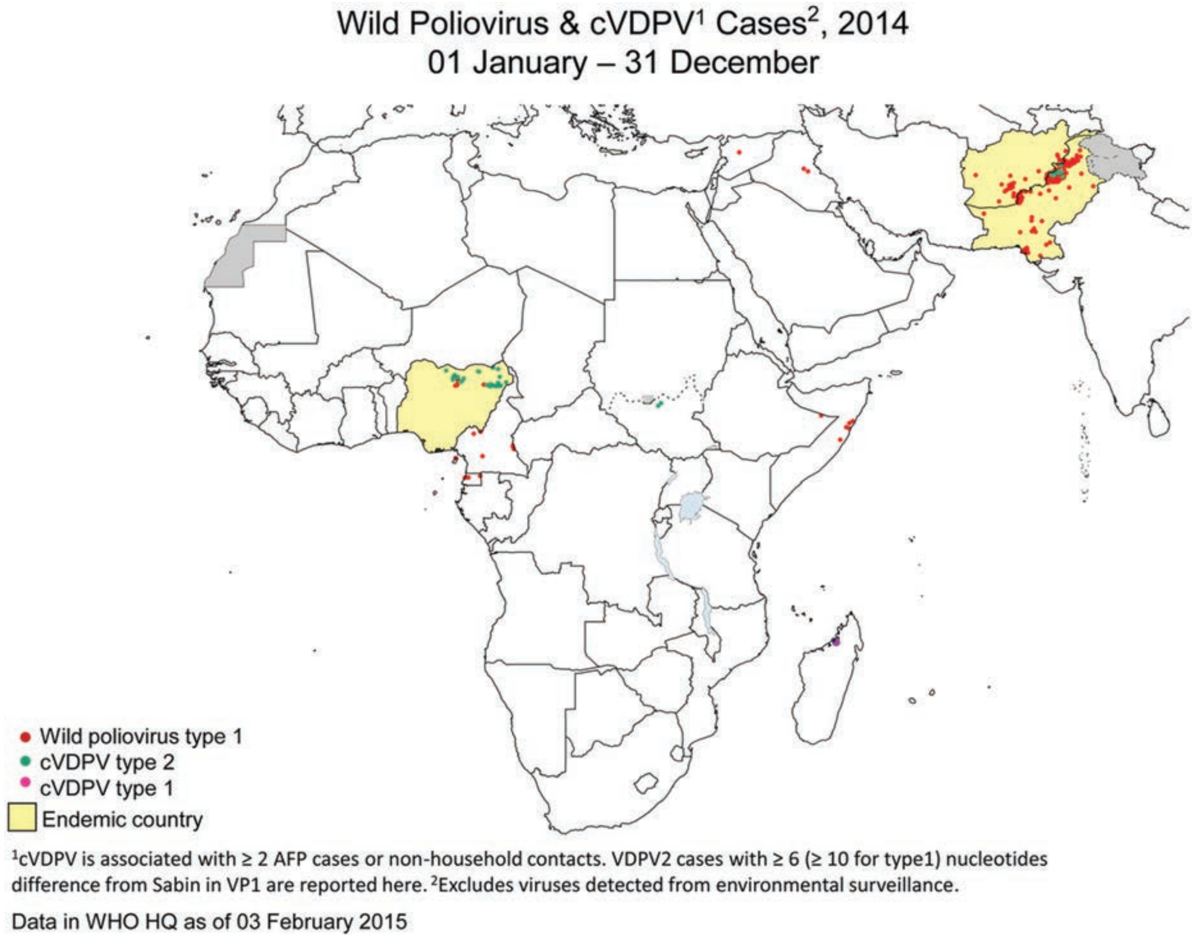
In general, mortality is not common with EV infections, with death being reported for 3.3% of cases with known outcomes. Infections with CVB4 and HPeV1 were associated with higher risk of death, and infections with E9 were associated with lower risk of death (23).

The Global Polio Eradication Initiative began in 1988. By 2006, indigenous transmission of wild poliovirus (WPV) type 2 infection had been interrupted globally (27), and by early 2012, endemic circulation of WPV types 1 and 3 had been controlled globally with the exception of Afghanistan, Nigeria, and Pakistan (28, 29). Despite this success in controlling indigenous transmission, importations of WPV, with subsequent ongoing transmissions in some cases, have been occurring continuously into previously polio-free countries in Africa and Asia (28) (Fig. 3). In 2011, three countries (Angola, Chad, Democratic Republic of the Congo) had reestablished transmission either of WPV1 or of both WPV1 and WPV3, and an additional nine countries had been affected by outbreaks of WPV1 or 3. Efforts toward global polio eradication have resulted in the decrease of polio cases from an estimated 350,000 cases annually to 650 cases in 2011 (28).

CLINICAL SIGNIFICANCE

EVs and HPeVs cause a wide array of both localized and systemic infections that affect many organ systems in patients of all ages (Table 2). Generally, no disease is uniquely associated with any specific serotype, and no serotype is uniquely associated with any one disease. This is true even of paralytic poliomyelitis, which has been associated with numerous NPEV serotypes. However, different serotypes of EV have been associated with more severe disease and/or death in different age groups of children (30). Likewise, HPeV3 is also associated with more-severe disease (10, 16, 31).

The majority of EV and HPeV infections are asymptomatic. The most common symptomatic manifestation of infection is acute nonspecific febrile illness with or without an exanthem. This so-called viral syndrome is one of the most numerically important causes of fever among infants and children. Although this illness itself is benign, it is of great concern because it mimics other more serious illnesses in infants and young children. Distinguishing EV and HPeV infections from those due to common bacteria and other viruses is often difficult on clinical grounds alone, and



¹cVDPV is associated with ≥ 2 AFP cases or non-household contacts. VDPV2 cases with ≥ 6 (≥ 10 for type1) nucleotides difference from Sabin in VP1 are reported here. ²Excludes viruses detected from environmental surveillance.

Data in WHO HQ as of 03 February 2015

FIGURE 3 Number of wild poliovirus (WPV) cases worldwide, 1 Jan 2014 to 31 Dec 2014. *, data for WPV from 2014 reported to the World Health Organization. †, excludes polioviruses detected by environmental surveillance and VDPV. Reprinted from the Global Polio Eradication Initiative (29). doi:10.1128/9781555817381.ch88.f3

hence, leads to unnecessary treatment. During summer and fall, the EVs are responsible for 50 to 60% of hospital admissions for the evaluation of acute febrile illness in children and infants (25).

By far the most vexing clinical EV/HPeV syndrome that the physician encounters is aseptic meningitis. The EVs are the most common cause of meningitis in the United States and account for over 80% of all viral meningitides (32,33).

TABLE 2 Clinical syndromes associated with EV and HPeV infection

Organ system	Disease
Neurologic	Aseptic meningitis, encephalitis, acute flaccid paralysis
Respiratory	Common cold, stomatitis, hand-foot-and-mouth disease, herpangina, lymphonodular pharyngitis, tonsillitis, rhinitis, bronchiolitis, bronchitis, pneumonia
Cardiovascular	Myocarditis, pericarditis
Miscellaneous	Acute hemorrhagic conjunctivitis (AHC), pleurodynia (Bornholm disease), febrile exanthematous illness, neonatal sepsis

More recently, HPeV3 has been recognized to cause a significant number of cases of neonatal sepsis and aseptic meningitis in children <2 years of age (16, 34). The onset of symptoms is usually sudden and generally includes a biphasic fever, headache, occasionally photophobia, vomiting, rash, and myalgias. In young children, symptoms include fever, lethargy, and anorexia (35), again mimicking bacterial infection. Currently, treatment is supportive with illness generally resolving after a week with no long-term sequelae. An estimated 30,000 to 50,000 hospitalizations for NPEV meningitis occur each year. The echoviruses and CVBs, particularly E4, E6, E9, E11, E16, and E30, and CVB2 and CVB5, constitute the principal EVs associated with this syndrome.

The term poliomyelitis refers to the inflammatory damage due to infection of the anterior horn cells of the spinal cord. Recognized clinically as acute-onset lower motor neuron paralysis of one or more muscles, the case fatality rate in the prevaccine era ranged from 5 to 10% (36), and in recent epidemics, as high as 48% (37). Historically, this disease was most commonly associated with the PVs; however, in most countries, this is no longer the case. In regions of the world where the polioviruses have been eradicated, the NPEVs and circulating vaccine-derived PVs (cVDPVs) are now the principal causes of acute flaccid paralysis.cVDPVs are circulating strains derived from the vaccine

(Sabin) strains of PV that have regained the capacity to cause paralytic poliomyelitis. Multiple NPEV serotypes are known to cause acute flaccid paralysis, and at least six poliomyelitis outbreaks have been caused by cVDPV (1). It is also important to point out that despite efforts towards polio eradication, these viruses are still endemic in some countries and have served as reservoirs for the reintroduction of polio to other areas.

Studies conducted in the United States prior to the development of molecular diagnostic techniques identified the EV as the cause of 11 to 20% of viral encephalitides (38). Larger, more recent studies of the etiology of encephalitis have identified EVs in approximately 3 to 5% of all cases, accounting for 27 to 37% of identified viral etiologies (39, 40). Serotypes from the HEV-B and HEV-A species are the predominant EV subgroup associated with this syndrome (25, 38–40). HPeV has also been shown as a causative agent (31). Rhombencephalitis, a severe and often fatal form of brainstem encephalitis, has been associated with EV71 infections in small children from several countries in the Asia-Pacific region (41–43); however, cases in the United States have also been reported (44, 45). Chronic EV meningoencephalitis in agammaglobulinemia occurs and is caused most frequently by the echoviruses, particularly E11.

Myocarditis was once an often fatal disease associated with EV; however, mortality rates are lower (~10%) with current medical care. Symptoms range from nonspecific (fever, myalgias, palpitations, or exertional dyspnea) to fulminant hemodynamic collapse and sudden death. Myocarditis has been implicated in 8.6 to 12% of cases of sudden cardiac death of young adults (46, 47). CVBs are responsible for one-third to one-half of all cases of acute myocarditis and pericarditis (48), with CVB2 and CVB5 being the most predominant serotypes identified in clinical studies. The echoviruses and PV have also been associated with myopericarditis, but significantly less so than CVB.

Neonatal systemic EV disease is associated with EV and HPeV acquisition *in utero*, perinatally or within the first 2 weeks of life (16, 22). This syndrome is characterized by multi-organ involvement with symptoms including lethargy, feeding difficulty, vomiting, tachycardia, dyspnea, cyanosis, jaundice, and diarrhea, with or without fever. Typically, neonatal systemic EV disease is associated with two severe clinical presentations (although infants may present with combinations or organ system involvement): encephalomyocarditis, often with heart failure; and hemorrhage-hepatitis syndrome with hepatic failure and disseminated intravascular coagulation (16, 22, 30). The morbidity and mortality associated with this syndrome are significant, with death occurring rapidly. Recently, three distinct clusters of severe EV illness due to CVB1, including six neonatal deaths, were reported in the United States from 2007 to 2008 (49, 50). However, other CVB and echoviruses, particularly serotype 11, are also frequently associated with this syndrome.

Although originally described for agammaglobulinemic patients, persistent, life-threatening EV infections can also occur in patients with combined variable immunodeficiency, severe combined immunodeficiency, hypogammaglobulinemia, or hyper-immunoglobulin M (hyper-IgM) syndrome, and in those undergoing bone marrow and solid organ transplant. Manifestations almost always include meningoencephalitis.

Hand-foot-and-mouth disease (HFMD) has historically been associated with CVA16. However, EV71, which is closely related to polioviruses and CVA16, has been the cause of large outbreaks of HFMD with neurological and systemic complications in the Asia-Pacific region (51). The neurological manifestations range from aseptic meningitis to acute flaccid paralysis and brainstem encephalitis, which is associated with systemic features, such as severe pulmonary edema and shock. Although serious disease due to EV71 has been described worldwide, high rates are observed in Asia; the reason for the geographic disparity in presentations is largely unknown but may be related to viral strain differences or to host HLA antigens (51). CVA6, which has been associated with herpangina, has now been linked to outbreaks of HFMD in the United States (52).

Upper respiratory tract signs and symptoms may accompany many of the enteroviral syndromes. The enteroviruses may be causal agents of upper and lower respiratory tract syndromes (e.g., summer cold, pharyngitis, tonsillitis, laryngotracheobronchitis, bronchiolitis, and pneumonia) (53, 54). Enteroviruses have been etiologically linked in up to 15% of cases of upper respiratory tract syndromes (54). The use of nucleic acid amplification methodologies has demonstrated that, depending on the country, the enteroviruses are responsible for up to approximately 19% of lower respiratory tract infections in hospitalized children (55, 56). Several of the EV C types (C104, C109, C117) have been isolated from individuals with pneumonia or respiratory tract disease (57–60).

In recent years, EV-D68 has become recognized as a cause of significant respiratory disease. Originally isolated from four children with pneumonia and bronchiolitis in 1962 (61), the overwhelming majority of reports of its isolation since then (and recently) have been from the respiratory tract as sporadic isolations or as clusters of infections or epidemics (60, 62–67). EV-D68 is phenotypically anomalous among the enteroviruses in that it is acid-labile and replicates poorly at 37°C, characteristics commonly found among the RVs (21, 68, 69). Furthermore, EV-D68 has been uncommonly encountered until recently (60, 65, 67); a 36-year review of nonpolio EV isolates reported to the CDC revealed that only 26 out of 52,812 isolates were EV-D68 (23). Phylogenetic analysis of EV-D68 isolates from around the world has indicated that, over the past 20 years, multiple clades have emerged and spread rapidly (70), possibly accounting for the increase in reported clusters and epidemics worldwide (62–66, 71).

Clinically significant lower respiratory tract disease occurs primarily in young children and infants (62–67, 71) but has been reported in adolescents and adults (60, 62, 65). Underlying conditions such as asthma or wheezing have been reported in approximately 70 to 80% of cases (62, 71). Reported clinical syndromes associated with EVD-68 include pneumonia, bronchiolitis, asthmatic bronchitis, asthma exacerbation, and wheezing (63, 64, 66, 67). Pulmonary signs and symptoms include cough, wheezing, dyspnea, tachycardia, and inter- and subcostal retractions (62, 63, 66, 71). Surprisingly, approximately one-half to three-quarters of children are afebrile. Hypoxia requiring supplemental oxygen is very common and can be severe enough to require admission to intensive care. Some children require bilevel positive airway pressure ventilation, mechanical ventilation, or even extracorporeal membrane oxygenation. Deaths are uncommon but have been reported. Chest radiographs may show infiltrates and atelectasis.

Other acute, clinical EV syndromes of significance include acute hemorrhagic conjunctivitis (predominantly caused by EV70 and a variant CVA24), herpangina, and pleurodynia (Bornholm disease). Over the past several years, data have been presented suggesting a link between EV infections and several chronic illnesses, including amyotrophic lateral sclerosis, chronic fatigue syndrome, polymyositis, congenital hydrocephalus, and attention-deficit/hyperactivity disorder, but definitive proof of causation is lacking (72). However, recent reports strengthen the possible association between enteroviruses and type 1 insulin-dependent diabetes mellitus, although human genetic factors also play an important role (73–75).

In general, the spectrum of diseases caused by HPeV is similar to that of EV (16). Diarrhea or respiratory symptoms have been described alone or in combination with other syndromes. Evidence linking HPeVs as the etiologic cause of epidemic diarrheal disease is lacking, but they may be associated with endemic cases of diarrhea (16). Outbreaks of respiratory disease have been reported, but data for the role of HPeV in endemic community respiratory disease are scant. In a cohort of 2,200 persons with respiratory symptoms, HPeV was detected in respiratory samples of only 1.2% (76). As with the EV neonatal sepsis, acute flaccid paralysis, aseptic meningitis, encephalitis, and myocarditis have been reported as a result of HPeV infection (particularly type 3). Interestingly, Ljungan virus, a parechovirus previously believed to infect rodents exclusively, has more recently been linked to intrauterine fetal death in humans (77).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Specimen selection is important for making a diagnosis of EV infection, as asymptomatic shedding, especially in stool, is common. The specimen collected should correlate with the clinical syndrome (Table 3). Typically, EV infection begins with viral replication in the epithelial cells of the respiratory or gastrointestinal tract and in the lymphoid follicles of the small intestine, followed by viremia, leading to a secondary site of tissue infection. Hence, for patients with aseptic meningitis, cerebrospinal fluid (CSF) is generally the specimen of choice; however, testing serum can yield better results early in the disease process. Likewise, if CSF is obtained >2 days after the onset of symptoms, the diagnostic yield may be lower. Prescreening of CSF for pleocytosis (lymphocytosis) is not recommended, as lack of pleocytosis, particularly in infants, is common (78–80). Furthermore, in the early stage of the disease, neutrophils may predominate for a few hours. On the other hand, patients with EV encephalitis often have negative CSF PCR results even when they have CSF pleocytosis; in these patients, throat and/or rectal swabs may be the specimen of choice. However, since EVs and HPeVs are excreted from the throat and gastrointestinal tract for prolonged periods (81, 82), their presence from these sites is not necessarily proof of cause. Furthermore, viruses detected from sterile sites can differ by 10 to 20% from viruses detected in throat and rectal swabs, as a result of dual infection (83). In cases of myocarditis and pericarditis, myocardial tissue is recommended over fluid (84). Analyses of blood samples by PCR have been almost uniformly negative (85). However, one report suggests that, at least in pediatric patients, testing of tracheal aspirates may be an alternative to myocardial biopsy (86). For cases of herpangina and HFMD, vesicle fluid is an efficient sample for diagnostic testing, with greater yields observed when multiple vesicles are swabbed.

The EVs are quite stable in liquid environments and are able to survive for many weeks in water, body fluids, and sewage; hence, rapid transport of specimens to the laboratory is generally not necessary. CSF and serum may be submitted to the laboratory in their original containers. Optimal specimens for virus isolation include stool, throat swabs, or throat washings, and swab specimens are best transported and stored in viral transport media. Stool is preferred over rectal swabs because of significantly higher virus yield. Viral infectivity for cell culture is preserved for long periods (years) at −70°C.

Specimen-processing procedures for nucleic acid amplification methods should preserve viral capsid integrity so that the EV RNA genome is protected from nucleases that are ubiquitous in body fluids and the environment. Excessive freeze-thaw cycles should be avoided. Residual samples found in clinical laboratory refrigerators may be suitable for nucleic acid-based detection of the EVs because of the inherent environmental stability of the EVs. Specimens that have been handled by automated chemistry or hematology analyzers should be considered compromised because of the potential for cross-contamination with other specimens.

EVs are highly stable; they can survive at room temperature for several days, and at 4 to −20°C for up to a year (87). However, at least for contrived specimens, room temperature storage for more than seven days negatively impacts reverse transcription (RT-PCR) detection of EVs (87). Long-term storage at −70°C or colder is recommended to prevent degradation of viral RNA.

Certain clinical specimens require processing prior to inoculation of cell cultures. Feces (1 ml if liquid, and approximately pea-sized samples if solid) are added to viral transport medium, vortexed, and centrifuged (1,000 × *g*, 30 min, 4°C). One milliliter of supernatant is removed and added to more viral transport media. Monolayers are inoculated with 0.2 to 0.3 ml of diluted supernatant. Nasopharyngeal aspirates and wash specimens are centrifuged (400 × *g*, 15

TABLE 3 EV and HPeV diseases and specimen selection

Disease	Acceptability of specimen[a]					
	Throat[b]	Rectal/stool[b]	Serum	CSF	Tissue	Conjunctival
Aseptic meningitis			±	+		
Encephalitis	+	+	±	±		
Poliomyelitis	+	+	±			
Respiratory disease	+					
Myocarditis					+	
AHC[c]						+
Febrile illness			+			
Neonatal sepsis			+			

[a] +, specimen is appropriate for testing; ±, specimen may be appropriate for testing.

[b] Associated with low specificity.

[c] Acute hemorrhagic conjunctivitis.

min, 4°C), and cell cultures are inoculated with 0.2 to 0.3 ml of supernatant. Serum, CSF, and urine need no pretreatment and may be inoculated directly onto the cell cultures. However, serum and urine can be toxic to monolayers. Cell death can often be prevented by replacing the culture medium within 24 hours of inoculation. Acid dissociation of antibodies bound to virus may improve detection when attempting to isolate EVs from serum and CSF (88).

Sera for antibody testing are best collected 2 to 4 weeks apart at both acute and convalescent stages. The sera should be frozen at −20°C and tested simultaneously.

DIRECT EXAMINATION

Antigen Detection

Historically, immunofluorescent techniques have not been used for the detection of respiratory infections caused by EVs because these viruses were believed to be associated with self-limited upper respiratory tract infection only.

Nucleic Acid Detection

Currently, nucleic acid amplification tests (NAATs) are preferred for the detection of EV and HPeV. The time required for viral isolation by traditional cell culture (up to 10 days for EV and 21 days for HPeV) is too prolonged to provide clinical utility. Some serotypes within the EV group C (i.e., CVA1, CVA19, and CVA22), as well as HPeV7-16, fail to grow in cell culture. Finally, some specimen types (i.e., 25 to 35% of CSF specimens) have titers that are too low to be detected by cell culture (89). Furthermore, studies have demonstrated that the use of EV NAAT testing has a favorable impact on patient care. Outcomes studies have demonstrated a correlation between time to result and length of stay (80). Antibiotic usage was reduced, fewer ancillary tests were performed, and hospitalization was shortened and less costly when positive EV results were reported within 24 h (79, 90, 91).

NAATs (RT-PCR or nucleic acid sequence-based amplification [NASBA]) are sensitive, specific, rapid, versatile, and clinically useful methods for the detection of EVs (92–94). NAAT of CSF is more sensitive than cell culture, which has a sensitivity of 65 to 75% (94–96). Interestingly, the sensitivity of NAAT for the detection of EV in cases of aseptic meningitis ranges from 70 to 100% despite indications that EVs are the primary causative agent (94, 97–100). One partial reason for this discrepancy is the fact that HPeV, which can account for approximately 5% of cases of neonatal sepsis and aseptic meningitis in the U.S., is not detected by universal EV RT-PCR assays (6).

A limited number of studies have evaluated the sensitivity and specificity of NAAT with other sample types. For serum, the sensitivity and specificity of RT-PCR range from 81 to 92% and from 98 to 100%, respectively, for the diagnosis of EV infection (95, 101). In contrast, 100- to 1,000-fold greater sensitivity of RT-PCR over culture has been reported for detection of EV and HPeV from throat swabs or stool specimens (102, 103). In addition to the enhanced sensitivity of NAAT testing, this technology has the added benefit of rapid turnaround times, with results available in as little as 2.5 hours including extraction (104).

Prior to being tested by NAAT, EV RNA must be extracted from specimens to eliminate ubiquitous RNases and to remove potential amplification inhibitors. Only a small sample size, generally 100 to 200 μl of fluid or 1 mg of tissue, is required. The most common methods for RNA extraction are gel membrane or silica binding. Multiple automated extraction methods have been utilized, including MagNA Pure LC and Compact (Roche Diagnostics System); easyMAG (bioMérieux); and M48, QIAcube, EZ One and QIAsymphony (Qiagen). An EV RT-PCR assay on the GeneXpert system includes extraction, amplification, and detection all in one cartridge (104). All aspects of NAAT testing, from extraction to detection, are discussed in more detail in chapter 6, Molecular Microbiology.

Currently, two Food and Drug Administration (FDA)-approved real-time assays are available for the detection of EV from CSF: Xpert EV and NucliSENS EasyQ Enterovirus (Table 4). In a multicenter analysis, the Xpert EV had

TABLE 4 NAATs for EV and HPeV Detection

Assay (manufacturer)	Method	Target	Regulatory status	Comment	References
Xpert EV (Cepheid)	Real-time RT-PCR	5′ NTR	IVD[a]	Extraction, amplification, detection in a single cartridge	104, 105
NucliSENS EasyQ Enterovirus (bioMérieux)	NASBA	5′ NTR	IVD	Separate extraction required; sensitivity comparable to Xpert EV	106–109
MGB Alert Enterovirus (ELITech)	Real-time RT-PCR	5′ NTR	ASR[b]	Uses patented chemistry	
Multicode-RTx (Luminex Corporation)	Real-Time RT-PCR	5′ NTR	ASR	Uses labeled primer and proprietary chemistry	
Enterovirus Consensus kit (bioMérieux-Argene)	Conventional RT-PCR	5′ NTR	CE	ELISA[c]-based detection	107, 122
Non-commercial EV reagents	Real-time & Conventional RT-PCR	5′ NTR, VP2	LDA[d]		100, 111–113, 121
HPeV Consensus kit (bioMérieux-Argene)	Conventional RT-PCR	5′ NTR	CE	ELISA-based detection	
Non-commercial HPeV reagents	Real-time	5′ NTR	LDA	Detects HPeV1–16	127–130

[a] IVD, *in vitro* diagnostic (FDA-approved test).
[b] ASR, analyte-specific reagent.
[c] ELISA, enzyme-linked immunosorbent assay.
[d] LDA, laboratory-developed assay.

a sensitivity of 95% and specificity of 100% (105). The sensitivity of the NucliSENS assay ranges from 89 to 93% (106–108); however, a limited head-to-head comparison suggests that the sensitivities of the two assays are similar (109). Additionally, 510K-cleared highly multiplexed respiratory virus assays that include the detection of EV and RV are currently available from Luminex Corporation (xTAG Respiratory Viral Panel) and BioFire Diagnostics (FilmArray Respiratory Panel). The Luminex and BioFire kits are only FDA-approved (as *in vitro* diagnostic tests) for nasopharyngeal specimens, and they do not differentiate EV from RV. Limited data have been reported on direct comparisons of EV in these assays, but comparisons have been performed on the overall sensitivity and specificity of all the targets, showing varying results (110).

Analyte-specific reagents (ASR) and laboratory-developed tests (LDT) for EV real-time RT-PCR are also widely used (Table 4). LDTs routinely target conserved sequences within the 5′ NTR or VP2, for nearly universal amplification of EVs (Table 4), with the most frequent 5′ NTR target reported by Rotbart (111) and Chapman et al. (112). Additionally, recent reports have separated the real-time RT-PCR into a two-step assay with cDNA being made with random priming. This modification has improved the sensitivity of the overall detection 10-fold compared with other commercially available kits (113). While these primers do not detect HPeV (discussed below), they do amplify the genomes of several RVs (106, 114, 115) and may miss some strains of EV (116). As with all ASR and LDT, laboratories must develop and validate the assays for themselves.

Despite the increased availability and benefits of real-time PCR, conventional RT-PCR is primarily used for viral typing (117, 118). There is a wide array of detection systems for conventional PCR, including agarose gel electrophoresis, reverse transcription loop-mediated isothermal amplification (119, 120) and colorimetric assays using enzyme-linked immunosorbent assay-type formats (100, 121, 122).

With regards to quality control, EV NAATs should include internal controls to detect amplification inhibitors and to assess nucleic acid recovery, as do the FDA-approved EV detection kits. EV RNA assay-verification experiments and quality-assurance performance demonstrate the need for a universal, nominal EV standard; however, none exists. Instead, most laboratories use either quantified EV isolates or transcripts from clones of target regions, both of which have to be created and validated in the laboratory. As an alternative, some companies offer EV and HPeV viruses for validation and control material. Unfortunately, these viruses are not quantified.

Multicenter proficiency testing programs for LDT EV NAATs indicate that properly designed tests can be equally effective in the detection of EV, regardless of the format used (123). However, other studies have reported marked variation in testing proficiency between centers. In one survey, one-third of participating laboratories were nonproficient in the detection of EV by RT-PCR (87). In another report, 6.8% of participating laboratories recorded false-positive results, pointing to the need for fastidious attention to methods for preventing cross-contamination (123). Overall, these findings underscore the importance of rigorous quality control and periodic proficiency reassessment to ensure uniformly high-quality testing.

Reports detailing the detection of HPeVs by RT-PCR have been published (10, 114, 124). Although the original methods were limited to the detection of HPeV1 and/or 2 (124–126), more recent reports detect at least types 1 to 6 (Table 4) (127–130) and some will detect all 16 (129). To date, an FDA-approved assay for the detection of HPeV has not been released, but LDTs and successful multiplexing of HPeV and EV PCR targets have been reported (131, 132).

ISOLATION PROCEDURES

A combination of human and primate cell lines are typically used for EV and HPeV isolation since no single cell line supports the growth of all types. The general susceptibilities of commonly used cell lines are summarized in Table 5. Isolation times for EVs from CSF using traditional cell culture techniques range from 4 to 8 days, but are shorter (1 to 3 days) from sites with higher viral titers. Recovery time for HPeV is variable and dependent upon type (HPeV1 and 2, 1 to 8 days; HPeV3, 14 to 17 days).

Infections of susceptible cell monolayers with EVs result in a characteristic cytopathic effect (CPE), consisting of shrinkage and rounding of individual cells within the monolayers. The nuclei of infected cells exhibit pyknosis. As infection progresses, the cells degenerate and separate from the monolayer. Often, the CPE is so characteristic that a presumptive diagnosis of EV infection can be made (Fig. 4). However, experience is necessary, as toxic effects from primary specimens, such as *Clostridium difficile* toxin, can mimic CPE (133). Although the CPEs for EV and HPeV are very similar, differences have long been noted. The CPE for HPeV can appear as larger, regularly shaped spheres compared to small irregular shapes caused by EVs (Fig. 4) (134).

The relatively low sensitivity of viral culture from CSF (65 to 75%; reviewed in reference 135) is likely due to the presence of neutralizing antibody, inadequate CSF volume for a comprehensive culture (i.e., inoculate 3 to 5 cell types), low viral load, and resistance of some serotypes to culture. In particular, many serotypes within the EV group C (i.e., CVA1, CVA19, and CVA22) and HPeV7-14 are noncultурable. Historically, stool cultures have been thought to have greater sensitivity for EV/HPeV detection than CSF culture (136); however, in comparison with PCR testing of stool specimens, the sensitivity of stool culture is lacking (103).

Shell vial culture (SVC), in which virus is detected with monoclonal antibodies in the absence of CPE, reduces the time to detection to 2 to 3 days. The sensitivity of SVC may or may not be higher than that of traditional cell culture (137–139), and is probably laboratory- and cell-line dependent (140). SVC using a mixture of cell lines in a single vial (Super E-Mix containing decay-accelerating-factor-expressing BGMK cells and A549 cells, Diagnostic Hybrids Inc. [DHI], Athens, OH) improves sensitivity (139, 140). Evaluation of the capacity of Super E-Mix to isolate HPeV has not been performed; however, it is presumed that at least HPeV1 and 2 would grow in this cell mix because A549 cells are susceptible to infection. The greatest limitation of SVC is the monoclonal antibody used in the detection steps (discussed below).

IDENTIFICATION

Broadly reactive and serotype-specific EV monoclonal antibodies have been developed (88) and applied to cell culture confirmation by immunofluorescence. While preliminary studies suggest that these reagents, used singly and in blends (DAKO-Enterovirus 5-D8/1 [DAKO, Glostrup, Denmark] and Pan-Enterovirus blend [Chemicon International Inc.]), demonstrated an important role in serotype identifications,

TABLE 5 Susceptibilities of cell lines commonly used for isolation of EV and HPeV

Cell line	Susceptibility to virus[a]									
	Polio	Coxsackie		Echo	HPeV					
		A[b]	B		1	2	3	4	5	6
Simian (monkey kidney)										
Primary rhesus (pRhMK)	+++	+	+++	+++	+	+	unk	unk	unk	unk
Primary cynomolgus (pCMK)	++++	+	+++	+++	+	+	unk	unk	unk	unk
Buffalo green (BGMK)	+++	+	++++	++	++++	+	+++	−	−	−
African green (Vero)	+++	+	+++	++	++++	+	+++[c]	++	+	++
Rhesus (LLC-MK2)	+++	+	+++	+++	+++	++	+++	−	−	−
Cynomolgus (tMK)	+++	+	+++	+++	++	++	+	++	+	−
Human										
Human cervix adenocarcinoma (HeLa)	+++	+	+++	+	++	+	−	+	unk	unk
Human colon carcinoma (HT-29)	+++	+	+++	+++	+++	+++	−	+++	+++	+++
Human colon carcinoma (CaCo-2)	+++	+	+++	+++	++	−	+++	+	−	−
Human embryonic lung fibroblast (HEL)	+++	++	+	+++	−	−	−	−	−	−
Human embryonic lung fibroblast (MRC-5)	+++	+	+	+++	+	+	−	unk	unk	unk
Human embryonic lung fibroblast (WI-38)	++	++	+	+++	+	+	−	unk	unk	unk
Human epidermoid carcinoma (HEp-2)	+++	+	+++	+	++	++	−	unk	unk	unk
Human kidney epithelial (293)	+++	+	++	+++	++	++	−	unk	unk	unk
Human lung carcinoma (A549)	+++	+	+++	+++	+++	+	++	+	+	−
Human rhabdomyosarcoma (RD)	+++	+++[d]	+	+++	+	+	−	+	+	−
Super E mix (combination of BGMK-hDAF and CaCo-2)[e]	++	unk	++++	++++	++	++	unk	unk	unk	unk

[a] Relative susceptibilities: +, minimally susceptible; ++++, maximally susceptible; −, non-susceptible; unk, unknown (no published reports). Some EVs are difficult to isolate even on minimally susceptible cell lines.

[b] Some CV-A (A1, A19, and A20) are not readily isolated.

[c] Improved yields after passage.

[d] Many CV-A only grow in RD cells.

[e] BGMK-hDAF, BGMK expressing human decay-accelerating factor; CaCo, human colon adenocarcinoma cells.

further studies have identified several limitations (137, 138, 141). The most notable limitations are cross-reactivity with the Chemicon blend (137) and the lack of EV71 detection with the Dako blend. A pool of anti-EV antibodies has been developed (D3 IFA Enterovirus Identification, DHI) which reportedly has good sensitivity, including the detection of EV71 with little or no cross reactivity. However, no peer-reviewed data on this product have been reported. The concordance of results for identification of clinical EV isolates to the species level using monoclonal blends versus the neutralization assay has demonstrated the latter may be significantly superior for the identification of the EVs (141). These studies appeared to indicate that monoclonal antibodies for EV identification should be used as a preliminary screen for species or serotype identification. With regards to HPeV, typing reagents are only available for types 1 and 2, and not all HPeV-2 isolates are neutralized by specific antisera (142).

TYPING SYSTEMS

This virus family exhibits a considerable amount of genetic variability driven by both mutation and recombination (143, 144). However, serotype determination is usually unnecessary for clinical purposes because the diseases caused by the EVs are not serotype specific. Currently, the identification of EV type is most useful for distinguishing between VDPV and NPEVs to facilitate the interpretation of viral culture results in areas where the Sabin PV strains are used for vaccination, as the live vaccine can be shed for 1 to 2 weeks from the throat and can remain in the feces for several

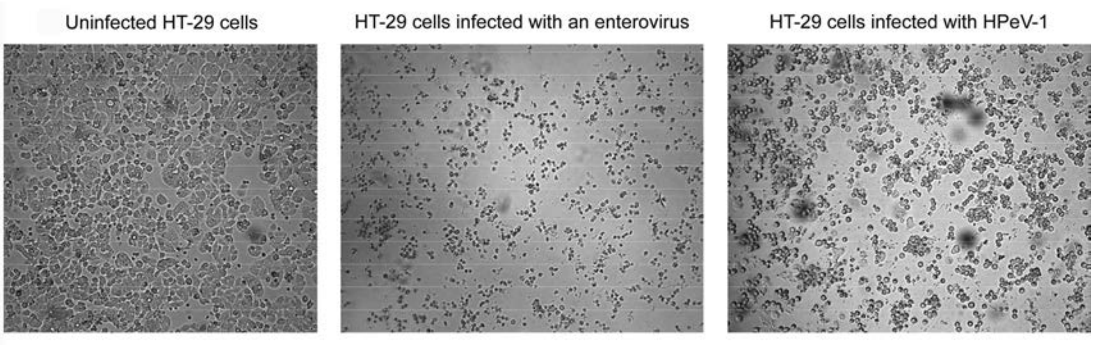

Uninfected HT-29 cells
HT-29 cells infected with an enterovirus
HT-29 cells infected with HPeV-1

FIGURE 4 Cytopathic effects observed 3 days after infection of HT-29 cells with an EV or HPeV1 isolate after passage on this cell line. Reprinted from reference 134.
doi:10.1128/9781555817381.ch88.f4

weeks to months (145). Identification of NPEV serotypes is useful for epidemiological purposes and for determination of serotypes associated with unusual or novel clinical manifestations, such as flaccid paralysis (1).

Historically, EV serotype determination involved neutralization with the Lim and Benyesh-Melnick (LBM) pools of antisera; however, several limitations exist for these methodologies. The pools identify only 40 of the 64 originally described EV serotypes, and genetic drift of the EVs over time has given rise to antigenic variants. Furthermore, the procedure is labor-intensive and the supply of LBM pools available from the World Health Organization is limited (distribution is restricted to reference laboratories).

Sequencing of the *VP1* gene is now the primary method used for typing. Specifically, a 340-bp region that encodes the serotype-specific neutralization epitopes of *VP1* is amplified, sequenced, and analyzed against a sequence database for known EVs (118, 146). A threshold of 25% nucleotide and 12% amino acid divergence generally corresponds to the previous serological division of EVs into members of the same or different serotypes (4). A pyrosequencing method based on the assay reported by Nix et al. (118) has also been developed (147). Molecular typing methods reduce the testing time by weeks (146) over classical serotyping. In addition, these methods of "molecular serotyping" have been extremely useful in typing the isolates that could not be neutralized by traditional LBM pools and were therefore classified as "nontypeable" EVs and in identifying multiple new EV serotypes and novel HPeVs.

Various other molecular methods have been described for either species-specific typing (i.e., PV) or for the detection of multiple EV types. PV-specific typing includes PCR restriction fragment length polymorphism analysis (148), probe hybridization (149), reverse transcription-loop-mediated isothermal amplification (120), and PV type-specific PCR assays (150). Line blot hybridization (151) and microwell oligonucleotide arrays (152) have been reported for rapid typing of multiple EV types.

SEROLOGIC TESTS

In the clinical setting, situations may arise where EV serology is requested; such is the case for myocarditis and determination of congenital infection in pregnant women. A recent study by Mahfoud et al. (153) showed that endomyocardial biopsy should be used instead of EV serology for patients with myocarditis. Complement fixation testing is available from a few commercial laboratories in the United States but is generally not considered a relevant test for the diagnosis of EV infection as these assays lack specificity (24). Classically, serologic diagnosis of EV infection involved comparing antibody titers in acute- and convalescent-phase serum in a neutralization assay, with a 4-fold or greater rise in type-specific antibody titer considered as diagnostic of recent infection. However, acute-phase sera are often not obtained because of nonspecific presentations early in EV disease.

Type-specific enzyme-linked immunosorbent assays for the detection of EV serotype-specific IgM antibodies (i.e., CVB- and EV71-specific IgM), as well as heterotypic assays for the detection of IgA, IgM, and IgG antibodies against EVs in general, have been developed (154, 155). Homotypic assays are more relevant to epidemiologic studies than to clinical diagnosis as they appear to be less sensitive than PCR for the diagnosis of EV infection but they have been successfully applied in such research settings (1, 156). Furthermore, the IgM response to EVs can be nonspecific and can lead to false-positive results (157, 158). Although cross-reactivity with non-EV pathogens has not been studied thoroughly, sera from patients infected with acute hepatitis A virus (a member of the *Picornaviridae* family) have been reported to produce a significant number of false-positive results in an enzyme-linked immunosorbent assay for detection of enterovirus IgM antibodies (155). Recently, PCR-enhanced immunoassays for the detection of EV-specific IgM have been evaluated (159). Such systems improve the sensitivity and specificity of immunoassays because the ligand is further amplified by PCR after immune capture (160). However, these methods are technically challenging to establish in the laboratory and are clearly investigational.

ANTIVIRAL SUSCEPTIBILITIES

There is no FDA-approved antiviral agent available for the treatment of EV or HPeV infections. However, several compounds targeting viral capsid proteins, proteases, polymerase, and other proteins are being developed, including some agents in phase-III clinical trials (161). A promising investigational drug, the WIN compound pleconaril, underwent extensive *in vitro* susceptibility testing against EVs, and clinical trials, but was not licensed for use by the FDA (162, 163). An antiviral compound with potent activity against the polioviruses is currently undergoing evaluation (164, 165).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

An understanding of the sites where asymptomatic shedding and disease-induced replication occur is critical to the interpretation of EV and HPeV test results. Detection of these viruses in the CNS, bloodstream, lower respiratory tract, and genitourinary tract implies a true invasive infection and a high-level likelihood of association with current illness. In contrast, they can be shed asymptomatically from the nasopharynx and the gastrointestinal tract for weeks to months. Detection of EVs by virus isolation or NAAT at these sites must be interpreted cautiously because their presence alone does not establish a diagnosis. EV in the feces or nasopharynx of a patient with meningitis may represent residual shedding from an infection from weeks before and may be unrelated to the current illness.

In young children, an additional factor potentially complicating the evaluation of results from these body sites is the administration of live attenuated oral PV in the first years of life. While the vaccine is no longer used in the United States (145), the majority of countries worldwide, including some in Europe, still employ oral PV as part of their vaccination regimes. Reporting an EV isolate in a setting where oral PV is used without specifying whether it is a PV or an NPEV can lead the physician to wrongly discontinue antibiotic or anti-herpes therapy in the belief that an EV etiology has been established. The distinction between PVs and NPEVs from the CNS, bloodstream, lower respiratory tract, and genitourinary tract is less important because VDPVs have rarely been isolated from such specimens, and may actually be causing the illness in question in those rare instances (166, 167).

A further cautionary note is warranted with regard to coinfections of the central nervous system with EVs and bacteria (142). In these rare instances, illness was clinically compatible with bacterial meningitis and EV was later isolated incidentally. Because of the severity of disease, antibiotics would have been used regardless of the sequence of pathogen detection. When the clinical presentation is typical of viral meningitis, coinfection with a clinically "silent" bacterium would be extraordinarily unlikely. Hence, identification of an EV from a sterile site in a patient with a clinically compatible illness is sufficient evidence for establishment of EV causality. HPeV NAAT testing should be considered if negative results are obtained for EVs in the setting of clinically compatible disease.

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Rhinoviruses

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TAXONOMY

Human rhinoviruses (HRVs) are members of the family *Picornaviridae*. Previously a separate genus, HRVs have been reclassified into three separate species (A, B, C) within the *Enterovirus* genus (1). Other *Picornaviridae* genera that are pathogenic for humans include *Parechovirus*, members of which have been associated with respiratory disease, and *Hepatovirus*. Recently, the *Cardiovirus* genus has been found to contain human pathogens as well (2).

HRVs derive their name from the predominant site of their replication and symptomatology, the nose. HRV isolates were originally classified into 99 serotypes (HRV1 through -99) based on neutralization with type-specific antisera; subsequent direct sequencing studies have shown that there are in fact many more types that have not been cultured and typed serologically (see below). While some cross-neutralization occurs, there is no group antigen (3, 4). HRV serotypes were further classified into two species—HRV-A, containing 74 serotypes, and HRV-B, containing 25 serotypes—based on sequence relatedness. The original 99 HRV prototype strains isolated in the 1960s and 1970s have been propagated and maintained by the American Type Culture Collection. One former HRV serotype, HRV87, has been shown by sequence analysis to be an acid-sensitive strain of human enterovirus (HEV) 68 (5, 6).

HRV serotypes can be further divided into two groups based on receptor binding (7, 8): (i) a major group (63 HRV-A and 25 HRV-B serotypes) that binds intercellular adhesion molecule 1 (ICAM-1), a member of the immunoglobulin supergene family that is expressed on the surface of many different cells (9); and (ii) a minor group (11 HRV-A serotypes) that binds to members of the low-density lipoprotein receptor family (8). Two different drug-reactivity groups of HRVs have also been identified based on composite sensitivities across a panel of antiviral compounds (10).

The wider use of molecular methods in viral diagnostics has led to the discovery of a novel group of HRVs identified in patients with lower respiratory tract disease. Classified as a new species, "C," these viruses are not new or recently emerging viruses (11) but have been circulating unnoticed due to their lack of growth in standard cell culture systems used for HRV isolation. Recently, HRV-C viruses were grown in human sinus epithelium organ cultures, where they were shown to utilize an as-yet-unknown cellular receptor other than ICAM-1 or low-density lipoprotein receptor (LDLR) (12).

Sequencing of the complete genomes of the 99 HRV-A and -B serotypes and available HRV-C strains confirmed clustering of all strains into three distinct phylogenetic groups (13). Because serotyping HRV-C was not possible due to its refractoriness to growth in cell culture, a genetically based classification system was developed for assigning HRV genotypes while retaining the serotype numbering system (14). To date, 51 HRV-C genotypes have been assigned using a threshold of 13% nucleotide differences in the *VP1* coding region from all HRV prototype strains or a 10% nucleotide difference in the *VP4/VP2* region if the VP1 sequence is unavailable; thresholds of 13% and 12% nucleotide differences in *VP1* for HRV-A and HRV-B type assignments, respectively, were also proposed (15). Designation of an additional six HRV-A and three HRV-B genotypes using the above thresholds brings the total number of currently recognized HRV types to 159.

DESCRIPTION OF THE AGENT

The HRV genome is a single-stranded, positive-sense RNA about 7,200 nucleotides in length. The genome is composed of a 5′ untranslated region (5′ UTR) coupled with a short peptide (VPg) that serves as a primer for genome replication (16), followed by a single open reading frame that terminates in a 3′ poly (A) tail (Fig. 1). Secondary structures within the 5′ UTR, a cloverleaf domain and an internal ribosomal entry site (IRES), are necessary for protein translation. The single polypeptide product of the open reading frame is subsequently cleaved into 11 viral proteins: 4 structural proteins that form the viral capsid (1A-1D or VP4-VP1) and 7 nonstructural proteins that are involved with virus replication or polyprotein processing (2A-2C and 3A-3D). The 3C protease participates in cleavage of polypeptide and is a target for antiviral drugs.

The HRV virion is a small (20 to 27 nm in diameter), nonenveloped icosahedron composed of 60 protomeric units, each consisting of the four structural protein subunits (VP1-VP4). VP1, VP2, and VP3 reside on the exterior of the virion and make up its protein coat. VP1 is the largest, most external and immunodominant protein of the virus capsid (17). A number of major neutralization sites reside in the VP1 proteins. Located within VP1 is a hydrophobic pocket into which antiviral compounds such as pleconaril bind (18). VP4 resides inside the protein shell. X-ray crystallography and cryoelectron microscopy studies have identified large depressions or "canyons" on each of the 60

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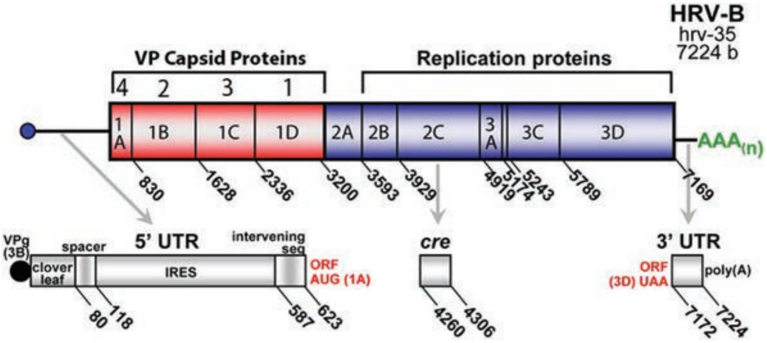

FIGURE 1 HRV genome structure. The genome consists one single open reading frame flanked by 5′ and 3′ untranslated regions (UTR). A small viral protein, VPg, is covalently linked to the 5′ UTR. VP, viral protein; IRES, internal ribosome entry site; cre, *cis*-acting replication element. Reproduced with permission from reference 22. doi:10.1128/9781555817381.ch89.f1

protomeric units, which appear to be sites for cell receptor binding and also play a critical role in conformational changes that follow attachment (19). Conformational changes in the canyon floor can also be induced by certain antiviral agents, thus inhibiting virus attachment to cells or virus uncoating (18).

HRVs replicate in the cytoplasm of infected cells, producing infectious virions that sediment at a buoyant density of 1.40 g/liter in cesium chloride. Empty capsids and particles lacking one or more structural polypeptides are also produced.

Mutation and recombination are two mechanisms that drive the extensive genetic diversity of HRVs and serve as the basis for HRV evolution. The RNA-dependent RNA polymerase, designated 3D polymerase, which facilitates synthesis of new viral genome, has no proofreading capacity and frequently generates mutations. Evidence of recombination between HRV-A and HRV-C within the 5′ UTR has also been shown: on phylogenetic analysis, the 5′ UTR sequences of some HRV-C genotypes cluster within the HRV-A clade (20, 21). Although HRV-B and HRV-C show little evidence of recombination within the genome coding region, recent studies have shown that HRV-A sequences have undergone extensive recombination during the early stage of diversification into types, and several contemporary HRV-A strains have been formed by recombination (22, 23).

As they have no lipid envelope, HRVs are fairly resistant to inactivation by organic solvents such as ether, chloroform, ethanol, and 5% phenol. Although HRVs are relatively thermostable, heating at 50 to 56°C progressively reduces infectivity. HRVs are traditionally differentiated from HEVs by their loss of infectivity on exposure to pH 3 for 3 hours at room temperature, though this may not be the case with all serotypes and strains.

EPIDEMIOLOGY AND TRANSMISSION

HRV infections are widespread and occur year-round. In temperate zones, transmission typically peaks in autumn and late spring (24). Some recent studies using molecular methods have also found substantial infections in summer and winter (24–26).

Seasonal fluctuations have been attributed to improved survival of HRV in conditions of high relative indoor humidity and herding together of children when school opens in the fall (27). HRV-A and HRV-C may cocirculate in equal proportions or may alternate as the predominant species (28–30). HRV-B is consistently detected less often (30). As the disease burden and diversity of HRV infections have become evident, the interest in HRV genotyping, molecular epidemiology, and virulence has intensified (11).

Studies in volunteers have shown that inoculation of virus into the nose or conjunctivae is the most efficient way to initiate infection, although virus can also be transmitted by aerosol (31, 32). HRV is present in highest titers in the noses of infected persons and commonly contaminates their hands. Consequently, investigators have found that HRV can be transmitted via hand-to-hand contact (33) or by contaminated fomites, followed by self-inoculation of virus into the nose or conjunctivae (34). Furthermore, transmission can be interrupted by using tissues impregnated with virucidal agents for nose blowing, by treating surfaces with disinfectant, or by applying iodine to the fingers (35). Recent studies have found ethanol hand sanitizers to be more effective than soap and water, and virucidal activity of the sanitizer can be enhanced and prolonged by the addition of organic acids (36). However, a reduction in transmission in a natural setting has not been established (154).

HRVs replicate primarily in airway epithelium, but also in the middle ear and sinuses, with an incubation period of 1 to 4 days (37). Peak of virus shedding coincides with the acute rhinitis. Symptoms last 7 days, on average, but can persist for 12 to 14 days or more (38, 39) and typically include profuse watery discharge, nasal congestion, sneezing, headache, mild sore throat, cough, and little or no fever. By culture, virus may become undetectable at 4 to 5 days or may be present in low titers for up to 2 to 3 weeks (40). Asymptomatic HRV infections are also common, occurring in 20 to 30% of infected persons (24, 41–43). Using reverse transcription PCR (RT-PCR), HRV RNA has been detected by some investigators 4 or 5 weeks after the onset of symptoms and, surprisingly, for 2 to 3 weeks prior to onset of symptoms (42, 44). However, genotyping has revealed that such prolonged shedding represents a series of sequential HRV infections with different HRV types, some asymptomatic (45). As many as 74 distinct HRV types have been found to be cocirculating, including multiple types in the same household (45, 46). Chronic shedding of a single HRV type has been documented only in immunocompromised hosts (47).

HRV causes minimal pathology in the nasal epithelium, and the symptoms of HRV infection parallel the rise and fall of chemical mediators of inflammation (34, 35). Psychological stress and inadequate sleep appear to increase susceptibility and the development of clinical symptoms (48–50), whereas exposure to a cold environment does not (51). Early studies concluded that immunity is type specific and correlates best with local production of IgA (52, 53).

CLINICAL SIGNIFICANCE

HRVs cause approximately two-thirds of cases of the upper respiratory syndrome known as the common cold and thus are responsible for more episodes of human illness than any other infectious agent (24, 35). Although considered a trivial illness, the common cold is acutely disabling and its cost, in days lost from work, cold remedies, and analgesics, is estimated at $40 billion annually in the United States (54).

With the use of molecular methods, more severe consequences of HRV infections have been increasingly recognized. HRVs can be the sole etiology of sinusitis and otitis media, as well as facilitating secondary bacterial infections (55, 56). HRVs have been implicated as the major viral cause of exacerbations of asthma, cystic fibrosis, and chronic obstructive pulmonary disease (COPD) (57–59). Indeed, HRV wheezing illness in infancy has been linked to childhood-onset asthma in genetically susceptible children (60). The impact of disease in the elderly is also substantial. In a Canadian study, 59% of outbreaks of respiratory disease in long-term care facilities were found to be due to HRVs, and some disease was severe (61).

HRV has been increasingly detected in patients with lower respiratory tract infections (62, 63); in patients hospitalized with wheezing or pneumonia (64–66), including school-age children (67); the elderly (27, 68); and those with chronic illnesses, cancer, immunosuppressive illnesses, transplants (69–74), or underlying pulmonary disease (75). The clinical manifestations of HRV in hospitalized infants are similar to those caused by respiratory syncytial virus (RSV); however, the mean age of HRV-affected infants tends to be slightly older (66). HRVs are commonly found either singly or in combination with other viruses. HRVs have been shown to impair the innate immune response (76, 77) and may predispose to invasive pneumococcal disease in children and bacterial superinfections in COPD (77, 78).

HRV-C viruses have been implicated in some studies as the predominant HRV species linked to hospitalizations for fever, wheezing, and lower respiratory tract disease, especially in young infants and asthmatic children (28, 79–84). In contrast with classic HRV-A and HRV-B, HRV-C replicates equally well at 37 and 34°C, facilitating infection in the lower respiratory tract (85). However, additional population-based studies are needed before definitive conclusions can be made about the prevalence and virulence of HRV species (37).

Although HRV viremia has rarely been identified by culture, HRV RNA has been detected using sensitive molecular methods in the blood of HRV-infected young children with severe respiratory illness (37), including 25% of those with HRV-associated asthma exacerbations (43). HRVs have also been detected in fecal specimens of children with meningitis, pericarditis, and gastroenteritis and even in a healthy child (86–89).

Because HRVs have historically been difficult to culture and serotype, little information is available on the relationships between serotype, clinical manifestations, and virulence (90). With the development of genotyping methods and increased awareness of the disease burden of HRV, this currently is an area of intense study (24, 37, 89).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

In natural infections, HRV can generally be isolated in culture from 1 day before to 6 days after onset of cold symptoms but is shed in highest concentration on day 1 or 2 of illness. In upper airway disease, HRV is excreted in highest titers from the nose; thus nasal rather than throat specimens should be obtained for diagnosis.

Early comparisons of nasal wash, nose swab, throat gargle, and throat swab specimens for the isolation of HRV from clinical specimens revealed nasal washes to be the best (53, 91), which are obtained as follows. Tilt the patient's head backwards and instill 1 ml of sterile phosphate-buffered saline (PBS) into one nostril. Then ask the patient to lean forward and allow the washing to drip into a sterile petri dish or other collection container. Repeat with the other nostril until each nostril has been washed with 5 ml of PBS. The washings are then transferred into a sterile container. However, nasal wash samples are cumbersome to obtain in most clinical settings.

For many years, nasopharyngeal (NP) aspirates have been the standard for infants and young children, but the most convenient and commonly collected sample at present is an NP swab placed in viral transport medium (VTM). Well-collected NP swabs can provide detection rates comparable to aspirates and are simpler to obtain (92, 93). A recent study found that, while nasal wash detected more HRV positives than NP swabs, the difference was not statistically significant when flocked swabs were used (94). Flocked swabs have been shown in some but not all studies to provide superior samples to cotton and some other swab types. However, due to the small, flexible shaft, flocked NP swabs are consistently rated as more comfortable for the patient than rigid-shaft, large-tipped collection swabs (94). Mid-turbinate flocked swabs provided a yield similar to NP swabs results in one report and are amenable to both self-collection and parents' collection of samples from young children (95, 96). Sputum has been reported to be comparable in yield to NP aspirates for respiratory viruses (97).

To diagnose lower respiratory tract infection, endotracheal aspirate, bronchoalveolar lavage, bronchial wash, or lung biopsy samples should be collected. Sputum, including induced sputum in children, has been recently found to be useful for diagnosing viral lower respiratory tract disease (93, 98).

All specimens should be transported promptly to the laboratory. For isolation, best results are obtained with prompt inoculation of cell cultures; however, specimens can be held up to 24 hours at 4°C in VTM with neutral pH. If longer delays are necessary, specimens should be frozen at –70°C and thawed just before inoculation. Freezing does not appear to be detrimental to virus recovery (91). Similar sample handling is recommended for molecular analysis; however, the compatibility of various VTMs with different extraction reagents must be confirmed in each laboratory, and manufacturers' instructions should be followed for all commercial kits.

DIRECT EXAMINATION

Antigen Detection

Due to the large number of serotypes and the lack of a common group antigen, antigen detection assays are not used in clinical practice.

Nucleic Acid Detection

Conventional RT-PCR Assays

Wider use of molecular diagnostics has dramatically increased HRV detection and greatly enhanced our appreciation of the role of these viruses in respiratory disease. RT-PCR assays have proven more sensitive than culture methods, doubling or even tripling the number of HRV infections detected (41, 99–102), and are clearly superior for detection of noncultivable HRV strains. Nevertheless, culture can occasionally recover some HRVs missed by RT-PCR due to variability in techniques and primer or probe mismatches (100, 102, 103).

The first RT-PCR diagnostic assay described for HRVs was reported by Gama et al. in 1988 (104). Since then, numerous molecular assays have been described. These assays typically target the 5′ UTR of the viral genome that contains highly conserved sequences suitable for molecular assay development. However, most of these assays also detected HEVs because the primer sets used could not effectively distinguish between these two virus groups. Earlier strategies to distinguish between HRVs and HEVs included differential amplicon sizing (105, 106), restriction enzyme digestion of a common-sized amplicon (107), internal probe hybridization (103, 108), and nested PCR using HRV-specific primers (84) or annealing and extension times optimized for HRV detection (101). Most of these reported methods failed to detect or accurately differentiate all viruses tested, and none evaluated all HRV and HEV types. RT-PCR and sequencing of amplicons to differentiate HRVs from HEVs and identify virus types (see below) are now commonly used by specialized reference laboratories but are still not easily implemented for routine clinical diagnosis (109).

At present, molecular methods are not standardized, and those published have differed in types of samples tested, methods of RNA extraction used, primer selection, and amplification and detection conditions. A comparison of the relative sensitivity of 11 published HRV primer pairs using clinical specimens positive for the three recognized HRV species demonstrated an overall discordance in performance, especially for specimens with lower virus loads (110).

Real-time RT-PCR Assays

More recently, real-time RT-PCR (rRT-PCR) assays for HRVs using SYBR Green, TaqMan hydrolysis probes, or molecular beacons have been developed (39, 72, 87, 109, 111–113). Real-time amplification methods have the advantages of simplicity, reduced risk of amplicon contamination, ability to estimate viral load, and a shortened time to result because detection occurs concurrent with amplification. Although SYBR Green and melt-curve analysis are less expensive than probe-based methods, results obtained using these techniques can be difficult to interpret (39).

A recently developed rRT-PCR assay (39) targeting the 5′ UTR was shown to detect all recognized HRV prototype strains as well as the novel species C viruses; it has been used successfully in studies to determine the epidemiology and clinical features of HRV infections in different populations (28, 114, 115). Although much effort has been devoted to improving the specificity of this assay, it still reacts with some HEVs when present at high titer in the clinical specimen.

Several HRV studies have demonstrated a positive correlation between viral load and disease severity, suggesting that virus quantitation may help clarify the clinical relevance of a positive test result (88, 116–119). Although a recent study has shown that rRT-PCR assays can be used to semiquantify HRV RNA in clinical material, accurate absolute HRV RNA quantification in respiratory specimens is complicated by (i) the lack of an accurately quantified international reference RNA standard, (ii) sequence variation among HRV strains that may differentially affect rRT-PCR amplification efficiency, and (iii) variability in sample collection procedures (120).

Multiplexed RT-PCR Assays

Numerous laboratory-developed and commercial multiplex RT-PCR assays based on different amplification platforms and combining multiple respiratory pathogens, including HRV, have been described in recent years. There are currently four commercial multiplex tests that include HRV that have been cleared for *in vitro* diagnostic use in the United States by the U.S. Food and Drug Administration (FDA): the Luminex xTAG RVP and Luminex xTAG RVP Fast (Luminex Molecular Diagnostics, Austin, TX); the FilmArray Respiratory Panel (RP) (BioFire Diagnostics, Salt Lake City, UT); and the eSensor Respiratory Viral Panel (RVP) (GenMark Dx, Carlsbad, CA) (Table 1).

The Luminex xTAG RVPv1 and xTAG RVP Fast utilize multiplex RT-PCR followed by amplicon identification using a fluid-microsphere-based array flow cytometry (121, 122) for detection of 12 and 8 viruses and subtypes, respectively. The FilmArray RP integrates sample preparation, amplification, and detection into one simple system using a preloaded blister pouch solid array and endpoint melting curve analysis to detect and identify 20 different respiratory pathogens in about 1 hour. The eSensor RVP uses competitive DNA hybridization and electrochemical detection to identify 14 different respiratory virus types and subtypes. Neither the FilmArray RP nor the LuminexTAG assays distinguish between HRV and HEV, while the eSensor RVP claims to be specific for HRV only (123, 124). Most commercial molecular assays require purchase of a dedicated instrument to perform. In contrast, the FTD respiratory pathogens multiplex assay (Fast-track Diagnostics, Luxembourg) uses standard real-time hydrolysis probe chemistries and common real-time PCR instrumentation for detection of 21 respiratory pathogens, allowing easy integration into the workflow of laboratories already using standard real-time PCR platforms (125).

At present, few clinical laboratories are either interested in or able to set up homebrew RT-PCR assays for HRV; even if available commercially, these assays may not be used unless they are part of a more comprehensive panel of respiratory pathogens.

These comprehensive multiplex assays have confirmed the frequent detection of HRVs in culture-negative clinical samples and have revealed many mixed pathogen infections. However, most are not quantitative, and all are costly to implement and maintain. Each commercially available system has its unique advantages and disadvantages, and each user should determine which system is appropriate for his or her specific diagnostic needs.

Although much effort has been devoted to distinguishing HRVs from HEVs in the clinical setting, a single assay that detects both virus groups could ultimately prove to be advantageous since some antivirals may be effective against both virus groups (38). Moreover, some HEVs (e.g., HEV-68, CoxA21) can also cause respiratory diseases (126–130). Thus, some multiplexed assays have not attempted to separate the 2 genera but instead target both (Table 1).

TABLE 1 Commercial multiplex molecular assays for respiratory pathogens, including HRV

Company	Assay	U.S. FDA[a]	Dedicated instrument	RT-PCR	Detection	Post-PCR handling	HRV, HEV, HRV/HEV[b]	No. viral/ bacterial pathogens identified	Company website	Key references
AutoGenomics	INFINITI RVP Plus	RUO	Yes	Conventional	Solid array	No	HRV (A/B), HEV	25	www.autogenomics.com/	145
bioMérieux	FilmArray Respiratory Panel	IVD	Yes	Real-time	Melting curve analysis	No	HRV/HEV	20	www.biofiredx.com/	146
Fast-track Diagnostics	FTD Respiratory pathogens 21	RUO	No	Real-time	Hydrolysis probe	No	HRV/HEV	21	www.fast-trackdiagnostics.com/	125
GenMark Dx	eSensor Respiratory Viral Panel	IVD	Yes	Conventional	Electrochemical	Yes	HRV	14	http://www.genmarkdx.com/	124
iCubate	Respiratory-V Cassette	RUO	Yes	Conventional	Solid array	No	HRV, Cox/EcV	20	www.icubate.com/	
Luminex	x-TAG RVP & RVP Fast	IVD	Yes	Conventional	Liquid bead array	Yes	HRV (A/B/C)/ HEV	USA (12 & 8); Europe (18/16)	www.luminexcorp.com/	147–150
Nanosphere	Verigene Respiratory Pathogens	RUO	Yes	Conventional	Solid array	No	HRV (A/B/C)/ HEV	15	www.nanosphere.us/	
PathoFinder	RespiFinder SMART 22 & SMART 22 Fast	RUO	No	Real-time	Melting curve analysis	No	HRV/HEV	21	www.pathofinder.com/	151, 152
Qiagen	ResPLEX II	RUO	Yes	Conventional	Liquid bead array	Yes	HRV, Cox/EcV	16	www.qiagen.com/	151
Seegene	Anyplex II RV16	RUO	No	Real-time	Melting curve analysis	No	HRV (A/B/C), HEV	16	www.seegene.com/	149
	Seeplex RV15 OneStep ACE Detection	RUO		Conventional	Amplicon size	Yes		15		

[a] RUO, research use only; IVD, FDA approved for use as *in vitro* diagnostic.

[b] Based on company literature: HRV, detects HRV (species, if not indicated); HEV, detects HEV; HRV/HEV, detects both HRV and HEV but indistinguishable; Cox/EcV, detects coxsackievirus types A and B and echovirus.

ISOLATION PROCEDURES

Cell Culture

Rhinovirus groups A and B grow only in cells of human or monkey origin. HRV group C has not yet been grown in cell culture. Although the original isolation of rhinoviruses was in primary monkey kidney cells, these cells have not been consistent in yielding a broad range of isolates. The most commonly used cells in clinical laboratories are the human embryonic lung fibroblast strains WI-38 and MRC-5. WI-38 cells are significantly more sensitive than MRC-5 (91), but MRC-5 cells are more commonly available. Human embryonic kidney (HEK) can also support rhinovirus replication. Unfortunately, different lots of normally sensitive cell lines have been found to vary over 100-fold in sensitivity to rhinovirus (10); the reasons for this variation are not known. Therefore, for optimal results, simultaneous use of at least two sensitive systems is recommended.

In the research setting, several HeLa cell clones, such as HeLa M or Ohio HeLa cells, HeLa H, HeLa R-19, and HeLa I cells, have been shown to support the replication of rhinoviruses to high titers (91). HeLa I cells were found to be more sensitive than WI38, MRC-5, fetal tonsil, or HeLa H or M cells for recovery of rhinovirus from clinical specimens (131). These specialized cell lines can be obtained only from research laboratories.

After inoculation, cultures are incubated in standard cell culture medium such as Eagle minimum essential medium (MEM) with 2% fetal calf serum and antibiotics at a neutral pH. To mimic conditions of the nose, cultures should be incubated at 33 to 35°C with continuous rotation in a roller drum to provide aeration of the monolayer. Rhinovirus cytopathic effects (CPE) can be observed as early as 24 to 48 hours after inoculation and are often detected by day 4. Passage of infected HeLa cell cultures may be necessary for some isolates before CPE is apparent. In fibroblasts, cellular changes are easier to read and are often detected earlier than in epithelial cell lines. Both large and small rounded, refractile cells with pyknotic nuclei are observed in foci that also contain cellular debris (Fig. 2). Rhinovirus CPE are similar to HEV CPE but may sometimes be confused with nonspecific changes. The CPE progresses over a 2- or 3-day period, with the degree of cellular change depending on the serotype and the inoculum dose. It should be noted that rhinovirus CPE can regress, or virus may inactivate if left too long. Therefore, cultures should be promptly passaged. Passage is also necessary to increase viral titers prior to the performance of identification tests.

Human embryonic lung fibroblast cell cultures should be observed for 14 days, with refeeding at 7 days to increase recovery of virus. HeLa cell cultures can be observed for only up to 7 to 8 days, when passage becomes necessary due to nonspecific cell degeneration and rounding.

Rapid centrifugation cultures using the HuH7 hepatocellular carcinoma cell line have been reported to detect respiratory viruses including HRV. After 4 days of incubation, HRV can be detected in the amplified culture by RT-PCR. While results were faster, HuH7 cells were not as sensitive as MRC5 conventional cultures incubated for 14 days (99).

Organ Culture

Organ cultures of human fetal nasal epithelium or trachea were used in the past to isolate rhinoviruses not grown in standard cell cultures but are no longer used for this purpose. Recently, however, cultures of nasal, sinus, and tracheal mucosa obtained from biopsy material have been used for research studies of rhinovirus pathogenesis (12, 85, 132, 133).

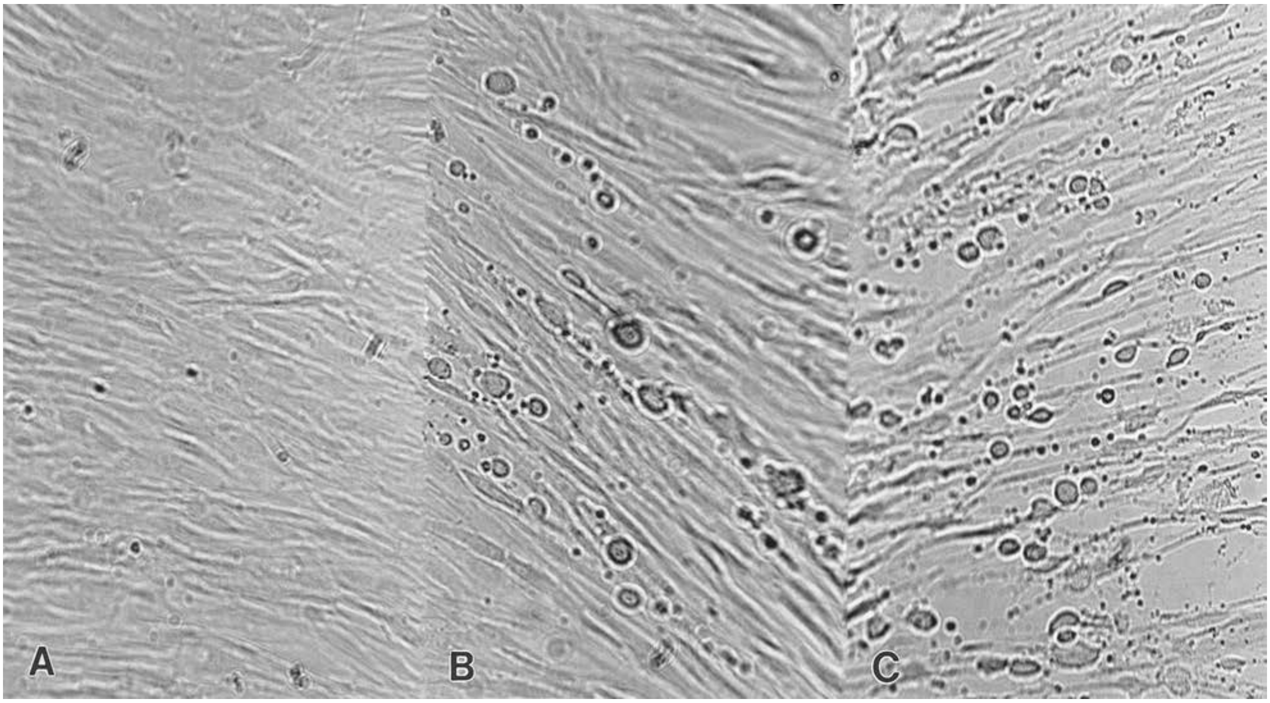

FIGURE 2 HRV CPE in human embryonic lung fibroblasts. (A) Uninfected cells; (B) early focus of rhinovirus CPE; (C) more advanced rhinovirus CPE. Magnification, ×100.
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IDENTIFICATION

A presumptive diagnosis of HRV isolation in cell culture is made by the appearance and progression of characteristic CPE in the appropriate cell lines. In clinical laboratories, further identification of a presumed HRV isolate is usually limited to differentiating it from HEVs by determining sensitivity to acid pH, although this practice has declined in recent years. Virus neutralization testing with type-specific antisera is time-consuming, costly, and not routinely available. In addition, the recently recognized HRV-C strains do not grow in culture, and no neutralizing sera are available.

Acid pH Stability

HRVs are sensitive to low pH and are inactivated, whereas HEVs remain viable. Thus, a reduction in virus titer of 2 to 3 $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$) can be expected upon exposure of HRV to low pH. To perform the test, first passage the isolate to obtain a minimum titer of 10^3 $TCID_{50}$/ml. Then prepare two solutions of a buffer, such as HEPES, one at pH 3.0 and one at pH 7.0. Add 0.2 ml of unknown virus suspension to 1.8 ml of HEPES pH 3.0 and 0.2 ml of virus to 1.8 ml of HEPES pH 7.0. Keep the mixtures at room temperature for 3 hours, adjust the pH to 7.0, make serial dilutions of the mixtures, and inoculate into cell culture. If the unknown virus is an HRV, a minimum 2 $\log_{10}$ reduction in viral titer should be evident in the acid-treated sample. As a control, a known HRV and a known HEV should be treated in a similar fashion.

As an alternative to the acid lability test, staining of isolates in parallel with both a pan-enterovirus immunofluorescence reagent that detects both HEV and HRV and an HEV-specific reagent that detects only enterovirus has been shown to rapidly and correctly identify 11 HRV isolates in a clinical setting (134).

Temperature Sensitivity

Since HRVs often grow best at 33°C, they may be distinguished from HEVs by inoculation of parallel serial dilutions of the unknown virus and incubation at 33 and 37°C. The onset of CPE should be more rapid and the titer of virus obtained should be higher at the lower temperature. Some HRV isolates may not show this temperature sensitivity, so this test is no longer used clinically (63).

Virus Identification by RT-PCR

RT-PCR targeting highly conserved regions of the 5′ UTR can be used for confirmation of HRV isolates in cell culture. However, cross-reactions with HEV can occur, especially with high-titered culture isolates (39, 135).

TYPING SYSTEMS

Serotyping HRVs by Virus Neutralization

Serotyping of HRV isolates by neutralization with type-specific antisera used to be the gold standard for HRV identification. However, the neutralization test is expensive and labor-intensive, results can be delayed for weeks, and few laboratories have the antisera necessary to perform the procedure. Moreover, HRV-C strains cannot grow in standard cell culture, and interpreting assay results can be difficult given cross-reactions between some serotypes. Serotyping requires the neutralization of 30 to 100 $TCID_{50}$ of virus-induced CPE by 20 units of antiserum. Hyperimmune antisera for HRVs are available through the ATCC. Intersecting serum pools similar to those used for HEV identification have been prepared to help narrow the identity of the virus isolate. Identification of the specific serotype is then performed using monospecific antisera. Detailed procedures are described elsewhere (136).

Genotyping HRVs by Sequencing

Molecular typing based on RT-PCR and amplicon sequencing has essentially replaced serotyping for type-specific identification of HRVs. Because the HRV VP1 capsid protein contains a number of neutralization domains that correlate with serotype, genotypic identification of HRVs based on complete *VP1* sequencing has been proposed using type assignment nucleotide differences thresholds of 13, 12, and 13% for HRV-A, -B, and -C, respectively (23). This change has led to only minor revisions of existing serotyping designation (such as reclassification of serotype pairs A8/A95 and A29/A44 as single serotypes). However, because of the high degree of sequence variation in the *VP1* gene and surrounding regions, it has been difficult to design universal primers that will successfully amplify all HRV strains, often requiring new primer designs and assay optimization when new HRVs are recognized (137). In contrast, genotyping based on sequencing the capsid *VP4/VP2* protein genes has been widely used for HRV genotyping in large epidemiological studies (64, 84) due to the availability of conserved regions for primer design that bracket the *VP4/VP2* region. Overall, HRV species and type identification based on nucleotide sequences of *VP4/VP2* correlate well with *VP1* genotyping (137).

The HRV 5′ UTR contains both highly conserved and variable sequences and has been reported as the most sensitive target for detection and typing of HRVs directly from clinical specimens (81, 90). However, sequence from the 5′ UTR cannot unequivocally determine HRV species or genotype due to the tendency of some HRV-A and HRV-C strains to recombine in this region, resulting in incongruent phylogenetic clustering compared with the capsid coding regions (20, 21, 137).

In a study to determine the relative contribution of the different HRV species to hospitalization among young children with acute respiratory infection (114), typing was first performed on all HRV RT-PCR–positive specimens using primers targeting a partial region of the *VP1* gene (137). *VP1* RT-PCR and sequencing identified a HRV genotype in 74% of the specimens. Specimens that were negative by *VP1* RT-PCR were then tested with primers targeting *VP4/VP2*, and an additional 18% were genotyped. The remaining specimens were then tested with primers targeting the 5′ UTR, and an additional 3% were genotyped. The remaining 5% of RT-PCR-positive specimens did not yield interpretable sequence data by any method. The presence of multiple HRV types in clinical specimens may complicate obtaining unambiguous sequence data when using classical Sanger sequencing methods. Next-generation sequencing methods may prove useful for discriminating among mixed HRV infections and studying HRV recombination and quasispecies.

SEROLOGIC TESTS

Determination of antibody response is impractical for the diagnosis of rhinoviruses in the clinical setting. The number of HRV serotypes and the lack of a common antigen make blind serologic testing impractical. Diagnosis by serology is also retrospective because antibody is usually not detectable until 1 to 3 weeks after onset of illness, with IgA predominant in nasal secretions and IgG predominant in serum (136). In research studies, antibody determination by neutralization test is the gold standard for serology (138). Enzyme-linked immunosorbent assay (ELISA) using specific serotype antigens has been used to detect serum and nasal IgG and IgA in volunteers inoculated with known HRV serotypes, and it was 100 to 10,000 times more sensitive than neutralization (52). Detailed procedures can be found elsewhere (136).

ANTIVIRAL SUSCEPTIBILITIES

Many antivirals show activity against HRVs in the laboratory (139). However, inadequate drug delivery to the site of infection has reduced clinical benefit, and treatment remains experimental. Studies have included intranasal administration of soluble ICAM-1 in HRV-infected volunteers; use of intranasal ipratropium bromide, an anticholinergic agent; intranasal imiquimod; or administration of antivirals and antimediators in combination (34, 140–142). Efficacy studies in humans of echinacea, an herbal remedy, and zinc tablets have been mixed (143). Although the *Picornavirus* capsid binding agent, pleconaril, showed some benefit in clinical trials of natural colds (38), it was not approved for clinical use. A more recent phase II trial of a pleconaril nasal spray remains unpublished (139). Another capsid-binding agent, BTA-798 or vapendavir, has had a successful phase II clinical trial in 2010–2011 (89). Although inhibitors of HRV 3C protease have shown potent antiviral activity, trials of rupintrivir and compound 1 have been halted (139). Due to numerous HRV types and apparent lack of cross-protective immunity, prospects for a vaccine have been considered negligible. However, it is hoped that full-genome sequencing will usher in a new era of antiviral and vaccine design (13).

EVALUATION, INTERPRETATION AND REPORTING OF RESULTS

A comparison of HRV diagnostic methods is shown in Table 2. Nucleic acid amplification assays have revolutionized HRV detection, revealed both the ubiquity of HRV infections and the association of HRV with serious disease, fos-

TABLE 2 Comparison of diagnostic methods for rhinoviruses

Method	Advantages	Disadvantages/limitations	Clinical applicability	Key references
RT-PCR or other NAAT[a]	Much more sensitive and rapid than culture methods for HRVs. Detects newly recognized rhinovirus HRV-C strains. Broadly reactive primers that are most sensitive for detection of HRVs in clinical specimens may also detect HEVs. Real-time assays are more rapid, simpler to perform, and less prone to contamination than conventional or nested PCR assays. Multiplex assays can detect multiple respiratory viruses. Instrumentation can automate many steps.	Differentiation of HRV from HEV can be difficult. Since the same clinical syndrome may be caused by other viruses, samples must be tested for additional viruses. Assays vary in sensitivity for various HRV types. Frequent HRV detection by highly multiplexed assays can lead to problems in interpreting clinical relevance.	The standard for detection	See Table 1
Virus isolation	Can recover in culture when rhinovirus not specifically requested. HRV-A and -B replicate in conventional cell cultures used in clinical laboratories (e.g., WI-38, MRC-5, HEK). These cultures may detect other viruses as well as rhinovirus (eg., CMV, VZV, HSV, adenovirus, HEV, RSV)[b].	Optimal recovery of multiple HRV serotypes requires use of additional sensitive cell cultures not routinely used in diagnostic laboratories (e.g., HeLa Ohio cells, HeLa I cells, fetal tonsil). Normally sensitive cells can vary over 100-fold in sensitivity. Differentiation of HRV from HEV isolates is time-consuming (e.g., quantitative acid sensitivity test). Recently recognized novel HRV-C does not grow in culture.	For clinical laboratories not using molecular methods	131, 153
Organ culture	Useful in studies of pathogenesis Biopsy or surgically removed tissues can be used.	Surgically removed and discarded sinus tissues can be used. Fetal nasal or tracheal tissues are not readily available.	Used in pathogenesis research	85
Serology to detect antibody response	May detect some infections not detected by virus isolation. Research tool used after infection of volunteers with a known HRV serotype or in epidemiologic studies	HRV antibody testing for clinical diagnosis is impractical due to the large number of HRV serotypes, the lack of a common antigen, and the need for acute and convalescent sera.	Used only in research settings	52, 138

[a] NAAT, nucleic acid amplification test.
[b] CMV, cytomegalovirus; VZV, varicella-zoster virus; HSV, herpes simplex virus; RSV, respiratory syncytial virus.

tered clinical research, and led to the discovery of HRV-C viruses.

In the clinical laboratory, screening for HRVs is rarely specifically requested. Historically, HRVs were isolated in conventional culture when the suspected virus was influenza or RSV. Following the 2009 influenza pandemic, demand for user-friendly, FDA-approved respiratory virus molecular assays greatly increased, and many of the commercial highly multiplexed respiratory viral panels (RVPs) target HRV. In clinical laboratories that utilize one of these RVPs, HRV is often the most commonly identified virus. The frequency of detection can even lead to the impression that HRVs are "normal flora" and can be ignored.

Thus, the interpretation of a positive HRV PCR result can be problematic. HRVs are extremely common, occur in asymptomatic persons, are prone to cause serial infections, can be shed for weeks after symptoms resolve, and can occur as coinfections with other viruses or bacteria. Interpretation of a positive HRV result in an individual patient often relies on risk factors and recovery of other pathogens. While viral load quantification by rRT-PCR may help determine its role in the acute illness, quantitative standards and guidelines are not available and may not correlate in an individual patient (88, 117–120, 144). Furthermore, the RVPs that include HRV detection do not generally provide quantitative results.

Molecular tests may also not accurately distinguish HRV from HEV (Table 1), and several HEVs have been recognized as important respiratory pathogens (126, 128, 130). Although therapy may not differ, more information is needed to determine whether differences in transmission, infection control measures, virus dissemination within the host, samples for diagnostic testing, and presence of extra-respiratory disease merit distinguishing HRV from HEV.

For laboratories relying on culture methods, isolation of HRV-A and -B, although insensitive, can be accomplished using commonly available conventional cell systems such as WI-38, MRC-5, or HEK. Incubation at 33 to 35°C and rotation of cultures provide optimal conditions for virus replication. Differentiation of HRV from HEV, with which their CPE can be confused, is based on acid-stability testing of isolates or confirmation by RT-PCR.

Specific identification of HRV serotypes by neutralization tests or genotypes by sequence-based molecular methods is reserved for epidemiologic research studies. Serologic testing is also not available outside the research setting.

Molecular techniques are essential for detection of HRV-C and to elucidate the role of HRV as lower respiratory tract pathogens and as significant causes of asthma and COPD exacerbation. However, HRV detection may not become a high priority for clinical laboratories until the cost effectiveness and impact of diagnosis on the management of hospitalized patients can be demonstrated, or effective therapy becomes available.

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Coronaviruses*

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TAXONOMY

Coronaviruses (CoVs) are so called due to their striking crown of surface projections, reminiscent of the solar corona, which can be seen by electron microscopy (Fig. 1). The *Coronaviridae* is one of four families within the order *Nidovirales* and is divided into two subfamilies, *Coronavirinae* and *Torovirinae*. Within the *Coronavirinae*, there are four genera, *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus* (1, 2). Species within the *Betacoronavirus* genus are further differentiated into lineages A, B, C, and D, although these lineages do not have formal taxonomic status. Previously, CoVs were separated into three serologically distinct groups: groups 1 and 2 contained human and animal CoVs and group 3 contained only avian CoVs. However, many new CoVs have recently been described and categorized genetically, and several hundred CoV genome sequences are now available (2). This has led to a reorganization of the taxonomic structure and the current phylogenetic relationship of the 20 recognized species within the *Coronavirinae* is illustrated in Fig. 2 (3). To date, six human CoVs (HCoVs) have been described. The *Alphacoronavirus* genus (previously CoV group 1) contains two HCoVs, HCoV-229E and HCoV-NL63, along with a number of other CoVs including those from bats, pigs, dogs and cats. The *Betacoronavirus* genus (previously CoV group 2) contains the remaining HCoVs and many other mammalian CoVs. HCoV-OC43 and HCoV-HKU1 are in *Betacoronavirus* lineage A, the causative agent of severe acute respiratory syndrome (SARS-CoV) is in lineage B, and the newly described Middle East respiratory syndrome CoV (MERS-CoV) is found in lineage C (4). The *Gammacoronavirus* genus (previously CoV group 3) and the newly proposed *Deltacoronavirus* genus predominantly comprise avian CoV species, with some CoVs found in mammalian species, but none thus far in humans (2, 5, 6).

DESCRIPTION OF THE AGENT

Structure

CoVs have very large, linear, positive-stranded RNA genomes, ranging from approximately 25 to 32 kb in size (6). The 5′ two-thirds of the genome is a replicase gene encoding 15 to 16 nonstructural proteins in two overlapping open reading frames, ORF1a and ORF1b. The remaining third of the genome encodes four to five structural proteins, translated from a nested set of subgenomic mRNAs, and additional accessory genes specific to particular CoVs. The spherical or pleomorphic virions are enveloped and contain a helical nucleocapsid of nucleoprotein (N) associated with the RNA genome. Embedded in the envelope are 20-nm-long trimers of spike glycoprotein (S), also called peplomers, which have a club-shaped morphology and facilitate attachment to cells. The envelope also contains integral membrane (M) and envelope (E) proteins. CoVs of *Betacoronavirus* lineage A also have 5- to 7-nm spikes of an additional membrane glycoprotein, hemagglutinin-esterase (HE). The molecular biology of CoVs has recently been reviewed (7). MERS-CoV is distinct from SARS-CoV in its use of a different cellular receptor for entry (8).

Origin

CoVs have been found in a wide range of domestic and wild mammals and birds; however, the particular diversity of strains found in birds and bats suggests that these animals are the natural reservoirs of CoVs, although this may also be the result of current sampling bias (2, 6). HCoV-229E and HCoV-NL63 were initially considered more closely related to each other than to the other alphacoronaviruses (9, 10). However, more recent genetic analysis of CoV from bats suggests zoonotic origins for both viruses (11–13). HCoV-OC43 is a subspecies of *Betacoronavirus 1* closely related to other domestic animal CoVs, particularly bovine CoV, and it is postulated that zoonotic transmission may have occurred from cattle to humans (14). HCoV-HKU1 is distinct from HCoV-OC43 within *Betacoronavirus* lineage A and its origin is unknown at present (15). SARS-CoV is the sole species in *Betacoronavirus* lineage B, comprising only SARS-CoV from humans and related CoVs from civets, ferrets, badgers, and bats (2, 16). The zoonotic origins of SARS-CoV are still unclear; however, it is likely that it emerged from a natural reservoir in bats and that zoonotic transmission occurred via animals such as civets in the exotic animal markets of southern China. The existence of other intermediate animal hosts is still debated due to the relative divergence of human and civet SARS-like CoVs from the bat CoVs found to date (17). The origin of MERS-CoV is still obscure but is also likely to be zoonotic; closely related CoV sequences have been found in bats (18, 19), raising the possibility of direct transmission to humans or indirect transmission via an unknown intermediate host or

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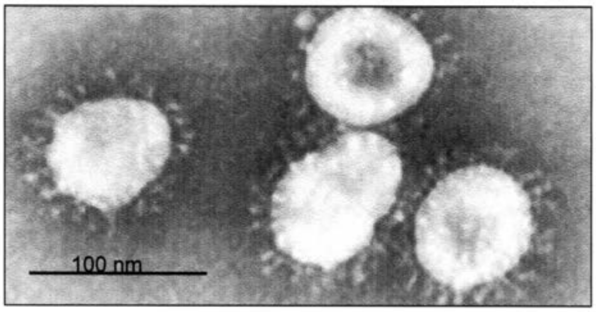

FIGURE 1 Electron micrograph of HCoV-OC43 showing pleomorphic shape and characteristic coronas made up of surrounding peplomers. doi:10.1128/9781555817381.ch90.f1

hosts, potentially camels (3, 20, 21). Recent genomic analyses suggest multiple sporadic introductions of MERS-CoV into the human population have occurred in Saudi Arabia, as well as human-to-human transmission (22).

Discovery

In the 1960s, a number of virus strains that had a cytopathic effect, morphology, and biology unlike those of known respiratory viruses were isolated in culture from individuals with respiratory illness in the United States and United Kingdom (23–25). Isolation of these viruses was difficult and generally required the use of primary organ cultures. They were morphologically and biologically similar and appeared most closely related to infectious bronchitis virus and mouse hepatitis virus. Together, they formed a new group of viruses recognized as coronaviruses in 1968. From this group, HCoV-229E and HCoV-OC43 were subsequently well characterized, owing in part to their existing ability (HCoV-229E) or subsequent adaptation (HCoV-OC43) to propagate *in vitro* (23, 24, 26). In 2003, SARS-CoV was isolated from cell cultures inoculated with respiratory specimens from affected patients; it had CoV-like morphology on electron microscopy and was identified as a novel CoV by sequence analysis (27–32). Experimental infection of cynomolgus macaques demonstrated it was the etiological agent of SARS (33). Shortly after the SARS epidemic, two further HCoVs were identified, HCoV-NL63 in 2004 (10) and HCoV-HKU1 in 2005 (34). HCoV-NL63 was isolated using a novel cDNA-AFLP-based technique to identify an unknown virus from culture (10) (Fig. 3). HCoV-HKU1 was identified solely using nucleic acid-based methods because it was refractory to growth in conventional cell lines (34). Retrospective analysis using stored samples has shown that both these "newly discovered" HCoVs have been circulating

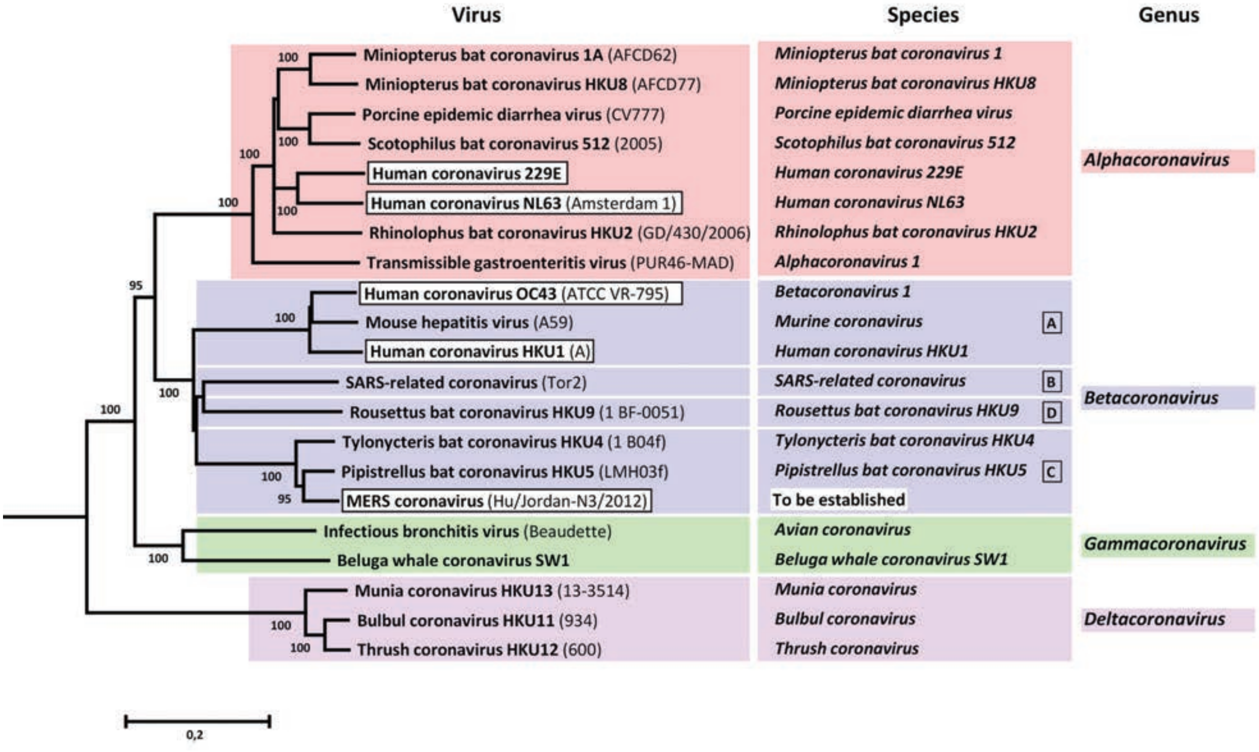
Virus
Species
Genus
Miniopterus bat coronavirus 1A (AFCD62)
Miniopterus bat coronavirus HKU8 (AFCD77)
Porcine epidemic diarrhea virus (CV777)
Scotophilus bat coronavirus 512 (2005)
Human coronavirus 229E
Human coronavirus NL63 (Amsterdam 1)
Rhinolophus bat coronavirus HKU2 (GD/430/2006)
Transmissible gastroenteritis virus (PUR46-MAD)
Human coronavirus OC43 (ATCC VR-795)
Mouse hepatitis virus (A59)
Human coronavirus HKU1 (A)
SARS-related coronavirus (Tor2)
Rousettus bat coronavirus HKU9 (1 BF-0051)
Tylonycteris bat coronavirus HKU4 (1 B04f)
Pipistrellus bat coronavirus HKU5 (LMH03f)
MERS coronavirus (Hu/Jordan-N3/2012)
Infectious bronchitis virus (Beaudette)
Beluga whale coronavirus SW1
Munia coronavirus HKU13 (13-3514)
Bulbul coronavirus HKU11 (934)
Thrush coronavirus HKU12 (600)
Miniopterus bat coronavirus 1
Miniopterus bat coronavirus HKU8
Porcine epidemic diarrhea virus
Scotophilus bat coronavirus 512
Human coronavirus 229E
Human coronavirus NL63
Rhinolophus bat coronavirus HKU2
Alphacoronavirus 1
Betacoronavirus 1
Murine coronavirus
Human coronavirus HKU1
SARS-related coronavirus
Rousettus bat coronavirus HKU9
Tylonycteris bat coronavirus HKU4
Pipistrellus bat coronavirus HKU5
To be established
Avian coronavirus
Beluga whale coronavirus SW1
Munia coronavirus
Bulbul coronavirus
Thrush coronavirus
A
B
D
C
Alphacoronavirus
Betacoronavirus
Gammacoronavirus
Deltacoronavirus
100
100
100
100
100
95
100
100
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FIGURE 2 Phylogenetic relationships among members of the subfamily *Coronavirinae*. A rooted neighbor-joining tree was generated from amino acid sequence alignments of the replicase proteins encoded by polymerase ORF1b for 20 CoVs, each a representative of a currently recognized CoV species, and for the newly recognized MERS-CoV strain Hu/Jordan-N3/2012; bovine torovirus strain Breda served as an out-group. Only bootstrap values equal to or larger than 95% are indicated. Virus names are given with strain specifications between parentheses; species and genus names are in italics as per convention. The tree shows the four main monophyletic clusters, corresponding to genera *Alpha-*, *Beta-*, *Gamma-*, and *Deltacoronavirus*. Also indicated are *Betacoronavirus* lineages A through D (corresponding to former CoV subgroups 2A through D). (Modified from reference 3, kindly provided by RJ de Groot, and used with permission). doi:10.1128/9781555817381.ch90.f2

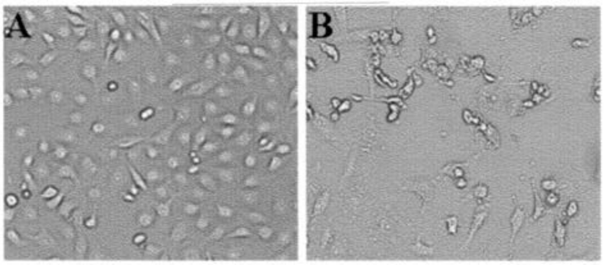

FIGURE 3 HCoV cytopathic effect at 5 days postinfection. (A) Uninfected LLC-MK2 cells. (B) HCoV-NL63-infected LLC-MK2 cells. doi:10.1128/9781555817381.ch90.f3

in the human population for many years (35). MERS-CoV was identified in 2012 by pan-CoV reverse transcription (RT)-PCR and genetic analysis (36). Rhesus macaques experimentally infected with MERS-CoV developed pneumonia, thus establishing a causal relationship between virus and disease (37).

EPIDEMIOLOGY AND TRANSMISSION

HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1 are endemic in humans. In contrast, SARS-CoV has not been reported since early 2004 and MERS-CoV appears to have only recently emerged. The four endemic HCoVs have a worldwide distribution (38–42). Serological surveys carried out several decades ago showed that HCoV infection fluctuated between years, with a higher frequency of serological responses in the winter compared to summer months. With the advent of RT-PCR detection, the substantial temporal and geographical variation in HCoV detection, and in the predominance of individual HCoVs, has been confirmed. This variability makes it hard to draw conclusions about the burden and periodicity of infection from studies describing HCoV epidemiology over only 1 or 2 years. Furthermore, due to their relatively recent discovery, few studies have looked for all four HCoVs simultaneously over long periods of time. From multiyear molecular studies in children and adults, detection by RT-PCR of all four endemic HCoVs typically peaks in the winter and spring months in temperate countries, and HCoV-OC43 and HCoV-NL63 appear to be the most commonly detected (35, 38, 39, 43–48) (Fig. 4). A 2- to 3-year periodicity has been described for HCoV-NL63, HCoV-229E and HCoV-OC43 in some RT-PCR and serological studies (49–51) but not in others (35, 38, 46). The combined HCoV detection rate in individuals with acute respiratory illness (ARI) in multiyear studies varies from 1 to 18% and coinfections with other respiratory viruses are common (38, 39, 44–46, 48). The highest HCoV detection rates are typically seen in children less than 2 years of age (Fig. 4).

Historical serological surveys demonstrated that infection with HCoV-229E and HCoV-OC43 could occur on a background of preexisting neutralizing antibody and that apparent reinfection with the same virus species was common (49, 50, 52, 53). Reinfection could also be achieved in experimental inoculations of healthy adults, although it generated subclinical infections under these conditions (54). In longitudinal studies in children, approximately half of the serological responses to HCoV-229E and HCoV-OC43 occurred in the absence of symptoms, suggesting the occurrence of natural subclinical infection (49, 50). Contemporary serological studies have shown that infection with HCoVs is a common event in early childhood, with seropositivity to all four HCoVs generally increasing with age. Seroconversion to at least one HCoV was observed in 24 out of 25 healthy infants by the age of approximately 20 months (44). However, seroconversion was more frequent with HCoV-OC43 and HCoV-NL63 than HCoV-229E and HCoV-HKU1, in line with individual detection frequencies determined by RT-PCR (44). The sequence of HCoV infection in infancy appears to be important, as prior HCoV-OC43 or HCoV-NL63 infection may elicit immunity that protects from subsequent HCoV-HKU1 or HCoV-229E infection, respectively, but not vice versa (44). In two independent cross-sectional seroepidemiologic studies in different populations, the proportion of infants with antibodies to both HCoV-229E and HCoV-NL63 decreased significantly from 0 to 6 months of age, but then increased rapidly, so that by the age of 4 years, more than 50% of children were seropositive for both viruses (55, 56). It is likely that this pattern illustrates the waning of maternal antibody and the development of new antibody in response to infection in early childhood. Some studies in adults have reported seroprevalences as high as 90 to 100% for HCoV-229E, HCoV-NL63, and HCoV-OC43, with a lower seroprevalence for HCoV-HKU1 of 59 to 91% (57). However, other studies have found much lower seroprevalences for all four HCoVs (56, 58–60). Differences in study population and geographic location as well as in assay sensitivity and specificity may account for the variation.

The major transmission route of HCoV is likely to be respiratory, although many animal CoVs also have a fecal-oral route of transmission and can replicate in epithelial cells in both the respiratory and gastrointestinal tracts. The isolation of HCoVs from fecal specimens has been described, but it is unclear to what extent HCoVs are transmitted feco-orally. In addition, a pilot study identified potential materno-fetal transmission of HCoV-229E, but further studies are awaited. HCoV can be detected by RT-PCR in respiratory secretions for over 3 weeks after the onset of illness, particularly in immunocompromised patients and neonates; however, it is not known if this represents viable virus (61, 62). HCoVs may survive on inanimate surfaces (63), in suspension (64), or as aerosols (65). Therefore, contact-mediated and aerosol-mediated transmission from the environment is a possibility. Nosocomial outbreaks of HCoV have been associated with severe illness in elderly-care facilities and neonatal and pediatric intensive care units (66). The typical incubation period of HCoV-229E, HCoV-OC43, and related strains was shown to be 2 to 5 days in intranasal inoculation experiments in healthy adults; however, it has not been well described for other common HCoVs or in different populations, such as children.

SARS-CoV emerged as a zoonosis in Guangdong province, China, in 2002 and then spread globally via human-to-human transmission to cause the SARS pandemic. Mutation in the receptor-binding domain of the S protein may have contributed to adaptation of the virus for human-to-human transmission (67). Global transmission appeared to be largely related to super-spreading events involving symptomatic individuals in places such as hospitals, hotels, and residential complexes, and asymptomatic seropositivity in the community was low (17, 30). Initial epidemiological investigations suggested that respiratory droplet secretion and direct or indirect contact were the most likely routes of transmission (31). The generation of aerosols, particularly in hospitals, was also important. SARS-CoV was shed in respiratory specimens, stool, urine, and other bodily fluids but, unusually for a respiratory virus, shedding peaked in

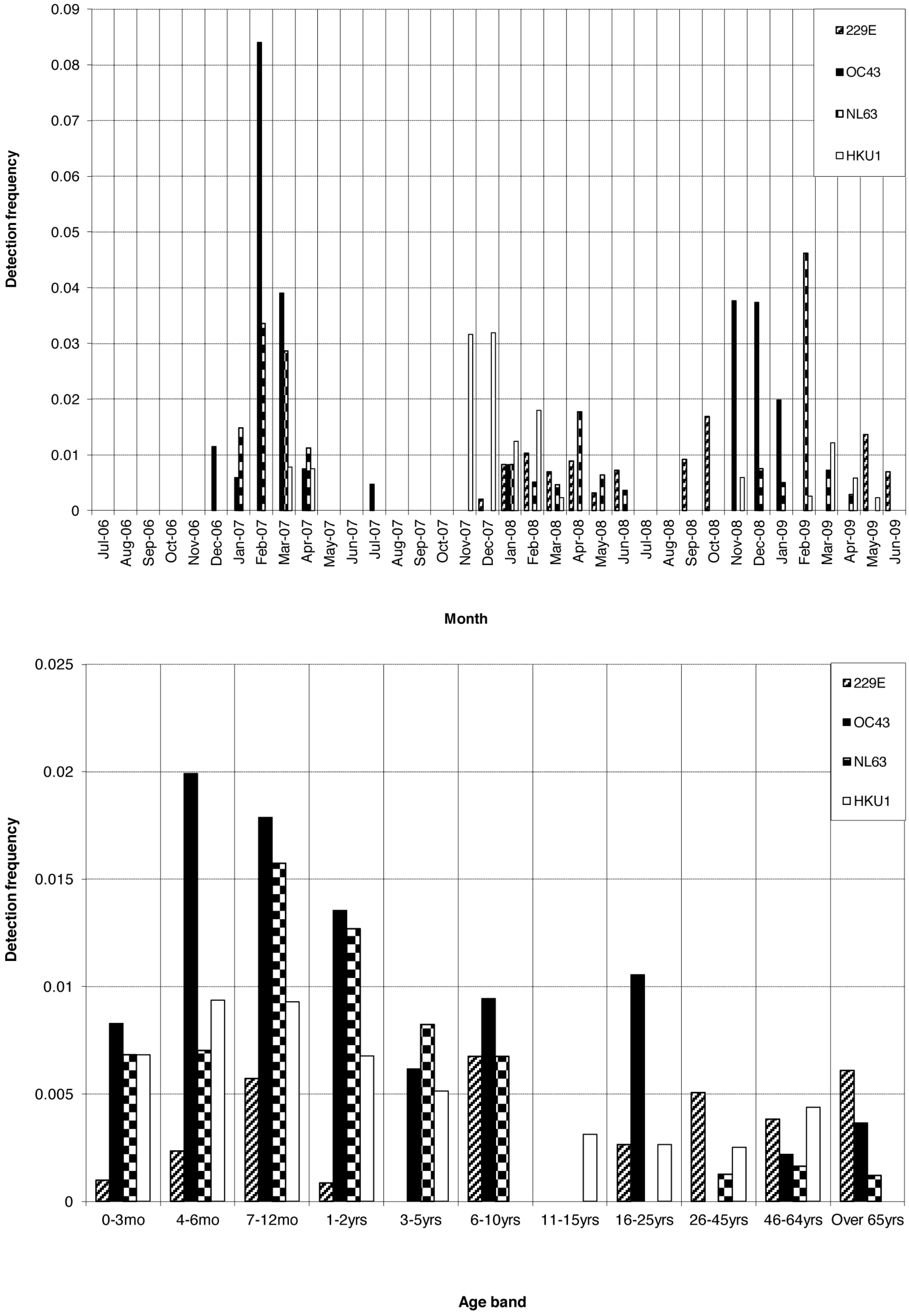

FIGURE 4 HCoV detection frequencies by month and age band over a 3-year period between 2006 and 2009 in Edinburgh, United Kingdom, using multiplex real-time RT-PCR. During this time, 11,661 specimens were tested, with 61 specimens positive for HCoV-HKU1, 99 for HCoV-OC43, 35 for HCoV-229E, and 75 for HCoV-NL63 (adapted from reference 39).
doi:10.1128/9781555817381.ch90.f4

the second week of illness (68). This meant the peak of infectivity occurred at the time when the patient was already likely to be symptomatic and in hospital. Public health measures, including rapid identification and quarantine of cases and improved infection control, brought transmission to a halt in mid-2003. SARS-CoV briefly re-emerged in Guangdong province in late 2003 from a probable new zoonotic transmission event and there were also later incidences of laboratory transmission. The incubation period of SARS was typically 2 to 14 days, with a mean of 4 to 5 days, although occasional cases with longer incubation periods were reported (30, 31).

MERS-CoV was first isolated from a patient with severe pneumonia hospitalized in the Kingdom of Saudi Arabia (KSA) in June 2012 (36). Further cases have since been reported from the Arabian Peninsula, particularly KSA, or linked to travel or residence in the region, or to close contacts of cases. MERS-CoV was found retrospectively in samples associated with a nosocomial outbreak of severe pneumonia in Jordan in April 2012 (69), but tests on stored sera have not shown evidence of infection in the general population (36). Cases have occurred sporadically or in clusters, but sustained human-to-human transmission has not been observed to date. Limited person-to-person transmission has been identified among close contacts and in health care facilities in a number of clusters; however, the vast majority of contacts have tested negative (70–72). The mode of transmission is currently unknown, and contact and droplet-based precautions are recommended, with airborne precautions for aerosol-generating procedures. The incubation period for MERS-CoV is estimated at a median of 5 days. Although 9 to 12 days has been reported, in most cases the incubation period appears to be less than one week (70, 71).

CLINICAL SIGNIFICANCE

The endemic HCoVs, HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1, are associated with upper respiratory tract infection (URTI) in individuals of all ages and occasionally with lower respiratory tract infection (LRTI). HCoV-229E and HCoV-OC43 were originally isolated from adults with mild URTI and were demonstrated to cause the symptoms of the "common cold" in human experiments (23, 24, 73, 74). Such experiments have not been carried out with HCoV-NL63 and HCoV-HKU1, so a causal relationship cannot be formally demonstrated. The four different endemic HCoVs are associated with similar URT symptoms such as fever, cough, sore throat, nasal obstruction, and rhinorrhea (40); HCoV-NL63 has been particularly associated with croup in children (42, 51, 75–77). Numerous epidemiological studies have indicated a temporal association with all four of the endemic HCoVs and ARI, and some studies have shown higher rates of HCoV detection in patients symptomatic with ARI compared to asymptomatic individuals (38). However, other studies have found similar detection rates in hospitalized cases compared to controls, typically 2 to 10% (42, 78). Drawing firm conclusions is difficult because in the majority of case-control studies, detection rates for HCoV are relatively low, and coinfection with other respiratory viruses is common. However, significant LRTI, such as community-acquired pneumonia and bronchiolitis, has been described in hospitalized adults and children from whom an endemic HCoV was the sole etiologic agent detected (76, 79, 80). HCoV has also been associated with significant morbidity due to LRTI in immunocompromised patients, patients with other underlying conditions, and those at the extremes of age (35, 39, 45, 79, 81). Overall, however, it is likely that the burden of severe illness due to endemic HCoVs is relatively minor in comparison to other respiratory viruses such as adenovirus, influenza virus, and respiratory syncytial virus (82, 83).

Several animal CoVs are known to cause diarrheal disease, but the link between HCoV infection and enteric infection is unclear. At present, HCoVs are thought to play a minor role, if any, in gastrointestinal disease. CoV-like particles have been seen in fecal specimens by electron microscopy, and human "enteric" CoVs, some apparently closely related to the betacoronaviruses bovine CoV and HCoV-OC43, were also isolated from patients with gastrointestinal disease (62, 84, 85). In recent years, work has focused mainly on RT-PCR detection of the four endemic HCoVs; although these have been found occasionally in cases of acute gastroenteritis, they have been equally rarely detected in controls (86–88). Some animal CoVs are also neurotrophic, and both HCoV-OC43 and HCoV-229E RNA has been detected in brain tissue, with HCoV-OC43 found significantly more frequently in patients with multiple sclerosis than controls (89). However, the clinical significance of these findings is currently unknown.

SARS-CoV and MERS-CoV cause severe respiratory illness with high mortality rates. During the SARS epidemic, the virus spread to 26 countries, resulting in approximately 8,000 clinical cases and over 700 deaths. The typical SARS clinical presentation was one of initial viral respiratory illness with fever, malaise, and nonproductive cough, followed by an atypical pneumonia with rapid respiratory deterioration. Diarrhea and lymphopenia were also features. Symptoms were milder in children than in adults and the overall crude case-fatality rate was 10% (90). MERS-CoV has been predominantly seen in adults with underlying comorbidities and, although a spectrum from mild to severe disease has been reported, most cases to date have been clinically severe (70, 91, 92). At the time of writing, there had been 160 laboratory-confirmed cases of infection with MERS-CoV including 68 deaths, giving a current crude case-fatality rate of around 40%. The typical clinical course comprises initial viral prodrome with fever, cough, subsequent pneumonia, and respiratory deterioration over several days (91). Severe complications include renal failure and acute respiratory distress syndrome with shock. Gastrointestinal symptoms are commonly reported, along with lymphopenia and other hematological abnormalities (36, 70, 71, 72, 91).

The key to the management of severe HCoV infection is good supportive care, as there is no specific treatment available. Several potential therapeutic agents against SARS-CoV, such as viral protein inhibitors, interferons, and antibodies, have been evaluated *in vitro* and in animal models (reviewed in reference 93). During the SARS outbreak, several experimental treatments were used in humans, but a retrospective review found no conclusive positive effects of any substance, and some potentially harmful effects (94). Studies have already begun to try to identify agents capable of treating MERS-CoV infection (95–97); however, the biology of MERS-CoV differs in certain important aspects from that of SARS-CoV.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

HCoVs can be detected in a range of routine URTand LRT specimens, such as nasopharyngeal swabs, nasopharyngeal aspirates (NPA), nasal washes, and bronchoalveolar lavage

(BAL) fluid. For SARS-CoV and MERS-CoV, higher viral loads or RT-PCR positivity rates have been found in LRT specimens compared to URT specimens. Both should be taken where possible, with LRT specimens the priority. Appropriate infection control precautions must be put in place for sample collection, particularly where aerosol-generating procedures are required. Fecal specimens were useful in the diagnosis of infection with SARS-CoV, particularly in the second week of illness, but MERS-CoV may have a different shedding pattern (68, 90). Detection of CoVs in urine, blood, lung biopsy, and postmortem tissue has also been described. For both SARS-CoV and MERS-CoV, samples should be taken from multiple sites and at several time points to increase the chances of detecting the virus, especially in the first week of illness when viral loads are relatively low. Acute- and convalescent-phase sera should also be taken for retrospective serological diagnosis because of the risk of false-negative RT-PCR results, particularly on URT specimens.

The transport of specimens should be carried out under transport category UN3373 Biological Substance, Category B, with packaging complying with instruction P650 (141). Specimens for the direct detection of HCoV can be handled in Biosafety Level 2 (BSL2) facilities using BSL2 practices, including the use of a biological safety cabinet for potentially aerosol-generating procedures. However, where there is a suspicion of SARS-CoV, MERS-CoV, or other novel CoV infection, virus isolation in culture should not be attempted unless laboratories have the relevant expertise, and work should only be carried out in a BSL3 facility using BSL3 practices. To reduce the risk of laboratory-acquired infection in a novel CoV scenario, nucleic acid-based detection is recommended because it does not require virus propagation and nucleic acid-extraction procedures inactivate the virus. Furthermore, respiratory specimens can be collected, transported, and processed in lysis buffer, thus inactivating the virus at the earliest stage in the diagnostic process.

DIRECT DETECTION

Microscopy

CoVs can be identified by their characteristic morphology on electron microscopy (EM); virions are spherical or pleomorphic, approximately 80 nm in diameter, and with projections around the virus envelope forming a “corona” (Fig. 1). Thin-section EM and negative-stain EM have typically been used for the identification and characterization of HCoVs propagated *in vitro*, rather than for direct detection (23, 24, 28). Cell culture enables amplification of the virus to levels sufficient for detection by EM, so therefore EM is not typically carried out directly on respiratory specimens. However, some laboratories do carry out direct detection of CoVs in fecal specimens by EM, although other direct methods are likely to be more suited to the routine diagnostic setting. EM is most relevant as a complementary method in the search for novel CoVs.

Antigen Detection

Antigen detection assays have the potential to be the most rapid of all detection methods. HCoV antigen is identified directly in clinical specimens by labeled monoclonal antibodies coupled to a detection system such as microscopy. This process may be more convenient than most nucleic acid-based methods where a laboratory does not have molecular experience. However, assays have been developed predominantly in-house to detect SARS-CoV, with the majority of assays targeting the abundant nucleocapsid (N) protein in enzyme immunoassay (EIA)-based formats. EIAs based on chemiluminescent detection and monoclonal antibodies may be particularly effective (98), with SARS-CoV N antigen detectable in the blood of approximately 60% patients in the 3rd week of illness (99). Other enzyme-linked immunosorbent assay (ELISA)-based antigen-detection assays have found SARS-CoV N antigen in NPA and stool specimens, particularly after 10 days of illness onset, but rarely in urine (100). The use of immunofluorescence by indirect fluorescent assay (IFA) on cellular preparations from throat washes has also been described (101). The sensitivity of endemic HCoV monoclonal antibody IFA or direct fluorescent assays varies depending on the species and is generally lower than RT-PCR (102). More recently, an ELISA-based method was developed for the detection of the N protein from HCoV-229E and HCoV-NL63, but has not yet been clinically evaluated (103).

Nucleic Acid Detection

Direct detection of nucleic acids in clinical specimens is the most common diagnostic method in use today for HCoV. Detection of nucleic acids is typically more sensitive and specific than other methods. It relies neither on the binding of antigen to potentially cross-reactive antibodies, nor on the detection of a timely and specific immune response in individuals. Two strategies have been employed in nucleic acid amplification tests (NAATs) for HCoV. The first is to select a target that is as conserved as possible amongst all known HCoVs, a so-called “pan-CoV” assay; the second is to select a region of the genome that is distinct between the different HCoV species, to generate a series of “species-specific” assays. Ideally, all four endemic HCoVs should be sought in diagnostic specimens, given our increasing knowledge of their roles as respiratory pathogens. Recent significant advances in our understanding of HCoVs have been made through nucleic acid-based methods, particularly the ability to randomly amplify and sequence nucleic acids from cultures and to compare them to databases to detect novel CoVs (36). Whole-genome sequencing can be carried out directly on respiratory samples (34), although it is not commonplace at this time in most clinical laboratories.

Pan-CoV Assays

Pan-CoV assays enable the detection of CoV infection without anticipation of the species involved. Regions of the conserved polymerase gene within the replicase ORF1a/b have been successfully used in pan-CoV assays, following alignment of known sequences and generation of consensus primers. Some pan-CoV assays use degenerate primers (48), some utilize multiple primer sets (79), and others employ a single set of nondegenerate primers (34). The more degenerate the primers, the lower the sensitivity for known HCoVs, but the higher the likelihood of detecting more distantly related CoVs. Different pan-CoV assays have been shown to vary in their capacity to detect known HCoVs (104), although some have sensitivity equivalent to individual species-specific assays (79). Therefore, the choice of approach will depend on the diagnostic objectives. Pan-CoV assays have been used as an initial screening test (79) and also for the detection of unknown viruses growing in cell culture, leading to the discovery of new HCoVs (34). To identify the CoV species, amplicons from pan-CoV assays need to be sequenced and subjected to restriction enzyme digestion or probe hybridization; positive specimens require further testing with species-specific assays.

Species-Specific Assays

Species-specific NAATs are very sensitive and provide species-level identification without sequencing or other post-amplification processing. The disadvantage of species-specific assays is that they are likely to miss emerging CoVs that are divergent in sequence, though this may also be the case with pan-CoV NAATs. It is possible to multiplex individual species-specific assays into a single reaction without a loss in sensitivity, thus reducing costs and hands-on time (39). However, some authors using other species-specific NAATs have found sensitivity is greater with individual assays (41, 105). Therefore, when setting up HCoV assays, it is wise to assess performance in both formats, in order to find the best arrangement for the individual laboratory. Regions of the N gene have been most commonly used for detection of individual HCoVs; however, several assays have been described targeting the membrane protein (M) gene, polymerase gene (replicase ORF1a/1b), and occasionally the spike protein (S) gene. Due to a transcription gradient across the CoV genome, which is a function of its complex biology, structural genes such as N, M, and S may be transcribed at higher levels than nonstructural genes such as the polymerase.

In-House NAATs

NAATs for HCoV mainly utilize PCR technology. As HCoVs have an RNA genome, an initial RT step is required; this can either be carried out separately (two-step RT-PCR) or combined with the PCR assay (one-step RT-PCR). In order to increase sensitivity and specificity, several HCoV RT-PCRs in current diagnostic use are "nested" so that the products of an initial round of amplification using outer primers are reamplified with a set of inner primers (39). Detection of amplified products has been traditionally carried out using gel-based electrophoretic systems and UV visualization of DNA bands of the expected size. The use of hybridization probes on a low-density microarray has also been described for a pan-CoV assay (106). However, real-time detection of amplified products, so-called real-time RT-PCR, is now possible. This is achieved through the use of fluorescent dyes that intercalate nonspecifically into dsDNA (76), or fluorescent probes which bind to the nascent DNA in a sequence-specific manner and emit light through a variety of mechanisms, the most common being hydrolysis (39). Real-time RT-PCR has the advantage of a closed-tube format; the reaction vessel is never opened after amplification, thereby reducing the opportunity to contaminate the laboratory environment with DNA template, and decreasing the potential for false-positive detection. As it combines amplification and detection in a single step, it is also significantly faster and requires less hands-on time than traditional RT-PCR methods. The sensitivity of real-time RT-PCR for HCoV detection has been shown to be equivalent or superior to that of conventional RT-PCR (107) and nested RT-PCR (39, 108). Examples of real-time RT-PCR assays used for the detection of the four endemic HCoVs are given in Table 1. Real-time RT-PCR can also give a quantitative output; although HCoV viral loads are unlikely to be required in the routine diagnostic setting, viral load profiles are useful in the description of severe disease associated with newly emerging CoVs, where viral load may be a prognostic marker. Non-PCR-based NAATs are infrequently described, although those using isothermal methods such as loop-mediated isothermal amplification (LAMP) have been successful and may be very suitable in resource-limited settings. A simple, low-cost LAMP assay for HCoV-NL63 has been shown to have comparable sensitivity to real-time PCR (109). In most cases, HCoV NAATs have been developed in-house by the diagnostic laboratory or adapted from the published method of another laboratory. In either case, this requires a significant initial time commitment from skilled scientific staff in terms of assay development, validation, and evaluation, as well as ongoing support in the routine use of these assays. However, in-house assays are economical to run and can be readily adapted to take into account oligonucleotide binding-site variation in existing HCoVs, or for the detection of emerging HCoVs, such as SARS-CoV and MERS-CoV, as soon as sequence data are available.

Commercial NAATs

The last 5 years have seen a large increase in the number and variety of commercial NAATs for HCoV detection, driven mainly by the development of respiratory virus panels for a syndromic diagnostic approach (Table 2). All respiratory viruses can cause a range of presenting symptoms and complications, and clinical features of ARI caused by different viruses are often indistinguishable. Large commercial panel assays that enable a specimen to be tested for an array of viruses simultaneously may be a viable alternative to the use of multiple in-house real-time PCR assays for routine detection, as well as in outbreak situations, despite their higher unit costs (110). Commercial assays vary significantly in hands-on time, some require more than one reaction tube per sample, and some are particularly suited to high-throughput (e.g., xTAG RVP/RVP Fast) or low-throughput settings (e.g., Filmarray Respiratory panel). Commercial tests are particularly attractive as an alternative to traditional culture and IF methods in laboratories that require the use of regulatory-body-approved *in vitro* diagnostic tests and/or in laboratories without molecular experience. However, for HCoV detection in particular, although many tests are CE marked (in Europe), few as yet have U.S. Food and Drug Administration (FDA) approval and not all assays will detect all four endemic HCoVs (Table 2). Direct comparison between commercial and in-house NAATs for HCoV detection has shown a range of performance. However, most comparisons are limited by the overall scarcity of HCoV-positive samples and individual species particularly, making it difficult to draw firm conclusions about sensitivity for individual HCoVs (110–113). Other commercial assay formats such as PCR-electrospray ionization mass spectrometry (ESI-MS) are also on the horizon. This is a rapidly changing field and it is likely that new commercial tests or modifications to existing ones will continue to come onto the market in the near future.

SARS-CoV Detection

Both in-house real-time and nested RT-PCR assays targeting the polymerase (replicase ORF1b gene) were quickly available for SARS-CoV, along with synthetic transcripts to act as positive control material and to enable quantification (27, 28, 30, 31). Later, other NAATs targeting the polymerase and N genes became available, and assays were revised to increase sensitivity of detection, particularly in the first week of illness (114). Isothermal NAATs such as nucleic acid sequence-based amplification and LAMP assays were also described (115), and commercial assays were developed with variable clinical sensitivities and specificities compared to RT-PCR and serology (116, 117). Pan-CoV assays that also detect SARS-CoV are available, but species-specific NAATs are preferred for first-line screening tests (118). A detailed review of NAATs for SARS-CoV, including a comparison of oligonucleotide sequences and assay performance, has been published elsewhere (116).

TABLE 1 Examples of published real-time RT-PCR assays for the detection of endemic HCoVs using different target genes[a]

Reference	Virus	Target gene	Oligonucleotide sequences	Assay format	Reported analytical sensitivity
Gaunt et al., 2010 (39)	HCoV-229E	M	For: CATACTATCAACCCATTCAACAAG Rev: CACGGCAACTGTCATGTATT Probe: ATGAACCTGAACACCTGAAGCCAATCTATG	Multiplex 1-step real-time RT-PCR on ABI 7500, hydrolysis probe detection	Detection limit 66 copies/reaction
	HCoV-OC43	M	For: CATACYCTGACGGTCACAATAATA Rev: ACCTTAGCAACAGTCATATAAGC Probe: TGCCCAAGAATAGCCAGTACCTAGT		Detection limit 18 copies/reaction
	HCoV-NL63	N	For: GTTCTGATAAGGCACCATATAGG Rev: TTTAGGAGGCAAATCAACACG Probe: CGCATACGCCAACGCTCTTGAACA		Detection limit 69 copies/reaction
	HCoV-HKU1	N	For: TCCTACTAYTCAAGAAGCTATCC Rev: AATGAACGATTATTGGGTCCAC Probe: TYCGCCTGGTACGATTTTGCCTCA		Detection limit 9 copies/reaction
Mackay et al., 2012 (41)	HCoV-229E	N	For: ACAACGTGGTCGTCAGGGT Rev: GCAACCCAGACGACACCT Probe: CATCTTTATGGGGTCCTG (MGB)	Individual 1-step real-time RT-PCR on Rotor-Gene 3000/6000/Q, hydrolysis probe detection	Detection limit ≤10 copies/reaction
	HCoV-OC43	N	For: GAAGGTCTGCTCCTAATTCCAGAT Rev: TTTGGCAGTATGCTTAGTTACTT Probe: TGCCAAGTTTTGCCAGAACAAGACTAGC		
	HCoV-NL63	N	For: GAGTTCGAGGATCGCTCTAATA Rev: TGAATCCCCCATATTGTGATTAAA Probe: AAAATGTTATTCAGTGCTTTGGTCCTCGTGA		
	HCoV-HKU1	ORF1b (pol)	For: GTTGGGACGATATGTTACGTCATCTT Rev: TGCTAGTACCACCAGGCTTAACATA Probe: CAACCGCCACACATAA (MGB)		
Kuypers et al., 2007 (79)	HCoV-229E HCoV-OC43 HCoV-NL63 HCoV-HKU1	ORF1b (pol)	F1: TGGTGGCTGGGACGATATGT F2: TTTATGGTGGTTGGAATAATATGTTG F3: TGGCGGGTGGGATAATATGT F-OC: CCTTATTAAAGATGTTGACAATCCTGTAC R1: GGCATAGCACGATCACACTTAGG R2: GGCAAAGCTCTATCACATTTGG R3: GAGGGCATAGCTCTATCACACTTAGG R-OC: AATACGTAGTAGGTTTGGCATAGCAC P1: ATAATCCCAACCCATRAG P2: ATAGTCCCATCCCATCAA P-OC: CACACTTAGGATAGTCCCA	Individual 1-step real-time RT-PCR, hydrolysis probe detection: 229E: F3/P2/R3 NL63: F2/P1/R2 OC43 & HKU1: F1/P1/R1 OC43: F-OC/R-OC/P-OC	10 viral copies/reaction in both consensus and subtype-specific reactions

Dare et al., 2007 (105); van Elden et al., 2004 (108)	HCoV-229E	N	For: CAGTCAAATGGGCTGATGCA Rev: AAAGGGCTATAAAGAGAATAAGGTATTCT Probe: CCCTGACGACCACGTTGTGGTTCA	Individual 1-step real-time RT-PCR on iCycler iQ, hydrolysis probe detection	100% detection at 50 copies/reaction, 93% detection at 5 copies/reaction
	HCoV-OC43	N	For: CGATGAGGCTATTCCGACTAGGT Rev: CCTTCCTGAGCCTTCAATATAGTAACC Probe: TCCGCCTGGCACGGTACTCCCT		100% detection at 50 copies/reaction, 33% detection at 5 copies/reaction
	HCoV-NL63	N	For: GACCAAAGCACTGAATAACATTTTCC Rev: ACCTAATAAGCCTCTTTCTCAACCC Probe: AACACGCT"T"CCAACGAGGTTTCTTCAACTGAG [internally quenched with BHQ1 indicated by "T"]		100% detection at 50 copies/reaction, 80% detection at 5 copies/reaction
	HCoV-HKU1	ORF1b (pol)	For: CCTTGCGAATGAATGTGCT Rev: TTGCATCACCACTGCTAGTACCAC Probe: TGTGTGGCGGTTGCTATTATGTTAAGCCTG		100% detection at 50 copies/reaction, 33% detection at 5 copies/reaction
Esposito et al., 2006 (140)	HCoV-229E	N	For: CGCAAGAATTCAGAACCAGAG Rev: GGGAGTCAGGTTCTTCAACAA Probe: CCACACTTCAATCAAAAGCTCCCAAATG	Individual 2-step real-time RT-PCR on ABI 7700/7500, hydrolysis probe detection	Sensitivity estimated at <500–1,000 copies/ml
	HCoV-OC43	N	For: GCTCAGGAAGGTCTGCTCC Rev: TCCTGCACTAGAGGCTCTGC Probe: TTCCAGATCTACTTCGCGCACATCC		
	HCoV-NL63	N	For: AGGACCTTAAATTCAGACAACGTTCT Rev: GATTACGTTTGCGATTACCAAGACT Probe: TAACAGTTTTAGCACCTTCCTTAGCAACCCAAACA		
	HCoV-HKU1	N	For: AGTTCCCATTGCTTTCGGAGTA Rev: CCGGCTGTGTCTATACCAATATCC Probe: CCCCTTCTGAAGCAA (MGB)		

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[a] Further RT-PCR assays for HCoV-HKU1 have been recently reviewed (15).

TABLE 2 Examples of currently available commercial assays incorporating the detection of endemic HCoVs[a]

Manufacturer	Product	Registration status for endemic HCoV detection	Method	Platform(s)	Endemic HCoV detected	Other targets detected	Approved specimen types	Turnaround time
Seegene	Magicplex RV Panel Real-time Test	CE-IVD	Multitube multiplex real-time PCR	AB7500 (Life Technologies), CFX96 (Bio-Rad), Rotor-Gene Q (Qiagen), SmartCycler II (Cepheid)	229E, OC43, NL63	FA, FA pandemic H1, FA H1, FA H3, FB, RSV A/B, hMPV, AdV A/B/C/D/E/F, RhV/EV, hBoV 1/2/3/4, PIV 1/2/3/4	NPS, NPA, BAL	4–5 h
PathoFinder	RealAccurate Respiratory RT-PCR kit v2.0	CE-IVD	Multitube multiplex real-time PCR	Real-time PCR instruments	229E, OC43	FA, FB, RSV A/B, PIV 1/2/3/4, RhV/EV, hMPV, AdV	NPA, nasopharyngeal lavage, swab, BAL, sputum	2.5–3 h
Fast-Track Diagnostics	FTD Respiratory Pathogens 33/21/21 plus	CE-IVD	Multitube multiplex real-time PCR	AB7500 / 7500 Fast (Life Technologies), Rotor-Gene 3000/6000/Q (Qiagen), CFX96 / DX with CFX software (Bio-Rad), LightCycler 480 (Roche), SmartCycler with Life Science software 2.0d (Cepheid)	229E, OC43, NL63, HKU1	FA, FA H1N1, FB, FC[b], RhV, PIV 1/2/3/4, hMPV A/B, hBoV, *M. pneumoniae*, RSV A/B, AdV, EV, PeV, *C. pneumoniae*[c], *S. aureus*[c], *S. pneumoniae*[c], *H. influenzae*[b], CMV[b], *P. jirovecii*[b], *H. influenzae* type B[c], *B. pertussis*[b], *M. catarrhalis*[b], *K. pneumoniae*[b], *Legionella* spp.[b], *Salmonella* spp.[b]	Nasal/throat swabs, BAL, sputum	1.5–2 h

Seegene	Seeplex RV15, ACE/RV15 One-Step, ACE/RV12 ACE Detection	CE-IVD, Health Canada-IVD	Multitube multiplex endpoint PCR: size fractionation	Thermocycler plus gel-based electrophoresis or autodetection instrument: ScreenTape System (Agilent Technologies), MCE-202 MultiNA (Shimadzu), LabChip Dx (Caliper Lifesciences)	229E, OC43, NL63	FA, FB, RSV A/B, hMPV, AdV, RhV A/B/C[d], EV[d], hBoV[d], 1/2/3/4, PIV 1/2/3/4[d]	NPS, NPA, BAL	4.5 h[e]
PathoFinder	RespiFinder 19/19 Cy5/22	CE-IVD	Multitube multiplex endpoint PCR: size fractionation	ABI Genetic Analyzers (Applied Biosystems),[f] CEQ systems (Beckman Coulter)[g]	229E, OC43, NL63, HKU1[h]	FA, FB, FA H1N1v[h], FA H5N1[i], RSV A/B, hMPV, *L. pneumophila*, *B. pertussis*, *M. pneumoniae*, *C. pneumoniae*, RhV/EV[h], PIV 1/2/3/4, AdV, hBoV[h]	NPA, nasopharyngeal lavage, swab, BAL, sputum	5–6 h
Seegene	Anyplex II RV16 Detection	CE-IVD	Multitube multiplex real-time PCR: melt-curve analysis	CFX96 (Bio-Rad)	229E, NL63, OC43	AdV, FA, FB, PIV1/2/3/4, RhV A/B/C, RSV A/B, hBoV 1/2/3/4, hMPV, EV	NPS, NPA, BAL	Not stated
PathoFinder	RespiFinder SMART 22/22 Fast	CE-IVD	Multitube multiplex real-time PCR: melt-curve analysis	Lightcycler 480 / II (Roche), Rotor-Gene 3000/6000 (Corbett), Rotor-Gene Q (Qiagen)	229E, OC43, NL63, HKU1	FA, FB, FA H1N1v, RSV A/B, hMPV, *L. pneumophila*, *B. pertussis*, *M. pneumoniae*, *C. pneumoniae*, RhV/EV, PIV 1/2/3/4, AdV, hBoV	NPA, nasopharyngeal lavage, swab, BAL, sputum	Within 4 h (SMART 22 Fast) or 6 h (SMART 22)
Luminex	xTAG RVP/RVP Fast/RVP Fast v2	CE-IVD, Health Canada-IVD	Suspension microarray	Luminex 100/200	229E, OC43, NL63, HKU1	RSV A/B, FA, FA H1, FA H3, FA H1N1[j], FA H5[k], FB, PIV 1/2/3/4, hMPV, RhV/EV, AdV, hBoV[l], SARS-CoV[k]	NPS, NPA, BAL	Within 4 h (RVP) or 8 h (RVP Fast)

(Continued on next page)

TABLE 2 Examples of currently available commercial assays incorporating the detection of endemic HCoVs[a] *(Continued)*

Manufacturer	Product	Registration status for endemic HCoV detection	Method	Platform(s)	Endemic HCoV detected	Other targets detected	Approved specimen types	Turnaround time
Idaho Technology Inc.	BioFire Film Array Respiratory Panel Pouch	CE-IVD, FDA	Single pouch combined extraction and multiplex PCR with endpoint melt-curve analysis	Film Array (Idaho Technology Inc.)	229E, OC43, NL63, HKU1	AdV, hMPV, RhV/EV, FA, FA H1, FA H1-2009, FAH3, FB, PIV 1/2/3/4, RSV, *B. pertussis*, *C. pneumoniae*, *M. pneumoniae*	NPS	About 1 h
Autogenomics	Infiniti RVP Plus	CE-IVD	Low density microarray	Infiniti Plus Analyzer (Autogenomics)	229E, OC43, NL63, HKU1	FA, FA swine H1N1, FB, PIV 1/2/3/4, RhV A/B, EV A/B/C/D, hMPV A/B, RSV A/B, AdV A/B/C/E	Not stated	Not stated
Genomica	CLART Pneumovir	CE-IVD	Low density microarray	Clinical Array Reader (Genomica)	229E	AdV, hMPV A/B, PIV 1/2/3/4a/4b, RhV, RSV A/B, hBoV, EV, FAH1N1, FA H3N2, FA H1N1v, FB, FC	Nasopharyngeal wash, nasopharyngeal exudate, BAL	5–7 h

[a] Abbreviations: FA, influenza A; FB, influenza B; FC, influenza C; PIV, parainfluenza virus; RSV, respiratory syncytial virus; AdV, adenovirus; hMPV, human metapneumovirus; RhV, rhinovirus; hBoV, human bocavirus; EV, enterovirus; PeV, parechovirus; CMV, cytomegalovirus; NPS, nasopharyngeal swab; NPA, nasopharyngeal aspirate; BAL, bronchoalveolar lavage. *M. pneumoniae*, *Mycoplasma pneumoniae*; *C. pneumoniae*, *Chlamydophila pneumoniae*; *S. aureus*, *Staphylococcus aureus*; *S. pneumoniae*, *Streptococcus pneumoniae*; *H. influenzae*, *Haemophilus influenzae*; *P. jirovecii*, *Pneumocystis jirovecii*; *B. pertussis*, *Bordetella pertussis*; *M. catarrhalis*, *Moraxella catarrhalis*; *K. pneumoniae*, *Klebsiella pneumoniae*.

[b] FTD 33 only.

[c] FTD 33 and 21 plus only.

[d] RV15 only.

[e] RV15 One-Step.

[f] RespiFinder 19 and 22 only.

[g] RespiFinder 19 Cy5 only.

[h] RespiFinder 22 only.

[i] RespiFinder 19 and 19 Cy5 only.

[j] RVP Fast v2 only.

[k] RVP only.

[l] RVP Fast and Fast v2.

MERS-CoV Detection

An in-house real-time PCR assay based on a target upstream of the envelope protein (upE) was quickly available for MERS-CoV, along with an alternative but less sensitive real-time PCR assay based on the ORF1b gene, and positive control transcripts (119). Shortly thereafter, a real-time RT-PCR assay based on ORF1a was demonstrated to have higher sensitivity than ORF1b and is now recommended for confirmation, along with two RT-PCR assays for amplification and sequencing of the N and polymerase genes (120, 121). In June 2013 the U.S. FDA authorized the emergency use of an rRT-PCR assay panel targeting the N and upE genes as an *in vitro* diagnostic test for MERS-CoV (122).

ISOLATION PROCEDURES

Isolation of HCoVs is not effective as a routine diagnostic tool because many commonly used cell lines are not permissive for growth or do not show obvious cytopathic effect (CPE). CPE is generally nonspecific and reagents for immunofluorescent detection in culture are not widely available. The use of RT-PCR to detect HCoV in cell culture supernatant has been described, but it may be more efficient in a routine context to carry out NAATs directly on the specimen. HCoV-229E was isolated originally in human embryonic kidney cells but later propagated in a number of human embryonic lung fibroblast cell lines such as WI-28, L132, and MRC-5 (23, 54, 73, 102, 108, 123). HCoV-OC43 was originally isolated in human embryonic tracheal organ cultures (24) and later adapted to suckling mouse brain and then to cell culture in lines including HRT-18, RD, and HEL (102, 107, 108, 124). HCoV-NL63 was originally isolated in tertiary monkey kidney cells and subsequently propagated in Vero and LLC-MK2 monkey kidney epithelial cell lines (10). Although some reports described HCoV-NL63 CPE by day 4 or 5 of inoculation (Fig. 3), other studies have found low viral titers with slowly developing CPE and sustained growth proving difficult (10, 104, 124, 125). HCoV-NL63 growth appears to be more efficient in CaCo-2 cell lines, and this has enabled the development of a cytopathogenic plaque assay (124). The continuous epithelial cell line HuH7 has been demonstrated to be permissive for HCoV-229E, HCoV-OC43, and HCoV-NL63 (60, 104, 126). However, HCoV-HKU1 has not yet been propagated in any common cell line (34). All four HCoVs, including HCoV-HKU1, were recently successfully propagated on primary human bronchial epithelial cells subpassaged to form pseudostratified human airway epithelial (HAE) cell cultures (127, 128). In this system, HCoV-NL63 was still difficult to isolate and there was no visible CPE for any of the viruses. SARS-CoV grows in Vero and fetal rhesus kidney (FRhK-4) cells, and in contrast to other HCoVs, focal CPE occurs and rapidly spreads across the monolayer within 2 to 6 days (27, 28, 31). Human cell lines such as HuH7, HEK-293, and Hep-G2 are also permissive for SARS-CoV infection (129). MERS-CoV was originally isolated in LLC-MK2 and Vero B4 cells (36), but also appears capable of infecting primate, pig, and bat cell lines. Inadvertent isolation of a novel CoV in cell culture is therefore a possibility, so where there is a suspicion of SARS-CoV, MERS-CoV, or other novel CoV infection, virus isolation should not be attempted without BSL3 facilities and procedures. NAATs should be used as a first-line test because this involves virus inactivation. Accidental transmission of SARS-CoV to laboratory workers has been described.

SEROLOGIC TESTS

Serological testing for diagnosis of the endemic HCoVs has largely been replaced by more sensitive and specific NAATs. However, serology is useful in epidemiological studies, enhancing our understanding of HCoV infection and aiding the investigation of outbreaks. It has been particularly important in the diagnosis of cases of novel and emerging HCoV infections such SARS-CoV. In these situations, affected patients may not test positive for viral RNA, particularly in the early phase of disease, but retrospectively can be shown to have developed an immune response. The use of viral lysate antigens in ELISA-based serological assays requires propagation of the CoVs to high titers, which is difficult for HCoV-NL63 and not possible for HCoV-HKU1 without HAE cultures. Furthermore, serological assays may lack specificity due to the antigenic relatedness amongst HCoVs, particularly between the alphacoronaviruses HCoV-229E and HCoV-NL63 and between the betacoronaviruses HCoV-OC43 and HCoV-HKU1. The viral nucleocapsid protein N induces a good immune response, and recombinant N protein has been widely used in ELISA-based assays as it can be readily expressed and purified without the need for viral culture. Unfortunately, cross-reactivity of serological assays using the full-length or truncated N protein has been reported, particularly between HCoVs of the same genus (56, 59, 130). However, the carboxy-terminal region of the N protein appears to be significantly less homologous between HCoVs, and recombinant N protein-based ELISAs utilizing this region have been successfully developed for all four endemic HCoVs (44, 56, 59). This may be at the expense of some sensitivity, though, as the carboxy-terminal region of the HCoV-OC43 N protein was recently demonstrated to be less immunogenic than the central region (131). The use of recombinant S-protein-based ELISAs has also been reported for HCoV-HKU1 (58). The difficulty in culturing some HCoVs is an obstacle to the development of neutralization assays; however, neutralization assays using S-pseudotyped HCoV-HKU1 and HCoV-NL63 viruses can circumvent this problem (58, 60).

As with other HCoVs, most serological assays for SARS-CoV were initially based on antibody detection in acute- and convalescent-phase sera by IFA using fixed infected cells and by ELISA using lysates from cell culture (28, 30). Recombinant N protein-based ELISAs were then developed to remove the requirement for BSL3 containment, with confirmation by Western blots targeting antibodies to both the N and S proteins. Neutralization assays also required cultivation of SARS-CoV and were less practical for routine diagnosis. A first-generation, biologically safe IFA was quickly available for MERS-CoV, but due to cross-reactivity with endemic HCoVs, positive results require confirmation by neutralization assay under BSL3 containment (119, 132). A further serological test has since been described, using a recombinant S protein subunit in a protein microarray format, for the detection of antibody to MERS-CoV and SARS-CoV (133). However, additional validation of MERS-CoV serological tests is required, and cases with positive serological test results in the absence of PCR testing or sequencing are currently considered probable cases (121).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The cornerstone of management of all HCoV infections is the rapid diagnosis of affected individuals because symptoms may be similar to those caused by other respiratory pathogens. Accurate and timely diagnosis aids surveillance, ensures that appropriate infection control and public health procedures are instigated, and contributes to antibiotic stewardship by providing a viral diagnosis. The generation of a diagnostic test result in which the laboratory and the end user can feel confident requires, among other things, the proper development and validation of HCoV diagnostic tests. Comprehensive guidelines for the development and validation of NAATs are available (134, 135). Participation in internal and external quality assurance (IQA, EQA) programs is also essential. Performance in the detection of HCoV by NAATs has been shown to vary between laboratories using Quality Control for Molecular Diagnostics (QCMD) EQA panels (136, 137). EQA enables comparison of proficiency between other diagnostic laboratories and identification of problems with sensitivity and specificity. This is particularly relevant for NAATs in which ongoing sequence variation in viral RNA genomes, reaction inhibition, and potential contamination are specific challenges. CoV NAATs should be frequently assessed in the light of new sequence information for their ability to detect new viral variants. Furthermore, the use of internal controls added at the specimen-extraction stage, which are copurified with viral RNA and are detected by NAAT alongside the HCoV gene target(s), is essential for the detection of RT-PCR inhibition. Finally, the use of good laboratory practice when performing molecular amplification assays is crucial and readers are directed to guidance on this subject (135, 138).

It is particularly important in the case of novel or emerging HCoVs such as SARS-CoV and MERS-CoV that nationally or internationally agreed protocols for specimen collection, handling, testing, and reporting should be followed, including the confirmation of positive diagnostic test results at designated reference laboratories and the involvement of public health specialists (121). Errors in the interpretation and reporting of results can have particularly significant adverse consequences in this context. The rapid reaction of the international diagnostic laboratory community to the emergence of SARS-CoV was mirrored in the recent response to MERS-CoV. In a matter of days after notification of the first case of MERS-CoV, an internationally coordinated effort had produced validated NAATs, which were then widely deployed in specialist laboratories (139). This was a testament to the effectiveness of collaborative international laboratory networks, although standardization and quality control of tests for emerging CoVs is a key aspect that must be adequately managed in the roll-out process. In these situations, the development of commercial tests lags behind because of the regulatory approval process, illustrating the importance of maintaining in-house molecular expertise in diagnostic virology laboratories. The widespread occurrence of CoV in mammals and birds and the potential for interspecies transmission events emphasize that we should be vigilant for the emergence of further human pathogenic CoVs in the future.

In summary, the four endemic HCoVs make up a small but significant proportion of the viruses found in patients with ARI. Including HCoVs alongside the more common respiratory viruses such as influenza, respiratory syncytial virus, and adenovirus in diagnostic screening panels will increase the diagnostic pick-up rate in ARI, particularly in a pediatric setting. It will also help to further our understanding of the role of HCoVs in respiratory infection.

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Hepatitis A and E Viruses

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TAXONOMY

Hepatitis A Virus

Hepatitis A virus (HAV) is the type species of the *Hepatovirus* genus within the family *Picornaviridae*. HAV is distinguished from other picornaviruses (e.g., poliovirus) by its tropism for the liver, high thermal stability, and a unique assembly process (Fig. 1). There are two major genotype groups found in humans, and a third genotype that is considered to be a true simian strain (AGM-27) (1). Although there is significant genetic diversity between HAV strains, all strains of HAV represent a single serotype and this diversity does not affect the performance of serological assays. Genotyping of HAV is based on sequence analysis around the VP1-2A region, and is primarily useful as a research tool for outbreak investigations (2).

Hepatitis E Virus

Hepatitis E virus (HEV) is the type species of the *Hepevirus* genus within the family *Hepeviridae* (3). Four genotypes of HEV have been described in humans, with genotypes 3 and 4 representing zoonotic infections originating from viruses endemic in swine, deer, and possibly other mammals (4–6) (see reference 7 for a comprehensive review), while genotypes 1 and 2 are endemic in humans. Additional strains of HEV have been recognized in a wide range of mammals, including rats, rabbits, bats, and ferrets, although there is no evidence that these strains infect humans. All mammalian genotypes demonstrate extensive antigenic similarity, but some studies suggest that differences between the prototype human HEV genotype 1 and other genotypes may affect the sensitivity of some diagnostic assays (8), while others have shown no significant differences (9). HEV has also been detected in chickens (10) and an HEV-like virus has been detected in fish (11). Further subclassification of HEV genotypes and subtypes is likely in the future based on extensive analysis of both viral sequences and genome arrangements (12).

DESCRIPTION OF THE AGENTS

Hepatitis A Virus

HAV is a small virus with a nonenveloped, icosohedral capsid of approximately 27 nm in diameter. The viral genome is composed of linear, positive sense RNA and is about 7,500 nucleotides (nt) in length (Fig. 1A). The structure of HAV is characteristic of all picornaviral genomes, being organized into an uncapped 5′ nontranslated region (NTR), then a single open reading frame encoding all known viral proteins, and a short 3′ NTR. Viral particles have a sedimentation coefficient of around 156S and buoyant density of 1.325 g/cm^3 in cesium chloride. HAV is resistant to low pH (<3.0) and shows remarkable thermal stability (13). Thorough cooking is necessary to inactivate HAV in contaminated foods.

An outline of the viral replication mechanism is shown in Fig. 1A. The positive-strand RNA genome encodes a single open reading frame (ORF), which is translated to yield a single polyprotein. The virus-encoded 3C protease liberates eight individual proteins including the three capsid proteins, VP0, VP1-2A (also known as PX), and VP3 (14). Five copies of each of the three proteins associate to form pentamers, and 12 pentamers are assembled into empty capsids or combined with RNA to form virions.

Following assembly, HAV is exported from infected hepatocytes, although the precise mechanism by which this occurs is unknown. *In vivo*, hepatocytes are organized into a complex three-dimensional structure with surfaces facing both the bile (apical surface) and blood (basolateral surface; Fig. 2). Studies have shown that secretion of HAV is towards the hepatic blood supply from the basolateral surface (15), which implies that HAV must traverse the hepatocyte for delivery to the gastrointestinal tract via bile, possibly via transcytosis (Fig. 2, insert). This indirect transport for apical-destined cargo is a common mechanism in hepatocytes and may also be relevant in export of HEV.

Diagnosis and immunization for hepatitis A are not complicated by strain differences because HAV exists as a single serotype worldwide, including the highly divergent simian AGM-27 (1). The antigenic sites of HAV are formed through the complex interactions of the proteins within and between pentamers (16), which has hampered the production of HAV antigenic material through recombinant DNA techniques. As a result, cell culture remains the only reliable source of HAV antigens for serological diagnostic tests and for vaccines. The replication of HAV in cell culture is rather inefficient compared to most picornaviruses, which necessitates very large-scale cell culture production, but the virus is highly immunogenic. A number of highly effective, licensed vaccines are available against HAV: Havrix (Glaxo SmithKline) and Vaqta (Merck) within the United States, together with Avaxim (Sanofi Pasteur MSD)

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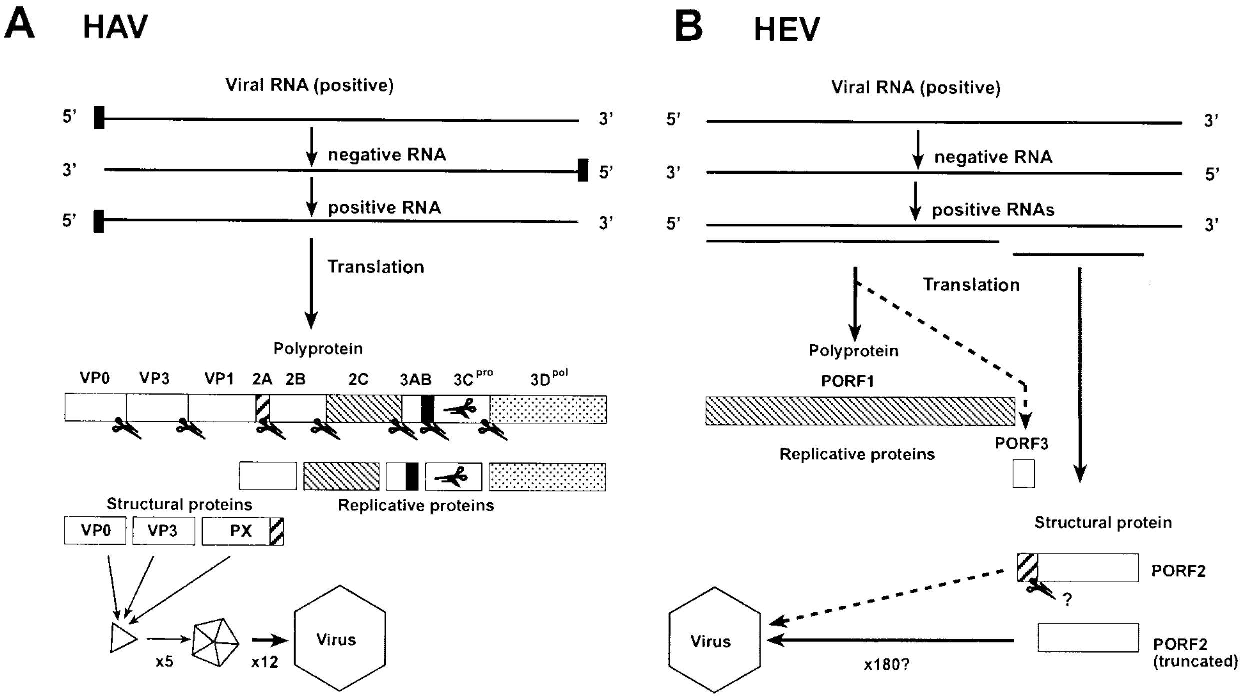

FIGURE 1 Genome replication and proteins of HAV and HEV. (A) Both viruses have positive-strand RNA genomes of around 7,200 to 7,500 nt. HAV replication proceeds via transcription from the genome to give full-length negative- and then positive-strand RNA, which can either be assembled into the virus particle or be used to translate further copies of a single, giant polyprotein which is processed by viral protease to yield the replicative proteins and capsid proteins. Assembly of five copies of each of the three capsid proteins (VP0, VP3 and PX) into pentamers and then 12 pentamers into capsids is required to form the antigenic sites of the virus. (B) Details of HEV replication are unknown, but it most likely produces a full-length negative-strand RNA, then full-length positive-strand RNA (new viral genomes), as well as subgenomic mRNAs which are used to translate the ORF2 (capsid) and ORF3 proteins plus the ORF1 polyprotein which is cleaved at unknown sites to yield the replicative proteins. Cleavage of full-length PORF2 results in assembly of virus-like particles from the truncated product, but the size of the authentic viral capsid protein is unknown. Modified from reference 118 with permission. doi:10.1128/9781555817381.ch91.f1

and Epaxal (Crucell/Berna Biotech) worldwide. A combination hepatitis A and hepatitis B vaccine, Twinrix (Glaxo SmithKline), is also widely available. Epaxal (also sold as HAVpur and Virohep-A in different countries) is formulated with virosomes as adjuvant, while the other HAV vaccines use alum adjuvants.

Hepatitis E Virus

Infectious virions of HEV are nonenveloped, icosahedral particles of approximately 32 to 34 nm diameter, comprising the PORF2 viral protein which encapsidates the single-stranded, positive-sense RNA genome of approximately 7,200 nt. Virions of HEV show much greater sensitivity to heat than HAV (17), although inadequate cooking of pork and deer products is likely to be a major factor in locally acquired HEV infections in developed countries. Virions have a sedimentation coefficient of 183S and a buoyant density of 1.29 g/cm^3 (18). The single capsid protein is encoded by ORF2, expected to yield a PORF2 protein of 660 amino acids. Various truncated forms of PORF2 can form virus-like particles; however, the capsid protein of authentic viral particles has not been characterized.

In contrast to HAV, heterologous expression systems for HEV viral proteins have been instrumental in the development of HEV diagnostics and vaccines, whereas continuous cell culture systems for HEV have been less robust than for HAV. The first of these used a subclone of hepatocyte-derived HepG2 cells, and while virus yields remain low, this cell culture system has allowed some key questions of virus replication to be addressed (19–21), and provided a system for the study of virus neutralization (22). An alternative and possibly more efficient cell culture system has been described in PLC/PRF/5 and A549 cells, with isolation of HEV genotypes 3 and 4 from patients with fulminant hepatitis (23, 24) and from samples of swine liver sold as food (25). Infectious cDNA clones of a number of strains of HEV have been described (26, 27) and have been important in elucidating the role of various HEV proteins in replication, including the interesting role of the ORF3 protein (PORF3) in release of progeny virus from cells (28).

A schematic of the HEV genome and encoded proteins is shown in Fig. 1B. The infectious cDNA clone of the SAR-55 strain of HEV described by Emerson and colleagues (26) is 7,204 nt, and most isolates of HEV from humans and swine are close to this length. The avian HEV is substantially smaller at 6,654 nt due to deletions in both ORF1 and ORF2 (29).

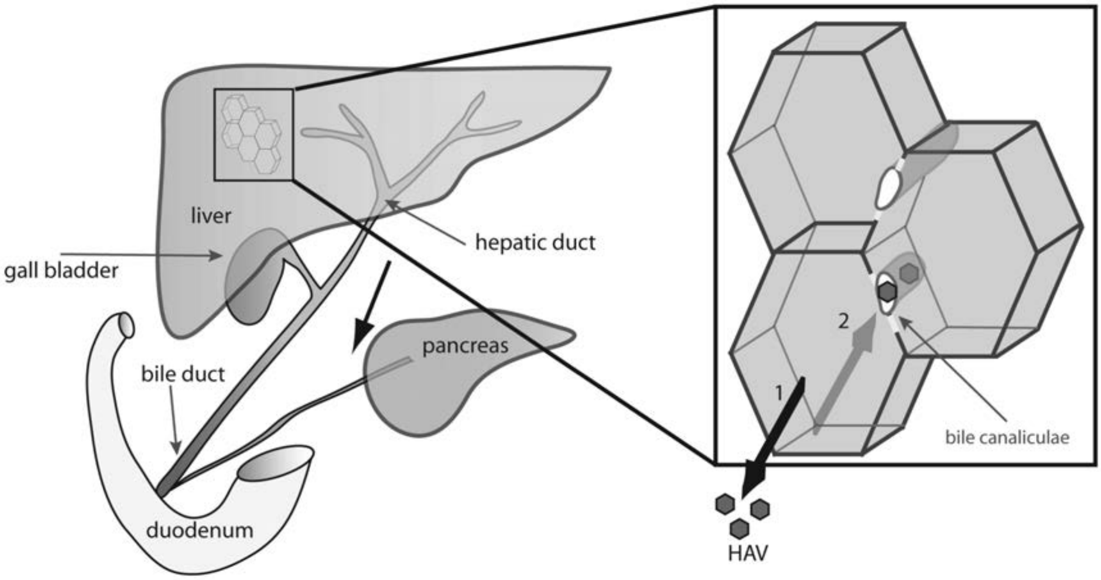

FIGURE 2 Export of HAV from hepatocytes. Polarized hepatocytes are organized in complex structures within the liver, with bile canaliculi representing grooves formed from the apical domains of adjacent hepatocytes (insert). Bile is secreted into the canaliculi via the apical membranes and then flows into the small intestine via a network of ducts. The basolateral surfaces of hepatocytes are in contact with the hepatic blood supply and underlying tissue. Following replication in hepatocytes, HAV is exported via basolateral membranes towards the blood supply (step 1, arrow). The virus is then transported back through the cell (step 2, arrow), possibly by transcytosis, to the apical surface and secreted into bile canaliculi. doi:10.1128/9781555817381.ch91.f2

The 5′ end of the genome has a 7-methylguanosine cap, while both 5′ and 3′ ends of the viral RNA have short, highly conserved untranslated regions (UTRs). Three ORFs, organized as 5′-ORF1-ORF3-ORF2-3′, encode the viral proteins (described here as PORFs) PORF1 (replicative polyprotein), PORF3 (virus export, other functions), and PORF2 (capsid protein), respectively (Fig. 1B).

Strains of HEV show a much greater degree of genetic diversity than HAV, with four putative genotypes identified as infecting humans (6). Genotype 1 is most common in the Indian subcontinent, Middle East, Eastern Europe, and much of the African continent, and it likely causes the majority of endemic human infections. Genotype 2 is most common in central Africa, cocirculating with genotype 1, and was first identified in Mexico. Genotypes 3 and 4 are endemic in swine worldwide, with genotype 3 predominant in North America, Western Europe, Australia, and New Zealand and genotype 4 in India, Taiwan, and Indonesia; both are common in China and Japan (30). Genotypes 3 and 4 represent the predominantly occurring genotypes in humans in North America, Japan, and Western Europe, where zoonotic transmission greatly outweighs ongoing human transmission, while in China and Eastern Europe the "human" genotype 1 is equally common in humans, suggesting mixed patterns of transmission.

The most recent analysis of available sequence data suggests four major groups of HEV (12): HEV infecting humans and swine (genotypes 1 to 4 plus an additional two genotypes from wild boar, and rabbit HEV); HEV infecting rodents and ferrets; HEV from bats; and HEV from chickens. The HEV-like virus from fish was suggested as a possible second genus on this basis (12).

In contrast to HAV, the genotypic differences between HEV strains are associated with significant antigenic differences, with corresponding effects on the sensitivity of first-generation serological assays. However, HEV in humans appears to exist as a single serotype, with experimental vaccines providing strong cross-genotype protection (31, 32). Improved serological tests are also able to detect all HEV strains (33, 34), although some studies suggest there may be reduced sensitivity for detection of genotype 3 infections when using some assays based on genotype 1 antigens (8, 35).

Two candidate HEV vaccines have shown positive results in phase III trials. The first, based on a truncated HEV ORF2 protein (amino acids 112–607) expressed in insect cells, showed strong efficacy in studies conducted in Nepal during 2003 (36), but has not been made commercially available. The second, based on amino acids 368–606 expressed in *Escherichia coli* (HEV 239) and now known as Hecolin (Xiamen Innovax Biotech, China), showed 100% efficacy in a very large phase III study in China, with no cases of HEV among 48,693 subjects who received three doses of vaccine versus 15 cases of HEV among 48,663 placebo recipients over 12 months (37). Hecolin was approved for use in China at the end of 2011 for individuals 16 years and older, but is not yet available in other countries.

EPIDEMIOLOGY AND TRANSMISSION

Despite sharing the primary mode of transmission via the enteric route, HAV and HEV have very different distributions of both disease and infection worldwide. Contributing factors include the presence of zoonotic hosts of HEV strains, the changing incidence of HAV in many countries due to effective use of vaccine, and the higher efficiency of HAV transmission. Infectious particles of HAV are very stable and excreted in very high titers, allowing both efficient person-to-person spread as well as waterborne infections, including foodborne outbreaks associated with importation of contaminated fresh produce. In developing countries with the highest disease burden, HEV is more commonly seen in waterborne outbreaks, with minimal person-to-person spread, while in developed countries zoonotic sources of HEV are more common, with transmission most often linked to consumption of meat products from infected animals. However, unresolved questions remain regarding the true prevalence of HEV infection in areas of high endemicity such as Nepal, where infection with HAV is almost universal and yet less than 50% of the population show evidence of exposure to HEV (38, 39), and in regions such as Western Europe, where high levels of antibody prevalence are detected by some assays (8) and yet clinical disease is rare. This is likely to be related to relatively high rates of subclinical infection with these presumably zoonotic infections, but more studies with consistent use of improved serological tests are required to better understand the epidemiology of HEV infection.

Hepatitis A Virus

Transmission

The generalized course of infection and serological responses for both HAV and HEV is shown in Fig. 3. Due to its enteric route of transmission, HAV is assumed to infect cells lining the alimentary tract. However, studies suggest that progeny HAV from intestinal epithelial cells is released back into the lumen of the gut rather than into the circulation (40), and the virus is around 10,000-fold more infectious when administered by the intravenous route rather than the oral route (41). This is consistent with early studies that suggested the liver as the primary site of replication, with amplification in the alimentary tract making little or no contribution (42). During the incubation period of 4 to 6 weeks after first exposure to the virus, HAV spreads throughout the liver without causing any cytolytic effects, but liver damage eventually results and is most likely mediated by the cellular immune response to the virus. Virus is excreted through bile to the gut, and very high titers of infectious HAV will be present in most patients at the onset of illness. The virus is generally cleared within several weeks, although prolonged excretion of HAV has been reported in a significant proportion of cases. In general, titers of excreted virus will be highest before the onset of obvious symptoms.

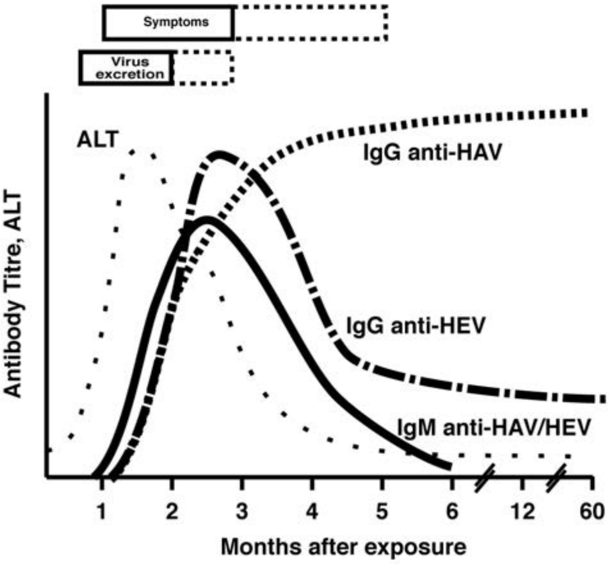

FIGURE 3 Serological and virological courses of infection with HAV or HEV. High levels of HAV-specific IgG provide lifelong protection from reinfection, but HEV-specific IgG declines rapidly during the first 6 months and might not persist at protective levels for life. Modified from reference 118 with permission. doi:10.1128/9781555817381.ch91.f3

Although the vast majority of progeny virus is excreted in the feces, HAV patients will display a viremia that may last for some weeks around the time of clinical presentation. Transmission of HAV via blood products has been demonstrated, and inactivation procedures to eliminate HAV are clearly required in the manufacture of blood products (43). Fortunately, such processes have now been validated for HAV, and further transmission is unlikely (44, 45).

Epidemiology: High, Low, and Intermediate Rates of Infection

HAV is found worldwide, but with widely varying epidemiological patterns that largely reflect sanitation standards (46). In countries or areas with poor sanitation, high levels of HAV exposure result in most individuals becoming infected at an early age, but in these circumstances HAV is not a major public health problem because the symptoms tend to be mild or absent in children and very few of the adult population are susceptible. However, HAV can certainly cause clinical disease in children, most obviously in foodborne outbreaks in schools of developed countries (47, 48) but also observed in countries such as Turkey, where HAV was the most commonly identified cause of fulminant hepatic failure in children from 1 month to 17 years age (49).

Conversely, in countries or populations with high standards of personal and public sanitation, relatively few individuals are exposed at an early age. In many countries of Western Europe, uniformly high standards of sanitation result in very low rates of HAV disease even though the vast majority of the population is susceptible. Prior to the widespread use of vaccines, sporadic HAV infection continued at a significant level in the United States, Australia, and other countries with generally high sanitation levels but a wide diversity of socioeconomic circumstances, especially in the very young and in disadvantaged communities where standards of personal hygiene are low. Acute infection rates in the United States have been reduced considerably since 1996 due to the progressive introduction of vaccination to at-risk groups, at-risk communities, and the general pediatric population (50). Focal circulation of this highly transmissible virus provides an ongoing source of virus for sustained transmission throughout the broader community, and the most profound reductions in overall attack rates followed the introduction of nationwide pediatric immunization, as observed progressively in the United States since 2006.

In the United States in 2007, international travel was the most frequently identified risk factor for HAV (17.5%), while household or sexual contact with an infected person was next at 7.8%, but risk factor data were not available for 67.7% of reported cases (50). Children in day-care centers were identified as a risk factor in 4.6% of cases, but children are likely to be a factor in a large proportion of the cases where no risk factor was identified (50, 51). An important aspect in the epidemiology and transmission of

HAV is the high titer of virus produced, combined with the very great physical stability of the viral particle, which is relatively insensitive to extremes of heat: infectious virus is readily recovered after heating for 10 minutes at 60°C (13).

HAV disease burden in the United States was estimated at more than 140,000 cases and 80 deaths annually in 1990 (52), but has declined rapidly since then (see reference 53 for a comprehensive review of the early phase of vaccine control). This decline was initially most pronounced in children, from a peak incidence of 39 cases/100,000 in 1990 to around 2 cases/100,000 in 2002, coincident with the introduction of targeted immunization of children from high-risk ethnic and racial groups and geographic areas, but the rate in adults (>18 years) also fell from around 17 cases/100,000 to 2 cases/100,000 during the same period (53). The public health problem of HAV in the United States and other countries with low endemicity is compounded by the potential for large outbreaks of HAV spread by contaminated foods. Food- and waterborne infection with HAV was identified as a factor in 6.5% of cases in the United States in 2007 (50), and a single multistate outbreak associated with pomegranate seeds imported from Turkey led to 162 confirmed cases in 2013. Similarly, frozen berries were implicated in a recent outbreak with 71 cases across Denmark, Finland, Sweden, and Norway (54).

In the past, foodborne outbreaks were largely confined to shellfish, which concentrate viruses such as HAV from large volumes of water and which are generally eaten raw or only lightly cooked. Examples are provided by outbreaks seen in Shanghai in 1988 (55), the United States in 1991 (56), and Australia in 1997 (57). However, the increased trade in foodstuffs between countries now creates additional sources of infection. A number of outbreaks within the United States and other countries have been traced to fresh and frozen foods that had presumably been exposed to contaminated water during production or to contaminated water or infected food handlers during food processing (47, 54, 58, 59).

Intravenous and other illicit drug use is also positively associated with a higher risk of HAV infection and may play a major role in the epidemiology of HAV in some societies (60). Men who have sex with men are at increased risk, and outbreaks in this population in different countries have been described (53).

Residents from developed countries who travel to regions of endemicity, especially individuals visiting friends and relatives in such regions, are at greatly increased risk of HAV infection (61). The relative contribution of travel to the HAV disease burden may be underestimated, because travel history is often overlooked in diagnosis and the majority of HAV cases do not have an identified risk factor.

The greatest disease burden for HAV worldwide falls on countries that have previously had high rates of HAV infection, but are now undergoing rapid economic growth and improvements in public sanitation and infrastructure, resulting in low to intermediate rates of HAV infection and antibody prevalence. Improved sanitation has the effect of sparing many young children from infection, but this results in a much larger pool of susceptible older children and young adults, who experience more pronounced disease. HAV may continue to circulate widely within households and other close contacts, resulting in high rates of clinical disease. Routine infant immunization is likely to have the most impact on disease burden by interrupting transmission cycles as seen in other countries, but the cost of universal immunization programs is a major barrier to control of HAV in many countries.

Hepatitis E Virus

Transmission

Hepatitis E is primarily transmitted via the fecal-oral route in developing countries, but direct ingestion of meat or offal from infected animals is clearly a significant factor in regions such as Western Europe and Japan. HEV RNA is detectable in the feces of 50% of patients 2 weeks after onset (62), and in one uncomplicated case was detected 52 days after onset (63). HEV RNA and infectious HEV have also been identified in fresh liver destined for human consumption (7, 25). HEV infection is generally self-limiting, but chronic infection has been characterized in patients on immunosuppressive therapy (64), and this has now been recognized as a significant clinical problem in developed countries with increasing rates of solid organ transplantation in particular (see below), but is unlikely to contribute significantly to ongoing transmission. Household contact appears to be much less important in transmission of HEV than for HAV (65).

Travel to areas of HEV endemicity is a major source of HEV infections in nonendemic areas such as the United States and Western Europe, but locally acquired (autochthonous) HEV infections also occur, primarily representing zoonotic infections from pigs, deer, or other unidentified hosts (66–69), and HEV should be considered in cases of acute hepatitis regardless of travel history. Cross-species infection has been experimentally established for some strains of swine (genotype 3) and human (genotype 1) HEV (70), although other strains are unable to cross species (71). In Japan and Western Europe, it is clear that consumption of raw or partially cooked liver or meat from swine or deer harboring corresponding strains of HEV is the source of transmission (72, 73); however, the significance of this route in other countries is unclear. Direct occupational contact with swine does not appear to be a significant disease risk although it may result in increased subclinical infections (74).

HEV exhibits a significant viremia, with the majority of patients being positive by reverse transcription-PCR (RT-PCR) at the time of disease onset, and there have been several reported cases of hepatitis E associated with transfusion in both endemic and non-endemic countries (75, 76).

Epidemiology: Areas of Endemicity

In countries where HEV is endemic, sporadic HEV infection is often the most common form of viral hepatitis on an annual basis, accounting for around 70% of cases in Kathmandu, Nepal (77), but above this background, major epidemics also occur with a periodicity of around 7 to 10 years. For example, HEV was shown retrospectively to be responsible for 16 of 17 epidemics of enterically transmitted hepatitis in India (78), but HEV is also responsible for at least 25% of sporadic hepatitis between epidemics (79). Epidemics are most often associated with the wet season; however, one study has shown an increased rate of infection associated with a period of unusually low rainfall, presumably due to human wastes concentrating in a riverine ecology (80).

Clinical HEV infection in developing countries is most common in adolescents and young adults but occurs to a lesser extent from childhood (81) to late ages. There appear to be considerable differences in exposure rates between countries in which HEV is considered endemic: in Egypt, around 60% of children were exposed to HEV by the age of 10 (82), whereas in Nepal only 16% of 12-year-olds had evidence of exposure to HEV, peaking at 31% later in life (39). The lack of correlation between many serological assays is also likely to contribute to these differences in observed exposure rates, and further studies are needed.

It is likely that the risk of clinical disease increases with age, as for hepatitis A, but while clinical HAV infection is uncommon in many developing countries because of high exposure rates in children, HEV exposure rates are never as high as for HAV and a large proportion of the population remains susceptible through to adulthood.

In developing countries, exposure to water contaminated with human waste is a clear risk factor for disease, and close contact with domestic animals may also be a risk. Importantly, boiling of drinking water appears to be protective (83), but while this will not be practical for all residents in many developing countries, it is advisable for pregnant women because of the high risk of fulminant hepatitis E in this population (see below). The low rate of person-to-person spread of HEV is likely to be due to a combination of lower particle stability (17) and lower virus titers in excreta compared to HAV.

Reported seroprevalence rates for anti-HEV IgG in countries of presumed endemicity vary widely, at least partly due to the different assays used, which show very poor concordance (8). Experimental infections of macaques have demonstrated a strong dose response for the development of clinical HEV disease (84) and the unexpectedly high rate of HEV seroprevalence in countries where clinical HEV is very rare may represent subclinical infection with low doses of zoonotic strains of HEV. There is some evidence for increased exposure to HEV in groups with occupational exposure to swine (74).

Travel to endemic countries remains the greatest risk factor for clinical HEV infection in residents of developed countries. This risk can be assumed to be especially great for travelers visiting friends and relatives, as for HAV (61). However, with the very clear evidence for autochthonous HEV infection from a large number of studies across many different developed countries, HEV infection must be considered in cases of unexplained hepatitis regardless of travel history and is especially important in immunosuppressed patients, where it may become chronic and more severe.

CLINICAL SIGNIFICANCE

Clinical Presentation and Course

Acute infections with any of the hepatitis viruses cannot be distinguished on clinical characteristics or pathological examinations, and the diseases caused by HAV and HEV will be considered together here. However, HEV infection is unique among the hepatitis viruses in being associated with a high mortality during pregnancy due to fulminant hepatitis with a very rapid onset, approaching 30% in the third trimester, and the rate of fulminant hepatitis for HEV outside of pregnancy is probably around 10-fold higher than for HAV (1% versus 0.1%). In addition, there is some evidence that HEV infection may have more severe sequelae in patients who are deficient for G6PD (85). The reasons for such severe outcomes in HEV infection are not known, but it is clear that women should take all possible precautions to avoid exposure to HEV during pregnancy. A major risk factor in this regard is travel from nonendemic countries to areas of endemicity such as India and Pakistan.

Infection with HAV or HEV can result in a broad range of clinical outcomes, from subclinical infections (especially in children, or with low doses of HEV) through to fulminant hepatitis. Disease severity shows a general increase with age; however, clinical and even fulminant hepatitis A and E do occur in children, and there is no reason to exclude the enterically transmitted hepatitis viruses from diagnostic consideration on the basis of patient age. A retrospective study in Turkey has shown HAV to be the most common identifiable cause of fulminant hepatitis in children (49).

Clinical presentation of acute viral hepatitis commonly begins with nonspecific, “flu-like” symptoms such as fever, headache, anorexia, nausea, and abdominal discomfort. Physical examination will usually reveal an enlarged, tender liver. The first distinctive sign of hepatitis is usually dark urine (excretion of conjugated bilirubin), followed by pale feces and jaundice (yellow discoloration of the skin and sclera), but many patients will not show visible signs of jaundice despite severe symptoms, even when raised levels of serum bilirubin are present. However, it must be stressed that these nonspecific symptoms alone are not sufficient to infer acute viral hepatitis in a patient without jaundice, and liver function tests are an important adjunct to diagnosis (86), with raised levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) detectable at the time of onset. Unless there are strong epidemiological grounds for suspicion (such as contact with an index case), hepatitis A or E should not be considered in the absence of one of these biochemical markers or jaundice.

In most developed countries, hepatitis A is much more likely than hepatitis E unless there are specific indications to suggest HEV, such as chronic elevation of liver enzymes in immunosuppressed patients or recent travel to developing countries combined with a history of receiving inactivated HAV vaccine. Patients with proven evidence of past HAV infection will not have HAV, other than those very rare cases that represent relapses after recent HAV infection (87). In developing countries with poor sanitation, clinical cases of HEV are often more common than HAV due to almost universal childhood exposure to that virus, although HEV appears to be relatively infrequent in many parts of South America. Coinfection with HAV and HEV has also been reported, with 14 of 33 outbreaks investigated in Cuba during the period 1998 to 2003 showing strong serological evidence of exposure to both viruses, although a higher proportion of individual patients were infected exclusively with HAV (88), consistent with its more efficient secondary transmission.

Serum aminotransferase levels usually resolve after a period of 3 to 4 weeks. Normalization of liver enzymes usually marks complete recovery; however, many patients will report an intolerance to fatty foods which may last for years. Relapses are rare for hepatitis A, occurring in approximately 7 to 12% of patients beyond 1 month (87). Protracted hepatitis A has been associated with a particular HLA allele (89). Prolonged disease with hepatitis E has also been observed in some areas of endemicity and is frequently characterized by cholestasis with jaundice and itching. Cholestasis can occur with both hepatitis A and hepatitis E.

While both HAV and HEV (outside pregnancy) have low overall rates of fulminant hepatitis, the onset of encephalopathy can be quite rapid (around 7 days from onset of dark urine for hepatitis A). HEV has been found responsible for around 62% of fulminant hepatitis in studies from India and Bangladesh (90), where the virus is highly endemic. Acute HAV infection in individuals with chronic hepatitis C or hepatitis B is associated with more severe outcomes (91).

While chronic HEV infection has rarely been reported in healthy individuals, a significant number of cases have been reported in patients receiving immunosuppressive therapy after solid organ transplantation (64, 92, 93). There is no suggestion that transplantation was the source of infection in these cases, with cases occurring many months or years after transplantation. Rather, these represent sporadic cases of HEV infection that have progressed to chronicity due to ongoing immunosuppression. In the largest study, of 217 French patients with elevated liver enzymes and with no evidence of drug toxicities or other viral causes, 14 patients (6.5%) tested positive for serum HEV RNA and 8 of these progressed to chronic infection (64). Chronic HEV infection has also been noted in HIV-infected patients, along with higher overall rates of HEV infection (94, 95). In addition to chronic hepatitis with persistent elevation of liver enzymes, neurological symptoms are sometimes evident during chronic HEV infection (96). The chronic infection can have severe or fatal sequelae, further complicated by the poor sensitivity of some available commercial serological assays in order to provide timely antiviral treatment (see below) (97).

Dose Response to Infection

HAV and HEV differ markedly in the relationship between infecting dose and the course of infection and disease. For HAV, studies in tamarins have shown a very clear correlation between higher infectious doses and reduced incubation period to develop disease, but no correlation with disease severity (41). Low-dose infections of cynomolgus macaques with HEV have also demonstrated an increased time to seroconversion, but HEV disease severity showed a positive correlation with high infectious doses (84, 98). This attenuation of low-dose infections may help to explain the low secondary attack rates of HEV, and especially the rarity of clinical HEV disease in developed countries despite seroprevalence estimates that range as high as 18 to 29% using assays that include antigens derived from genotype 3 strains of HEV (99).

Vaccines and Antiviral Agents

Passive immunization with human gamma globulin has been used for many years to provide short-term protection against hepatitis A and is still used for postexposure prophylaxis (<2 weeks post exposure, including household contacts), especially in individuals at risk of severe disease due to age or pre-existing liver disease and for infants less than 1 year of age. As noted above, there are now a number of highly efficacious, inactivated vaccines available against hepatitis A (Havrix and Vaqta in the United States, plus Avaxim and Epaxal worldwide) or hepatitis A and B (Twinrix), and these have replaced the use of gamma globulin for protection of individuals with future risk of HAV infection. Preliminary studies also suggest that HAV vaccines may be useful for prevention of household transmission when administered no more than 8 days after exposure, but not in those most at risk of severe disease, where gamma globulin is still preferred (100). In the United States, progressively expanded programs of active immunization against hepatitis A have been implemented, beginning in 1996 with children (>2 years of age) living in communities with elevated rates of hepatitis A, travelers to countries where standards of hygiene and sanitation are low, men who have sex with men, recipients of clotting factors, injecting drug users, and patients with chronic hepatitis (where disease severity is exacerbated) (86). Extension of this program in 1999 to include children in all states/counties reporting more than 10 cases/100,000 population contributed to the rapid decline in HAV disease rates in the United States over the subsequent 7 years, and since 2006, the inclusion of children nationwide from 12 to 24 months of age (following the licensure of vaccines for use beginning at 12 months of age) has led to further reduced disease rates, from 3,579 cases reported in 2006 to only 1,398 in 2011 with an incidence of 0.4 per 100,000 (see Centers for Disease Control, http://www.cdc.gov/hepatitis/Statistics).

Two different vaccines have now shown efficacy in phase III trials for protection against HEV infection: a 56-kDa subunit antigen (amino acids 112–607 of PORF2 expressed in insect cell culture) (36) and a particulate subunit antigen, HEV 239 (amino acids 368–606 of PORF2 expressed in *E. coli*) (37). However, the HEV 239 vaccine is the only commercially available product, with regulatory approval for the vaccine, known as Hecolin (Xiamen Innovax Biotech, China), granted in China in December 2011 for subjects 16 years of age and older.

The progress of vaccine development against HEV was underpinned by studies by Purcell, Emerson, and colleagues at the National Institutes of Health, which first demonstrated that antibody to PORF2 is sufficient to protect against hepatitis E (101). Active immunization of macaques with the candidate 56-kDa subunit antigen vaccine was also shown to confer protection against viral challenge with both homologous and heterologous HEV strains (31, 101, 102).

The 56-kDa vaccine was subsequently acquired by Glaxo SmithKline and completed a phase III clinical trial in Army recruits in Nepal in 2003, with results published in 2007 (36). In that study, 1,794 healthy HEV seronegative adults received three doses of either the 53-kDa vaccine or placebo at months 0, 1, and 6 and were monitored for a median of 804 days. Hepatitis E developed in 66/896 placebo recipients versus 3/898 vaccine recipients, yielding an efficacy of 95.5% (36). However this vaccine has not progressed to licensure, presumably because of concerns about its market viability.

HEV 239 (Hecolin) followed a similar path of development from preclinical studies demonstrating safety and induction of antibody responses (103, 104) before the definitive phase III trial that showed 100% efficacy (37). In this study, 97,356 healthy adult subjects received three doses of either 30 μg of Hecolin with alum adjuvant or placebo (hepatitis B vaccine) at months 0, 1, and 6 and were monitored for 12 months after the final dose. Hepatitis E developed in 15/48,663 placebo recipients, versus 0/48,693 vaccine recipients (37). Notably, patients with pre-existing antibody to HEV were not excluded from this trial, and follow-up at 2 years was able to show that both naturally acquired and vaccine-induced antibodies were protective against infection (105). Hecolin was approved for use in China at the end of 2011 but is not yet available in other countries. While questions remain about the duration of protection provided by Hecolin (as well as the 56-kDa antigen), it is to be hoped that HEV vaccine may become more widely available, especially in high-burden countries and for use in the face of outbreaks, and further follow-up from the Hecolin trial should clarify the duration of protection. In low-burden countries, vaccine would be most useful for travelers (including the military, Peace Corps, etc.) and potentially for protection of organ transplant recipients and HIV-infected patients to prevent chronic HEV infection associated with immunosuppression (see above).

Numerous recent studies have provided a strong evidence base for the use of ribavirin for the antiviral treatment of chronic HEV infection in transplant recipients and other immunosuppressed patients (106–108). Initial studies used combinations of ribavirin with pegylated interferon alpha (as for treatment of hepatitis B and hepatitis C), but the most recent findings suggest that 3 months of therapy with ribavirin alone can provide virological clearance in 95% of patients, with a sustained response in 78% of patients (108).

In contrast, no antivirals are available for treatment of hepatitis A. Experimental inhibitors have been developed that are active against the 3C protease of HAV but clinical development of these is unlikely, as the acute disease is largely immunopathogenic in mechanism and chronic infection is not significant.

No specific symptomatic or anti-inflammatory treatments can be advised for acute hepatitis A or E. Bed rest and attention to diet (avoiding fatty foods) are commonly recommended to minimize symptoms and speed recovery; alcohol intake should be minimized. Pruritis is a feature of cholestatic hepatitis that may justify the cautious use of corticosteroids.

Intensive supportive medical care, as for acute liver failure due to other causes, is required in cases of fulminant hepatitis A or E. Fulminant hepatitis A has a better prognosis for spontaneous recovery than hepatitis B (69 versus 19% [109]). Liver transplantation is less frequently performed, and there is some risk of graft reinfection (110).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Standard methods for collection, transport, and storage of sera or plasma are adequate for the detection of both IgG and IgM antibodies to HAV and HEV. Sera or plasma can be stored at 4°C for weeks, but should be frozen under other circumstances (–20°C or –70°C for storage; dry ice for shipping). Shipping is most often an issue with HEV testing, which is not available in many routine diagnostic laboratories. IgM is more sensitive to freezing and thawing than IgG is, and repetitive cycles should be avoided. Feces may be collected for epidemiological studies of HAV (genotyping of strains) and are stable as a solid mass or slurries in phosphate-buffered saline or water for years at −70°C. HEV is difficult to detect in feces, and serum is preferred for RT-PCR and genotyping. Because HEV is more labile than HAV, sera for HEV RT-PCR studies must be tested within 24 hours or frozen and shipped at −70°C.

Rapid point-of-care (RPOC) tests for HEV-specific IgM are now available outside the United States (33, 34), which can overcome the need for collection and shipping of venous blood. These tests can be used either for fresh whole blood or for serum and plasma as for laboratory assays.

DIRECT EXAMINATION

Electron Microscopy

Electron microscopy, and especially immunoelectron microscopy, are of historical importance for their roles in identifying HAV (111) and HEV particles (18) before the characterization and molecular cloning of the viruses and development of specific diagnostic tests, but they are insensitive and technically difficult and have no diagnostic value.

Antigen Detection

HAV

HAV antigen (HAAg) is very stable and is readily detected in the acute-phase fecal samples collected from many patients. Viral antigen is detected by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies and may be present in very high titers in some patients, but antigen detection is less sensitive than RT-PCR or follow-up serology for detecting the uncommon cases where IgM has not yet developed at initial presentation. There are no commercially available tests for HAAg.

HEV

HEV antigen is less stable than HAV, and no reliable tests are available (either commercially or on research-only basis) for the detection of antigen in clinical samples. It is unlikely that antigen detection would have any clinical value relative to serology or RT-PCR performed on sera (below).

Nucleic Acid Detection

HAV

HAV patients are viremic in the prodromal phase, and one study from Brazil has reported around 12% of children during a common-source outbreak to have been RT-PCR positive but IgM negative ("window period") at the time of testing (112). The high proportion of window period cases found in this study contrasts with the high sensitivity of HAV IgM detection in other studies and may be related to high-dose infections with short incubation periods, and/or early case detection due to active surveillance of the outbreak. The authors also reported the detection of HAV RNA in 26% of patients initially classified as acute, sporadic non-A, non-C hepatitis (112). All patients available for follow-up seroconverted, highlighting the utility of follow-up IgM serology or RT-PCR in the uncommon cases of acute hepatitis with unknown etiology, especially in a country where HAV is endemic. Serum is the preferred specimen for nucleic acid detection, but plasma is acceptable provided that anticoagulants do not interfere in the reactions; feces are least suitable, with variable levels of virus and high levels of inhibitors. While RT-PCR is useful for detection of very early (window period) infections and the confirmation of questionable IgM results, accredited assays are not commercially available for routine diagnostic testing of possible acute hepatitis A. In the absence of a mandate for screening of the blood supply for HAV RNA, there may be insufficient test numbers for such assays to be commercialized; conversely, many manufacturers now routinely conduct HAV RT-PCR screens on pools of source plasma for blood products, although virus inactivation methods provide the most important protection for such products. HAV RNA can also be used for genotyping of virus for research purposes using RT-PCR primers in the region of VP1-2A (Fig. 1) (113).

HEV

RT-PCR remains the gold standard in the diagnosis of acute HEV infection. However, levels of HEV RNA are generally low and the sensitivity of RT-PCR for diagnosis is very dependent on patient presentation early in disease, and rapid and careful processing of serum or plasma samples and appropriate transport at –70°C. Routine tests for HEV RNA have only recently been commercialized (114) and are not widely available, and reference laboratories are therefore generally needed for RNA testing and quality control. Serological tests are preferable for routine diagnosis, especially in regions with high prevalence of HEV (see below). However, RT-PCR has provided the majority of information on zoonotic infections with HEV around the world (4–6, 73, 75) and is likely to remain useful for HEV testing in areas of low endemicity such as the United States and Western Europe.

ISOLATION PROCEDURES

Many primary isolates of HAV have been adapted to cell culture, but virus isolation is insensitive and is not useful in diagnosis or epidemiological studies. Similarly, some primary strains of HEV appear to replicate well in cell lines (23, 115), but virus isolation is not useful for diagnosis.

IDENTIFICATION AND TYPING SYSTEMS

As described above, the direct detection of HAV and HEV RNA by RT-PCR and sequence-based genotyping has been used for the identification of potential sources of sporadic cases of HEV in nonendemic countries and for the investigation of common-source outbreaks of HAV. HEV genotyping is usually based on the ORF1 region and to a lesser extent on the ORF2 region (4–6), while HAV strains and genotypes are determined by sequencing in the VP1-2A region of the genome (113).

SEROLOGIC TESTS

Despite the very good specificity of most serologic tests for HAV and HEV, their appropriate use and interpretation are dependent on patients meeting the clinical criteria for acute hepatitis, particularly in areas where there is low incidence of the viruses, such as the United States and other developed countries. The clinical criteria for the HAV surveillance case definition in the United States define “An acute illness with discrete onset of symptoms (e.g. fatigue, abdominal pain, loss of appetite, intermittent nausea, and vomiting) and jaundice or elevated serum aminotransferase levels” (86). Serologic testing should be limited to persons who meet the clinical criteria or to persons likely to have been exposed to HAV (86), and in light of the nonspecific nature of the symptoms, the importance of jaundice or elevated ALT or AST in meeting the case definition must be stressed. These same criteria are clearly relevant to HEV. In the absence of these clinical criteria for patients in nonendemic regions, the positive predictive value of the tests will be severely compromised. This is especially relevant given the very low rates of clinical HAV disease in countries with successful immunization programs, and of HEV disease in most developed countries.

Hepatitis A Virus

Tests for HAV-specific IgM are commercially available from numerous sources in both manual and automated enzyme immunoassay formats. The technology for detection of HAV-specific IgM is sufficiently well established that there are no significant differences between the diagnostic performance (sensitivity and specificity) of the laboratory-based testing platforms. Choices of diagnostic assays for HAV may reasonably be made on the basis of convenience for the testing laboratory, with a range from single-strip ELISAs for small numbers of specimens, through to completely automated systems for high-volume laboratories.

Test principles are either IgM class-capture or indirect ELISA. In the IgM class-capture assays, plates coated with anti-human IgM are used to capture IgM from patient samples, with HAV-specific IgM being detected by binding of labeled hepatitis A antigen (HAAg; inactivated HAV derived from cell culture). Indirect ELISAs use plates coated with HAAg, to which HAV-specific IgM will bind, and bound IgM is then detected using anti-human IgM conjugates. In both cases, assay reactivity and cutoffs are determined from internal controls supplied with the kits. All commercially available HAV serological assays use inactivated, whole HAV derived from cell culture and thus detect the same antibody specificities.

Total or IgG anti-HAV does not have diagnostic value because antibody persists for life, although a rising titer of IgG (greater than 4-fold in specimens taken 2 weeks apart) is indicative of acute HAV. Total HAV-specific antibody is a marker of immunity and can be used for prevaccination screening to identify those who require immunization, but the cost-effectiveness of screening depends on many factors (116). Postvaccination tests are not required, due to the very high efficacy of available HAV vaccines. Tests for the measurement of total anti-HAV use a competitive ELISA format, where HAAg is coated on plates and specific antibody in patient serum binds to the antigen, resulting in a proportionately reduced assay signal from the binding of labeled monoclonal antibodies to HAAg.

In addition to serum or plasma, saliva can be used for detection of anti-HAV IgM or total antibody (117), but the use of saliva or other samples, such as urine, falls outside the manufacturers’ recommendations for current tests.

RPOC tests for HAV-specific IgM have been developed (118), but are not yet commercially available. In common with the RPOC test for HEV-specific IgM (33, 34) (below), such tests could prove especially useful in outbreak investigations.

Hepatitis E Virus

A number of research and commercial immunoassays for HEV-specific IgG and IgM are available in various countries, but with significant differences in their sensitivity and specificity (see below). Only IgG ELISA tests are currently approved for routine diagnostic use in the United States; in-house IgM ELISAs are available in reference laboratories. The appropriate use and interpretation of current serological assays for HEV infection must take into account differences in assay sensitivity and specificity, the pattern of serological responses (IgG and IgM, and in some cases IgA) to various antigens, and the widely varying prevalence of clinical HEV infection worldwide.

Laboratory-Based HEV Serological Tests

Many recombinant and synthetic peptide antigens have been used in diagnostics for HEV over the past 20 years, contributing to the uncertainty regarding seroprevalence rates around the world, as studies using different antigens cannot be compared. However, the general pattern of antibody responses to HEV infection can be implied from a large number of studies (Fig. 3). High titers of IgG are found during acute infection, but can remain at high levels for many years and HEV-specific IgM is therefore preferable for diagnosis, especially in areas with high prevalence of past HEV infection. IgM (and probably IgA) are usually elevated at the time of clinical presentation and remain detectable for some months; however, a small proportion of acute-phase patients will have HEV RNA in serum before antibody responses can be detected.

Improved commercial IgM and IgG tests have now been developed based on well-defined recombinant antigens in both ELISA and immunoblot formats. In studies with patients from developing countries, the MP Biomedicals HEV IgM ELISA 3.0 showed an overall sensitivity of 98.7% (149 positive of 151 confirmed HEV patients) and specificity of 97.6% (203 negative of 208 patients with other forms of viral hepatitis or other diseases) (34). The same antigen is also used in a double-antigen sandwich assay for total HEV-specific immunoglobulin (HEV ELISA 4.0), which is notable in being useful for detection of antibody in potential zoonotic hosts such as pigs, as well as in humans (119). These assays are based on a genotype 1 HEV antigen that is highly conserved between strains, but the HEV ELISA 3.0 has shown variable sensitivity and specificity for genotype 3 infections in different studies (8, 9).

Alternative commercial tests for HEV-specific IgM and/or IgG have now been developed, including some with incorporation of genotype 3 antigens. Studies focused on sensitivity and specificity for detection of genotype 3 infections have generally demonstrated good sensitivity for an immunoblot for separate detection of IgG and IgM and using genotype 1+3 antigens (Mikrogen recomline, Germany) and for ELISAs from Beijing Wantai (China), Fortress (United Kingdom), and DiaPro (Italy) (8, 9, 120, 121). As noted above, the MP Biomedical ELISA 3.0 showed reduced sensitivity for genotype 3 infections in one study (8), but equivalent in another (9). More comprehensive studies are still required to define the most appropriate serological tests for detection of IgM for diagnosis, and IgG for prevalence of HEV infection, especially in low-prevalence countries. HEV-specific IgA has also been reported to have utility in HEV diagnosis (122, 123) but further studies are required.

RPOC HEV serological tests

The major disease burden of HEV falls in developing countries and is especially severe among displaced populations such as refugees, with major epidemics observed during 2004–2005 in the Greater Darfur region of Sudan (124) and during 2012–2013 in Kenya (125) and what is now South Sudan (126). Timely access to sophisticated laboratory facilities is not available in these situations, highlighting the need for rapid diagnostic assays that can provide reliable diagnosis at the point of care, with minimal training or infrastructure. RPOC tests for HEV-specific IgM have now been developed in a novel format of immunochromatographic test (Fig. 4) (33, 34): these tests are commercially available in many countries outside the United States.

The HEV IgM RPOC test (MP Biomedical, Singapore) has been evaluated in two separate studies. In the first study (34), the RPOC test showed sensitivity of 96.7% (146 of 151) and overall specificity of between 98.6% (205 of 208) and 96.9% (220 of 227) depending on exclusion or inclusion of sera having high levels of rheumatoid factor (RF), a common source of false-positive reactivity in rapid assays. In the second study (33), the RPOC test showed slightly lower sensitivity of 93% (186 of 200), but specificity of 99.7% (320 of 321) against a panel including RF-positive sera and patients with acute Epstein-Barr virus infection, a further source of problems in many RPOC tests. The positive predictive value (PPV) and negative predictive value (NPV) of the test were estimated at 98.0 to 99.5% and 97.6 to 95.0%, respectively (33, 34). These results suggest that the RPOC tests will certainly prove useful for the diagnosis of HEV in field situations of endemic regions. An HEV IgM RPOC test has also been developed by Beijing Wantai (HEV-IgM Rapid Test), but there are no published reports on performance. RPOC tests offer the further benefit of reduced collection of venous blood.

Serological Diagnosis of HEV Infection in Areas of Low Prevalence

In areas with a low but significant incidence of clinical HEV infection, such as the United States and Western Europe, assay specificity will have a very large impact on the predictive value of HEV serologic tests. In order to be useful, HEV diagnostic assays will need to demonstrate very high specificity, high sensitivity, and the ability to detect infections caused by all four genotypes of HEV present worldwide. Assays for HEV-specific IgG are generally unsuitable for definitive serological diagnosis because of high background prevalence and should be restricted to initial screening and prevalence studies. For example, if the incidence of HEV infection among acute hepatitis patients in the United States was 0.2% versus a minimum estimate of HEV prevalence of 2%, then only 1 in 10 patients reactive in a test for HEV-specific IgG would be true positives, leading to an unacceptable PPV. Notably, some assays detect much higher HEV IgG prevalence rates among healthy populations with an even lower PPV for acute HEV infection.

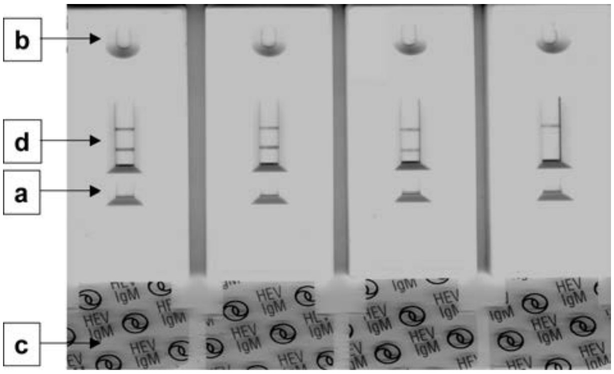

FIGURE 4 RPOC tests for HEV-specific IgM. Undiluted serum (25 µl) was added to the specimen window (a) and allowed to migrate through part of the membrane. Approximately 30 seconds later, 3 drops of buffer was added to the buffer window (b), the separator was removed by pulling the protruding end (c), and 1 drop of wash buffer was added to the specimen window. Results were visible through the viewing window (d) after 8 minutes. Samples are considered positive for IgM anti-HEV if two colored lines appear in the viewing window, and negative for IgM anti-HEV if a colored line appears only at the control line. Test kits as described (33, 34), kindly provided by MP Biomedical, Singapore. Data from the authors' laboratory. doi:10.1128/9781555817381.ch91.f4

The detection of HEV-specific IgM is therefore the method of choice for diagnosis of acute HEV infection in areas of low prevalence, along with HEV RNA where testing is available. As noted above, a variety of assays are commercially available for this purpose, including an IgM immunoblot using genotype 1+3 antigens (Mikrogen recomline); IgM ELISAs from Beijing Wantai (China), Fortress (United Kingdom), MP Biomedical (Singapore), and DiaPro (Italy); and the HEV IgM RPOC assay (MP Biomedical, Singapore). Many of these new tests appear to be suitable for use in nonendemic regions, but on the balance of evidence published to date, the Mikrogen recomline immunoblot and Beijing Wantai and Fortress IgM ELISAs may have the best sensitivity for genotype 3 infections that are most common in these regions (8, 9, 120, 121).

Serological Diagnosis of HEV Infection in Areas of High Prevalence

The detection of HEV-specific IgG is of little use for diagnosis of acute infection in developing countries where HEV is endemic and large numbers of patients will have IgG from past infections. Detection of HEV-specific IgM must therefore be the method of choice in areas of endemicity, especially since RNA assays are rarely available.

As HEV accounts for as much as 70% of the acute sporadic hepatitis in countries where hepatitis is endemic, the specificity of assays is less critical in these settings, and assay sensitivity is of primary concern. Because HEV genotypes 3 and 4 are less significant in these settings, all of the commercial IgM ELISAs and the HEV IgM RPOC assay (above) are likely to be suitable. The robustness of assays should also be considered, which may make the IgM immunoblot less suitable in these settings.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

In many developed countries, including the United States, the rarity of HEV infection and rapid decline in rates of HAV infection, coupled with increasing rates of hepatitis C virus infections, provides a challenge in the correct diagnostic test selection for individual patients. In particular, testing for HAV IgM in the absence of clinical or epidemiological features consistent with acute hepatitis A is likely to result in false-positive results (86), and the same is true of HEV. Close attention should be paid to the clinical characteristics and patient history, including overseas travel and immunosuppression, in both the selection of tests to be performed and the interpretation of test results.

Hepatitis A is a reportable disease in the United States, Australia, and many other developed countries, where patient contacts are generally managed by active or passive immunoprophylaxis. In routine laboratories, interpretation of HAV and HEV IgM serological tests is based on reference to internal test controls according to manufacturers' specifications. Many laboratories test for total HAV Ig, and if positive, retest for HAV IgM, which may be more convenient for sample throughput in areas of low endemicity. There are no standard confirmatory tests for HAV and HEV, highlighting the need for test requests to be justified on clinical and/or epidemiological grounds in order to reduce false-positive results. False-negative results are uncommon for clinically apparent cases of HAV, with IgM almost always detectable at the time of clinical onset, but if false negatives are strongly suspected, for example a negative HAV IgM test in a patient with raised ALTs and contact with a confirmed case of HAV, it may be appropriate to request a second specimen for retesting. Negative results for HEV IgM (or IgG) in immunosuppressed patients with biochemical evidence of hepatitis should be interpreted with care, because a false-negative result can result in delay of appropriate antiviral therapy for chronic HEV infection (97); RNA testing should be considered.

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Hepatitis C Virus

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TAXONOMY

Hepatitis C virus (HCV) is classified within the family *Flaviviridae* in its own genus, *Hepacivirus*. Phylogenetic analysis of helicase sequences has been used to probe its relatedness to other viruses in the family (1). The data suggest that HCV is most closely related to the nonpathogenic human virus GBV-C. The next closest relatives appear to be the pestiviruses, viruses that infect nonhuman hosts (bovine viral diarrhea virus and hog cholera virus). HCV is more distantly related to arthropod-borne viruses in the *Flavivirus* genus that infect humans (yellow fever virus, dengue virus, West Nile virus).

DESCRIPTION OF THE AGENT

HCV has an ~9.6 kb positive-sense RNA genome composed of a long open reading frame (ORF) flanked by terminal 5′ and 3′ untranslated regions (UTRs, Fig. 1). The 5′ UTR is highly conserved and the 3′ UTR has a short variable sequence, a poly(U) tract, and a highly conserved element. Core, p7, E1, and E2 are structural protein genes that encode nucleocapsid, transmembrane, gp33, and gp72 proteins, respectively.

During replication, the HCV ORF is translated into a single polyprotein (approximately 3,000 amino acids) that is subsequently cleaved by host and viral proteases encoded by NS2, 3, and 4A genes. The NS3 gene also encodes a helicase. The p7 region encodes a protein that is essential for replication of infectious virus (2); however, its specific function is unknown. The RNA-dependent RNA polymerase (RDRP) NS5B lacks efficient proofreading activity, resulting in extensive genome mutation during replication and quasispecies generation within an infected individual. NS5A is a multifunctional, nonstructural phosphoprotein (3). It is a key component of the replication complex and binds other constituents of the complex, including RDRP, RNA, and cyclophilin A, a host protein required for HCV RNA replication. NS5A also appears to play a role in virion assembly.

Currently, there are seven major genotypes of HCV and more than 50 known subtypes based on genomic sequence heterogeneity (4). Genotypes and subtypes differ by 30 to 35% and by 20 to 25% of nucleotides, respectively. Genotypes 1 to 3 (Gt1 to Gt3) have a worldwide distribution and account for most HCV infections in Europe and North America. In the United States, the majority of HCV infections in all age groups are Gt1 (75%), followed by Gt2 (13.5%) and Gt3 (5.5%). Gt4 is most prevalent in the Middle East and in North and Central Africa; Gt5 is found primarily in South Africa; Gt6 occurs throughout Asia; Gt7 has been detected in an immigrant from the Congo, and its region of endemicity is unclear. This chapter focuses on Gt1 to Gt4, given their worldwide prevalence.

EPIDEMIOLOGY AND TRANSMISSION

HCV is a globally significant pathogen, infecting over 150 million individuals. In the United States, it is the most common blood-borne infection, causing an estimated 3 million chronic infections (5). Acute hepatitis C occurs in the United States at a rate of approximately 0.3 per 100,000, with estimates of 19,000 new infections per year after adjusting for underreporting and asymptomatic infection (6). In the blood-screening era, most transmission occurs after exposure to a low viral inoculum through intravenous drug use, multiple sexual partners, sex with a chronically infected partner, iatrogenic exposure, and occupational exposure to blood such as needlestick (7). Rates of acute hepatitis C in HIV-infected men who have sex with men (MSM) are rising and greater than those in HIV-uninfected MSM, suggesting that the former is a high-risk group that merits screening (8).

CLINICAL SIGNIFICANCE

The clinical features of acute infection are depicted in Fig. 2. The majority of individuals are thought to be asymptomatic. Spontaneous clearance is observed in approximately 25% of acute infections; the remaining individuals become chronically infected. Signs of hepatitis during acute infection are actually positive indicators, as they represent early, vigorous antiviral T-cell responses associated with spontaneous virus clearance. These responses are minimal or absent in individuals who progress to chronicity (9).

Why some individuals clear their infection while others progress to chronic HCV infection is unknown. To investigate the mechanisms governing clearance, genome-wide association studies (GWAS) have been used to identify genetic determinants that predominate in individuals with spontaneous clearance versus progression to chronicity. This approach has identified a single nucleotide polymorphism (SNP) in the *DQA1-DQB2 HLA* class II region (10) found on reference SNP (rs) 4273729, and multiple SNPs upstream

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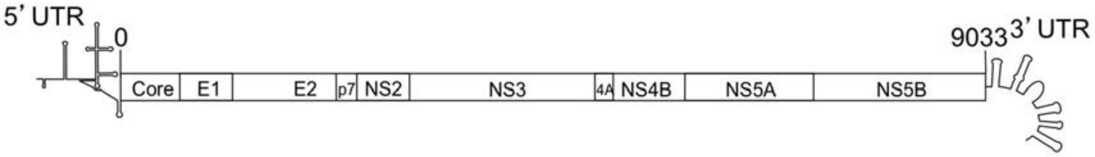

Protein	Role	nuc pos	aa pos	aa length	size
Core	Encapsidation	342	1	191	p21
E1	Receptor binding, entry?	915	192	192	gp31
E2	Receptor binding, entry?	1491	384	363	gp70
p7	viroporin?	2580	747	63	p7
NS2	NS2/3 Zn-dependent protease	2769	810	217	p21
NS3	NS2/3 Zn-dep protease, NS3/4A protease, helicase-NTPase	3420	1027	631	p70
NS4A	cofactor for NS3-4A protease	5313	1658	54	p8
NS4B	membranous web organization	5475	1712	261	p27
NS5A	phosphoprotein	6258	1973	448	p58
NS5B	RNA-dependent RNA polymerase	7602	2421	591	p68

FIGURE 1 HCV genome and protein coding scheme. UTR, untranslated region; E, envelope; NS, nonstructural gene; nuc, nucleotide; pos, position; aa, amino acid; gp, glycoprotein. Numbering is according to references 108 and 109. doi:10.1128/9781555817381.ch92.f1

from the *IL28B* gene (11, 12; reviewed in reference 13), of which the most strongly associated are variants on rs12979860 (10) that are independently associated with clearance and additively effective. The homozygous G allele on *HLA* class II rs4273729 and homozygous C allele on *IL28B* rs12979860 are associated with clearance. The homozygous C allele on *HLA* class II rs4273729 and the homozygous T allele on rs12979860 are associated with chronic infection. Heterozygotes tend more toward chronic infection than clearance. *IL28B* encodes interferon λ3, an interferon that stimulates a signaling cascade similar to interferon α and interferon β, but through a distinct receptor. The worldwide geographic distribution of *IL28B* genotypes appears to explain in part the observed racial differences in acute infection outcome (12, 14). Haplotypes associated with resolution are found more commonly in East Asian populations in whom rates of spontaneous clearance are high. Haplotypes associated with progression to chronic infection occur with higher frequency in individuals of African ethnicity in whom rates of chronic infection are high. How genetic determinants in *HLA* class II and *IL28B* regulate immune responses that mediate HCV clearance is not yet understood.

Disease progression occurs in a relatively small proportion of patients with chronic hepatitis C infection. Over decades, progressive liver damage produces cirrhosis in 10 to 20% of chronic infections and liver failure or hepatocellular carcinoma in approximately 5% of chronic infections (15). However, the overall disease burden is significant due to the number of infected individuals. In fact, liver failure due

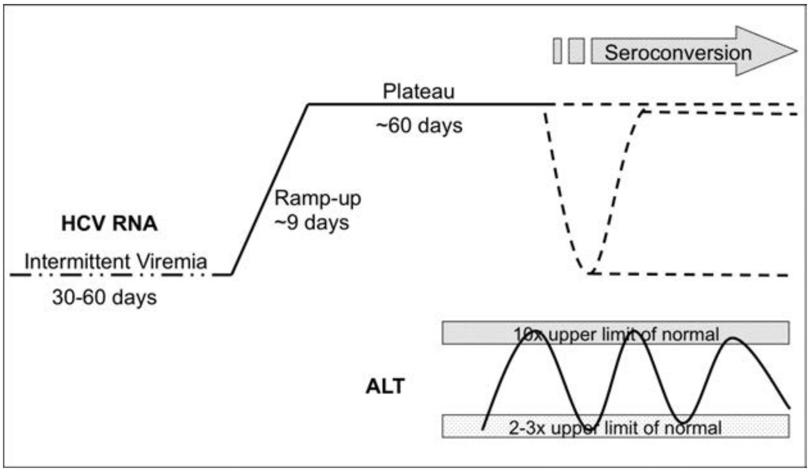

FIGURE 2 Clinical features of acute hepatitis C following exposure to low viral inoculum such as occupational needlestick exposure or community-based exposure (49, 76, 110). Characteristics following higher-dose exposure (transfusion with contaminated blood products) may be different. Intermittent viremia phase estimated from needlestick exposure (76). Kinetics of other characteristics derived from seroconversion panels (49). ALT, alanine aminotransferase. Dashed lines indicate potential viremia patterns as defined by HCV RNA levels in peripheral blood. Adapted from reference 49. doi:10.1128/9781555817381.ch92.f2

to chronic hepatitis C infection is the leading cause of liver transplantation in the United States. (16). Risk factors for disease progression include diseases or behaviors that induce additional hepatic injury (such as concomitant hepatitis B virus infection and alcohol consumption) or impair antiviral immunity (such as HIV infection). Epidemiologic descriptors such as male gender and older age at infection are also associated with higher risk and faster rate of disease progression.

Unlike other chronic viral infections such as HIV and hepatitis B virus, virologic parameters including viral load and genotype do not predict disease progression or indicate disease severity in chronic hepatitis C (17, 18). Viral load remains fairly constant once chronic hepatitis C infection is established (18), and rates of progression have been found to correlate more with disease severity in the liver, as manifested by the extent of fibrosis on initial liver biopsy (19), than with the level of hepatitis C virus replication represented by viremia (20).

The HCV treatment landscape has historically been dominated by interferon α, which, in its pegylated form in combination with ribavirin, had reasonable efficacy for Gt2 and Gt3 (~80%) but much lower efficacy for Gt1 (~45%). Genetic determinants of treatment response to interferon α-containing treatment regimens appear to vary from those associated with clearance of acute infection. The *DQA1-DQB2 HLA* class II region SNP was not found to be associated with therapeutic responsiveness of Gt1 infections (10), whereas loci upstream of *IL28B* appear to be important (10, 11, 21–23). The allele on rs12979860 appears to be the most significant; the CC haplotype was found to be the strongest pretreatment predictor of response, was associated with more rapid virus clearance kinetics in the first 48 hours of treatment, and occurred in a high proportion of individuals in whom viremia was no longer detectable after only 4 weeks of treatment (24). The role of *IL28B* SNPs in responsiveness of Gt2 and Gt3 infections to pegylated interferon/ribavirin is controversial; some studies demonstrate supportive data while others have failed to find an association (25).

The overall poor efficacy of pegylated interferon/ribavirin has spurred the development of direct-acting antiviral drugs (DAAs) for the treatment of chronic hepatitis C virus infections. Approval of the first-generation Gt1-specific protease inhibitors boceprevir and telaprevir was greeted with a good deal of optimism given the findings of clinical trials that demonstrated substantially improved efficacy of approximately 70% (26, 27). However, once these drugs were placed into general clinical use, high rates of adverse reactions were observed that limited tolerability and led to premature discontinuation, and enthusiasm for them dampened quickly. With the approval of second-generation DAAs with equivalent (or better) efficacy and apparently improved side-effect profiles, these first-generation agents are no longer recommended for use in U.S. treatment guidelines (http://www.hcvguidelines.org). They figured prominently in European treatment guidelines published in early 2014 (28) but have been dropped in more recent Web-based recommendations (http://www.easl.eu/_clinical-practice-guideline).

Newly approved second-generation DAAs include sofosbuvir and simeprevir. Sofosbuvir, an NS5B inhibitor with pan-genotypic activity, is a significant advance in HCV treatment. It is highly effective (response rates of >90% for Gt1, Gt2, and Gt4; 29), can be administered in an oral, interferon-free regimen along with ribavirin for treatment of Gt2 and Gt3, and requires the briefest treatment duration to date for previously untreated patients (12 weeks in combination with pegylated interferon plus ribavirin for Gt1, 12 weeks in combination with ribavirin for Gt2, and 16 weeks in combination with ribavirin for Gt3). It is the first drug approved for use in Gt4 infections. Simeprevir is an NS3/4A (protease) inhibitor with activity against Gt1 and Gt4 that was recently approved for use in combination with pegylated interferon α plus ribavirin in Gt1-infected patients, including treatment-naive individuals and those who have previously failed treatment. Response rates of >80% were observed in treatment-naive individuals (30) and patients who responded to pegylated interferon α plus ribavirin during treatment, but relapse occurred after treatment cessation (31). Treatment duration is reduced by half compared to the standard regimen of pegylated interferon plus ribavirin (24 versus 48 weeks).

Therapy for hepatitis C virus infection continues to evolve. The trend, initiated by sofosbuvir treatment for Gt2 and Gt3, is toward highly potent DAAs that can be dosed orally (interferon-free), once daily, and for short duration (24 weeks or less). Several new combination regimens with these features are notable as they have completed phase III clinical trials and will likely soon be available for clinical use. For Gt1 treatment, response rates of >90% have been observed with therapy as short as 12 weeks for several two-drug regimens and a quadruple-drug regimen (32, 33). The two-drug regimens include simeprevir plus sofosbuvir and NS5a inhibitors plus sofosbuvir (daclatasvir plus sofosbuvir [34] and ledipasvir plus sofosbuvir [35, 36]). This treatment success is particularly remarkable considering the typically low response rates (~40%) of Gt1 infections to standard 48-week pegylated interferon α plus ribavirin regimens. Similar high efficacy was observed with a 24-week treatment course of daclatasvir plus sofosbuvir for Gt2 and Gt3 (34).

Numerous DAAs in the classes of N3/4A protease inhibitors, NS5B polymerase inhibitors (nucleotide and non-nucleotide inhibitors), NS5A replication complex inhibitors, and inhibitors of host cell proteins required for HCV genome replication, such as cyclophilin A, are currently in clinical trials. Given the plethora of agents and drug combinations that are expected to gain approval in the next few years, detailed description is beyond the scope of this chapter, and instead the reader is referred to several excellent reviews (37, 38) and Web-based resources containing the latest recommendations on all aspects of HCV treatment (http://www.hcvguidelines.org, http://www.easl.eu/_clinical-practice-guideline).

Treatment for chronic hepatitis C has changed from a “one size fits all” paradigm, in which all patients were treated for 48 weeks with pegylated interferon plus ribavirin, to one of tailored therapy based on genotype and response to treatment as assessed by viral load (termed “response-guided therapy” and used primarily during treatment of Gt1 infections with pegylated interferon/ribavirin and with first-generation DAAs telaprevir and boceprevir). With the advent of highly potent, pan-genotypic DAA combination therapy, the pendulum may eventually swing back to “one size fits all,” resulting in considerably simplified treatment-associated testing. In the interim, diagnostic testing is evolving as new drug regimens are introduced. Change has occurred in the types of tests that are useful and in how they are used. The types of assays that are useful, and their utility during treatment with currently available drugs, are summarized in Table 1 and discussed further in the diagnostic testing sections below.

TABLE 1 Utility of HCV RNA tests in different treatment regimens

Drug regimen	Viral genotype/subtype	Viral load	IL28B genotype (rs12979860 haplotype determination)	Resistance mutation detection
Pegylated interferon α/ribavirin	Genotype required to determine duration of therapy and probability of response; subtype (Gt1a vs Gt1b) of no utility	All genotypes: baseline, end of treatment, end of follow-up (to determine SVR) Gt1 and Gt4: on-treatment monitoring for response-guided therapy and futility rules	Gt1: helpful in predicting response to therapy Gt2 and Gt3: not useful	Not useful
Telaprevir or boceprevir plus pegylated interferon α/ribavirin	Genotype required to determine treatment eligibility (Gt1-specific protease inhibitors); subtype (Gt1a vs Gt1b) potentially useful in predicting moderate differences in therapeutic response	Baseline, on-treatment monitoring for response-guided therapy, futility rules, end of treatment, end of follow-up (to determine SVR)	Helpful in predicting response to therapy	Not useful
Simeprevir plus pegylated interferon α/ribavirin	Genotype required to determine treatment eligibility (simeprevir approved solely for Gt1); Gt1 subtype necessary (Gt1a should have resistance testing for NS3 Q80K mutation)	Baseline, on-treatment monitoring for futility rules, end of treatment, end of follow-up (to determine SVR)	Helpful in predicting response to therapy	Gt1a: detection of NS3 Q80K mutation at baseline to determine treatment eligibility
Sofosbuvir plus pegylated interferon α/ribavirin (Gt1 and Gt 4) Sofosbuvir plus ribavirin (Gt2 and Gt3)	Genotype required to determine treatment regimen and treatment duration (Gt3 treated for longer than Gt2)	Prescribing information lacks directives; baseline, end of treatment, and of follow-up helpful in determining response. On-treatment testing likely to be performed to assess compliance	Not useful	Not useful
Next-generation combination DAAs	Genotype likely necessary to determine regimen and duration; subtype (Gt1a vs Gt1b) potentially useful for protease inhibitors to predict moderate differences in therapeutic response	Baseline, end of treatment, and of follow-up helpful in determining response. On-treatment testing likely to be performed to assess compliance	Unknown but likely not useful	Unknown but likely not useful

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Serum and plasma are acceptable for HCV NATs and serology tests. Plasma collected in heparin-containing tubes is not acceptable for PCR-based assays since *Taq* polymerase is inhibited by this anticoagulant. Serum and plasma should be obtained from whole blood as per standard technique, described in chapter 79. Requirements for specimen processing and storage, such as recommended intervals between collection/processing, storage conditions, and freeze-thaw cycles, vary by assay; package inserts should be consulted for specific information. Generally, whole-blood specimens can be transported at 25°C prior to processing. Liver tissue is not typically tested, except for histologic assessment of fibrosis in Gt1-infected individuals.

DIRECT EXAMINATION

Microscopy

By electron microscopy, HCV virions are 55- to 65-nm spheres with 6-nm surface projections. HCV antigens and nucleic acids are detectable in liver sections by immunohistochemistry and *in situ* hybridization, respectively. However, these methods are insensitive and nonspecific, and therefore HCV detection by microscopy has minimal clinical utility.

In the pre-DAA era, liver fibrosis was usually assessed histologically in Gt1 infections to determine liver disease status to justify the need for therapeutic intervention. Gt1 infections are poorly responsive to pegylated interferon/ribavirin treatment and typically required longer treatment duration. Therefore, therapy for Gt1 infections was usually reserved for patients with advanced fibrosis, in an attempt to prevent cirrhosis. With the availability of highly effective therapy for all HCV genotypes, the rationale for histologic assessment will likely shift from treatment justification to ensuring appropriate management for individuals with bridging fibrosis/cirrhosis such as screening for hepatocellular carcinoma, treatment of cirrhosis-related sequelae, and selection of an appropriate DAA regimen, since infections in these individuals have historically been more difficult to treat (39).

In response to the disadvantages of liver biopsy (its invasive nature, potential complications, and sampling error), noninvasive tests have been developed, including assessment of liver stiffness through ultrasound (transient elastography) and surrogate markers of fibrosis that can be assessed in peripheral blood. Noninvasive tests are useful for detecting advanced fibrosis but are poor at distinguishing intermediate fibrosis stages. Biopsy is therefore preferred if this level of accuracy is necessary. Otherwise, noninvasive tests for the detection of advanced fibrosis/cirrhosis have been advocated in the latest treatment guidelines (http://www.hcvguidelines.org, http://www.easl.eu/_clinical-practice-guideline).

Antigen Detection

A core antigen detection/quantification chemiluminescent microparticle immunoassay is marked for use in Europe (Conformité Européenne) (Architect HCV Ag Test, Abbott, Germany) and is available globally. In this test, microparticles coated with anti-core antibodies are used to detect core antigen in pretreated serum or plasma samples. This test is potentially useful in resource-limited settings because of target stability (eliminating the need for sample storage at ultra-low temperatures), simplified instrumentation, and easier technical protocols that reduce labor/training pressures. The analytical sensitivity ranges from 3 to 13 fmol/liter depending upon genotype (corresponding to approximately 3.0 $\log_{10}$ IU/ml HCV RNA measured by NAT), the assay is linear between 0.5 and 4.0 $\log_{10}$ fmol/liter, and good quantitative correlation with HCV RNA quantification tests has been observed (40–42). For the diagnosis of chronic infection, this test is useful as a surrogate for NAT, to identify seropositive individuals who are viremic (43, 44). However, its inferior performance compared to NAT using seroconversion panels suggests that it is too insensitive for the diagnosis of acute infection (42). This test may be of some use in predicting response to pegylated interferon α/ribavirin at early times (week 1 and week 2) after treatment initiation (45), but it lacks adequate sensitivity to assess response at end of treatment. It is also unlikely to be of much utility in determining therapeutic response to DAAs, where assays with limits of detection of 10 to 15 HCV RNA IU/ml are required at end of treatment and end of follow-up.

Nucleic Acid Detection/Quantification Tests

Clinical Utility

NAT Usage in the Diagnosis of Acute Hepatitis C

HCV NATs are useful in establishing the diagnosis of acute HCV infection in seronegative individuals because HCV RNA can be detected as early as 1 week after exposure via needlestick or transfusion (46–48) and at least 4 to 6 weeks prior to seroconversion in a number of transmission settings (49). Testing by sensitive qualitative or quantitative NATs (Tables 2 and 3) is recommended (<15 IU/ml in European guidelines, ≤ 50 IU/ml in U.S. guidelines [28, 50]) since viral loads are low and HCV RNA in peripheral blood is intermittently detected early after known exposure (49). Establishing this diagnosis is useful due to the efficacy of therapy. Without therapy, approximately 75% of infected individuals will develop chronic infection and may require future therapy. Sustained response rates of 70 to 80% have been reported when treatment with interferon α monotherapy is initiated within 12 weeks of acute HCV diagnosis (51, 52). The decision to initiate interferon α therapy immediately versus waiting to allow for spontaneous clearance is complex; deferral can be considered if factors associated with spontaneous resolution, such as favorable *IL28B* genotype and symptoms of hepatitis, are present (53, 54). The potential availability of highly efficacious DAAs that require short treatment courses may significantly simplify this decision in the future.

NAT Usage in the Diagnosis of Chronic Hepatitis C

Given the asymptomatic nature of most acute HCV infections, the majority of patients will present symptomatically in the chronic phase. The diagnosis of chronic HCV is established with antibody screening tests to document infection and with HCV RNA NAT to demonstrate viral replication. NAT is particularly important in identifying chronic HCV infection in the setting of HIV, where the prevalence of HIV/HCV coinfections is high. Approximately 5% of HIV-1-infected individuals have HCV viremia but no HCV antibodies as detected by second- and third-generation serologic assays; low CD4 cell counts (<200 cells/μl) have been found to be a risk factor (55, 56). NAT should therefore be performed regardless of serology results to accurately diagnose chronic infection in coinfected patients whose humoral responses may be impaired.

NAT Usage in the Management of Chronic Hepatitis C Therapy

NATs continue to be diagnostically important as new DAAs are introduced; however, their roles are changing (summarized in Table 1). HCV genotype and viral load should be determined at baseline. Genotype is used to determine treatment regimen and duration, depending upon the drug. In the pegylated interferon-α/ribavirin era, genotype was also useful in predicting response to therapy; however, response rates to DAAs are generally uniformly high across genotypes. Three viral load assessments are common to all therapeutic regimens: baseline, end of treatment, and end of follow-up. Baseline testing is critical as it is the starting point in documenting treatment-induced elimination of viremia. End-of-treatment testing is performed to ascertain that adequate clearance of viremia occurred during drug treatment. End-of-follow-up testing is performed months

TABLE 2 Commercial HCV RNA qualitative tests[a]

Test (manufacturer)	Test format	Extraction chemistry	Amplification chemistry	Limit of detection ($\log_{10}$ IU/ml)
Aptima HCV (Hologic)	Manual	Target capture (HCV RNA)	TMA	1.0
Cobas Amplicor v2.0 (Roche)	Semiautomated[b]	Guanidine HCl (total RNA)	Reverse transcription/PCR	1.7
Cobas Ampliprep/Cobas Amplicor v2.0 (Roche)	Automated	Silica-based (total nucleic acid)	Reverse transcription/PCR	1.7
Versant HCV RNA (Siemens)	Manual	Target capture (HCV RNA)	TMA	1.0

[a] All tests are FDA approved and Conformité Européenne (CE) marked according to European *In Vitro* Diagnostic Directive 98/79/EC.
[b] Manual extraction, automated reaction setup.

TABLE 3 Commercial HCV RNA quantification tests[a]

Test (manufacturer)	Extraction chemistry	Amplification method	Measurable range (log$_{10}$ IU/ml)[b]	Limit of detection (log$_{10}$ IU/ml)	Reference(s)
Cobas AmpliPrep/Cobas TaqMan v1.0 (Roche)	Silica-based (total nucleic acid)	RT-real-time PCR	1.6 to 7.8[c]	1.2	102
Cobas AmpliPrep/Cobas TaqMan v2.0 (Roche)	Silica-based (total nucleic acid)	RT-real-time PCR	1.2 to 8.0	1.2	64
High Pure/Cobas TaqMan v2.0 (Roche)	Guanidine HCl/glass fiber column (total viral nucleic acid)	RT-real-time PCR	1.4 to 8.5	1.3	112, 113
RealTime HCV (Abbott)	Magnetic microparticle/total nucleic acid	RT-real-time PCR	1.1 to 8.0	1.1	59, 93, 94, 100, 113
Versant HCV RNA 3.0 (Siemens)	Lysis without extraction	bDNA	2.8 to 6.9	2.8	101

[a] All tests are FDA approved and Conformité Européenne (CE) marked according to European *In Vitro* Diagnostic Directive 98/79/EC.
[b] Lower limit of quantification to upper limit of quantification of undiluted specimens.
[c] Analytical measuring range. Expanded clinical reportable range (1.6 to 9.8 log$_{10}$ IU/ml) can be obtained by maximum dilution of 1:100.

after the end of treatment, to assess durability of clearance in the absence of drug. Individuals who have no detectable viremia by NAT at end of follow-up are defined as having achieved sustained virologic response (SVR) and are considered cured (57). Viral load monitoring for SVR was conventionally performed 24 weeks after end of treatment with pegylated interferon α/ribavirin and first-generation DAAs. A follow-up period of 12 weeks has been shown to be comparably predictive of SVR at 24 weeks (58); the attainment of SVR after 12 weeks of follow-up is now incorporated into analysis of all DAA trials and is being used for patient management with all new DAA combinations.

The need for therapeutic monitoring during treatment is regimen-dependent. Less potent therapies requiring long treatment courses are administered under the paradigm of response-guided therapy, in which viral load assessments at various times during treatment are used to differentiate responders from nonresponders and to tailor treatment duration accordingly. In rapid responders, shorter drug courses are effective, while longer courses are required for individuals who clear virus more slowly. Treatment is stopped early in nonresponders, defined as those who fail to meet clearance milestones as defined by each drug regimen. The newest highly potent DAA combinations are used for short courses and are so effective that on-treatment monitoring to assess therapeutic efficacy is largely unnecessary. In the future, it is likely that on-treatment testing will be performed solely to assess compliance and to allow counseling of individuals with residual on-treatment viremia regarding the requirement for adherence to therapy.

NATs that are acceptable for use in management with DAAs are more sensitive and have lower limits of quantification than those that are acceptable for pegylated interferon α/ribavirin. Assays that were used to define efficacy in phase III clinical trials of DAAs had performance characteristics that included a lower limit of quantification of at least 25 HCV RNA IU/ml and a limit of detection of at least 15 HCV RNA IU/ml. Tests that conform to these characteristics should be used to determine response to DAA treatment (Table 3).

Nucleic Acid Preparation for HCV NAT

Nucleic acid extraction is the first step in almost all HCV NATs. Some extraction methodologies are HCV specific while others target total RNA, total nucleic acids, or total viral RNA in clinical samples (Tables 2 and 3). Although semiautomated assays that consist of manual extraction with automated downstream processes are still available, most testing is currently performed with automated commercial test systems that include instrumentation for front-end nucleic acid extraction, reaction setup, and back-end qualitative detection or quantification (Tables 2 and 3). There have been no comprehensive comparisons of these automated extraction platforms using a single HCV NAT to determine extraction efficiencies and overall performance.

Qualitative Tests

Qualitative NATs that employ reverse transcription/conventional PCR and transcription-mediated amplification (TMA) are available (Table 2; see chapter 4 for description). TMA is more sensitive than conventional RT-PCR. Qualitative NATs are approved for the detection of HCV RNA in seropositive individuals to document chronic infection. None of the available assays is approved for use in assessment of treatment response to DAAs; however, TMA-based tests are adequately sensitive (limit of detection, <15 IU/ml) for use in accurately determining clearance of viremia at end of treatment and SVR at end of follow-up.

Quantitative Tests

A World Health Organization international calibration standard has been available since 1997; HCV RNA is therefore quantified in International Units per milliliter (IU/ml). The 4th International Standard is currently in use. It consists of plasmas from three different Gt1a-infected individuals collected prior to seroconversion that have been pooled and then further diluted in HCV-seronegative/HCV RNA-negative pooled human plasma. It is a biological standard whose concentration was derived by testing at multiple sites with currently approved quantitative HCV NATs (Table 3), plus one additional commercial assay not approved for use in the United States (Versant HCV kPCR v1.0). To permit longitudinal consistency in quantification, the 4th International Standard has been calibrated in parallel with the 2nd and 3rd International Standards and has been assigned a value of 5.4 log$_{10}$ IU/ml.

Most quantitative NAT testing is performed with commercially available tests that have obtained regulatory approval (Table 3); some large reference laboratories use their own proprietary laboratory-developed NATs. Results are reported as IU/ml. The Versant HCV RNA 3.0 is a branched DNA (bDNA)-based microwell plate assay that does not require nucleic acid extraction. HCV virions are lysed and viral genomes are captured with probes. Genomes are quantified by hybridization using a series of probes that contain iso-C and iso-G oligonucleotides to reduce nonspecific hybridization and increase assay sensitivity. Luminescent signal emission is enhanced through preamplifier and amplifier probe hybridization. Quantification is performed using external calibrators that are included on each plate. The advantages of this assay are its simple genomic isolation procedure, high upper limit of quantification (ULQ), genotype inclusiveness, and reproducibility. The greatest drawback is its lower limit of quantification/limit of detection that is greater than that of real-time RT-PCR assays. This test is insufficiently sensitive for monitoring response to DAA therapy.

HCV RNA quantification can be performed by real-time PCR with commercially available reagents (Table 3) that amplify 5′ UTR sequences but employ slightly different amplicon detection chemistry. The probe in the Abbott RealTime HCV ASR assay is labeled with a 5′ fluorophore and a 3′ quencher. Unhybridized probe is randomly coiled and fails to fluoresce due to fluorophore and quencher proximity. Major fluorescence occurs upon probe hybridization; a minor amount of fluorescence is produced due to cleavage of the 5′ labeled nucleotide by *Taq* polymerase. Reduced genotype bias is achieved through low-temperature hybridization that permits binding of probes despite probe-target mismatches. Quantification is performed via an internal calibrator. This assay has a limit of detection and measurable range suitable for use with DAAs (59).

In the Cobas AmpliPrep/Cobas TaqMan HCV real-time PCR test, total nucleic acid extraction and reaction setup are performed by the AmpliPrep, and amplification occurs on the TaqMan instrument. Two versions are available. The first test version demonstrated genotype bias, with an inability to detect some Gt4 infections and underquantification of others (60–62). A second, newer version has numerous modifications from sample preparation through target amplification to improve genotype inclusivity and increase sensitivity while allowing lower sample input volume (650 µl versus 1 ml for version 1.0 [63, 64]). A third real-time PCR test combines viral RNA extraction with manual spin column (High Pure) with TaqMan 2.0 real-time PCR. These assays are calibrated externally by the manufacturer; lot-specific calibration coefficients are used by system software to calculate HCV RNA concentrations. An internal quantitative standard is added prior to extraction to quantitatively correct for potential inhibitors within individual samples. The 2.0 versions have limits of detection and measurable ranges that are suitable for use with DAAs (Table 3).

ISOLATION PROCEDURES AND IDENTIFICATION

HCV is a fastidious virus that is not recoverable with mammalian cells (primary or transformed) carried in routine or reference clinical virology laboratories. Virus can be propagated in specialty laboratories using systems for recovery of infectious virus after transfecting permissive cells with HCV molecular clones (65–67) or using a newly reported hepatoma cell line (68). These advances are powerful tools for the development of DAAs and vaccines, but they have not been adopted for diagnostic use.

GENOTYPING

Genotyping of HCV is useful for determining drug treatment regimen (Table 1). Genotype determination is also useful for counseling patients on the likelihood of therapeutic response to pegylated interferon/ribavirin. For highly effective DAAs, the prognostic value of genotyping is likely to be more limited and regimen dependent. For example, SVR rates to sofosbuvir/ribavirin are at least 30% greater for Gt2 than for Gt3; larger differences were observed in cirrhotics and individuals who had failed prior interferon α/ribavirin treatment (29).

Subtyping of Gt1 (determination of Gt1a versus 1b) is important in the DAA era. Response to certain protease inhibitor regimens can be subtype dependent. For example, SVR rates to protease inhibitors are 15% higher for Gt1b than for Gt1a (26, 27, 69). Gt1 subtyping is also important in the selection of certain DAA regimens. Individuals with Gt1a infections can harbor viruses with preexisting mutations that confer resistance to protease inhibitors, specifically the point mutation in NS3 resulting in glutamine-to-lysine change at amino acid 80, or in Q80K, which confers resistance to simeprevir. In prescribing information for simeprevir, it is recommended that individuals with Gt1a infections be further tested for Q80K to determine suitability for treatment with regimens containing this protease inhibitor.

HCV genotypes and subtypes can be determined by commercially and user-developed NATs based on a variety of biochemical methods (summarized in Tables 4 and 5). Assays based on 5′ UTR sequences are generally acceptable for genotype determination but must be carefully designed for subtyping because of the degree of sequence conservation among different viruses. More accurate subtype determination can be achieved through analysis of NS5b, core, and/or core-E1 genes.

As of this writing, there is one U.S. Food and Drug Administration (FDA)-approved HCV genotyping product available in the United States, the RealTime HCV Genotype II test. This assay employs reverse transcription/real-time PCR using TaqMan chemistry and multiple hydrolysis probes in three different reactions to detect all HCVs, to identify Gt1-5, and to differentiate Gt1a from Gt1b. The 5′ UTR is used as the target for genotype discrimination, and NS5B sequences are used to for Gt1 subtyping. The test is highly accurate compared to direct sequencing (70). Indeterminate rates of approximately 2% have been reported, with most issues arising from Gt3 determination; an inability to distinguish Gt1 subtypes was observed for approximately 5% of samples (71).

Direct (Sanger) sequencing is considered the gold standard in accuracy for HCV genotype and subtype determination. Sequences generated from samples are compared to reference genotype and subtype sequence libraries. Mixed genotype infections can be difficult to detect when the proportion of one genotype greatly exceeds the other. Although it is too laborious for use in clinical laboratories, amplicon cloning may help to detect minority genotype populations (below 10%); however, analysis of large numbers of clones may be required. Sequencing methods require specialized instrumentation and analysis software. These methods are generally labor-intensive, and time to result is usually longer than with other methods.

Reverse hybridization is probably the most common method adopted by clinical laboratories. It is more reliable than direct sequencing for the detection of mixed infections. Low-throughput formats use paper strips; higher-throughput microwell plate and microarray formats have been developed (Table 4).

For tests that have received regulatory approval, laboratories must verify accuracy of test performance in-house. More extensive assay verification is required for laboratory-

TABLE 4 Commercial HCV RNA genotyping tests[a]

Test	Method	Target(s)	Subtyping	Comment	Reference(s)
eSensor HCV Genotyping Test (GenMark)[b]	Amplicon capture with oligonucleotide probes; electrochemical detection with ferrocene-labeled oligonucleotide signal probes	5′ UTR	No	Suitable for use with Roche Cobas Ampliprep/Cobas TaqMan amplicons; subtyping unreliable due to 5′ UTR conservation	114
Trugene HCV[b] (Siemens)	Direct bidirectional sequencing using forward/reverse primers labeled with unique fluorophors	5′ UTR	No	Suitable for use with Roche Amplicor HCV or Amplicor HCV Monitor test amplicons; subtyping unreliable due to 5′ UTR conservation; resolution of mixed genotypes (9:1 ratio) reported	115
Versant HCV Genotype (LiPA) 2.0 (Siemens)[c,d]	Reverse hybridization with detection on strips (line probe); automated blot processor and band interpretation instrumentation available	5′ UTR and core gene	Yes	In contrast to direct sequencing, mixed genotype infections are readily detected; compared to version 1.0 (5′ UTR analysis only) version 2.0 demonstrates improved genotype and subtype accuracy (particularly Gt1, 6); some difficulty in reliably distinguishing Gt2 and Gt4 subtypes reported	116–118
Gen-Eti-K DEIA (Sorin Biomedica)	Reverse hybridization with detection on multiwell plate	Core gene		Performance reported as similar to LiPA 2.0	119, 120
HCV DNA Chip v2.0 (Bio-Core)	Amplicon hybridization to chip	5′ UTR	Yes	Agreement with direct sequencing reported as 100% and 95% for genotype and subtype	121
Invader (Hologic)[e]	RT-PCR plus Invader detection	5′ UTR	No	Two genotypes detected in single well using distinct genotype-specific probes; invader oligonucleotides and FRET probes with different fluorophores; HCV genotype determination therefore requires three wells per sample	122
RealTime HCV Genotype II[c,f] (Abbott)	Real-time RT-PCR	NS5b (Gt1a and Gt1b), 5′ UTR (Gt1 through Gt5)	Yes	96% genotype agreement with LiPA 2.0 and NS5b-based direct sequencing	123

[a] Genotypes 1 through 6 can be determined with all assays.
[b] Available globally as Research Use Only product.
[c] CE-marked and approved according to European *In Vitro* Diagnostic Directive 98/79/EC.
[d] U.S. regulatory status, Research Use Only.
[e] Available outside the U.S. only, as Research Use Only.
[f] Approved for use by FDA.

developed tests. From the perspective of accuracy, genotype determination of the more common viruses (Gt1 to Gt3) is fairly straightforward because these samples are readily accessible. Gt4 through Gt6 are more problematic as they are found less commonly. Samples containing HCV are available commercially (Gt1 through Gt3 from ProMedDx, Norton, MA; Gt1 through Gt4 from Acrometrix, Benicia, CA; Gt1 through Gt6 from BocaBiolistics, Coconut Creek, FL, and SeraCare, Milford, MA) for use in preimplementation accuracy studies. One strategy for samples found to putatively contain these less common genotypes is to refer them to a reference laboratory for genotype confirmation.

SEROLOGIC TESTS

The diagnosis of chronic HCV infection is usually established with serology assays. In addition to risk-based testing found in previous hepatitis C screening recommendations, new screening recommendations include one-time serology testing for individuals born between 1945 and 1965 (72), the cohort with the projected highest prevalence of chronic hepatitis C (Table 6). Birth-cohort screening has been recommended because previous recommendations were ineffective in identifying what is thought to be the bulk of chronic infections.

Serologic testing for HCV has undergone considerable evolution. The first serologic assay comprised a single NS4 peptide (c-100-3) and was introduced in 1990 shortly after HCV was identified as a major cause of non-A, non-B hepatitis. This assay represented a significant advance, particularly in identifying contaminated blood products and preventing transfusion-transmitted hepatitis C; however, its flaws of low sensitivity and specificity were soon apparent (sensitivity, ~70%; positive predictive values in low- and

TABLE 5 Laboratory-developed HCV RNA genotyping methods[a]

Test	Method	Target(s)	Genotyping	Subtyping	References
Direct sequencing	Sanger and CLIP biochemistries	NS5b, core, core-E1	1 through 6	Yes	124–127
Reverse hybridization	Amplicon detection on multiwell plates (enzyme-linked oligosorbent nucleotide assay) or microarrays	5′ UTR	1 through 6	Yes	128, 129
RFLP	RT-PCR with restriction enzyme digestion of amplicons; more sensitive than subtype-specific PCR	5′ UTR	1 through 6	Yes	130, 131
Heteroduplex mobility analysis	Differential electrophoretic mobility of matched versus mismatched duplexes	5′ UTR, NS5b	1 through 4, 6	Yes	132, 133
PSEA or PSMEA	Fluorescent PCR products of different lengths detected on DNA sequencer; highly sensitive detection of mixed genotypes (level of minority genotypes detected by PSEA, ~3%; PSMEA ~1%).	5′ UTR	PSEA, Gt1a, 1b, 2a/c, 2b, 3, 4; PSMEA, Gt1a, 1b, 2a, 2b, 3a, 3b, 4, 6a	Partial	134, 135
Subtype-specific PCR	PCR with subtype-specific primers; amplicons of different size detected by gel electrophoresis	Core, NS5b	Gt1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, 6a	Yes	136, 137
Real-time PCR	TaqMan or FRET chemistries with specific probes or melt curve analysis	5′ UTR	1 through 4	Yes	138–142

[a] Abbreviations: RFLP, restriction fragment length polymorphism; PSEA, primer-specific extension analysis; PSMEA, primer-specific mispair extension analysis.

high-prevalence populations, 30 to 50% and 70 to 85%, respectively [73]). Increased sensitivity and specificity were achieved in second-generation blood screening tests. These tests (one of which is still marketed) were better able to detect acute infections due to the incorporation of NS3 and core antigens (Table 7 and Fig. 3), two targets of early antibody responses. The reasons for improved performance of third-generation blood screening assays that incorporate NS5 and contain reconfigured NS3 antigen (Fig. 3) are unclear. Improved antigenicity of the alternate NS3 antigen leading to increased detection during early seroconversion has been argued (74). However, the finding of HCV RNA-positive donors who were consistently reactive by EIA 3.0 but remained unreactive by EIA 2.0 for over 6 months suggests that more complex immunologic factors may be responsible (75).

Using seroconversion panels in which infection has been documented by serology only, the seroconversion window has been documented to decrease from approximately 16 to 10 to 8 weeks with the introduction of first-, second-, and third-generation serology tests, respectively. It is important to note that when infection is documented with NAT, the seroconversion window using second- and third-generation serology screening tests is much longer (Fig. 2) (49, 76). Table 7 contains information on HCV serology screening and diagnostic tests available in the United States; numerous other assays are also available globally (77).

TABLE 6 Recommendations for chronic hepatitis C screening

- One-time serology should be performed for all individuals born between 1945 and 1965 (72).
- Serology should also be performed in individuals with the following risk factors (50):
  - Injection drug use (recent or remote; single or multiple episodes)
  - Conditions associated with hepatitis C prevalence
    - HIV
    - Hemophilia with receipt of clotting factors prior to 1987
    - Hemodialysis
    - Unexplained aminotransferase elevations
  - Blood transfusion or organ transplant prior to 1992
  - Children born to HCV-infected mothers
  - Needlestick injury or mucosal exposure to HCV-positive blood
  - Current sexual partners of HCV-infected persons

Despite improvements in performance characteristics, spurious serology results can still be observed. Second-generation assays are still available (Abbott HCV EIA 2.0 [Fig. 3 and Table 7]) and can yield false-negative results due to low sensitivity compared to third-generation assays. Therefore, in the appropriate clinical setting (for example, an individual with sudden-onset hepatitis, or an occupational exposure such as needlestick from an HCV-infected individual), alternative testing (third-generation EIA or NAT) should be considered in a patient with a negative EIA 2.0 result. Alternative testing should also be considered for any negative serology test result if acute hepatitis C is suspected (see “Evaluation, Interpretation, and Reporting of Results/Interpretation of Results in Acute Hepatitis C” below for additional discussion). False-negative results have also been observed in immunocompromised patients (HIV with CD4 <100, chemotherapy-treated oncology patients); therefore, NAT should be performed to definitively exclude infection in these patients (56, 78).

For most antibody screening assays, a positive result is considered initially reactive and repeat testing in duplicate is required. Results are reported as reactive if two or more replicates are positive. One advantage of certain chemiluminescent and microparticle immunoassays (AxSYM Anti-HCV [Abbott]; Architect anti-HCV [Abbott]; VITROS Anti-HCV [Ortho]; Advia Centaur HCV [Siemens]) (Table 7) is that initial positive results can be reported as reactive; repeat testing is not required due to the high sensitivities and specificities of these tests.

HCV serologic tests were originally developed as screening assays, with an emphasis on sensitivity and consequent potential for false-positive results. Early recommendations

TABLE 7 Serologic assays for the detection of anti-HCV antibodies[a]

Test (manufacturer)	Assay type	S/CO threshold[b]	Sensitivity (%)	Specificity (%)	Approved for donor screening	References
Abbott HCV EIA 2.0 (Abbott)	Enzyme immunoassay	≥3.8	85.6	98.4	Yes	143
AxSYM Anti-HCV (Abbott)	Microparticle immunoassay	≥10.0	100[c]	94[d]	No	Package insert
Architect Anti-HCV (Abbott)	Chemiluminescent microparticle	≥5.0	99.7[c]	97.7[d]	No	Package insert
Abbott Prism HCV (Abbott)	Chemiluminescent immunoassay	NA[e]	100%	100%	Yes	Package insert
Ortho HCV version 3.0 EIA (Ortho)	Enzyme immunoassay	≥3.8	96.9 to 100	100	Yes	144
Vitros Anti-HCV (Ortho)	Chemiluminescent immunoassay	≥8.0	100	96.5 to 98.1	No	145, 146
Advia Centaur HCV (Siemens)	Chemiluminescent immunoassay	≥11.0	100	99.9	No	147

[a] All assays approved for diagnostic use by U.S. Food and Drug Administration.

[b] >95% samples with S/CO ratios above indicated threshold predicted to be confirmed positive.

[c] Expressed in package insert as percent positive agreement.

[d] Expressed in package insert as percent negative agreement.

[e] NA, not available.

(79) therefore advised confirming all positive results by recombinant immunoblot assay (RIBA) before proceeding to NAT to avoid erroneous diagnoses. Confirmation of all reactive screening test results was never widely adopted by clinicians or laboratorians outside the blood-screening arena largely due to the availability of more definitive NATs and the conviction that RIBA did not represent an adequate confirmatory test as it employed the same antigens as antibody screening tests. Subsequent recommendations included a complex algorithm and advised the confirmation of screening results with low signal/cutoff (S/CO) ratios by RIBA, since false positives were most likely to occur in these samples (80). RIBA is no longer being manufactured, and current screening algorithms for chronic HCV (outlined

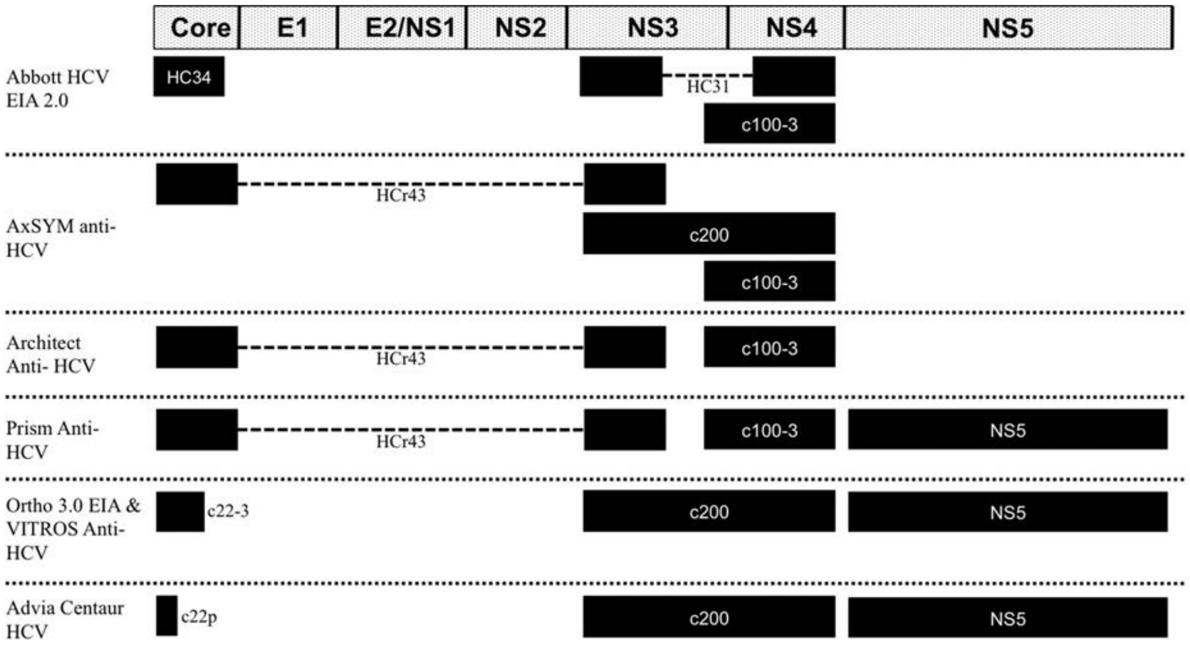

FIGURE 3 Antigens in serology tests currently available commercially in the United States. Abbott HCV EIA 2.0 is the only second-generation screening test currently available. AxSYM, Architect, Ortho 3.0 EIA, Ortho Vitros, and Advia Centaur are third-generation HCV antibody screening tests. HC34, recombinant antigen containing HCV core protein (amino acids [aa] 1 to 150); HC31, recombinant antigen containing NS3 (aa 1192 to 1457) and NS4 (aa 1676 to 1931) separated by an 8-aa linker. c100-3, recombinant antigen containing NS3-4 (aa 1569 to 1931). HCr43, fusion protein of two noncontiguous antigens, c33c and core protein (aa 1 to 150). c200, recombinant antigen containing NS3-4 (aa 1192 to 1931). NS5, recombinant antigen (aa 2054 to 2995). c22-3, recombinant antigen containing core protein (aa 2 to 120). c22p, peptide containing core protein major epitope (aa 10 to 53). doi:10.1128/9781555817381.ch92.f3

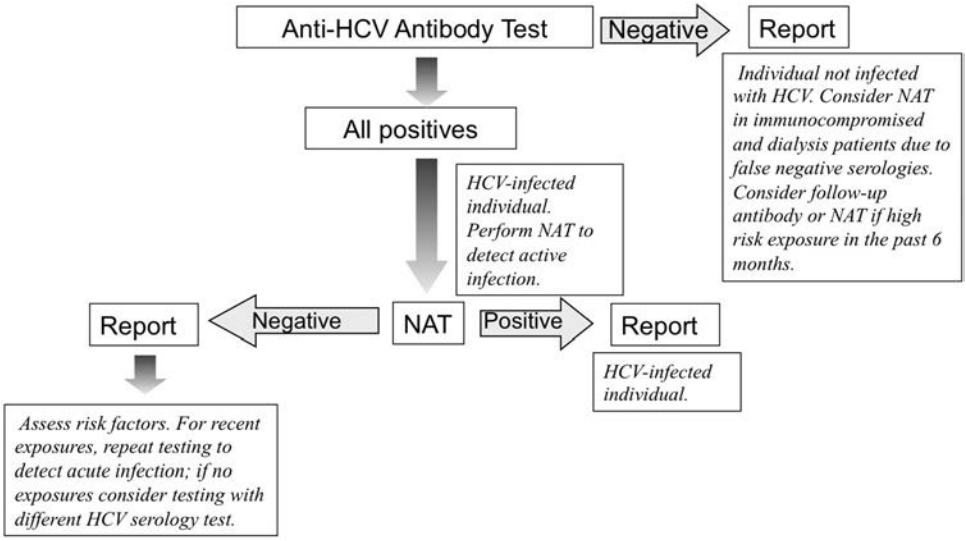
Anti-HCV Antibody Test
Negative
Report
Individual not infected with HCV. Consider NAT in immunocompromised and dialysis patients due to false negative serologies. Consider follow-up antibody or NAT if high risk exposure in the past 6 months.
All positives
HCV-infected individual. Perform NAT to detect active infection.
Report
Negative
NAT
Positive
Report
HCV-infected individual.
Assess risk factors. For recent exposures, repeat testing to detect acute infection; if no exposures consider testing with different HCV serology test.

FIGURE 4 Recommended testing algorithm for detection of HCV infection. Derived from reference 111. doi:10.1128/9781555817381.ch92.f4

in Fig. 4) have largely jettisoned the concept of confirmatory testing unless a spurious positive result is suspected in an individual with no risk factors. In these instances, S/CO ratios are still useful as thresholds for confirmatory testing although they are not formally mentioned (see Table 7 for assays available in the United States and Europe; S/CO ratios for other assays are described in reference 81).

ANTIVIRAL SUSCEPTIBILITY

To date, there is limited clinical utility of antiviral susceptibility testing in the treatment of chronic hepatitis C. The viral genetic determinants associated with suboptimal response to pegylated interferon/ribavirin were poorly understood and were never directly determined. Protease resistance mutations were readily detectable after treatment with first-generation DAAs; however, testing for mutations was never used in pretreatment to guide drug regimen selection in Gt1-infected patients. The findings of a number of clinical studies of resistance in treatment-naive individuals undergoing therapy with first-generation protease inhibitors plus pegylated interferon α/ribavirin demonstrated that this testing was not clinically useful for the first DAAs. For example, SVR could be achieved in treated individuals with preexisting mutations at baseline (82). Some patients who failed therapy did not have drug-resistant mutations at baseline, even with detection methods as sensitive as next-generation sequencing (83). Not all patients who failed protease inhibitor therapy had resistant virus that dominated in the posttreatment quasi-species; in fact most had wild-type virus (84). Even in those with preexisting mutations who failed triple therapy, other more highly resistant variants emerged *de novo* (83).

Currently, baseline drug resistance testing for next-generation DAAs is limited to simeprevir, based on phase III clinical trial data demonstrating that Gt1a-infected patients with the point mutation resulting in glutamine-to-lysine change at amino acid 80, or Q80K, had diminished cure rates to simeprevir/pegylated interferon/ribavirin compared to patients without this mutation (31). The prevalence of this mutation varies geographically, appearing in approximately 10% of Gt1as in South America, 20% in Europe, and almost 50% in North America (85). Prescribing information for simeprevir therefore recommends that individuals with Gt1a infections be further tested for Q80K to determine suitability for treatment with regimens containing this protease inhibitor. Detection of the Q80K resistance mutation is performed by direct sequencing and is available through specialty reference laboratories. The need for the development of Q80K detection tests for more general use is questionable given that simeprevir, a drug approved for use with interferon α for the treatment of Gt1 infections only, is likely to have limited appeal and longevity in a marketplace that is shifting toward pan-genotypic, interferon-free drug regimens.

IL28B haplotype testing to detect host genetic markers of interferon refractoriness was important in Gt1-infected individuals before initiating treatment with interferon α-containing regimens, to determine probability of response. Real-time PCR and direct sequencing methods have been described (86–88), and testing is available at large centers and in reference laboratories. The utility of *IL28B* haplotype determination prior to treatment with next-generation interferon-free combination DAA regimens is unclear. This test may have a limited lifespan.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Interpretation of Results in Acute Hepatitis C

Establishing the diagnosis of acute hepatitis C can be challenging. Most cases of acute hepatitis C are asymptomatic; therefore, serum aminotransferase elevations can be helpful if present but should not be relied upon as the sole indicator of infection. Additionally, seroconversion can be delayed and viremia can be sporadic. Serology and RNA assessment should therefore both be performed to optimally detect infection. Diagnostic accuracy also requires testing at multiple time points. Single HCV NAT results do not reliably predict exposure outcome. Transient HCV RNA negativity has been documented early postinfection in individuals who become chronically infected, and later, at the time of seroconversion

(Fig. 2) (89). Transient positive results have been found in those who spontaneously clear virus (49, 90).

Antibody screening has been recommended 4 to 6 months after a known exposure, since the majority of individuals seroconvert by this time (91). These public health guidelines have not been updated since the accumulation of evidence regarding early treatment efficacy and the increasing adoption of acute HCV treatment into clinical practice. More recent practitioner recommendations advocate serologic (and NAT) testing at multiple time points prior to 6 months because it may provide complementary and confirmatory information that is helpful in shaping the decision to start treatment.

Interpretation of Results in Chronic Hepatitis C

Issues regarding NAT interpretation in chronic hepatitis C include reporting of quantitative data, sources of variability in quantification, interpreting changes in viremia, and using HCV RNA quantification to define therapeutic response.

Reporting Quantitative Data

Linearity is one of the important parameters used to define the measurable range of a quantitative assay. With currently available quantitative tests, linearity can only be achieved using logarithmically transformed (base 10) data. HCV RNA levels should therefore be reported and interpreted in $\log_{10}$ format. This requirement is fairly unique to viral load measurement and is difficult to grasp by care providers since most quantitative interpretations are performed with arithmetic data. Laboratorians should nonetheless strive to educate care providers regarding the need for logarithmic interpretation, to facilitate proper patient management. Quantitative data and assay precision can be reported in logarithmic and arithmetic formats to assist with this educational effort.

Sources of Variability in HCV RNA Quantification

Despite the implementation of an international standard for calibration of quantitative assays, HCV RNA measurement is not equivalent between assays (92–94). Patients should therefore be tested with a single viral load assay to avoid inconsistent results that could potentially be interpreted erroneously and lead to management errors.

The factors that contribute to variability in interassay quantification are complex. The WHO international standard and the properties of individual assays, such as inherent genotype bias, are likely contributors. The development of a WHO standard that functions as a common calibrator has been extremely useful in improving the comparability of different assays. However, it is an imperfect calibrator from the perspective of physical standardization since it is a single genotype (Gt1a), and its value represents a consensus concentration obtained by diverse assays in numerous laboratories rather than a concentration that is traceable to a physically quantifiable substance such as phosphorus content.

Sequence diversity between HCV genotypes can also affect HCV RNA quantification, resulting in genotype-dependent quantitative bias. For example, assays based on primers and probes that have varying, genotype-dependent degrees of complementarity may favor the quantification of one genotype over another, or extraction methods may favor the amplification of certain genotypes. Evidence of such genotypic bias in quantification has been reported and has been serious enough to require assay reformulation. Genotype-dependent quantification variability has been reported in multiple studies comparing the performance of different quantitative tests (60, 95–99). These findings underscore the importance of monitoring treatment efficacy with a single assay.

Parameters such as interlaboratory variability, specimen type, and tube type do not seem to play a major role in quantification variability, although the available data are not exhaustive. In a study of four different quantitative assays (Versant HCV RNA 3.0, Cobas Amplicor Monitor, Cobas TaqMan, and RealTime HCV), reasonable quantitative agreement was observed among four labs that performed testing on a standardized panel (100). No significant differences in quantification have been reported between serum and plasma specimens for Versant HCV RNA 3.0 and Cobas AmpliPrep/Cobas TaqMan tests (101, 102). Laboratories that are planning to offer testing from a sample or tube type that has not been approved for use with a given platform should establish assay performance (including quantitative equivalence) to prevent the introduction of unanticipated quantification variability.

Interpreting Changes in Viremia

Changes in viremia must be interpreted in the context of assay precision and variability in viremia over time in untreated chronic infection. Using a quantitative assay with excellent precision, viral load was observed to vary up to 10-fold in stable, untreated chronic hepatitis C (18). During treatment, a viral load change of at least this magnitude must therefore occur in order to be biologically significant.

Using HCV RNA Quantification to Define Therapeutic Response

Determination of response to treatment has evolved over time as more is understood regarding response to a given regimen, as more sensitive tests with broad measurable ranges have become available, and as these newer tests are incorporated into clinical trials of new drugs. Testing during treatment became an essential feature of treatment with pegylated interferon α/ribavirin as clinical studies determined that response-guided therapy (successfully shortening treatment in Gt1-infected patients who responded rapidly) and futility rules (early treatment discontinuation when certain quantitative milestones were not achieved) were effective in therapeutic management. Phase III clinical trials of first-generation DAAs defined response-guided therapy criteria and futility rules that were unique to each drug. Testing during treatment was therefore an essential management tool as soon as these drugs were approved. In the future, the need for on-treatment testing is likely to be highly variable and drug dependent. For the new, highly potent next-generation DAAs, testing during treatment is becoming less necessary as inhibition of replication and virus elimination are rapid. For example, directions for simeprevir use include stopping rules but not response-guided therapy, and no testing is indicated in sofosbuvir prescribing information. Prescribing information should be consulted to determine the need for testing during treatment with future drug regimens. If on-treatment testing is applicable, the assay used to determine response during clinical trials should be noted, performance should be compared to the laboratory's test of record, and interpretive guidance should be provided if differences exist. Such appraisals are necessary because disparate results can be obtained with tests that vary in performance, which can lead to inappropriate management decisions. Clinical samples with low HCV RNA concentrations near the limits of detection can be particularly problematic since assays that have comparable analytical sensitivity can have differing sensitivity in clinical samples that manifest as variable HCV RNA detectability (103, 104). For example, thresholds and definitions of rapid response to pegylated interferon α/ribavirin and to first-generation DAA regimens were defined with one real-time PCR test; a significant proportion of subjects who met qualifications for shortened therapy during the trials failed to qualify when on-treatment samples were retested retrospectively with a different, more sensitive assay (105, 106). Interestingly, almost all of these subjects attained SVR after shortened therapy, as dictated by initial testing, demonstrating that individual tests can greatly impact management.

Definitions of end-of-treatment response and SVR have also changed over time according to drug regimen and available tests. For pegylated interferon α/ribavirin, conventional qualitative PCR tests with a limit of detection of ≤ 50 IU/ml were suitable to determine treatment efficacy; end-of-treatment response and SVR were defined as HCV RNA undetectable (<50 IU/ml). Many DAA clinical trials have been performed exclusively with one real-time PCR test, the Cobas TaqMan assay using High Pure extraction. This test has a limit of detection that is less than its lower limit of quantification (Table 3). For first-generation DAAs, response definitions at end of treatment and SVR were <25 IU/ml, without further stipulating "HCV RNA undetected," due to the ultimate clinical trial snafu—a contamination event in one testing laboratory that led to "<25 IU/ml, HCV RNA detected" results at end of treatment and traditional end of follow-up (when SVR is determined) in a number of subjects who, upon extended follow-up, were found to have cleared infection (107). For future DAA regimens, end of treatment and SVR response definitions are likely to be <25 IU/ml, HCV RNA undetected.

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Gastroenteritis Viruses*

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Acute infectious gastroenteritis is a common illness affecting infants, children, and adults worldwide, resulting in a substantial public health burden with significant morbidity and mortality. Children under 5 years of age, the elderly, and individuals with compromised immune systems are particularly prone to more severe illness. The disease is characterized by inflammation of the stomach and intestines and major symptoms include some combination of diarrhea, vomiting, nausea, and cramping abdominal pain. Many different microorganisms can cause gastroenteritis, including viruses, bacteria, and parasites. However, in developed countries, viruses account for most of the infectious gastroenteritis. The major enteric viruses are a genetically and antigenically diverse group of RNA- and DNA-containing viruses belonging to four separate families, and include rotaviruses, caliciviruses (noroviruses and sapoviruses), astroviruses, and the enteric adenoviruses. Rotaviruses are the most important cause of severe acute gastroenteritis in young children, while noroviruses are the most widely recognized cause of nonbacterial epidemics of gastroenteritis attacking people of all ages. Most disease caused by sapoviruses, astroviruses, and enteric adenoviruses is observed in young children. Other viruses, such as coronavirus, torovirus, Aichi virus, picobirnavirus and bocavirus, have been implicated in human gastroenteritis, but their etiologic role is not well established and these viruses will not be discussed in detail in this chapter.

TAXONOMY

Rotaviruses

Rotaviruses are classified as members of the *Rotavirus* genus within the family *Reoviridae* (Table 1), which contains 10 other distinct genera. Based on group-specific antigens of the major viral structural protein VP6, rotaviruses are divided into seven groups (A to G) (1). Group-specific epitopes are also found on other structural proteins as well as on some nonstructural proteins. Group A to C rotaviruses infect both humans and animals, with the group A rotaviruses infecting humans most frequently and causing disease mainly in young children. Groups B and C cause infections in swine but have occasionally been associated with foodborne or waterborne outbreaks of human disease in China (group B) and Japan (group C). Group D to G rotaviruses are mainly animal pathogens. Within each group, rotaviruses are further classified into serotypes based on neutralization assays using antibodies against two major outer capsid proteins, VP4 and VP7. VP4 is a spike protein on the capsid surface which is sensitive to protease cleavage; therefore, the types based on the VP4 protein are also called P types (protease sensitive). VP7 is a glycoprotein and the types based on this protein are also called G types. Both P and G types are also classified as genotypes based on the sequences of the VP4 and VP7 genes. In 2008, a new nucleotide-sequence-base, complete genome classification system was recommended (2, 3) and a Rotavirus Classification Working Group composed of specialists in molecular virology, infectious diseases, epidemiology, and public health was formed to assist in delineation of new genotypes. This system assigns a specific genotype to each of the 11 genome segments of a particular rotavirus strain, and scientists discovering a potentially new rotavirus genotype are invited to send the novel sequence to the Rotavirus Classification Working Group, where the sequence will be analyzed and the new strain will be assigned the complete descriptor of Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx. G and P typing are still most commonly used for monitoring rotavirus genetic evolution and vaccine development. To date, 26 G-genotypes and 35 P-genotypes have been reported (4). Genotypes G1P[8], G2P[4], G3P[8], and G4P[8] are most common in North America, and genotypes do vary from one geographic location to another in different parts of the world.

Caliciviruses

Caliciviruses belong to the family *Caliciviridae* (Table 1) and are a diverse group of human and animal viruses which includes five classified genera, *Norovirus* (formerly called Norwalk-like viruses), *Sapovirus* (formerly called Sapporo-like viruses), *Nebovirus*, *Lagovirus*, and *Vesivirus* and four newly proposed genera, *Bavovirus*, *Nacovirus*, *Recovirus*, and *Valovirus* (5–7). Caliciviruses within the *Norovirus* and *Sapovirus* genera mainly cause gastroenteritis in humans. Within each genus, strains are further grouped into genogroups (G) and genotypes or clusters. Noroviruses are classified into five genogroups (GI-V), with GI, GII, and GIV found mainly in humans, GII and GIII in pigs and cattle, and GV in mice (8). Based on sequence variations in open reading frame 1 (ORF1) and ORF2, at least 14 genotypes

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TABLE 1 Taxonomy and virologic properties of the major gastroenteritis viruses[a]

Virus	Family	Size (nm)	Appearance under EM	Viral genome	Genetic or antigenic types
Rotavirus	*Reoviridae*	70	Wheel-shaped, triple-layered capsid	Segmented dsRNA	7 antigenic groups (A-G) and 27 G and 35 P types
Norovirus	*Caliciviridae*	30–38	SRSV	Linear ss(+)RNA	5 genogroups and 14 types of GI and 29 types of GII
Sapovirus	*Caliciviridae*	30–38	SRSV, with Star of David appearance	Linear ss(+)RNA	5 genogroups and 14 types
Enteric Adenoviruses	*Adenoviridae*	70–100	Icosahedral capsid	Linear dsDNA	HAdV40 and HAdV41
Astrovirus	*Astroviridae*	28–30	SRSV, 5- or 6-pointed star shaped	Linear ss(+)RNA	8 serotypes

[a] dsRNA, double-stranded RNA; ss(+)RNA, single-stranded positive-sense RNA; dsDNA, double-stranded DNA; SRSV, small, round, structured virus.

of GI and 29 genotypes of GII have been reported (9, 10). A single genotype of norovirus (genogroup II, genotype 4, [GII.4]) has been found to be the most predominant in the past decade, causing up to 80% of all norovirus gastroenteritis outbreaks (11) in many countries. Norovirus GII.4 has demonstrated a much faster evolution than other strains, and new GII.4 clusters or variants appear to emerge every 2 to 5 years; the latest variant, called the "Sydney strain," caused global epidemic outbreaks during 2012 (12–14). Similar to noroviruses, sapoviruses are grouped into 14 genotypes within five genogroups (15, 16). Genogroups GI, GII, GIV, and GV contain human strains of sapovirus and GIII contains the porcine strains (15).

Enteric Adenoviruses

Human adenoviruses (HAdVs) are members of the family *Adenoviridae* (Table 1) and are divided into seven species (A to G). The different species have different tissue tropisms and propensity for causing different clinical syndromes; species F is the group most clearly associated with gastroenteritis. Recently, the Human Adenovirus Working Group has proposed using whole-genome sequence analyses to characterize and name human adenoviruses as "type" instead of "serotype" to rectify the misclassification of human adenoviruses previously characterized by the serological methods of serum neutralization and hemagglutination inhibition that target two small conservative regions on the hexon gene (17, 18). There are now 68 types of HAdVs based on serological assays, whole-genome sequencing, and phylogenomics (17–19). Many AdVs are readily isolated from human stools, but their role in the etiology of acute gastroenteritis is unclear. There are two types, however, AdV40 and AdV41 in species F, which are definitively associated with infantile diarrhea and are referred to as enteric adenoviruses (EAdVs). These EAdVs are also known as "fastidious adenoviruses" because they are difficult to grow in cell culture. More recently, other HAdV types, namely HAdV-52 belonging to species G and HAdV-67 belonging to species D, have been implicated as potentially causing gastroenteritis (19, 20).

Astroviruses

Astroviruses belong to the *Astroviridae* family (Table 1) and are so named because of their characteristic star-like surface structure. Two genera of astroviruses, *Mamastrovirus* and *Avastrovirus*, have been described according to their host species; the first group infects mammals and the latter group infects birds (21). Within each genus, there are multiple species of astroviruses named after the hosts in which they replicate. Human astroviruses have been divided into eight types based on antigenic and genetic typing (22). Type 1 appears to be most common, but all eight types have been detected throughout the world.

Other Viruses Causing Gastroenteritis

Other enteric viruses that have been implicated in acute gastroenteritis of humans include coronaviruses and toroviruses, two genera in the *Coronaviridae* family (23), Aichi virus in the genus *Kobuvirus* of the *Picornaviridae* family (24), picobirnaviruses in the *Picobirnaviridae* family (25), and human bocavirus (HBoV) in the genus *Bocavirus* within the *Parvoviridae* family (26). A detailed discussion of these viruses is beyond the scope of this chapter.

DESCRIPTION OF THE AGENTS

Rotaviruses

Rotaviruses are nonenveloped and are among the few human viruses that possess a segmented, double-stranded RNA genome (Table 1). Mature viral particles are approximately 70 nm in diameter with a wheel-like appearance (*rota* = wheel in Latin) and possess a triple-layered icosahedral protein capsid composed of an outer layer, an intermediate layer, and an inner core (Fig. 1). The double-stranded RNA genome contains 11 segments ranging in size from ~3,300 (segment 1) to ~660 (segment 11) base pairs (bp) and each segment codes for a single gene product. The gene coding assignments of the 11 genome segments has been determined; these segments encode six major structural proteins (VP1, VP2, VP3, VP4, VP6, and VP7) that appear on the matured viral particles and six nonstructural proteins (NSP1-NSP6) that are expressed in infected cells and play important roles in viral genome replication, protein synthesis, capsid assembly, and maturation. The outermost layer of the nucleocapsid contains VP4 and VP7, the middle layer is composed of VP6, and the innermost core is composed of VP2 and contains a polymerase complex composed of VP1 and VP3. One of the nonstructural proteins, NSP4, is considered to be a viral enterotoxin (27). Similar to influenza viruses, having a segmented genome allows for genetic reassortment of rotavirus strains, resulting in the possible formation of new virus types.

Caliciviruses

Caliciviruses are small (30–38 nm), round, nonenveloped viruses possessing a single-stranded, positive-sense RNA ge-

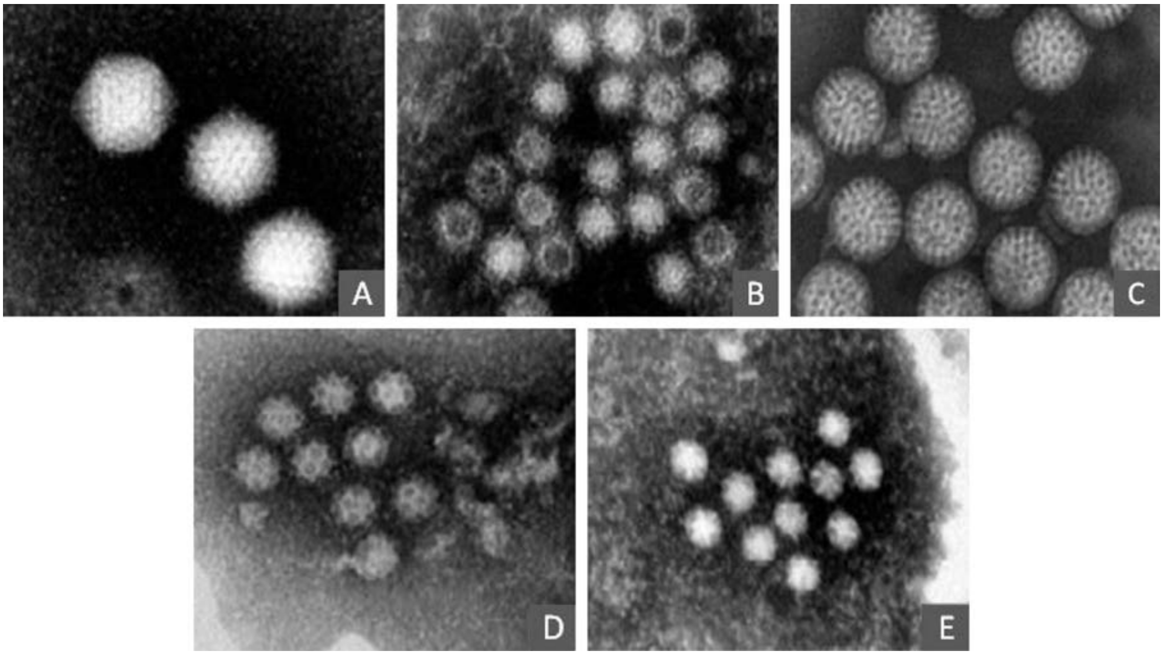

FIGURE 1 Electron micrographs of the major gastroenteritis viruses. (A) rotavirus, (B) noroviruses, (C) sapovirus, (D) astrovirus, (E) adenoviruses. doi:10.1128/9781555817381.ch93.f1

nome (Table 1). Sapoviruses have the typical morphology of caliciviruses with the "star of David" appearance with cup-shaped depressions (*calyx* = cup in Latin) on the surface of the virus (Fig. 1). Noroviruses possess a smoother surface structure and therefore also have been called "small round structured viruses" (28). Cryo-electron microscopy (EM) and X-ray crystallography analysis of recombinant viral-like particles (VLPs) have revealed that noroviruses possess a T = 3 icosahedral capsid composed of 180 proteins that form 90 dimeric capsomers. Each capsid protein possesses two major domains, the shell (S) and the protrusion (P) domains. Expression of the P domain in *Escherichia coli* has resulted in self-formation of a subviral particle, the P particle, which maintains the VLP's properties of receptor binding and antigenic recognition (29). This P particle is highly stable and easy to make and has been proposed as a potential subunit vaccine for noroviruses.

The RNA genome of noroviruses is poly(A) tailed and is ~7.5 kb in length (30). It is organized into three open reading frames (ORFs), with ORF1 encoding the nonstructural proteins, ORF2 the VP1 capsid protein, and ORF3 a minor structural protein. The sapovirus genome is slightly different in that sequences encoding the nonstructural and capsid proteins are fused into one large ORF.

Enteric Adenoviruses

AdVs were named from the Greek word *aden*, meaning "gland," after their original isolation from adenoid tissue and were also called "adenoid-associated viruses." HAdVs have been linked to a number of diseases, including respiratory illness, conjunctivitis, and diarrhea (31). AdVs are nonenveloped, icosahedral particles that are 70 to 100 nm in diameter (Table 1). Mature virions consist of a DNA-containing core surrounded by a capsid composed of hexon, penton, and fiber proteins (Fig. 1). The genome is a linear, nonsegmented, double-stranded DNA of 30 to 38 kb which varies in size from group to group. The capsid is composed of 252 capsomeres, of which 240 are hexons and 12 are pentons. There are a total of 11 viral structural proteins, of which the hexons, penton bases, and fibers are of important clinical relevance. These proteins are involved in viral entry (fiber) and intracellular transportation. The fiber proteins extend from the surface of the capsid and interact with primary cellular receptors, which for the majority of AdVs, including the EAdVs, is the coxsackievirus B-adenovirus receptor, a transmembrane protein belonging to the immunoglobulin family. The hexon and fiber proteins also contain the major neutralization epitopes.

Astroviruses

Astroviruses are small (28 to 30 nm in diameter), round, and nonenveloped viruses with a typical five- or six-pointed star-like appearance (*astron* = star in Greek) (Fig. 1). As determined by X-ray crystallography and computer modeling, the virus has some structural resemblance to the caliciviruses, but even more strongly resembles the hepatitis E virus. The virions contain a single-stranded, positive-sense RNA genome of about 6.8 kb in length (Table 1). The viral genome contains three ORFs, with ORF1a and ORF1b encoding the nonstructural proteins. ORF1a has a protease motif, transmembrane helices, nuclear localization signals, and a ribosomal frameshifting signal, whereas ORF1b has an RNA polymerase motif. ORF2 encodes the capsid precursor protein, which is approximately 87 kDa in size and is further cleaved to smaller peptides of 25 to 34 kDa to form mature virions.

EPIDEMIOLOGY AND TRANSMISSION

Viral gastroenteritis occurs in all age groups and socioeconomic classes and in all parts of the world. Important epidemiological features include age, season of the year, duration of symptoms, severity of disease, and exposure and vaccination history (Tables 2 and 3). Severe illness is seen most commonly in children younger than 5 years of age, with the peak incidence observed in children between the ages

TABLE 2 Transmission and epidemiology of the major gastroenteritis viruses

Virus	Mode(s) of transmission	Target population(s)	Occurrence
Rotavirus	Fecal-oral, ?respiratory, ?food and water	Children <5 yr old (peak activity at 6 mo to 2 yr of age)	Fall/winter months in temperate climates, year round in tropical regions
Norovirus	Fecal-oral, foods, water, ?respiratory	All age groups	Winter peak, year-round outbreaks common in variety of settings
Sapovirus	Fecal-oral, ?respiratory	Mainly children (primarily infants and toddlers), less so in adults	Sporadic year round, outbreaks occur
Enteric Adenoviruses	Fecal-oral, ?respiratory	Mainly infants and young children < 5 yrs old	Sporadic year round, outbreaks occur
Astrovirus	Fecal-oral, water, ?respiratory	Mainly young children	Sporadic year round, outbreaks occur

of 6 months and 2 years. Viruses account for 50 to 90% of acute gastroenteritis requiring hospitalization and 3 to 10% of all pediatric hospital days in this age group. Sporadic illness and outbreaks in healthy adults, elderly populations, and immunosuppressed patients have been described.

Gastroenteritis viruses are transmitted by the fecal-oral route through close contact with infected persons or indirectly by contact with contaminated fomites (e.g., shared eating utensils, environmental surfaces, toys in playrooms). Transmission is highly efficient because of the physical hardiness of gastrointestinal viruses, the high amounts of virus particles concentrated and shed in stools, the small infectious dose required, and resistance of these viruses to various environmental conditions (32). Enteric viruses are stable over wide pH and temperature ranges and even after drying, heating, or freezing, and they are able to survive on human hands and inanimate objects for extended times. Certain viruses, particularly the noroviruses, are readily transmitted by eating or drinking contaminated foods or beverages, which usually results in large outbreaks. Foods may be contaminated by food preparers or handlers who have viral gastroenteritis; water and shellfish may become contaminated by sewage, and eating raw or undercooked shellfish or drinking contaminated water may lead to illness. Enteric viruses are also thought to be transmitted through respiratory secretions, but this has not been fully proven. Most viral diarrheal diseases are endemic, with significant disease burden in both developing and developed countries. Caliciviruses, especially the noroviruses, also cause epidemics of acute gastroenteritis, which is an important public health concern. In countries with temperate climates, viral gastroenteritis does not have the typical summer peak of most bacterium- and parasite-caused gastroenteritis. Instead, viral gastroenteritis is more common in colder seasons, with a typical winter-spring peak. Hospital-associated spread of the enteric viruses, particularly for rotavirus and norovirus, is common without the implementation of appropriate infection prevention and control measures.

Rotaviruses

Rotaviruses are the leading cause of severe dehydrating diarrhea in infants and children less than 2 years of age and are a major cause of childhood deaths in developing countries. The mortality caused by rotavirus infection is much lower in developed countries, but in the prevaccine era, the disease burden was high, representing approximately 2.7 million diarrheal episodes each year in the United States, with significant numbers of physician visits and hospitalizations and substantial medical and societal costs. Infants usually acquire the disease from their siblings or from their parents, who may have subclinical infection. Shedding of rotavirus in stools can occur prior to onset of disease and continue following cessation of diarrhea. Cross-species transmission of rotaviruses has been demonstrated, indicating a potential for zoonotic disease between animals and humans (33).

TABLE 3 Selected clinical features of the major gastroenteritis viruses

Virus	Clinical significance	Incubation time, days	Vomiting, days	Diarrhea, days	Virus shedding, days
Rotavirus	Acute severe dehydrating gastroenteritis, hospital-acquired infections, subclinical infections occur	1–2	2–3	5–8	8–10
Caliciviruses	Noroviruses: moderate to severe acute gastroenteritis, hospital-acquired infections, prolonged shedding and chronic disease in immune compromised patients, subclinical infections occur Sapoviruses: mild to moderate gastroenteritis, subclinical infections occur	1–2	0.5–1	1–2	1–21
Adenovirus	Moderate to severe gastroenteritis, hospital-acquired infections, persists in immune compromised patients	8–10	2–3	4–12	8–13
Astrovirus	Mild to moderate gastroenteritis, subclinical infections occur	1–2	1–4	1–4	8–10

Caliciviruses

Noroviruses are highly contagious and have been recognized as the most important cause of nonbacterial acute gastroenteritis in both developing and developed countries (34). Both common-source outbreaks and sporadic disease can occur. The disease is endemic in children, but all ages can be infected due to potential incomplete protective immunity following a childhood infection or reinfection with antigenically different strains. Noroviruses are now recognized as the leading cause of acute gastroenteritis in children in the United States since the number of cases of acute gastroenteritis associated with rotavirus has declined due to introduction of the rotavirus vaccines (35). Noroviruses commonly cause large outbreaks in closed communities and in a variety of settings, such as hospitals, child care centers, schools, restaurants, nursing homes for the elderly, cruise ships, and military communities or camps. Particular attention has been paid to foodborne outbreaks occurring in nursing homes for the elderly and on cruise ships in the United States and European countries. Chronic diarrhea, increased mortality, and prolonged fecal shedding of noroviruses is often seen in immunocompromised hosts. In recent years, noroviruses have been reported to recognize the ABH (secretor) and Lewis histo-blood group antigens as receptors (36), and susceptibility of individuals to norovirus infection is based on the expression of these antigens on the gut epithelium; persons devoid of H type 1 epitopes are resistant to infection with noroviruses. Direct evidence of zoonotic transmission of noroviruses remains absent, although noroviruses closely related to human strains have been detected in domestic and wild animals.

Sapoviruses cause disease mainly in children, and sporadic cases and outbreaks in daycare centers and institutional settings have been reported. However, these viruses were recently found to be very common in adult gastroenteritis outbreaks in North American and Europe (37–39).

Enteric Adenoviruses

While the role of EAdVs in acute gastroenteritis is widely accepted, the incidences of AdV-related gastroenteritis differ considerably in various studies and locations, with an average of ~5% of cases of pediatric diarrhea being caused by EAdVs. Outbreaks of EAdV-associated diarrhea have been reported in hospitals and child day care centers. Human EAdV infection does not have the typical winter seasonality of the rotaviruses and caliciviruses, and sporadic cases are reported year round. Like other enteric pathogens, human EAdVs are frequently detected in stools of children without gastroenteritis. As many as 41% of children acquire EAdV infection during hospitalization for other diseases (40). EAdVs also cause prolonged or chronic diarrhea in immunocompromised patients, especially in hematopoietic stem cell transplant recipients, which is an increasing concern in the clinical care of these patients (41, 42).

Astroviruses

Human astroviruses cause mainly pediatric gastroenteritis. Cases in caregivers of sick children, immunocompromised adults (43), military troops (44), and nursing homes (45) have been reported. Astrovirus infection can be linked to 2 to 10% of cases of pediatric gastroenteritis worldwide, depending on settings and diagnostic tests used. Asymptomatic infections are common in all ages. There is a 70 to 90% seroprevalence of astroviruses among school-aged children, indicating frequent infections during childhood.

CLINICAL SIGNIFICANCE

Acute viral gastroenteritis is characterized by a variety of symptoms and signs, including fever, diarrhea, vomiting, abdominal cramps, irritability, lethargy, and dehydration, with rotavirus gastroenteritis considered to be the most severe. Although symptoms of gastroenteritis due to noroviruses, sapoviruses, astroviruses, and the enteric adenoviruses are considered to be milder that those of the rotaviruses, increasing data show that noroviruses are frequently detected in hospitalized children and emergency room visitors with acute gastroenteritis, indicating that noroviruses may also cause severe gastroenteritis (35). Headache, anorexia, malaise, myalgia, and nausea may also be observed. Clinical illness is similar for all gastrointestinal viruses, making it difficult, if not impossible, to distinguish which virus is causing the disease based on symptoms alone. The clinical presentation can vary widely and symptoms can appear alone or together and can mimic noninfectious conditions. In general, with the exception of adenoviruses, viral gastroenteritis has a sudden onset following a short incubation period of 1 to 2 days (Table 3). The duration of symptoms is normally less than 7 days, and virus shedding in stools of infected individuals is approximately 1 to 2 weeks in most cases but may last longer depending on the virus and host. The stools are usually watery or loose with no mucus or blood, and fecal leukocytes are typically absent or present only in minimal numbers. Viral gastroenteritis is normally self-limited in well-nourished, immunocompetent individuals, and asymptomatic infections or mild disease are common. Severe illness can be seen among infants, younger children, the elderly, and immunocompromised individuals who are unable to effectively rehydrate following loss of fluids due to vomiting and diarrhea, and can result in significant morbidity and hospitalizations. For rotaviruses, severe dehydration can be lethal and remains an important cause of infant mortality in developing countries.

Rotaviruses can cause chronic infection in immunocompromised individuals, such as patients who have undergone organ transplantation and those infected with human immunodeficiency virus (HIV), and in rare cases, the viruses can disseminate systemically and involve other organ systems. Norovirus illness can also become debilitating and life-threatening in immunocompromised patients (46). Increased numbers of studies have shown that noroviruses are among the pathogens associated with immunocompromised hosts, causing up to 18% of chronic diarrhea, with prolonged viral shedding in the stools of recipients who underwent allogeneic hematopoietic stem-cell and solid organ transplantations (47). Noroviruses also can cause necrotizing enterocolitis in children (48, 49). Enteric adenovirus infections in immunodeficient individuals, such as hematopoietic stem-cell and solid organ transplant recipients, HIV patients, and other individuals with immunosuppressive therapy, are also of growing clinical importance (41). AdV infection in these individuals can lead to mild gastroenteritis, asymptomatic infection, or severe disease, including gastroenteritis, pneumonitis, hemorrhagic cystitis, hepatitis, and disseminated infection associated with high mortality. Patients can excrete AdVs in stool for weeks to months after infection. Potential treatment of AdV infection in such clinical settings includes reduction of immunosuppressive drugs, intravenous immunoglobulin, and intravenous antiviral therapy with cidofovir (50).

There are no effective antivirals that can be used for the treatment or prevention of infection and disease caused by gastroenteritis viruses. Treatment involves supportive care and rehydration therapy; restoration of electrolyte balance is the most important procedure for severely dehydrated cases and for reducing the mortality in developing countries.

The lack of antiviral drugs against rotaviruses and the significant disease burden of rotavirus gastroenteritis were the main driving forces behind the development of rotavirus vaccines. After decades of research and the failure of some earlier vaccine products, two live attenuated rotavirus vaccines were licensed for use in the United States and are now being used worldwide; the pentavalent bovine-human reassortant vaccine containing G1-G4 and P[8] types (RotaTeq; Merck & Co., Inc.) was licensed in 2006 and the monovalent attenuated human G1P[8] vaccine (Rotarix; GlaxoSmithKline, Inc.) was licensed in 2008. Both vaccines have been shown to be effective and safe in children, providing 80 to 100% protection against severe disease and 70 to 80% protection against rotavirus gastroenteritis of any severity in high- and middle-income countries. A relatively low protection with the two current vaccines against rotavirus gastroenteritis was observed in low-income countries. A major reason for the decreased efficacy of the vaccines was due to high prevalence of heterotypic strains circulating in these countries (51). Both vaccines were not associated with intussusception in large trials prior to its licensure (52). The Advisory Committee on Immunization Practices (ACIP) recently updated its recommendations for rotavirus vaccination to include the use of Rotarix and RotaTeq for prevention of rotavirus gastroenteritis (53). However, it was reported in very recent postlicensure studies that both Rotarix and RotaTeq vaccines had increased rates of intussusception after vaccination in U.S. populations, suggesting that the potential risks and benefits of these vaccines should be considered (54, 55).

Since noroviruses cause the great majority of epidemics of viral gastroenteritis, development of vaccines and antivirals against noroviruses is needed. Approaches to develop a norovirus vaccine include transgenic plant-, baculovirus-, or *E. coli*-expressed recombinant VLPs or P particles as a subunit vaccine. Challenges in development of the vaccines above are the lack of understanding of herd immunity after infection, particularly the mechanism of the short duration of protective immunity following a natural infection, and the lack of a cell culture and suitable animal model for efficacy studies. The high genetic and antigenic diversity of noroviruses also makes it difficult to select vaccine targets for broad protection. A randomized, double-blinded, and placebo-controlled multicenter clinical trial of norovirus genotype GI.1 VLP vaccine was recently reported. In this trial, immunization with the GI.1 VLP vaccine significantly reduced the frequencies of norovirus gastroenteritis (56). Although the GI genotype is not the major epidemic genotype of norovirus-associated gastroenteritis around the world, this trial shed light on the potential of vaccination against norovirus infection using this unique approach.

At present, preventive and control strategies for gastrointestinal virus infections are aimed at identifying and eliminating the source of infection by promoting standard and contact precautions and good personal hygienic practices (e.g., careful and thorough hand washing with soap and water), performing proper disinfection of environmental surfaces, and identifying and avoiding sick contacts and other potential sources of infection and spread. Certain gastrointestinal viruses (e.g., noroviruses) may be resistant to some common disinfectants and cleaning solutions; sodium hypochlorite is most effective at inactivation of the enteric viruses.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Gastroenteritis viruses replicate and cause disease mainly in the intestine, and stool is the foremost specimen of choice for laboratory diagnosis. It is preferable to collect specimens within the first 48 hours of illness since viral shedding is at its maximum at this time and increases the likelihood of virus detection. Specimens collected later in the course of disease have a lower detection rate, although prolonged shedding of some gastroenteritis viruses has been reported after the onset of illness. Stool specimens of a few grams are sufficient for detection by EM and antigen or nucleic acid detection methods. The samples can be collected with a bed pan and then transferred into smaller containers or test tubes. Specimens can be recovered from a diaper with a wooden tongue depressor which is then placed in an appropriate container for transport to the laboratory. Rectal swabs may be easy to obtain, but they are poor specimens for viral diagnosis and are not recommended. If it is necessary to collect a rectal swab, a sufficient quantity of fecal material (at least a pea-sized amount) should be obtained. If fecal material is not clearly visible on the swab, the specimen is most likely inadequate. Rectal swabs should be immediately placed in a suitable viral transport medium. Vomitus material can be used for the detection of gastrointestinal viruses as a supplement to stool specimens, but is not routinely recommended for diagnostic testing. Additional details of specimen collection and processing are given in chapter 79.

In general, the best results are obtained by testing fresh stool samples, particularly when viewing viral size and morphology by EM. Stool samples should be stored at 4°C immediately after collection and promptly transported to the laboratory for processing. Single-use aliquots of processed specimens should be placed in multiple cryovials for testing and storage, particularly if multiple tests are planned for different types of pathogens or for confirmation of results. By processing in this manner, specimens are not frozen and thawed repeatedly and are never returned to the original specimen cryovial, thereby avoiding possible degradation of viral particles, proteins, and/or nucleic acids and decreased detection rates and cross-contamination of specimens, respectively. If not tested immediately, specimens should be promptly frozen and stored at −70°C. Stool samples can be shipped on wet or dry ice, depending on their storage conditions (e.g., short- or long-term storage). Unnecessary freezing and thawing between shipment and storage should be avoided.

Serum samples can be used for detection of IgG antibodies against the gastroenteritis viruses. Documenting seroconversion between acute- and convalescent-phase serum samples has been particularly useful for screening for viral infection in outbreak investigations. However, such testing is performed mainly in research laboratories and is not suitable for clinical diagnosis of acute infection since the results are obtained retrospectively and are not always predictive of active disease. The acute-phase serum sample should be collected as soon as possible after onset of illness and tested simultaneously with the convalescent-phase serum sample. Processed serum specimens may be stored at 2 to 8°C for several days pending completion of testing or can be stored frozen at −20°C or colder for prolonged storage. Repeated freezing and thawing should be avoided and specimens should not be stored in frost-free freezers.

Drinking water, foods, other beverages, or other environmental specimens are normally collected and used to investigate outbreaks of viral gastroenteritis, mainly those caused by noroviruses. If a food item or water is suspected as the source of an outbreak, samples should be obtained as early as possible in the outbreak and stored under appropriate conditions as described above, depending on when the testing will be performed. Since testing of these specimen types is not routinely performed in clinical laboratories and often requires special handling and processing (e.g., filter concentration of 5 to 100 liters of water and processing of food to remove food debris), a laboratory with the capability to test these specimens should be contacted (e.g., a local or regional health department or the Centers for Disease Control and Prevention in Atlanta, GA).

DIRECT DETECTION

Electron Microscopy

EM has been successfully used for the rapid examination of stool samples for the simultaneous detection and identification of viral agents of gastroenteritis based on characteristic size and shape of intact viral particles when visualized by negative staining on a support grid. It offers the main advantages of speed and simplicity, but is limited by the availability and high cost of the instrument, the requirement for specialized facilities and expertise, and a moderate to low sensitivity and specificity. To be detected by EM, gastrointestinal viruses must be present in high concentrations, usually 10^5 to 10^6 viral particles/ml of processed stool. Liquid stool samples can be applied to the grid after clarification by low-speed centrifugation at 2,000 × *g* for 10 min. For a solid stool sample, a 10 to 20% suspension can be prepared first using either distilled water or a 1% ammonium acetate solution. One drop (5 to 10 μl) of the preparation is added directly to a 300- to 400-mesh copper support grid, or a sample drop can be placed on a piece of Parafilm and the grid is then floated on the top of the drop. After incubation for approximately 1 min, excess specimen is drawn off the surface of the grid with absorbent filter paper. A longer incubation may be used to facilitate the capture of more virus particles, but the sample should not be allowed to completely dry to the surface of the grid. If the resulting stool suspension is too thick and difficult to examine by EM, the sample may require further dilution. The grid is then stained with a drop of 2% phosphotungstic acid (pH 7.0) for 10 to 30 s and then blotted with an absorbent filter paper. The dried grid is then ready for EM examination.

Under EM, negatively stained virus particles appear as lighter structures surrounded by a dark background (Fig. 1) (57). The most definitive of the viral particles are the adenoviruses and rotaviruses, whose icosahedral capsids are readily discerned. Each of the smaller round viruses, including the noroviruses, sapoviruses, and astroviruses, has its unique surface appearance: sapoviruses have a rigid surface structure with cup-like depressions of a typical "star of David" appearance with a dark center to the star shape; noroviruses have a less rigid surface appearance, lacking of the star of David structure; and astroviruses possess a distinct five- to six-point star appearance (Fig. 1). Other viral particles of different shapes and sizes and particles lacking well-defined features are also commonly seen and can pose a diagnostic dilemma. Bacteriophages in stool specimens may also be confused with small gastroenteritis viruses, especially if their tails are absent. Thus, a confident diagnosis relies on repeated observations of viral particles of identical morphology and size in multiple EM fields on the surface of the support grid.

For stool specimens containing low amounts of virus, a concentration step using ultracentrifugation or agar diffusion may be required. High-speed centrifugation at 100,000 × *g* for 1 h is used to pellet most of the viral particles. Following the centrifugation, the pellet is resuspended in distilled water and processed for making the grids as outlined above. A Beckman Airfuge with an EM-90 rotor is useful to deposit the viral particles onto support grids when using small-volume specimens. For agar diffusion, a drop of prepared stool suspension is placed on a small block of 1% agar prepared in distilled water and the grid is floated on the surface of the drop. The support grid is not removed until the drop is completely absorbed into the agar; it is then processed for EM as described above. This procedure not only concentrates the virus particles but also removes excessive quantities of salts that may precipitate on the grid and obscure visualization of the viruses.

Immunoelectron microscopy (IEM) has played an important role in the discovery of a number of gastroenteritis viruses, including the prototype strain of norovirus (57, 58). The principle of the technique is to allow viral particles to form aggregates when stool suspensions are mixed with virus-specific antibodies. These aggregates are much easier to identify by EM than single particles alone. A solid-phase IEM can be used to reduce the background when reading the prepared specimen grid by capturing the viral particles using virus-specific antibodies precoated to the surface of the grid (59).

In general, EM has been largely replaced by alternative methods for the diagnosis of gastroenteritis viruses and is seldom available in diagnostic laboratories in the United States.

Antigen Detection Assays

Immunologic assays have been used for the direct detection of gastrointestinal viruses in processed stool samples and commercial kits are available for some of the viruses (Table 4). Common formats of antigen-detection assays include conventional 96-well microplate enzyme immunoassays (EIA), latex agglutination tests, and rapid membrane-based immunochromatographic assays. These antigen assays are rapid, inexpensive, and simple to perform and are highly suitable for use by most, if not all, clinical laboratories. Stool suspensions of 10 to 20% are normally prepared in phosphate-buffered saline (pH 7.4) or sodium carbonate buffer (pH 9.5) for use in the tests. Overall, the number of available antigen-detection assays is limited, and tests remain unavailable for some of the enteric viruses because of the lack of specific antibodies used to capture and detect appropriately conserved viral antigens. In addition, even the performance of existing assays needs to be vastly improved for better sensitivity and specificity. Because of the high degree of genetic and antigenic diversity of enteric viruses, the sensitivity of many antigen-detection assays is relatively low to moderate, although still higher than the sensitivity of EM. Monoclonal or hyperimmune antibodies against specific viral capsid proteins, such as the VP6 protein of rotaviruses and the capsid protein or the P domain of the capsid protein of noroviruses, have been used in the development of these antigen-detection tests. Due to the lack of a cell culture for human noroviruses, recombinant viral capsid proteins expressed in an *in vitro* system have been used as the source of reagents for assay development.

The more recently developed membrane-based immunochromatographic assays are valuable for small- to moderate-sized laboratories with a demand for diagnosis of sporadic viral gastroenteritis (60). These immunochromatographic kits are designed as dipsticks or self-contained cassettes and usually include built-in positive controls to ensure that the

TABLE 4 Selected commercial antigen-detection tests available for diagnosis of viral gastroenteritis

Manufacturer (URL)	Product name(s)	Test format(s)	Availability
ANI Biotech Oy (www.anibiotech.fi)	Biocard Rotastick, Biocard Adenostick	Immunochromatographic card test (dipstick); adenovirus test not specific for types 40/41	Not available in United States; CE[a] marked kits in Europe
Bio-Rad Laboratories, Inc. (www.bio-rad.com)	Pastorex Rotavirus	Latex agglutination test	Not available in United States; CE marked kit in Europe
Denka-Seiken Co., Ltd. (www.denka-seiken.com)	NV-AD Norovirus Test	96-well microplate enzyme immunoassay	Available in Japan and some other countries; not available in United States
Meridian Bioscience (www.meridianbioscience.com)	ImmunoCard STAT! assays for rotavirus, adenovirus, and norovirus	Immunochromatographic card tests (cassettes); adenovirus test not specific for types 40/41	All tests are CE marked in Europe; only rotavirus assay is FDA licensed in United States
	Premier Rotaclone, Premier Adenoclone, Premier Adenoclone – Types 40/41	96-well microplate enzyme immunoassays	All tests are FDA licensed and CE marked for use in United States and Europe
Oxoid Limited - Thermo Fisher Scientific (www.oxoid.com)	Remel Xpect Rotavirus	Immunochromatographic card test (cassette)	Not available in United States; CE marked kit in Europe
	Remel ProSpecT assays for rotavirus, adenovirus, and astrovirus; IDEIA norovirus assay	96-well microplate enzyme immunoassays	Not available in United States; CE marked kit in Europe
R Biopharm AG (www.r-biopharm.com)	RIDAQUICK assays for rotavirus, combined rotavirus and adenovirus and norovirus	Immunochromatographic card tests (both dipsticks and cassettes available); adenovirus test not specific for types 40/41	All tests are CE marked in Europe; not available in United States
	RIDASCREEN Rotavirus, RIDASCREEN Adenovirus, RIDASCREEN Norovirus (3rd Gen), RIDASCREEN Astrovirus	96-well microplate enzyme immunoassays; adenovirus test not specific for types 40/41	All tests are CE marked in Europe; only norovirus assay is FDA licensed in United States
SA Scientific (www.sascientific.com)	SAS Rota Test, SAS Adeno Test	Immunochromatographic card test (cassettes)	Both tests are FDA licensed and CE marked for use in United States and Europe
Sekisui Virotech (www.sekisuivirotech.com)	Serozyme assays for rotavirus, adenovirus, norovirus, and astrovirus	96-well microplate enzyme immunoassays; adenovirus test not specific for types 40/41	Not available in United States; CE marked kits in Europe

[a] CE, Conformité Européenne.

reaction has been performed and interpreted appropriately. The tests require minimal equipment and the results can be readily visualized. Because of their speed, simplicity, and low cost, immunochromatographic kits are amenable for point-of-care testing. In general, the immunochromatographic assays have a slightly lower sensitivity and specificity than the more conventional EIA-based 96-well microplate systems for detection of rotavirus and norovirus (61–63).

The latex agglutination tests use latex beads coated with virus-specific antibodies and are based on the agglutination of these beads when virus particles or viral antigens present in the specimens bind to the antibody-coated beads. Since some fecal specimens can cause nonspecific agglutination, latex particles coated with nonviral antibodies are also included in the kits. Particulate matter found in some prepared stool suspensions may interfere with the correct reading of the agglutination, and the stools are normally clarified by low-speed centrifugation before the supernatants are mixed with the latex reagent. A positive reaction is indicated by visible clumping (agglutination) occurring within minutes of performing the test. The assays are generally less sensitive than EIAs and immunochromatographic assays but are most useful in small- to moderate-sized diagnostic laboratories (61).

Conventional 96-well microplate EIAs for rotaviruses and AdVs have been on the market for years and are still widely used due to their reasonably high sensitivity and specificity and consistent test results. Immunochromatographic tests for rotaviruses and AdVs were introduced later and have been readily adopted for diagnostic use. In a cross-comparison of seven commercially available immunochromatographic assays with an EIA for the detection of group A rotaviruses in fecal samples, the results of six immunochromatographic assays were shown to be comparable with those of the EIA, and only one immunochromatographic assay had a significantly lower positive detection rate (62).

Immunochromatographic tests for the detection of multiple pathogens, such as both rotaviruses and AdVs, have also been developed. Direct-comparison studies have shown that these assays have the sensitivity and specificity comparable to those of the more traditional EIAs and are useful for rapid diagnosis in ambulatory practice (64, 65). Of note, most commercially available AdV antigen-detection assays are not specific for the EAdV types 40 and 41 with the exception of the Premier Adenoclone-Types 40/41 EIA from Meridian Bioscience (Cincinnati, OH).

Although a number of antigen-detection tests are now being developed and commercialized for noroviruses and astroviruses, most of these assays are only CE marked in Europe for *in vitro* diagnostic use and are not available in the United States. Only a single norovirus assay (RIDASCREEN Norovirus 3rd Generation EIA) from R-Biopharm AG (Darmstadt, Germany) has been licensed by the U.S. Food and Drug Administration for preliminary identification of norovirus when testing multiple specimens during outbreaks. Due to their limited sensitivity and specificity, currently available norovirus antigen assays are used mainly for research and are not recommended for routine clinical diagnosis of individual cases of sporadic disease (66, 67). Several studies on evaluation of commercial EIA and immunochromatographic kits for detection of norovirus antigens showed much lower sensitivity compared to results obtained using reverse transcription-PCR (RT-PCR), resulting in the detection of significantly lower numbers of GI and GII viruses (66–68).

Molecular Detection Assays

Diagnosis of viral gastroenteritis based on the identification of viral genomes includes direct detection of viral RNA or DNA by electrophoresis, amplification of viral DNA or RNA by PCR or RT-PCR, and DNA sequencing. Such molecular methods are extremely sensitive and play an ever-increasingly important role in the rapid and accurate detection of the enteric viruses (69). Nucleic acid amplification tests like PCR are now the method of choice for the detection of viruses causing gastroenteritis, and a growing literature now exists to demonstrate the overall utility of these assays.

Direct Staining of Nucleic Acid following Electrophoresis

The segmented, double-stranded RNA genome of rotaviruses has traditionally been the target for direct detection and genotyping using electrophoretic methods (70). The viral RNA is extracted from stool specimens and then subjected to separation by polyacrylamide gel electrophoresis followed by silver staining for detection. This technique has been available for many years and is used primarily in research laboratories; the method is as sensitive as EM. It also has been applied to the classification of rotaviruses, in which the appearance of an unusual electropherotype may denote a novel strain or group of rotaviruses. Schematic diagrams of electropherotypes of group A to G rotaviruses have been compiled as a reference. Direct detection and genotyping of viral DNA using this technique has also been applied to the diagnosis of human EAdVs following digestion of the adenoviral genomic DNA with restriction enzymes.

PCR and RT-PCR

Extraction of Nucleic Acids for PCR or RT-PCR

One of the major challenges of viral detection in stool specimens by PCR or RT-PCR is the presence of inhibitors in the samples, which could lead to false-negative results.Such inhibitors can be monitored by adding an internal control to the sample during the nucleic acid extraction and purification process. Approaches to reduce inhibition include dilution of stools and/or extracted nucleic acids; treatment of samples with chelating agents, detergents, or denaturing chemicals during RNA extraction (71); and inclusion of amplification facilitators such as bovine serum albumin and betaines during the PCR (72). Most commercial nucleic acid extraction protocols now use silica- or magnetic bead-based extraction technologies that are simple and efficient and provide appropriate amounts of nucleic acids of high quality that are essentially free of contamination with cellular proteins, carbohydrates, and lipids that may act to inhibit or interfere with downstream amplification.

Nucleic acids extracted from stool specimens usually contain large amounts of nonviral nucleic acids from different microorganisms and host cells that may be nonspecifically amplified by virus-specific primers. These nonspecific PCR products cannot be completely eliminated even under the high stringency conditions of the reactions. In this case, hybridization with virus-specific probes is usually required as part of the PCR process to confirm the results.

Conventional PCR

Conventional PCR (cPCR or cRT-PCR) refers to the more traditional amplification process in which amplified product is allowed to accumulate and plateau as the thermal cycling goes to completion or end-point before the detection or analysis steps are performed. This type of PCR normally relies on the detection of amplified PCR products by size fractionation using agarose or polyacrylamide gel electrophoresis. By using type-specific primers, cPCR has been widely used to determine the molecular epidemiology of viral gastroenteritis. For example, cRT-PCR is used for genotyping of group A rotaviruses using primers specific to the gene regions that code for the two major viral surface neutralization proteins VP7 (G types) and VP4 (P types) (73). cPCR followed by sequencing of the PCR products also has been commonly used for studies of the molecular epidemiology of noroviruses, the assessment of genetic variation among the various norovirus strains, and the investigation of outbreaks of gastrointestinal disease (8). The major disadvantages of cPCR include the requirement for multiple complex, labor-intensive, and time-consuming steps; low throughput and high variability; and the significant risk of contamination due to the open system and manipulations of amplified products. While cPCR is still commonly used in many research laboratories that study enteric viruses, it does not have wide applicability in diagnostic laboratories.

Real-time PCR

Currently, real-time PCR is the most common approach for establishing a diagnosis of viral gastroenteritis and, to this end, a number of assays have been developed and implemented by individual laboratories for the detection of the major enteric viruses (Table 5). Simply put, real-time PCR allows for simultaneous detection of specific nucleic acid sequences in real time by using nonspecific intercalating dyes or fluorescent dyes bound to target-specific probes. With real-time PCR, the amplified product is being measured at each PCR cycle. Advances in this technology include preoptimized universal reagents and universal conditions for amplification, simplified assay development and design of primers and probes, multiple amplicon-detection chemistries

TABLE 5 Selected molecular assays for detection of the major viruses known to cause gastroenteritis

Virus	Primers	Probes	Target gene	Length(bp)	Instrument	Reference(s)
Rotavirus	NSP3F/R NSP3F2	TaqMan	NSP3	87	ABI:7700/7000/7300/7500	74
	RotaAF1.2/RotaA R1.2	MGB TaqMan	VP6	145	ABI:7000	75
	VP2F1-5/VP2R1.2	MGB TaqMan	VP2	79	ABI:7900HT	76
	JVKF/JVKR	TaqMan	NSP3	131	Bio-Rad: iCycler	77
Norovirus	COG1F/G1R COG2F/G2R Multiplex PCR	TaqMan	ORF1-ORF2 junction region	85-GI 98-GII	ABI:7700/7000/7300/7500	78, 79
	NV192/193-G1 NV107a,c/NV119-GII Multiplex RT-PCR	MGB TaqMan	ORF1-2 junction region	98-GI 94-GII	ABI PRISM:7700	80
	JJV1F/R-GI JJV2F/COG2R-GII	TaqMan	ORF1-2 junction region	96-GI 98-GII	Bio-Rad: iCycler Cepheid: SmartCycler ABI:5700	78, 81
	COG1F/G1R-GI COG2F/G2R-GII Mon4F/R-GIV	TaqMan	ORF1-2 junction region	85-GI 98-GII 98-GIV	Roche: LightCycler 1.0	82
	GIF/GIR-GI GIIF and GIVF/GII&IVR	MGB TaqMan	ORF1-2 junction region	85-GI 97-GII & IV	ABI:7000	83
Sapovirus	sapoFa&Fb/sapoR	MGB TaqMan	Polymerase	104	ABI:7000	83
	SaV124F, SaV1F, SaV5F/SaV1245R	MGB TaqMan	Polymerase; capsid junction	104	ABI:7500 Fast	84
	SapoF/R	TaqMan	Polymerase	N/A	ABI:7500	85
	CU-SVF1 and CU-SVF2/Cu-SVR	TaqMan	Polymerase; capsid junction	104	Bio-Rad: iCycler	86
Astrovirus	AV1/AV2	TaqMan	Capsid	90	ABI:7700/7000	87
	AstU1-4/AstL1-2	TaqMan	Capsid	218	ABI:7000	88
	HastF/HastR	MGB TaqMan	Capsid	67	ABI:7000	83
Enteric adenovirus	AdenoF/AdenoR	MGB TaqMan	Hexon	130	ABI:7000	75
	EadVF/R	TaqMan	Hexon	135	ABI:7500	89

and many choices of thermal cyclers, higher throughput capabilities and enhanced reproducibility, flexibility to multiplex assays to detect multiple viruses in a single reaction, a low risk of contamination because of the closed system, increased sensitivity and specificity over the more traditional detection methods, and software-driven operations.

Laboratory-developed real-time PCR assays for the detection of gastroenteritis viruses have been shown to have superior performance characteristics and are much better than any combination of EM and/or antigen-detection methods (for references, see Table 5). These assays can readily detect coinfections with different enteric viruses and can be coupled with other assays that detect nonviral enteric pathogens. Their specificity and sensitivity are high, with detection limits of 1.5 to 3.6 viral particles per PCR for rotavirus (77) and less than 10 RNA copies per PCR for noroviruses (82). The primers of these assays have been selected according to highly conserved regions of the viral genomes, such as those in the VP6, VP2, and NP3 genes of rotaviruses. Primers targeting the rotavirus NP3 gene are particularly useful for broad detection and are capable of detecting most of the common genotypes (G1 to G4 and G9) as well as some rare genotypes, such as G10 and G12 of rotaviruses (77, 90).

The ORF1-ORF2 junction region is the most conserved region of the norovirus genome, and primers targeting this region have been used for the development of real-time PCR assays that are highly sensitive for broad detection of many genotypes of noroviruses (78). With primers specific to individual genogroups (GI and GII), a multiplex real-time PCR which is highly sensitive and useful for typing noroviruses has also been developed (78). The multiplexed assay increases testing efficiency by decreasing test time by 50% and reducing reagent costs compared with those of other real-time PCR methods. A LightCycler real-time RT-PCR assay with additional primers and a probe targeting the GIV norovirus has also been developed (82). Currently, this and the multiplex assays are widely used in clinical and research laboratories worldwide, especially in North America.

Laboratory-developed real-time PCR assays for the detection of sapoviruses, astroviruses, and the EAdV also have been developed and have been shown to be highly sensitive and specific with increased detection rates over EM and available antigen-detection assays (75, 83–89).

Isothermal Amplification Assays

Nucleic acid sequence-based amplification (NASBA) and loop-mediated isothermal amplification (LAMP) assays have been applied to the detection of noroviruses from stool specimens and are categorized as isothermal amplification methods. These assays involve single-temperature amplification with no thermal cycling. Similar to real-time PCR, the assays have been shown to be extremely sensitive. NASBA has been used to detect norovirus RNA, but the detection specificity does not appear to be acceptable for clinical diagnostic use because of the decreased stringency when amplifying viral RNA at relatively low temperatures of ~40°C (91). The LAMP technology has the added advantages of speed and simplicity, with the reaction being performed in a single tube and requiring no more than 1 hour to complete. Reactions normally show a high tolerance to biological products so that extensive nucleic acid extraction is often not necessary and assays require no large equipment and only simple heat blocks or water baths since amplification occurs in low and unchanged temperature condition, normally at 60 to 65°C. The by-product of LAMP is the production of magnesium pyrophosphate. In positive samples, it precipitates out of solution, causing a turbid reaction that can easily be read using a small turbidity reader. A fluorescent metal indicator can also be added to enhance the readability of the produced turbidity. The simplicity of LAMP assays makes them applicable to laboratories with limited resources and experience in performing molecular methods and which desire rapid turnaround times or testing at point of care using a robust assay system. Laboratory-developed LAMP assays have been applied to the detection of norovirus GI and GII and have demonstrated compatible results with real time RT-PCR (92–94). Recently, a commercial LAMP kit for detection of norovirus GI and GII developed by Eiken Chemical Co. (Tokyo, Japan) has improved the detection sensitivity in comparison with laboratory-developed LAMP assays (94). With further development and validation, LAMP assays may prove to be an acceptable alternative to current real-time PCR assays for the detection of enteric viruses.

Application of Nanotechnology to Nucleic Acid Testing

The field of nanotechnology is growing considerably and being aggressively applied to the commercial development of molecular diagnostics. Advances in microelectronics, microfluidics, and microfabrication have paved the way for new and more standardized technologies with the ultimate goals of offering ever simpler and cost-affordable molecular platforms that use smaller quantities of reagents and samples. These assays are capable of highly multiplexed testing for comprehensive syndrome-specific assessment of the etiology of specific diseases and sample-in/answer-out testing for all laboratories regardless of size, resources, or capacity.

To this end, the first commercial highly multiplexed, syndrome-specific molecular testing assay for the detection of enteric organisms, the xTAG gastrointestinal pathogen panel (GPP), was developed by Luminex (Austin, TX) and recently licensed by the U.S. Food and Drug Administration (FDA) for the simultaneous detection of 11 pathogens, including 7 enteric bacteria, 2 parasites, and 2 viruses (rotavirus and noroviruses GI/GII) associated with gastroenteritis (69, 95). The *in vitro* diagnostic product licensed in Canada and Europe also includes the detection of EAdV types 40/41 and two additional bacteria and one parasitic pathogen. The assay couples PCR with a suspension microarray to readily screen for multiple enteric pathogens and has been shown to have a better sensitivity compared to rapid immunochromatographic tests for detecting rotavirus, noroviruses, and AdV 40/41 (96), and comparable results to laboratory-developed molecular methods (97).

Another highly multiplex assay, the FilmArray Gastrointestinal Panel, is currently under development by BioFire Diagnostics (Salt Lake City, UT). The goal of this assay is to provide rapid sample-in/answer-out results for the simultaneous detection of 23 gastrointestinal pathogens from stool samples, including rotavirus type A, norovirus types GI and GII, EAdV types 40/41, human astrovirus serotypes 1 to 8, and sapovirus serotypes I, II, IV, and V. The FilmArray platform is a fully closed and automated system for sample preparation, nested multiplex PCR, and result analysis that uses integrated electropneumatics and chemical circuits in a reaction pouch with an approximate turnaround time of 1 hour and 2 to 5 minutes of total hands-on time. This system shows great potential for use at point of care for the identification of enteric pathogens associated with acute gastroenteritis. Similar devices are being developed by other companies including GenMark Dx (Carlsbad, CA).

ISOLATION PROCEDURES

Cell Culture

While growth in cell culture can be used to detect and identify many different viruses from clinical specimens, it is generally time-consuming and not considered sufficiently rapid to contribute to meaningful management of a disease like acute gastroenteritis. In addition, most of the enteric viruses causing human gastroenteritis are fastidious in cell culture and require multiple passages before they can readily grow in cell culture from their primary isolation. Therefore, cell culture is not routinely used in the clinical diagnosis of viral gastroenteritis.

Cell lines for isolation of rotaviruses include MdBK, PK-15, BSC-1, llC-MK2, Ma104, CaCo-2, and HRT-29. To grow the viruses in cell culture, the culture medium must be supplemented with proteases such as trypsin or pancreatin. This approach has been adapted for titration of viruses by plaque assay and serotyping by virus neutralization.

Propagation of human caliciviruses in cell culture has not yet been achieved. A method of using a three-dimensional organoid model of human small-intestinal epithelium for cultivation of human noroviruses was reported but has not been reproduced by other laboratories. The porcine enteric calicivirus, a sapovirus, has been successfully adapted in porcine kidney cell lines but only in the presence of intestinal contents of pigs as a supplement in the culture medium. The murine norovirus has been reported to replicate in primary dendritic cells and macrophages (98).

EAdV types 40 and 41 grow best in the Graham 293 human embryonic kidney cell line, which has been transformed by AdV type 5 DNA. A plaque assay has been developed recently for the detection of EAdVs types 40 and 41 (99).

Isolation of astroviruses from clinical samples is difficult, although most of the astrovirus serotypes have been adapted in HEK or llC-MK2 cell culture (100). Propagation of astroviruses in cell culture requires the presence of trypsin in the culture medium.

ANTIGENIC AND GENETIC TYPING SYSTEMS

Both antigenic and genetic typing methods are important in understanding the classification and epidemiology of many gastroenteritis viruses and for developing preventive strategies against the diseases caused by these pathogens. Typing of gastroenteritis viruses is used mainly in research laboratories since typing does not affect clinical care and management decisions.

According to antigenic and genetic variations in the two major surface proteins of rotaviruses, VP7 and VP4, a dual system of antigenic (serotyping) and genetic typing has been used for the classification of human group A rotaviruses. The antigenic typing is accomplished by characterizing the specific interaction of a rotavirus with a panel of monoclonal antibodies representing individual G (VP7) and P (VP4) types of rotaviruses. The genetic typing is performed by RT-PCR using type-specific primers targeting unique regions of the VP7 and VP4 genes. Using this classification system, each strain of rotavirus is dually assigned to a G[P] type by either the antigenic or the genetic typing method. The antigenic typing results are highly correlated with the genetic typing for the G types, while the correlation between antigenic and genetic typing for the P types is low. Due to a limited supply of type-specific antibodies, P genotyping is commonly used. Frequent genetic and antigenic drifting of rotaviruses may result in newly emerging variants that are no longer detectable by the current typing assays (73). It becomes necessary to update the methods and reagents continuously, such as with type-specific monoclonal antibodies for the antigenic typing and primers for the genotyping.

Rotaviruses are also typeable based on the genetic variations in the major structure protein VP6 and the putative viral enterotoxin nSP4 genes. The recently recommended new classification system based on sequence information for all 11 genomic RNA segments is an extension of the previous classification systems (3). While this new system is challenging due to the requirement of sequencing all 11 genomic segments, it will eventually influence our understanding of the genetic variation, host-pathogen interaction, evolution, and potentially zoonotic nature of human rotaviruses.

Genetic typing has been used mainly for human norovirus identification and classification due to the lack of an efficient cell culture or animal model for a neutralization assay. The wide genetic diversity of human noroviruses also restricts the methods used for the G and P typing based on type-specific primers, although genogroup-specific RT-PCR using the GI and GII group-specific primers has been developed (101). Thus, genetic typing of human noroviruses is based mainly on sequencing of the amplified DNA products following RT-PCR detection. Primers from the highly conserved RNA-dependent RNA polymerase genes have been commonly used. However, primers targeting the viral capsid proteins are recommended because the viral capsids are directly involved in host-receptor interaction and immune responses. Sequence alignment followed by phylogenetic analysis has been commonly applied for the classification of human noroviruses, and there are ~40 genotypes associated with acute human gastroenteritis in two major genogroups (GI and GII) of noroviruses.

Genomic DNA restriction enzyme analysis was commonly used before monoclonal antibody-based EIA typing was developed for the detection and typing of EAdVs. In addition, typing PCR or PCR in combination with restriction enzyme analysis has also been developed for the detection and typing of AdVs (102, 103). Recently, a quantitative real-time PCR assay was described for detecting HAdVs and identifying EAdVs of types 40 and 41 (104). Field surveillance using various methods suggests that the choice of diagnostic method may influence the epidemiologic picture and disease burden attributed to EAdV infections. There are 68 types of human adenovirus (HAdVs) grouped into 7 subgroups (A to G) based on serological, whole-genome sequencing, and phylogenomics assays (17–19).

For typing of astroviruses, immunologic assays, such as IEM and a typing ELISA, have been described but are not commercially available (59). The most commonly used RT-PCR typing methods for astroviruses are summarized in a recent review by Guix et al. (105).

SEROLOGIC TESTS

Antibody-neutralization tests based on plaque reduction or epitope-blocking assays using type-specific monoclonal antibodies have been described for rotaviruses. There is no neutralization-based serologic test for most other gastroenteritis viruses. However, ELISAs for antibody detection using specific viral proteins as the capture antigens have been used in epidemiology studies of gastroenteritis viruses. Recombinant viral capsid proteins of caliciviruses and astroviruses generated in a baculovirus vector and other systems are an excellent source of viral antigens for these studies (106). Application of these assays in sero-surveillance against gastroenteritis viruses has played an important role in understanding the importance of these viruses in different populations. Monitoring seroconversion based on a collection of paired acute- and convalescent-phase sera has been used in outbreak investigations.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

There are many issues involved in the laboratory diagnosis of viral gastroenteritis and detection of gastroenteritis viruses can be quite difficult. As a result, much of viral gastrointestinal disease goes unrecognized. Enteric viruses do not grow well, if at all, in conventional cell culture systems used in clinical laboratories, and there is a definite lack of available reagents and assays for clinical use. Commercial rapid antigen-detection tests are available for rotavirus and, to a much lesser extent, for adenovirus types 40 and 41, noroviruses, and astroviruses; they offer speed in the diagnosis of viral gastrointestinal disease but are not completely sensitive or specific and only detect the pathogen of interest. In recent years, PCR has offered great promise for the diagnosis of viral gastroenteritis and has emerged as the most sensitive and specific method for the detection of viral causes of diarrhea. Real-time PCR is the most widely used molecular method for detecting the viral agents of gastroenteritis.

There are many reasons to attempt a laboratory diagnosis in individuals suspected of having a viral gastrointestinal disease. A prompt diagnosis of viral gastroenteritis may provide benefit in the management of individual patients by limiting unnecessary antibiotics, laboratory tests and hospital procedures; reducing hospital stay and sequelae; guiding treatment decisions; and providing earlier informed-decision making for better care. Increasing data have also shown that the major gastroenteritis viruses, such as rotaviruses, noroviruses, and EAdVs, can cause chronic gastroenteritis with prolonged shedding of viruses in the stools of recipients of transplanted organs, HIV patients, and other immunocompromised individuals. Prompt diagnosis in these clinical settings is important to adjust immunosuppressive therapy, to assess prognosis, and to stop the transmission of the disease. Rapid identification and monitoring of the source of infection in outbreaks of acute gastroenteritis, such as water, food, and environmental surfaces, is also important for disease control and prevention in the community. Early identification of food handlers with subclinical infection or chronic shedding of viruses is believed to be important for the prevention of food-borne outbreaks.

EM remains a simple and rapid method for clinical diagnosis of viral gastroenteritis, although it is less sensitive than most molecular diagnostic methods. It also requires experienced individuals who are familiar with the morphologies of different gastroenteritis viruses and who are able to differentiate atypical viral particles from unrelated cellular and microbial debris and structures commonly seen in stool specimens. The use of reference grids (57) containing viral particles with typical morphologies of the different gastroenteritis viruses is helpful for a person who is new to the field to gain such experience. IEM is useful for identifying unknown viral pathogens that may be an etiology of acute gastroenteritis, but this method is not widely used in diagnostic laboratories.

The antigen-detection methods, although moderate in sensitivity, may be a practical and inexpensive choice for the initial screening of stool samples if tests are available for a specific virus. Commercial antigen-detection assays for rotaviruses, EAdVs, and astroviruses can be applied to clinical diagnosis (Table 4). Also, antigenic tests based on type-specific monoclonal antibodies against the G and P types of rotaviruses and various types of astroviruses are widely used in research laboratories. Commercial antigen-detection assays for human noroviruses suffer from a lack of sensitivity and specificity and are not recommended for clinical diagnosis, although they may be useful for outbreak investigations.

Nucleic acid-based assays, particularly real-time PCR, are highly sensitive and specific and are increasingly used as the primary test of choice for clinical diagnosis. While less useful in clinical diagnosis, cPCR with type-specific primers and sequencing of the amplified product is appropriate for determining the molecular epidemiology of viral gastroenteritis.

Most gastroenteritis viral families are genetically diverse, which makes clinical diagnosis using PCR and RT-PCR difficult. Human noroviruses have over 40 recognized genetic clusters within three genogroups (9, 10). Using primers targeting highly conserved regions of the genome, the majority of known human noroviruses are detected, but there is probably no single primer pair that can detect all strains. In this case, multiple primer sets targeting different regions of the genome can be used to enhance the detection rates. In addition, degenerate primers based on sequence variations of known viral family members have also been used (15).

Diagnosis of viral gastroenteritis remains difficult because of the lack of readily available commercial kits and reagents for many viral families. The fact that a variety of microbial pathogens—including bacterial and parasitic agents in addition to viruses—as well as noninfectious conditions can cause acute gastroenteritis in humans further complicates the situation. Furthermore, many viral pathogens are known to cause subclinical infection. Others, such as AdV, can be shed for prolonged periods in stools. In some cases, more than one pathogen is detected in the same clinical sample or during the same episode of clinical illness, complicating efforts to determine the true etiology. Thus, care needs to be taken in the interpretation of laboratory results for gastroenteritis viruses. Proper epidemiologic case-control studies are necessary to address these issues.

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Rabies Virus*

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TAXONOMY

There are numerous etiologic agents in the genus *Lyssavirus* which result in an acute, fatal viral encephalomyelitis known as rabies in both humans and animals (1). These agents are single-stranded, bullet-shaped RNA viruses of the order *Mononegavirales* and family *Rhabdoviridae*. The genus includes 14 species, the prototype *Rabies virus*, and the less commonly known species of rabies-related lyssaviruses: *Aravan virus*, *Australian bat lyssavirus*, *Duvenhage virus*, *European bat lyssavirus 1*, *European bat lyssavirus 2*, *Irkut virus*, *Khujand virus*, *Lagos bat virus*, *Mokola virus*, *West Caucasian bat virus*, *Shimoni bat virus*, *Bokeloh bat lyssavirus*, *Ikoma lyssavirus*, and a proposed new species, *Lleida bat virus*, http://www.ictvonline.org/index.asp (Fig. 1) (2–5). The lyssaviruses were first characterized by serologic neutralization, which was useful in classifying them into related or more distant groups (6). More recently, differences have been defined by nucleotide sequence analysis, and lyssaviruses are grouped as different genotypes or species (7). Current commercial human and animal vaccines are based on the rabies virus and provide adequate crossprotection against most other lyssaviruses, with the exception of those that are highly divergent from rabies virus, *Lagos bat virus*, *Mokola virus*, *West Caucasian bat virus*, *Ikoma virus*, and by inference, *Lleida bat virus* (since no virus isolate is available for analysis).

DESCRIPTION OF THE AGENT

The virions are rod-shaped, approximately 180 by 75 nm, and consist of five structural proteins: the glycoprotein (G), matrix protein (M), nucleoprotein (N), phosphoprotein (P), and large polymerase protein (L) (Fig. 2). The virus is contained in an envelope bilayer derived from the host cell cytoplasmic membrane during budding. Peplomers, G protein trimeric spike inserts of approximately 10 nm, are found within the surface of the virus envelope. The rabies virus G protein binds with host cell surface receptors (adsorption), initiating a cascade in the infectious cycle and replication. Virus-neutralizing antibodies produced to the G protein after vaccination or natural infection may inhibit this process. The inner surface lining of the envelope is formed by the M protein, which binds with the G protein and envelope and the ribonucleoprotein (RNP) core.

The RNP is composed of tightly wound RNA, encapsidated by the phosphorylated N protein and associated with the P and L polymerase proteins. All lyssaviruses contain nonsegmented genomes (RNA) of approximately 12 kb. The RNA-nucleoprotein complex is responsible for transcription of genomic RNA to five polyadenylated mRNAs, which are translated into the structural proteins, and for viral replication by providing a template to synthesize complementary full-length (negative sense) genomic RNA. During assembly, the coiling of RNP-M protein binds with the G protein as the completed virus buds from the plasma membrane (8). From a diagnostic viewpoint, the N and G proteins and viral RNA have been the focus of most laboratory research evaluations. Rabies diagnosis in postmortem brain tissues is based on the ability to detect intracellular viral inclusions, which are collections of RNP. Because the rabies virus G protein and host cell receptor interactions initiate the infection cycle, the G protein is directly involved in pathogenesis and virulence, induction of immune responses, and binding of neutralizing antibodies. Vaccines prepared from whole virions, purified rabies virus G proteins, and recombinant viral vaccines encoding rabies virus G proteins have been used to successfully immunize and protect animals from rabies virus infections (9, 10). In addition, detection of antibody to the G protein has been used as a method to evaluate vaccine potency. More detailed reviews of virus replication and virus pathogenesis have been published (11–13). Descriptions of the rabies virus epidemiology, transmission, clinical signs, and diagnosis are generalizable to the other lyssaviruses.

EPIDEMIOLOGY AND TRANSMISSION

Rabies is a zoonotic disease (primarily an animal disease that may be transmitted to humans). All mammals are susceptible. Although rabies viruses are endemic on five of seven continents, the geographic distributions of the other lyssaviruses are more localized: *Lagos bat virus*, *Shimoni bat virus*, *Mokola virus*, *Duvenhage virus*, and *Ikoma virus* have been detected only in Africa; *European bat lyssavirus*, *Bokeloh bat lyssavirus*, and *Lleida bat virus* only in Europe; *Australian bat lyssavirus* exclusively in Australia; and *Aravan bat virus*, *Khujand bat virus*, *Irkut bat virus*, and *West Caucasian bat virus* restricted to single isolations in Eurasia. In the United States, more than 6,000 rabies cases are diagnosed annually in animals. The predominant terrestrial reservoir species are raccoons in the Eastern states, skunks in California and the

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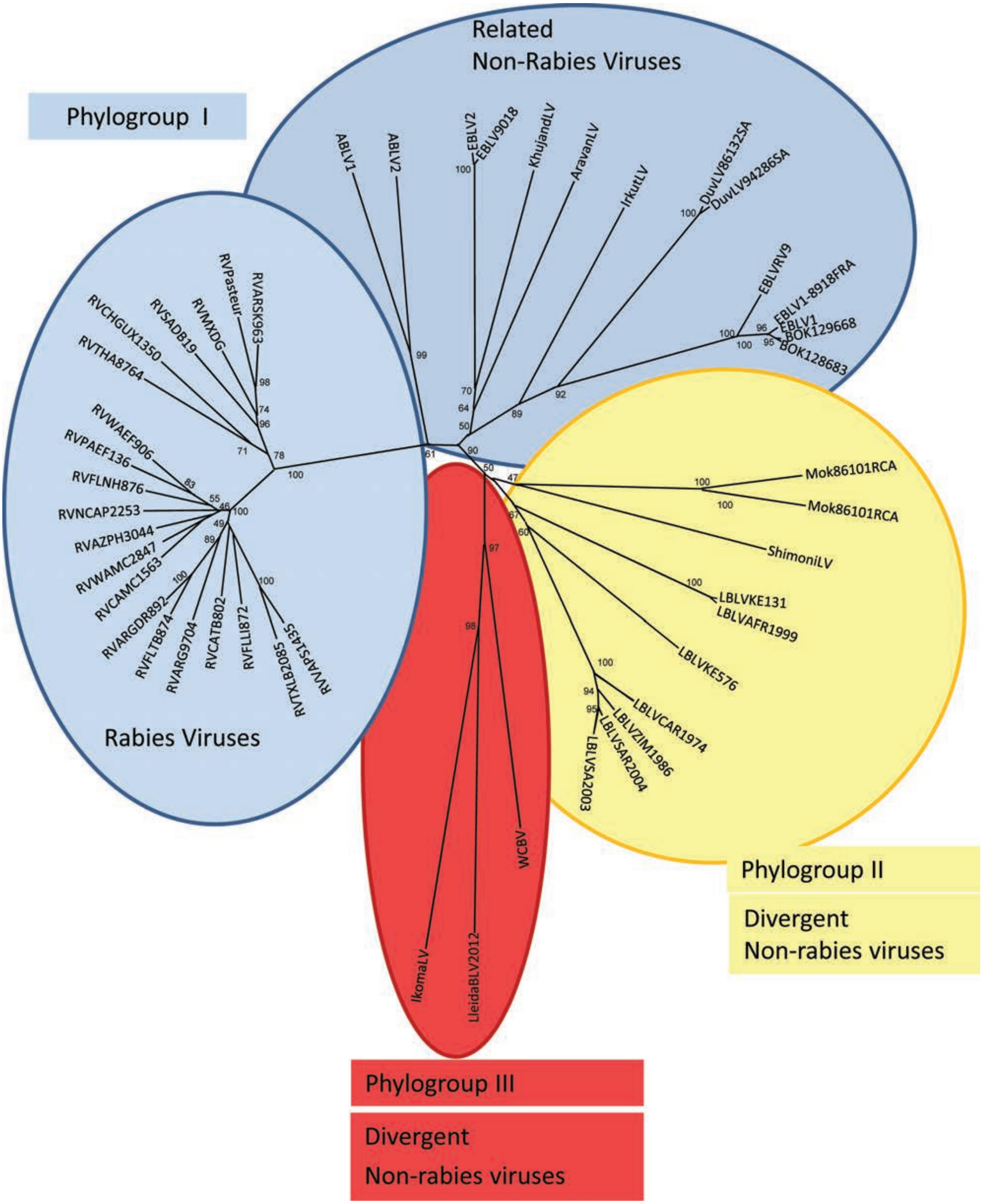
Related
Non-Rabies Viruses
Phylogroup I
ABLV1
ABLV2
EBLV2
EBLV9018
KhujandLV
AravanLV
IrkutLV
DuvLV86132SA
DuvLV94286SA
EBLVRV9
EBLV1-8918FRA
EBLV1
BOK129668
BOK128683
RVARSK963
RVPasteur
RVMXDG
RVSADB19
RVCHGUX1350
RVTHA8764
RVWAEF906
RVPAEF136
RVFLNH876
RVNCAP2253
RVAZPH3044
RVWAMC2847
RVCAMC1563
RVARGDR892
RVFLTB874
RVARG9704
RVCATB802
RVFLLI872
RVTXLB2085
RVVAPS1435
Rabies Viruses
Mok86101RCA
Mok86101RCA
ShimoniLV
LBLVKE131
LBLVAFR1999
LBLVKE576
LBLVCAR1974
LBLVZIM1986
LBLVSAR2004
LBLVSA2003
WCBV
IkomaLV
LleidaBLV2012
Phylogroup II
Divergent
Non-rabies viruses
Phylogroup III
Divergent
Non-rabies viruses

FIGURE 1 Lyssavirus diversity was demonstrated by limited sequence analysis (400 nucleotides) of gene sequences performed using neighbor joining and maximum likelihood methods and bootstrap analysis of 1,000 trees. Full gene sequences were available for all lyssaviruses in GenBank, except for Lleida virus. Phylogroup 1 is composed of rabies viruses and closely related nonrabies lyssaviruses against which the currently available rabies biologics are effective. These biologics are, however, not effective with the divergent lyssaviruses in phylogroups II and III.
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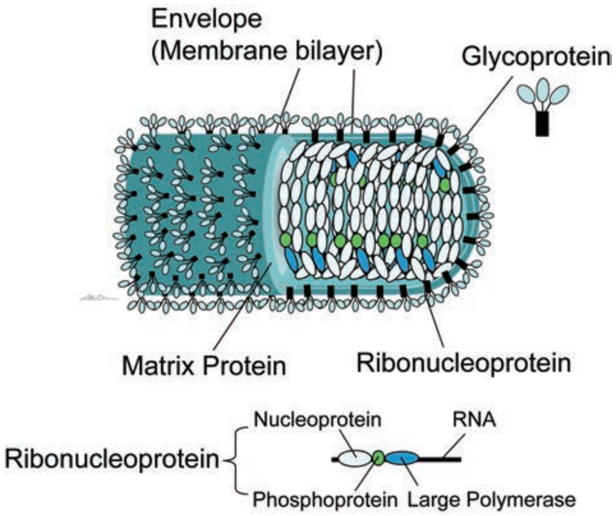

FIGURE 2 Diagram of lyssavirus morphology and structural proteins. doi:10.1128/9781555817381.ch94.f2

North Central and South Central states, gray foxes in Texas and Arizona, Arctic and red foxes in Alaska, and mongooses in Puerto Rico. In addition, there are more than 16 distinct rabies viruses associated with unique species of insectivorous bats throughout their ranges in North America. Although dogs represent a major reservoir species in most developing nations, enzootic canine rabies transmission was pushed to extinction in the United States and other developed countries, primarily due to mandatory dog vaccination and stray animal control (14, 15).

Human rabies cases are rare in developed countries where canine rabies is controlled and access to prophylactic biologics is ideal. Fewer than eight cases are reported annually in the United States. Despite the relative ease of disease prevention in an exposed human, it is estimated that more than 55,000 preventable human deaths worldwide occur annually. Beyond avoiding contact with wild animals and unvaccinated, stray, or domestic animals with unknown immunization histories, access to appropriate human rabies postexposure prophylaxis remains an urgent global public health need to avert this preventable human mortality.

The main route of transmission is via the bite of a rabid animal. Virus is shed in the saliva, often in high amounts. Human rabies cases due to nonbite exposures are extremely rare. Nonbite exposures include scratches, open wounds, and mucous membranes contaminated with a source of rabies virus such as infected saliva or central nervous system (CNS) tissues from a rabid animal. Nosocomial infection (inhalation of accidentally aerosolized rabies virus) and subsequent mucous membrane contact is a potential source of nonbite exposure. Infection by this route is rare but has been documented (16, 17). No human rabies cases have been substantiated from fomite (surface contamination) exposures, most likely because the virus is readily inactivated by drying and agents such as detergents. In the event of an exposure, immediate wound cleansing with soap and water is indicated, followed by appropriate urgent medical care; delays in initiation of human rabies postexposure prophylaxis may result in death.

Lyssaviruses are susceptible to a number of common laboratory disinfectants such as 1:256 quaternary ammonium compound, 0.5% sodium hypochlorite (10% bleach), 70% isopropanol, and 70% ethanol. Chemical disinfectants are less effective when used on items contaminated with brain tissues or heavy suspensions of brain tissue. Decontamination of instruments, laboratory glassware, and disposable waste is best achieved by autoclaving at a minimum of 121°C at 15 lb for 60 minutes (18).

CLINICAL SIGNIFICANCE

The initial clinical presentation of rabies in humans and animals may be rather unremarkable and nonspecific. The majority of incubation periods range from several weeks to several months after exposure. Following the prodromal period, an acute neurological phase develops. The majority of rabies cases manifest as an encephalitic form, and less than 20% are observed as a paralytic presentation. Among patients exhibiting classical encephalitic symptoms, generalized arousal or hyperexcitability, including periods of confusion, hallucinations, agitation, or aggressive behavior, may occur, with intermittent lucid periods. Clinical progression may be characterized by difficulty swallowing, hypersalivation, lacrimation, sweating, dilated pupils, and autonomic dysfunction. Cranial nerve dysfunction may include anisocoria and facial or tongue paralysis. Hydrophobic and aerophobic spasms can be severe. In the end stage, multiple systems may be unstable, often leading to coma and invariably death. Rabies continues to have one of the highest case fatality rates of all known infectious agents.

The paralytic form of presentation includes flaccid muscle weakness, frequently spreading in an ascending pattern to the other extremities. In some cases, laryngeal and facial muscle weakness or bilateral deafness occur. These cases may resemble Guillain-Barré syndrome. Other symptoms include urinary incontinence and priapism. Hydrophobia is less common with the paralytic form, but mild inspiratory spasms may be present. Cardiopulmonary complications and instability usually result in cardiac arrest and death (19).

There is no definitive treatment for rabies after clinical signs are apparent. Appropriate management of a patient with rabies is supportive, usually progressing rapidly to respiratory protection through sedation and intubation, as well as complex medical management in attempts to stabilize cardiopulmonary and other major organ system function. Despite the successful outcome of intensive medical management of a high school student from Wisconsin in 2004, specific rabies antiviral treatment does not exist, and the prognosis remains inexorably grim. The latest information on experimental management of rabies cases may be found on the Medical College of Wisconsin website: http://www.mcw.edu/Pediatrics/InfectiousDiseases/PatientCare/rabies.htm.

Administration of rabies vaccine and human rabies immune globulin (RIG) to patients with laboratory-confirmed rabies has no proven efficacy, may complicate antemortem testing, and may accelerate the disease process and patient demise.

All potential rabies virus exposures need to be evaluated on a case-by-case basis. True exposures (bite or mucous membrane) to suspect animals or animals proven rabid should receive prompt medical intervention and postexposure prophylaxis as outlined in the ACIP guidelines.

Unvaccinated individuals exposed to a rabid animal should receive RIG at dose of 20 international units (IU)/kg of body weight. The RIG should be infiltrated around the wound, and any remaining volume should be given at a site distant from where the vaccine was administered. In addition, four or five doses of a human rabies vaccine licensed for use in the United States should be administered on days 0, 3, 7, and 14 and, if indicated, 28 following exposure. Individuals who previously received immunization against rabies require only two doses of vaccine on days 0 and 3, regardless of the current rabies virus-neutralizating antibody titer (20).

Individuals at high risk for rabies virus exposure (laboratorians, field biologists, veterinarians) are recommended to receive pre-exposure rabies prophylaxis. According to the current ACIP guidelines for Prevention of Rabies in Humans, a series of three doses of modern cell culture rabies vaccine (licensed for use in the United States) should be administered intramuscularly in the deltoid on days 0, 7, and 21 or 28. For all individuals in the high-risk group, determination of rabies virus-neutralizing antibody (VNA) titer is recommended every 2 years. Individuals working under somewhat higher risk for rabies exposure, such as in research or high-volume diagnostic laboratories, typically have their VNA levels monitored more frequently, such as every 6 months. Pre-exposure vaccination and monitoring of detectable neutralizing antibodies does not ensure protection from a particular exposure. On exposure, cleansing of the wound and postexposure rabies vaccine boostering (days 0 and 3) are essential for aversion of clinical disease.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

A summary of tests, required samples, and shipping and storage conditions may be found in Table 1. Diagnosis of rabies in animals requires postmortem examination of the brain. An adequate sample consists of a full cross-section of the brain stem (pons, medulla, or midbrain areas) and cerebellum (vermis and right and left lateral lobes inclusive). Removal of CNS tissues should be performed by trained, vaccinated personnel wearing personal protective equipment (gown or lab coat with sleeves, double latex or heavy rubber gloves, N95 respirator, face shield). Information on sample collection for postmortem rabies diagnosis in animals may be found at http://www.cdc.gov/rabies/pdf/rabiesdfaspv2.pdf.

Information regarding samples for diagnosis of rabies in humans is available at http://www.cdc.gov/rabies/specific_groups/doctors/ante_mortem.html. The antemortem diagnosis of rabies in humans requires the collection of fresh saliva (no preservatives or transport media), a full-thickness skin biopsy from the nape of the neck (including hair follicles placed on saline moistened gauze) (no preservatives or transport media), and serum and cerebrospinal fluid (CSF). The saliva is subjected to nested reverse transcription PCR (RT-PCR). Part of the skin sample is also subjected to nested RT-PCR, but most is cryostat sectioned and a fluorescein-tagged anti-rabies virus reagent is applied for antigen detection. The serum and CSF samples are analyzed by several methods for antibody analysis; this typically includes rabies virus neutralization using the rapid fluorescent focus inhibition test (RFFIT) and analysis for human IgG and IgM that may bind to rabies-infected cells. Other samples such as brain biopsy (for rabies virus antigen detection) and corneal impressions may be useful in diagnosis of rabies but are inferior to the recommended battery of four samples (i.e., saliva, skin biopsy, serum, and CSF). For the optimal sensitivity and specificity of diagnosis, all four samples, and sometimes repeated sequential sets, may have to be tested. Patient submission forms may be obtained on the Centers for Disease control and Prevention (CDC) website http://www.cdc.gov/rabies/pdf/rorform.pdf. The CDC Rabies Laboratory should be consulted before sample collection and submission (phone, 404-639-1050).

For postmortem diagnosis of rabies in humans, the brain samples consisting of a cross-section brain stem and cerebellum, vermis, and right and left lobes are necessary. These samples are tested for antigen, and if present, further antigenic and genetic characterization. Formalin-fixed tissues can be analyzed for histopathology and antigen detection. However, these samples are not recommended for primary diagnosis of rabies unless fresh brain tissues are unavailable; these types of samples require lengthy preparation for processing, embedding in paraffin, and sectioning prior to high-complexity experimental diagnostic techniques. Postmortem or retained antemortem serum and CSF may be quite useful in confirming a postmortem diagnosis.

The shipment of samples to a testing laboratory for rabies diagnosis should follow the U.S. Department of Transportation's *Guide to Transporting Infectious Substances Safely* (https://hazmatonline.phmsa.dot.gov/services/publication_documents/Transporting%20Infectious%20Substances%20Safely.pdf). Packaging of clinical samples for rabies diagnosis should fulfill International Air Transport Association (IATA) regulations for shipment of biological substances, category B, UN 3373. Packaging of cell culture isolates and stock virus suspensions carry a higher infection risk and should fulfill the IATA regulations for category A, UN 2814 (infectious substance affecting humans) and UN 2900 (affecting animals). Samples for rabies testing should be shipped on dry ice (or ice packs for same-day delivery) to the diagnostic laboratory by the most expedient transport or courier method.

The validity of laboratory diagnostic tests depends on optimal collection and storage of the samples. The ideal storage condition for fresh brain tissues for rabies virus antigen detection, virus isolation, and RT-PCR is at −80°C. Storage for short intervals such as 48 hours or less at 4°C or 4 weeks or less at −20°C may be adequate for samples analyzed for rabies virus antigen. Tissues placed in 10% buffered formalin should remain in the fixative a minimum of 24 to 48 hours at ambient temperature. Formalin-fixed brain tissues should be stored in 70% ethanol at room temperature for long-term storage and never frozen. Paraffin-blocked tissues and tissue sections (slides) should be stored at ambient temperatures and never frozen.

The ideal storage and shipping conditions of saliva samples for RT-PCR and virus isolation are at −80°C or below. Skin biopsy samples for RT-PCR and antigen detection should also be stored at −80°C. Samples for antigen detection may be stored for short intervals at −20°C before testing. Serum and CSF samples for rabies serologic testing should be stored at −20°C or below. Samples may be stored for short intervals prior to testing at 4°C. Whole blood samples should be centrifuged and the serum removed prior to freezing. Whole blood should never be shipped in the same container with samples on dry ice, because there is a risk of freezing and hemolysis regardless of packing insulation. Hemolyzed and chylous serum samples are unsatisfactory for testing. Other biological fluids (e.g., vitreous fluids, tracheal washings, tears) for RT-PCR or virus isolation require storage at −80°C or below.

DIRECT DETECTION METHODS

Direct methods may be used to detect histopathological changes and viral antigen (inclusions) and to observe virion morphology. These methods provide rapid diagnosis within minutes to hours without the need for amplification of virus isolation such as in cell culture or amplification of genome via RT-PCR.

TABLE 1 Routine tests for rabies diagnosis[a]

Analysis	Test method	Detection	Sample	Source	Requirements	Provider	Results
Microscopic examination	Specialized stains (e.g., H&E and Seller)	Eosinophilic intracytoplasmic inclusions	Postmortem CNS	Human or animal	Brain stem, cerebellum, hippocampus	Pathology laboratories (hospital, veterinary)	Detects eosinophilic intracytoplasmic inclusions, low sensitivity (60–80%)
Antigen detection by immunofluorescence	DFA	Rabies virus antigen	Postmortem CNS	Human or animal	Full cross-section of brain stem and cerebellum	State public health, veterinary laboratories, CDC	Detects rabies virus specific antigen, very high sensitivity (100%)
Antigen detection by immunofluorescence	DFA (cryostat)	Rabies virus antigen	Antemortem nuchal biopsy	Human	Full thickness of skin; 5- to 6-µm cryosections	CDC (some regional or national labs)	Detects rabies virus specific antigen
Antigen detection by immunofluorescence	FF DFA	Rabies virus antigen	FF postmortem CNS	Human or animal	Full cross-section of brain stem and cerebellum	CDC (some regional or national labs)	Detects rabies virus specific antigen, very high sensitivity (~100%)
Antigen detection by IHC	IHC	Rabies virus antigen	FF Postmortem CNS	Human or animal	Full cross-section of brain stem and cerebellum	CDC (some regional or national labs)	Detects rabies virus specific antigen, very high sensitivity (~100%)
Antigen detection by IHC	DRIT	Rabies virus antigen	Postmortem CNS	Human or animal	Full cross-section of brain stem and cerebellum	CDC, U.S. Department of Agriculture (some regional or national labs)	Detects rabies virus specific antigen, very high sensitivity (~100%)
Nucleic acid	RT-PCR	Rabies virus RNA	Postmortem CNS	Human or animal	50 mg of brain stem and cerebellum	CDC (some regional or national labs)	Produces specific RT-PCR amplicons; very sensitive (detects ~1 IU), but related to primer specificity
Nucleic acid	Nested RT-PCR	Rabies virus RNA	Antemortem saliva, throat, skin, other	Human	100 µl of fluid samples or ≤50 mg of tissue	CDC (some regional or national labs)	Produces specific RT-PCR amplicons; very sensitive (detects ~1 IU), but related to primer specificity
Virus isolation	Cell culture (e.g., MNA) and animal inoculation (e.g., MI)	Infectious rabies virus	Postmortem CNS	Human or animal	20% brain homogenate (brain stem and cerebellum)	State public health, veterinary laboratories, CDC	Detects infectious rabies virus
Serology (virus neutralization)	RFFIT, FAVN, MNT	Rabies virus-neutralizing antibodies	Antemortem or postmortem serum or CSF	Human or animal	Serum or CSF	CDC (some commercial, regional or national labs)	Detects neutralizing antibodies. NT are useful in determining immunization status.
Serology by indirect IFA	IFA	Rabies virus IgG or IgM antibodies	Antemortem or postmortem	Human or animal	Serum or CSF	CDC (some regional or national labs)	Detects specific rabies virus IgM and IgG antibodies from serum or CSF. Very sensitive as a diagnostic technique.

[a] H&E, hematoxylin and eosin; FF, formalin fixed; NT, neutralization tests.

Microscopic Methods

Microscopic examination methods may utilize routine stains (e.g., hematoxylin and eosin) to examine abnormal histopathology consistent with encephalomyelitis, or more specialized stains (e.g., Sellers, Mann's, and Giemsa) to observe typical viral eosinophilic intracytoplasmic inclusions such as classical Negri bodies within neurons (21, 22). Historically, Sellers stain (2:1 methylene blue and basic fuchsin) was most often used for Negri body detection. In contrast with more advanced, sensitive antigen detection methods, this staining procedure is most successfully performed on brain tissues (hippocampi, cerebral cortex pyramidal cells, cerebellum, and Purkinje cells) during the later stages of disease, when inclusions are most abundant and more easily detected. Brain impression slides are stained for 2 to 10 minutes in Sellers stain (the time needed varies depending on the thickness of the impression) and then rinsed with tap water. Typically, rabies virus infection demonstrates intracytoplasmic magenta inclusions that are oval or round with dark blue basophilic granules inside. Although of historical importance, this method has limitations in specificity and sensitivity. For example, when properly performed, the Sellers staining technique may identify Negri bodies in 50 to 80% of rabid animals. Observation of virions by electron microscopy (EM) allows examination of the ultrastructure, shape, and size. The technique provides supportive evidence of a rhabdovirus infection but requires careful examination by expertly trained personnel of numerous observational fields of the sample. As such, it is impractical and too costly for routine diagnosis (23).

Antigen Detection

Direct Fluorescent Antibody Test

The standard test for rabies virus antigen detection in CNS tissues is the direct fluorescent antibody (DFA) test. This test is relatively easy to perform and highly specific, approximates 100% sensitivity, and can be completed in 3 or 4 hours. The "Protocol for Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing" may be obtained at http://www.cdc.gov/rabies/pdf/rabiesdfaspv2.pdf. In contrast to the nonspecific staining of viral inclusions observed with histologic stains, the rabies virus diagnostic conjugates consist of antibodies to the whole virion or the RNP, labeled with fluorescein isothiocyanate (FITC). Within infected brain cells there are abundant collections of rabies virus proteins (antigen), especially RNP. The conjugates may be hyperimmune polyclonal or monoclonal antibodies directed against highly conserved rabies virus epitopes.

Impression slides are prepared from a cross-section of brain stem and cerebellum (right, left, and vermis) or hippocampi (right and left) and are fixed in acetone for a minimum of 1 hour at −20°C. The brain impression slides are tested with two different anti-rabies virus conjugates to ensure antigen detection of the diverse rabies virus variants. When the conjugates are added to rabies virus-infected brain impressions and incubated for 30 minutes at 37°C, the labeled antibodies bind with the rabies virus proteins (antigens) and form specific antigen-antibody-FITC complexes. After the impression slides are washed in phosphate-buffered saline (PBS) twice for 3 to 5 minutes, specific antigen-antibody complexes remain. These complexes fluoresce an intense sparkling apple-green color when observed with a fluorescence microscope equipped with an FITC filter combination. Morphologically, rabies virus antigen may appear as fluorescing large or small, oval or round inclusions, dustlike particles, or strands (Fig. 3).

Direct Rapid Immunohistochemistry Test

The direct rapid immunohistochemistry test (DRIT) is an alternative procedure for rabies virus antigen detection (24). The DRIT uses a cocktail of purified biotinylated anti-rabies virus nucleocapsid monoclonal antibodies to detect rabies virus antigen. To date, the test has demonstrated sensitivity and specificity equal to that of the DFA test in detecting rabies virus antigen. Anticipated use for this test method includes confirmatory DFA testing as well as augmentation of passive public health surveillance since, in contrast with fluorescence microscopy, light microscopy is adaptable to field use. The test is currently applied to augment surveillance in samples from animals that have not exposed humans to the virus. Advantages of the procedure include rapid procedure (1 hour to completion); inactivation of virus in samples through fixation in formalin, whereas acetone fixation in the standard protocol does not; and minimized equipment requirements (ambient incubation temperatures, standard light microscope). The sample requirements for DRIT are the same as those of DFA and other antigen-detection methods. (Refer to the section "Collection, Transport, and Storage" of this chapter for detailed information.) The DRIT is performed on brain touch impressions prepared the same as with the DFA. Slides are fixed in 10% buffered formalin for 10 minutes, rinsed in PBS with 1% Tween 20 (TPBS), then pretreated with hydrogen peroxide for 10 minutes before primary antibodies are applied. The test is a multistep process (biotinylated primary antibodies, streptavidin-peroxidase, and 3-amino-9-ethylcarbazole [AEC]): first an incubation with biotinylated rabies virus monoclonal antibodies for 10 minutes followed by rinsing in TPBS; then incubation with streptavidin-peroxidase for 10 minutes followed by rinsing with TPBS; then incubation with peroxidase substrate (AEC) for 10 minutes to initiate (red) color development followed by rinsing with distilled water. Slides are counterstained with Gill's hematoxylin for 2 minutes, rinsed with water (and a cover glass added with water-soluble mounting media), and observed with a standard light microscope. Rabies virus antigen appears as bright red, large or small, oval or round inclusions, dust-like particles, or strands against a contrasting blue background (Fig. 3).

Formalin-Fixed Tissues

Formalin-fixed CNS tissue samples cannot be tested by the standard DFA test. The fixation process causes chemical crosslinking of proteins. Formalin-fixed tissue samples that have been processed, embedded in paraffin, and sectioned may be tested by a modification of the DFA for formalin-fixed samples. Rabies virus antigen detection, whether by standard DFA, DRIT, or modified DFA on formalin-fixed samples, requires the same tissue sampling of a complete cross-section of brainstem and cerebellum. Modifications of the standard DFA for formalin-fixed samples include heating the slides to 55 to 60°C to melt the paraffin, deparaffinization in xylene, and rehydration of tissue sections in graded alcohols. In addition, a proteinase K digestion for 30 minutes at 37°C is needed, presumably to disassociate chemical bonds formed during formalin fixation such that rabies virus epitopes are available for detection. Incubation with anti-rabies virus FITC conjugate is longer than the standard DFA incubation (i.e., 1 hour instead of 30 minutes), and the wash times in PBS (twice at 15 minutes) are longer to clear unbound conjugate from tissue sections (25). The reliability of this method depends on the availability of

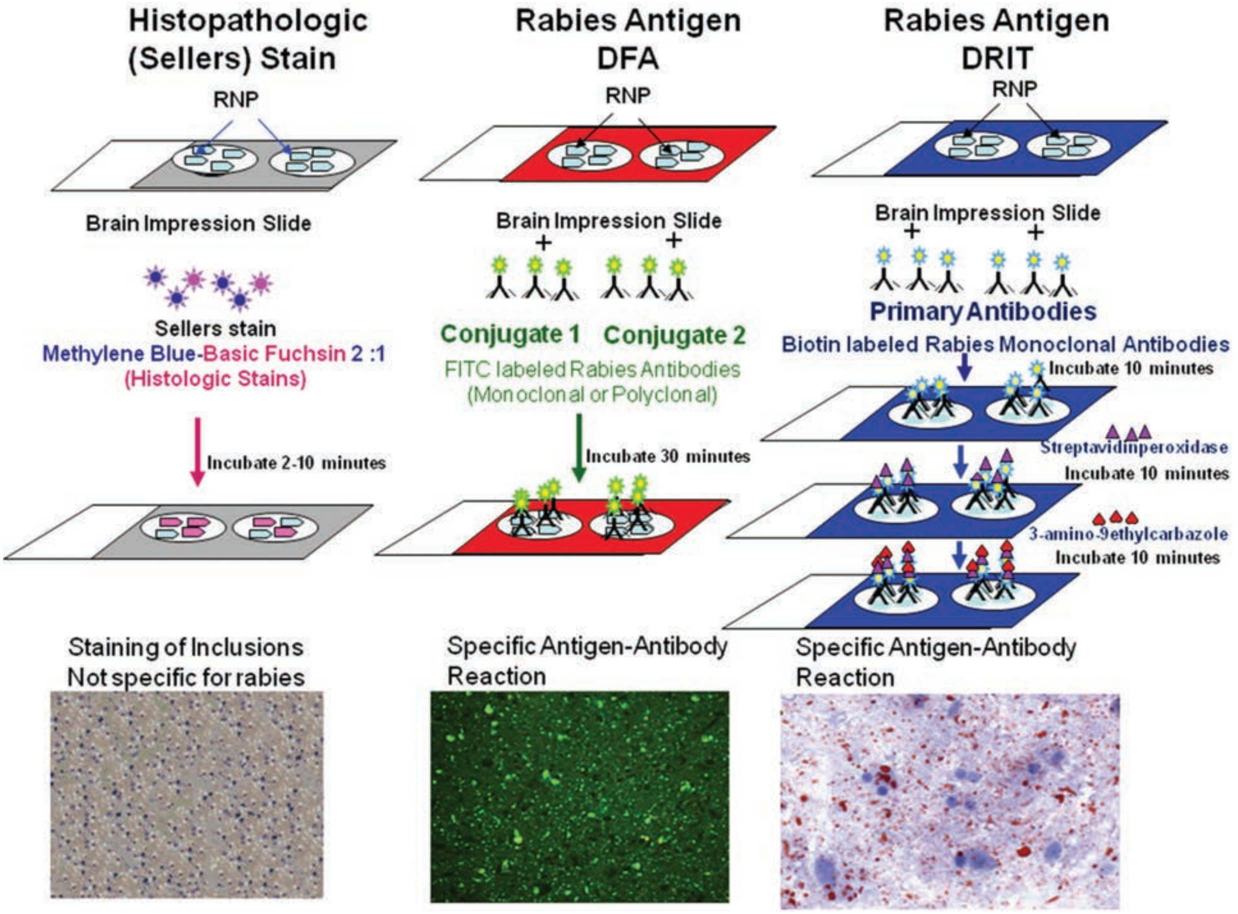
Histopathologic (Sellers) Stain
RNP
Brain Impression Slide
Sellers stain
Methylene Blue-Basic Fuchsin 2 :1
(Histologic Stains)
Incubate 2-10 minutes
Staining of Inclusions
Not specific for rabies
Rabies Antigen DFA
RNP
Brain Impression Slide
+
+
Conjugate 1
Conjugate 2
FITC labeled Rabies Antibodies
(Monoclonal or Polyclonal)
Incubate 30 minutes
Specific Antigen-Antibody Reaction
Rabies Antigen DRIT
RNP
Brain Impression Slide
+
+
Primary Antibodies
Biotin labeled Rabies Monoclonal Antibodies
Incubate 10 minutes
Streptavidinperoxidase
Incubate 10 minutes
3-amino-9ethylcarbazole
Incubate 10 minutes
Specific Antigen-Antibody Reaction

FIGURE 3 Comparison of nonspecific histologic staining and antigen detection methods. doi:10.1128/9781555817381.ch94.f3

a high-affinity, highly concentrated polyclonal anti-rabies virus conjugate, because the modified standard DFA test for formalin-fixed tissues may require a working dilution of rabies virus conjugate as much as 5 to 10 times more concentrated for sensitive and specific detection of rabies virus antigen in these tissue samples.

An immunohistochemistry (IHC) test for rabies virus antigen detection is an alternative method for formalin-fixed tissues samples that have been processed, embedded in paraffin, and sectioned (26). The IHC protocol also requires enzyme digestion but with pronase instead of proteinase K to disassociate crosslinking of protein bonds. The IHC test is similar to the DRIT, but it is performed on paraffin-embedded tissues. It is a multistep process: first digestion with pronase followed by a rinse in TPBS, then 3% hydrogen peroxide for 10 minutes to remove endogenous peroxidase activity. Then normal goat serum is added and slides are incubated for 15 minutes to block nonspecific binding of the primary antibody. The primary anti-rabies virus monoclonal or polyclonal antibodies are added and incubated for 60 minutes. The secondary antispecies biotinylated antibody is added and incubated for 15 minutes, followed by a rinse in TPBS and incubation with streptavidin-peroxidase for 15 minutes. After rinsing with TPBS, slides are incubated with peroxidase substrate, AEC, for 5 to 10 minutes to initiate (red) color development, followed by rinsing with distilled water. Finally, slides are counterstained with Gill's hematoxylin for 2 minutes, rinsed with water, and cover slips are added with water-soluble mounting media. Slides are observed with a standard light microscope. Rabies virus antigen appears as bright red, large or small, oval or round inclusions or dustlike particles within the cytoplasm of infected neurons against a light blue background of the hematoxylin-stained tissue. Advantages of the IHC test over the standard DFA modified for formalin-fixed samples include the ability to test for rabies virus antigen and other etiologies simultaneously by including antibodies to the other agents; the ability to examine the histpathology of tissues; and the ability to observe slides with a standard light microscope rather than fluorescence microscopy. Disadvantages are the time required for the procedure (approximately 6 hours) and the high complexity of the method, requiring optimization of multiple antibodies and reagents.

DFA Test of Nuchal (Neck) Biopsy Specimens

Antemortem DFA tests for rabies virus antigen are performed on serial 5- or 6-μm tissue cryosections of neck skin biopsies and provide a rapid method for viral antigen detection in nerves at the base of hair follicles. The DFA test on tissue sections is performed exactly as the standard DFA test on CNS tissues. In its antemortem application, the method has optimized sensitivity but may remain negative (false negative) in early stages of the clinical disease or in later clinical stages, presumably if antigen is masked or bound by the endogenous antibodies sometimes present in the terminal patient. On rare occasions, nested RT-PCR on the same sample may confirm the diagnosis while rabies antigen is not present in sufficient quantity to visualize with the DFA on skin sections.

Nucleic Acid Detection

RT-PCR methods are the most sensitive tests for rabies virus diagnosis. The sensitivity of RT-PCR depends in part on the sample (type and condition), the integrity of sample handling and storage to preclude contamination and degradation, the particular method of RNA extraction and RT-PCR, the primers selected for amplification, the quality of reagents, individual technical expertise, interpretation of the results, and confirmation methods. The usefulness of RT-PCR as a routine diagnostic test on postmortem (fresh and fixed) CNS tissues is limited. Highly sensitive, broadly reactive, less expensive, and less time-consuming procedures for antigen detection by DFA are more efficient routine tests for rabies diagnosis. RT-PCR is an essential tool for molecular analysis and, in some cases, to confirm a suspected diagnosis on CNS tissues (e.g., those that are compromised in quality), but its maximum utility is the detection of nucleic acid in non-CNS samples (e.g., antemortem saliva, skin, cornea impressions, tears, eye swabs, throat swabs, postmortem vitreous fluid) when fresh CNS tissues may be unavailable (27). When all conditions are optimal, RT-PCR can detect as little as 1 infectious unit of rabies virus. Nested RT-PCR increases the sensitivity (10- to 100-fold) to below 1 infectious unit of virus (28). RNA may be limited or degraded when examining non-CNS samples, and nested (or heminested) RT-PCR is usually required. Until recently, the diversity among lyssaviruses and the lack of specific nondegenerate universal primers have discouraged use of real-time PCR for detection of rabies virus RNA in non-CNS samples when the rabies virus variant or lyssavirus species is unknown. A real-time RT-PCR for human antemortem diagnosis has been developed with degenerate primers and probes, with reported sensitivity to detect all major rabies virus variants (genotype 1), including *Aravan virus* and *Khujand virus*. The future incorporation of real-time techniques that detect all lyssaviruses will allow more rapid diagnosis, less chance of cross-contamination, and test automation (29, 30).

ISOLATION OF RABIES VIRUS

Isolation methods are useful in detecting infectious virus in samples and are sometimes used as a confirmatory test alternative to the standard DFA. The classical methods include *in vivo* isolation in animals (usually intracerebral inoculation of suckling mice) and *in vitro* virus isolation in cell cultures. For most routine diagnostic needs, the inoculation of cell cultures such as mouse neuroblastoma (MNA; Biowhittaker, Walkersville, MD) or a similar alternative cell culture line, CCL 131 (ATCC, Rockville, MD), provides the same sensitivity as animal inoculation but with quicker results and without the maintenance required for the use of laboratory animals. For these purposes, 0.4 ml of supernatant from a 20% brain suspension prepared in tissue culture medium with 10% fetal calf serum (MEM10) is inoculated into a suspension of 4×10^6 MNA cells/2 ml and incubated for 1 hour at 37°C. To maximize infection of the cells, gentle mixing of the suspension is performed every 15 minutes. The cell suspension is diluted to a volume of 10 ml with MEM10, 6 ml is transferred to one 25-ml cell culture flask, and the remaining suspension (4 ml) is transferred to 12 Teflon-coated 6-mm slides (or other cell culture slides). At least one slide should be acetone-fixed daily and examined by DFA for rabies virus antigen. At least one additional passage of the cell culture (performed in 3 to 5 days) is required to rule out rabies. Ideally, *in vivo* testing use should be reserved for efficacy and safety studies for biologics or virulence and pathogenesis studies. Cell cultures may be useful in the propagation, amplification, and quantification of virus and antibodies; the production of vaccines; determination of the safety of vaccine lots; and the study of rabies virus pathogenesis in particular cells (31, 32).

IDENTIFICATION

As previously described in detail, identification of a *Lyssavirus* infection is made typically by direct examination of brain impressions and demonstration of specific viral inclusions (antigen) by DFA. Isolates are also identified in brains of inoculated mice and cell cultures by DFA. Electron microscopy may be used to determine the morphologic identification of lyssaviruses by examination of the virion ultrastructure in cell cultures or CNS tissues.

Further secondary characterization of *Lyssavirus* infection may be made by antigenic typing with monoclonal antibodies, genetic typing with sequence analysis, or investigating patterns of cross-neutralization.

TYPING SYSTEMS

Rabies viruses may be readily identified by antigenic and molecular methods as being associated with the broad groups of carnivore and bat variants. Through antigenic analysis, at least five major reservoirs are detectable among carnivores in the United States. Genetic typing adds resolution and identifies seven distinct virus lineages among the current variants. Antigenic typing is less useful in certain cases involving rabid bats. Nucleotide sequence analysis adds resolution when studying these samples. Typing methods are useful in a variety of circumstances, such as determining the rabies virus variants in human cases with unclear or unknown exposure histories, discovering the emergence of new viruses, monitoring the epidemiologic spread or re-emergence of virus in defined geographical areas, detecting spillover or host-switching of variants from the predominant host species to another species, and monitoring the success of rabies vaccination programs through characterization of positive samples.

Antigenic Typing

Antigenic typing with monoclonal antibodies (MAbs) can be performed by an indirect fluorescent antibody test (IFA) on acetone-fixed brain impression slides and rabies virus-infected cell culture slides or by the indirect rapid immunohistochemistry test (IRIT) on formalin-fixed brain impression slides or cell culture slides (33, 34). If direct brain impressions are used, the best results are obtained if 75 to 100% of the microscope fields contain viral antigen. If insufficient antigen is present, it is necessary to amplify the virus by inoculating cell cultures or animals. A panel of seven commercially available murine anti-rabies-virus-N MAbs is used to distinguish rabies virus variants by the different reaction patterns. Antigenic typing methods are inexpensive, rapid, and easily performed to determine rabies virus variants in a few hours. Limitations include the necessity for amplification when antigen amounts are inadequate and a lack of resolution for certain terrestrial and bat rabies virus variants. If antigenic typing results are inconclusive, additional testing can be performed at reference laboratories such as the Centers for Disease Control and Prevention (CDC), which has a more extensive panel of MAbs and resources for sequence analysis (35).

Nucleotide Sequence Analysis

Genetic typing methods for molecular epidemiologic studies have become routine since more laboratories are able to extract RNA, perform RT-PCR tests, and sequence viruses. The N protein gene has been the one most frequently used in molecular epidemiology studies. Studies have focused the analyses on short sequences of less than 400 nucleotides; however, current technologies have expanded the focus from partial gene sequences to whole viral genomes (5). Currently, there are thousands of N gene sequences (complete and partial) in GenBank for comparison. Lyssavirus researchers typically focus on the G, P, and L genes (4, 28). These data may assist in understanding evolutionary relationships between lyssaviruses (Fig. 1), understanding specific gene functions in host species, and predicting viral pathogenesis, replication, and virion formation.

SEROLOGIC TESTS

The serologic tests for rabies virus antibody include rabies virus neutralization assays and antibody-binding assay methods. Each varies in sensitivity, specificity, type of antibody detected, and the viral antigen (protein) recognized. These methods, most robust when applied in parallel on sequential samples, are routinely used to diagnose rabies in humans (36, 37).

Neutralization Tests

Neutralization tests are largely embraced as the most powerful for prediction of an adequate response to immunization against rabies and for the specific diagnosis of rabies during the later clinical stages. Neutralization of rabies virus relies on antibodies directed to the outer glycoprotein antigens; these are functional assays measuring performance against active viral infection of cells in culture, or historically, in mice intentionally infected with rabies, along with dilutions of test serum. The historic mouse neutralization (MNT) has been largely replaced by the rapid fluorescent focus inhibition test (RFFIT) and a simplification of it, the fluorescent antibody virus neutralization test (FAVN). All of these test methods measure the ability of rabies virus antibodies in serum or CSF samples to neutralize a known standard challenge virus dose. The RFFIT and FAVN, a modification of the RFFIT microneutralization test performed in microtiter plates instead of chamber slides, are most frequently used to determine the immunization status of vaccinated humans and animals, respectively. When performed in qualified laboratories by qualified personnel, both test methods demonstrate acceptable and similar sensitivity and specificity in determining rabies virus neutralizing antibodies; tests results are best compared when converted to international units per milliliter on the basis of *a priori* criteria specifying acceptable performance of an international reference standard in each particular run of an assay method (36–39).

IFA Tests

IFA tests are sensitive methods for detection of IgM and IgG antibodies that have bound to rabies virus antigen within infected cells. Binding activity may be present in human serum and CSF and is thus a useful component of the battery of tests applied for antemortem diagnosis (27, 37). It appears that the IFA is useful for the measurement of antibodies against internal rabies virus proteins such as RNP rather than G. Serum or CSF samples are titrated and added to acetone-fixed cell culture slides infected with rabies virus (CVS-11). The endpoint antibody titer is the last dilution demonstrating specific fluorescence. The IFA titers are not comparable with levels of *in vivo* viral neutralizing antibody to the rabies virus G surface proteins.

ANTIVIRAL SUSCEPTIBILITIES

As previously described, no specific rabies antiviral biologics are currently licensed, and the prognosis remains inexorably grim. Once clinical signs are present, rabies remains fatal in greater than 99% of cases. For the most current information on experimental recommendations and approaches, consult the rabies registry at the Medical College of Wisconsin website: http://www.mcw.edu/Pediatrics/InfectiousDiseases/PatientCare/rabies.htm. Antiviral drugs previously used in human treatment regimens include ribavirin, ketamine, amantadine, and interferon alpha. Although effective against RNA virus infections, ribavirin is contraindicated for rabies treatment due to the depression of the immune response. However, the use of ketamine (anesthetic and antiviral) and amantadine (neuroprotectant and antiviral) is included in current human rabies treatment. Interferon alpha has demonstrated toxicity and is now contraindicated.

EVALUATION, INTERPRETATION, AND REPORTING RESULTS

Written protocols, which include quality assurance and quality-control measures, are essential for all diagnostic tests. All reagents should be optimized before use with known positive samples from two or more rabies virus variants endemic to the geographical region and known negative control samples. The accuracy and limitations of each diagnostic test should be understood before interpretation of the test results (Table 1). The national standard protocol in the United States for rabies diagnosis in postmortem brain tissues is the DFA test. Procedural requirements of the standard DFA, DRIT, RT-PCR, and isolation methods maximize sensitivity by testing the CNS tissues most likely to be positive in rabid animals (brain stem and cerebellum). Problems of crosscontamination in direct detection and amplification methods can be avoided by processing necropsy samples separately, using separate containers for acetone fixation and washing of DFA tests, and using different dedicated laboratory areas for processing RNA and cDNA samples for RT-PCR. Multiple readers are required to evaluate each of the diagnostic tests and provide quality assurance. Since there are no universal primers for all lyssaviruses, multiple broadly reactive or degenerate primers are needed to rule out rabies by RT-PCR. Confirmatory testing is required for all rabies diagnostic tests with weak reactions or unusual results (atypical morphology, atypical reactions patterns, and epidemiologic inconsistencies). Samples with nonspecific reactions and inconclusive results should be sent to a reference laboratory for confirmation and alternative testing methods. The timeliness of reporting results directly affects medical intervention in humans and management of exposed animals. Ideally, a primary rabies diagnosis from CNS tissues of suspect animals (or humans) should be made in 24 to 48 hours so that potentially exposed humans and animals may be appropriately managed for the optimal prevention of human and animal rabies.

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Arboviruses

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TAXONOMY

Arthropod-borne viruses (arboviruses) transmit to a vertebrate host through the bite of an infected arthropod and cause more than 80 diseases in humans. These viruses have been grouped based on transmission by their arthropod vector: mosquito, tick, sand fly, midges, and others (Table 1). There are more than 500 taxonomically diverse viruses listed from seven distinct families of viruses: *Togaviridae*, *Flaviviridae*, *Bunyaviridae*, *Reoviridae*, *Rhabdoviridae*, *Orthomyxoviridae*, *and Asfaviridae*. The medically important arboviruses are primarily from *Togaviridae*, *Flaviviridae*, *and Bunyaviridae*, and this chapter focuses on these three families. The complete list and characterization of the first discovery and isolation of these viruses can be found in The International Catalog of Arboviruses (http://wwwn.cdc.gov/arbocat/index.asp).

DESCRIPTION OF THE AGENT

All arboviruses are RNA viruses of either positive or negative polarity (with the exception of African swine fever virus in the family *Asfaviridae*). Typically, they are enveloped viruses containing single-strand and/or segmented RNA genomes and are capable of replication in both arthropod and mammalian cells. Arboviruses range from 40 to 100 nm in size and have a RNA genome of approximately 10 to 12 kb. Many arboviruses have been genetically sequenced (full length or partial, e.g., structural genes) and available on GenBank for diagnostic test and vaccine development.

Some arboviruses have developed divergent genomic sequences within species based on geographical location and are referenced as genotypes, subtypes, or lineages. These genotypes result from the evolution of these virus species within restricted ecological niches, allowing for evolutionary divergence for improved adaptation within these niches. Genotypes have been described for virus species in all three families/genera: (i) *Flaviviridae*, *Flavivirus*: Japanese encephalitis virus (JEV), dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV); (ii) *Togaviridae*, *Alphavirus*: eastern equine encephalitis virus (EEEV), western equine encephalitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV); and (iii) *Bunyaviridae*, *Bunyavirus*: LaCrosse virus (LACV).

EPIDEMIOLOGY AND TRANSMISSION

The epidemiology of arboviruses is dependent on the virus, vector, and geographical constraints based on the presence of the vector and amplifying host. Many arboviruses are geographically restricted and have limited circulation confined to certain continents or specific regions of the world (e.g., tick-borne encephalitis virus [TBEV] circulates in Europe and parts of Asia). Environmental changes favorable to the vector or virus can cause either epidemics or expansion of arboviruses into new regions. Changes in the vector ecology or number of susceptible individuals in a given population also play an important role in arbovirus transmission. Worldwide globalization has been implicated as the major cause of the introduction of new vectors into regions where not previously present. This includes increases in travel of infected humans, animals, or vectors into new regions, providing a unique environment in which the pathogen adapts and thrives due to the abundance of susceptible hosts (1). For example, chikungunya virus (CHIKV) is transmitted principally by the mosquito vector *Aedes aegypti*; however, a single point mutation in the membrane fusion glycoprotein increased its adaptation to the tiger mosquito vector *Aedes albopictus* and led to the outbreaks observed in tropical areas of the Indian Ocean (2, 3). Genetic analysis suggested that the emergence of WNV in the Americas was due to importation of WNV into North America from the Middle East. The method of introduction either by mosquito or host was not fully determined. Nevertheless, WNV swept across the United States at alarming speed following its emergence in 1999 in New York because the New World avian hosts were more susceptible than the Old World avian host. Hence, the epizootic transmission of WNV in North America caused an alarming number of avian deaths, particularly among corvids and especially the American crow (*Corvus brachrhynchos*) (4). Current transmission of most medically important arboviruses worldwide is available on the Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO) websites (http://www.who.org and http://www.cdc.gov).

The transmission cycle of all arboviruses includes an arthropod vector and a vertebrate animal reservoir as the amplifying host. Some viruses have evolved an urban and a sylvatic transmission cycle as observed with CHIKV and YFV. Arboviruses may cause seasonal epidemics often linked to the abundance of the vector, intermediate amplifying host, susceptible hosts, and introductions of new genotypes, subtypes, or lineages. Humans have become the principal amplifying host without the need for an intermediate vertebrate amplifying host for certain arboviruses, such as DENV, YFV, and CHIKV.

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TABLE 1 Characteristics of arboviruses affecting humans

Family/genus	Virus common name	Vector	Vaccine	Geographical distribution[a]	Tropism/complications	Other risk factors
Bunyaviridae						
Nariovirus	Crimean-Congo hemorrhagic fever	Tick	None	A	Viscerotropic/hemorrhage	Potential nosocomial
Orthobunyavirus	Bwamba	Mosquito	None	A	Viscerotropic	
	Bunyamwera	Mosquito	None	A	Viscerotropic/encephalitis	
	Guaroa	Mosquito	None	CSA	Viscerotropic	
	Ilesha	Mosquito	None	A	Viscerotropic	
	La Crosse	Mosquito	None	NA	Neurotropic/encephalitic	
	Tahyna	Mosquito	None	E	Viscerotropic	
	Oropouche	Midge	None	CSA	Viscerotropic/hemorrhage, encephalitis	
	Tataguine	Mosquito	None	A	Viscerotropic	
Phlebovirus	Toscana	Sandfly	None	E	Neurotropic/aseptic meningitis	
	Hartland	Tick	None	NA	Possible viscerotropic	
	Sandfly fever (Naples, Sicilian)	Sandfly	None	E, I/SEA	Viscerotropic	
	Rift Valley fever	Mosquito	None	A	Viscerotropic/hemorrhage, encephalitis	
Flaviviridae						
Flavivirus	Bussuquara	Mosquito	None	CSA	Viscerotropic	
	Dengue	Mosquito	None	A,NA, CSA, I/SEA, O	Viscerotropic/hemorrhage, encephalitis	Transfusion; transplacental; sickle-cell anemia
	Japanese encephalitis	Mosquito	SJE-VAX/IXIARO	I/SEA	Neurotropic/encephalitis	
	Kyasanur Forest	Tick	None	I/SEA	Viscerotropic/hemorrhage, encephalitis	
	Tick-borne encephalitis	Tick	Ecepur/TBE-Immun	E	Neurotropic/encephalitis	Unpasteurized dairy milk
	West Nile/Kunjin	Mosquito	None	NA, CSA, E, I/SEA, O	Neurotropic/encephalitis	Transfusion; organ donation; transplacental
	Powassan	Tick	None	NA	Neurotropic/encephalitis	
	Murrey Valley	Mosquito	None	O	Neurotropic/encephalitis	
	Yellow fever	Mosquito	YF-VAX	A, CSA	Viscerotropic/hemorrhage, encephalitis	
	Rocio	Mosquito	None	CSA	Neurotropic/encephalitis	
	Ilheus	Mosquito	None	CSA	Encephalitic	
	Omsk hemorrhagic fever	Tick	None	R	Viscerotropic/hemorrhage	
	St Louis encephalitis	Mosquito	None	NA, CSA	Neurotropic/encephalitis	
	Zika	Mosquito	None	A/I/SEA	Viscerotropic	

(Continued on next page)

TABLE 1 Characteristics of arboviruses affecting humans *(Continued)*

Family/genus	Virus common name	Vector	Vaccine	Geographical distribution[a]	Tropism/complications	Other risk factors
Reoviridae						
Coltivirus	Colorado tick fever	Tick	None	NA	Viscerotropic/hemorrhage, encephalitis	
Togaviridae						
Alphavirus	Barmah Forest	Mosquito	None		Viscerotropic	
	Chikungunya	Mosquito	None	A, I/SEA[b]	Viscerotropic	
	Eastern equine encephalitis	Mosquito	None	NA, CSA	Neurotropic/encephalitis	
	Western equine encephalitis	Mosquito	None	NA	Neurotropic/encephalitis	Transplacental
	Venezuelan equine encephalitis	Mosquito	TC-83	NA, CSA	Neurotropic/encephalitis	
	O'nyong-nyong	Mosquito	None	A	Viscerotropic	
	Mayaro	Mosquito	None	CSA	Viscerotropic	
	Ross River	Mosquito	None	O	Viscerotropic	
	Semliki Forest	Mosquito	None	A	Neurotropic/encephalitis	
	Sindbis	Mosquito	None	A	Viscerotropic	
Rhabdoviridae	Vesicular stomatitis	Sandfly	None		Viscerotropic	
	Piry	Mosquito	None	CSA	Viscerotropic	
Orthomyxoviridae	Thogoto	Tick	None	A	Neurotropic/encephalitis	
	Dhori	Tick	None	I/SEA	Viscerotropic/encephalitis	

[a] Abbreviations: A, Africa and Middle East; NA, North America; CSA, Central and/or South America; E, Europe and/or continental Asia; I/SEA, India and Southeast Asia, including China and Japan; O, Oceania; R, Russia.

[b] 2013 emergence in Caribbean islands.

Other sources of arbovirus transmission include infected blood and/or organ donations. This method of infection was best described for WNV following its emergence in the U.S., where transplantation of infected organs caused fatalities in organ recipients (5). These studies prompted the investigation of other arbovirus infections via blood and organ donations, especially DENV, which causes a high rate of asymptomatic infections (75%) with sufficient viral titers to infect transfusion or organ recipients (6).

CLINICAL SIGNIFICANCE

Many of the medically important arboviruses that are human pathogens cause varied clinical presentations from mild flu-like symptoms, such as fever, malaise, and headache, to severe disease with hemorrhage and encephalitis, occasionally leading to death. These clinical symptoms can be categorized by viruses that are primarily either viscerotropic or neurotropic. Viscerotropic viruses cause febrile illnesses that often present with rash and arthralgia. Additional reported clinical symptoms include hepatitis, jaundice, respiratory symptoms, photophobia, and others. A summary of viral tropism and associated complications resulting from severe disease in humans for the commonly reported arboviruses is listed in Table 1. Although published reports often focus on severe cases, those clinical cases classified as mild forms of disease can cause significant morbidity and sequelae. For example, individuals with dengue fever (a mild form of the disease) may be absent from work or school for 7 days or more with occasional sequelae of fatigue and general malaise that can last for months (7).

Commonly observed clinical symptoms for neurotropic arboviruses include coma, meningitis, flaccid paralysis, encephalitis, and Guillain-Barré syndrome. Neurotropic arboviruses include the genera *Flavivirus* (JEV and TBEV subcomplex), *Alphavirus* (VEEV, EEEV, and WEEV), and *Bunyavirus* (LACV). Neuropathogenesis includes two important yet distinct factors: neuroinvasiveness and neurovirulence. Neuroinvasiveness is the capacity of the virus to invade the central nervous system (CNS) and/or the peripheral nervous system (PNS). Neurovirulence is defined as the replication of the virus within neuron or glial cells in the CNS and/or PNS. Within the virus genus *Flavivirus*, JEV and TBEV serocomplex groups have adapted to replicate in the nervous system. Although many cases of encephalitis involving nonneurotropic arboviruses have been reported, these viruses are not neurotropic in nature and the reported encephalitis is often observed as an element of multiorgan failure associated with fatal cases (Table 1). The envelope glycoprotein is an important determinant for neurotropism in the genus *Flavivirus* and is linked to the glycosylation variants of the envelope gene product. The most important host risk factors for severe flavivirus infection are age, genetics, immunocompromise, and preexisting flavivirus immunity. Most flavivirus infections cause either an asymptomatic or an inapparent infection. The rate of neuroinvasive disease is often 0.1% but varies with the prevalence of the virus in the population. For example, during the peak of the WNV transmission season in the United States, the rate of neuroinvasive disease was 1 in every 150 infections with a mortality rate of 5 to 10% (8, 9). WNV epidemics in 2012 had increased rates of neuroinvasive disease compared to previous nonepidemic years, with neuroinvasive rates comparable to rates observed in the 2002 U.S. epidemic (10).

Age is the most common host risk factor documented in both human clinical studies and animal models. Younger individuals have a higher susceptibility to neuroinvasive disease, implying that the developing nervous system is more susceptible to the virus. Children are more susceptible to JEV, and thus encephalitis and severe disease are primarily observed in children younger than 9 years of age. For WNV, younger patients have a higher risk for aseptic meningitis, whereas elderly patients have a higher risk of encephalitis with poor outcome. For the elderly, the risk of neuroinvasive disease is most likely related to a compromised immune system or preexisting medical conditions. Preexisting systemic conditions such as autoimmune diseases, diabetes, and obesity are some of the risk factors associated with WNV severe disease (11). These preexisting systemic conditions resulting in a less functional and responsive immune system are also associated with the reduction in vaccine responsiveness in elderly individuals.

Viscerotropic arboviruses include DENV, YFV, Rift Valley fever virus (RVFV), and others (Table 1). Common symptoms for these viruses are fever, malaise, headache, photophobia, and occasionally rash and arthralgia. Other symptoms include hepatitis and jaundice, which is sometimes mistaken for hepatitis virus infection. A distinct clinical manifestation for these viruses is hemorrhage and shock syndrome. However, only a relatively small percentage of patients will develop hemorrhage during the course of illness, followed by multiorgan failure and eventually death. Other viruses that present with viral hemorrhagic syndrome, such as Lassa virus (arenavirus) and Ebola and Marburg viruses (filoviruses), can be confused with hemorrhagic arbovirus syndromes. However, there are clinical differences between arboviral and other hemorrhagic viral infections as well as geographical constraints that would facilitate proper clinical diagnosis.

Since there are few pathognomonic symptoms associated with each arbovirus, there is often misdiagnosis due to nonspecific clinical symptoms during the early acute stage of an infection. Other diseases that have available prophylaxis or are vaccine preventable, such as rubella/measles, influenza, leptospirosis, and malaria, have been misdiagnosed as arboviral infections. Clinical misdiagnosis will delay appropriate medical treatment and triage of patients. Common misdiagnoses for viscerotropic arboviruses are malaria and leptospirosis. Many patients, particularly in the African continent, are treated with antimalaria medication prior to laboratory confirmation of *Plasmodium* infection. There is no known negative impact of malaria treatment of an arbovirus infection; however, there are no data to support this observation (12). Encephalitis and hemorrhagic symptoms associated with certain arbovirus infections can aid in accurate clinical diagnosis.

Vaccines and antiviral medications for prophylaxis or treatment are limited for most arboviruses (Table 1). Clinicians principally rely on supportive care including fluid replacement, analgesics, and transfusion of blood products. Diagnosis of arboviruses should always consider the geographical location where the infection was acquired. Patient information such as vaccine history, travel history, and exposure to vector should be considered. While many arboviruses circulate in restricted geographical regions, other arboviruses, such as DENV, now circulate in most tropical and subtropical regions of the world due to its rapid geographical expansion. Clinical diagnosis can be considerably narrowed by using known incubation periods, risk of infection based on geographical distribution of the virus, and presence of vector. Concurrent infections either with cocirculating arboviruses or with other infectious or parasitic diseases (e.g., leptospirosis, influenza, and malaria) can occur (13–15).

There is no clear evidence that concurrent infections have higher morbidity or mortality rates, due to the small sample size that prohibits studies with sufficient statistical power.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Human specimens for laboratory testing include sera, whole blood, and/or cerebrospinal fluid (CSF) from acutely infected patients. Virus can be detected in serum specimens of infected patients for most arboviruses during the first 48 hours following symptoms or fever onset. Nucleic acid amplification tests (NAATs) are the most sensitive tests for case confirmation. Specimens collected during the viremic phase (days post onset of illness [DPO], 0 to 5) should be stored at −80°C. Freeze-thaw of these specimens usually degrades the RNA rather quickly and can significantly reduce viral titers by as much as 1 log following a single freeze-thaw cycle. When humans are the primary amplifying host such as for DENV and YFV, viral titers in blood and tissue are approximately 10^3 to 10^9 genome equivalent copies/ml and readily detected by most molecular diagnostic tests. Other arboviruses such as WNV, St. Louis encephalitis virus (SLEV), and JEV have a vertebrate amplifying host, such as avian or swine, and humans are dead-end incidental hosts. For these viruses, identification in blood is less probable since viral titers range from approximately 10 to 1,000 PFU/ml, which is often below the limit of detection of many reverse transcriptase PCR (RT-PCR) tests (16). These neurotropic viruses can be successfully isolated in culture or RT-PCR amplified from CSF, particularly in patients clinically diagnosed with encephalitis or meningitis. However, in neuroinvasive cases, identification in blood must be performed prior to the development of encephalitis. A few studies have indicated arbovirus persistence in humans and include WNV and Colorado tick fever virus (CTFV), where viral RNA was detected for weeks following initial infection (17).

Serum specimens tested for immunodiagnostic tests should be collected during both the acute phase (DPO, 0 to 5) and convalescent phase (DPO, 6 to 14). Paired specimens provide the highest likelihood of a confirmed test result by seroconversion. For CSF specimens obtained from patients suspected of a neurotropic arboviral infection, IgM antibodies are detected earlier in the course of infection than in sera; however, both specimens (serum and CSF) should be obtained for best results. A single positive serum specimen provides a diagnosis of a recent arbovirus infection; however, acute infection cannot be confirmed due to persistence of the IgM response for some arboviruses (e.g., WNV) (18).

Postmortem tissue specimens are collected for direct detection methods from fatal cases. Other specimens collected for diagnostic testing include arthropods such as ticks, mosquitoes, sandflies, and midges. These specimens are used for virus isolation and/or tested for the presence of viral nucleic acids. Arthropod field tests for virus identification in the vector include rapid tests, such as dipstick, using antibodies directed against viral antigen (e.g., envelope glycoprotein). These tests are commercially available for WNV, DENV, EEEV, and RVFV (19–21).

DIRECT EXAMINATION

Microscopy

Viral identification using immunohistochemistry on biopsy or tissue specimens is primarily performed postmortem. The types of tissue specimens obtained are dependent on the suspected arbovirus infection. Because autopsies are not practiced and/or culturally accepted for many of the regions of the world where arboviruses circulate, pathology studies for arbovirus-related fatalities are few. The most complete pathology information from fatal cases is primarily from flavivirus-related deaths (JEV, WNV, YFV, and DENV). The specific tissue sampled is determined by viral tropism and varies by arbovirus family and species. For neurotropic viruses that infect the CNS, tissue specimens are collected and tested for the presence of viral antigens or nucleic acid from regions of the CNS including the cortex, cerebellum, and brain stem. For JEV, brain autopsies primarily test tissue specimens with NAAT and cell culture virus isolation techniques for confirmation (22, 23). For viscerotropic viruses, such as DENV and YFV, other organs are considered for immunohistochemistry and RT-PCR testing, such as liver, kidney, lymph nodes, spleen, and heart (24, 25). Few pathology studies have been published for genera *Alphavirus* and *Bunyavirus*. Another method of detection is nested RT-PCR techniques in formalin-fixed, paraffin-embedded human tissue to detect viral genome in tissue specimens (26, 27).

Antigen Detection

Viral Antigen Enzyme-Linked Immunosorbent Assay

Viral antigen detection tests have been primarily developed for rapid virus identification field testing for arthropod specimens, in contrast to highly sensitive RT-PCR used primarily for testing of human specimens. Recently the nonstructural protein 1 (NS1) antigen test for DENV has been used for diagnosis from human serum specimens successfully, due to the microplate enzyme-linked immunosorbent assay (ELISA) format and ease of use compared to RT-PCR. The DENV NS1 ELISA can be implemented in many resource-poor countries for virus surveillance. The NS1 protein is a glycoprotein expressed by all flaviviruses and is essential for viral replication. During viral replication, NS1 is secreted as a hexamer composed of dimer subunits. NS1 produces a humoral response and is known as a complement-fixing antigen.

The sensitivity of the first DENV NS1 antigen-capture ELISA ranged from 1 to 4 ng/ml; however, commercial tests have sensitivities of 50 ng/ml of serum (28–30). Results from earlier studies suggested that NS1 levels in blood correlated with disease severity among DENV patients, although these results were not corroborated by later studies (29, 30). The NS1 ELISA has the capacity to distinguish between DENV serotypes (31). The NS1 antigen test is less sensitive in secondary DENV infections due to an immune complex formed from multiple DENV exposures. The NS1 test is commercially available for DENV, and many investigators have evaluated its sensitivity and specificity (32–35) (Table 2). Because of the relatively higher specificity of the NS1 test compared to the IgM test, it may also be useful in differentiating between flaviviruses. However, many of the viruses in the JE serocomplex group and other flaviviruses do not develop sufficiently high viral titers such as those observed in DENV infections; therefore, it is unlikely that the NS1 antigen detection test for these flaviviruses will be used for diagnostics. Other antigen ELISAs include RVFV and EEEV tests, but these tests have only been used for mosquito specimens.

Nucleic Acid Detection

There are various NAAT methods to detect arbovirus nucleic acid, including RT-PCR (standard and real time) and

TABLE 2 Commercial diagnostic tests available for arboviruses

Family	Virus	Name of test	Manufacturer	Country of origin	Type of test	Sensitivity[a] (%)	Specificity[a] (%)
Flavivirus	Dengue	Simplexa Dengue Kit	Focus Diagnostics	USA	Molecular		
		LightMix Kit Dengue Virus	Roche/TIB MOBIOL	Germany	Molecular		
		Truenat Dengue	Molbio Diagnostics PVT, Ltd.	India	Molecular		
		Sentosa SA Dengue I-IV RT-PCR Test	Vela Diagnostics	Singapore	Molecular		
		Dengue Virus II Real Time RT-PCR Kit CE Marked	Vacunek	Spain	Molecular		
		Geno-Sen's Dengue 1–4 Real Time PCR Kit	Genome Diagnostics Pvt, Ltd.	Netherlands	Molecular		
		Dengue Virus (Multiplex)	Mikrogen Diagnostik	Germany	Molecular		
		Dengue Virus	Genesis Diagnostics	United Kingdom	Molecular		
		abTES DEN 5 qPCR I Kits	AIT biotech	Singapore	Molecular		
		DiaPlexQ Dengue Virus Detection Kit	SolGent	Korea	Molecular		
		Dengue RNA Real Time PCR Kit	Co-Diagnostics	USA	Molecular		
		RealStar Dengue RT-PCR Kit	Altona Diagnostics	Germany	Molecular		
		Dengue Virus Real Time RT-PCR Kit	Centena Biomed	India	Molecular		
		Dengue Virus	TIB MolBiol	Germany	Molecular		
		PLATELIA Dengue NS1 Ag	BioRad	France	Antigen		
		Dengue NS1 Ag ELISA	Standard Diagnostics, Inc.	Korea	Antigen		
		Dengue Early ELISA	Panbio	Australia	Antigen		
		DENV Detect IgM MAC ELISA	InBios International, Inc.	USA	Antibody		
		Dengue Virus IgM Human ELISA Kit	Abcam	China	Antibody		
		Dengue Duo IgM IgG Capture ELISA	Iverness	Australia	Antibody	55–94	100
		AlphaWell Dengue IgM	Mikrogen Diagnostik	Germany	Antibody	90	97.6
		AlphaWell Dengue IgG	Mikrogen Diagnostik	Germany	Antibody	>90	93
		NovaLisa Dengue Virus IgM U Capture	NovaTec Immunodiagnostics GmbH	Germany	Antibody	>98	98
		NovaLisa Dengue IgG	NovaTec Immunodiagnostics GmbH	Germany	Antibody	>90	93
		NovaLisa Dengue IgM	NovaTec Immunodiagnostics GmbH	Germany	Antibody	90	98
		DENV JE MACE	Venture Technologies Sdn Bhd	Malaysia	Antibody		
		Focus DENV IgM Capture/IgG DxSelect	Focus Diagnostics	USA	Antibody		
		PATHOZYME-DENGUE IgM/IgG and IgM	Omega	United Kingdom	Antibody		
		ImmunoComb Dengue IgM & IgG BiSpot	Orgenics	Israel	Antibody		
		Dengue Virus IgM ELISA	Genway Biotech, Inc.	USA	Antibody	90	98
		Dengue Virus IgG ELISA	Genway Biotech, Inc.	USA	Antibody	>90	93
		ImmunoLISA Dengue IgM/IgG Capture	Orgenics	Israel	Antibody		
		ELISA IgM and IgG Formats	Panbio	Australia	Antibody		
		EUROIMMUN DENV Mikrotiter ELISA	Euroimmun	Germany	Antibody		
		Dengue IgM capture ELISA	Standard Diagnostics, Inc.	Korea	Antibody		
		Dengue virus IgM ELISA	IBL International	Germany	Antibody		
		Dengue virus IgG ELISA	IBL International	Germany	Antibody		
		Dengue virus ELISA kit (HRP)	Novus Biologicals	USA	Antibody		
		EUROIMMUN DEN 1–4 IIFT Biochip	Euroimmun	Germany	IFA-Antibody		
		Dengue Virus Type 2 Antibody Detection	Progen Biotechnik	Germany	IFA-Antibody		

(Continued on next page)

TABLE 2 Commercial diagnostic tests available for arboviruses *(Continued)*

Family	Virus	Name of test	Manufacturer	Country of origin	Type of test	Sensitivity[a] (%)	Specificity[a] (%)
	West Nile	Geno-Sen's West Nile Virus Real Time PCR kit	Genome Diagnostics Pvt, Ltd.	Netherlands	Molecular		
		RealStar WNV RT-PCR kit	Altona diagnostics	Germany	Molecular		
		Artus WNV LC RT-PCR Kit and TM kit	QIAGEN GmbH	Germany	Molecular		
		West Nile Virus	TIB MolBiol	Germany	Molecular		
		West Nile Virus Real Time RT-PCR kit	Centena Biomed	India	Molecular		
		West Nile Virus (multiplex)	Mikrogen Diagnostik	Germany	Molecular		
		Panbio WNV ELISA	Panbio	Australia	Antibody		
		West Nile Detect IgM Capture ELISA	InBios International, Inc.	USA	Antibody	95	95
		West Nile Virus DxSelect ELISA kit	Focus Diagnostics	USA	Antibody	100	100
		EUROIMMUN WNV Microtiter ELISA	Euroimmun	Germany	Antibody		
		Panbio WNV IFA Slides	Panbio	Australia	IFA-Antibody		
	St. Louis encephalitis	Arbovirus IFA IgM and IgG	Focus Diagnostics	USA	IFA-Antibody		
	Yellow fever	FTD Tropical Fever Africa (YFV multiplex)	Mikrogen Diagnostik	Germany	Molecular		
		IFA IgM and IgG	Euroimmun Biochip technology	Germany	IFA-Antibody		
	Japanese encephalitis	Japanese Encephalitis Virus Real Time RT-PCR kit	Vacunek	Spain	Molecular		
		Japanese Encephalitis Virus Real Time RT-PCR kit	Centena Biomed	India	Molecular		
		Geno-Sen's JEV Real Time PCR kit	Genome Diagnostics Pvt, Ltd.	Netherlands	Molecular		
		FTD Tropical Fever Asia (JEV multiplex)	Mikrogen Diagnostik	Germany	Molecular		
		JE Detect IgM MAC ELISA	InBios International, Inc.	USA	Antibody		
		Panbio Japanese Encephalitis - Dengue IgM	Panbio	Australia	Antibody		
		EUROIMMUN JEV IgG/IgM IIFT	Euroimmun	Germany	Antibody		
	Tick-borne encephalitis	SERION ELISA Classic TBE Virus IgG/IgM	Virion/Serion	Germany	Antibody		
		IMMUNOZYM FSME IgG All species	Progen Biotechnik	Germany	Antibody	95	98
		IMMUNOZYM FSME (TBE) IgM	Progen Biotechnik	Germany	Antibody	99	99
		EUROIMMUN TBEV Mikrotiter ELISA	Euroimmun	Germany	Antibody		
		EIA TBE Virus IgM / EIA TBEV IgG+avidity	TestLine Clinical Diagnostics	Czech Republic	Antibody		
		TBEV/FSME IgM ELISA	IBL International	Germany	Antibody		
		TBEV/FSME IgG ELISA	IBL International	Germany	Antibody		
		Virotech FSME IgG/IgM ELISA	Sekisui Virotech GmbH	Germany	Antibody		
		NovaLisa TBEV IgG	NovaTec Immunodiagnostics GmbH	Germany	Antibody	>98	>98
		NovaLisa TBEV IgM	NovaTec Immunodiagnostics GmbH	Germany	Antibody	>90	>95
		IBL TBE IgG ELISA and IgM ELISA	IBL International	Germany	Antibody		

Alphavirus	Chikungunya	RealStar Chikungunya RT-PCR kit	Altona Diagnostics	Germany	Molecular		
		Chik Virus	TIB MolBiol	Germany	Molecular		
		abTES CHIKU qPCR I Kits	AITbiotech	Singapore	Molecular		
		Sentosa CHIKV RT-PCR Test	Vela Diagnostics	Singapore	Molecular		
		Chikungunya Virus Real Time RT-PCR Kit	Centena Biomed	India	Molecular		
		Geno-Sen's Chikungunya Virus Real Time PCR Kit	Genome Diagnostics Pvt, Ltd.	Netherlands	Molecular		
		Chikungunya Virus (Multiplex)	Mikrogen Diagnostik	Germany	Molecular		
		Chikungunya IgG/IgM Human ELISA Kit	NovaTec Immundiagnostica GmbH	Germany	Antibody	>90	>90
		Chikungunya IgM ELISA	Standard Diagnostics, Inc.	Korea	Antibody	93.60	95.90
		Chikungunya Virus IgM Human ELISA Kit	Abcam	China	Antibody	>90	>90
		Chikungunya IgM ELISA Test Kit	Genomix Biotech	India	Antibody		
		Chikungunya Virus IgG Capture ELISA	Genway Biotech, Inc.	USA	Antibody	>90	>90
		Chikungunya IgM U-Capture ELISA	IBL International	Germany	Antibody		
		Chikungunya IgG Capture ELISA	IBL International	Germany	Antibody		
		Chikungunya Virus IgM U-Capture ELISA	Genway Biotech, Inc.	USA	Antibody	>90	>90
		EUROIMMUN CHIKV IgG/IgM IIFT	Euroimmun	Germany	IFA-Antibody		
	Ross River	Ross River TAB ELISA	IBL International	Germany	Antibody		
		Panbio Ross River Virus IgG/IgM	Panbio	Australia	Antibody		
		Arbovirus IFA IgM and IgG	Focus Diagnostics	USA	IFA-Antibody		
	Eastern encephalitis	Arbovirus IFA IgM and IgG	Focus Diagnostics	USA	IFA-Antibody		
	Western encephalitis	Arbovirus IFA IgM and IgG	Focus Diagnostics	USA	IFA-Antibody		
Bunyavirus	La Crosse	Arbovirus IFA IgM and IgG	Focus Diagnostics	USA	IFA-Antibody		
Phleboviruses	Toscana	TOSCANA VIRUS IgG/IgM	Diesse	Italy	Antibody		
		Toscana Virus (TOSV) Real Time RT-PCR kit	Vacunek	Spain	Molecular		
		EUROIMMUN Sandfly Viruses IIFT BIOCHIP	Euroimmun	Germany	IFA-Antibody		
	Naples	EUROIMMUN Sandfly viruses IIFT BIOCHIP	Euroimmun	Germany	IFA-Antibody		
	Rift Valley	Rift Valley Fever Virus PCR kit	Centena Biomed	India	Molecular		
		RVF Diagnostics Kits IgG, IgM, Antigen	BDLS Diagnostic Test Kits	United Kingdom	Antibody		
	Crimean-Congo hemorrhagic fever	CCHF IgM, Human	BDLS Diagnostic Test Kits	United Kingdom	Antibody		

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[a] As reported by manufacturer.

reverse transcription-loop mediated isothermal amplification (LAMP). The main advantages of NAATs are confirmation of the infecting virus and rapid turnaround time for results. The potential for laboratory contamination with target analyte or amplicons is the main disadvantage of these techniques. When laboratories are unable to provide separate rooms dedicated to template-free procedures and nucleic acid amplification procedures, there is a higher probability of contamination. Contamination by exogenous template provides false-positive results that may have a public health impact and possibly become life threatening to the patient.

RT-PCR

Currently the most routinely used technique is real-time RT-PCR that utilizes fluorescently labeled probes. This is due to its simplistic format (tubes are not opened after amplification, decreasing the risk of contamination), generally high sensitivity, multiplex capacity, and the ability to quantify the amount of virus present. Primers and fluorescent probes are designed to target the conserved regions of the genome for the most specific result. Some tests are designed to detect any virus within a particular genus, using consensus sequences (e.g., *Flavivirus*, *Alphavirus*, and *Bunyavirus*) referred to as "pan or universal" format (36, 37). These pan/universal primers are useful for emerging infectious disease detection where the pathogen is a suspected arbovirus. Once the genus of the unknown virus is determined, then specific primers for each species within that genus are used to identify and confirm the infecting pathogen. The fluorescence is measured and captured as cycle threshold values (C_T) that are often correlated to either genome equivalent copies or PFU determined with a standard curve.

Standard RT-PCR uses gel electrophoresis, in which the molecular size (kilobases) of DNA fragments generated from test samples is compared with that of amplified DNA fragments of known viral standards. This test cannot be used for nucleic acid quantification since gel electrophoresis uses ethidium bromide as a method of detection. The sensitivity of the test is dependent on ethidium bromide limit of detection (1 to 5 ng of nucleic acid).

LAMP

The loop-mediated isothermal amplification (LAMP) nucleic acid detection technique has been developed for various arboviruses. The assay requires only one standard temperature (64°C) and is considered a low-cost alternative to standard and real-time RT-PCR because the technique does not require thermal cycling. The reaction is a strand displacement reaction that uses four different primers that recognize six different regions of the target gene using *Bst* polymerase instead of *Taq* polymerase. The amplified product can be visually identified by either white precipitate or SYBR green yielding a green or yellow color change. This test has been developed for WNV, JEV, CHIKV, DENV, and RVFV (38–42).

Isolation Procedures

Isolation of arboviruses is generally performed for virus characterization studies and as a confirmatory test for RT-PCR positive specimens. Most arboviruses can be isolated from blood or tissue if the integrity of the specimen is maintained by appropriate storage at −80°C. These viruses require various levels of biological containment (biosafety level 2 [BSL2] or BSL3) and laboratories should consult the Biosafety and Microbial and Biomedical Laboratories (BMBL) manual for proper biosafety practice (http://www.cdc.gov/biosafety/publications/bmbl5/). The virus isolate can generate enough material for sequencing purposes to determine origin of the virus, and these viral isolates then become reference viruses for future characterization studies. Low-passage virus is preferred for phylogenetic analysis since these viruses mutate at a rate of 10^{-3} substitutions/site/year. Interestingly, arboviruses' mutation rates are 0.21 magnitudes lower on average and their rates of synonymous substitutions are lower than those of all other RNA viruses (43). Virus isolation can identify novel viruses especially when an RT-PCR positive sample cannot be categorized by a species-specific RT-PCR assay. For example, a positive specimen by panflavivirus NAAT that cannot then be amplified in a flavivirus species-specific RT-PCR can then be isolated and sequenced to determine whether the virus is a new species within this family/genus.

Arbovirus isolation is performed in either primary cell culture or cell culture lines. Primary cultures typically include primary duck, chicken embryo, monkey, and hamster kidney cells. The classic mammalian cell lines include Vero, LLCMK2, BHK-21, CER, SW13, and PK cells. The infecting virus causes characteristic cytopathic effects (CPE) (e.g., plaques, cell fusion, and/or syncytium formation). CPE patterns can assist in determining the genus and possibly the family of virus. Another viral amplification technique includes the intracerebral inoculation of specimen into 2- to 3-day old suckling mouse brain (SMB). If virus is present, the mice will develop paralysis and die within 2 weeks of inoculation. Occasionally viruses require multiple passages in SMB for neuroadaptation of the viral isolate. Other organ specimens should also be harvested from mice, including liver and kidney. For example, certain orthobunyaviruses replicate at a higher titer in liver compared to brain. Adaptation of the viral isolate in cell culture requires inoculation using infected homogenized infected tissue followed by multiple passages of the virus isolate in cell culture.

Many arboviruses, including DENV and Sindbis virus (SV), can also be amplified by direct injection into competent mosquitoes such as *Aedes*, *Culex*, and *Toxorhynchites* species (reviewed in reference 44), or in mosquito cell lines (C6/36 and AP61) (45, 46). Occasionally these viruses can cause persistent infections in mosquito cell lines with no apparent CPE. Careful quality control is required for maintenance of mosquito cell lines to monitor persistent infection by a virus contaminant.

When specimens are submitted to a reference laboratory for virus isolation, multiple cell lines or methods of virus isolation are used depending on where the specimen is obtained, based on common circulating viruses in the region. Typically, the specimen is first inoculated into cell culture using at least one mosquito cell line and various mammalian cell lines, particularly Vero and BHK21. The inoculated cell culture flask is monitored daily for CPE. To maintain virus infectivity, a neutral pH is maintained in the cell culture flasks throughout the virus growth procedure and checked daily. Acidic environments (pH <6) cause conformational changes in the virus structural proteins and render the virus noninfectious in cell culture. In the absence of CPE, a sample of the culture is tested for the presence of viral antigens using an immunofluorescent assay (IFA) that probes with cross-reactive monoclonal antibodies (mAbs) to identify viral family/genus. The virus isolate is usually stored in cell culture medium containing at least 10 to 20% fetal bovine serum and stored at −70°C for future characterization studies. Currently, inoculation in SMB is not permitted in many labs due to Institutional Animal Care and Use Committee (IACUC) rules that prohibit this method of virus amplification. For culture of human or animal serum, undiluted sera, and two additional sera dilutions of 10^{-1} and 10^{-2} (to avoid viral interference for high-titered virus specimens) are inoculated. If a tissue specimen is received, it is homogenized and diluted by weight to volume basis (10 to 20%).

IDENTIFICATION

Virus detected in cell culture is identified by IFA using virus-specific mAbs that target the structural gene products, because they are the most divergent yet conserved within species. Antigenic characterization using species-specific mAbs cannot differentiate between genotypes or lineages and is only useful for viral species-specific identification. Polyclonal antibodies are also used to determine the virus family followed by testing with species-specific mAbs. Some of the targeted mAbs cross-react with epitopes identified in all species within a family/genus of viruses. These broadly cross-reactive mAbs or polyclonal antibodies identify the family of virus, which can then be tested with specific mAbs that target epitopes within the structural proteins that are conserved between species. Some limitations of IFA are false-positive results due to background and nonspecific antibody reactivity. Additionally, IFA is a subjective test and highly vulnerable to operator error. Depending on the tissue specimen, the background from the fluorescently tagged antibody can vary significantly. Autofluorescence can occur in certain specimens with high lipid content or an abundance of chitin protein found in insects. Sequence analysis provides finer differentiation between viruses within families and within serocomplex groups that determines the origin and the molecular epidemiology of the virus. Phylogenetic analysis can assist in tracking the virus movement and transmission within a region or throughout the world by spatial and temporal analyses (reviewed in reference 47). This analysis can determine the introduction rate of specific viruses in a region and the rate of evolution. Arbovirus evolution is interdependent on the host and the vector; thus, phylogenetic analysis cannot predict the dominant evolutionary change for increased adaptation to either host or vector (reviewed in reference 1).

Novel methodology for virus identification, such as deep sequencing, ultra-deep sequencing, or amplicon sequencing (sequencing the same DNA target amplicons many times to find rare mutations), may enhance the identification of new species or families of viruses from tissue, arthropod, or blood. Sequencing provides the advantage of identifying new subtypes of existing viruses or new species in vectors or vertebrate host prior to their emergence in the human population. Viruses that may rapidly evolved to infect humans as their amplifying or dead-end incidental host can be rapidly identified for prevention and control measures. For example, Chiu et al. reconstructed a newly discovered virus in less than 24 hours, which was significantly faster than conventional sequencing techniques (48). These researchers identified the Lone Star virus in the genus *Bunyavirus*, carried by *Amblyomma americanum* tick and related to the Heartland virus, an illness recently reported among farmers in Missouri (http://www.cdc.gov/ncezid/dvbd/heartland/index.html.)

SEROLOGIC TESTS

Hemagglutination

The hemagglutination inhibition assay (HIA) is a classic test used for arbovirus diagnostics and was the basis for virus family classification prior to genome sequencing. The test was first described in 1958 by Clarke and Casals and later adapted to a microtiter plate format in 1980 (49). HIA is not antibody isotype specific; hence, it does not differentiate between IgM and IgG. The presence of anti-arbovirus host antibodies in serum specimens inhibits the agglutination of the goose erythrocytes as observed by a button-like appearance of the erythrocyte in the 96-well microtiter plate. Defining a positive HIA titer is influenced by (i) the titer of the positive control used in the test and (ii) whether the infection is a primary or secondary arbovirus infection within the same genus. The results are subjective and vary by the experience of the laboratory technician performing the test.

The main advantage of HIA is that it can be used for surveillance involving multiple hosts since the test is not species-specific. Public health laboratories located in regions where multiple arboviruses are cocirculating routinely use HIA for vertebrate sentinel surveillance. ELISAs have essentially replaced the HIA in human specimen testing due to the difficulty in standardizing HIA reagents and buffers and the availability of goose erythrocytes. Additionally, the interpretation of the results for HIA is subjective and cross-reactivity between viruses within the same group or subgroup yields nonspecific results.

Enzyme-Linked Immunosorbent Assay

IgM

One component of the acquired immune response following an arbovirus infection is the synthesis of IgM antibodies. IgM is detectable in the late acute and the convalescent phase of the infection and is an indication of an acute or recent arbovirus infection. During the acute phase of infection (the first 5 days post onset of symptoms), a relatively small percentage (~<30%) of patients have detectable IgM antibodies; however, this varies by patient and virus. The peak IgM antibody titer occurs in the convalescent phase of the infection (6 to 14 days post onset of fever) and ELISA testing during this period yields the most reliable results. Commercially manufactured ELISAs for some arboviruses are available with varied sensitivities and specificities (Table 2).

IgM antibody capture ELISA (MAC-ELISA) format is preferred by most diagnostic laboratories and commercial diagnostic kits. There are two main reasons for this widespread use. First, the MAC-ELISA is based on capturing IgM antibodies on a microtiter plate using an anti-human IgM antibody, thus minimizing the interference of the high-avidity IgG antibodies binding to the antigen. Second, because many antigens are unpurified heterogenous protein products and often are composed of a mixture of viral antigens, the MAC-ELISA removes nonspecific antibody binding, allowing for many different sources of antigen to be used. In MAC-ELISA format, the antigen can be used in excess in combination with broadly cross-reactive mAbs that are generally arbovirus group-specific. Another advantage of the MAC-ELISA format is that antigens can be interchanged to compare antibody response to many different arboviruses within the same family, mainly due to the use of broadly cross-reactive monoclonal antibodies conjugated to a detector molecule. The MAC-ELISA is a sensitive test; however, the specificity of the test varies and is dependent on other arboviruses circulating that can cause cross-reactivity in the test. For example, in countries where DENV and JEV cocirculate, there is a high degree of cross-reactivity in the IgM ELISA results that may require confirmation with a neutralization test.

The sensitivity and specificity of the MAC-ELISA are dependent on the source of the antigen, the detector antibody, the conjugated chromogen, serum condition (unacceptable conditions include hemolyzed, lipemic, or contaminated with bacteria), and the dilution of the specimen used for the test. The viral antigens used in the MAC-ELISA are produced by virus inoculation in SMB, virus inoculation in cell culture, and/or recombinant antigens produced in bacteria expression vectors. SMB-derived antigen provides the highest sensitivity in the MAC-ELISA because it is composed of a high-titer mixture of all viral antigens, yet it is the least specific due to its impurity. Conversely, recombinant antigens, developed from immunogenic structural gene products, can be less sensitive yet provide better test specificity. Recombinant antigens may maintain the native conformation or glycosylation of arbovirus structural proteins, which are essential for antibody binding. Those recombinant antigens that maintain the native form of the protein tend to provide the most sensitive and specific result.

IgM kinetics can vary by arbovirus family, genera, and species and by primary versus secondary infections. For example, secondary DENV infections (>1 DENV infection) had lower IgM titers compared with primary DENV infections (50). In these unique cases, IgG seroconversion is the serologic confirmatory test (51). In other atypical cases, the IgM response may persist for at least 3 months or as long as a year, as observed with WNV and JEV infections (52–54). IgM persistence observed with some arboviruses infections has prompted the development of IgG-antibody avidity tests to assess the infection status (acute, recent, or past) (55).

For encephalitic arboviruses, the detection of IgM antibodies in CSF differs from detection in serum. For JEV infections, anti-JEV IgM is detectable in serum approximately 9 to 13 days following onset of symptoms; however, in the CSF IgM is detectable as early as 1-day post onset of symptoms (56). When testing serum specimens with HIA, Burke et al. assessed that this test was inadequate to confirm 34% of the encephalitis cases as JEV infection when compared to MAC-ELISA (57). In addition, anti-JEV IgM antibodies can persist in the CSF for as long as a year, suggesting persistent JEV infection of the CNS (58).

IFA has also been used as an indirect IgM test to assess reactivity against arboviruses. Fixed arbovirus-infected cells are spotted onto a slide and patient serum specimen is added. Following several washes to remove excess antibodies, a fluorescently labeled anti-human IgM- or IgG-specific mAb is then added to the slide. The result is a subjective determination of fluorescence observed by microscopic analysis (Table 2).

Reference and public health diagnostic laboratories may use in-house developed IgM tests; however, many commercial IgM and rapid tests are available for some of the medically important arboviruses including WNV, JEV, YFV, and DENV (59–61) (Table 2). Some of these commercial tests have very good sensitivity and specificity when compared to gold standard or reference tests. Prior to implementing a commercial assay, each diagnostic laboratory should perform a comprehensive independent evaluation (62).

IgG

The presence of anti-arbovirus IgG antibodies is an indication of a long-term acquired immunity from past arbovirus infection. These IgG antibodies can be detected for up to 60 years after the initial infection, as determined for DENV (63). There are two primary methods to measure IgG, direct and indirect ELISA. Direct IgG ELISA is less sensitive than the indirect method and requires purified viral antigen. The indirect IgG ELISA is more sensitive and uses a capture antibody to immobilize the viral antigen to a solid surface. This capture step is especially important for unpurified antigens generated from virus infection of SMB, since this antigen is less immunogenic unless it has been captured and concentrated (64). The specimen is added to the captured viral antigen and then detected with a secondary anti-human IgG-enzyme conjugated antibody. The indirect IgG ELISA has essentially replaced HIA because of its ease of use and improved specificity. The IgG ELISA results are reported as qualitative, quantitative, or as end-point dilutions (51, 64). However, due to cross-reactivity within families and between species of viruses, the IgG ELISA lacks specificity and may require additional confirmatory testing such as a neutralization test for virus identification. The IgG ELISA will bind all available anti-arbovirus IgG (neutralizing or nonneutralizing antibodies); hence, the test tends to be more sensitive and less specific than the plaque reduction neutralization test (PRNT). Another test for IgG detection in serum specimens is the IFA as previously described (Table 2).

Some investigators have explored the use of other structural antigens besides the envelope protein to increase the specificity of the IgG ELISA for flaviviruses. Cardosa et al. demonstrated that the IgG response to premembrane (prM) protein has greater specificity when differentiating between DENV and JEV (65). Other researchers have improved the specificity in the IgG ELISA by using a recombinant polypeptide located in the N-terminal portion of the envelope protein (66). IgG avidity ELISAs have proven useful in differentiating primary versus secondary flavivirus infection (67, 68). The avidity assay uses stringent 3 to 6 M urea wash buffer in order to remove nonspecific IgG binding. This stringent wash step has been useful in differentiating acute from past arbovirus infections during unique circumstances in which the IgM response persists (55, 69).

Analyses of IgG by subclass have been studied as markers of disease severity (70–72). For JEV, subclass IgG1 is the most abundant antibody in the CSF following confirmed cases of clinical encephalitis. Thakare et al. found undetectable levels of IgG2 subclass in sera or CSF of JEV patients and postulated that the IgG1 subclass is more cytophilic than other IgG subclasses and may be more effective in viral clearance (70). For DENV patients, IgG1 and IgG4 subclasses are risk markers for the development of dengue hemorrhagic fever and dengue shock syndrome (the more severe presentation of the disease) (71, 72).

Neutralization

PRNT is the most specific serological tool for the determination of type-specific antibodies and is typically used to confirm the infecting arbovirus in convalescent-phase sera (73). This biological assay is the specific interaction of virus and antibody *in vitro*, resulting in the inactivation of the virus such that it is no longer able to infect and replicate in cell culture. PRNT results are presented as the endpoint titer of neutralizing antibodies from sera to a specific virus and may suggest the level of immune protection against the infecting virus. Two main uses of PRNT are (i) for vaccine studies to determine immune protection and (ii) for virus confirmation in IgM- or IgG-positive specimens.

PRNT is the confirmatory test used to assess the infecting virus when cross-reactivity is observed in IgM and IgG tests. Because PRNT requires from 4 to 10 days for a result (depending on the virus), it is not typically used for clinical diagnosis. Additionally, this test is not routinely performed in most diagnostic laboratories because it is expensive and requires a tissue culture facility and highly skilled technical staff. To obtain reliable and reproducible PRNT results, laboratories must standardize viral strains and cell lines. Unfortunately, there is no consensus of the percent of antibody-induced plaque reduction that determines a positive result in the test. Some laboratories set a positive test result at 70% reduction of the total input plaquing virus and others use 90% reduction. Although a 90% reduction in plaques provides higher specificity, it is often less sensitive. In vaccine studies, 50% reduction in plaques provides the sensitivity needed because of the relatively low neutralization titer produced following vaccination with attenuated strains of viruses in naive subjects. The challenge of PRNT is the standardization among all end users. As stated previously, three variables account for most of the inconsistent results observed between laboratories: interpretation of the results (PRNT 90%, 70%, 50% reduction), the cell lines used for plaquing the virus, and the laboratory viral strains. In an attempt to standardize the PRNT worldwide for DENV, subject matter experts from around the world published guidelines for PRNT standardization (74).

Antibody neutralization has been studied extensively in animal models, natural infections in humans, and *in vitro* cell culture experiments. Neutralizing antibodies are primarily directed against the structural proteins of the virus. The neutralization epitopes for genus *Flavivirus* are located throughout the E glycoprotein, including domain I, domain II, and the surface of domain III (75–77). For the *Alphavirus* genus, the neutralizing epitopes have been mapped primarily to the E2 envelope protein, which is also important for receptor binding and cell entry. Similar E2 epitope clustering in the glycoprotein has been identified for many of the members of the genus *Alphavirus* (e.g., VEE and SV) and Ross River virus (RRV) (78, 79). Lastly, for the genus *Bunyavirus*, the neutralizing epitopes are found within the G1 glycoprotein (80).

Viral neutralization requires that multiple antibodies occupy the surface of the virion to inhibit the viral infection. This is referred to as the multihit mechanism (81, 82). A homologous antibody response would be more effective in virus neutralization than a heterologous response. A combination of neutralizing, nonneutralizing, and subneutralizing antibodies has a synergistic effect. In other instances, heterologous antibodies are hypothesized to potentiate or enhance viral entry as observed with DENV and WNV (83–85).

Flaviviruses have both group-specific and type-specific antibody neutralization determinants. The type-specific determinants are cross-reactive when tested in heterologous reactions, causing the cross-reactivity observed in serological IgM and IgG tests. The neutralizing antibody titer required for protective immunity is still not well understood. Vaccine studies have provided information on protective antibody titers for flaviviruses, such as YFV, JEV, and DENV; however, many of these studies were from primary infections with attenuated vaccine strains.

Commercial Tests

Many commercial tests have been developed for the identification of arbovirus infections (Table 2). These tests include molecular tests, antibody tests, and IFA. The formats vary from standard microtiter plate tests to rapid diagnostic tests with variable sensitivity and specificity as evaluated by the manufacturer or by independent investigators. Advantages of commercial tests include (i) standardization of the ancillary reagents, which allows for reproducible results between testing laboratories, and (ii) production of tests under good manufacturing practice (GMP). Commercial tests may provide the end user with higher quality assurance often not associated with in-house developed tests. However, commercial assays can vary in quality (lot-to-lot variations) and in reliability (test availability). Companies on occasion discontinue manufacturing these tests when the market is not profitable, leaving the end user, in particular reference laboratories, with no other option than to maintain laboratory-developed tests (LDTs). Additionally, commercial tests may be cost-prohibitive in comparison to LDTs, resulting in a preference to use LDTs despite the fact that LDTs may not be as sensitive as their commercial counterparts. Other aspects of the tests unrelated to their performance that may determine their use in laboratories are technical time needed to perform the test, storage requirements, reagent stability, and ease of use.

Another factor to consider is commercial test quality. Each country has different regulatory agencies of manufacturing and distribution that determine test quality. U.S. commercial laboratories require Food and Drug Agency (FDA) approved tests for clinical diagnostics. In order to reduce duplicative efforts, some countries rely on the U.S. FDA regulatory approval process for the use of commercially available tests for clinical diagnosis. Other countries have developed their own internal assessment requirements. The risk of a false-positive or false-negative result is dependent on the disease and clinical treatment. There are limited available commercial tests for most arboviruses, with the exception of DENV, JEV, CHIKV, and WNV, and even fewer FDA approved tests (Table 2). Although many of these tests claim very good performance for sensitivity and specificity, independent evaluations are important corroboration of the company's results. Recent studies involving DENV diagnostics for both IgM tests and NS1 tests determined that the performance for some of the companies was below the acceptable range for use. This study also determined that tests that depend on subjective reading of results, such as rapid diagnostic tests, often have large variation due to reader-to-reader variation (59).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The testing algorithm for diagnosing acute arboviral infection is a matrix divided into acute and convalescent specimen collection (Fig. 1). The acute serum or CSF specimen (DPO, ≤5) is tested for the presence of virus by NAAT, virus isolation, or viral antigen tests, or for IgM in CSF for encephalitic viruses. The convalescent-phase serum or CSF specimen (DPO, >5 to <14) is tested for the presence of IgM and IgG antibodies. An acute arbovirus infection is defined as the detection of virus by isolation or NAAT or seroconversion by IgM and/or IgG. A single specimen with an IgM positive test result suggests a recent infection. Rare cases of viral RNA persistence have been reported, notably for WNV and CTFV. The detection of only IgG in a specimen is an indication of a past infection unless paired specimens indicate seroconversion. IgM seroconversion in paired specimens is defined as a negative test result in the acute-phase specimen followed by a positive test result in the convalescent-phase specimen. IgG seroconversion is the same as for IgM (negative in acute, positive in convalescent) or a 4-fold rise in IgG titer from the acute specimen to the convalescent specimen with a minimum of 5 to 7 days between specimens. Because of the high degree of cross-reactivity observed within arbovirus genera, serology results are generally

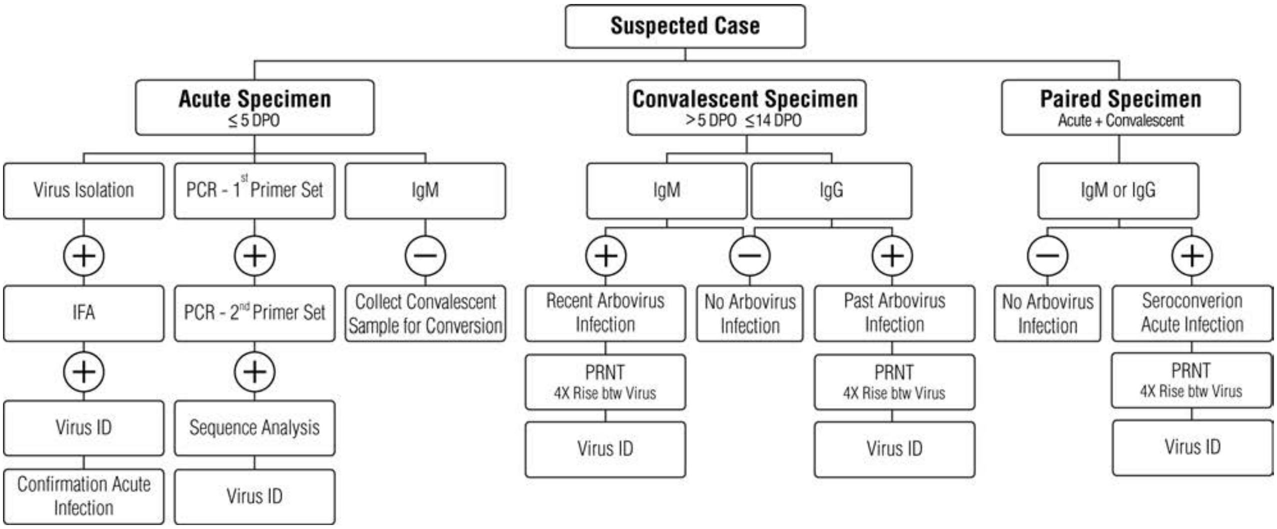
Suspected Case
Acute Specimen
≤ 5 DPO
Convalescent Specimen
> 5 DPO ≤ 14 DPO
Paired Specimen
Acute + Convalescent
Virus Isolation
PCR - 1st Primer Set
IgM
IgM
IgG
IgM or IgG
+
+
−
+
−
+
−
+
IFA
PCR - 2nd Primer Set
Collect Convalescent Sample for Conversion
Recent Arbovirus Infection
No Arbovirus Infection
Past Arbovirus Infection
No Arbovirus Infection
Seroconversion Acute Infection
+
+
PRNT
4X Rise btw Virus
PRNT
4X Rise btw Virus
PRNT
4X Rise btw Virus
Virus ID
Sequence Analysis
Virus ID
Virus ID
Virus ID
Confirmation Acute Infection
Virus ID

FIGURE 1 General laboratory testing algorithm for human serum specimens from suspected arbovirus infection. doi:10.1128/9781555817381.ch95.f1

confirmed with neutralization tests when definitive diagnosis is required. The testing algorithm for other specialized epidemiological studies, including seroincidence and/or serosurveys to measure the prevalence in the population, is different than that for defining acutely ill patients, and testing must be modified. Serosurveys and seroincidence studies assess the transmission of arboviruses and the prevalence within the population typically used for vaccine or public health intervention studies. Because these epidemiology studies rely on measuring acute, recent, and past infection, the testing algorithm and the interpretation of the test results differ and require other relevant study subject information, such as travel history, vaccination history, age, and other demographics, to accurately determine the prevalence of specific arbovirus infections within a particular population.

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Hantaviruses

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Hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS, also referred to as hantavirus cardiopulmonary syndrome, HCPS) are rodent-borne zoonoses caused by certain members of the virus family *Bunyaviridae*, genus *Hantavirus*. A hallmark of HFRS and HPS is a reversible increase in the permeability of small blood vessels. Diagnosis early in the course of disease is critical to the successful management of HFRS and HPS.

TAXONOMY

The International Committee on Taxonomy of Viruses currently recognizes 24 species in the genus *Hantavirus* (http://www.ictvonline.org/index.asp). Strains of 12 of these species and 11 other named hantaviruses have been causally associated with HFRS or HPS (Table 1).

Specific rodents (i.e., one or two closely related members of the order Rodentia) are the principal hosts of the hantaviruses known to cause human disease. Other natural hosts of hantaviruses include shrews (2) and moles (3), which are members of the order Soricomorpha. The geographical distribution of human disease caused by a particular hantavirus subsumes the geographical range of its principal rodent host(s) (Table 1).

The rodent-borne hantaviruses are divided into three groups based upon the taxonomic assignment of their principal host(s): family Muridae, subfamily Murinae (Old World rats and mice); family Cricetidae, subfamily Arvicolinae (voles and lemmings); and family Cricetidae, subfamilies Neotominae and Sigmodontinae (New World rats and mice). Murine rodents and voles are the principal hosts of the hantaviruses known to cause HFRS; neotomine rodents and sigmodontine rodents are the principal hosts of the hantaviruses known to cause HPS (Table 1). Many rodent-borne hantaviruses, particularly those associated with voles or lemmings, have not been associated with human disease. The degree of genetic and antigenic relatedness among rodent-borne hantaviruses typically correlates with the degree of (phylo)genetic relatedness among their respective principal hosts.

DESCRIPTION OF THE AGENTS

Hantavirus is pleomorphic, averages 100 nm in diameter, and possesses a lipid-bilayer envelope (1). The envelope displays a grid-like pattern that is apparent when negatively stained virions are viewed by electron microscopy (Fig. 1) (4). Protruding from the lipid envelope are spikes, approximately 6 nm in length, which are formed from the virus glycoproteins Gn and Gc (1). The virion interior contains ribonucleocapsids—segments of single-stranded genomic RNA complexed with nucleocapsid protein and L protein (an RNA-dependent RNA polymerase).

The genomes of hantaviruses consist of three unique negative-sense, single-stranded RNA molecules, designated L (large, approximately 6.5 to 6.6 kb), M (medium, 3.6 to 3.7 kb), and S (small, 1.7 to 2.1 kb) (1). These RNA molecules encode the L protein, the glycoprotein precursor (which is cotranslationally cleaved to yield the envelope glycoproteins Gn and Gc), and the nucleocapsid protein, respectively. The S segments of some hantaviruses appear to encode a functional nonstructural (NSs) protein (5). The ribonucleocapsids display helical symmetry and form circular structures as a result of base pairing by highly conserved, inverse complementary nucleotide sequences at the termini of each genomic segment (1).

EPIDEMIOLOGY AND TRANSMISSION

Hantaan virus, the prototypical member of the genus *Hantavirus*, is the cause of a severe form of HFRS in South Korea, China, and eastern Russia; Dobrava-Belgrade virus is an agent of a severe form of HFRS in the Balkans, Greece, and Russia; Seoul virus is an agent of a relatively mild form of HFRS in Asia, Europe, and the Americas (6); and Puumala virus is the agent of nephropathia epidemica, which is a relatively mild form of HFRS in Europe and western Russia. Other hantaviruses known to cause HFRS include Saaremaa virus in Europe (7) and Amur virus in eastern Russia (8). The case-fatality rate of HFRS ranges from 0.2% (Puumala virus) to 15% (Hantaan virus). Approximately 30,000 HFRS cases occur worldwide each year (9); the majority of these cases occur in Asia and are caused by Hantaan or Seoul virus.

The geographical distribution of HPS includes Canada, the contiguous United States, Panama, and eight South American countries (Table 1). Arguably, Sin Nombre virus and Andes virus are the major causes of HPS in North America and South America, respectively. From 1993 through 2009, 510 HPS cases in the United States were reported to the National Notifiable Diseases Surveillance

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TABLE 1 Hantaviral species associated with human disease[a]

Virus species	Natural rodent host(s)	Known geographical distribution	Human disease[b]
Principally associated with members of the rodent family Muridae, subfamily Murinae[c]			
Dobrava-Belgrade virus	*Apodemus flavicollis* (yellow-necked field mouse) and other *Apodemus* spp.	Balkans, Greece, Russia	HFRS
Hantaan virus	*Apodemus agrarius* (striped field mouse)	China, Korea, Russia	HFRS
Saaremaa virus	*Apodemus agrarius*	Europe	HFRS
Seoul virus	*Rattus norvegicus* (Norway rat) and *Rattus rattus* (black rat)	Worldwide	HFRS
Principally associated with the family Cricetidae, subfamily Arvicolinae[c]			
Puumala virus	*Myodes glareolus* (red bank vole) and other *Myodes* spp.	Scandinavia, western Europe	HFRS
Principally associated with the family Cricetidae, subfamily Neotominae or Sigmodontinae[c]			
Andes virus	*Oligoryzomys longicaudatus* (long-tailed pygmy rice rat)	Argentina, Chile	HPS
Bayou virus	*Oryzomys palustris* (marsh rice rat)	United States	HPS
Black Creek Canal virus	*Sigmodon hispidus* (hispid cotton rat)	United States	HPS
Laguna Negra virus	*Calomys laucha* (small vesper mouse) and *C. callosus* (large vesper mouse)	Argentina, Bolivia, Brazil, Paraguay	HPS
New York virus	*Peromyscus leucopus* (white-footed mouse)	United States	HPS
Rio Mamoré virus	*Oligoryzomys* spp. (rice rats)	French Guiana, Peru	HPS
Sin Nombre virus	*Peromyscus maniculatus* (deer mouse)	United States, Canada	HPS

[a] Other named hantaviruses that have been associated with human disease include Amur virus (eastern Russia, HFRS); Monongahela virus (United States, HPS); Choclo virus (Panama, HPS); Central Plata virus (Uruguay, HPS); Bermejo, Lechiguanas, and Oran viruses (Argentina, HPS); and Anajatuba, Araraquara, Castelo dos Sonhos, and Juquitiba viruses (Brazil, HPS). The Korean field mouse (*Apodemus peninsulae*) is the principal host of Amur virus; the white-footed mouse (*P. leucopus*) is the principal host of Monongahela virus; sigmodontine rodents are natural hosts of Anajatuba, Araraquara, Bermejo, Central Plata, Choclo, Juquitiba, Lechiguanas, and Oran viruses; and the natural host(s) of Castelo dos Sonhos virus are not known.

[b] HFRS, hemorrhagic fever with renal syndrome; HPS, hantavirus pulmonary syndrome.

[c] Murinae, Old World rats and mice; Arvicolinae, voles and lemmings; Neotominae and Sigmodontinae, New World rats and mice.

System or registered by the Centers for Disease Control and Prevention, and the majority of these cases were attributed to Sin Nombre virus (10). Similarly, more than 1,000 HPS cases were reported in Argentina from 1995 through 2008 (11) and the majority of these cases were attributed to Andes virus. Although there are far fewer HPS cases than HFRS cases each year, outbreaks of HPS usually are highly lethal, with case-fatality rates of 20 to 60%.

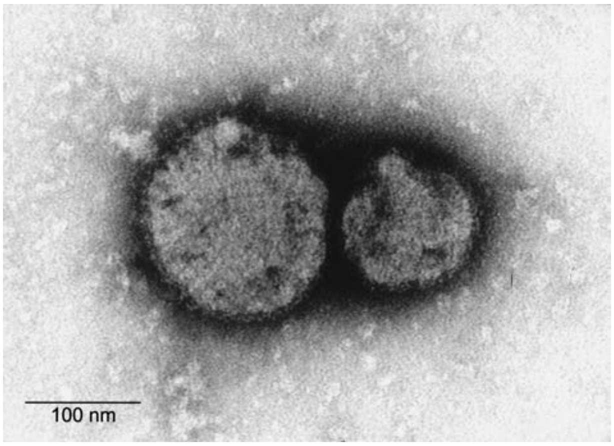

FIGURE 1 Electron micrograph of negatively stained Sin Nombre virus (2% phosphotungstic acid stain, pH 6.5). Courtesy of Charles Humphrey, Centers for Disease Control and Prevention. doi:10.1128/9781555817381.ch96.f1

Humans usually become infected with hantaviruses by inhalation of aerosolized droplets of urine, saliva, or respiratory secretions from infected rodents or by inhalation of aerosolized particles of feces, dust, or other organic matter contaminated with secretions or excretions from infected rodents. The aerosol transmission of hantaviruses from rodents to humans has been well documented (12, 13). Other means of infection include contamination of cutaneous injuries with infectious virus, contact of infectious materials with mucous membranes, ingestion of food contaminated with infectious rodent secretions or excretions, and—only in the case of Andes virus—contact with an HPS case during the acute phase of illness (14). Note that person-to-person transmission of hantavirus has never been documented in Europe, Asia, or North America.

In nature, the risk of infection in humans depends upon occupational or recreational activities, ecological factors that affect the abundance of infectious rodents, and other variables that influence the frequency and intensity of human exposure to infected rodents and their secretions or excretions. The cleaning of closed quarters occupied by infected rodents has been repeatedly associated with an increased risk of infection (15–17), and a recent outbreak of HPS among visitors to a national park in the western United States created far-reaching (i.e., global) challenges with regard to locating persons potentially exposed to hantavirus-infected rodents immediately before or during the outbreak (18).

CLINICAL SIGNIFICANCE

The clinical and pathological features of nephropathia epidemica, severe HFRS, and HPS were reviewed recently (19). Both syndromes are associated with acute thrombocytopenia and a reversible increase in microvascular (capillary) permeability. A major difference between the two syndromes is that the retroperitoneum is the major site of the vascular leak in HFRS, whereas the lungs and thoracic cavity are the major sites of the vascular leak in HPS.

The length of the incubation period in HFRS and HPS usually is 2 to 4 weeks but can range from a few days to 2 months. The clinical course of HFRS can be divided into five phases: prodrome (typically 3 to 7 days), hypotensive (several hours to 4 days), oliguric (4 to 5 days), diuretic (7 to 11 days), and convalescent (weeks to months). Similarly, the clinical course of HPS can be divided into four phases: prodrome (3 to 6 days), cardiopulmonary (7 to 10 days), diuretic (1 to 3 days), and convalescent (weeks to months). Death in HFRS is usually due to shock in the hypotensive or diuretic phase. Death in HPS is usually attributed to hypoxia (pulmonary edema) or shock in the cardiopulmonary phase.

In severe HFRS caused by Hantaan virus or Dobrava-Belgrade virus, the prodrome usually begins with an abrupt onset of high fever, chills, headache, blurred vision, malaise, and anorexia, then includes severe abdominal or lumbar pain, gastrointestinal symptoms, facial flushing, petechiae, and an erythematous rash or conjunctival hemorrhage. The hypotensive phase begins with a characteristic drop in platelet number followed by defervescence and abrupt onset of hypotension, which may progress to shock and more apparent hemorrhagic manifestations. Other abnormalities may include elevated serum levels of aspartate transaminase (20). In the oliguric phase, blood pressure returns to normal or becomes high, urinary output falls dramatically, concentrations of serum creatinine and blood urea nitrogen increase, and severe hemorrhage may occur. Spontaneous diuresis, with polyuria greater than 3 liters per day, heralds the onset of recovery. Distinct clinical phases are less obvious in HFRS caused by Seoul virus and nephropathia epidemica, and visible superficial hemorrhages usually do not occur in nephropathia epidemica (21). Pathological findings in HFRS at autopsy include effusions in body cavities, retroperitoneal edema, and enlarged, congested, hemorrhagic kidneys (22).

HPS was first recognized in 1993 as a highly fatal disease in the southwestern United States (23). The original description of HPS (24) subsequently was modified to include mild infections that do not result in radiographic evidence of pulmonary disease (25). It is now recognized that HPS sometimes includes renal impairment and, at least in South America, bleeding manifestations (26, 27). The prodrome in HPS is characterized by fever, myalgia, and malaise. Other symptoms that may occur during the prodrome include headache, dizziness, anorexia, abdominal pain, nausea, vomiting, and diarrhea. Nonproductive cough and tachypnea usually mark the onset of pulmonary edema. Fully developed HPS is characterized by rapidly progressive (time span of 4 to 24 hours) noncardiogenic pulmonary edema, hypoxemia, large volumes of pleural effusion, and cardiogenic shock. Hypotension and oliguria are the result of shock. Myocardial depression may occur and contribute to shock (28). The diuretic phase is characterized by rapid clearance of pulmonary edema and resolution of fever and shock. Hematologic abnormalities in HPS at hospitalization include thrombocytopenia, hemoconcentration, and the presence of large, reactive (immunoblastic) lymphocytes (29, 30). Other laboratory abnormalities may include elevated levels of hepatic enzymes, hypoalbuminemia, metabolic acidosis, and, in severe cases, lactic acidosis. The gross abnormalities in fatal HPS at autopsy include copious amounts of frothy fluid in bronchi and other airways; heavy, edematous lungs; and large volumes of pleural fluid (30, 31).

Therapy

Successful management of HFRS and HPS begins with prompt recognition of the disease and hospitalization of the patient. Shock in HFRS usually can be effectively managed by judicious administration of vasopressors and intravenous fluids. Intravenous ribavirin given within the first few days of the onset of clinical disease has been shown to significantly decrease morbidity and mortality in HFRS in the People's Republic of China (32). The oxygen status of an HPS patient should be closely monitored so that oxygen supplementation and mechanical ventilation can be provided if required. Transfer to a state-of-the-art critical care facility with the capacity for extracorporeal membrane oxygenation should be an early consideration since life-threatening pulmonary edema and cardiogenic shock can develop rapidly in HPS (28). Immune (neutralizing) serum may prove beneficial in the treatment of HPS because (i) survival in HPS cases has been positively correlated with neutralizing antibody titers at admission (33) and (ii) post-exposure passive antibody therapy has protected Andes virus-infected laboratory rodents against lethal HPS-like disease (34). The effect of ribavirin on the course and outcome of HPS has not yet been rigorously investigated (see "Antivirals" below).

Vaccines

There are no World Health Organization-approved hantavirus vaccines available; however, inactivated vaccines have been developed in Asia and used locally in South Korea and China for the protection of humans against HFRS (35). These vaccines were prepared from the brains of suckling rats or mice or from cell cultures infected with Hantaan virus or Seoul virus. Hantavax (GreenCross Vaccine Corp., Seoul, Korea) and other inactivated vaccines that have been tested in humans yielded only low levels of neutralizing antibodies at 1 year after the last vaccine dose, raising concern about the duration of protection afforded by these vaccines (36–38). Optimization of vaccination schedules and advances in adjuvant technology may increase the duration of immunity elicited by inactivated vaccines.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

The clinician should consult with local health authorities and national (or international) reference laboratories that maintain diagnostic capability for hantaviruses before shipping clinical specimens to a diagnostic laboratory.

Infectious hantavirus has been isolated from blood, serum, urine, and cerebrospinal fluid collected soon after the onset of clinical disease (39). Thus, blood, serum, urine, respiratory secretions, and other biological materials from HFRS and HPS patients, especially specimens collected during the acute phase of illness, should be considered potentially infectious to humans. Laboratorians should note that infections with cell culture-adapted Hantaan virus have occurred in individuals who performed centrifugation of concentrated virus (39) and that dried, cell culture-grown virus maintained at room temperature has been infectious for up to 2 days (40). The U.S. Department of Health and Human Services has recommended the following precautions for the handling of hantavirus clinical specimens: (i) sera from potential HFRS or HPS cases should be handled at biosafety level 2 (BSL-2), (ii) potentially infectious tissue specimens should be handled at BSL-2 using BSL-3 practices, (iii) all procedures that could result in splatter or aerosolization of human body fluids should be done inside a certified biological safety cabinet, and (iv) propagation of virus in cell culture and virus purification should be carried out in a BSL-3 facility using BSL-3 practices (40). Hantaviruses are thermolabile (41) and can be inactivated by acids, alcohols, bleach, paraformaldehyde, detergents that disrupt lipid membranes, many commercial disinfectants, and UV irradiation (7, 39, 42, 43).

Blood, serum, and plasma samples for serology may be stored at 4°C and shipped to the diagnostic laboratory on cold packs if there is no significant delay between collection and testing. Otherwise, these specimens should be stored at −20°C or colder and shipped on dry ice. Blood, blood clots, solid tissues, and other samples intended for RNA extraction or virus isolation should be stored continuously at −70°C or colder, subjected to as few freeze–thaw cycles as possible, and shipped on dry ice in order to preserve the integrity of the viral RNA and infectivity of the virus. Samples shipped by air should be packaged, documented, and shipped in accordance with International Air Transport Association Dangerous Goods Regulations (http://www.iata.org). In the United States, ground shipments must comply with regulations issued by the U.S. Department of Transportation (49 CFR parts 171 to 178).

DIRECT EXAMINATION

Laboratory assays that have been used for diagnosis of hantaviral infections in humans have not been standardized; as such, no hantavirus diagnostic assays have received approval from the U.S. Food and Drug Administration. Diagnostic test kits that are commercially produced in Europe are sold “for research use only” in the United States.

Microscopy

Direct electron microscopic examination of tissues is of limited diagnostic value but has been used to detect virions and viral replicative structures in autopsy samples. Electron microscopic examination of autopsy tissues from HFRS and HPS patients found that mature virions are infrequent in tissues and can be difficult to identify due to considerable polymorphism in size and shape. Structures determined to be hantaviral inclusion bodies were seen more often than intact virions (31, 44).

Antigen Detection

Immunohistochemistry has been used to detect hantaviral antigens in tissues from fatal HFRS cases, biopsy materials from nephropathia epidemica cases, and tissues from fatal HPS cases (Fig. 2) (31). Polyclonal antibodies (e.g., immune sera from humans, experimentally infected rabbits, experimentally or naturally infected rodents) and murine monoclonal antibodies have been used as primary antibodies in immunohistochemistry assays. Fatal HPS is associated with widespread distribution of hantaviral antigen in lung, liver, spleen, kidney, and heart tissues, with antigen primarily localized within endothelial cells of capillaries and other small blood vessels (31). Immunohistochemistry assays for diagnosis of hantaviral infections in humans are limited to the few institutions that have access to the appropriate primary antibodies and control (comparison) tissues.

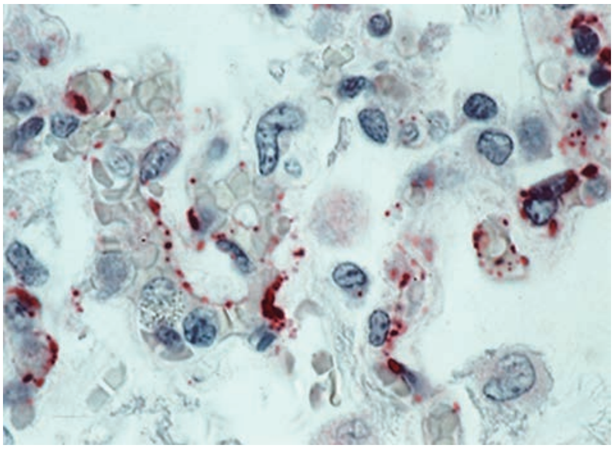

FIGURE 2 Photomicrograph of human lung tissue showing hantaviral antigen in pulmonary microvasculature, with hantaviral antigen stained in red by using immunohistochemistry. Courtesy of Sherif Zaki, Centers for Disease Control and Prevention. doi:10.1128/9781555817381.ch96.f2

Nucleic Acid Detection

Since the 1993 outbreak of HPS in the southwestern United States, reverse transcription PCR (RT-PCR) assays have been employed extensively to (i) detect hantaviral RNA in clinical samples from HPS and HFRS patients and (ii) obtain amplicons for viral characterization by DNA sequencing. Total RNA can be extracted from blood, plasma, serum, and solid tissues (e.g., kidney and lung), and can be protected from RNase degradation by using methods that employ chaotropic salts such as guanidinium isothiocyanate. Other specimens that may yield measurable levels of hantaviral RNA include urine and saliva. Sets of oligonucleotide primers have been designed to anneal to regions of the S and M genomic segments that are highly conserved among the hantaviruses (23, 45, 46). RT of hantaviral RNA and subsequent PCR amplification of the resulting cDNA usually can be accomplished by using a “one-step” single-tube format in which a single set of oligonucleotides is used to prime both enzymatic reactions. Clinical specimens rarely yield an RT-PCR product that can be visualized by UV transillumination of agarose gels stained with ethidium bromide or other DNA-binding dyes; consequently, a second (i.e., nested) PCR amplification usually must be performed to obtain amplicons that can be detected on gels and purified for sequencing. The lower limit of detection for nested RT-PCR assays for hantaviruses has been reported to be less than one viral fluorescent focus unit or (approximately) 316 median cell culture infectious doses of virus per 1.0 ml (47, 48). RT-PCR assays have been used to detect hantaviral RNA in fixed, paraffin-embedded tissues (49). Real-time RT-PCR assays have been developed for some hantaviruses and are being used increasingly for routine diagnosis (47, 50–54). These assays match or exceed the sensitivity of nested RT-PCR assays (with less manipulation of reaction components and shorter times to detection compared to conventional RT-PCR assays) and can provide precise estimates of viral load in clinical samples.

ISOLATION PROCEDURES

Isolation of infectious hantavirus from blood, serum, urine, or solid tissues using cultured cells or live laboratory animals is significantly less sensitive and requires considerably more time than for established RT-PCR assays for hantavirus-specific RNA. Thus, virus isolation is not commonly used for the diagnosis of hantaviral infections in humans.

The Vero E6 cell line (ATCC CRL-1586) has been used to isolate infectious hantaviruses from blood, solid tissue, and/or urine samples from HFRS patients and from serum and urine of HPS cases caused by Andes virus (55, 56). Typically, monolayer cultures of Vero E6 cells are inoculated with a crude or clarified tissue homogenate and then maintained under a fluid overlay for 10 to 14 days. Successful virus isolation may require repeated blind passages of inoculated cell culture material. Hantaviruses usually are neither cytopathic in cultured cells nor pathogenic in laboratory rodents; consequently, detection of infection in cultured cells and in tissues of laboratory rodents often requires an indirect method (e.g., fluorescent antibody test for viral antigen or RT-PCR assay for hantavirus-specific RNA).

IDENTIFICATION

Serologic Methods

Historically, serologic methods were used to define taxonomical relationships among the hantaviruses. Neutralization of infectivity *in vitro*, immunofluorescent antibody assays (IFA), enzyme-linked immunosorbent assays (ELISA), and a variety of other serologic methods have been used to characterize hantaviruses isolated from clinical samples and rodents. The antibodies used in these assays have included immune sera from HFRS or HPS patients, immune sera from experimentally or naturally infected rodents, and immune mouse ascitic fluids. Overall, serologic cross-reactivity within the genus *Hantavirus* is greatest between hantaviruses principally associated with phylogenetically closely related rodent species, and usually is higher in antigen-binding assays such as IFA and ELISA than in neutralization assays. Neutralization of infectivity has been measured by plaque reduction in monolayer cultures of Vero E6 cells maintained under an overlay containing agarose and by focus reduction in monolayer cultures of Vero E6 cells maintained under an overlay containing agarose (57) or methylcellulose (58). The focus-reduction neutralization test is used more widely than the plaque-reduction neutralization test because some hantaviral strains do not consistently produce readily discernible plaques in monolayer cultures of cells stained with neutral red. Foci of infected cells (viral antigen) in the focus-reduction neutralization test can be revealed by immunochemical staining (59) or chemiluminescence (60).

Genetic Methods

The use of genetic sequence data to define taxonomic relationships within the genus *Hantavirus* has become standard practice, in part because a majority of hantaviruses have never been adapted to growth in cultured cells and because our knowledge of the serologic relationships among some hantaviral species is based on one strain per species. Since an RT-PCR or nested PCR product can be amplified directly from RNA extracted from clinical materials and then sequenced, analysis of sequence data (particularly from the nucleocapsid protein gene or Gc region of the glycoprotein precursor gene) usually provides the most rapid and specific means for determination of the species identity of a hantavirus.

TYPING SYSTEMS

Analysis of nucleotide sequence data provides the fastest and most discriminating method for typing of hantaviruses. In pairwise comparisons, nonidentites among the complete glycoprotein precursor gene sequences and among the complete nucleocapsid protein gene sequences of strains of different hantaviral species ranged from 17.5% (*Saaremaa virus* [GenBank accession no. AJ009774] and *Dobrava-Belgrade virus* [AJ410616]) to 49.1% (*Bayou virus* [GQ244521] and *Thottapalayam virus* [DQ825771]) and from 19.3% (*Bayou virus* [GQ200820] and *Black Creek Canal virus* [L39949]) to 46.5% (*Seoul virus* [AY273791] and *Thottapalayam virus* [AY526097]), respectively (Maria N. B. Cajimat, unpublished data). An advantage of sequence data is that it is not affected by variation between lots of antibodies or lots of antigens used in serologic typing. Furthermore, analysis of nucleotide sequence data enables subtyping of hantaviruses, which often can identify geographic variants and variants associated with particular rodent-host subspecies or populations.

SEROLOGIC TESTS

Virtually all HFRS and HPS patients have high levels of anti-hantavirus immunoglobulin M (IgM) in serum or plasma at or soon after the onset of clinical disease (61–64). Many of these patients also have measurable levels of anti-hantavirus IgG during the acute phase of their illnesses (62, 65–68). The IgM and IgG responses are directed first against the nucleocapsid protein and then against the glycoproteins (62, 69–72). Anti-hantavirus IgM may persist as long as 6 months after the end of the acute phase of illness in nephropathia epidemica (67) and longer than 2 months after the end of the acute phase of illness in HPS (65). The level of anti-hantavirus IgG increases through the end of the acute phase of illness, remains high for months or years, and then declines gradually (72). Anti-hantavirus IgG may persist as long as 10 years in HFRS cases (72) and more than 3 years in HPS cases (33).

Neutralizing antibodies may appear during the acute phase of HFRS and HPS and are reactive against the glycoproteins Gn and Gc. A strong neutralizing antibody response early in the course of infection has been positively associated with successful recovery from HPS caused by Sin Nombre virus (58). High titers of neutralizing antibodies have been found 10 to 20 years after infection in nephropathia epidemica cases (73) and as long as 11 years after infection in HPS cases (74).

Antibodies (IgM and IgG) from patients infected with one hantavirus may cross-react with the nucleocapsid proteins or glycoproteins of other hantaviruses (66, 69, 75, 76), but IgG against Gn usually is more specific than anti-nucleocapsid protein IgG (62, 77). Neutralizing antibody, even in acute- or early convalescent-phase sera, can efficiently neutralize strains of several different hantaviral species and thus may not yield a species-specific diagnosis (78).

A variety of methods have been used to detect antibodies against hantaviruses in serum or plasma. These methods include high-density particle agglutination, IFA, immunoprecipitation, radioimmunoassay, hemagglutination inhibition, plaque- and focus-reduction neutralization, Western (immuno)blotting, IgM-capture (μ-capture) ELISA, and IgG ELISA. The most widely accessible and perhaps the best serologic method for diagnosis of HFRS and HPS in the face of disease is the IgM-capture ELISA, which is done by many national public health laboratories, some state and regional public health laboratories, and some commercial reference laboratories. Note that ELISA for anti-hantavirus IgM, which employs hantaviral antigen bound to a solid substrate, appears to be significantly less accurate (i.e., less specific, with a higher rate of false-positive results) than IgM-capture ELISA (79).

The IgM-capture ELISA typically uses a lysate of hantavirus-infected cells for the test antigen and should include an uninfected cell lysate for the control (comparison) antigen and appropriate positive and negative control sera (63, 64, 80, 81). The IgM-capture ELISA can be highly sensitive for detection of anti-hantavirus IgM but may not be specific for the virus that was used to prepare the test antigen. For example, an IgM-capture assay for detection of antibody to a Sin Nombre virus antigen has detected IgM to all known agents of HPS in the Americas (63, 69, 82–84).

The antigens used in serologic assays for anti-hantavirus antibodies traditionally were prepared from cultures of virus-infected cells, concentrated virus, or tissues of naturally or experimentally infected rodents. Antigens must be rendered noninfectious prior to use. Inactivation of hantavirus usually is accomplished by gamma irradiation (^{60}Co source). Heat treatment is an alternative method for making hantavirus antigens noninfectious prior to use in serologic assays (41).

Recombinant nucleocapsid proteins or glycoproteins expressed in bacteria, yeasts, insect cells, or mammalian cells, as well as synthetic peptides, have been used as antigens in IFA and ELISA for the detection of anti-hantavirus antibodies. The recombinant protein and synthetic peptide antigens can be more specific than native viral proteins in distinguishing between etiological agents of HFRS or HPS.

ANTIVIRAL SUSCEPTIBILITIES

Currently, there are no antiviral compounds approved by the U.S. Food and Drug Administration for the prevention or treatment of HFRS or HPS. However, ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) has been shown to have a significant therapeutic effect on the severity of HFRS in clinical studies done in South Korea and the People's Republic of China (32, 85), and protected ANDV-infected laboratory rodents against lethal HPS-like disease when treatment was initiated early in the course of infection (86). In contrast, an open-label study done in the United States failed to demonstrate an appreciable effect of intravenous ribavirin therapy on the lethality of HPS (87).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Early diagnosis is critical to the successful management of HFRS and HPS and, at least in the case of HPS caused by Andes virus, to implementation of appropriate isolation procedures to prevent virus transmission to health care providers and other persons. Diagnosis early in the course of disease is difficult because the hantaviral prodrome is similar to the prodrome of many other diseases. Fever and severe myalgia are prominent during the prodrome in HFRS and HPS. These symptoms, especially in patients who develop thrombocytopenia, should lead health care providers in regions of endemicity to suspect hantaviral disease.

The laboratory criteria for diagnosis of HPS established by the Centers for Disease Control and Prevention (see http://www.cdc.gov/hantavirus/health-care-workers/hps-case-definition.html) are as follows:

- Presence of hantavirus-specific IgM in persons who meet the case definition for HPS (see http://www.cdc.gov/hantavirus/health-care-workers/hps-case-definition.html) or a 4-fold or greater increase in titers of hantavirus-specific IgG in paired acute- and convalescent-phase serum samples
- Positive RT-PCR assay for hantavirus-specific RNA in plasma or other types of biological specimens
- Positive immunohistochemistry assay for hantaviral antigen in lung, spleen, kidney, or other solid tissues

These criteria could be used for laboratory diagnosis of HFRS as well.

As indicated previously, many HFRS cases and HPS cases have measurable levels of anti-hantavirus IgG during the acute phase of illness (62, 65–68). As such, confirmatory tests (i.e., assays for hantaviral RNA) should be done on diagnostic specimens from cases that are positive for anti-hantaviral IgM and negative for anti-hantaviral IgG.

The extreme sensitivity of RT-PCR assays, especially those employing nested PCR or real-time PCR, predisposes these tests to generating false-positive results as a consequence of template contamination. Accordingly, nucleic acid detection assays should include the proper negative controls and be supported by the results of tests for hantavirus-specific antibodies or antigen. Also, the proper controls for IgM-capture ELISA and IgG ELISA (e.g., sera from laboratory-confirmed HPS cases) and immunohistochemistry assays are essential for correct interpretation of the results of these assays. Most local laboratories do not have direct access to these materials. Thus, diagnostic testing is often limited to federal laboratories, research institutions that have a specific interest in HFRS or HPS, and a small number of commercial laboratories.

As noted previously, testing procedures for the detection of hantaviral infections in humans have not been standardized. Thus, confidence in the results reported from individual laboratories varies substantially according to the tests used and the rigor of the standards for diagnosis. In an external quality control study for serological diagnosis of hantavirus infections involving 18 laboratories in Europe and Canada, only 53 and 76% of IgM- and IgG-positive samples, respectively, were diagnosed correctly (88).

The requirement for reporting HFRS and HPS varies among local health agencies and from country to country. In the United States, reporting of HPS cases is mandated at the state level, and HPS is listed as a notifiable disease in the National Notifiable Diseases Surveillance System maintained by the Centers for Disease Control and Prevention, although reporting is not compulsory. Confirmatory laboratory tests (i.e., μ-capture ELISA for anti-hantavirus IgM, ELISA for anti-hantavirus IgG, and assays for hantavirus-specific RNA) at the Centers for Disease Control and Prevention typically are completed 24 to 48 hours after delivery of diagnostic specimens to the Viral Special Pathogens Branch (Centers for Disease Control and Prevention, 1600 Clifton Road, Atlanta, GA 30333; phone, [404] 639-1115; fax [404] 639-1118) (Pierre E. Rollin, personal communication). The results of these assays likely enhance the accuracy of the national surveillance system. Undoubtedly, the results of confirmatory tests on diagnostic specimens from all persons in the United States who are diagnosed with HPS would improve the epidemiological value of the national surveillance system.

The findings and conclusions in this chapter are those of the authors and do not necessarily represent the views of the CDC. The use of product names in this manuscript does not imply their endorsement by the U.S. Department of Health and Human Services.

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Arenaviruses and Filoviruses

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This chapter focuses on the viral hemorrhagic fever (VHF) viruses from two taxa, the families *Arenaviridae* (1) and *Filoviridae* (2). Some, but not all, members of these virus families cause severe, frequently fatal VHF in localized areas of the world. Although exotic in North America, these viruses are important public health problems in Africa and South America and have the potential of being introduced into the United States by travelers returning from these areas. The arenaviruses and, particularly, the filoviruses have been associated with serious nosocomial outbreaks involving health care workers and laboratory personnel. However, more recent outbreaks have generally been in settings where infection control has been lax. The lack of nosocomial transmission associated with Lassa or Marburg HF cases imported into the United States or Europe would suggest that the implementation of standard infection control practices are sufficient to prevent virus spread within the medical care setting. During the ongoing West African Ebola outbreak, several infected health care workers have been evacuated to the United States and Europe. The U.S. guidelines for patient evaluation and management, sample collection, shipping and processing have been updated (http://www.cdc.gov/vhf/ebola/index.html). Unfortunately, such practices are not always possible in resource-poor regions within Africa or South America (http://www.cdc.gov/vhf/ebola/hcp/international/managing-patient-flow.html).

Continuing detection and isolation of new arenavirus and filovirus species from new geographic locations remain important for public health. The similarity of initial isolation, clinical management, and viral diagnostic procedures for patients with suspected arenavirus or filovirus infections is the rationale for grouping these taxonomically distinct viruses in this chapter. Viruses from other taxonomic families, including *Bunyaviridae* and *Flaviviridae* (chapters 95 and 96), have also been associated with VHF (3).

TAXONOMY AND DESCRIPTION OF AGENTS

Arenaviridae

The family *Arenaviridae* comprises 43 named viruses which have unique morphologic and physiochemical characteristics. Antigenic relationships are established mainly on the basis of broadly reactive antibody binding assays, historically the complement fixation test (4), the indirect fluorescent-antibody (IFA) test (5), and, more recently, the enzyme-linked immunosorbent assay (ELISA). Both serologic and phylogenetic analyses of the viruses divide the arenaviruses into two complexes. The lymphocytic choriomeningitis (LCM) or Old World complex contains LCM virus and the Lassa viruses, including a number of apparently benign Lassa-like but unique viruses, Mopeia virus from Mozambique and Zimbabwe and Mobala and Ippy viruses from the Central African Republic. All have been isolated from rodents of the family *Muridae*. More recently, new arenaviruses have been isolated, or identified through molecular detection and sequencing, from rodents, for example, Merino Walk virus (6), Menekre and Gbagroube viruses (7), Kodoko virus (8), Morogoro virus (9), Lemniscomys and Mus minutoides viruses (10), and Luna virus (11), or from humans, in the case of Lujo virus from an outbreak of human fatal HF with person-to-person transmission (12, 13). The Tacaribe or New World complex includes Tacaribe, Junin, Machupo, Amapari, Cupixi, Parana, Latino, Pichinde, Tamiami, Flexal, Guanarito, Sabia, Oliveros, Whitewater Arroyo, Pirital, and Bear Canyon viruses (14); Ocozocoautla de Espinosa virus (15); Allpahuayo virus (16); Tonto Creek and Big Brushy Tank viruses (17); Real de Catorce virus (18); Catarina virus (19); Pampa virus (20); Skinner Tank virus (21); and Chapare virus (22). Some of the viruses have not yet been isolated and are known only from molecular sequence data. All the rodent isolates of New World complex viruses have been from rodents of the family *Muridae* subfamily *Sigmodontinae*, and Tacaribe virus was originally isolated from bats. Highly divergent arenaviruses have recently been identified in boas and boid snakes with snake inclusion body disease (23, 24). The Old and New World arenavirus complexes are distantly related; only when very-high-titer antisera are used can cross-reactions be observed. Monoclonal antibodies with specificities for structural proteins of arenaviruses suggest that the N protein is the group-reactive determinant, whereas the envelope glycoproteins (G1 and G2) are responsible for type specificity (1, 25).

The morphology of arenaviruses is distinctive in thin-section electron microscopy (26) and was the basis for first associating LCM virus with Machupo virus and ultimately associating these viruses with all the viruses in the present family. Three major virion structural proteins are usually found (25). The virus glycoprotein precursor (GPC) is processed

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into two glycoproteins, G1 (50,000 to 72,000 Da) and G2 (31,000 to 41,000 Da), which constitute the virion envelope and spikes and which both serve as highly type-specific neutralization targets. In addition, a stable signal peptide is retained as an essential subunit in the mature glycoprotein complex (27). The third major protein is the N protein (63,000 to 72,000 Da), which is clearly associated with the virion RNA and is considered the nucleocapsid protein. Four RNA species can be isolated from intact arenavirus virions. Two are virus specific and are ambisense in coding strategy: the small (S) RNA (22S) (encoding the N and GPC), and the large (L) RNA (31S) (encoding the viral L polymerase and the Z protein, a regulatory element) (1). In addition, ribosomal 28S and 18S species are isolated in virions in different proportions depending on external conditions.

Arenaviruses mature by budding at the cytoplasmic membrane, and host proteins are incorporated into the virion envelope. Vero cells infected with each of the viruses contain distinctive intracytoplasmic inclusion bodies, immunoreactive with anti-N but not anti-G1 or anti-G2 antibody. All arenaviruses are readily inactivated by ethyl ether, chloroform, sodium deoxycholate, and acidic media (pH less than 5). β-Propiolactone (28) and gamma irradiation (29) are both reported to inactivate arenavirus infectivity while preserving reactivity in standard serologic tests.

Filoviridae

The filoviruses, including both Ebola viruses and Marburg viruses, have a common morphology and similar genomic organizations and complements of structural proteins (30). Marburg virus and Ebola virus virion RNAs are nonsegmented, negative-sense, and single-stranded RNAs, 19.1 kb long, and 4.0×10^6 to 4.5×10^6 Da. The viral genomes are linearly arranged in a manner consistent with other nonsegmented, negative-sense, and single-stranded RNA viruses. Some sequence similarity to the paramyxoviruses, especially in the nucleocapsid and polymerase proteins, was noted. However, comparison with other filovirus protein sequences confirms that filoviruses are highly distinct. Furthermore, filoviruses are sufficiently distinct by ultrastructural and serologic criteria to warrant separate taxonomic status as members of the family *Filoviridae* (2, 31, 32).

Marburg viruses and Ebola viruses have at least seven virus-specific structural proteins, expressed from seven genes (2). For Ebola viruses, the ribonucleoprotein complex contains L (180 kDa), N (104 kDa), VP30 (30 kDa), and VP35 (35 kDa) in loose association. L is an RNA-dependent RNA polymerase, and VP35 may play a role similar to that of the P protein of paramyxoviruses and rhabdoviruses. GP (125 kDa) is the major spike protein; VP40 (a matrix protein) plus VP24 make up the remaining protein content of the multilayered envelope (31, 33). The GPs of Ebola viruses and Marburg viruses can be differentiated by the presence or absence of N- and O-linked glycans and by the lack, in Marburg viruses, of the second open reading frame coding for the small soluble glycoprotein expressed during Ebola virus infection *in vitro* and *in vivo* (34). When grown in Vero (or Vero E6) or MA-104 cells, the GP of Marburg viruses is totally devoid of terminal sialic acid, whereas that of Ebola viruses has abundant (2–3)-linked sialic acid. Phylogenetic analysis of GP genes from filoviruses clearly separates these viruses into 2 genera, *Ebolavirus* and *Marburgvirus*. The *Ebolavirus* genus contains five species: *Zaire ebolavirus*, *Sudan ebolavirus*, *Reston ebolavirus*, *Taï Forest ebolavirus* (formerly known as *Ivory Coast ebolavirus*), and *Bundibugyo ebolavirus* (35), each containing one virus (Ebola virus, Sudan virus [SUDV], Reston virus [RESTV], Taï Forest virus, and Bundibugyo virus [BDBV], respectively). The *Marburgvirus* genus has just a single virus species, *Marburg marburgvirus*, which contains two viruses, Marburg virus and Ravn virus (36).

Despite their unusual long rod-like morphologic properties, filoviruses resemble the other lipid-enveloped viruses, including the arenaviruses, in being susceptible to heat, lipid solvents, β-propiolactone (28), formaldehyde, UV light, and gamma radiation (29). These viruses are stable at room temperature for several hours but are inactivated by incubation at 60°C for 1 h.

EPIDEMIOLOGY AND TRANSMISSION

Arenaviridae

Arenaviruses are maintained in nature by association with specific mammalian hosts (Table 1), in which they produce chronic viremia and/or viruria. The viruses are routinely isolated from blood and urine samples of their specific rodent host. Naturally occurring human disease can usually be traced to direct or indirect contact with infected rodents. Aerosol infectivity is thought to be an important natural route of infection as well. Attempts to implicate arthropod vectors have been unsuccessful, but ectoparasites taken from viremic mammalian hosts have occasionally yielded arenavirus isolates. Of the 39 named mammalian members of the family *Arenaviridae*, 9 are known to be human pathogens. Nosocomial transmission is well described for Lassa and Machupo viruses. Lujo virus was identified during a nosocomial outbreak involving five persons, four of whom died (12). Because of the high level of virus circulation in West Africa (mostly Nigeria, Sierra Leone, Guinea, and Liberia), Lassa fever is the VHF most frequently exported to areas where VHF is not endemic, including several instances in the United States.

Filoviridae

Marburg and Ebola HFs are caused by taxonomically distinct viruses which form two genera, *Ebolavirus* and *Marburgvirus*, within the family *Filoviridae* (2). Marburg virus was first recognized in 1967, when 31 persons in Germany and Yugoslavia became infected following contact with monkey kidneys, primary tissue cultures derived from monkeys imported from Uganda, or sick patients (37). Ebola viruses first emerged in two major disease outbreaks, which occurred almost simultaneously in Zaire and Sudan in 1976. Over 500 cases were reported, with case-fatalities of 88% in Zaire and 53% in Sudan. Despite their simultaneous emergence, these viruses were shown to be two distinct viruses (now named Ebola virus and SUDV) representing two different species, *Zaire ebolavirus* and *Sudan ebolavirus*, based on serologic and sequence analysis criteria (38). In 1989 and 1990, a new distinct species of Ebola virus, RESTV, was isolated from cynomolgus monkeys being held in quarantine in Reston, VA, and Perkasie, PA, following their importation from the Philippines (39). RESTV reappeared in monkeys exported from the Philippines to Siena, Italy, in 1992 (40, 41) and to Alice, TX, in 1996 (42). In 1994, another genetically distinct species of Ebola virus, Taï Forest virus, was associated with disease in a human and deaths among chimpanzees in the Ivory Coast (Côte d'Ivoire) (43). In 1995, Ebola virus reappeared in Zaire, and subsequently (1996 and 2001 to 2005), a number of outbreaks have occurred in Gabon and adjacent areas of the Republic of Congo among apes and humans. Some human cases were associated

TABLE 1 Currently recognized arenaviruses and filoviruses and associated human diseases

Virus, date isolated	Natural host	Geographic distribution	Naturally occurring human disease	Human laboratory infections
Old World arenaviruses				
LCM, 1933	*Mus musculus* (house mouse)	Americas, Europe	Undifferentiated febrile illness, aseptic meningitis; rarely serious	Common; usually mild, but 5 were fatal
Lassa, 1969	*Mastomys* sp. (multimammate rat)	West Africa, imported cases in Europe, Japan, USA	Lassa fever; mild to severe and fatal disease	Common; often severe
Mopeia, 1977	*Mastomys natalensis* (multimammate rat)	Mozambique, Zimbabwe, Tanzania	Unknown	None; little experience
Mobala, 1983	*Praomys* sp. (soft-furred mouse)	Central African Republic	Unknown	None; little experience
Ippy, 1984	*Arvicanthis* sp. (grass rats)	Central African Republic	Unknown	None
Merino Walk, 1985	*Myotomys unisulcatus* (Busk Karoo rat)	Republic of South Africa	Unknown	None
Menekre, 2005 (only sequence available)	*Hylomyscus* sp. (African wood mouse)	Ivory Coast	Unknown	None
Gbagroube, 2005 (only sequence available)	*Mus* (*Nannomys*) *setulosus* (African pigmy mouse)	Ivory Coast	Unknown	None
Morogoro, 2007 (only sequence available)	*Mastomys natalensis* (multimammate rat)	Tanzania	Unknown	None
Kodoko, 2007	*Mus* (*Nannomys*) *minutoides* (savannah pygmy mouse)	Guinea	Unknown	None
Lujo, 2008	Unknown	Zambia, Republic of South Africa	Fatal HF	None
Lemniscomys, 2008 (only sequence available)	*Lemniscomys rosalia* (multimammate rat)	Tanzania	Unknown	None
Mus minutoides, 2008 (only sequence available)	*Mus minutoides* (savannah pygmy mouse)	Tanzania	Unknown	None
Luna, 2009	*Mastomys natalensis* (multimammate rat)	Zambia	Unknown	None
New World arenaviruses				
Tacaribe, 1956	*Artibeus* sp. bats	Trinidad, West Indies	Unknown	One suspected; moderately symptomatic
Junin, 1958	*Calomys musculinus* (drylands vesper mouse)	Argentina	AHF	Common; often severe
Machupo, 1963	*Calomys callosus* (large vesper mouse)	Bolivia	BoHF	Common; often severe
Cupixi, 1965	*Oryzomys gaeldi* (rice rat)	Brazil	Unknown	None detected
Amapari, 1965	*Neacomys guianae* (Guiana bristly mouse)	Brazil	Unknown	None detected
Parana, 1970	*Oryzomys buccinatus* (Paraguayan rice rat)	Paraguay	Unknown	None detected
Tamiami, 1970	*Sigmodon hispidus* (hispid cotton rat)	USA: Florida	Antibodies detected	None detected
Pichinde, 1971	*Oryzomys albigularis* (Tomes's rice rat)	Colombia	Unknown	Occasional; mild to asymptomatic
Latino, 1973	*Calomys callosus* (large vesper mouse)	Bolivia	Unknown	None detected
Flexal, 1977	*Oryzomys* spp. (rice rats)	Brazil	None detected	One recognized (severe)
Guanarito, 1989	*Zygodontomys brevicauda* (short-tailed cane mouse)	Venezuela	Venezuelan HD	None detected
Sabia, 1993	Unknown	Brazil	VHF	Two recognized (both severe)
Oliveros, 1996	*Bolomys obscuris* (Dark bolo mouse)	Argentina	Unknown	None detected

(Continued on next page)

TABLE 1 Currently recognized arenaviruses and filoviruses and associated human diseases *(Continued)*

Virus, date isolated	Natural host	Geographic distribution	Naturally occurring human disease	Human laboratory infections
New World arenaviruses *(Continued)*				
Whitewater Arroyo, 1997	*Neotoma* spp. (wood rats)	USA: New Mexico, Oklahoma, Utah, California, Colorado	Unknown	None detected
Pirital, 1997	*Sigmodon alstoni* (Alston's cotton rat)	Venezuela	Unknown	None detected
Pampa, 1997	*Bolomys* sp.	Argentina	Unknown	None detected
Bear Canyon, 1998	*Peromyscus californicus* (California mouse), *Neotoma macrotis* (large-eared woodrat)	USA: California	Unknown	None detected
Ocozocoautla de Espinosa, 2000 (only sequence available)	*Peromyscus mexicanus* (Mexican deer mouse)	Mexico	Unknown	None detected
Allpahuayo, 2001	*Aecomys bicolor* (bicolored arboreal rice rat), *Aecomys paricola*	Peru	Unknown	None detected
Tonto Creek, 2001	*Neotoma albigula* (white-throated woodrat)	USA: Arizona	Unknown	None detected
Big Brushy Tank, 2002	*Neotoma albigula* (white-throated woodrat)	USA: Arizona	Unknown	None detected
Real de Catorce, 2005 (only sequence available)	*Neotoma leucodon* (white-toothed woodrat)	Mexico	Unknown	None detected
Catarina, 2007	*Neotoma micropus* (southern plains woodrat)	USA: Texas	Unknown	None detected
Skinner Tank, 2008	*Neotoma mexicana* (Mexican woodrat)	USA: Arizona	Unknown	None detected
Chapare, 2008	Unknown	Bolivia	Single fatal HF case	None detected
Unclassified arenaviruses				
Golden Gate, 2009	*Boa constrictor* (boa constrictor)	USA: California	Unknown	None detected
Collierville, 2009 (only sequence available)	*Boa constrictor* (boa constrictor)	USA: California	Unknown	None detected
CAS, 2009 (only sequence available)	*Corallus annulatus* (annulated tree boa)	USA: California	Unknown	None detected
Filoviruses				
Marburg, 1967	*Rousettus aegyptiacus* (fruit bat)	Imported in Germany and Yugoslavia; Kenya, Zimbabwe, DRC, Angola, Uganda; imported single cases in Netherlands and USA	Fatal cases	Yes
Ebola, 1976	Bats?	DRC, Gabon, Republic of Congo; imported case in South Africa	Fatal cases	Yes
Sudan, 1976	Bats?	Sudan, Uganda	Fatal cases	None detected
Reston, 1989	Bats?	Philippines; imported infected primates in USA and Italy	Asymptomatic cases	Seroconversion in handlers of nonhuman primates, in pig farmers, and in slaughterhouse workers
Taï Forest, 1994	Bats?	Ivory Coast	Febrile illness, survived	None detected
Bundibugyo, 2007	Bats?	Uganda, DRC	Fatal cases	None detected

with exposure to infected nonhuman primates (44, 45). In 2000, SUDV was responsible for a large outbreak in Gulu, in the northwest of Uganda. Approximately 425 persons were infected, and 53% of them died (46, 47). In Yambio (South Sudan) in 2004, 17 persons were infected by SUDV, and 7 of them died (48). An outbreak due to Marburg viruses, involving gold miners and secondary contacts, started in 1998 and continued into 2000 in eastern Democratic Republic of Congo (DRC) (49). Several bats collected in the mine were found to be positive for Marburg viruses using reverse transcription (RT)-PCR (50). In spring 2005, a large outbreak of Marburg HF occurred in Uige, northern Angola. A total of 252 cases and 227 deaths from Marburg HF were reported as of 24 August (51–53).

In 2005, fruit bats positive for Ebola virus by RT-PCR were found in Gabon (54). In 2007 and 2008, Ebola virus was responsible for two small outbreaks in the same area of Kasaï-Occidental (DRC) (55; http://www.who.int/csr/don/2009_02_17/en/index.html). A new distinct species of Ebola virus, BDBV, was isolated during an outbreak centered in and around Bundibugyo in western Uganda (35). A total of 131 cases of suspected, probable, or confirmed cases were reported from August to December 2007. In 2008, RESTV was isolated from swine tissues during the laboratory investigation of samples from a 2007 outbreak of high mortality in swine in farms in the Philippines, which was thought to be atypical porcine reproductive and respiratory syndrome (PRRS) (56). PRRS and Circo 2 viruses were also isolated from the swine, leaving it unclear the extent to which RESTV was contributing to the disease symptoms. The exact role of RESTV in the clinical process is still under investigation through field surveillance. Experimental infections of swine with RESTV isolated from Philippine pigs did not cause clinically notable disease, although the virus was shed and detectable antibodies developed in experimental infections in the laboratory (123). This virus species appears to be less virulent for humans than are other filovirus species, since four workers at the U.S. quarantine facility in 1990 were found to have antibody to RESTV without having experienced disease and several slaughterhouse and pig farm workers from the more recent outbreak in the Philippines were also found with antibody without associated clinical disease (http://www.who.int/csr/resources/publications/HSE_EPR_2009_2.pdf).

In 2007, four workers from a lead ore mine near Ibanda, Uganda, were infected with Marburg viruses. Two patients died, and the subsequent investigations led to the identification of Marburg virus RT-PCR-positive *Rousettus aegyptiacus* fruit bats and, for the first time, the isolation of Marburg viruses from this potential reservoir (57, 58). In 2008, Marburg HF was diagnosed in 2 tourists who independently visited the same bat-inhabited cave (Python Cave, Queen Elizabeth National Park) in western Uganda. The first patient was a Dutch woman who subsequently died of Marburg HF in the Netherlands (59). The second patient was an American woman from Colorado who recovered and was diagnosed retrospectively (60). A single case of Ebola HF associated with SUDV was reported in 2011 in Uganda (61), but in 2012, four unrelated outbreaks occurred: SUDV in Kibaale (Uganda) in July and August, BDBV in Isiro (DRC) from August to October, Marburg virus in Kabale (Uganda) in October and November, and SUDV in Luwero (Uganda) in November (62). In the spring of 2014, the Guinean Ministry of Health reported an outbreak of Ebola HF in the forest region of the country. It is now (as of January 2015) the biggest outbreak ever, with more than 21,000 confirmed and probable cases mostly in Guinea, Sierra Leone, and Liberia, multiple medical evacuations of infected medical responders to Europe and the United States, and infected travelers with sometimes secondary cases reported in Senegal, Mali, Nigeria, and the United States (an update can be found at http://www.who.int/csr/disease/ebola/situation-reports/en/).

CLINICAL SIGNIFICANCE

Patients with VHF frequently present with similar, nonspecific clinical signs resembling malaria, typhoid fever, and pharyngitis. A detailed travel history, coupled with a high index of suspicion and availability of definitive virologic tools, should facilitate rapid diagnosis and the timely implementation of appropriate patient isolation, clinical management procedures, and public health measures. For Ebola, U.S. guidance documents are available online (http://www.cdc.gov/vhf/ebola/index.html).

Arenaviridae

Among the known arenavirus human pathogens, LCM virus produces the least severe infection (63). A modest proportion of LCM virus infections are subclinical. A "typical" LCM case is usually heralded by fever, myalgia, retro-orbital headache, weakness, and anorexia. Especially during the first week, prominent symptoms include sore throat, chills, vomiting, cough, retrosternal pain, and arthralgia. Rash occurs but is infrequent. In about one-third of the patients, fever recurs, coinciding with the onset of frank neurologic involvement, usually aseptic meningitis, but less frequently meningoencephalitis. Complete recovery is almost always the rule. Thus, LCM virus infections are temporarily debilitating but rarely fatal, even when neurologic complications arise. Although recognized only recently, *in utero* infections with LCM virus are a cause of significant birth defects of the central nervous and ocular systems (64, 65). Fatal forms of LCM infections were reported recently in several clusters following transplantation of organs from LCM virus-infected donors to immunosuppressed recipients (66–68).

Lassa, Junin, and Machupo virus infections are much more severe. Lassa fever patients usually present at the hospital within 5 to 7 days of onset and complain of sore throat, severe lower back pain, and conjunctivitis. These signs and symptoms usually increase in severity during the following week and are accompanied by nausea, vomiting, diarrhea, chest and abdominal pain, headache, cough, dizziness, and tinnitus. Later, pneumonitis and pleural and pericardial effusions with friction rub frequently occur. A maculopapular rash may develop, but frank hemorrhage is seen in only a proportion of the more severe cases. Bleeding from puncture sites and mucous membranes and melena are more common. Although mild or unapparent infections occur, approximately 15 to 20% of hospitalized patients die, usually as a result of sudden cardiovascular collapse resulting from hepatic, pulmonary, and myocardial failure. Few Lassa fever patients develop central nervous system signs, although tinnitus or deafness may develop as recovery begins. Lassa fever is a particularly severe disease among pregnant women, for whom mortality rates are somewhat higher. The disease course in children is similar to that in adults, but in infants a condition described as swollen-baby syndrome, characterized by anasarca, abdominal distension, and bleeding, is typical. Clinical laboratory studies are not usually helpful for Lassa fever; specific virologic testing is required, especially in a setting where Lassa fever is less common or where mild, atypical cases are occurring.

The clinical pictures for Argentinian HF (AHF) due to Junin virus and Bolivian HF (BHF) due to Machupo virus are well characterized; for Venezuelan HF due to Guanarito virus, Sabia virus in Brazil, or Chapare virus (with a single confirmed case in Bolivia), less information is available (3). However, all these infections are sufficiently similar to each other to be discussed as a single entity, South American or New World arenavirus HFs (SAHFs). Incubation periods range from 7 to 14 days, and very few subclinical cases are thought to occur. Following gradual onset of fever, anorexia, and malaise, i.e., over several days, constitutional signs involving the gastrointestinal, cardiovascular, and central nervous systems become apparent by the time patients present to the hospital. On initial examination, AHF and BHF patients are febrile, acutely ill, and mildly hypotensive. They frequently complain of back pain, epigastric pain, headache, retro-orbital pain, photophobia, dizziness, constipation or diarrhea, and coughing. Flushing of the face, neck, and chest and bleeding from the gums are common. Enanthem, petechiae or tiny vesicles spread over the erythematous palate and fauces, is almost invariably present. Neurologic involvement, ranging from mild irritability and lethargy to abnormalities in gait, tremors of the upper extremities, and, in severely ill patients, coma, delirium, and convulsions, occurs in more than half of the patients. During the second week of illness, clinical improvement may begin or complications may develop. Complications include extensive petechial hemorrhages, blood oozing from puncture wounds, melena, and hematemesis. These manifestations of capillary damage and thrombocytopenia do not result in life-threatening blood loss. However, hypotension and shock may develop, often in combination with serious neurologic signs, among the 15% of patients who subsequently die. Survivors begin to show improvement by the third week. Recovery is slow; weakness, fatigue, and mental difficulties may last for weeks, and a significant proportion of patients relapse with a “late neurologic syndrome,” which includes headache, cerebellar tremor, and cranial nerve palsies. In contrast to the case with Lassa fever, clinical laboratory studies are frequently useful. Total leukocyte counts usually fall to 1,000 to 2,000 cells/mm^3, although the differential remains normal. Platelet counts fall precipitously, usually to between 25,000 and 100,000/mm^3. Routine clotting parameters are usually normal or slightly abnormal; however, patients with severe cases may show evidence of disseminated intravascular coagulation.

In the single Lujo virus outbreak in Southern Africa, involving 5 confirmed cases, the patients presented with nonspecific febrile illness with headache and myalgia following an incubation ranging from 7 to 13 days (12). A morbilliform rash was evident in 3 Caucasian patients on days 6 to 8 of illness, but not in 2 African patients. In the four fatal cases, the disease course was biphasic. The second phase, starting around 1 week postonset, was characterized by a rapid deterioration with respiratory distress, neurologic signs, and circulatory collapse. All patients had thrombocytopenia on admission to hospital (platelet count range 20×10^9 to 104×10^9/liter). Three patients had normal white cell counts and two had leukopenia on admission, while four developed leukocytosis during the illness. The last patient (the only one surviving) was treated with the antiviral drug ribavirin.

In fatal arenavirus infections, histopathologic findings include multifocal hepatocellular necrosis with minimal inflammatory response, interstitial pneumonitis, myocarditis, and lymphoid depletion. Extensive arenavirus antigens are seen in parenchymal cells as well as cells of the mononuclear phagocytic system, much more than morphologic lesions would suggest (see Fig. 4).

Filoviridae

Marburg and Ebola HF cases are clinically similar, although the frequencies of reported signs and symptoms vary among individuals. Following incubation periods of 4 to 16 days, onset is sudden and is marked by fever, chills, headache, anorexia, and myalgia. These signs are soon followed by nausea, vomiting, sore throat, abdominal pain, and diarrhea. When first examined, patients are usually overtly ill, dehydrated, apathetic, and disoriented. Pharyngeal and conjunctival injection is usual. Within several days, a characteristic maculopapular rash over the trunk, cutaneous petechiae, and mucous membrane hemorrhages appear. Gastrointestinal bleeding, accompanied by intense epigastric pain, is common, as are petechiae and bleeding from puncture wounds and mucous membranes. In the current Ebola outbreak, hemorrhage is rare but profuse diarrhea is frequently observed and is responsible for severe dehydration (http://www.cdc.gov/vhf/ebola/hcp/clinician-information-us-healthcare-settings.html). Shock develops shortly before death, often 6 to 16 days after the onset of illness. Abnormalities in coagulation parameters include fibrin split products and prolonged prothrombin and partial thromboplastin times, suggesting that disseminated intravascular coagulation is a terminal event and is usually associated with multiorgan failure (69). Clinical laboratory studies usually reveal profound leukopenia early, sometimes moderately elevated at a later stage. Platelet counts decline to 50,000 to 100,000/mm^3 during the hemorrhagic phase. Severe hypokalemia can occur as a consequence of profuse vomiting and diarrhea. Liver enzymes and creatinine levels parallel the liver and kidney failure encountered in the latter stages of the disease.

In fatal filovirus infections, disseminated infection and necrosis in major organs such as the liver, spleen, lungs, kidneys, skin, and gonads are extensive. Extensive hepatocellular necrosis associated with the formation of characteristic viral inclusions is seen in fatal Ebola virus infections. Lymphoid depletion and microvascular infection and injury are seen in all filovirus infections. Abundant antigens and nucleic acids can be seen in all major organs by using immunohistochemistry and *in situ* hybridization (see Fig. 2 and 3).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Safety and Security

Some arenaviruses (Lassa, Junin, Machupo, Sabia, Guanarito, Chapare, and Lujo viruses) and all the filoviruses are classified as biosafety level 4 (BSL4) agents, as there is a high risk of infection of laboratory personnel and appropriate precautions must be taken (70) (http://www.cdc.gov/vhf/ebola/hcp/interim-guidance-specimen-collection-submission-patients-suspected-infection-ebola.html#update1). Most are also classified in the United States as select agents by the Department of Health and Human Services and should be handled as such (http://www.selectagents.gov/SelectAgentsandToxinsList.html and http://www.selectagents.gov/Regulations.html). Ebola viruses and Marburg viruses have recently been classified as tier 1 select agents, and additional regulations exist for possessing, using, or transferring tier 1 select agents and toxins (http://www.selectagents.gov/faq-general.html#sec1q6). At a minimum, barrier nursing procedures should be implemented and personnel caring for the patient and handling diagnostic specimens should wear disposable caps, gowns, shoe covers, surgical gloves, and face masks (preferably full-face respirators equipped with high-efficiency particulate air filters) (71, 72). Gloves should be disinfected immediately if they come in direct contact with infected blood or secretions. Manipulation of these specimens and tissues, including sera obtained from convalescent-phase patients, may pose a serious biohazard and should be minimized outside a BSL4 laboratory (70, 72). Use of Vacutainer tubes (e.g., BD Vacutainer) is considered safer than use of syringes and needles, which must be disassembled before their contents are transferred to another tube. Procedures which generate aerosols (e.g., centrifugation) should be minimized and performed only if additional protective measures, such as keeping the equipment in a class I or II laminar-flow hood, are taken. For specialized procedures, the infectivity of samples may be greatly reduced, if not totally inactivated, by the addition of Triton X-100 and heating. Heating to 60°C for 1 h renders diagnostic specimens noninfectious and is acceptable for measurement of heat-stable substances such as electrolytes, blood urea nitrogen, and creatinine. When the equipment is available, sterilization by ^{60}Co gamma irradiation is the preferred method of inactivation. Extraction of RNA from infectious samples by using guanidinium thiocyanate (with a proper ratio, at least 1 to 5 or 1 to 10, of lysis reagents) should be conducted in a laminar-flow hood. After this step, the extracted RNAs are no longer infectious.

Specimen Collection

For virus isolation, antigen detection, and RT-PCR, serum, heparinized plasma, or whole blood should be collected during the acute, febrile stages of illness and frozen on dry ice or in liquid nitrogen vapor. In the United States, the guidance for Ebola virus sample collection and submission has been updated (http://www.cdc.gov/vhf/ebola/hcp/interim-guidance-specimen-collection-submission-patients-suspected-infection-ebola.html). Storage at higher temperatures (above −70°C) leads to losses in infectivity. Blood obtained in early convalescence for diagnostic purposes may be infectious despite the presence of antibodies, and it should be handled accordingly (70–72). In addition to blood samples, throat wash specimens have also been used for virus isolation during infections with arenaviruses (Lassa, Junin, and Machupo viruses). LCM virus may be recovered from acute-phase serum samples obtained during the first week after onset but more likely from cerebrospinal fluid during the period of meningeal involvement, and from the brain at autopsy, but it is rarely, if ever, recovered from throat washings or urine specimens. LCM has been easily isolated from the blood of immunocompromised organ transplant recipients. Chapare and Lujo viruses were isolated from blood during the acute phase of the disease. Filoviruses are usually recovered from acute-phase serum samples; various specimens, including throat washings, oropharyngeal secretions, urine, soft tissue effusions, semen, and anterior eye fluid, have yielded filovirus isolates, even occasionally when the specimens were obtained late in convalescence.

Postmortem Specimens

Lassa, Machupo, and Junin viruses, Marburg viruses, and Ebola viruses are all readily isolated from specimens of spleen, lymph nodes, liver, and kidney obtained at autopsy but rarely, if ever, from brain or other central nervous system tissues. Notably, Lassa virus is usually isolated from the placentas of infected pregnant women. In the severe and fatal clinical forms observed after organ transplantation, LCM virus was isolated from several organs by biopsies taken before death or at autopsy. Lujo virus was isolated from liver at autopsy. Formaldehyde-fixed tissues and paraffin-embedded blocks are also suitable for histopathology and immunohistochemical identification of viral antigens and should be conserved and shipped at room temperature.

Shipping

If an arenavirus or filovirus infection is suspected on clinical and epidemiological evidence, and before sending the specimens, the clinicians should consult with the local health authorities and the relevant following laboratories that maintain BSL4 facilities and a diagnostic capability for these agents. For all testing of infectious material, samples should be packaged in accordance with current recommendations (International Airline Transportation Association [IATA]) (http://www.iata.org/index.htm). The package should be sent by rapid courier, and preliminary results could be expected within 24 to 48 h after reception of the specimens.

1. Centers for Disease Control and Prevention, Viral Special Pathogens Branch, 1600 Clifton Rd., Centers for Disease Control and Prevention, Atlanta, GA 30333. Phone: (404) 639-1115. Fax: (404) 639-1118. URL: http://www.cdc.gov/vhf/ebola/hcp/interim-guidance-specimen-collection-submission-patients-suspected-infection-ebola.html.
2. U.S. Army Medical Research Institute of Infectious Diseases, Headquarters, Fort Detrick, 1425 Porter St., Frederick, MD 21702-5011. Phone: (301) 619-2833. Fax: (301) 610-4625.
3. Center for Applied Microbiology and Research, Novel and Dangerous Pathogens, Manor Farm Road, Porton Down, Salisbury, Wiltshire SP4 0JG, United Kingdom. Phone: 0980-612224. Fax: 0980-611310.
4. National Institute for Communicable Diseases, Special Viral Pathogens Laboratory, 1 Modderfontein Road, Private Bag X4, Sandringham, Johannesburg, South Africa. Phone: 27 (11) 386 6376 or 082 903 9131. Fax: 27 (11) 882 3741.
5. National Microbiology Laboratory (NML), Public Health Agency of Canada, Winnipeg, Manitoba, Canada. Phone: 1-800-545-7661 or 1-866-262-8433.

Several BSL4 laboratories are available in Europe (http://www.enivd.de/index.htm), and in the United States, other BSL4 laboratories with diagnostic capability may be available in the near future.

DIRECT EXAMINATION

Electron Microscopy

Individual arenavirus virions are pleomorphic and range in size from 60 to 280 nm (mean, 110 to 130 nm) (Fig. 1A). A unit membrane envelops the structure and is covered with club-shaped, 10-nm projections. No symmetry has been discerned. The most prominent and distinctive feature of these virions is the presence of different numbers of electron-dense particles (usually 2 to 10), which may be connected by fine filaments. These particles, 20 to 25 nm in diameter, are identical to host cell ribosomes by biochemical and oligonucleotide analysis (26). Immunoelectron microscopy techniques also work well for diagnosis of arenavirus infections, although the morphology of the virions is less striking for arenaviruses than filoviruses.

Marburg viruses and Ebola viruses have been successfully visualized directly by electron microscopy of both heparinized blood and urine obtained during the febrile period as well as in tissue culture supernatant fluids. The combination of the size and shape of the virions is sufficiently characteristic

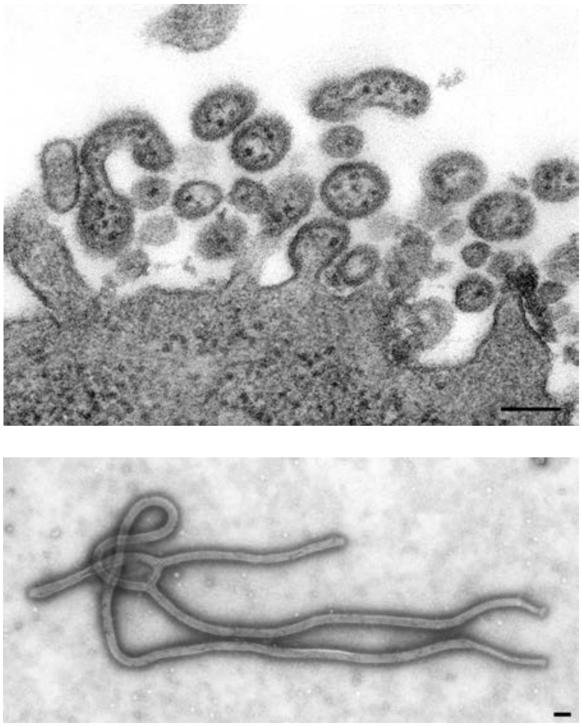

FIGURE 1 Ultrastructural characteristics of LCM virus and Ebola virus as seen in tissue culture cells. (A) LCM virus, an arenavirus, showing pleomorphic enveloped particles with internal ribosome-like granules. Scale bar, 100 nm. (Courtesy of C. S. Goldsmith.) (B) Isolate of Ebola virus, a filovirus, showing enveloped filamentous particles around 80 nm wide. Some filaments can occasionally measure up to 15,000 nm in length. Scale bar, 100 nm. (Courtesy of C. S. Goldsmith.) doi:10.1128/9781555817381.ch97.f1

to allow a morphologic diagnosis of filovirus (32, 39, 73). The virus particles are very large, typically 790 to 970 nm long and consistently 80 nm in diameter. Bizarre structures of widely different lengths are frequently found in negatively stained preparations, sometimes exceeding 14,000 nm, as well as branching, circular, or "6" shapes, probably resulting from coenvelopment of multiple nucleocapsids during budding (Fig. 1B) (32, 73, 74).

Ebola virus inclusions are often seen in thin-section electron micrographs of liver. The inclusions consist of viral nucleocapsids mostly seen in longitudinal section. Numerous viral particles are also seen in sinusoidal spaces (Fig. 2B). These virions can be differentiated and identified as Marburg viruses or Ebola viruses by immunoelectron microscopy techniques (74–76).

Antigen Detection

Immunohistochemistry

IFA staining of impression smears or air-dried suspensions of the liver, spleen, or kidney have been used successfully to detect cytoplasmic inclusion bodies associated with Marburg virus infection; clumps of Marburg virus antigen have also been observed by IFA examination of infected, dried, citrated blood smears. This approach was successfully adapted to the diagnosis of RESTV in impression smears from blood, tissues, and nasal turbinates, as it was to the detection of Junin virus-infected cells in peripheral blood and urinary sediment. Hematoxylin and eosin staining of the liver reveals hepatocellular necrosis and numerous intracytoplasmic and eosinophilic Ebola virus inclusions, as well as sinusoidal dilatation and congestion (Fig 2A). The development of immunohistochemical techniques for detection of filovirus and arenavirus antigens in formalin-fixed tissues has recently advanced to the point that their results are far more satisfactory than those of IFA examination of frozen, acetone-fixed sections (38, 77–80). Obtaining frozen sections for diagnosis is rarely worth the biohazard incurred, especially since the threshold sensitivity for detection exceeds 6 $\log_{10}$ PFU per g. For filoviruses, paraffin blocks of tissues are sectioned. Sections are deparaffinized, hydrated, digested with protease, and stained for the presence of viral antigens with immune rabbit serum or cocktails of murine monoclonal antibodies (39, 79, 80). Biotinylated secondary antibody is then reacted, and the product is developed with a streptavidin-alkaline phosphatase system. Substitution of other chromogens can further increase sensitivity while reducing background (77). In the 2007–2008 epizootic of Reston virus in swine in the Philippines (56), Ebola virus antigens were detected in different tissues, including lymph nodes (Fig. 3A). Remarkable success was reported in the application of immunohistochemistry to the demonstration of Ebola virus antigens in formalin-fixed skin biopsy specimens obtained from deceased Ebola HF patients in Zaire in 1995 (80, 81) (Fig. 3B). Immunohistochemistry was used to confirm the diagnosis of a fatal Lassa fever case in a New Jersey patient returning from West Africa (Fig. 4A). More recently, it was the immunohistochemistry assays on a liver biopsy specimen (Fig. 4B) which first indicated the arenavirus etiology of a disease observed in South Africa, leading to the description of Lujo virus (12). In the cluster of LCM cases occurring through organ transplants (66, 67), very abundant LCM virus antigen could be found in a number of organs (Fig. 4C).

Antigen Capture ELISA

Development of an antigen capture ELISA for quantitative detection of arenavirus and filovirus antigens in viremic sera and tissue culture supernatants has facilitated the early detection and identification of these agents (82–85). These tests reliably detect antigens in samples inactivated by heat-detergent treatment, β-propiolactone, or irradiation; therefore, they can be conducted safely without elaborate containment facilities. The threshold sensitivities for these assays are approximately 2.1 to 2.5 $\log_{10}$ PFU per ml, and so they are sufficiently sensitive to detect antigen in most acute-phase VHF viremias and to detect viruses at the concentrations present in throat wash and urine samples. Substitution of one or several monoclonal antibodies of high avidity and appropriate specificities for polyclonal sera (mostly against nucleoprotein and VP40) generally increases the sensitivities and specificities of these antigen capture ELISAs (85–88).

Nucleic Acid Detection

RT-PCR followed by genome analysis is rapidly replacing diagnostic methods based on antigen-antibody methods and has the advantage of complete inactivation of the samples in the first extraction step and lower limits of detection. However, as RT-PCR is more prone to cross-contamination problems, we would still encourage its use in combination with traditional methods (virus isolation and/or serology). Real-time RT-PCR assays have more recently supplanted older RT and amplification methods because of the automation and

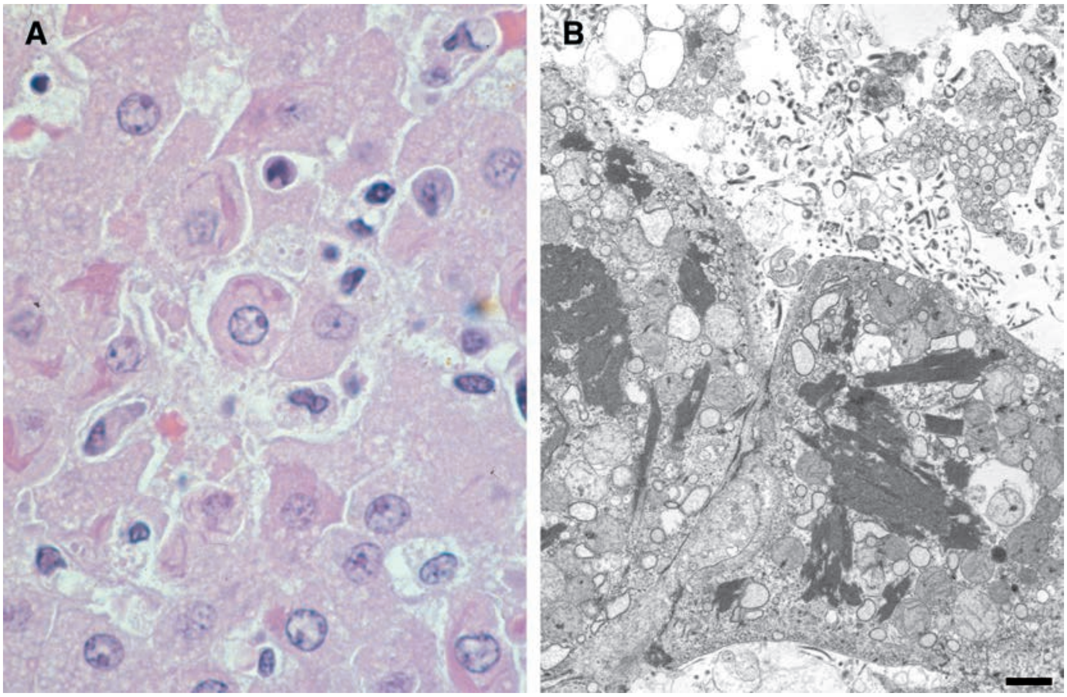

FIGURE 2 Ebola virus HF. (A) Section of liver showing hepatocellular necrosis, numerous intracytoplasmic, eosinophilic Ebola viral inclusions, as well as sinusoidal dilatation and congestion. Hematoxylin and eosin stain; original magnification, ×250. (B) Several Ebola viral inclusions are seen in a thin-section electron micrograph of liver. The inclusions consist of viral nucleocapsids mostly seen in longitudinal section. Numerous viral particles are also seen in sinusoidal spaces. Scale bar, 100 nm. (Courtesy of C. S. Goldsmith.) doi:10.1128/9781555817381.ch97.f2

machine reporting capabilities of the method. Real-time PCR combined with classical cell culture may also be used for testing antivirals against Lassa and Ebola viruses (89).

Arenaviridae

The considerable genetic variation among Lassa viruses requires caution in primer design if diagnosis of infections originating in more than one geographic area is being considered (90–93). To remediate this problem, primers targeting the 5′ region of the S RNA have been developed (94) and are used on a routine basis on a very large set of patients presenting at Irrua Specialist Teaching Hospital in Nigeria (95). In limited-resource settings, real-time PCR assays would reduce the reported low rate (13/1,650 samples [0.8%] in Irrua) of PCR contamination, but they still lack field validation. Likewise, various strategies for RT-PCR have been devised to detect Junin virus RNA in clinical materials (20, 90, 91, 96). Some of these are reputed to be far more sensitive than conventional isolation procedures, especially in the presence of antibody. Any method which promises to be a reliable substitute for procedures that entail the manipulation of infectious BSL4 virus deserves attention. However, the ability to isolate and retain the virus has obvious advantages and should not be abandoned. Even in instances in which the etiological agent has been identified, the shipment of clinical material to an appropriate reference laboratory should be highly encouraged.

Filoviridae

Protocols which maximize detection are usually based on conserved sequences within the N, VP40, VP35, or L genes, while fine discrimination and phylogeny are based on the more variable GP region (2, 36, 97, 98). The sensitivity of RT-PCR for various Ebola virus species is similar to that of conventional isolation. During the SUDV outbreak in Gulu, an RT-PCR-based assay was very valuable because of its ability to identify early patients prior to identification by any other available tests, and for early convalescent-phase specimens, which had already cleared detectable antigen. Using nucleoprotein-based real-time quantitative RT-PCR, the viral load was found to correlate with disease outcome (47, 99). During the 2005 Marburg HF outbreak in Angola and the 2012 outbreak in Uganda, Marburg virus real-time PCR assays were developed and effectively applied, respectively, in the laboratories (Luanda and Entebbe) and in the field (Uige and Kabale) (53, 62, 100). Other platforms are available (98, 101, 102), and recently, RT–loop-mediated isothermal amplification has been proposed, but it has not yet been field tested (103). In the current Ebola outbreak, real-time PCR, targeting NP and VP40, is used in the field in West Africa and also in suspected and confirmed cases in Europe and the United States.

ISOLATION PROCEDURES

Cell Culture

Virus culture should not be attempted in BSL2 laboratories, and if inadvertently inoculated, cultures must be autoclaved and destroyed. Clinical specimens and clarified tissue homogenates (usually 10% [wt/vol]) are diluted in a suitable maintenance medium and adsorbed in small volumes to cell monolayers grown in suitable vessels, such as T-25 tissue culture flasks. If no antigen is detected by IFA by 14 days, the sample is considered negative, but supernatant fluids

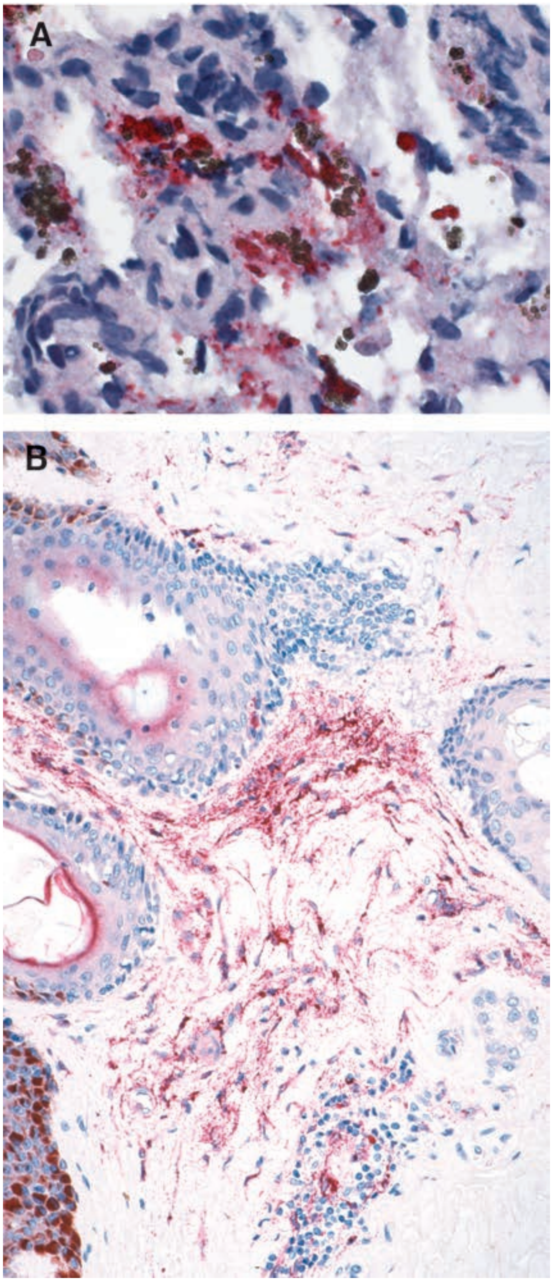

FIGURE 3 (A) Using immunohistochemistry, abundant Ebola virus antigens (in red) are seen in the lymph node of a pig infected by Ebola Reston virus. Original magnification, ×158. (B) Skin showing massive viral burden as seen in this section immunostained for Ebola virus antigens. Original magnification, ×50 (immunoalkaline phosphatase staining; naphthol fast red substrate with light hematoxylin counterstain). doi:10.1128/9781555817381.ch97.f3

should be blind passaged to confirm the absence of virus. To confirm the presence and identity of the virus, scraped cells are tested by immunofluorescence assay and supernatant fluids are tested by antigen capture ELISA or RT-PCR techniques.

Arenaviridae

Cocultivation of Hypaque-Ficoll-separated peripheral blood leukocytes with susceptible cells has increased the isolation frequency of Junin virus. Cocultivation of lymphocytes from spleens of experimentally infected animals has yielded Lassa virus late in convalescence, even after neutralizing antibody has appeared. The technique merits systematic development for the remaining arenavirus and filovirus pathogens.

Although historically Machupo and Junin viruses were isolated by inoculation of newborn hamsters and mice, respectively, Vero E6 cells are approximately as sensitive and are far less cumbersome to manage in BSL4 containment. Furthermore, Vero cells usually permit isolation and identification within 1 to 5 days, a significant advantage over the use of animals, since 7 to 20 days of incubation is required for illness to develop in the animals.

Filoviridae

The best general method currently available for isolation of filoviruses is the inoculation of appropriate cell cultures (usually Vero cells), followed by IFA or other immunologically specific or nucleic acid-specific testing of the inoculated cells for the presence of viral antigens or genomic RNA at intervals. Other cell lines, including human diploid lung (MRC-5) and BHK-21 cells, also work; MA-104 cells (a fetal rhesus monkey kidney cell line) may be more sensitive than Vero cells for some strains.

Animal Inoculation

Arenaviridae

Intracranial (i.c.) inoculation of weanling mice is still regarded by some as the most sensitive established indicator of LCM virus (104), although adequate cell culture systems exist for the isolation of LCM virus. Care must be taken to use mice from a colony known to be free of LCM virus. Many LCM virus isolates produce a characteristic convulsive disease within 5 to 7 days, which is nearly pathognomonic. Brains from dead mice may be used to prepare ELISA antigens or may be subjected to IFA or immunohistochemical staining to obtain presumptive identification. Clarified mouse brain may also be used as the antigen for confirmatory testing by neutralization or RT-PCR.

Most LCM virus strains are also lethal for guinea pigs. The pathogenicity of virulent Lassa virus strains for outbred Swiss albino mice inoculated i.c. seems to vary with different sources; mice should not be seriously considered for Lassa virus isolations. Strain 13/N guinea pigs develop hemorrhagic disease after Lujo virus inoculation (105).

For the New World arenaviruses, particularly Junin virus, young adult guinea pigs inoculated either i.c. or peripherally have been used. Guinea pigs die 7 to 18 days after Junin virus inoculation. Strain 13/N guinea pigs are exquisitely sensitive to most Lassa virus strains and uniformly die 12 to 18 days after inoculation; outbred Hartley strain guinea pigs are somewhat less susceptible. Newborn mice (1 to 3 days old) are highly susceptible to Junin virus inoculated i.c.; newborn hamsters are believed to be more sensitive to Machupo virus.

Filoviridae

Marburg viruses and the Ebola viruses and SUDV produce febrile responses in guinea pigs 4 to 10 days after inoculation; however, none of these viruses kill guinea pigs consistently on primary inoculation, and only Ebola virus and Marburg virus have been adapted to uniform lethality by sequential guinea pig passages. Ebola virus is usually pathogenic for newborn mice inoculated i.c., but SUDV, RESTV, and Marburg viruses are not.

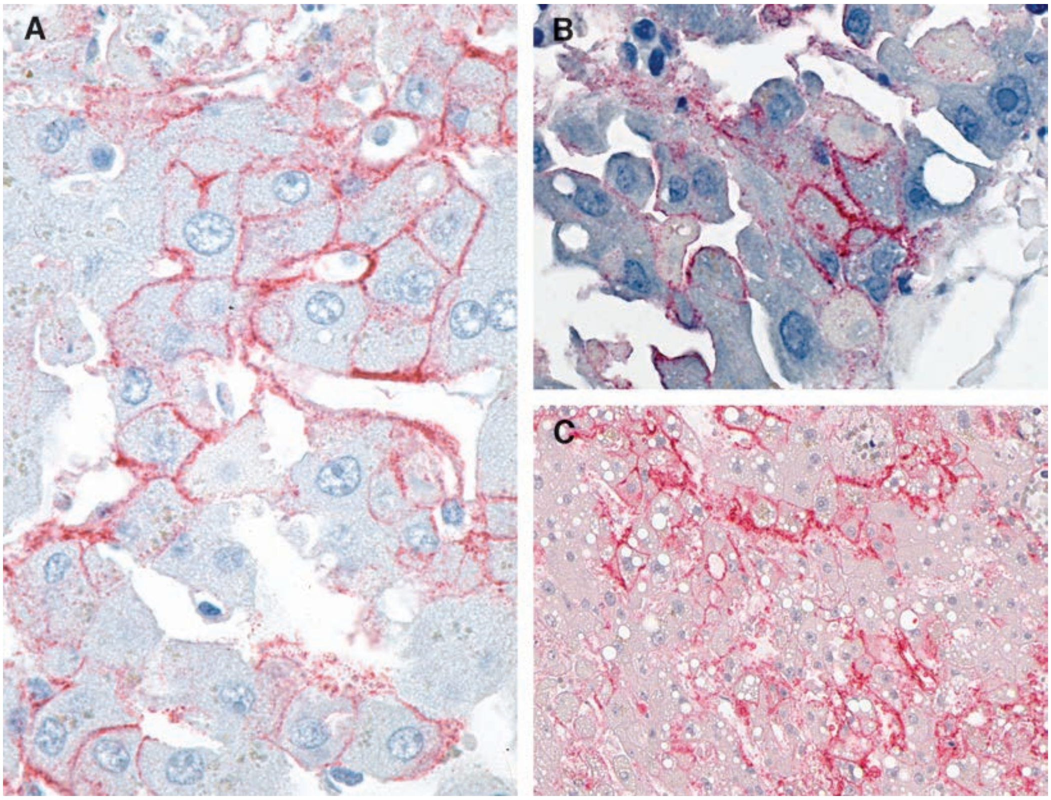

FIGURE 4 Using immunohistochemistry, abundant virus antigens are seen within the cytoplasm of hepatocytes and sinusoidal lining cells in the liver of patients infected by (A) Lassa virus (original magnification, ×158), (B) Lujo virus (original magnification, ×158), or (C) LCM virus (transplant patient) (original magnification, ×50) (immunoalkaline phosphatase staining; naphthol fast red substrate with light hematoxylin counterstain). doi:10.1128/9781555817381.ch97.f4

IDENTIFICATION OF VIRUS

Typing Antisera

Detection of viral antigens in infected tissue culture cells (usually Vero or MA-104) permits a presumptive diagnosis, provided that the serologic reagents have been tested against all the reference arenaviruses and filoviruses expected in a given laboratory, thus permitting an interpretation of virus cross-reactions. Virus isolates in cell culture supernatant fluids or tissue homogenates are presumptively or specifically identified by their reactivity with diagnostic antisera in various serologic tests (see below). Specific polyclonal antisera are prepared in adult guinea pigs, hamsters, rabbits, rats, or mice inoculated intraperitoneally with infectious virus. Rhesus and cynomolgus monkeys that are convalescent from experimental infections are also reasonable sources for larger quantities of immune sera. Polyvalent polyclonal "typing" sera or ascitic fluids made by immunization with a number of viruses within the families have also been useful in early identification of virus isolates and in immunohistochemistry on unknown patient materials. Diagnostic antisera produced by single injection of infectious virus are less cross-reactive and usually have higher titers than those produced by multiple injections of inactivated antigens. To further reduce the induction of extraneous antibodies, input virus should be derived from tissues or cells homologous to the species being immunized; likewise, the virus suspension should be stabilized with homologous serum or serum proteins. Sera produced for use in the IFA tests and ELISA should be collected 30 to 60 days after inoculation; sera for neutralization tests should be collected later. All sera must be inactivated and rigorously tested for the presence of live virus before being removed from a BSL4 environment.

Production and use of specific murine monoclonal antibodies with fine specificities for N and GP epitopes of LCM virus, Lassa virus, Junin virus (106, 107), and other arenaviruses have been reported, as have monoclonal antibodies against the different proteins of the filoviruses. Reference reagents for all of these viruses are not generally available outside the appropriately equipped specialized laboratories.

Immunofluorescence

To process infected cells for IFA examination and presumptive identification, inoculated cell monolayers are dispersed by using glass beads or a rubber policeman or trypsinization, washed, and placed onto circular areas of specially prepared Teflon-coated slides. These "spot slides" are air dried, fixed in acetone at room temperature for 10 min, and either stained quickly or stored frozen at −70°C. Although acetone fixation greatly reduces the number of infectious intracellular viruses, spot slides prepared in this manner should still be considered infectious and handled accordingly. Gamma irradiation has been used to render spot slides noninfectious (29), with no diminution in fluorescent-antigen intensity. Alternatively, infected cells may be biologically inactivated with β-propiolactone (28). Gamma irradiation is recommended if the appropriate equipment is available.

For direct FA tests, specific immunoglobulins conjugated to fluorescein are used with Evans blue counterstain. Specific viral fluorescence is characterized by intense, punctate to granular aggregates confined to the cytoplasm of infected cells. Specific Marburg virus and Ebola virus fluorescence may include large, bizarrely shaped aggregates up to 10 μm across. Nonspecific fluorescence is rarely a problem in IFA procedures for these viruses. Detection of Marburg virus, Ebola virus, Lassa virus, and LCM virus antigens by the IFA test is usually considered sufficient for a definitive diagnosis, although Lassa and LCM viruses cross-react at low levels in this test (5). Detection of Junin or Machupo virus antigens by the IFA test constitutes a presumptive diagnosis, since these viruses can be reliably distinguished from each other only by neutralization tests or sequencing. IFA formats for viral detection are more cumbersome and cross-reactive but can be used if direct conjugates are unavailable. Immunohistochemical techniques for detecting arenaviruses (77) and filoviruses (78, 79) with a variety of chromogens can also be applied.

SEROLOGIC DIAGNOSIS

IFA Test

Until the early 1990s, the IFA test was widely regarded as the most practical single method for documenting recent infections with filoviruses or for large prevalence studies (108). Preparation of spot slides with infected Vero cells is identical to the procedure described above. Some refinements to enhance reproducibility and quality between spot slides lots have been suggested (109). Although monovalent spot slides are usually desired and are prepared with cells optimally infected with a single virus, polyvalent spot slides can also be prepared by mixing cells infected with different viruses selected from these or other taxonomic groups which have similar geographic distributions (110). The requirement for a BSL4 facility to produce the spot slides could be avoided by using recombinant-antigen-expressing cells (111). Discrepancies in titers determined by different laboratories, or even different investigators, are common. In addition, in most of the severe and fatal forms of these diseases, the patients may not develop a humoral response and die without detectable antibodies. This technique is not recommended for acute diagnosis of HFs.

ELISA for Detection of IgG and IgM Antibodies

For all intents and purposes, the IgG and IgM ELISAs have replaced the more subjective IFA tests as the serologic tests of choice (83, 112). ELISA procedures for Lassa virus-specific IgG and IgM have been developed and successfully used on field-collected human sera (82, 87). When this ELISA is used in combination with the Lassa antigen capture ELISA described above, virtually all Lassa fever patients can be specifically diagnosed within hours of hospital admission. A simplification of this procedure, which entails the use of infected Vero cell detergent lysate as the antigen, diluted in phosphate-buffered saline and adsorbed directly to the microtiter plate wells, has been developed for Ebola viruses (83, 84, 113). Test sera are serially diluted, with a 1:100 initial dilution, and incubated with antigen in a format analogous to the antigen capture ELISA described above. Following incubation, washing, and addition of horseradish peroxidase-conjugated anti-human antibodies, 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) substrate is added for color development. Species-specific conjugates allow testing of other animal species during epidemiological studies. To avoid the use of several conjugates during epidemiological studies, a protein A-protein G conjugate can be used. More recently, a competition assay using antibody-phage indicator has been proposed, but it has never been used in the field (114). Samples are considered positive if the optical density at 410 nm exceeds the mean plus 3 standard deviations for the normal-serum controls. This procedure can be further modified to detect virus-specific IgM by coating the plates with anti-human IgM followed by test serum dilutions and cell slurry antigens and then using the antigen capture protocol. These IgG and IgM ELISA procedures have worked well with specimens obtained from humans during natural infection and from animals experimentally infected with Lassa, Machupo, and Junin viruses and filoviruses. A simplification of the ELISA plate procedure, substituting filter paper disks, has been reported for RESTV (115); it appears to sacrifice some sensitivity and precision in comparison with the more established procedures but may find application in a field setting. All of these developmental assays are sufficiently robust to warrant field testing. Recombinant full or truncated proteins have also been proposed and used as antigens for ELISAs (116, 117).

Neutralization Tests

For the arenaviruses, plaque reduction tests with Vero cells are generally used. For measuring the levels of neutralizing antibody to Lassa and LCM viruses, which are both difficult to neutralize and are poor inducers of this antibody, test sera are diluted, usually 1:10, in medium containing 10% guinea pig serum as a complement source and mixed with serial dilutions of challenge virus. Titers are expressed as a $\log_{10}$ neutralization index, defined as $\log_{10}$ PFU in control minus $\log_{10}$ PFU in test serum. For Junin and Machupo viruses, the more conventional serum dilution-constant virus format is normally used, although the constant serum-virus dilution format is equally useful for distinguishing among virus strains. Neutralizing-antibody responses require weeks to months to evolve but persist for years (118). Performance of these tests is restricted to laboratories equipped to handle the infectious viruses.

In survivors, neutralizing antibodies to Lassa virus first appear very late in convalescence (6 weeks or later), long after the viremia has disappeared. This pattern of early IFA and delayed neutralizing-antibody response is similar for LCM virus infections. Neutralizing antibodies against Junin and Machupo viruses become detectable 3 to 4 weeks after onset, soon after the termination of viremia. While these antibodies are thought to be important in protection against reinfection, their role in resolution of acute infections is less firmly established (119, 120).

As described above, reliable tests for measuring the levels of neutralizing antibody to filoviruses are not currently available.

Western Blotting

Western blotting is feasible for demonstrating antibodies to arenaviruses and filoviruses. However, it has never been applied systematically or routinely to diagnosis, although it was proposed as a confirmatory test to supplement the IFA test for filovirus antibodies (109). Detection of the nucleocapsid (N) band plus either VP30 or VP24 was taken as diagnostic. The Western blotting procedure was further refined by miniaturization, using the Phast system sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Trans-Blot apparatus (Phast Western blot system). This test has not been routinely used for antibody detection.

Other Serologic Tests

Other serologic tests have been applied to diagnosis but have largely been abandoned: gel diffusion precipitation test for arenaviruses, the complement fixation test, reverse passive-hemagglutination (and inhibition) test utilizing Lassa virus antibody-coated erythrocytes, and a radioimmunoassay using ^{125}I-labeled staphylococcal protein A for Ebola virus. Western blotting and radioimmunoassay procedures are used in specialized laboratories to determine the fine specificities of monoclonal antibodies and occasionally to confirm the results of individual sera found to be positive by ELISA.

ANTIVIRAL SUSCEPTIBILITIES

Therapy of VHF follows general principles: symptomatic treatment with careful maintenance of fluid balance and management of bleeding diasthesis. The antiviral drug ribavirin, if used early in the course of the disease, is effective in Lassa HF in reducing mortality and duration of disease (3). Ribavirin is also effective in Junin HF and has been used to treat BHF, Lujo virus infection, and LCM virus infection in immunocompromised patients (3, 12, 67). The side effects are limited because of the short duration of therapy, but the drug is teratogenic. No antiviral drugs against filovirus are yet commercially available, but some postexposure treatment has showed some efficacy in nonhuman primate models, such as recombinant human activated protein C, small interfering RNA, or immunotherapy using cocktails of monoclonal antibody (121). During the current Ebola outbreak, convalescent-phase immune plasma, cocktails of monoclonal antibody, and antivirals have been administered to patients through compassionate-use programs, and clinical trials are starting in West Africa.

EVALUATION AND INTERPRETATION OF RESULTS

Early diagnosis of arenavirus and filovirus infections is desirable since specific immune plasma and appropriately selected antiviral drugs are in certain cases effective when treatment is initiated soon after onset. Early recognition of these infections should also trigger strict isolation procedures to prevent the spread of disease to patient contacts. In areas where specific viruses are endemic, the index of suspicion is often high, and experienced clinicians may be remarkably accurate in rendering an accurate diagnosis of fully developed cases on clinical grounds alone. However, even in these areas, specific virologic and serologic tests are required to confirm clinical impressions, since many other diseases, including malaria, typhoid, rickettsial infections, idiopathic thrombocytopenia, and viral hepatitis, may masquerade as an arenavirus or filovirus infection. In other countries where evacuated suspected (or confirmed) patients or recent travelers showing symptoms are evaluated, a rapid confirmation is needed and RT-PCR or real-time PCR is the test of choice.

Since patients with most of the severe and fatal forms of these diseases can die without developing detectable levels of antibody, timely diagnosis requires a means of detecting RNA, infectious virus or antigen in the field. The RNA detection assays are definitively the most sensitive assays and are widely used. The antigen capture ELISA holds promise for use in detecting clinically relevant concentrations of virus in infectious or inactivated sera, body fluids, and tissues of humans and nonhuman primates infected with Machupo, Lassa, Ebola, and Marburg viruses (39, 42, 82–84).

RT-PCR assays may eliminate the need to isolate infectious virus to establish a definitive diagnosis, but virus isolation remains important for subsequent genetic and pathogenesis studies. The ability to amplify viral genomes from infected tissues and even from formalin-fixed tissues, and to sequence the reaction products, has eclipsed serologic methods of identification and classification of arenaviruses (90–92) and filoviruses (35, 47). With the exception of LCM virus, which may be handled at a lower containment level, attempts to isolate these viruses should not be made outside a BSL4 laboratory (70). A combination of several laboratory techniques should be used to confirm any clinical suspicion of HF.

Among arenaviruses, Lassa virus is usually able to be detected (virus isolation, antigen detection, and RT-PCR) from acute-phase sera of hospitalized patients soon after admission, frequently in the presence of specific IgM antibody, and a detectable antibody response does not necessarily signal imminent recovery; viremia frequently persists, and some patients die despite an antibody response. Junin, Machupo, and LCM viruses are recovered less frequently, and diagnosis is usually based on the detection of specific antibodies later in the course of the disease. The presence of specific IgM ELISA-detectable antibodies in the cerebrospinal fluid of LCM patients constitutes a definitive diagnosis, and for all arenavirus infections, the presence of specific IgM antibodies is indicative of recent infection. The extent to which heterologous arenavirus infection and/or reinfection broadens antibody specificity has not been systematically evaluated for any of the available serologic tests. Neutralizing antibodies against arenaviruses persist for long periods, perhaps for life, and thus provide the most reliable basis for determining the minimum resistance of a population to reinfection. The role of neutralizing antibody in acute recovery is less clear. The protective efficacy of passively administered immune plasma is believed to be a direct function of neutralizing-antibody titers, and plasma should be selected on this basis, especially for Junin and Lassa fever (119, 120).

For filoviruses, RT-PCR, antigen detection, and IgM are the more valuable techniques for acute-case diagnosis. Because of the time required for culture and the biohazard, isolation data for these viruses are usually available only retrospectively. Marburg virus and Ebola viruses are usually easily isolated from acute-phase serum samples. A rising IgM or IgG ELISA titer constitutes a strong presumptive diagnosis. Since IgM titers do not persist for long, a decreasing titer suggests a recent infection which occurred perhaps only within several months. In general, IgG and IgM antibodies show a stronger reactivity to homologous Ebola virus antigens. Because of the low sensitivity of the IgM ELISA to heterologous antigen, there are some limitations in using the IgM ELISA prior to identification of the virus species. In contrast, IgG antibodies are relatively cross-reactive to heterologous antigens (122).

The IFA test has yielded misleading results when applied in diagnostic settings and population-based surveys and should be abandoned. For unknown reasons, the filoviruses are notoriously poor inducers of neutralizing antibody. The role of neutralizing antibodies for Ebola virus protective immunity is unclear; passively administered IgG with very high neutralizing-antibody titers conferred only partial protection to experimentally infected primates (120).

The highest priority for future development is refinement of the available diagnostic tools to permit definitive diagnosis and virus identification in the field. PCR-based assays add another dimension to the capability of field laboratories to diagnose acute disease almost in real time. Proper tailoring of primers should permit the design of tests with the proper degree of specificity. The emergence of new arenaviruses, as well as BDBV, in the past decade serves as a reminder that broadly reactive, grouping reagents are still required to augment the newly evolving tools of conventional and real-time PCR and capture ELISAs based on extremely specific monoclonal antibodies and gene sequences.

An investment in rapid diagnosis should result in more timely intervention with effective implementation of appropriate public health measures and use of evolving treatment regimens. The implementation of appropriate infection control measures has been demonstrated to greatly reduce the transmission and dissemination of these highly virulent viral pathogens.

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Herpes Simplex Viruses and Herpes B Virus

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TAXONOMY

Herpes simplex virus (HSV) types 1 and 2, formally designated human herpesvirus 1 and human herpesvirus 2, respectively, are members of the family *Herpesviridae*. Along with varicella-zoster virus (human herpesvirus 3) and a number of viruses primarily affecting nonhuman hosts, they comprise the subfamily *Alphaherpesvirinae*.

DESCRIPTION OF THE AGENT

The herpesviruses are thought to have coevolved with their hosts (1, 2), and thus the earliest documentation of presumptive herpes simplex infection appears shortly after the development of writing and clinical observation. The infectious nature of HSV was demonstrated in 1919 (3). The concepts of seropositivity and recurrence developed in the 1930s (4, 5). In the 1960s, antigenically distinct strains (HSV-1 and HSV-2) were identified. HSV-1 and HSV-2 are 2 of 8 known human herpesviruses (6). In addition, herpes B virus ("herpesvirus simiae"), a herpesvirus infecting macaques, is an important zoonotic pathogen in humans.

The linear, double-stranded genome is 152 kbp for HSV-1 and 155 kbp for HSV-2. HSV-1 and HSV-2 share 83% nucleotide identity within their protein-coding regions. The genome is organized into a unique long and unique short region, each of which is flanked by inverted-repeat regions. Although significant stretches of sequence are conserved between unrelated clinical isolates, identification of isolates can be achieved by sequencing (7–9), PCR (10), or restriction endonuclease digestion (11).

The HSV virion is 120 to 300 nm in diameter, with a central electron-dense core containing the DNA, an icosahedral capsid consisting of 162 capsomeres surrounding the core, and a tegument layer surrounded by a spiked envelope containing viral glycoproteins that aid in attachment, penetration, and immune evasion (6). The envelope is a trilaminar, lipid-rich layer derived largely from the nuclear membrane of the infected cell. Because the viral envelope is lipid rich, the virus is readily inactivated. Lipid solvents, such as 70% ethanol or isopropanol (but not alcohol >95%), Lysol, or bleach, or exposure to a pH of <5 or >11 or temperatures of >56°C for 30 min will all eliminate infectivity (12, 13).

Viral replication begins with attachment to the target cell via cell type-dependent host receptors (14, 15). After attachment, the viral envelope and cell membrane fuse, releasing the capsid into the cell. The capsid is translocated to the nuclear pores (16, 17), and DNA is released into the nucleus. The tegument protein VP16 induces expression of the immediate early, or alpha, proteins which in turn transactivate the expression of the early, or beta, genes. The early genes are maximally expressed 5 to 7 h after infection and are involved in the synthesis of progeny viral DNA. DNA replication is required for optimal synthesis of the late, or gamma, genes which code for mainly structural proteins. Progeny DNA is processed and assembled into preformed capsids within the cell nucleus. The DNA-containing capsids attach to patches of the nuclear membrane that contain viral tegument proteins and viral glycoproteins. In sensory ganglion neurons, the virus establishes latency, and episomal HSV DNA is maintained for the lifetime of the infected individual. Upon reactivation, nucleocapsids are transported anterograde to the axon terminus, where the final assembly of enveloped virus occurs (18), typically with infection of surrounding epithelial cells. The replication cycle is complete within about 20 h in epithelial cells.

Innate immune mechanisms such as activation of macrophages, NK cells, and interferon production play a predominant role in the control of primary infection, while both innate and adaptive immunity are important in the control of recurrences. Although both cellular and humoral immunity are thought to be important, persons with defective cellular immunity often show severe HSV infections, while those with agammaglobulinemia do not (19). $CD4^+$ T cells infiltrate mucocutaneous lesions early in lesion development (20), followed by cytotoxic T lymphocytes (21). HSV-specific T cells can be detected early in lesion development and persist at the lesion site after healing (22, 23). T cells also play an important role in controlling viral reactivation from the dorsal root ganglia, via noncytotoxic mechanisms that do not result in neuronal death (24).

Functional antibody responses include complement-independent and complement-dependent viral neutralization (25). Immunoglobulin G (IgG), IgM, and IgA responses to individual viral proteins arise within the first weeks after infection (19). IgM wanes after 2 to 3 months, appearing sporadically thereafter, and in about a third of patients, after recurrent genital herpes episodes. Antibody titer may or may not rise after recurrences (26). However, IgG titers are maintained for years after primary infection.

Most epitopes are shared between HSV-1 and HSV-2. As a result, it is difficult to distinguish antibodies to HSV-1

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from those to HSV-2 (27). In particular, in HSV-1-seropositive patients, seroconversion to HSV-2 is accompanied by brisk anamnestic responses to HSV-1, resulting in the predominant antibody response being directed toward HSV-1 rather than to HSV-2 for prolonged periods (27). While type-specific epitopes have been demonstrated on the major viral proteins, only glycoprotein G (gG) elicits predominantly type-specific responses.

EPIDEMIOLOGY AND TRANSMISSION

HSV infections occur worldwide with no seasonal distribution. The virus is spread by direct contact with virus in secretions. Incubation periods range from 1 to 26 days. The prevalence of HSV-1 infection increases gradually from childhood, reaching 80% or more in later years (28). In contrast, the seroprevalence of HSV-2 remains low until adolescence and the onset of sexual activity. The incidence of antibodies to HSV-2 in the United States reached 21% in the period 1988 to 1994 (29); however, in more recent years seropositivity rates have apparently declined to 17% for the period 1999 to 2004 (30). The rate of seropositivity varies widely, reaching more than 50% in some demographic groups (30, 31). In general, the seroprevalence of HSV-2 is higher in the United States than in other developed countries (32, 33).

Importantly, a large percentage of individuals seropositive for HSV-1 and/or HSV-2 are unaware of the infection (29, 30). Such persons comprise an important reservoir of infection. The risk of genital HSV transmission has been reported to be reduced by 30 to 50% through the use of condoms (34, 35), and a more recent case-crossover analysis of previous study data suggests that the reduction of transmission may be substantially more than previously estimated (36). Transmission is also reduced approximately 50% by disclosure of HSV infection status to sexual partners (37) and by approximately 50% with suppressive antiviral therapy in the source partner (38). It is likely that combining these approaches further reduces risk.

CLINICAL SIGNIFICANCE

Primary Infection

Most HSV-1 infections are acquired early in childhood as subclinical or unrecognized infections (39). Young children may present with classic primary HSV-1 infection characterized by gingivostomatitis, fever, and marked submandibular lymphadenopathy. Oral lesions progress to ulceration and heal without scarring over 2 to 3 weeks. Adolescents may present with pharyngitis and mononucleosis.

Primary infection with HSV-2 classically presents as herpes genitalis, with extensive, bilateral vesicles, fever, inguinal lymphadenopathy, and dysuria (40). Lesions ulcerate and heal without scarring within 3 weeks. Secondary lesions may develop in the second to third week. Subclinical or unrecognized primary infection with HSV-2 is common.

The proportion of primary genital infections due to HSV-1 is increasing (41), from about 10% in 1983 to 32% in 1995 (40, 42), and now represents the major cause of first-episode anogenital infections at least some populations (43–45). In a large study of young women, 18 to 30 years of age, primary infection with HSV-1 was more than twice as common as infection with HSV-2 (45), and of clinically recognized primary HSV-1 infections, more than 75% were genital rather than oral. This trend is thought to result from changing sexual practices, including increased oral-genital exposure (43). The clinical presentation of primary genital HSV-1 cannot be distinguished from that of HSV-2. However, recurrent disease (see below) is less common with genital HSV-1 than HSV-2 (40, 46), and thus, determining the infecting virus type is useful for prognostic purposes.

Latency and Recurrent Disease

Primary infection with HSV-1 or HSV-2 is followed by the establishment of latency in the dorsal root ganglia, typically the trigeminal ganglia for orolabial disease and the lumbosacral ganglia for genital disease. Periodically, the virus reactivates and travels via the nerve axon to oral or genital sites, resulting in release of infectious virus and, in some cases, lesion formation. Recurrent disease has milder symptoms and a shorter time to lesion healing than primary episodes (39, 40). The frequency of HSV-2 genital recurrences varies widely among individuals, ranging from none to 12 or more per year (47), with a mean rate of 0.33 recurrences/month. Orolabial HSV-1 recurrence rates are lower, with a mean of 0.12 episodes/month, while genital HSV-1 infection occurs even less frequently (mean, 0.02 episodes/month) (48). While HSV-2 may be isolated from the pharynx during primary genital herpes episodes, orolabial HSV-2 recurrences are extremely infrequent (48).

Asymptomatic or Subclinical Infection

Approximately 70 to 90% of individuals with HSV-2 antibodies have not been diagnosed with genital herpes (29, 30). Over half of such persons recognize and present with symptoms after education regarding manifestations of HSV disease (49, 50). In addition, about 20% of patients presenting with first episodes of genital herpes have serologic evidence of having been infected for some time (51). These episodes most closely resemble recurrent disease, with relatively mild symptoms. The risk of recurrence is similar between patients presenting with true primary and first recognized recurrent episodes (26). Most patients with genital herpes have episodes of subclinical virus excretion from anogenital sites; these constitute an important source of transmission. The copy number of HSV DNA can sometimes be as high during subclinical episodes as when symptoms occur (52). In general, oral shedding of HSV-1 occurs somewhat less frequently than genital shedding of HSV-2 (53). Oral shedding of HSV-2, or genital shedding of HSV-1, are comparatively rare, particularly outside the setting of newly acquired disease (48, 53, 54).

Neonatal Herpes

The most serious consequence of genital HSV infection is neonatal herpes (55). Infection usually occurs during vaginal delivery when the infant is exposed to HSV in maternal secretions. The risk of maternal-to-infant transmission is 10-fold higher in mothers experiencing unrecognized primary infection during the time of labor than in those shedding HSV as a result of recurrent, subclinical reactivation. This conclusion was drawn in part from the finding that the neonatal infection rate is 54 per 100,000 among HSV-seronegative mothers, 26 per 100,000 among mothers with only HSV-1 antibody, and 22 per 100,000 among all mothers with HSV-2 antibody (56, 57). Transmission of HSV-1 occurs at a significantly higher rate than that of HSV-2 (57, 58). The high rate of transmission during primary disease means that 50 to 80% of all cases of neonatal herpes occur in children of women who acquire genital HSV infection near term (59, 60). Pregnant women who present with HSV infection should undergo both a type-specific serologic assay and viral typing to identify those infants at highest risk for infection (61).

Infected neonates can present with HSV disease localized to the skin, eyes, and mucosa or more serious central nervous system (CNS) or disseminated disease but often have nonspecific presentation (55, 62). The mortality rate for untreated neonates with disseminated disease exceeds 70%. Early diagnosis and antiviral therapy while disease is localized to the skin can substantially reduce the morbidity and mortality associated with neonatal infections (63, 64). PCR should be used to test samples from lesions, other mucosal sites, cerebrospinal fluid (CSF), and blood (55, 65–67). Although culture has continued to be advocated for surface specimens (68), the superior sensitivity of PCR for surface specimens is well established (52).

Herpes Central Nervous System Disease in the Immunocompetent Host

HSV is the most common cause of fatal sporadic encephalitis in the United States (69). HSV encephalitis presents as fever, behavioral changes, and altered consciousness, resulting from localized temporal lobe involvement. Without treatment, mortality exceeds 70%, and few survivors recover normal neurologic function. Early treatment with acyclovir reduces morbidity and mortality; however, residual neurologic impairment is common (70). HSV PCR of CSF is the test of choice (71, 72). Culture of CSF is not acceptable for diagnosis of HSV encephalitis.

Genital HSV may be followed by sporadic meningitis or recurrent meningitis (Mollaret's syndrome) characterized by headache, fever, photophobia, and lymphocytic pleocytosis (73, 74). The condition is self-limiting and typically resolves within one week (73). HSV also can be associated with myelitis, radiculitis, ascending paralysis, autonomic nerve dysfunction, and possibly Bell's palsy.

Systemic HSV Infection in Hospitalized Adults

HSV can sometimes be detected by PCR in the blood during primary HSV disease (75) and in recurrent herpes labialis (76). It can also occasionally be detected in the blood of hospitalized adults, either immunocompetent or immunocompromised (77, 78). Hospitalized patients with detectable HSV viremia frequently have clinical symptoms, including hepatitis, fever, CNS alterations, skin lesions, abdominal pain, or sepsis, although it has not been established that HSV is the cause of these symptoms. Nevertheless, hospitalized patients with detectable HSV viremia do have a high rate of mortality, suggesting a role for testing.

Ocular Herpes Infections

HSV is the most common viral cause of corneal infection in the United States, affecting 400,000 to 500,000 people (79, 80). Most corneal HSV infections are limited to the epithelial layer, causing characteristic branching ulcerations, pain, photophobia, and blurred vision. HSV epithelial keratitis responds well to oral or topical therapy (see below). Such superficial infections heal without loss of vision. HSV infection may extend to the corneal stroma, leading to scarring and opacification of the cornea. Stromal HSV infections respond favorably to a combination of corticosteroids and topical or oral antivirals (81, 82).

Herpes in the Immunocompromised Host

Immunosuppressed individuals with defective cell-mediated immunity frequently develop symptomatic HSV disease. HSV infections in such individuals can be severe, with extensive mucocutaneous necrosis and involvement of contiguous tissues leading to esophagitis or proctitis. Disseminated HSV, which can lead to meningoencephalitis, pneumonitis, hepatitis, and coagulopathy, may be more common in immunocompromised and hospitalized patients than is generally recognized (77, 83). Disseminated infections require intravenous antiviral therapy and monitoring for development of antiviral resistance.

Antiviral Therapy

Several antiviral drugs, including acyclovir, valacyclovir, penciclovir, and famciclovir, are now widely used to treat mucocutaneous and genital herpes (84, 85) and for long-term suppression of recurrent episodes. These drugs are selectively activated by the viral thymidine kinase and have minimal side effects. Suppressive therapy reduces the risk of viral transmission between HSV-discordant partners (38). *N*-Docosanol, a nonprescription topical medication, and topical acyclovir are used for the treatment of herpes labialis. HSV conjunctivitis, blepharitis, and dendritic keratitis are either treated topically with trifluridine, idoxuridine, or vidarabine or orally with acyclovir, valacyclovir, or famciclovir (81). In cases with stromal involvement, the addition of topical corticosteroids reduces the persistence of inflammation (81). Neonatal herpes, herpes encephalitis, and severe infections in immunocompromised patients require prompt intravenous antiviral therapy (86). The alternative agents foscarnet, cidofovir, and virdarabine are generally reserved for acyclovir-resistant herpes infections (85, 87). New compounds targeting the HSV helicase/primase complex (88) have shown efficacy in clinical trials (89) and are likely to provide additional therapeutic options in the future.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Samples for DNA amplification (PCR) require careful collection and handling, which are essential to ensure adequate specimen and avoid contamination of the sample with exogenous viral DNA. For lesions or mucocutaneous sites, a dedicated specimen should be collected with a Dacron swab in viral transport medium (VTM) or digestion buffer (90). Serum, plasma, and CSF do not require special handling. Blood from neonates is collected in lavender-topped tubes (EDTA) for PCR of peripheral blood mononuclear cells and plasma (65). Heparin can inhibit PCR and therefore is not an acceptable anticoagulant. Specimens for PCR can be maintained at 4°C for up to 72 h, but longer storage should be at −20°C (91, 92).

For culture, specimens from lesions or mucocutaneous sites should be placed in VTM and kept at controlled temperature to retain optimal infectivity. VTM is made of balanced salt solutions such as Hanks balanced salt solution, Stuart's medium, Leibovitz-Emory medium, veal infusion broth, or tryptose phosphate broth buffered to maintain a neutral pH. Protein stabilizing agents such as gelatin or bovine serum albumin are added as well as antibiotics to prevent bacterial overgrowth (93). Most VTM are inappropriate for bacterial or chlamydial transport. However, multimicrobe M4 media from Remel (Lenexa, KS) and universal transport medium (UTM) from Copan (Murrieta, CA) are suitable for viruses, *Chlamydia*, *Mycoplasma*, and *Ureaplasma*. It is important that laboratories validate the suitability of the selected media for their particular applications, as performance can vary. For example, in one study, M4 or the

UTM system from Copan supported detection of low-titer HSV better than other media (94).

Necrotic debris or exudate should be removed from mucosal sites (endocervix, anogenital areas, conjunctiva, and throat) or lesions with a cotton swab prior to sampling. The area must be vigorously rubbed with Dacron, rayon, or cotton swabs on aluminum shafts to ensure that infected cells at the base of the lesion are collected. Calcium alginate swabs and swabs with wooden shafts inhibit infectivity (93, 95). Swabs are then placed into VTM for transport. Several manufacturers offer combined swab/media packaging, such as the Copan UTM system and the Remel multimicrobe M4 system.

Samples should be shipped cold (on ample ice packs) but not frozen. Prolonged (>48 h) storage should be at −70°C. HSV stability is reduced significantly at 22°C compared with 4°C; in one study, median half-lives of virus in Copan UTM were 6.8 days (HSV-1) and 4.9 days (HSV-2) at 4°C but only 1.9 (HSV-1) and 3.8 (HSV-2) days at 22°C (94). With other transport media, reported half-lives of HSV-1 have ranged from 0.5 to 4 days at 22°C and from 1 to 180 days at 4°C (96).

A single transport vial may provide specimen for both viral culture and direct antigen detection tests. Alternatively, slides for direct florescent antibody detection can be prepared immediately after collection by gently spreading the material on a swab in a thin layer over a clean microscope slide. The slide is air-dried and fixed in cold acetone before transport.

Fluids such as tracheal aspirates should be collected aseptically and transported without VTM. Urine should be collected by clean catch and refrigerated before transport. Once in the laboratory, urine is diluted 1:1 with culture medium. Corneal samples are obtained with a scalpel blade, and cells are suspended in VTM. Tissue is placed in VTM in a sterile container. Prolonged storage of tissue requires immersion in sterile 50% neutral glycerol in saline or, alternatively, in culture medium with 5% fetal bovine serum. Fresh tissue samples can also be frozen, sectioned, applied to slides, and fixed in acetone.

DIRECT DETECTION

Detection of virus in patient samples without an intervening culture amplification step provides the most rapid diagnosis. PCR or other DNA amplification techniques provide the best sensitivity of the direct detection approaches and have become the method of choice for many laboratories. Immunostaining methods to detect antigen require less expertise than cytopathic effect (CPE)-based culture methods and are usually less expensive than culture or DNA amplification.

DNA Amplification

PCR or other amplification approaches are the most sensitive methods for direct detection of HSV (97). This is especially important in situations where sensitivity is critical, such as CSF, but recently, PCR has been increasingly adopted for anogenital and other specimen types as well. For many years, DNA amplification for HSV was available in the United States only through laboratory-developed tests. In 2010, however, the FDA granted the first of several clearances to DNA-based HSV tests, and the availability of FDA-cleared assays has accelerated the trend from culture toward DNA testing.

Whether testing is done in an FDA-cleared or laboratory-developed test format, precautions to prevent contamination of the sample and the inclusion of frequent negative controls within the assay are critical (98, 99). Some laboratories include isopsoralens or uracil-*N*-glycosylase in their PCR amplifications, preventing the amplification of PCR products in subsequent reactions. The likelihood of laboratory contamination by amplicon is reduced by using real-time PCR detection systems that eliminate the need for postamplification manipulation of the PCR product. Laboratories performing PCR testing should participate in a regular proficiency testing program, such as those provided by the College of American Pathologists (www.cap.org) or Quality Control for Molecular Diagnostics (www.qcmd.org).

Depending on the primers and detection methods, PCR can be set up to detect both HSV-1 and HSV-2 (90, 100), or to allow distinction of HSV-1 from HSV-2 (90, 101). PCR primers have been described for amplifying portions of HSV genes encoding thymidine kinase (U_L23); DNA polymerase (U_L30); DNA binding protein (U_L42); glycoproteins B, C, D, and G (U_L27, U_L44, U_s6, U_s4, respectively); and many others (102). The choice of target gene is probably not a critical factor for HSV PCR, and efficient and sensitive assays have been developed using a variety of target genes. Instead, laboratories should ensure that the primers and probes in any contemplated assay follow the principles of efficient PCR assay design (103). Distinction of HSV-1 and HSV-2 can be achieved using type-specific primers or probes, melting curve analysis, restriction enzyme analysis, or direct sequencing. At our institution, we detect both HSV-1 and HSV-2 by PCR using a real-time detection system (see below) with type-common gB primers and probe (92). To differentiate HSV-1 from HSV-2, we use a multiplex PCR assay containing type common gB forward primers that amplify both viruses with equal efficiency. Differentiation is achieved using an HSV-1 probe labeled 5′ with VIC and 3′ with TAMRA (6-carboxytetramethylrhodamine), and an HSV-2 probe labeled 5′ with FAM (6-carboxyfluorescein) and 3′ with TAMRA (104). Test results are available on the same day as receipt of specimen.

Traditional methods to detect PCR products include intercalating agents such as ethidium bromide, Southern blot hybridization, and liquid hybridization. A major improvement was the development of HSV assays using real-time detection of PCR products (90, 105–108). Real-time systems allow constant monitoring of the amount of PCR product by detection of a fluorescent signal generated as PCR product accumulates. These systems decrease the likelihood of laboratory contamination. Laboratory contamination is of particular concern for CSF specimens, where reliable detection of low viral loads is of major clinical significance, and can occur when such specimens are processed together with lesion or other specimens with high viral load. Careful technique and systematic monitoring of negative controls is essential.

Real-time PCR allows more accurate quantification than is possible with older quantitative-competitive assays (90). Quantitation of virus may be useful in monitoring response to antiviral therapy, particularly in HSV encephalitis or neonatal herpes infections (109). Using these or other primer/probe sets, a large number of labs offer in-house validated assays with real-time detection and quantitation.

Despite the lack of FDA-approved tests, PCR is the gold standard for detection of HSV in CSF, neonatal, and ocular specimens and is increasingly used with other sample types as well. Substantial differences in performance have been reported in interlaboratory comparisons, particularly in sensitivity for detection of low-positive specimens (110). There is no consensus on the required sensitivity for CSF testing, and interlaboratory differences are exacerbated by the current lack of an international reference standard for HSV. Thus, it is important to carefully consider the laboratory's reputation, performance data, and test turnaround times before selecting a provider of DNA amplification testing.

As stated above, only recently have commercial molecular assays for HSV achieved FDA approval (Table 1), while CE-marked assays have been available in Europe for several years. Importantly, none of the commercial assays are FDA-cleared for use with CSF specimens. The Eragen Multi-Code-RTx HSV 1 & 2 kit was FDA-cleared in 2010 for qualitative detection and typing of HSV in vaginal lesion swabs from symptomatic female patients only and is now available from Luminex Corp (Austin, TX). The assay is based on PCR amplification using a synthetic isoC:isoG DNA base pair. It was initially cleared for use on samples extracted using the Roche MagNA Pure LC instrument and amplified on the Roche LightCycler real-time PCR instrument (Basel, Switzerland). FDA clearance was subsequently obtained for specimens extracted using the bioMérieux NucliSens easyMag extraction system.

The ProbeTec herpes simplex viruses (HSV 1 & 2) Q^X amplified DNA assays (BD, Franklin Lakes, NJ) use strand displacement amplification for the qualitative detection and differentiation of HSV-1 and -2. The assays are performed on the fully automated BD Viper system in extracted mode and were FDA cleared in 2011 for testing of clinician-collected external anogenital lesion specimens. In a large head-to-head comparison, the assay proved to be far more sensitive than culture and had a specificity and sensitivity of 95.1 to 100% compared to the University of Washington PCR assay (111).

Finally, BioHelix Corp (Beverly, MA) offers the IsoAmp HSV assay, which was FDA cleared for detection of HSV-1 and -2 in genital and oral lesion specimens from symptomatic patients. The IsoAmp HSV assay is based on a helicase-dependent amplification technology and a unique single-use handheld disposable detection device, allowing rapid (1.5 h) turnaround time. The assay does not differentiate between HSV-1 and -2. In a comparison with viral culture, the IsoAmp HSV assay had a sensitivity of 100% and a specificity of 96.3%; the analytical sensitivities for HSV-1 and HSV-2 were 5.5 and 34.1 copies per reaction, respectively (112).

Other Nucleic Acid-Based Tests

In situ hybridization or solution hybridization methods are not as widely used as DNA amplification, indirect fluorescent antibody assay, or direct fluorescent antibody assay (DFA). These tests use DNA or RNA probes, some of which are type specific. The sensitivity of direct hybridization methods is limited (approximately 1×10^5 copies/ml), and thus, these approaches have been largely replaced by PCR or other amplification methods.

Microscopy

Changes that are characteristic for HSV are sometimes visible in fixed, stained cells from lesions (Tzanck preparations) or cervical scrapings (Papanicalou stains) or hematoxylin and eosin stains of fixed tissue (113). Enlarged or degenerating cells, syncytium formation, chromatin margination, a "ground glass" appearance of the cytoplasm, and nuclear inclusions are typical of HSV-infected cells. These methods are widely available but lack sensitivity and specificity (114). Virus-specific methods are preferred.

Antigen Detection

Slides are prepared with cells from the patient's specimen, fixed, and coated with antibody preparations against HSV-1 and HSV-2 or against type common epitopes. Some manufacturers offer dual anti-HSV and anti-varicella-zoster virus (anti-VZV) reagent combinations. If the antibodies are linked to a fluorophore such as fluorescein isothiocyanate, the test is a DFA. If bound anti-HSV antibodies are detected with secondary antibodies conjugated to fluorophore, the test is an indirect fluorescent antibody assay. Same-well testing can be performed using antibody conjugates with different fluorophores for HSV-1 and HSV-2 (115). DFA is 10 to 87% as sensitive as culture with higher sensitivity from vesicular lesions and poor sensitivity from healing lesions (48). DFA is far less sensitive than PCR (116), and validated commercial kits for DFA are not readily available.

TABLE 1 FDA-cleared molecular amplification tests for HSV

Test	Source	Amplification technology	Approved specimen types	Qualitative or quantitative	HSV typing?	Instrumentation
Multi-Code-RTx HSV 1 & 2 kit	Luminex, Austin, TX	PCR	Vaginal lesion swabs from symptomatic female patients	Qualitative	Yes	Extraction using Roche MagNA Pure LC or bioMérieux NucliSens easyMag; amplification on Roche LightCycler
ProbeTec herpes simplex viruses (HSV 1 & 2) Q^x amplified DNA assays	BD, Franklin Lakes, NJ	Strand displacement amplification	Clinician-collected external anogenital lesion specimens	Qualitative	Yes	BD Viper System in extracted mode
IsoAmp HSV assay	BioHelix Corp., Beverly, MA	Helicase-dependent amplification	Genital and oral lesion specimens from symptomatic patients	Qualitative	No	Single-use handheld disposable detection device

An effective modification of DFA employs a cytocentrifuging step to apply cells from the sample to slides for subsequent DFA testing. The cytospin step results in better adherence of cells to the slides and fewer inadequate specimens than drip application methods (117) while allowing for a less-than-2-h turnaround time. The reported sensitivity of cytospin DFA has varied widely; in one study, it was higher than culture for detecting HSV from swab specimens (117), while another study, using different DFA antibody and cell culture systems, reported sensitivity of 31% versus culture (118). The Light Diagnostics SimulFluor assay (Millipore, Billerica, MA) has been reported to have a sensitivity of 80.0% and a specificity of 98.8% compared to culture (115) and a sensitivity of 86.2% in a multimethod comparison including two PCR-based assays (119).

Antibodies give their own distinct patterns depending on the viral antigens recognized (Table 2). DFA can vary from lab to lab, depending on sample quality, the method of preparation of slides, the reagent used, and other factors. Staining patterns require skill to interpret; nonspecific staining needs to be distinguished from staining of viral antigen by a trained reader. It is important to ensure that antibody reagents are validated for use directly on clinical samples; some antibodies intended for culture confirmation can give spurious results in other settings.

Enzyme immunoassay (EIA) is rapid and amenable to automation. Sensitivity can be as good as culture for diagnosis from lesions (120) or as low as 35% in asymptomatic patients (121, 122). EIA for direct detection of virus has been largely supplanted by other methods.

ISOLATION PROCEDURES

Virus detection methods, including culture and modified culture, are used to diagnose mucocutaneous, genital, and ocular lesions (Table 3). Conventional culture uses cells that are permissive for HSV-1 and HSV-2 as well as other viruses that may be of diagnostic importance. Inoculated cells are examined frequently for CPE (see below). In our laboratory, 95% of HSV isolates produce CPE by day 5; only 5% require 5 to 14 days. Mink lung cells, rhabdomyosarcoma cells, human diploid fibroblasts such as MRC-5 and WI-38, and human epidermoid carcinoma lines such as Hep-2 and A549 are commercially available. Direct comparisons suggest that test sensitivity can be markedly affected by the cells used, particularly with a low-titer HSV inoculum. Mink lung cells are reportedly more sensitive than MRC-5 cells, while Vero cells have had low sensitivity reported even with high-titer isolates (123).

Infected cells develop cytoplasmic granulation and then become large, round, and refractile. Clusters or "foci" of infected cells appear early after inoculation in diploid fibroblast or epidermoid carcinoma lines (Fig. 1). CPE in A549 cells is characterized by syncytium formation, particularly with HSV-2 (124). Cells then lyse and detach from the substrate with eventual destruction of the monolayer.

IDENTIFICATION AND TYPING

Other viruses, as well as toxic factors in specimens, can mimic the CPE of HSV (Fig. 2A). Confirmation of putative HSV CPE is accomplished using commercially available polyclonal or monoclonal antibodies against type common or type-specific epitopes. The most widely used reagents are fluorescein conjugated for use in fluorescent antibody tests (Table 2 and Fig. 2B). Trinity Biotech (Bray, Ireland) and Diagnostic Hybrids, Inc. (Athens, OH), both supply these reagents. SimulFluor HSV1/2 (Millipore, Billerica, MA) can detect and type HSV from a single sample. Peroxidase-conjugated antibodies against HSV-1 and HSV-2 are also used in *in situ* EIA formats (123).

Spin amplification methods reduce the time required to detect HSV in culture by detecting viral proteins before CPE develops (123). The most widely used technique involves centrifuging the sample onto monolayers of cells on coverslips that are placed in the bottom of small vials ("shell vials") or in the wells of flat-bottomed plates. After 16 to 48 h of incubation, the coverslips or plates are subjected to antigen detection tests for HSV (115). When compared to standard culture, spin amplification sensitivity ranges from 71 to 97% (125–127). Sensitivity is affected by time of incubation before staining with 48 to 72 h yielding more positives than 16 to 24 h. The cell line also affects sensitivity, with mink lung or A549 cells having greater sensitivity than MRC-5 cells (125).

The ReadyCells HSV system (Diagnostic Hybrids, Inc., Athens, OH) is a mixture of cryopreserved CV-1 and MRC-5 cells (128) in a shell vial format supporting growth of HSV-1 and HSV-2. Combined with immunodetection, this approach allows rapid definitive diagnosis and differentiation of HSV-1 and HSV-2 from lesions.

TABLE 2 Monoclonal antibodies for HSV-1 and -2 detection, confirmation, and typing by immunofluorescence

Reagent	Source	Intended use	Comments
Light Diagnostics SimulFluor HSV 1/2	Millipore, Billerica, MA	Confirmation of CPE in cell culture; direct examination of clinical specimens prepared by cytospin	HSV-1: whole-cell stain (apple green); HSV-2: cytoplasmic or membrane stain (yellow); simultaneous testing for both HSV-1, HSV-2
Light Diagnostics SimulFluor HSV/VZV	Millipore, Billerica, MA	Confirmation of CPE in cell culture; direct examination of clinical specimens prepared by cytospin	Simultaneous testing for HSV-1, HSV-2, and VZV
Light Diagnostics HSV1 and HSV2 DFA typing kit	Millipore, Billerica, MA	Differentiation of HSV-1 and HSV-2 in direct specimens and cell culture	Requires separate spots for HSV-1 and HSV-2
D^3 DFA identification and typing kit	Diagnostic Hybrids, Athens, OH	Confirmation of CPE and typing in cell culture	HSV-1: strong focal nuclear staining; HSV-2: strong nuclear and perinuclear staining
MicroTrak HSV 1 & 2 culture identification/typing test	Trinity Biotech, Jamestown, NY	Confirmation of CPE and typing in cell culture; antigen detection prior to CPE by shell vial	HSV-1: nuclear staining; HSV-2: strong nuclear stain, with speckled cytoplasmic staining

TABLE 3 Selected diagnostic tests for HSV

Syndrome	Sample	Lab Tests	Comment(s)
Oral or genital lesion	Swab in VTM	Culture, shell vial	Viability must be preserved
		DFA, cytospin DFA	Cell architecture must be maintained
		PCR	Better stability than culture
Recurrent genital symptoms; culture-negative	Serum	Western blot	Limited availability; good confirmatory test
	Serum	gG-specific ELISA	FDA approved
	Capillary blood	gG-based point-of-care tests	FDA approved
	Swab in VTM	PCR	Better sensitivity than culture
Neonatal herpes	CSF; blood in EDTA	PCR	Collect separate CSF sample for PCR
	Eye, mouth, nasal, and rectal swabs	Culture, DFA, PCR	Blood in EDTA (purple top)
Ocular herpes	Swab in VTM	PCR, culture, DFA	Clinical presentation may be sufficient for diagnosis
Conjunctivitis, dendritic corneal ulcers	Corneal scraping	PCR, culture, DFA	
Encephalitis	CSF, brain tissue	PCR	Culture of CSF should not be performed
Recurrent lymphocytic meningitis	CSF	PCR	Culture is far less sensitive than PCR and is not recommended

The enzyme-linked virus inducible system (ELVIS) is based on BHK cells with a reporter gene for β-galactosidase that is driven by the promoter from the HSV-1 UL39 gene (129). Other viruses do not transactivate this promoter. Infected cells express the reporter gene, resulting in a color change visualized by light microscopy. The ELVIS HSV test system (Diagnostic Hybrids) allows HSV-positive cells to be further tested to distinguish HSV-1 from HSV-2. ELVIS-based systems can have comparable sensitivity to standard culture (130) and spin-amplified cell culture (131).

SEROLOGIC TESTS

Because nearly all HSV structural proteins have extensive antigenic cross-reactivity, only IgG tests based on the type-specific HSV glycoprotein gG accurately distinguish HSV-1 and HSV-2 antibodies (27, 132, 133). Tests based on crude antigen mixtures are still marketed but have unacceptably low sensitivity and specificity, especially for detecting new HSV-2 infections in those with prior HSV-1 (27, 132, 134, 135). Type-specific IgM tests are not available. FDA-approved IgM tests decrease the time to detecting seroconversion in new infections but cannot accurately distinguish new from established infections (136). IgM-based tests can also be prone to false-positive results (137, 138) and thus should be confirmed by other approaches. Tests for HSV-2 antibody avidity can discriminate accurately between first episodes (low avidity) and recurrent episodes (high avidity) in most cases (139). However, these tests are not commercially available.

Western Blot

The HSV Western blot (WB) used by the University of Washington laboratory uses nitrocellulose blots prepared with human diploid fibroblast-infected cell proteins. WB detects antibodies to multiple viral proteins, including those to the type-specific glycoproteins gG-1 and gG-2 (140). About 20% of sera must be preabsorbed against Sepharose beads coated with HSV-1 or HSV-2 proteins and retested to give definitive results. This combination of tests gives a very accurate determination of HSV-1 versus HSV-2 antibody status (140) and is considered the gold standard for comparison of other serologic assays.

Commercial Type-Specific gG-Based Assays

A number of manufacturers now offer FDA-approved assays using gG as the target antigen. As noted above, gG-based assays are preferred over those based on crude antigen mixtures, due to their ability to distinguish antibodies to HSV-1 versus HSV-2. Microplate format EIAs include the HerpeSelect enzyme-linked immunosorbent assay (ELISA) from Focus Diagnostics (Cypress, CA), the Captia HSV 1 and HSV 2 IgG type-specific ELISA test kits (Trinity Biotech, Bray, Ireland), the anti-HSV-1 and anti-HSV-2 ELISA IgG kits from Euroimmun (Lubeck, Germany), and the Zeus ELISA HSV-1 and -2 test systems (Raritan, NJ). The Roche Elecsys HSV-1 IgG and HSV-2 IgG assays are FDA-cleared for use on several automated immunoassay instruments. Some newer assays utilize microparticle-based detection technology, which provides the possibility of multiplexing and offers potential advantages in assay throughput and reproducibility. The Plexus HerpeSelect 1 and 2 IgG test kit (Focus, Cypress, CA), the BioPlex 2200 HSV 1 and 2 kit (Bio-Rad), and the AtheNA Multi-Lyte HSV-1&2 (ZEUS Scientific, Raritan, NJ) utilize Luminex detection technology (Austin, TX). The LIAISON HSV-1 and HSV-2 type-specific IgG tests are bead-based chemiluminescent immunoassays requiring the use of the Liaison analyzer (DiaSorin, Saluggia, Italy). Finally, simple gG-based lateral flow assays are available that are designed for point-of-care testing. These include the HerpeSelect Express IgG (Focus Diagnostics), which distinguishes antibodies to HSV-1 and HSV-2, and the Biokit HSV-2 rapid test (Biokit, Barcelona, Spain).

The commercial gG-based tests perform well in clinical use in North America. When compared with the University of Washington WB, HerpeSelect ELISA had 91 to 96% sensitivity with 92 to 95% specificity for HSV-1 and 96 to 100% sensitivity with 96 to 97% specificity for HSV-2. HerpeSelect Immunoblot had 99 to 100% sensitivity with 93 to 96%

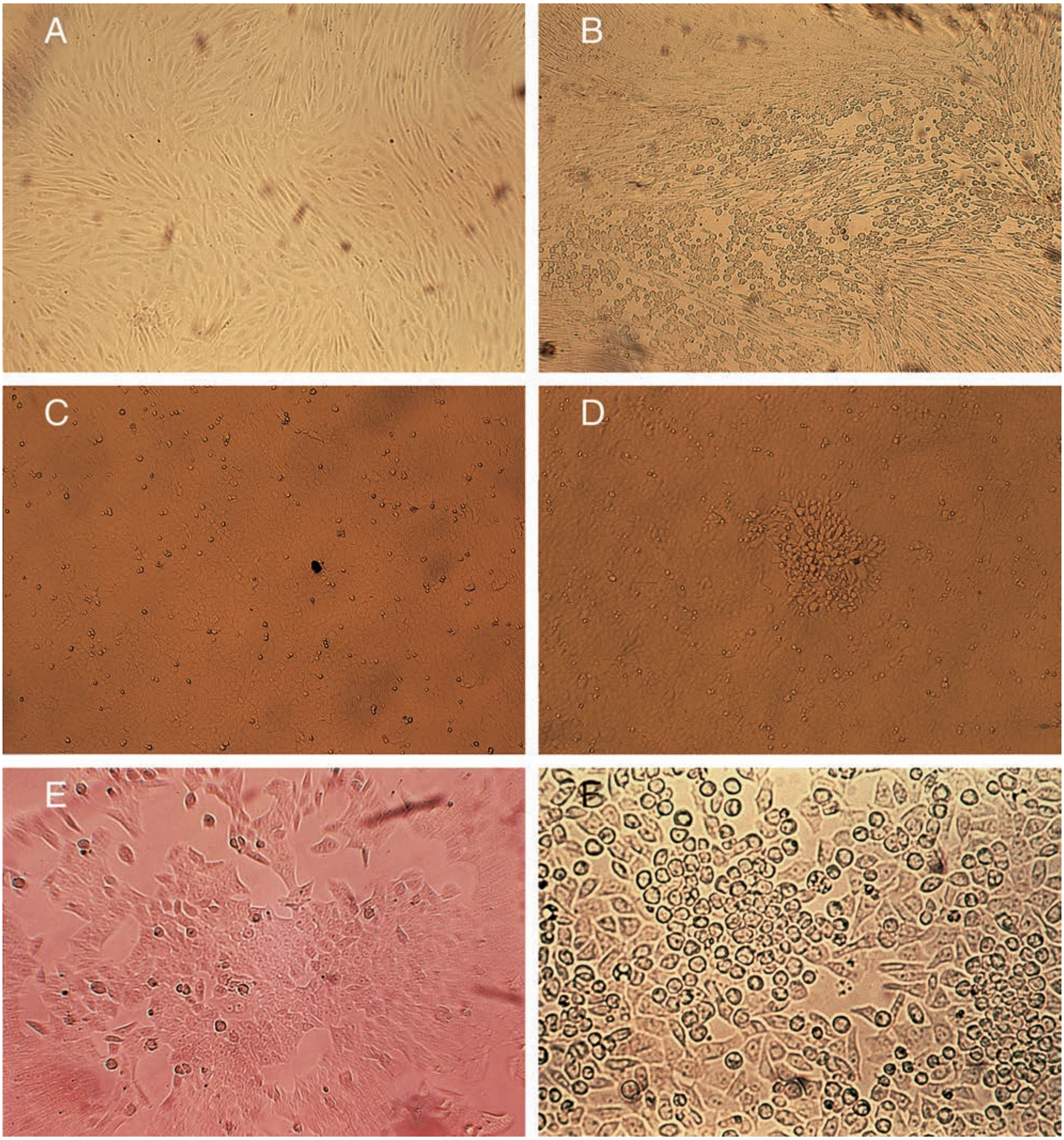

FIGURE 1 (A) Normal human diploid fibroblasts. Magnification, ×400. (B) HSV-1 in human diploid fibroblasts. Magnification, ×400. (C) Normal mink lung. Magnification, ×100. (D) HSV-2 in mink lung cells. Magnification, ×100. (E) Normal Hep-2 cells. Magnification, ×400. (F) HSV-2 in Hep-2 cells. Magnification, ×400. doi:10.1128/9781555817381.ch98.f1

specificity for HSV-1 and 97 to 100% sensitivity and 94 to 98% specificity for HSV-2 (133). The BioPlex assay had sensitivities and specificities of 85 and 98% for HSV-1 and of 100 and 95% for HSV-2 when compared to WB (136). The Luminex-based assays show good agreement with the Focus HerpeSelect EIA: 94.9% for the AtheNA Multi-Lyte HSV-1&2, 97.8% for the BioPlex 2200 HSV 1 and 2 kit, and 97.4% for the Plexus HerpeSelect 1 and 2 IgG test kit (141). For point-of-care tests, the POCkit-HSV-2 test (now Biokit HSV-2 rapid test) had 93 to 96% sensitivity with 95 to 98% specificity in comparison tests (133). While performance characteristics against WB are not available, the HerpeSelect Express IgG assay had 100% sensitivity and 97.3% specificity when compared to the HerpeSelect ELISA (142). The median time to seroconversion is approximately 2 to 3 weeks for HerpeSelect HSV ELISA and the Biokit HSV-2 rapid test, the only FDA-approved tests for which extensive data are available (143, 144).

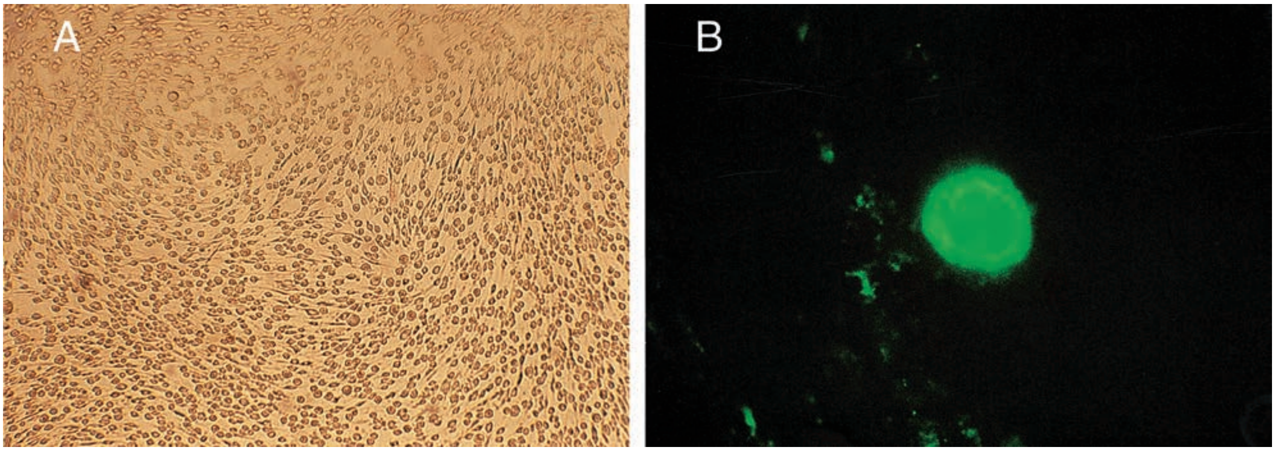

FIGURE 2 (A) Toxicity in human diploid fibroblasts. Magnification, ×100. (B) HSV-2 direct fluorescent antibody confirmatory test. doi:10.1128/9781555817381.ch98.f2

ANTIVIRAL SUSCEPTIBILITIES

Acyclovir-resistant strains of HSV typically result from mutation of the gene encoding the viral thymidine kinase and, more rarely, from mutations in the HSV polymerase. Prolonged use of antivirals can lead to selection of resistant strains, especially in immunocompromised individuals and neonates (145). Strains with detectable *in vitro* resistance can also be isolated from patients who have never received acyclovir (146). Such isolates are rarely of clinical significance in immunocompetent individuals, and these persons generally respond well to acyclovir (147). However, resistance can occasionally be observed in immune-privileged sites even in immune-competent individuals (148). Acyclovir-resistant HSV can lead to treatment failure (147), and thus laboratory confirmation of resistance can inform patient management decisions (149). Some patients with suspected acyclovir-resistant virus respond to an increased dosage of the drug. For most patients, second line therapy requires the use of less desirable drugs such as foscarnet or cidofovir. However, since some mutations in the HSV polymerase cause acyclovir resistance but do not affect sensitivity to penciclovir, testing for susceptibility to individual antivirals may be warranted in certain circumstances (145), as it may allow use of a less toxic drug.

Susceptibility testing can be performed by genotyping or phenotypic assays. A major drawback of genotypic assays is that frameshift or nonsense mutations are possible throughout the thymidine kinase gene, requiring complete sequencing of the gene for definitive results (145). Among phenotypic assays, plaque reduction is considered the gold standard (145, 147). Alternative phenotypic assays include antigen reduction by EIA and genome reduction by DNA hybridization (145). In general, a 50% inhibitory concentration of <2 μg/ml is used as the threshold for susceptibility and correlates relatively well with clinical response (150). However, false determinations of resistance are common in the various phenotypic assays, and interlaboratory variability is significant (145). Thus, clinical correlation of testing results is essential.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Laboratory results are best interpreted in the context of the patient's presentation and history and with the knowledge of the natural history of HSV infection.

Interpretation of Virus Detection Tests

Interpretation of positive test results depends upon the specificity of the test. Specificity of culture is extremely high if confirmatory tests are performed. *In situ* techniques for antigen detection (FA or immunoperoxidase staining of infected cells) have better specificity than other antigen detection methods because the staining pattern allows the technologist to confirm that the signal is specific (Table 2). Given its extreme sensitivity, PCR is quickly becoming the test of choice in many situations. PCR is most reliable when closed system methods are used and lab practices are strictly met to avoid contamination of specimens with exogenous HSV DNA. Given these caveats, in practice, positive culture or PCR results are highly diagnostic in patients with lesions due to gingivostomatitis, genital herpes, or ocular infections. Positive FA tests from lesions or ocular swabs are considered reliable. HSV found in the CNS, tissues, blood, or the eye by culture or PCR is diagnostic. Positive cultures or positive PCR tests in symptomatic infants at 1 to 3 weeks of life are highly diagnostic (151).

For other patients, interpretation of a positive viral detection test requires caution. One potential confounding issue is the simultaneous presence of multiple potential pathogens. For example, respiratory secretions from immunocompromised patients may contain HSV in addition to the presumably causative agent such as respiratory syncytial virus, influenza, parainfluenza, or adenovirus (152, 153). HSV may also be shed concurrently with other pathogens from oral or anogenital sites or may not be the cause of the episodic syndrome that triggered testing. Syphilis and chancroid are the most likely alternative causes of genital ulcers in the United States. In a series of patients with genital ulcers other than those typical of herpes (vesiculopustular lesions), 65% had only herpes detected while 20% had both syphilis and herpes detected (154). HSV results from such patients need to be interpreted in the context of laboratory testing for other pathogens in the differential diagnosis. Symptoms of HSV gingivostomatitis or genital infection can be mimicked by noninfectious causes, such as Stevens-Johnson syndrome, or by other infectious agents. Because of the sporadic nature of asymptomatic HSV shedding (155), swabs of oral or anogenital sites can also yield positive HSV culture, PCR, or antigen tests when the underlying cause of disease is not herpes.

Positive cultures or PCR results from skin, conjunctival, mouth, or nasal swabs from neonates less than 24 h old may reflect maternal virus rather than neonatal infection. Neonates born to mothers shedding HSV from the genital tract at labor and delivery may be tested 24 to 72 h after birth, the likely time frame for receiving maternal culture or PCR results. Most babies developing neonatal herpes present between 9 and 14 days of life (156), well beyond the time frame where maternal viral contamination would be a problem. However, early initiation of therapy is strongly associated with a favorable outcome (157), and thus, current opinion favors aggressive monitoring and treatment (55).

False-negative PCR, culture, or DFA tests may occur because of poor technique in sampling, resulting in the failure to obtain virus or infected cells. For culture, false negatives can result because of prolonged transport time, exposure of samples to high ambient temperature, or because the specimen has low levels of virus; these are less problematic for PCR testing. Recurrent herpes episodes yield lower levels of virus for shorter periods (40). In many cases, a combination of tests or repeated testing is the most sensitive approach. Performing both culture and FA can increase sensitivity by 20% or more from genital lesions; repeating testing at a later recurrence nearly doubles the sensitivity (158). PCR is the most sensitive test available and is the test of choice for herpes encephalitis, disseminated neonatal herpes, and increasingly, diagnosis of anogenital herpes (52, 159). However, in areas without local PCR testing, direct or indirect FA methods and culture may yield more timely results. Antiviral therapy should be started empirically if disseminated herpes, neonatal herpes, or encephalitis is suspected.

PCR has adequate sensitivity to detect shedding of HSV in individuals with asymptomatic infection or negative cultures (160–162) and also to detect HSV in CSF, a specimen with a low yield of HSV by culture. The sensitivity of HSV PCR for HSV encephalitis has been reported to be about 96% and the specificity 99% (163). Since the viral load in CSF can be quite low in HSV encephalitis, it is critical that PCR assays are optimized to provide the best possible limit of detection. Importantly, none of the commercial molecular tests for HSV have been approved for use on CSF. PCR has much greater sensitivity than culture for detection of genital HSV-2 both from lesions and during periods of subclinical shedding (52). PCR can detect virus early in lesion development before culture positivity and can remain positive for several days after HSV lesions become negative by culture (160, 164). PCR positivity in such cases probably represents infectious virus below the limit of detection by culture, and PCR positivity is quickly lost in the absence of active shedding (161). False negatives can occur in PCR, due to reaction failure or the presence of inhibitors in clinical specimens (165–167). It is therefore critical that only negative reactions accompanied by positive internal controls be accepted as true negatives (168). In addition, PCR false negatives can occur due to sequence variation in the primer- or probe-binding regions (119). Importantly, a negative PCR result for HSV does not rule out HSV disease, since specimens taken very early or late in the course of disease may not have viral DNA (169). This is especially true in pediatric populations; for example, in one series, nearly 25% of infants with CNS HSV infections had negative CSF PCR results (151). The specificity of PCR for detection of HSV encephalitis has been reported to be greater than 98% (170); thus, all HSV-positive PCR results demand immediate attention. In patients with CNS HSV infections, HSV PCR may become negative after about 7 days of antiviral therapy (163). The persistence or reemergence of virus after antiviral therapy has been associated with a poor clinical outcome (171).

Quantitative PCR may be useful in monitoring the response to antiviral therapy. Successful therapy is associated with a decline in viral load in the CSF (109, 166, 172, 173), and a long duration of viral detectability is associated with poor outcome (174). Quantitation of HSV may also have prognostic value. Patients with higher viral loads have been reported to have worse clinical outcomes (175); in one study, those with >100 copies/μl (100,000 copies/ml) of CSF had worse outcomes than patients with lower levels (173). However, other groups have reported no association between CSF viral load and clinical outcome (166, 172, 174, 176).

Interpretation of Type-Specific Serology

Type-specific serology is useful when culture or other virus detection methods are not available, when specimen collection or transport is inadequate, or for evaluation of serostatus in the absence of clinical disease. Serology based on gG-1 and gG-2 is vital for diagnosing subclinical and unrecognized HSV infections. In fact, many experts believe that better recognition of genital HSV-2 infections by increased use of accurate gG-based serologic tests could help slow the spread of genital herpes (177–179).

As with other herpes laboratory tests, serology tests based on gG must be ordered and interpreted with care. First, due to the low specificity of non-gG-based commercial tests, it is important to ensure that a gG-based test is used. If so, a positive test for HSV-2 antibodies in a patient with genital lesions is highly likely to be a true positive. A positive HSV-2 antibody test in a patient without a history compatible with genital herpes may be a false positive and should be confirmed by testing with a different type-specific test (180). Negative HSV-2 test results in a patient with symptomatic genital disease may indicate genital HSV-1. No test can distinguish between antibodies elicited by oral versus genital HSV-1, and definitive diagnosis in such cases rests on direct detection of virus. False-negative test results may also occur during seroconversion; sera drawn 4 to 6 weeks later should also be tested. While “index values” in HSV-2 EIAs have been shown to rise during seroconversion, index values alone are not reliable indicators of early versus established infection (181). IgM tests based on gG-1 and gG-2 are not available commercially. The available IgM tests cannot distinguish new from established symptomatic episodes with sufficient accuracy and are not recommended (136).

As noted above, type-specific serology is critical for identifying pregnant women with new HSV infections, who are at high risk for transmitting virus to their neonates (55, 179). Determining HSV-2 serostatus early in pregnancy has been recommended so that treatment options can be considered (179). If a herpes culture from the anogenital region is positive at labor or delivery, a negative maternal type-specific serology indicates high risk (30 to 50%) for neonatal herpes. A positive maternal serology by Western blot or equivalent test indicates a lower risk of transmission (1 to 3%).

HERPES B VIRUS

Description of the Agent

Herpes B virus, also known as Macacine (formerly Cercopithecine) herpesvirus 1 or monkey B virus, is similar to HSV in terms of genome size and structure and in terms of virion morphology. Herpes B virus DNA has 161 ± 12 kbp (182) and is extremely (approximately 75%) G+C rich.

The replication cycle of herpes B virus is very similar to HSV. Herpes B virus is detectable as soon as 6 h postinfection, and titers of virus stabilize by 24 to 36 h (183). At least nine herpes B virus glycoproteins have been identified, and at least two of these share antigenic determinants with glycoproteins B and D of HSV (184). As an enveloped virus, herpes B virus can be inactivated by lipid solvents, UV light, or heat, and thus, cell-free virus is inactivated rapidly in the environment.

Clinical Significance

Herpes B virus is the simian counterpart to HSV in Old World monkeys of the genus *Macaca*, including the rhesus, cynomolgus, Japanese, Taiwan, and stump-tail macaques. The virus can affect the oropharynx or genital areas and is spread between animals via biting, sexual activity, or other close contact (185, 186). The seroprevalence of herpes B virus among adult macaques in the wild is nearly 75% (187). Among animals housed in outdoor breeding corrals, the seroprevalence is approximately 22% before 2.5 years of age, rising to more than 97% among animals 2.5 years or older (188). Primary infection in the macaque is often asymptomatic, but the virus establishes latency and can reactivate. Reactivation can be triggered by stress, such as the transition of animals from freedom to captivity, or crowded conditions. Recurrent disease in macaques is typically characterized by vesicular lesions of the tongue and buccal mucosa, progressing to ulceration. Encephalitis is rare but can occur. Asymptomatic shedding of virus can occur.

In 1932, a researcher was bitten by a macaque and developed ascending myelitis and encephalitis culminating in death. Herpes B virus was subsequently described (189). Sporadic cases of herpes B virus infection in humans have subsequently been described in the literature. Humans can be infected via animal bites, mucosal or eye exposure (190), inoculation of broken skin, needle sticks, or potentially, via aerosols. The possibility of herpes B virus transmission makes macaques unsuitable as pets (191). Safety precautions for workers with close contact with macaques and recommendations for postexposure management have been published (192). Primary cell cultures from macaques also represent a potential source of virus (192).

Herpes B virus disease is severe in humans with a mortality rate of 70% or higher without treatment. The first symptoms usually appear 3 to 5 days after exposure (although they can appear up to several weeks later) and include a localized vesicular lesion at the site of inoculation, erythema, and edema. Lymphangitis and lymphadenopathy follow, with fever, myalgia, vomiting, and cramping. Neurologic signs develop quickly, starting with meningeal irritation, diplopia, and altered sensation, progressing to paralysis, altered mentation, respiratory depression, seizures, and death within 10 days to 6 weeks. Human-to-human transmission appears to be extremely rare (193). Asymptomatic infection in humans also appears to be rare. In one study of over 300 primate handlers (of whom more than 150 had a history of exposures), none had antibody to herpes B virus (194).

Acyclovir is effective against herpes B virus, although the 50% effective dose is 10-fold higher than for HSV. Ganciclovir is somewhat more effective than acyclovir (195), although clinical experience with ganciclovir is limited. Foscarnet has also been used in some cases (190). Postexposure therapy can often prevent the development of acute disease (196). The preferred drug for postexposure prophylaxis is oral valacyclovir (192), due to its improved bioavailability (192). Clinical disease necessitates intravenous antiviral therapy. Intravenous therapy should be continued until symptoms resolve and two or more viral cultures are negative after having been held for 10 to 14 days, at which time therapy can be switched to oral antivirals (192). Reactivation and relapse has occurred in some patients upon cessation of antiviral therapy. Many clinicians therefore recommend continuing oral antiviral therapy indefinitely.

Collection, Transport, and Storage of Specimens

Because herpes B virus should be propagated only under biosafety level 4 conditions, diagnostic specimens are best handled by specialized reference laboratories (see below). Precautions should be taken by other laboratories to ensure that herpes B virus specimens are not confused with HSV specimens and thus cultured under lower biosafety conditions, since amplification of virus in culture increases the risk to laboratory personnel. Lesion swabs should be taken from each collection site using a separate, sterile Dacron or cotton swab with a wooden or plastic shaft. Swabs or biopsy tissue should be placed into separate tubes containing 1 to 2 ml of VTM. Specimens can be stored in the refrigerator for up to 1 week or at <−60°C indefinitely.

Reference Laboratories for Herpes B Virus

Advice regarding proper specimen collection and submission of specimens for herpes B virus diagnosis can be obtained from the CDC (http://www.cdc.gov/herpesbvirus/index.html) or from the National B Virus Resource Laboratory, Atlanta, GA [phone, (404) 413-6550; http://www.gsu.edu/bvirus].

Identification of Virus and Serodiagnosis

Herpes B virus can be cultured in monkey kidney and chick embryo cells. A characteristic cytopathic effect similar to that of HSV is seen, with polykaryon formation and intranuclear Cowdry type A inclusion bodies. Confirmation of herpes B virus in culture can be done using monoclonal antibodies or molecular techniques. PCR for herpes B virus (197–199) is generally preferred over culture for diagnostic purposes, since it is comparatively rapid and highly sensitive and specific and avoids the need to amplify infectious virus to high titers.

Serodiagnosis of herpes B virus infections has been complicated by extensive cross-reactivity with HSV-1 and HSV-2. However, careful ELISA and Western blot tests have been developed that allow discrimination of antibodies to herpes B virus, HSV-1, and HSV-2 (200–202). Serologic testing can be useful for evaluation of potentially infected animals involved in human exposures and for screening research animals. Serial determinations of serostatus from potentially exposed individuals are also useful adjuncts for diagnosis (190, 192).

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Varicella-Zoster Virus

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TAXONOMY

Varicella-zoster virus (VZV) belongs to the family *Herpesviridae*, based on morphological criteria, and is one of the eight human-pathogenic herpesviruses identified so far. On the basis of its biological properties, it is classified, together with herpes simplex virus (HSV), as a member of the subfamily *Alphaherpesvirinae* (genus *Varicellovirus*, species *Human herpesvirus 3* [HHV-3]).

DESCRIPTION OF THE AGENT

VZV is an enveloped virus with a diameter of about 180 to 200 nm (Fig. 1). It contains an icosahedral nucleocapsid surrounded by a tegument structure, a lipid envelope that allows the virus to be degraded by lipid solvents, and a linear double-stranded DNA genome (1). The viral genome has an approximate length of 125,000 bp, making it the smallest of the human herpesviruses (2), and it carries at least 70 viral genes. The VZV genome consists of a unique long region (UL), a unique short region (US), and flanking internal and terminating repeat regions, and it can exist in four isomeric forms (Fig. 2).

VZV may infect susceptible cells either by fusion at the cell surface or by endocytosis. Virus replication takes place in the nucleus of the infected cell, and similar to other herpesviruses, the progression of viral gene expression is highly regulated. Not all VZV gene functions are yet known. The especially well-characterized ones include some transcription regulator proteins (IE 61 to IE 63), viral enzymes such as thymidine kinase (TK, open reading frame [ORF] 36), the DNA polymerase (ORF 28), and various VZV glycoproteins, such as gB (ORF 31), gE (ORF 68), and gH (ORF 37), which are required for virus attachment and for inducing a host immune response.

VZV exhibits low genomic diversity among isolates in comparison to other herpesviruses (3). From full-genome sequencing, five major VZV clades and two provisional genotypes have been established (4, 5). There is no evidence that naturally circulating VZV strains differ significantly in virulence.

EPIDEMIOLOGY AND TRANSMISSION

VZV is ubiquitous and highly contagious, and primary infection with VZV therefore usually occurs in early childhood. Prevaccination seroepidemiological studies in 11 European countries have shown that in most areas, more than 90% of 10- to 15-year-olds were already seropositive for VZV (6). In the United States, the incidence of varicella has declined significantly since 1995, when the VZV vaccine was generally introduced (7).

Primary infection with VZV is most likely acquired by virus transmission through aerosols. Cell-free virus is also produced at high levels in the skin vesicles, and thus the fluid from these vesicles is also highly infectious. Infection occurs by inoculation of the respiratory tract mucosa and replication in tonsillar lymphoid tissue, from which the virus is then disseminated, most likely by T cells (1). The incubation period is 10 to 21 days. During this incubation time, the virus is spread by viremia, may also replicate in reticuloendothelial organs, and finally infects cutaneous epithelial cells, which are the major sites of virus replication (1). Eventually, the infected host begins to shed virus by the respiratory route. New vesicles containing infectious fluid may appear for several days after rash onset. Individuals are no longer infectious once the last set of vesicles have dried and crusted.

After primary VZV infection, the virus remains latent in ganglia. Upon reactivation, herpes zoster may develop. Herpes zoster rash vesicles are filled with virus-containing fluid and may be also a source of primary infection for susceptible, VZV-seronegative individuals. Virus can also be transmitted from mother to child during pregnancy (8).

CLINICAL SIGNIFICANCE

Varicella (chicken pox) is the manifestation of primary infection with VZV. The clinical appearance of chicken pox is usually dominated by a generalized vesicular rash. Sometimes, symptoms such as fever, malaise, or abdominal pain are seen as prodromal symptoms 24 to 48 hours before and during the first days after the onset of the rash. New vesicles develop during the first 3 to 6 days of varicella. Due to its characteristic clinical appearance, the diagnosis of varicella is often a clinical one and does not require laboratory confirmation. Clinical reinfection with VZV has been described for immunocompetent and immunosuppressed individuals (9) and might occur more frequently than usually supposed (10).

Complications associated with chicken pox can occur, and before vaccination was introduced, up to 4 of 1,000 cases of chicken pox in the United States required hospitalization each year (11). Older age, immunosuppression, and pregnancy are considered to be generally associated with a higher

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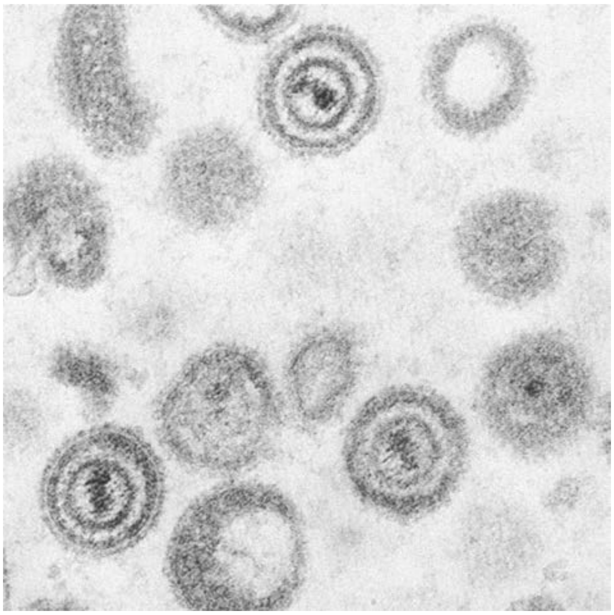

FIGURE 1 Electron micrograph of VZV in skin vesicle fluid from a patient with varicella; magnification, ×100,000. (Reprinted from reference 128.)
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complication rate with varicella. The most frequent complications seen with chicken pox are secondary bacterial infections of the skin lesions, which can lead to abscesses, lymphadenitis, and rarely, also to bacteremia and sepsis. In healthy adults especially, varicella may be complicated by VZV pneumonia, which is 25 times more frequent in these patients than in children and has been described to be especially severe in pregnant women (12). The hospitalization rate among adults with varicella is about 32 per 1,000 reported cases, which is more than 6 times higher than the rate for children (13).

VZV encephalitis can occur during chicken pox, due to primary infection or to postinfectious processes, and often presents with symptoms of acute cerebellar ataxia (14). Other varicella complications include hepatitis, nephritis, or acute thrombocytopenia. In immunosuppressed patients, extensive general dissemination can occur, leading to multiorgan infection and death if not treated early.

Primary infection with VZV during the first 21 weeks of gestation may lead to congenital varicella syndrome in the fetus, characterized by cutaneous scarifications, atrophy of the extremities, and in rare cases, seizures, microcephaly, and other sequelae. The association between the clinical syndrome and VZV has been confirmed by detection of viral DNA by PCR in fetal tissue (15). The incidence is low, <1% in the first 2 trimesters (16). Neonatal varicella can result in severe disseminated infection in babies born within 4 days before to 2 days after the maternal varicella rash appears.

After primary infection, VZV persists in the host in a latent state in sensory trigeminal and dorsal root ganglia. VZV reaches the sensory ganglia, most likely by retrograde axonal transport from skin lesions, and eventually also by hematogenous spread (1). Variable amounts of virus, ranging from 10 to about 55,000 viral genome copies per 100,000 ganglion cells, have been detected in latently infected hosts (17–19). During VZV latency, transcription and translation of different genes are observed (20). The virus is kept under control mostly by the host's VZV-specific T-cell immunity. When this immunity decreases due to immunosuppression or older age, reactivation of the virus may occur.

Reactivation of VZV can lead to limited subclinical local infection, or the virus may spread via neurons to the skin, resulting in the clinical syndrome herpes zoster. Herpes zoster is mostly characterized as a vesicular rash, typically limited in immunocompetent hosts to the dermatome innervated by a single sensory nerve (1). It is often preceded and accompanied by intense neuropathic pain due to sensory neuron involvement. The eye may be affected, resulting in zoster ophthalmicus. An increase in the VZV-specific T-cell response limits viral spread. The most frequent complication of herpes zoster is postherpetic neuralgia, which presents as severe pain and may last for up to several months after herpes zoster.

In immunocompetent hosts, the most severe complications of herpes zoster are associated with VZV infections of the central nervous system (CNS). Viral meningitis, myelitis, or encephalitis may be observed, with encephalitis probably occurring due to neuronal spread. Cases of facial palsy syndrome are also seen. In most cases, the diagnosis of herpes zoster is a clinical one, based on its characteristic appearance and the distribution of vesicles. In some cases, however, reactivation may also result in "zoster sine herpete," a syndrome of undefined local pain that is sometimes also associated with CNS infection that occurs in the absence of a

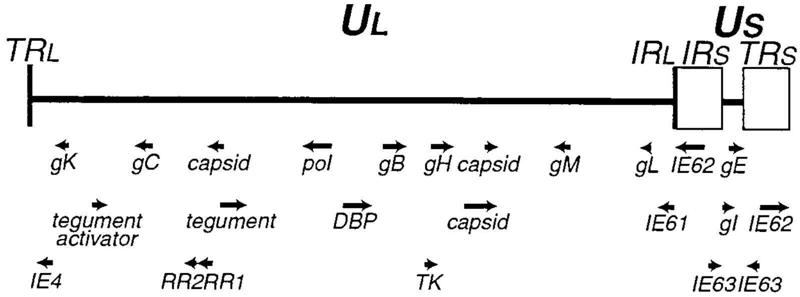

FIGURE 2 Schematic of the VZV linear double-stranded DNA genome. UL, unique long segment (100 kbp); US, unique short segment (5.4 kbp); TRs and TRL, terminal repeats; IRs and IRL, internal repeats. The arrows indicate the direction of the transcription of the viral genes indicated. RR, ribonucleotide reductase; DBP, DNA binding protein. (Reprinted from reference 129.)
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vesicular rash. The diagnosis of "zoster sine herpete" can be provided only by virological investigation. It may occur more commonly than previously thought. Approximately 25% of cases with CNS complications due to virologically confirmed VZV reactivation occurred in the context of zoster sine herpete (21).

In the immunosuppressed host, primary infection and reactivation can lead to severe and possibly life-threatening generalized infection. In the prevaccine era, severe disseminated primary infection associated with high mortality was a particular concern in immunosuppressed children. This remains an important entity in undervaccinated populations. After bone marrow or solid-organ transplantation, patients frequently exhibit episodes of VZV reactivation, which sometimes proceed to severe disseminated infection and to the development of visceral zoster, a clinical picture characterized by severe abdominal pain and associated with the involvement of internal organs, such as the liver, colon, or lung (22–25). Among solid-organ transplant recipients, lung transplant patients especially have an increased risk for VZV complications (26), but VZV can also cause substantial problems in patients with hematological malignancies or after hematopoietic stem cell transplantation (27). HIV-infected individuals may also undergo severe episodes of VZV reactivation and dissemination, especially during AIDS, and they may exhibit an unusual clinical presentation (28–30). VZV can also play a role in the development of immune reconstitution inflammatory syndrome, and mucocutaneous zoster may occur within 4 weeks after the initiation of highly active antiretroviral therapy (HAART) (31).

Disease caused by VZV can be prevented by vaccination. A live attenuated varicella vaccine derived from the Japanese Oka strain was developed in the early 1970s. It was approved in the United States in 1995 for persons who are susceptible to chicken pox and is currently also recommended in various European countries (32). The childhood vaccination policy in the United States has led to a substantial decrease in varicella incidence (33), disease severity, and associated complications (34). An Oka-derived vaccine has also been developed to boost T-cell immunity against VZV in older patients in order to prevent zoster reactivation (35).

Varicella-zoster immunoglobulin may prevent severe varicella and is thus given to high-risk patient populations to avoid the development of disease. Seronegative women during the first 21 weeks of pregnancy receive the commercially available VZV immunoglobulin up to 72 hours after VZV exposure, to avoid the development of congenital varicella syndrome in the fetus (8). In immunosuppressed VZV-seronegative patients, such as hematopoietic cell transplant patients, application of VZV immunoglobulin has been recommended up to 96 hours postexposure to inhibit the development of severe generalized infections (36).

Early and rapid diagnosis of VZV infection is important. Different detection methods are available (summarized in Table 1). Due to its sensitivity, PCR has largely supplanted culture and become the preferred method in many instances. Antigen detection is still useful, as it can be performed rapidly and in a random-access format. Serology is most useful to ascertain immunity.

Specific antiviral treatment of VZV infection and reactivation is possible. If treatment is necessary, it is usually performed with nucleoside analogues such as acyclovir or penciclovir or with the better orally bioavailable prodrugs valacyclovir and famciclovir (37, 38). Brivudine is another nucleoside analogue that has proven effective against VZV (39). Anti-VZV treatment is applied in immunosuppressed hosts and in immunocompetent patients when clinical complications arise. In addition, herpes zoster reactivation is also treated to limit the development of postherpetic neuralgia and to prevent ocular involvement.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Various specimens can be used for diagnosis (Table 1), depending on the clinical presentation and test method. Vesicular fluid contains cell-free virus, mostly at high concentration. Since nearly all patients with varicella and many patients with herpes zoster show a vesicular rash, and because collection of vesicular fluid is very convenient, laboratory diagnosis from vesicular fluid is a major diagnostic tool for detection or confirmation of VZV infection

TABLE 1 Diagnostic tests for VZV-induced disease

Method	Target(s)	Suitable specimens (disease)	Comment
PCR	ORF 17, 29, 31, 62, 69	Fluid, cells, or crust from lesions (varicella, herpes zoster) Blood (disseminated infection, zoster sine herpete) CSF (encephalitis, meningitis, myelitis, facial palsy, zoster sine herpete) Tissue (autopsy)	Most sensitive method. Faster time to result compared to culture. Quantification possible
DFA	gE	Cells from lesions (varicella, herpes zoster) Tissue (autopsy)	More sensitive than culture. Rapid time to results (<2 h); random access.
Culture	Infectious virus	Lesion fluid or cells (varicella, herpes zoster) Blood (disseminated infection, zoster sine herpete) Tissue (autopsy)	Positive early in disease (<2 days after rash onset). Recovery can be difficult, as virus is very labile. Traditional diagnostic standard now largely replaced by PCR due to lack of sensitivity and long time to result (7–10 days for CPE)
Serology	Anti-VZV IgM, IgG	Serum (primarily to assess immunity)	Not recommended for use in disease diagnosis; false-positive and false-negative IgM results may arise. To test IgG levels, 7–10 days required between acute- and convalescent-phase samples.

or reactivation. The vesicular fluid can be collected using capillary pipettes or syringes. For PCR analysis, vesicular fluid can also be collected on swabs and submitted to the laboratory in physiological saline or in viral transport medium. If further virus culture is planned, it should be kept in mind that virus collected on swabs is less stable and is also further diluted in the medium, which may decrease the efficiency of virus culture.

VZV DNA is detectable by PCR in plasma, serum, whole blood, and peripheral blood mononuclear cells (PBMCs). Plasma or serum is conventionally used due to ease of preparation. Whole-blood specimens should be submitted in anticoagulants other than heparin if PCR testing is to be performed, as heparin can inhibit *Taq* polymerases. Virus can be recovered from PBMCs early in disease; however, this is a method used primarily in research. Cerebrospinal fluid (CSF) should be submitted in a sterile container. Usually, uncentrifuged CSF is used for detection of VZV DNA, but virus can also be detected in the cellular fraction or in supernatant.

Tissue is tested primarily at autopsy. In disseminated disease, tissue testing has now largely been replaced by PCR of blood specimens due to test sensitivity and ease of specimen procurement. Cells from tissue can be stained by immunofluorescence. Touch preparations of cells are prepared by pressing tissue (typically 10 to 15 mm in size) against the clean surface of a glass slide multiple times, over a length of 30 to 40 mm. Slides are air dried, fixed in cold acetone, and stained with reagents used in antigen detection (see below). Tissue homogenates can be prepared as described in chapter 79 and tested by culture or PCR.

Generally, VZV DNA, which is ultimately amplified by PCR methods, is quite stable during collection, transport, and storage. VZV virions, however, are quite labile; therefore, specimens should be placed into culture as quickly as possible after collection if isolation is required. Samples for PCR can be stored at −20°C. Virus isolation from frozen samples is largely ineffective unless cellular fractions are stored in cryoprotectant medium at −80°C.

DIRECT EXAMINATION

Microscopy

One of the oldest and simplest direct detection methods is the Tzanck test. In this assay, cellular material is scraped from the base of vesicular lesion and put onto glass slides. These smears are then stained, examined under a microscope, and screened for multinucleated giant cells, which contain multiple eosinophilic intranuclear inclusions representing viral capsids (Fig. 3). However, since the presence of these cells is characteristic both of herpes simplex and of varicella-zoster virus infections, a specific diagnosis of VZV cannot be made with this test. VZV can be visualized by electron microscopy, but this method also does not allow a clear differentiation between the different herpesviruses (Fig. 1).

Antigen Detection

VZV antigen detection from cell-containing vesicle material by direct fluorescent-antibody assay (DFA) is sometimes still used as a first front-line diagnostic test for rapid detection of VZV infection in hospitalized patients. Cell suspensions obtained by skin scrapings from the base of the vesicle are applied onto glass slides, fixed with cold acetone, and dried. Then, cells are stained with fluorescently labeled monoclonal antibodies (MAbs) for 30 min at 37°C in a humidified chamber. After a washing step, cells are covered with a coverslip, and staining is visualized by fluorescence microscopy (40). VZV-specific monoclonal antibodies are commercially available (Merifluor, Meridian Diagnostics, Meridian Bioscience Inc., Cincinnati, OH; Light Diagnostics, Chemicon/Millipore, Billerica, MA). Antigen detection is clearly less sensitive than PCR-based methods for direct detection of virus from clinical material and can be performed only from samples containing intact cells, which limits its general applicability (41).

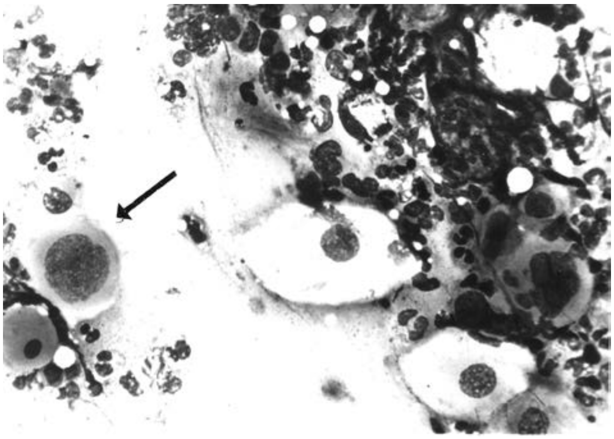

FIGURE 3 Giemsa-stained preparation of material from the base of a vesicular lesion. Magnification, ×102. The arrow indicates a giant cell with a folded nucleus characteristic of VZV or HSV. doi:10.1128/9781555817381.ch99.f3

Nucleic Acid Detection

Over the last decade, nucleic acid (NA) amplification-based techniques, especially PCR assays, have become standard tools for the diagnosis of VZV disease. These techniques have revolutionized the diagnosis of VZV disease of the CNS and of disseminated VZV infection in immunocompromised patients and the identification of herpes zoster in patients who do not develop the typical rash. The advantages of these molecular techniques are that they require only small volumes of input material and are highly sensitive and rapid.

Since the description of the first detection of VZV DNA in CSF by conventional PCR (42), the PCR techniques used have changed substantially. Real-time PCR methods (reviewed in chapter 6) that are more sensitive and can be quantitative have replaced older PCR techniques and are now routinely performed in many diagnostic laboratories. Numerous in-house PCR tests have been published for amplifying various gene segments of the VZV genome (Table 1). There is no generally preferred target sequence, and the genetic variability between virus strains is low. An increasing number of commercially available VZV nucleic acid test kits are being developed, for instance, VZV tracer (affigene; Cepheid), the VZV PCR kit (Abbott Laboratories), the LightCycler VZV Qual kit (Roche Diagnostics), or the Iam VZV Q-LAMP Qualitative assay (DiaSorin). Protocols have also been designed for simultaneous amplification of VZV together with various other viruses causing similar clinical pictures. Commercially available tests include the artus HSV LC-PCR kits (Qiagen; CE marked for *in vitro* diagnostic use in Europe, research use only in Canada, and not available in the United States) or HSV1 HSV2 VZV R-gene (Argene Inc.; CE marked for *in vitro* diagnostic use in Europe, research use only in the United States).

These multiplex PCRs are able to identify a variety of diagnostically important viruses simultaneously, either within one tube or by parallel detection in a single PCR run (43, 44). VZV detection and quantification by PCR can be performed in various clinical materials (for an overview, see Table 2). Quantified VZV DNA for use as nucleic acid detection and quantification test controls is provided by various manufacturers, for instance, Advanced Biotechnologies, Inc. (Columbia, MD), ZeptoMetrix Corp. (Buffalo, NY), or NIBSC (Hertfordshire, United Kingdom).

VZV DNA from vesicular fluid and skin scrapings can be detected easily by PCR. This helps to discriminate quickly between vesicular lesions caused by VZV and those due to various other causes, especially HSV-1 or HSV-2 infection.

TABLE 2 Quantitative VZV DNA results with different clinical materials from patients with different VZV-associated diseases[a]

Material	Clinical syndrome	No. investigated	PCR positive (%)	Viral load (median, mean, or geometric mean)	Viral load (range)	Reference
Vesicle	Varicella	3		1.0×10^8 co/ml		69
	Zoster	3		7.4×10^8 co/ml		69
Cerebrospinal fluid	Varicella	1		10^2 co/ml		60
	Varicella	1		91 co/ml		59
	Zoster	30		1.4×10^4 co/ml	$50–2.6 \times 10^8$ co/ml	21
	Zoster	16		3.1×10^3 co/ml	$1.1 \times 10^2–4 \times 10^4$ co/ml	59
	Meningitis	17		4.1×10^3 co/ml	$50–1.7 \times 10^6$ co/ml	21
	Meningitis	25		2.7×10^4 co/ml	$2 \times 10^3–5.2 \times 10^5$ co/ml	58
	Encephalitis	13		7.4×10^4 co/ml	$1 \times 10^3–2.6 \times 10^8$ co/ml	21
	Encephalitis	19		8.6×10^4 co/ml	$3 \times 10^2–2.1 \times 10^8$ co/ml	58
Saliva	Ramsay Hunt syndrome	25	52		$30–1.4 \times 10^6$ co/50µl	130
	Facial palsy without rash	31	55		$10–1 \times 10^5$ co/50µl	130
Whole blood	Varicella in adults	34	100	5×10^2 co/ml	$20–10^5$ co/ml	73
	Varicella	8	87.5	1.6×10^3 co/ml	$2 \times 10^2–1.1 \times 10^4$ co/ml	72
	Zoster dermatomal	9	90	2×10^2 co/ml	$10^2–9 \times 10^2$ co/ml	72
	Zoster multidermatomal	5	100	2.7×10^3 co/ml	$9 \times 10^2–3 \times 10^5$ co/ml	72
	Healthy control	20	0			72
	Blood donors	100	0			82
Peripheral blood mononuclear cells	Varicella	9	100	12 co/10^5 cells		68
	Varicella	19	73	4.9×10^2 co/10^5 cells	$5–5 \times 10^3$ co/10^5 cells	69
	Varicella	21	48		$1.4 \times 10^2–3.4 \times 10^3$ co/10^5 cells	70
	Zoster	10	20	10 co/10^5 cells		69
	Zoster	71	16		$10–10^2$ co/10^5 cells	70
	Zoster	130	78	9×10^2 co/10^5cells	$40–2.9 \times 10^4$ co/10^5cells	74
	Healthy controls	28	0			69
	Healthy controls (50 ml blood)	53	5	1.3×10^3 co/10^5cells	$6 \times 10^2–5 \times 10^3$ co/10^5cells	74
Serum/plasma	Varicella	18	88.9	2.0×10^3 co/ml	$100–2 \times 10^5$ co/ml	72
	Varicella	5	100	10^4 co/ml		68
	Disseminated varicella	1		1.2×10^7 co/ml		79
	Zoster dermatomal	9	66.7	100 co/ml		72
	Zoster multidermatomal	6	100	2.7×10^3 co/ml		72
	Zoster	9	100	1.7×10^3 co/ml	$1.8 \times 10^2–10^4$ co/ml	77
	Zoster	12		7×10^3 co/ml	$4 \times 10^2–2 \times 10^5$ co/ml	76
	Zoster disseminated	4	100	2.1×10^5 co/ml	$2.5 \times 10^3–7.4 \times 10^5$ co/ml	77
	Zoster visceral disseminated	1		2×10^5 co/ml		80
	Healthy control	10	0			72
Aqueous humor	Acute retinal necrosis	2			9×10^2 and 5.5×10^6 co/ml	131
	Anterior uveitis	8		2.5×10^5 co/ml	$3.8 \times 10^2–1.2 \times 10^7$ co/ml	85
Tissue	Postmortem	32	68		6–28 co/10^5 GC[b]	19
	Postmortem	14	79	2.6×10^2 co/10^5 GC		18
	Postmortem	17	100	9×10^3 co/10^5 GC	$5.8 \times 10^2–5.5 \times 10^4$ co/10^5 GC	17

[a] Abbreviations: co, copies; GC, ganglion cells.

PCR is more sensitive than virus isolation or direct immunofluorescence for the detection of VZV in vesicular lesions (45, 46). The benefits of VZV PCR were also shown for the diagnosis of vaccine-modified varicella disease (41). VZV DNA can be detected by PCR in crusting lesions (47) and in skin scrapings in VZV-associated facial palsy even in the absence of visible vesicular lesions (48). The easy access and the high virus detection rates make dermal lesions an ideal material to be used not only for diagnosis but also for further genotyping of vaccine and wild-type (WT) strains using genotype-specific PCR strategies (49, 50).

Analysis of CSF using nucleic acid amplification has become the method of choice for diagnosis of neurological disease associated with VZV, such as cerebellitis, aseptic meningitis, and encephalitis. Using PCR, it was found that neurological disease is an important and not infrequent complication in immunocompetent as well as immunosuppressed patients. VZV DNA was detected in CSF of up to 10% of patients presenting with clinical symptoms of aseptic meningitis and (meningo-) encephalitis (51–54). PCR analysis is also useful for the diagnosis of VZV-induced neurological symptoms in cases where the characteristic vesicular rash appears only after the start of CNS infection, or even in cases of zoster sine herpete (21, 55–58). No rash was observed in about one-quarter of all cases of patients suffering from VZV-associated neurological symptoms (21, 57, 58). Higher mean VZV DNA loads were found in the CSF of patients with herpes zoster-associated encephalitis than with meningitis (Fig. 4A) and in patients requiring intensive care treatment than in those who did not, although some overlap was observed among individual patients in each of the compared groups (Fig. 4B) (21). Virus is predominantly detected within 1 week after the onset of clinical symptoms, but in cases of VZV-induced neurological symptoms without rash, virus can be detected in the second week of disease (21, 57).

In cases of varicella-induced cerebellar ataxia, the amount of viral DNA was shown to be generally low (42, 59, 60), supporting the theory that these symptoms might be mediated by the antiviral immune response. After initiation of antiviral therapy, CSF usually becomes negative for VZV DNA in the follow-up in uncomplicated cases (42). In contrast, the continuous presence of viral DNA has been found in individual HIV-infected patients in spite of therapy, and this was associated with the death of these patients (61).

PCR testing plays an important role in the diagnosis of acute VZV-associated peripheral facial palsy occurring as a neurological complication in the course of VZV reactivation. In cases of Ramsay Hunt syndrome, virus can be detected not only in vesicles of the auricles and the oral cavity but also in the facialis nerve sheath, middle ear mucosa, and cerebrospinal fluid (62). As the rash is mostly hidden in the ear or mouth and may be faint or delayed, only sensitive testing for the presence of VZV by PCR has shown that a considerable number of "idiopathic" peripheral facial palsy or Bell's palsy cases are due to VZV reactivation. Using PCR methods, VZV was detected in saliva samples of 58% of patients with VZV-induced facial palsy, with 64% of those cases presenting without characteristic rash (63). Virus was shown to be present in oropharyngeal swabs until day 12 in patients with acute peripheral facial palsy (64). VZV DNA levels in saliva were about 10^4 copies higher, and recovery of facial function was worse in patients with facial palsy and oropharyngeal lesions than in those with facial palsy alone (64). VZV can be detected rarely in CSF samples of patients with facial palsy (62, 65), and lower amounts of virus are found in these patients than in those presenting with meningitis and encephalitis (Fig. 4C) (21, 58, 66).

VZV DNA is also detectable in PBMCs (67–70) and in whole blood (71–74), and although the viremia is assumed to be cell associated, viral DNA can also be detected in serum or plasma (68, 72, 75–77). Viremia is detected by PCR in up to 100% of cases of acute varicella, but virus can also be detected in the blood compartment in cases of herpes zoster. VZV DNA was detected in 47% of serum samples obtained within the first 8 days after the start of herpes zoster from otherwise healthy patients (75). In a small cohort of 9 immunocompetent patients with herpes zoster, all were found to be VZV DNA positive when the amount of plasma analyzed was increased to 1 ml (77). Quantitative analysis showed that viral loads may range from 5 to 5×10^3 copies/10^5 cells in PBMCs, from 20 to 10^5 copies/ml in whole blood, and from 100 to 2×10^5 copies/ml in plasma or serum. The virus load is higher in patients with acute varicella than in those with herpes zoster (72). A higher viral load correlates with a larger number of skin lesions and more-severe disease (73) as well as with the presence of multidermatomal zoster (72). The viral load was lower in samples taken only a few days after the start of varicella symptoms (69) and was found to be negative

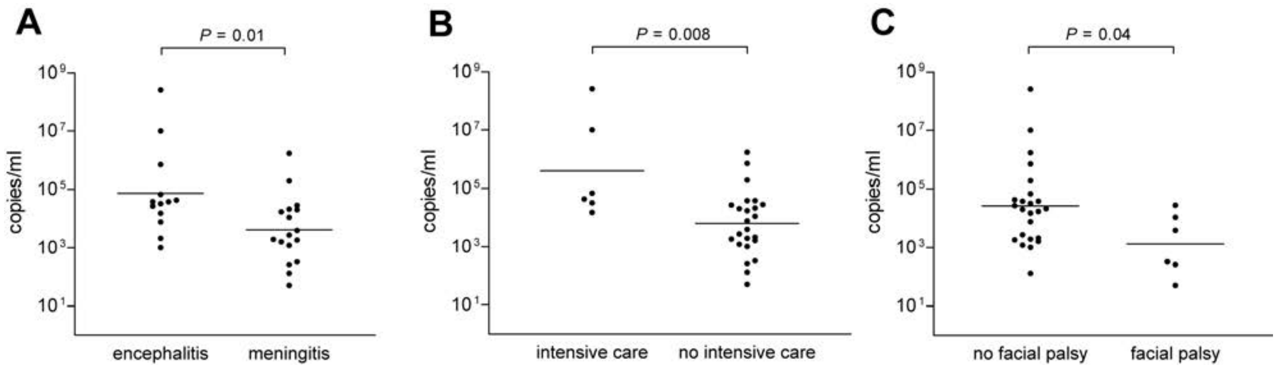

FIGURE 4 Comparison of the amount of VZV DNA in CSF according to the neurological diagnosis (A), the severity of disease (B), and the presence of acute peripheral facial palsy with meningeal signs (C). The geometric mean value is shown by a horizontal line. The *P* value was calculated using the Mann-Whitney *U* test. (Modified from reference 21, with permission.) doi:10.1128/9781555817381.ch99.f4

in convalescent-phase sera of patients with shingles (75). When applying a highly sensitive nested-PCR assay, VZV DNA was also detected in PBMCs of patients with facial palsy without dermal lesions (78).

Virus detection in blood is important for the diagnosis of complicated or clinically unclear courses of varicella or herpes zoster, especially in cases in which the appearance of a rash is delayed or completely lacking, and also in cases of visceral dissemination (77, 79–81). In immunocompromised patients, quantitative PCR analysis proved to be an important tool for monitoring the clinical course of disease and assessing therapeutic success (76, 77, 79, 80).

VZV is not detectable in the blood in the majority of healthy control patients, even with sensitive PCR methods (82), unless a very large volume of blood (50 ml) is used (74). In other studies, VZV was detectable in 2 to 3% of PBMC samples taken from immunocompetent individuals without clinical signs of VZV illness (70, 83). These findings support the assumption that asymptomatic reactivation of latent virus may occur and may be important for boosting host immunity to the virus (84).

VZV PCR can be performed from aqueous humor and has been found to be helpful in confirming VZV-associated ocular disease. The viral load in the aqueous humor of patients with anterior uveitis corresponds to the extent of iris atrophy (85).

Identification of VZV by PCR in bronchoalveolar lavage specimens has been reported and seems to be useful for the successful early diagnosis of severe VZV pneumonitis (86). The detection of VZV DNA in tissue samples by PCR is also possible and has been used to confirm the association between VZV and congenital varicella syndrome (15, 87). Generally, the use of highly sensitive PCR techniques may be especially important for confirming the diagnosis of VZV-related disease in cases of less disseminated or milder disease associated with lower viral loads (48, 70) or for samples obtained later in the course of disease after the initiation of antiviral therapy.

ISOLATION PROCEDURES

Virus isolation by cell culture methods provides the basis for phenotypic characterization of individual VZV strains, for generating VZV-infected cells for serological tests such as fluorescent antibody to membrane antibody (FAMA), and for the phenotypic analysis of VZV drug resistance. Many laboratories use human foreskin fibroblasts for isolation of VZV from clinical samples. Other sensitive host cells include diploid human cell lines, preferably derived from fetal kidney or fetal lung, human lung carcinoma (A549), or human melanoma cell lines, and nonhuman cell lines such as primary monkey kidney cells (40, 88). A cell mixture (CV-1 and MRC-5) is commercially available for the recovery of herpesviruses including VZV (H&V Mix; Diagnostic Hybrids, Quidel Corp.). In contrast to other herpesviruses, VZV maintains a high level of genetic stability during the culturing procedure. Cell cultures are performed at 37°C under sterile conditions, and the medium used is usually Eagle's minimal essential medium, prepared in Hanks' or Earle's balanced salt solution (BSS) containing neomycin and glutamine and supplemented with 10% fetal bovine serum (FBS) for the growth medium and 1 to 2% FBS for maintenance. The medium is heat inactivated at 56°C for 30 min. The need for a medium change is indicated by a drop in pH, and this is required at least once weekly. When virus culture is done for diagnostic purposes, the clinical material is adsorbed onto the cell monolayer or inoculated directly into the medium, and the cells are then incubated at 37°C and evaluated daily by microscopy. The development of a cytopathic effect (CPE) is variable but is usually visible from 4 days up to maximally 2 weeks. The CPE consists of small foci of rounded and swollen cells (Fig. 5). Confirmation of VZV infection is done by PCR or by staining monolayer cells with VZV-specific monoclonal antibodies (described below). Shell vial centrifugation cultures can provide results in 2 to 5 days and are more sensitive than conventional cultures for VZV but less sensitive than PCR.

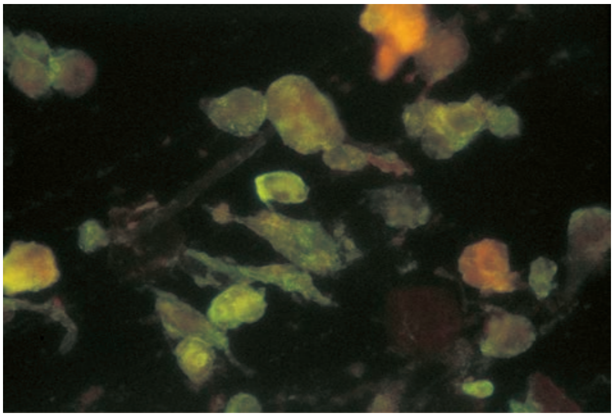

FIGURE 5 Immunofluorescence assay for the detection of VZV-positive cells from a patient with zoster. A smear of a skin vesicle was fixed and stained with monoclonal antibody to VZV gE. Magnification, ×200.
doi:10.1128/9781555817381.ch99.f5

IDENTIFICATION

Virus Identification from Cell Culture

Specific identification of VZV from clinical material in cell culture after the emergence of a CPE is necessary because it may be difficult to distinguish VZV from other herpesviruses. VZV-specific PCR analysis may be performed for identification. For this purpose, supernatant is taken from the culture, and DNA is extracted and subjected to PCR. Monolayers can also be stained with VZV-specific MAbs when approximately 50% of the monolayer demonstrates CPE suggestive of VZV. For this purpose, cell monolayers are washed with phosphate-buffered saline (PBS). Then, monolayer cells are scraped into 0.5 ml PBS. Slides are prepared by directly applying the suspension to a glass slide using a cytocentrifuge. Slides are air dried and then fixed in cold acetone for 10 minutes. Following washing with PBS, cells are stained with VZV-specific MAbs. Commercial reagents approved for use in culture confirmation include the D^3 DFA VZV Detection kit (DHI) as well as the MAbs listed in "Antigen Detection" above. After a 30-min incubation at 37°C, monolayers are washed in PBS and rinsed in distilled water. Excess moisture is removed by blotting around the wells. Stained cells should not be allowed to dry, as this produces artifactual fluorescence. Mounting medium and a coverslip are applied, and the staining is visualized by fluorescence microscopy.

Genetic Identification of VZV Strains

Genetic identification of VZV is performed by different molecular methods, directly from clinical specimens, but primarily on a research basis. Characterization of VZV strains is usually done by PCR assays followed by restriction fragment length polymorphism (RFLP) analysis or by sequencing of characteristic fragments and determination of single nucleotide polymorphisms (SNPs) (89). Genetic analysis of virus strains has proven that the VZV strains identified during varicella are identical within an outbreak and are also identical to those present in subsequent herpes zoster (90). It has been shown that VZV strains vary between geographic areas (3, 89).

Characterization of VZV strains may be especially important for differentiating between WT strains and Oka vaccine strains after vaccination, for determining the etiology of a postvaccination rash, for analyzing the association between vaccination and the development of herpes zoster, and also for assessing whether transmission of vaccine virus to susceptible persons has occurred. Different methods for discrimination between WT and vaccine strains have been published, and in this regard, much attention has been focused on the VZV ORF 62 because most nonsynonymous VZV vaccine mutations occur in this ORF. In particular, SmaI and NaeI sites in ORF 62 have been shown to allow the discrimination of vaccine strains from WT strains and also from the Oka parental strain (91). RFLP analysis has also been described for other ORFs, for example, ORF 6 (92), with differences in the AluI sites, and ORFs 38 and 54 in combination, with differences in the PstI and BglI sites (93).

Real-time PCR assays based on sequence polymorphisms, especially in ORF 62 or ORF 38, have also been established (50, 94–96). In addition, a considerable amount of sequence data for various ORFs in the VZV genome has been obtained so far in the search for SNPs that would allow further VZV strain discrimination (3).

The analysis of the Oka vaccine itself has shown that it contains a mixture of different strains that also differ in the R2 repeat region (97). The development of rash after vaccination seems to be associated with the emergence of certain strains that are closely related to the Oka parental strain (98).

SEROLOGIC TESTS

Antibodies against VZV develop in the course of primary infection, are directed against various proteins of the virus, and can be detected in most cases within 3 days after the appearance of the rash. Serologic testing can be used for confirmation of primary infections, where it is applied especially in clinically atypical cases. Detection of VZV-specific antibodies (Abs) is also routinely used for determining immune status. This may be required for patients who cannot remember their varicella history and is especially important in pretransplantation evaluations as well as after virus exposure to guide prophylaxis with varicella immunoglobulin in high-risk individuals such as solid-organ or bone marrow transplant patients, pregnant women, and health care workers. In addition, it may be used for monitoring a patient's immune status after vaccination, although assessment of vaccine efficacy is not routinely performed. A variety of serologic methods that differ in specificity, sensitivity, and utility are available for detecting VZV-specific Abs.

Specialized IgG Detection Tests

A number of different serologic assays are performed in specialized or referral laboratories often for research purposes. One such test is the FAMA test, considered to be the "gold standard" for serologic testing (99), and which correlates best with protection against varicella (100). A flow cytometry-adapted version of the FAMA assay has also been developed (101). The FAMA titer, however, is not predictive of long-term protection after vaccination. Although high seroconversion rates have been described after vaccination, a loss of FAMA-detectable Abs is generally observed over time (102). FAMA is labor-intensive and nonautomated. A noncommercial and apparently very sensitive enzyme immunoassay (EIA) developed by Merck, based on the detection of VZV glycoprotein (gp) preparations, has been used for extended postvaccination serologic studies. EIA titer levels of 5 gp EIA units/ml or more at 6 weeks after vaccination have been associated with a high degree of protection against breakthrough for the following 7 years (103). However, the fact that breakthroughs have also been observed after this Ab level has been achieved suggests that gp EIA levels correlating with long-term protection have not been adequately defined. Other IgG detection assays that are used in specialized settings include the anticomplement immunofluorescence test, the time-resolved fluorescence immunoassay (TRFIA) (104), and plaque reduction assays that detect neutralizing Abs against varicella, which are important tools for confirming that vaccine-induced Abs are in fact neutralizing circulating wild-type VZV strains (105).

IgG Detection Tests in Routine Diagnostic Laboratories

Clinical laboratories typically use commercial immunoassays, for which a variety of different formats are available, including EIA (colorimetric and fluorescence detection) chemiluminescence assays, and immunoassays. These tests have the advantages of being less laborious than the FAMA, not requiring any additional VZV cell culture procedures, being amenable to objective interpretation and, for many assays, to automation, and for some instruments, presenting a random-access format. The different tests use either whole VZV-infected cell lysate as the specific antigen or, in some cases, purified glycoprotein, and the test procedure is done according to the manufacturer's protocol. In some laboratories, noncommercial, in-house EIAs have been established for routine detection of VZV-specific Abs, mostly with whole viral lysates as test antigen (104, 106–108).

Validation of commercial tests is usually done by comparison to FAMA, and the results obtained show that these assays are generally less sensitive than FAMA. Commercial immunoassays have been shown to detect Abs in 43% to 92% of naturally infected individuals identified as seropositive by FAMA or an adapted FAMA protocol (109). In their specificity, commercial immunoassays are more similar to the FAMA (106). Considering that one aim of an Ab test is usually to determine if a person is susceptible to infection and thus a candidate for vaccination, the lower sensitivity of the commercial immunoassays leads to more unnecessary vaccinations. The risks associated with this, however, are low compared to the risk of infection of a person who has been falsely declared immune. Immunoassays are typically used to determine VZV-specific Abs in blood, but they may also serve to detect Abs in CSF.

IgG Avidity Assays

To discriminate recent from past infections or to assess the impairment of Ab development in immunosuppressed patients, VZV IgG antibodies may be further tested for their avidity (110–112). For this purpose, an EIA is run in duplicate, and one of the runs is treated with dilutions of sodium thiocyanate or with diethylamine (110, 111). The avidity index is calculated as the ratio of the treated-sample optical density (OD) to the untreated-sample OD.

IgM Assays

Testing for IgM antibodies is usually performed by commercially available EIAs and is applied to confirm primary VZV infection, especially when the clinical picture is not typical for varicella. In addition, detection of VZV-specific IgM Abs in CSF is sometimes performed retrospectively to confirm intrathecal VZV Ab production after VZV infection of the CNS.

Cellular Immunity

It has been shown that VZV-specific cellular immunity is the key factor in keeping VZV in a latent state. T-cell-mediated immunity consists of CD4 and CD8 effector and memory cells and is detected up to 2 weeks after the rash appears (113). A decrease in cellular immunity, which is observed with increasing age or during immunosuppression, facilitates VZV reactivation and development of herpes zoster (114). Cellular immunity against VZV can be measured with a gamma interferon enzyme-linked immunospot (ELISPOT) assay or intracellular cytokine staining followed by fluorescence-activated cell sorter (FACS) analysis (115–117). The identification of VZV-stimulated cytokines may be used to assess the VZV-specific immunocompetence in transplant recipients (112, 118) or to identify protection against VZV after vaccination, in patients negative for VZV-specific Abs (111). However, these methods are so far mainly investigational.

ANTIMICROBIAL SUSCEPTIBILITIES

Most VZV strains are fully susceptible to antiviral drugs. Resistance against acyclovir or penciclovir has been observed primarily in HIV-infected individuals in the pre-HAART era, in transplant recipients, and in hemato-oncological patients.

Development of resistance may lead to uncontrolled viral dissemination, visceral complication, and death of the patient (119, 120). Data from *in vitro* studies and investigation of resistant wild-type isolates have shown that acyclovir resistance is associated mostly with mutations in the VZV thymidine kinase (TK) gene and only rarely due to mutations in the VZV DNA polymerase (121). Foscarnet and cidofovir can be used for treatment of acyclovir-resistant strains. Resistant VZV strains may be detected only in specific compartments, and therefore, when screening for drug-resistant VZV strains is performed, samples from all affected body sites should be investigated (122). Antiviral susceptibility of VZV strains can be determined phenotypically. Changes in virus replication in the presence of different concentrations of various drugs are measured in most cases by plaque reduction assay in human diploid lung cells. The 50% effective doses of acyclovir have been shown to range from 2.06 μM to 6.28 μM (37). Other methods that have been described include the late-antigen synthesis reduction assay (123) and phenotypic characterization of the TK gene without the need for virus isolation (124). Phenotypic analysis has several disadvantages that limit its clinical utility: time to result typically is prolonged, limiting its use in clinical decision making; it cannot easily be performed from all clinical materials; and it may be not sensitive enough for detection of minor resistant VZV populations. Therefore, genotypic resistance testing is preferred for clinical use, as it is rapid and can be performed from different clinical materials. Genotypic resistance testing is done mostly by sequencing of certain regions of the VZV TK gene that are associated with resistance to acyclovir (125) and of parts of the polymerase gene. A number of defined VZV mutations have been described so far, whose influence on resistance against different drugs has been proven by phenotypic data (126). Resistance testing is typically performed only in research settings or in specialized reference laboratories but is not applied in routine diagnostics.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

For appropriate evaluation and interpretation of test results, knowledge about the clinical background of the individual patient is of utmost importance. The interpretation of the results is dependent on whether a primary infection with VZV is suspected or whether the patient has already had chicken pox. In addition, it is important to know whether a patient is immunosuppressed or not.

Primary infection is usually diagnosed based on clinical presentation. Virus can be detected from vesicles by PCR and antigen detection assays and from pustules and even from crusts by PCR. The results of serologic tests can be suggestive but are not definitively diagnostic of primary infection. For example, IgM (alone or in the presence of low IgG) or a 4-fold increase in IgG in convalescent-phase serum can be observed during primary infection; however, these serologic responses can also be detected during reactivation.

Herpes zoster can also be diagnosed clinically; however, confirmation may be necessary if vesicles are limited or otherwise indistinct in appearance from other types of infection. Detection of virus in vesicle fluid is preferred.

The presence of VZV in blood can be observed by PCR assays during chicken pox and sometimes during VZV reactivation. In most immunocompromised hosts, detection of VZV in blood by PCR is generally indicative of severe disease. It should be noted, however, that VZV DNA can also be detected in the blood of HIV patients with localized zoster. The same holds true for the detection of VZV-DNA in CSF, which is generally considered a pathological finding. Both clinical situations usually require immediate antiviral treatment.

However, highly sensitive detection of virus DNA does not always prove the causality of disease. VZV DNA may also be found due to secondary and/or subclinical reactivation but is then observed mostly at clearly lower levels than during VZV disease. In this context, the quantitative evaluation and reporting of VZV DNA levels are gaining significance. For immunocompromised patients, so far no defined VZV DNA thresholds in blood exist for distinguishing subclinical reactivation from clinically relevant and potentially fatal disease. However, severe visceral infections are clearly associated with higher virus load levels in blood (Table 2). Studies have also shown that high VZV DNA levels in CSF may be associated with the expression and severity of CNS disease (Fig. 4). It may therefore be useful to assess and report quantitative results in the setting of VZV CNS disease. In the pre-PCR era, Ab detection in CSF and quantitation in comparison to blood Ab levels were used for diagnosis of VZV infections of the CNS. The detection of IgM Abs is considered a proof for intrathecal Ab production, as is a significantly higher Ab level in CSF compared to that in blood when it is associated with an intact blood-brain barrier. The clinical significance of these tests in VZV infections of the CNS and their value for therapeutic decisions are limited, as they allow a diagnosis only late in the course of disease compared to PCR tests from CSF, which identify virus replication already at an earlier stage of disease. Serology from CSF was, however, described to be of certain value for detection of VZV vasculopathy (127).

The major importance of Ab assays, of which commercial immunoassays are currently the most commonly used, lies in their ability to identify previously infected individuals, as indicated by anti-VZV IgG Abs in the absence of IgM. This information can be used to guide further vaccination decisions. However, this may result in the vaccination of seropositive individuals since these tests are not as sensitive as other gold standard formats.

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Human Cytomegalovirus

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100

TAXONOMY

Human cytomegalovirus (CMV), formally designated human herpesvirus 5 (HHV-5) by the International Committee on Taxonomy of Viruses, is a member of the family *Herpesviridae*, which includes herpes simplex virus 1 (HHV-1) and 2 (HHV-2), varicella-zoster virus (HHV-3), Epstein-Barr virus (HHV-4), and human herpesviruses 6, 7, and 8. It is classified in the subfamily *Betaherpesvirinae* with cytomegaloviruses of other animal species based on its tropism for salivary glands, slow growth in cell culture, and strict species specificity. Human CMV is the type species of the genus *Cytomegalovirus*, and its name is derived from the enlargement of cells (*cyto* = cell, *mega* = large) infected by the virus. Regions of genome sequence homology between CMV and herpesviruses 6 and 7 have been identified, and HHV-6 and HHV-7 are now classified with CMV among the betaherpesviruses.

DESCRIPTION OF THE AGENT

Complete CMV particles have a diameter of 120 to 200 nm and consist of a core containing a large 220- to 240-kb linear double-stranded DNA genome, an icosahedral capsid with 162 capsomeres, an amorphous tegument or matrix, and a surrounding phospholipid-rich envelope. The CMV genome consists of more than 200 open reading frames that encode structural and regulatory proteins and proteins that function to modulate the immune system of the host. Electron microscopic features of CMV include virions morphologically indistinguishable from those of other herpesviruses, a high ratio of defective viral particles, and the presence of spherical particles called dense bodies. Viral replication occurs in the nucleus of the host cell and involves the expression of immediate early (α), early (β), and late (γ) classes of genes. The viral envelope is formed as assembled nucleocapsids bud from the inner surface of the nuclear membrane.

Molecular and immunologic techniques have been used to study variation among CMV strains. Although it has been shown that different strains of CMV are 95% homologous to the standard laboratory reference strains AD-169 and Towne, genetically distinct CMV genotypes that display polymorphisms in multiple coding and noncoding regions of the virus genome have been identified. Strain diversity has been associated with specific differences in geographic distribution, transmission, tissue tropism, immunopathogenesis, and clinical manifestations of disease, but the exact role and importance of CMV genotypes in infection and disease are largely unknown (for a review, see reference 1).

CMV is inactivated by a number of physical and chemical treatments, including heat (56°C for 30 min), low pH, lipid solvents, UV light, and cycles of freezing and thawing.

EPIDEMIOLOGY AND TRANSMISSION

CMV has a worldwide distribution and infects humans of all ages, with no seasonal or epidemic patterns of transmission. The seroprevalence of CMV increases with age in all populations and ranges from 40 to 100%; the virus is acquired earlier in life and the prevalence is highest among lower socioeconomic groups in crowded living conditions. CMV can be transmitted vertically and horizontally, and infections are classified as being acquired before birth (congenital), at the time of delivery (perinatal), or later in life (postnatal).

Most infections are acquired by direct close personal contact with individuals who are shedding virus. Since CMV has been detected in many body fluids, including saliva, urine, breast milk, tears, stool, vaginal and cervical secretions, blood, and semen, it is clear that transmission can occur in a variety of ways. Prolonged shedding of virus after congenital or acquired CMV infection contributes to the ease of virus spread; virus may be excreted for weeks, months, or even years following a primary infection.

Transplacental infection of the fetus can occur following primary or recurrent infection of a pregnant woman, but the risk of CMV transmission to the fetus and the rate of symptomatic fetal infection are much higher with primary maternal infection. The incidence of fetal damage is highest if infection occurs during the first 12 to 16 weeks of pregnancy. Maternal illness may be mild, with fever and nonspecific symptoms, but is not clinically apparent in most pregnant women. Newborns can also acquire infection at the time of delivery by contact with virus in the birth canal. Nearly 10% of women shed CMV in the genital tract at or near the time of delivery, and virus is transmitted to approximately 50% of the newborns. Such infants begin to excrete virus at 3 to 12 weeks of age but usually remain asymptomatic. Mother-to-infant transmission of CMV through breast milk is very common; low-birth-weight preterm infants are at greatest risk for developing disease. Of children who attend day care centers and enter as toddlers, 20 to 70% experience CMV infection over a 1- to 2-year

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period. Infection is usually asymptomatic, but the children may transmit CMV to their parents and other caregivers, posing a risk to an unborn fetus if a woman is pregnant at the time. Susceptible health care workers and employees of day care centers are at risk of occupational exposure. In adolescents and adults, sexual transmission of CMV may occur and is an important route of CMV spread.

Similar to infections with other herpesviruses, primary infection with CMV results in the establishment of a persistent or latent infection. The sites of latent infection are thought to include various tissues, endothelial cells, and leukocytes. Therefore, CMV can be transmitted through transfusion of blood products and by exposure to tissue and hematopoietic stem cells during transplantation. Reactivation of the virus can occur in response to different stimuli, particularly immunosuppression.

CLINICAL SIGNIFICANCE

CMV infections are common and usually asymptomatic in otherwise healthy children and adults; however, the incidence and spectrum of disease in newborns and in immunocompromised hosts establish this virus as an important human pathogen (for a review, see references 2 and 3).

Immunocompetent Host

The vast majority of immunocompetent children and adults who acquire CMV infection postnatally remain asymptomatic. Symptoms in young adults can mimic the infectious mononucleosis syndrome caused by Epstein-Barr virus and include prolonged fever (persisting for 2 to 3 weeks), malaise, an atypical lymphocytosis, and mild hepatitis without the production of heterophile antibody. Exudative pharyngitis is frequently absent, and lymphadenopathy and splenomegaly are less common with CMV-associated infectious mononucleosis. Organ-specific diseases, including colitis, hepatitis, encephalitis, pneumonia, and anterior uveitis, have also been described in the immunocompetent host but are rare. A link between CMV infection in the immunocompetent host and atherosclerosis has been suggested, and there is growing evidence that CMV is a contributing factor to vascular disease in certain individuals (for a review, see reference 4). More recently, there has been an increase in the number of reports of severe CMV infection associated with poor outcomes in hospitalized, critically ill immunocompetent patients (5, 6). Reactivation of CMV is common in these patients and is linked to increased length of stay in the hospital and/or intensive care unit, increased duration of mechanical ventilation, severe sepsis, high disease severity, and mortality (7).

Fetus and Newborn Infant

CMV infection has been detected in 0.2 to 2.5% of newborn infants and is the most common identified cause of congenital infection. Approximately 10 to 15% of congenitally infected infants develop symptoms during the newborn period; possible manifestations range from severe disease with any combination of intrauterine growth retardation, jaundice, hepatosplenomegaly, petechiae, thrombocytopenic purpura, myocarditis, pneumonitis, central nervous system abnormalities, deafness, and chorioretinitis to more limited involvement. Symptomatic infants may die of complications within the first months of life; more commonly, they survive but are neurologically damaged. It is now recognized that even congenitally infected infants who are asymptomatic at birth may develop progressive sensorineural hearing loss, visual impairment, or psychomotor and/or intellectual disabilities later in life. Perinatal or postnatal infection with CMV may occur in term and preterm infants as a result of exposure to maternal cervicovaginal secretions during delivery, ingestion of breast milk after delivery, and blood transfusions. In high-risk preterm newborns, morbidity and mortality can be significant, and hepatosplenomegaly, neutropenia, thrombocytopenia, atypical lymphocytosis, hemolytic anemia, pneumonitis, and necrotizing enterocolitis have been described. Although a large proportion of congenital CMV infections are attributed to primary maternal infection in the developed world, recent studies in resource-poor settings have shown similar rates of sequelae, especially hearing loss, following primary or reactivated maternal infection (see reference 3 for a review). Therefore, contrary to past beliefs, preexisting infection and development of CMV antibody in the mother may not eliminate the risk for significant infection in the infant. Thus far, it appears that perinatally or postnatally infected infants do not develop late neurologic sequelae of infection.

Immunocompromised Host

CMV infections are frequent and occasionally severe in children or adults with congenital or acquired defects of cellular immunity, such as patients with AIDS, cancer patients (particularly those with leukemia and lymphoma receiving chemotherapy), and recipients of solid organ and hematopoietic stem cell transplants. Infections in these patients may be due to reactivation of latent virus or primary infection or reinfection with exogenous virus, which may be introduced by blood transfusions or by the grafted organ. Symptoms tend to be most severe after primary infection; however, reactivation infection or reinfection in a severely immunocompromised host may also cause serious illness. Active infection usually occurs between 1 and 4 months after transplantation, when patients are at the height of their immunosuppression, or when $CD4^+$ lymphocyte counts drop below 50 to 100 cells/µl for individuals infected with HIV. The widespread use of antiviral drugs for prophylaxis or preemptive therapy following transplantation has resulted in the emergence of late-onset (>90 days posttransplant) CMV disease (for a review, see reference 8). The frequency and severity of CMV infection in organ transplant recipients are variable and depend on the type of transplant, the source of the donated organ, the immune status of the recipient, and the duration of the immunosuppressive therapy. The major symptoms in these patients represent a nonspecific "viral syndrome" and usually include fever, malaise, lethargy, myalgia or arthralgia, leukopenia, thrombocytopenia, and hepatitis. Specific organ damage may lead to pneumonitis in recipients of lung or heart-lung transplants; the development of myocarditis, retinitis, or accelerated vascular damage and atherosclerosis after cardiac transplantation; hepatitis and pancreatitis in liver and pancreas transplant recipients, respectively; and gastrointestinal disease. CMV infection in transplant recipients has also been associated with delayed or failed bone marrow engraftment, an increased incidence or severity of graft-versus-host disease, and an increased risk of graft rejection in solid organ transplants. Death may occur as a result of various complications, including bacterial and fungal superinfections. CMV infection, particularly when associated with pneumonitis, is an important cause of morbidity and mortality after hematopoietic stem cell transplantation. In patients infected with HIV, CMV is an important cause of fever, sight-threatening retinitis, encephalitis, polyradiculomyelopathy, and gastrointestinal infections including esophagitis, gastritis, and ulcerative colitis. However, there has been a significant decline in severe CMV disease in AIDS patients with the introduction of highly active antiretroviral therapy.

Treatment and Prevention

Antiviral drugs, including ganciclovir, valganciclovir, foscarnet, cidofovir, and fomivirsen, have been licensed by the U.S. Food and Drug Administration (FDA) for treatment of CMV retinitis in immunocompromised hosts, particularly in patients with AIDS. Ganciclovir, valganciclovir, valacyclovir, cidofovir, and foscarnet have been used with or without immunoglobulin products containing CMV-specific antibodies in various strategies as universal prophylaxis, preemptive therapy, and treatment for CMV disease in transplant recipients (for a review, see references 9 and 10). Newborns with congenital or perinatal CMV disease may benefit from treatment with ganciclovir or valganciclovir, but experience is limited and additional clinical trials are warranted. Use of antiviral drugs to treat CMV infections in immunocompetent adults is seldom indicated, and further studies are needed in hospitalized, critically ill immunocompetent patients with CMV to determine if treatment is beneficial. Several experimental CMV antiviral drugs are being studied and include brincidofovir (an orally bioavailable derivative of cidofovir formerly called CMX001), maribavir (an oral inhibitor of the CMV UL97 kinase), letermovir (a CMV UL56 terminase inhibitor), and leflunomide (an arthritis drug that inhibits CMV virion assembly). There are currently no licensed vaccines available for prevention of CMV disease. A number of candidate vaccines have been developed and are in various stages of preclinical and clinical evaluation (for a review, see reference 11). These include a live attenuated whole-virus vaccine and DNA plasmid, subunit, and recombinant vaccines.

Asymptomatic excretion with CMV is common, and standard precautions and good personal hygienic practices (e.g., careful hand washing) can help decrease the possibility of transmission. Common disinfectants containing alcohol, detergents, and chlorine are effective in inactivating the virus. Transmission of CMV by blood transfusion or through breast milk can be minimized by using CMV antibody-negative donors or treating the products to remove or kill the virus prior to use.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

At present, a variety of methods are available for use in the diagnosis and management of patients infected with CMV. These include isolation of the virus in cell culture, histologic and cytologic techniques, assays for the direct detection and/or quantification of viral proteins or nucleic acids, serologic tests, phenotypic and genotypic assays to screen for antiviral drug resistance, and tests to measure cellular immune function of the host and control of CMV infection. The selection of assays to perform and the choice of specimen(s) to be tested depend on the patient population and clinical situation and the intended use of the individual tests. The details of specimen collection and processing are given in chapter 79.

Specimens for Direct Detection

Tissue specimens, respiratory secretions, urine sediment, cerebrospinal fluid (CSF), umbilical cord blood, amniotic fluid, aqueous humor and vitreous fluid, and peripheral blood leukocytes have been used for the direct detection of CMV antigens or nucleic acids from various patient populations. Blood specimens have proven most useful for identification and monitoring of CMV disease in immunocompromised hosts. Purified blood leukocytes are used when performing the CMV antigenemia assay, while whole blood, plasma obtained from anticoagulated whole blood, serum obtained from clotted blood, or purified peripheral blood leukocytes have all been used to quantitate CMV DNA in molecular amplification assays (12, 13, 14, 15). EDTA is currently the preferred anticoagulant for molecular testing since it is considered to be the most effective stabilizer of nucleic acids in blood. The optimal frequency of blood collection remains to be established for surveillance of different patient groups, although it is common practice to submit blood specimens once a week while monitoring viral loads during preemptive antiviral therapy (9, 10). The first specimen should be collected at the start of therapy to establish a baseline viral load (16). There is considerable debate about which blood compartment is best suited for the detection of CMV DNA (14, 17, 18). Although peripheral blood leukocytes were commonly used in the past, whole blood and plasma are now considered the specimens of choice (19). Whole blood has been shown to yield the highest levels and most frequent and earliest detection of CMV DNA of all blood compartments examined (14) and may represent the most practical specimen for laboratories to process for use in the diagnosis and monitoring of CMV disease, since it requires little processing prior to extraction of the DNA and both cell-associated and extracellular viruses can be detected. Also, delays in sample preparation can result in lysis of leukocytes after blood collection, which may result in inaccurate quantitation of CMV DNA if either purified leukocytes or plasma is used as a specimen source (20). A potential limitation for the use of whole blood is the possibility of variation in leukocyte counts from patient to patient; this may lead to erroneous quantitative measurements if fluctuations in cell numbers are not taken into consideration. Also, detection of low levels of CMV DNA in whole blood may not always correlate with active CMV disease. Plasma may be preferable in neutropenic patients who may have inadequate numbers of leukocytes for testing and may be a better specimen source for predicting CMV disease. When monitoring patients over time, the same specimen type should be used to minimize the variations observed between different blood compartments. For the CMV antigenemia assay, a total of 4 to 7 ml of whole blood is usually recommended for collection, but at least 10 ml of blood may be required for patients with severe neutropenia (e.g., absolute neutrophil counts of less than 200/mm^3). Any type of anticoagulated blood can be used, including blood collected in heparin, EDTA, sodium citrate, or acid citrate dextrose, although the most extensive experience has been with using heparin or EDTA. The blood should be kept at 4°C during storage and transport and should be processed within 6 to 8 h of collection for accurate and reliable quantitation of the viral load and within 24 h and no later than 48 h for qualitative testing (21). A decrease in quantitative antigenemia levels after storage of blood specimens for 24 h to 48 h has been described, although most positive specimens remain positive after this time when held at 4°C (21, 22). As a general rule, specimens for molecular amplification should be stored at 4°C immediately after collection and then promptly transported to the laboratory for processing. CMV DNA has been shown to be stable in whole blood for up to 5 days when stored at 4°C, for 3 to 4 days when stored at room temperature, and for 14 days at 4°C when plasma is separated from whole blood (23, 24). Single-use aliquots of processed specimens should be placed in multiple cryovials for testing and storage.

By processing in this manner, specimens are not frozen and thawed repeatedly and are never returned to the original specimen cryovial, thereby avoiding possible degradation of the DNA and cross-contamination of specimens, respectively. If not tested immediately, specimens should be promptly frozen and stored at −70°C. Impression smears, frozen sections, or formaldehyde-fixed and paraffin-embedded material can be used for *in situ* hybridization or histopathologic examination of tissue specimens obtained from patients with pneumonia, gastrointestinal disease, hepatitis, myocarditis, retinitis, pancreatitis, nephritis, cystitis, or central nervous system disease. For prenatal diagnosis of congenital CMV infection, collection of amniotic fluid has largely replaced chorionic villus sampling or cordocentesis to collect fetal blood. Amniotic fluid should be collected after 21 to 23 weeks of fetal gestation for best sensitivity (25). Neonatal blood collected at birth and dried on paper (e.g., Guthrie cards) as blood spots has been described for neonatal screening and for retrospective diagnosis in patients beyond the neonatal period with a clinical suspicion of congenital CMV infection (for a review, see references 26 and 27). However, dried blood spots and blood in general are not as sensitive as urine or saliva for newborn screening. Dried blood spots have also been used for rapid genotyping of CMV strains in congenitally infected newborns (28) and for quantification of CMV DNA in solid organ transplant recipients (27), and both liquid- and dried-saliva specimens have proven useful for sensitive and specific detection of CMV DNA in newborns (29).

Specimens for Virus Isolation

CMV can be isolated from a variety of body fluids and tissues; however, urine, respiratory secretions (e.g., saliva, throat washings, and bronchoalveolar lavage fluid), and anticoagulated whole blood (leukocytes) are most common for diagnostic purposes. Urine specimens should be clean-voided specimens. Because excretion of CMV in urine is intermittent, increased recovery of the virus is possible by processing more than one specimen. Adjustment of urine specimens to pH 7.0 with 0.1 N NaOH or 0.1 N HCl is recommended to reduce toxicity to cell cultures. Centrifuging urine specimens to obtain sediment-enriched samples has been advocated but is usually unnecessary and may produce toxicity more frequently than do uncentrifuged urine samples. Blood leukocyte cultures have been used in the evaluation of immunocompromised patients. Detection of CMV in leukocytes is often a better indicator of symptomatic CMV infection than is shedding of virus in urine or respiratory secretions, although the sensitivity of cultures from blood is inadequate in comparison to using molecular methods or the antigenemia assay. Fresh blood collected in the presence of heparin, sodium citrate, acid citrate dextrose, or EDTA may be used. A number of procedures for obtaining peripheral blood leukocytes have been described. However, density gradient centrifugation with either Ficoll-Hypaque or a mixture of sodium metrizoate and dextran 500 is most suitable for a clinical laboratory. Both mononuclear cells and granulocytes are efficiently separated from erythrocytes in a single step. The procedure is rapid and easy to perform, and the reagents are commercially available. Compared with traditional sedimentation methods, the technique results in a greater number of virus isolates and an increased yield of infectious foci or plaques. Alternatively, the direct lysis procedure described below may be used, although a reduced ability to culture CMV following direct erythrocyte lysis has been reported (30). Bronchial washings and tissue biopsy and autopsy specimens, particularly of lungs, kidneys, spleen, liver, brain, the gastrointestinal tract, and retinas, also can be processed for virus isolation. Recovery of CMV from tissues is strong evidence for organ involvement. All tissue specimens should be placed in a suitable viral transport medium immediately after collection. Since CMV loses infectivity when subjected to freezing and thawing, specimens for virus isolation should be kept at 4°C in an ice-water bath or refrigerator until they can be used to inoculate cultures, preferably within a few hours after collection. When prolonged transport times are unavoidable, infectivity is reasonably well preserved for at least 48 h at 4°C. In general, it is never a good idea to hold specimens for CMV isolation at room temperature, and specimens should be transported to the laboratory as quickly as possible after collection to maintain viability of the virus. If freezing the specimen is necessary, an equal volume of 0.4 M sucrose-phosphate added to the specimen helps preserve viral infectivity. All frozen specimens should be stored at −70°C or colder or in liquid nitrogen. Loss of virus infectivity occurs if specimens are stored at −20°C. All specimens are treated with antibiotics before inoculation of cell cultures.

Specimens for Serologic Testing

Single serum specimens for immunoglobulin G (IgG) antibody are useful in screening for evidence of past infection with CMV and for identifying individuals at risk for CMV infection. This approach is especially helpful in testing sera from organ transplant donors and recipients before transplantation and from donors of blood products that are to be administered to premature infants or bone marrow transplant patients. Also, knowing the immune status of women prior to conception may be helpful in identifying those individuals who may be most susceptible to primary CMV infection following pregnancy. For serologic diagnosis of recent CMV infection, detection of IgM in a single serum specimen may be beneficial or paired sera should be obtained at least 2 weeks apart when testing for IgG antibody. The acute-phase serum sample should be collected as soon as possible after onset of illness and tested simultaneously with the convalescent-phase serum sample. If congenital infection is suspected, specimens from mother, fetus, and newborn can be submitted for the evaluation of IgG and IgM antibodies for the detection of prenatal, natal, and postnatal CMV infections. When testing for IgM in the fetus, blood should be collected after 22 weeks of gestation since fetal synthesis of antibodies starts at 20 weeks of gestation and may not reach detectable levels for 1 to 2 more weeks. Processed serum specimens may be stored at 2 to 8°C for several days pending the completion of testing or can be stored frozen at −20°C or colder for more-extended periods of time. Repeat freezing and thawing should be avoided, and specimens should not be stored in frost-free freezers. Testing saliva or oral fluids for CMV-specific antibodies has been suggested as a noninvasive alternative to the collection of blood from children (31). In patients with CMV neurologic disease, CSF may be tested for viral antibody if paired with a serum specimen collected on or close to the same date. However, the yield of such testing is low and limited by delays in intrathecal production of virus-specific antibody and passive transfer of serum antibodies across a damaged blood-brain barrier. As a general rule, screening for CMV-specific IgG and/or IgM antibodies to diagnose congenital CMV infection and antibody testing of CSF for the diagnosis of CMV neurologic disease have limited utility and have been largely replaced by more-direct methods like PCR.

Specimens for Measurement of Cell-Mediated Immunity

Whole blood in heparin as the anticoagulant is collected for assays that measure cell-mediated immune responses to CMV infection (32, 33). For enzyme-linked immunosorbent spot (ELISPOT) tests, a total of 15.0 ml of whole blood is typically used to purify and stimulate peripheral blood mononuclear cells using defined CMV antigens. The number of cells is adjusted to 2.0×10^5/reaction for use in the assay. For described Quantiferon assays, a total of 1.0 ml of whole blood is collected into a tube containing CMV antigens dried onto the inner wall of the tube. The blood is vigorously mixed by shaking for 5 seconds to ensure that the entire inner surface of the tube has been coated with blood and then immediately stored at 37°C for 16 to 24 hours to stimulate cells before processing by centrifugation to obtain plasma for testing. Plasma samples can be stored for up to 4 weeks at 2 to 8°C or for extended times at −20°C or below prior to testing.

DIRECT EXAMINATION

Histopathologic testing, antigenemia assays, and qualitative and quantitative molecular methods have been routinely used for the direct detection of CMV from clinical samples. Histologic or immunohistochemical staining of tissue biopsy specimens is most useful in the diagnosis of localized CMV tissue-invasive disease, while antigenemia assays and molecular amplification tests are now the standard of care for testing and monitoring patients at increased risk for severe CMV disease, diagnosing active CMV disease, and monitoring response to therapy (for a review, see references 34 to 36).

Histopathologic Testing

Characteristic large cells (cytomegalic cells) with basophilic intranuclear inclusions and, on occasion, eosinophilic cytoplasmic inclusions can be seen in routine sections of CMV-infected biopsy or autopsy material following staining with Wright-Giemsa, hematoxylin and eosin, or Papanicolaou stains (Fig. 1). The nuclear inclusion has the appearance of an "owl's eye" because it has marginated chromatin that is typically surrounded by a clear halo that extends to the nuclear membrane. The presence of characteristic cytologic changes by histopathologic testing suggests CMV infection and, while less sensitive than molecular methods like PCR, is more predictive of disease, especially in the gastrointestinal tract, and correlates with active disease in most cases. Overall, histopathologic diagnosis of CMV disease involves time and labor and is relatively insensitive, and since CMV can infect tissues without producing morphologic changes, failure to find typical cytomegalic cells does not exclude the possibility of CMV infection; additional virologic or serologic confirmation is suggested. The sensitivity of histopathologic testing can be increased somewhat by using immunohistochemical staining to detect CMV antigens or *in situ* hybridization to detect CMV nucleic acids. A major obstacle to tissue diagnosis of CMV is the need to perform invasive procedures to obtain specimens for testing.

Antigen Detection

The CMV antigenemia assay is a sensitive, specific, and rapid method for the early diagnosis of CMV infection and can be used for routine monitoring of patients at high risk for severe CMV disease, including recipients of solid organ and hematopoietic stem cell transplants, HIV-infected patients, and patients treated with immunomodulating drugs. The test is relatively simple to perform and is based on immunocytochemical detection of the 65-kDa lower-matrix phosphoprotein (pp65) in the nuclei of peripheral blood leukocytes. By using this assay, CMV can be detected before the onset of symptoms and the viral load can be quantified to assist in predicting and differentiating CMV disease from asymptomatic infection (37, 38). The procedure has also been used to evaluate the efficacy of antiviral therapy and to predict treatment failure and the development of viral resistance (39, 40), to prompt the institution of preemptive therapy (41, 42), to detect CMV in leukocytes of CSF from AIDS patients with infections of the central nervous system (43), and for diagnosis of congenital CMV infection (44), CMV gastrointestinal disease (45), and CMV infection in the immunocompetent host (5). The pp65 protein has also been found in endothelial cells circulating in the blood of immunocompromised patients, and some investigators have suggested that infection of these cells is associated with organ involvement and more-advanced disease (46, 47). The results of the antigenemia assay correlate well with the quantitative detection of CMV DNA in whole blood, leukocytes, or plasma in molecular amplification assays (17, 48–52).

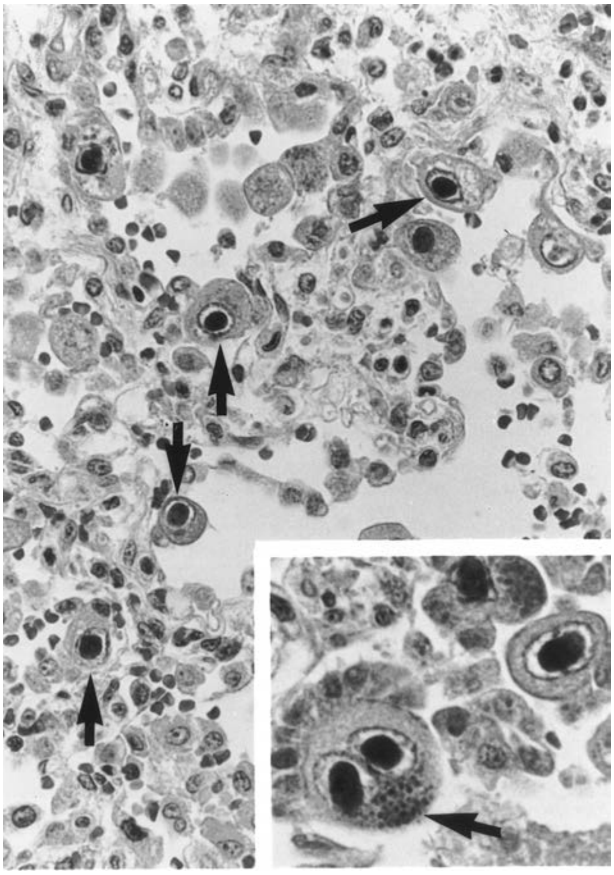

FIGURE 1 Fixed hematoxylin and eosin-stained lung tissue from a patient with interstitial pneumonia. Note the numerous giant cells that possess large intranuclear inclusions surrounded by characteristically clear halos (arrows). Less pronounced granular inclusions may also be present in the cytoplasm (inset). (Courtesy of Eduardo Ruchelli.)
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In the antigenemia assay, leukocytes (mainly polymorphonuclear leukocytes) are enriched from freshly collected whole blood by sedimentation with dextran. A method for direct lysis of erythrocytes and subsequent isolation of leukocytes from whole blood has been described (53) and is now routinely used by many laboratories. This modification allows for a shorter total assay time and the capability of processing more specimens. Following sedimentation, the remaining erythrocytes are lysed with ammonium chloride, the granulocytes are counted in a hemocytometer or automated cell-counting instrument, and a known number of cells (usually 2×10^5, although variations of 5×10^4 to 10^6 cells have been used) are cytocentrifuged onto microscope slides. The cells are fixed with formaldehyde or paraformaldehyde and then permeabilized with the nonionic detergent Nonidet P-40 and stained with suitable monoclonal antibodies directed against CMV pp65. These processes are followed by incubation of the cells with a fluorescein isothiocyanate-labeled secondary antibody diluted in a counterstain. Slides are read by microscopy at magnification of ×200 to ×400. Positive results are viewed as homogeneous yellow to apple green fluorescence within the nuclei of infected cells (Fig. 2). Quantitative results are usually expressed as the number of antigen-positive cells per total number of leukocytes evaluated. Absolute CMV antigenemia values or specific thresholds to predict symptomatic disease or prompt administration of preemptive antiviral therapy have not been fully established for interpretation of quantitative testing, and they appear to be different between patient populations. Therefore, it is more important to monitor patients and trend relative rises and/or falls in the level of antigen-positive cells in multiple blood specimens collected over time than it is to rely on a single test result. The presence of small numbers of antigen-positive cells generally indicates asymptomatic infection, whereas increasingly larger numbers are more strongly associated with clinically significant disease. In patients with severe immunosuppression (e.g., after allogeneic hematopoietic stem cell transplantation), however, even very small numbers of antigen-positive cells may be significant. For transplant recipients, the frequency and extent of monitoring vary with the institution and from one transplant population to another and depend on the medical approach taken to manage CMV infection and disease (for a review, see references 35 and 36).

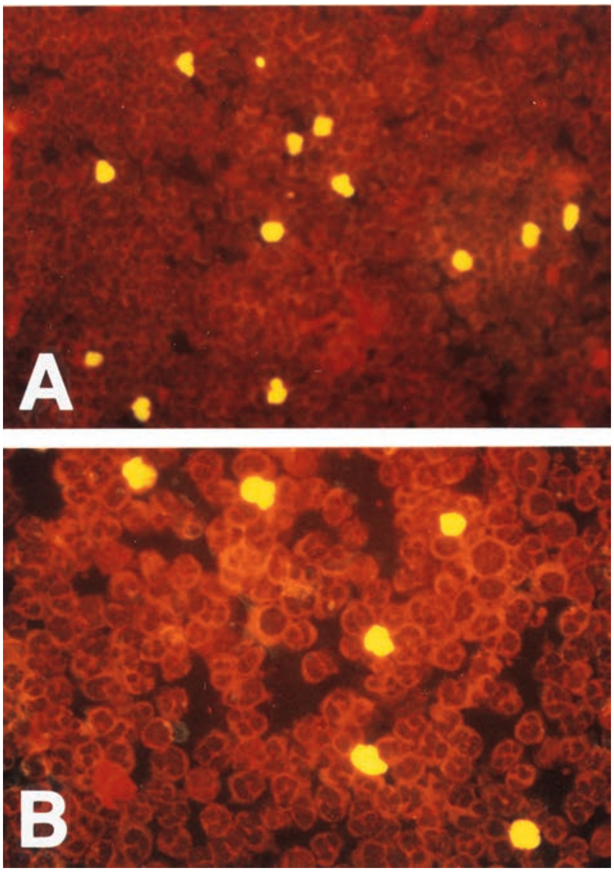

FIGURE 2 CMV antigen-positive polymorphonuclear leukocytes. Note the nuclear staining when a monoclonal antibody directed against pp65 is used. Magnification, ×100 (A) or ×400 (B). doi:10.1128/9781555817381.ch100.f2

The major advantages of the antigenemia assay are that it is more sensitive than either conventional tube or shell vial cultures for the detection of CMV from blood; it can be completed in 2 to 4 h, providing same-day turnaround of results; and the procedure can be readily modified for quantitative measure of the viral load. The assay has the disadvantages of being labor-intensive and time-consuming, particularly when large numbers of specimens are being processed; the blood must be processed in a timely manner for accurate results; and the test should be performed by personnel with experience in immunocytochemical techniques due to the subjective nature of the interpretation. Considerable time, effort, and expertise are needed in isolating and counting cells, adjusting the cell concentration to 10^6/ml before preparing the slides for staining, and then reading the stained slides. Also, interlaboratory variability exists with regard to the exact procedures used to perform the antigenemia assay and there is a definite need for assay standardization (54–56). Commercial kits containing monoclonal antibodies and other reagents needed to perform the antigenemia assay are available (Table 1). Several of these assays have been licensed by the FDA, and comparative studies have demonstrated equivalent performances. Because of the low throughput, the antigenemia assay is best suited for laboratories processing small numbers of specimens.

Nucleic Acid Detection

Over the years, molecular amplification assays have progressively replaced the antigenemia assay and other nonmolecular tests for the diagnosis and monitoring of CMV infections. PCR is currently the most preferred and widely used molecular method for the detection of CMV DNA and mRNAs, and the sensitivity and specificity of PCR for diagnosis of active CMV infection have been evaluated (63–67). Amplification has been performed with a variety of primer pairs from gene regions of the immediate early antigen (UL122-123 locus), the major immediate early antigen (UL122-123 locus), the DNA polymerase (UL54), glycoproteins B (UL55) and H (UL75), pp65 (UL83), pp67 (UL65), US17, HXFL4, the EcoRI D fragment, the HindIII X fragment, the pp150 tegument protein (UL32), the major capsid protein (UL86), and the junction between glycoprotein B and the major immediate early antigen. Based on published literature, the most common targets for CMV PCR include glycoprotein B, the immediate early antigen, the major immediate early antigen, and US17 followed by the pp65 and polymerase genes. Not all primers are equally sensitive in amplifying CMV DNA, and in several studies the sensitivity of the assay was increased by amplifying genomic regions from both the immediate early and the late CMV genes or by using nested primers to a single gene fragment. The use of both gene fragments enabled the detection of a variety of clinical isolates, indicating that

TABLE 1 Available commercial CMV antigenemia assays

Manufacturer[a]	Product name	Recommended blood collection and processing: Amt (ml)	Anticoagulant used	Storage conditions	Cell separation technique	No. of cells per slide	Monoclonal antibody used	Total assay time (h)	Reference(s)
Bio-Rad Laboratories, Inc., Hercules, CA	CMV Brite	5–10	Heparin or EDTA	20–25°C for 6–8 h	Dextran sedimentation	1.5×10^5	C10/C11	4–5	57, 58
	CMV Brite Turbo	3–5	EDTA	20–25°C for 6–8 h	Direct erythrocyte lysis with NH_4Cl	2.0×10^5	C10/C11	2	58–60
bioMérieux, Inc., Durham, NC	Argene CINAkit HCMV ppUL83 Rapid Antigenemia	7	Heparin or EDTA	2–8°C for up to 24 h	Dextran sedimentation or direct erythrocyte lysis with NH_4Cl	2.0×10^5	1C3 + AYM-1	4–5 or 2	58, 61
Millipore Corp., Temecula, CA	Light Diagnostics CMV pp65 Antigenemia	5–10	Heparin or EDTA	20–25°C for up to 24 h	Dextran sedimentation or direct erythrocyte lysis with NH_4Cl	2.0×10^5	Proprietary	4–5 or 2	62

[a] CMV Brite, CMV Brite Turbo, and CINAkit are FDA licensed for qualitative testing only; Light Diagnostics kit is not FDA licensed; all kits are CE marked.

strain variability is not a limiting factor for PCR diagnosis of CMV. PCR has been used successfully to detect CMV DNA in a variety of clinical specimens from transplant recipients, patients with AIDS, and infants with congenital infection. It also has been used for the continued surveillance of immunocompromised patients and for evaluation of the therapeutic efficacy of antiviral drugs (39, 68, 69).

Of concern with PCR for CMV diagnosis is whether the test can distinguish between active disease and asymptomatic infection or latency. CMV viremia is considered to be the best predictor of CMV disease, and CMV DNA has been successfully detected in whole blood, purified peripheral blood leukocytes, plasma, and serum by PCR (15, 66, 68, 69). However, PCR is extremely sensitive and the qualitative detection of CMV DNA in these specimens has limited value in predicting symptomatic disease and in monitoring the success of antiviral therapy in immunocompromised patients. CMV DNA can be detected in blood by PCR in the absence of disease and can be found for weeks or months after successful therapy of symptomatic patients (39, 70, 71). However, there are clinical settings in which qualitative PCR appears to be useful, including in the diagnosis of less common forms of CMV infection, such as detection of CMV in CSF of patients with encephalitis or polyradiculomyelitis; in urine, tissue, amniotic fluid, or fetal blood for diagnosis of congenital CMV infection; in the aqueous or vitreous humor in patients with CMV retinitis; and in the blood of patients at high risk of severe infection, such as CMV donor-positive, recipient-negative transplant patients, so that antiviral therapy can be started early in the course of infection. Also, a negative result from qualitative PCR of blood has a high negative predictive value for excluding systemic CMV disease, so qualitative PCR can be used as an initial screen of blood specimens before quantifying CMV DNA from only those samples that are positive. Qualitative PCR can also be used on tissue specimens from immunocompromised patients with suspected localized or compartmentalized tissue-invasive disease. However, careful consideration should be given to whether detection of CMV by highly sensitive molecular methods truly represents infection of the organ or possible contamination with the virus from infected extracellular fluids that bathe the tissue. While negative PCR results would suggest absence of CMV tissue-invasive infection, positive results may not be significant and should be correlated with clinical and histopathologic findings and detection of CMV viremia in blood. In this regard, *in situ* hybridization or immunohistochemical staining may be helpful in localizing the virus within the tissue.

Similar to the antigenemia assay, measuring the level of CMV DNA in blood appears necessary to predict and diagnose CMV disease in immunocompromised hosts (72, 73). Consequently, a number of quantitative and semiquantitative molecular assays have been developed over the years and have included target and signal amplification methods. Most laboratories now use quantitative real-time PCR (12, 74–80) because it allows for detection of amplified nucleic acids as they accumulate, and technological advances have provided for rapid and accurate quantification over a dynamic range of orders of magnitude, using a variety of different chemistries and platforms. Also, assays for CMV can be multiplexed with tests for other herpesviruses for simultaneous monitoring following transplantation (81, 82). The performances of individual PCR assays have been compared to the antigenemia assay and culture as well as to each other (48, 50, 51, 83–88). Overall, these molecular methods provide quantitative results comparable to those obtained by the antigenemia assay; they are also more sensitive than culture for detecting CMV infection and can detect CMV before the onset of clinical symptoms. It has been shown that transplant recipients and AIDS patients with active CMV disease have higher levels of CMV DNA and that a rapid rise in the CMV DNA copy number correlates with the presence of symptoms and drug failure during treatment (49, 89–91). Also, Rasmussen et al. (92) have determined that quantitation of CMV DNA from peripheral blood leukocytes can be used to identify HIV-infected patients at risk for development of symptomatic CMV retinitis. It has also been determined that immunocompromised patients with CMV disease have more CMV DNA in either whole blood, plasma, or leukocyte fractions than do patients without disease but that the level of CMV DNA in whole blood or leukocytes is higher than that in plasma (49, 93). Quantitation of CMV in plasma, therefore, may be less sensitive for monitoring CMV infection, although a positive result may correlate better with active disease. Although there is general agreement between PCR and antigenemia assays, the quantitative numerical relationship is not exact and differences should be expected since these assays measure different biological features of CMV replication and infection. PCR is extremely sensitive and may detect CMV earlier than the antigenemia assay and may continue to be positive after antigen testing is negative. Conversely, negative PCR results have been reported for patients with low numbers of positive cells in the antigenemia assay (51). Therefore, depending on the test and parameters used, these assays may be more or less sensitive when compared to one another and may have higher or lower positive predictive values for CMV disease. As in the antigenemia assay, absolute CMV DNA levels or threshold values for predicting symptomatic disease and initiating preemptive therapy have not been determined and may differ by specimen type and testing platform selected and from one laboratory and patient population to another. It is currently more important to monitor the relative changes in DNA levels from serial blood specimens collected over time and tested using the same assay and specimen type. If desired, specific cutoff levels should be prospectively determined based on the assay and specimen used and the type of patients being monitored. Because of the high sensitivity of PCR and depending upon the cutoff value selected, the positive predictive value for symptomatic CMV disease may vary and may affect clinical decision making and the length of antiviral therapy. Quantitative PCR methods for the estimation of CMV DNA levels in CSF, amniotic fluid, tissue, and urine of CMV-infected patients have been described (44, 83, 94–96), but more research is needed to assess the clinical relevance of quantitating CMV DNA from these specimens. It has been shown that the prognosis of congenital CMV infection may be directly related to the amount of CMV in the urine of an infected neonate and that quantitating CMV DNA in the CSF of AIDS patients may help determine if disorders of the central nervous system are attributed more to CMV than to the direct effects of HIV or other opportunistic pathogens.

In general, real-time PCR assays for the detection and quantification of CMV DNA are sensitive, specific, and reproducible and significantly reduce the time necessary to report results that may have an impact on the care and management of patients. The method offers the distinct advantages of being less technically demanding and less expensive to perform than more conventional assays, and quality reagents and automated instruments for nucleic acid extraction and amplification and detection are readily available and greatly improve the potential for standardization and increased accuracy of results. The assays also require less specimen volume for testing and can be easily performed on neutropenic patients with low leukocyte counts, the DNA is stable during extended specimen transport and storage times, and large numbers of specimens can be efficiently processed. Furthermore, the overall risk of amplicon contamination has been minimized, as real-time platforms are closed systems. Various commercial real-time and end-point PCR assays are available as either analyte-specific reagents or packaged as complete kits (Table 2). However, most real-time PCR assays are developed in the laboratory by the end user and differ in the types of specimens used, the nucleic acid target selected, the choice and design of suitable oligonucleotide primers and probes, optimization of specimen extraction and PCR amplification conditions, the controls and calibration standards used, the method chosen to detect and/or quantify the amplified product, and the quantitative units of measure and vial loads reported from one laboratory and method to another. Many of the assays require additional validation, and there is a definite need for traceable and commutable international reference materials that include both standards and controls for institutional comparison of results, since significant variability in CMV quantification has been observed between laboratories (118–121). To this end, the first international CMV standard was developed by the World Health Organization in 2010 and is available from the National Institute of Biological Standards and Controls in the United Kingdom. Secondary material calibrated against this standard now can be purchased from commercial manufacturers, and a second standard reference material has been made available from the National Institute of Standards and Technology in the United States (122). Use of these standard reference materials and the recommended reporting of viral loads in international units/ml (IU/ml) should allow for broader interassay agreement among different laboratories using different quantitative molecular assays (123) and should facilitate the establishment of defined testing algorithms and cutoff values that predict clinical disease and improve management and outcomes in CMV-infected patients. A commercial quantitative assay, the Roche COBAS AmpliPrep/COBAS TaqMan CMV Test, has been developed using this international standard and has been licensed by the FDA. Results are expressed in IU/ml, and the level of agreement has been shown to be high across different laboratories during evaluations of this system (124, 125). More recently, droplet digital PCR has been developed and has the advantages of direct quantification of CMV DNA without the requirement for standards and the use of a calibration curve to facilitate accurate measurements of viral load (126). Ultimately, the clinical utility of these assays depends on their accuracy, reproducibility, precision, ease of use, cost, availability, and predictive value. Molecular methods, however, are now considered to be invaluable additions to the analytical tools already being used to provide a rapid diagnosis of established CMV disease, identify patients at risk of developing disease, assess the progression of disease and the risk of relapse, direct the initiation of preemptive therapy, monitor the response to therapy, and predict viral resistance and treatment failure.

ISOLATION PROCEDURES

Cell Cultures

Human fibroblasts best support the growth of CMV and therefore are used for diagnostic purposes. Acceptable fibro-

TABLE 2 Selected commercial molecular assays available for qualitative and quantitative detection of CMV[a]

Manufacturer	Assay name	Method	Target	Measurement	Availability	Reference(s)
Abbott Molecular, Des Plaines, IL	Artus CMV PCR	Real-time PCR	UL123 MIE gene	Quantitative; calibrated against 1st WHO International Standard for CMV	ASR in United States; CE-marked kit in Europe	97–99, 100, 104, 105
Altona Diagnostics, Hamburg, Germany	RealStar CMV PCR kits 1.0 and 1.2	Real-time PCR	Proprietary	Quantitative; calibrated against 1st WHO International Standard for CMV	Not available in United States; CE-marked kit in Europe	
bioMérieux, Inc., Durham, NC	Argene CMV R-gene	Real-time PCR	UL83 pp65 gene	Quantitative	RUO in United States; CE-marked kits in Europe	101, 102
	Argene CMV HHV6,7,8 R-gene	Real-time PCR	CMV UL83 pp65 gene	Quantitative		
Cepheid Europe, Maurens-Scopont, France	Affigene CMV Trender	Real-time PCR	Proprietary	Quantitative	Not available in United States; CE-marked kit in Europe	103, 104
	Smart CMV	Real-time PCR	Proprietary	Quantitative		
ELITech North America, Princeton, NJ	MGB Alert CMV 3.0 Primers and Probes	Real-time PCR	UL123 IE 1 gene	Qualitative and quantitative	ASR for laboratory-developed assays	105
ELITech Group, Puteaux, France	Alert Q-CMV Real Time Complete	Real-time PCR	UL123 MIE gene	Quantitative	Not available in United States; CE-marked kit in Europe	106
	Alert CMV Early Complete	Nested endpoint PCR	UL123 MIE gene	Qualitative		
Focus Diagnostics, Cypress, CA	Simplexa CMV	Real-time PCR	UL83 gene	Quantitative; calibrated against 1st WHO International Standard for CMV	Not available in United States; CE-marked kit in Europe	105
Luminex Corp., Austin, TX	EraGen Multicode CMV Primers	Real-time PCR	UL54 pol gene	Qualitative and quantitative	ASR for laboratory-developed assays	105
PrimeraDx, Mansfield, MA	ICEPlex ViraQuant Panel (BKV, CMV, EBV, HHV-6, HHV-7)	Real-time PCR	Proprietary	Quantitative	RUO	107
Qiagen, Valencia, CA	Artus and EASY artus CMV PCR	Real-time PCR	UL123 MIE gene	Quantitative	ASR in United States; CE-marked kit in Europe	97, 102, 108
Roche Diagnostics, Indianapolis, IN	COBAS AmpliPrep/COBAS TaqMan CMV Test	Real-time PCR		Quantitative; calibrated against 1st WHO International Standard for CMV	FDA licensed in United States; marked for *in vitro* diagnostic use in Europe and Canada	109–112
	COBAS Amplicor CMV Monitor	Endpoint PCR	UL54 pol gene	Quantitative	Not available in United States; CE marked in Europe	113–116
	LightCycler CMV Quantitative Kit	Real-time PCR	UL54 pol gene	Quantitative	CE-marked kit in Europe	108, 117

[a] Abbreviations: pol, polymerase; pp65, phosphoprotein 65; MIE, major immediate early; IE1, immediate early 1; ASR, analyte-specific reagent; RUO, research use only.

blast cultures include those prepared from human embryonic tissues or foreskins and serially passaged diploid human fetal lung strains such as WI-38, MRC-5, or IMR-90. Diploid fibroblast cells should be used at a low passage number, since they may become less susceptible to CMV infection with increasing cell generations. Several of these fibroblast cell lines are commercially available. Culture systems for the detection of CMV in clinical specimens include conventional tube cultures and spin amplification shell vial assays. Although still performed in many laboratories, the use and clinical utility of cultures for CMV have diminished owing to a much better overall performance and ease of use of newer and more-efficient non-culture-based detection methods.

Conventional Tube Culture

Specimens to be tested are added in a volume of 0.2 ml to tubes of confluent fibroblasts maintained in Eagle minimal essential medium with 2% fetal bovine serum. Alternatively, the tubes are drained of medium, the inocula are absorbed for 1 h in a stationary position or by centrifugation at 700 × *g* for 45 min at 30 to 33°C, and then fresh medium is added. After inoculation, the tubes can be rolled or kept stationary at 37°C. Twenty-four hours later, the medium is changed in tubes inoculated with urine or leukocyte specimens. Thereafter, and for other types of specimens, the medium is changed once a week or more frequently as the pH of the culture medium changes or if toxicity appears. When toxicity necessitates passage of the culture, cells rather than culture medium should be passaged, since CMV remains mostly cell associated. Cells are removed by addition of 0.25% trypsin–0.1% EDTA to the monolayers and incubation at 37°C for approximately 1 min. When the cells detach, Eagle minimal essential medium with 2% fetal bovine serum is added, and the cells are used to inoculate fresh tubes. Tubes are examined for cytopathic effect (CPE) for at least 4 weeks for most specimens (6 weeks for leukocyte specimens). Control, uninoculated cultures are handled in the same manner as those inoculated with clinical specimens.

CMV isolates are normally identified solely on the basis of characteristic CPE and host cell range. The time of appearance and the extent of CPE depend on the amounts of virus present in specimens. In cultures inoculated with urine from a congenitally infected newborn, CPE may develop by 24 h and progress rapidly to involve most of the monolayer if the virus titer in the urine is extremely high. More commonly, foci of CPE, consisting of enlarged, rounded, refractile cells, appear during the first week, and progression of CPE to surrounding cells proceeds slowly (Fig. 3). In cultures inoculated with urine or respiratory specimens from older individuals, CPE usually appears within 2 weeks. Leukocyte cultures may not become positive until after 3 to 6 weeks. The usual slow progression of CPE in tube cultures inoculated with clinical specimens is due, at least in part, to limited release of virus into extracellular fluid. With strains of CMV that have been serially passaged, including laboratory-adapted strains, greater amounts of extracellular virus are released and CPE progresses more rapidly. Viruses such as adenovirus and varicella-zoster virus occasionally produce CPE indistinguishable from that of CMV, so suspected CMV isolates are best confirmed by an immunofluorescence assay (IFA) using monoclonal or polyclonal antibodies that are available from various commercial sources. The appearance of typical nuclear fluorescence of infected cells indicates the presence of CMV. PCR or other molecular amplification methods also can be used for confirmation of suspected isolates.

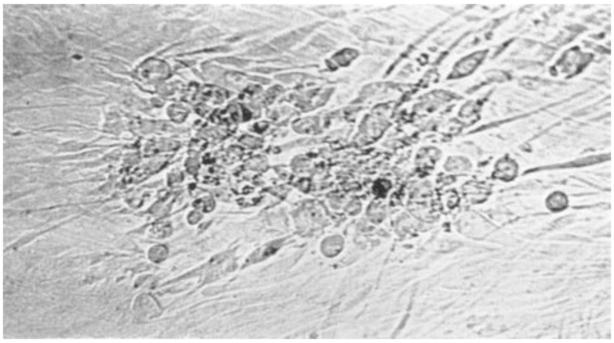

FIGURE 3 CPE produced by a CMV isolate in human skin fibroblasts 10 days postinoculation. Unstained preparation; magnification, ×100. (Courtesy of Sergio Stagno.) doi:10.1128/9781555817381.ch100.f3

For storage of fresh isolates, monolayers exhibiting CPE are treated with trypsin-EDTA, and the cells obtained are suspended in Eagle minimal essential medium with 10% fetal bovine serum and 10% dimethyl sulfoxide and then frozen at −70°C. Infectivity can be better maintained for long periods by storage in liquid nitrogen.

Spin Amplification Shell Vial Assay

The spin amplification shell vial assay has been used extensively as a rapid culture method for the detection of CMV in clinical specimens and has largely replaced the slower conventional tube cultures in most laboratories. The technique is based on the amplification of virus in cell cultures after low-speed centrifugation and detects viral antigens produced early in the replication of CMV, before the development of CPE. Even low titers of virus present in specimens are easily amplified and rapidly detected within 24 h. Monoclonal antibodies are commercially available and are used for the detection of CMV early antigens. *In situ* hybridization with DNA probes to CMV has also been used.

MRC-5 fibroblast cells are grown to confluency on 12-mm-diameter round coverslips in 1-dram (3.7-ml) shell vials and inoculated with 0.2 ml of specimen. Shell vials of MRC-5 cells can be obtained commercially or prepared in the laboratory. Monolayers should be inoculated within 1 week after preparation, since older monolayers demonstrate decreased sensitivity to CMV and increased toxicity. Two vials should be inoculated for urine, tissue, and bronchoalveolar lavage fluid specimens, and three vials should be inoculated for blood specimens (127). Alternatively, disruption of purified leukocytes by sonication before their use in the shell vial assay may increase the sensitivity of CMV detection from blood (128). Increasing the frequency of blood collection and the volume of blood obtained also may enhance the diagnostic yield of the shell vial assay from this specimen (129). After inoculation, the vials are centrifuged at 700 × *g* for 40 min at 25°C, and then 2.0 ml of Eagle minimum essential medium containing 2% fetal bovine serum and antibiotics is added. The cultures are incubated at 37°C for 16 to 24 h, fixed with acetone, and stained. A longer incubation time may be used, but the time should be determined by each laboratory on the basis of individual experience, reagents, staining technique used, and whether monolayers are purchased or prepared in the laboratory. Uninfected and CMV-infected monolayers are included as

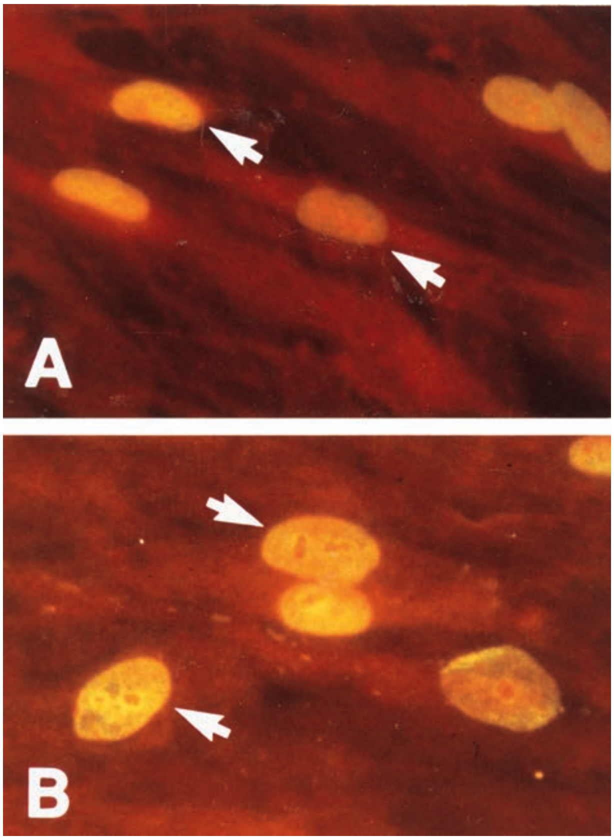

FIGURE 4 Demonstration of CMV early antigens in the nuclei (arrows) of infected MRC-5 cells following shell vial culture and IFA staining. (A) Staining of immediate early antigen appears as an even matte yellow to green fluorescence with specks of brighter yellow to green. (B) Viral inclusions (owl's eyes) may be visible in the nuclei. Magnification, ×400. doi:10.1128/9781555817381.ch100.f4

negative and positive controls, respectively. Mink lung (ML) cells, a nonhuman continuous cell line, are comparable to MRC-5 fibroblasts for the detection of CMV in clinical specimens by shell vial culture. A distinct advantage in using ML cells is that this cell line can be propagated and passaged in the laboratory for a long time without a decrease in susceptibility to CMV. Significantly less toxicity and an increase in the number of CMV-positive nuclei were also observed with ML cells. Coverslips are scanned at a magnification of ×200 to ×250, and specific staining is confirmed at ×400 to ×630. Positive cells contain yellow to apple green fluorescent nuclei against a red cytoplasmic background. Staining of immediate early antigen appears as an even matte yellow to green fluorescence with specks of brighter yellow to green (Fig. 4A). Viral inclusions (owl's eyes) may be visible in the nuclei (Fig. 4B).

The spin amplification shell vial assay has the important features of being rapid, sensitive, and specific, but skilled technical personnel and close attention to the quality of specimens, monolayers, and reagents are required for optimum performance.

SEROLOGIC TESTS

A variety of tests with high sensitivities and specificities are available for the detection of either IgM or IgG antibodies to CMV (for an overview, see reference 130). In deciding which test to perform, one should consider such factors as the number of specimens to be tested, the patient population, cost, turnaround time, equipment needs, and ease of performance. The method that is chosen depends on the needs of individual laboratories. For small-volume laboratories, IFA may be more cost-effective and practical, while enzyme immunoassays (EIA) may be more suitable for laboratories with higher volumes of specimens. Overall, detection of CMV-specific IgM or determination of a seroconversion from a negative to a positive IgG antibody response can be useful for the diagnosis of primary CMV infection in certain clinical settings, and the screening of blood and organ donors and recipients plays an important role in preventing the transmission of latent CMV to patients at high risk for severe CMV disease.

Enzyme Immunoassays

Over the years, EIAs have largely replaced other traditional methods for detecting antibodies to CMV. The main advantages of the EIA are that it is rapid, sensitive, and specific. In addition, multiple specimens can be handled daily at a relatively low cost. Kits that detect CMV IgG and IgM are available from a number of commercial sources (Table 3). The kits are easy to use, and the manufacturers have provided detailed instructions. All the materials necessary to perform the assay are included, and the reagents are stable with time. Some companies also provide a spectrophotometer and automated plate washer, which otherwise must be purchased separately at additional expense. The development of robotics technology has led to the commercial availability of both fully automated and semiautomated EIA instruments that include sample dispensers, diluters, washers, and spectrophotometers with complete computer programming and generation of electronic and written reports. More recently, an EIA that uses the envelope glycoproteins B and H to distinguish CMV strain-specific antibody responses has been developed (131). This assay may be useful for identifying strain diversity in different patient populations and may provide a better understanding of the implications of infection with multiple CMV strains and the role of strain-specific antibody in the protective immune response against CMV.

Immunofluorescence Assays

Indirect and anticomplement IFAs are commonly used methods for detecting CMV antibodies. In the indirect IFA, dilutions of test serum are incubated with virus-infected cells that have been fixed to a glass microscope slide. Specific antibody-antigen complexes are detected using an anti-human antibody conjugated with fluorescein isothiocyanate and fluorescence microscopy. Anticomplement immunofluorescence is similar to the indirect IFA. It differs, however, in that the test serum is first heat inactivated to remove endogenous complement activity and then incubated with virus-infected cells on glass slides. An exogenous source of complement is added and bound by any specific antigen-antibody complexes that have formed. A fluorescein-labeled anticomplement antibody is then added; it binds to the C3 component of complement, and the slides are read using a fluorescence microscope. Anticomplement IFA amplifies the fluorescent signal above what can be seen using an indirect IFA, allowing for the detection of small amounts of antibody or antibodies of low avidity. IFAs are useful and inexpensive methods that offer the advantages of speed and simplicity for the qualitative and quantitative detection of CMV antibodies. Commercial kits are readily available, or antigen-coated slides and labeled secondary antibodies can

TABLE 3 Immunoassay systems for CMV IgG and IgM antibody detection

Manufacturer	System(s)	CMV test availability	
		In the United States	Outside the United States
Abbott Diagnostics, Abbott Park, IL	Architect i1000SR, i2000SR, i4000SR	None	IgG and IgM, IgG avidity
Beckman Coulter, Inc., Brea, CA	Access/Access 2	None	IgG and IgM
	Unicel DxI 600, DxI 800, DxC 600i, DxC 660i, DxC 680i, DxC 860i, DxC 880i	None	IgG and IgM
bioMérieux, Inc., Durham, NC	VIDAS/Mini-VIDAS	IgG and IgM	IgG and IgM
Bio-Rad Laboratories, Hercules, CA	BioPlex 2200	IgG and IgM	IgG and IgM
	EVOLIS Premium/EVOLIS Twin Plus	IgG and IgM	IgG and IgM
DiaSorin, Inc., Stillwater, MN	ETI-MAX 3000	IgG and IgM capture	None
	LIAISON	IgG and IgM	IgG and IgM
Ortho Clinical Diagnostics, Raritan, NJ	VITROS ECi	None	IgG and IgM
	VITROS 3600/5600	None	IgG and IgM
Roche Diagnostics, Indianapolis, IN	Cobas 8000 (e602 module)	None; IgG and IgM and IgG avidity in development	IgG and IgM
	Cobas 6000 (e601 module)	None; IgG and IgM and IgG avidity in development	IgG and IgM
	Cobas 4000 (e411 module)	None; IgG and IgM and IgG avidity in development	IgG and IgM
Siemens Healthcare Diagnostics, Tarrytown, NY	Immulite 2000/2000 XPi	IgG and IgM	IgG and IgM
	Immulite 1000	IgG and IgM	IgG and IgM
Trinity Biotech, Jamestown, NY	Use of semiautomated washers and readers, Trinity Biotech DSX/DS2 automated open systems, or other open platforms (e.g., Awareness Technology Chem Well 2910, Bio-Rad PhD/PhD Ix, Diamedix Mago 4S/Mago Plus, Dynex Technologies Agility, Grifols Triturus, Inova Diagnostics Quanta-Lyser 240/160/2	IgG and IgM capture	IgG and IgM capture
Wampole (Alere), Waltham, MA	Use of semiautomated washers and readers, Alere DSX/DS2 automated open systems, or other open platforms (e.g., Awareness Technology Chem Well 2910, Bio-Rad PhD/PhD Ix, Diamedix MAGO 4S and MAGO Plus, Dynex Technologies Agility, GRIFOLS Triturus, Inova Diagnostics Quanta-Lyser 240/160/2	IgG and IgM	IgG and IgM
Zeus Scientific, Raritan, NJ	AttheNA Multi-Lyte	IgG	IgG

be purchased separately. The major disadvantages of IFA systems are that they require a fluorescence microscope and darkroom for examining slides and that extensive training is needed to read and interpret the test results.

CMV IgM Antibody Measurements

Commercial reagents and complete EIA and IFA diagnostic kits are available for measuring CMV IgM antibodies and are most useful in the diagnosis of CMV infection in newborns. The procedures are essentially the same as those used to detect IgG antibodies, except that anti-human IgM antibodies labeled with suitable markers are used to detect CMV-specific IgM bound to viral antigens on the solid phase. A recognized pitfall of CMV IgM assays is the occurrence of false-positive and false-negative reactions. False-positive reactions occur when sera contain unusually high levels of rheumatoid factor in the presence of specific CMV IgG. Rheumatoid factor is an immunoglobulin, usually of the IgM class, that reacts with IgG. It is produced in some rheumatologic, vasculitic, and viral diseases, including CMV infection. IgM rheumatoid factor forms a complex with IgG that may contain CMV-specific IgG. The CMV IgG binds to CMV antigen, carrying nonviral IgM with it; in this setting, a test designed to detect IgM will produce a false-positive result. False-negative reactions occur if high levels of specific IgG antibodies competitively block the binding of IgM to CMV antigen. Therefore, it is highly recommended to separate IgM and IgG fractions before testing to decrease the incidence of both false-positive and false-negative IgM test results.

Rapid and simple methods for the removal of interfering rheumatoid factor and IgG molecules from serum have been developed. These include selective absorption of IgM to a solid phase and removal of IgG by using hyperimmune anti-human IgG antibody, staphylococcal protein A, or recombinant protein G from group G streptococci. Serum pretreatment methods are now readily available and are incorporated in the procedures of commercially available IFA and EIA kits, which have resulted in more-reliable IgM tests. More recently, reverse capture solid-phase IgM assays have been used as an alternative approach to avoiding false-positive or false-negative results. This method uses a solid phase coated with an anti-human IgM antibody to capture the IgM from a serum specimen, after which competing IgG antibody and immune complexes are removed by washing. The bound IgM antibody is then exposed to specific CMV antigen, and an enzyme-conjugated second antibody and substrate are added. The development and use of recombinantly derived CMV proteins as a source of antigenic substrate also have greatly improved the performance of IgM assays (132, 133).

Although the detection of CMV-specific IgM may be beneficial in the determination of recent or active infection, the results should be interpreted with caution. Because IgM does not cross the placenta, a positive result from a single serum specimen from an infected newborn is diagnostic. However, there may be a lack of or delay in production of IgM in the newborn. Testing for the presence of CMV-specific IgM antibody beyond the newborn period is usually not recommended since IgM antibody can appear in both primary and reactivated CMV infections and can persist for extended periods after a primary infection. This complicates the interpretation of test results, particularly for pregnant women or immunocompromised patients, and may not be predictive of recent or active infection. Like newborns, immunocompromised individuals also may have a delay in IgM production or may be unable to mount a significant IgM antibody response. Lastly, patients with Epstein-Barr virus-induced infectious mononucleosis may produce heterotypic IgM responses, resulting in false-positive CMV IgM test results.

IgG Avidity Assay

Measurements of CMV-specific IgG avidity have proven useful for distinguishing primary from nonprimary infections in women suspected of having CMV during pregnancy and in solid organ transplant recipients. CMV-specific IgG of low avidity is produced during the first weeks to months following primary infection, while IgG antibody of increasingly higher avidity is produced with past or nonprimary infections. The detection of CMV-specific IgM in maternal serum is not predictive of virus transmission to the fetus, since fewer than 10% of pregnant women who are positive for CMV IgM actually give birth to a congenitally infected infant. More recently, it has been suggested that finding low-avidity CMV-specific IgG antibody in CMV-IgM positive pregnant women may improve the diagnosis of recent primary CMV infection and may have prognostic value with regard to fetal infection (134, 135). In solid organ transplant recipients, it has also been shown that a delay in the development of high-avidity CMV-specific IgG antibody correlates with prolonged antigenemia and a poor prognosis (136). Both commercial and user-developed CMV-specific IgG avidity assays are available, and the tests are performed by making simple modifications to conventional EIA protocols to effectively separate and differentiate antibodies of low and high avidity (137, 138).

Measurement of CMV-Specific Cell-Mediated Immunity

CMV-specific CD4$^+$ and CD8$^+$ T-cell responses are critical to the control of CMV replication and the onset of symptomatic infections. Recently, tests have been developed to assess the CMV-specific cell-mediated immune response and may have potential clinical application to identify transplant recipients at increased risk for CMV infection following transplantation, to guide decision making regarding prophylaxis and preemptive therapies, and for risk stratification of patients before transplantation (32, 33). Most assays measure the status of immune reconstitution by detecting the release of gamma interferon following stimulation of whole blood or peripheral blood mononuclear cells with CMV-specific antigens or peptides, and use of these tests has been recommended in the most recent updated international CMV consensus guidelines on the management of CMV in transplant patients. To that end, assays like the QuantiFERON-CMV (Cellestis Limited, Victoria, Australia) and T-Track CMV ELISpot (Lophius Biosciences, Regensburg, Germany) have been commercialized and have recently received CE marking in Europe (139).

ANTIVIRAL SUSCEPTIBILITY TESTING

Resistance of CMV to ganciclovir has been reported in immunocompromised patients, particularly patients with AIDS receiving long-term ganciclovir treatment for CMV retinitis. Ganciclovir-resistant clinical isolates have also been recovered from bone marrow and solid organ transplant recipients and a patient with chronic lymphocytic leukemia. Although foscarnet is the alternative therapy for ganciclovir-resistant CMV infections, clinical CMV isolates resistant to foscarnet alone or to foscarnet in combination with ganciclovir have been described. The mechanisms of resistance to ganciclovir in CMV include mutations in the catalytic domain of the UL97 phosphotransferase gene, leading to a deficiency in drug phosphorylation, and alterations in the UL54 viral DNA polymerase gene; the latter is also responsible for resistance to foscarnet (for a review, see references 140 to 142). Ganciclovir-resistant CMV isolates with mutations in the UL54 gene are generally cross-resistant to cidofovir, and isolates resistant to ganciclovir, foscarnet, and cidofovir have also been reported.

The described emergence of antiviral drug resistance has led to a definite need for *in vitro* antiviral susceptibility testing. Laboratory confirmation of drug resistance in the setting of rising, rebounding, or persistently high viral loads during prolonged therapy (median of 5 months) is essential for defining the mechanisms of antiviral resistance, for determining the frequency with which drug-resistant CMV mutants emerge in clinical practice, for predicting treatment failure and identifying cross-resistance to other antiviral agents, for instituting the most appropriate alternative therapy, and when evaluating new antiviral agents. A number of phenotypic and genotypic assays have been described for testing the susceptibility of CMV to antiviral agents (for an overview, see reference 143). Phenotypic assays, although considered to be the reference methods for many years, are rarely used in clinical practice today, as they are cumbersome, labor-intensive, and expensive and have turnaround times that are far too long to be clinically relevant. Genotypic assays are currently the method of choice and offer speed and efficiency in screening and analysis of whole-blood or plasma specimens or CMV isolates and allow for an earlier detection of the emergence of drug resistance than phenotypic assays. Phenotypic assays measure the ability of CMV to grow in cell culture in the presence of various concentrations of antiviral drug, and they generally require isolation and passage of the virus in cell culture before testing begins. They offer the distinct advantage of being a direct measure of CMV susceptibility to any antiviral drug and can provide data on the concentration of drug needed to inhibit viral replication. Genotypic assays have been used to screen clinical specimens or CMV isolates for the identification of mutations in the CMV UL97 phosphotransferase gene and the UL54 DNA polymerase gene that confer resistance to ganciclovir, foscarnet, and/or cidofovir (144–146). These assays normally involve using PCR for amplification of specific viral genes and sequencing of the amplified products; some assays use specific probes to detect selected mutations or use restriction endonuclease digestion of amplified products to identify alterations in the viral genome known to be associated with viral resistance to a given viral agent. Newer genotypic assays have been developed and involve automated sequencing methods performed directly from specimens without the need for PCR amplification (147–149); they usually require a minimum quantity of 1,000 copies/ml of CMV DNA for the most reliable results. Current genotypic assays, however, detect only known drug-resistant mutations, and the results may be confounded by the presence of mutations that have no bearing on drug resistance. Phenotypic assays are still required to identify drug-resistant viruses with novel mutations for antiviral resistance but now involve genetic transfer of the mutation sequences to a laboratory-adapted reference CMV strain or artificial cloning system for rapid phenotyping. The overall complexities of phenotypic and genotypic assays make either method less than routine for most clinical laboratories.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

There are a number of points to consider when choosing which tests to perform for the detection of CMV. Conventional tube cultures are highly specific and can detect unexpected viruses but are generally too slow and insensitive to have an impact on clinical decision making when considering CMV as the primary pathogen. Shell vial cultures decrease the time required for detecting CMV in culture, but the sensitivity varies by specimen type and from laboratory to laboratory. Cultures also are often positive in the absence of true CMV disease; this is particularly true when screening urine or respiratory specimens for CMV, in which shedding of CMV is common during asymptomatic infection and is usually not suggestive of more-severe illness. Isolation of CMV in culture may be useful for postpartum and *in utero* diagnosis of congenital CMV infection and tissue-invasive disease, and CMV isolates may be needed for select phenotypic antiviral susceptibility assays.

In the immunocompromised host, the use of direct detection methods to diagnose CMV disease is most beneficial for patient care. Virologic or serologic detection of CMV indicates infection but does not establish whether the infection is responsible for symptomatic illness. CMV is ubiquitous, and asymptomatic excretion is high in patients who never progress to disease. Reactivation of latent virus is also common, and other pathogens may be simultaneously present in patients with overt disease. To implicate CMV as the cause of an illness, laboratory confirmation of active disease in an appropriate clinical setting is required. In this regard, most clinical virology laboratories have largely replaced their conventional tube and shell vial culture systems with CMV antigenemia assays or, to a much greater extent, with molecular methods for accurate CMV detection and/or quantification from blood. Quantitative measures of CMV pp65 antigen or CMV DNA can provide a rapid diagnosis of established disease, identify patients at risk of developing disease, assess the progression of disease and the risk of relapse, direct the initiation of preemptive therapy, monitor the response to antiviral therapy, and predict treatment failure and the emergence of drug-resistant virus. Evaluating trends in viral loads over time is currently more useful than interpreting a single test result in relation to a defined cutoff value, since the initial viral load, the rate at which it rises, and its persistence with time may predict the risk of CMV disease and possible relapse and aid in establishing treatment failure. Serial monitoring of high-risk patients also may be beneficial with respect to aiding in the decreased use of and exposure to antivirals, leading to a more cost-effective and focused use of such agents. Interpretation should be done with caution, as not all changes in quantitative CMV DNA levels may reflect a substantial biological or clinical difference, especially if the changes are small and fall within the expected range of variability for the assay used. Also, an isolated result for CMV viremia may not be predictive of disease and the need for immediate antiviral treatment. Lastly, negative viral load results do not always exclude CMV disease in symptomatic patients; end organ involvement, particularly in the gastrointestinal tract and lungs, may occur in the absence of detectable levels of CMV viremia. Histopathologic examination and/or PCR can be useful in immonocompromised hosts for the diagnosis of specific organ invasive diseases. However, detection of CMV in tissue specimens must be interpreted with caution, particularly when infections with more than one organism are documented, as the relative importance of each pathogen in producing clinical illness may be difficult to determine. The degree of histological involvement may help in determining the role and severity of CMV in causing disease.

The isolation of CMV from urine, respiratory secretions, or other body fluids within the first 3 weeks of life is the traditional means of confirming the diagnosis of congenital infection in newborns. Urine is the preferred specimen because it contains greater amounts of virus, and the virus therefore grows quickly in culture. PCR has also been used to find CMV DNA in urine of congenitally infected infants (150), and viremia in infants with congenital infection has been detected by the antigenemia assay and PCR. Attempts to isolate or detect virus from maternal blood leukocytes and from amniotic fluid and fetal blood and tissue by molecular and/or antigen-based tests may provide useful information for prenatal diagnosis of congenital CMV infection (for a review, see references 25 and 151). While such prenatal testing is a sensitive indicator of maternal or fetal CMV infection, positive results do not predict which infants will have disease. In this regard, quantitative measures of viral load in congenital CMV infection may prove beneficial for predicting symptomatic outcome and for guiding patient management (44). It has been demonstrated that elevated levels of CMV DNA in blood during early infancy is associated with hearing loss in newborns with symptomatic and asymptomatic congenital CMV infection (152, 153). Also, quantitative real-time PCR has been used to quantify CMV DNA from amniotic fluid samples to predict fetal and neonatal outcomes (154) and to test umbilical cord blood to screen for neonatal CMV infection at delivery (69). Infants not previously tested but found to be excreting virus after 3 weeks of age may have either congenital or acquired infection. Standard serologic or virologic tests do not differentiate between these possibilities.

In general, serologic tests for CMV-specific IgG antibody have limited value for the diagnosis of acute infection since the results are obtained retrospectively and are not predictive of disease. Also, the transplacental passage of maternal IgG antibody begins at 18 weeks gestation and confounds the diagnosis of CMV infection in the neonate, and immunocompromised hosts may not mount a normal immune response or they may have been given blood transfusions or intravenous immunoglobulins that contain detectable CMV IgG antibodies. Nonetheless, a history of seroconversion from a negative to a positive IgG antibody response to CMV is diagnostic of primary infection and may be beneficial in evaluating a pregnant woman with symptoms of viral disease. For pregnant women with preexisting CMV-specific IgG and/or IgM antibody, however, IgG avidity testing may be more helpful in distinguishing primary from nonprimary infections and for predicting CMV transmission to the fetus. Use of the IgG avidity assay or tests that measure immune reconstitution in immunocompromised hosts may have value in detecting those at highest risk for CMV disease. Whenever possible, serologic diagnoses of CMV infection should be confirmed by other virologic methods. CMV-specific IgG serologic assays play a more important role in the determination of an individual's immune status to CMV. Detection of CMV-specific IgG in a single serum specimen indicates exposure to CMV at some time in the past. Negative serum antibody titers may exclude CMV infection. In the evaluation of an organ donor and recipient, a seronegative recipient who receives an organ or blood products from a seropositive donor is at increased risk for developing primary CMV infection and serious disease. Knowing the serostatus of the donor and recipient is therefore important in determining the treatment or prophylaxis to be used and in considering the type of donor to be selected and blood products to be given.

The application of virologic methods, including conventional and shell vial culture, rapid direct-detection assays, and serologic testing, should be combined with clinical assessment of the patient to provide an accurate, reliable diagnosis of CMV infection and disease and to allow subsequent prompt, appropriate patient management and timely intervention with specific antiviral therapy. There is a particular need to differentiate CMV infection from graft rejection in transplant patients, since the administration of potent immunosuppressive antirejection drugs during active CMV infection can result in life-threatening disease.

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Epstein-Barr Virus

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TAXONOMY

Epstein-Barr virus (EBV) is a member of the *Herpesviridae* and belongs to the *Gammaherpesvirinae* subfamily (1). EBV is closely related to another human pathogen, human herpesvirus 8, which also replicates in epithelial cells and establishes long-term latency in lymphocytes. However, EBV is the only human pathogen in the genus *Lymphocryptovirus*; human herpesvirus 8 belongs to the genus *Rhadinovirus*.

DESCRIPTION OF THE AGENT

Morphologically, EBV is a typical herpesvirus with 162 capsomeres in an icosahedral arrangement surrounded by a lipid-rich envelope. The virus has a double-stranded, 172-kbp DNA genome which exists in a linear form in the mature virion and in a circular episomal form in latently infected cells. The genome consists of a series of unique sequences alternating with internal repeats, all sandwiched between two terminal repeat elements which are joined during circularization. Integration of viral DNA into host chromosomal DNA occurs rarely and has been documented primarily for lymphoblastoid cell lines.

EBV primarily infects naïve B cells. This requires the binding of the major EBV outer envelope glycoprotein gp350/220 with the cellular complement receptor C3d (also known as CD21 or CR2) and gp42 with the major histocompatibility complex class II molecule. *In vivo*, EBV uses the highly regulated, sequentially expressed proteins described below to persist in the host and transmit infection. It is believed to do this by usurping normal B cell physiologic processes. EBV substitutes for antigen, T cell help, the B cell receptor, and other signals that result in cellular activation, proliferation, differentiation, and survival, mimicking pathways followed when naïve B cells encounter antigen with the end result of establishing latency in memory B cells (2). *In vitro*, EBV transforms B cells into lymphoblastoid cell lines. Although CD21-negative epithelial cells of the oropharynx are the most important site of viral replication and amplification (3), monocytes may also be productively infected by EBV (4, 5). In some clinical settings, T and NK (natural killer) cells also demonstrate evidence of infection (6). EBV nuclear antigens—EBNA 1, EBNA 2, EBNA 3 (also referred to as EBNA 3a), EBNA 4 (EBNA 3b), EBNA 5 (EBNA LP), and EBNA 6 (EBNA 3c)—and latent membrane proteins (LMP 1 and 2A and B) may be expressed in B cells. Four types of B cell latency have been defined, based on various levels of expression of the latency-associated proteins (7, 8). In latency type 0, only EBV-encoded RNAs (EBERs) and rightward transcripts from the BamHIA gene are expressed. EBER 1 and EBER 2 are nonpolyadenylated RNAs, are therefore not translated to a protein, and act to inhibit antiviral effects by interferon as well as apoptosis. EBERs are present in high copy numbers (10^6 to 10^7 copies) in virtually all EBV-infected cells. This expression prolife is seen in memory B cells of healthy carriers. In latency type I, in addition to the type 0 expression profile, EBNA 1 is detectable; this is observed in Burkitt's lymphoma. In latency type II, LMP 1 and 2 are expressed in addition to gene products of latency type I; this is observed in germinal center and memory B cells in the tonsil and malignancies, including Hodgkin's lymphoma, nasopharyngeal carcinoma (NPC), nasal NK cell lymphoma, chronic active EBV infection, and nasopharyngeal lymphoma. Finally, in latency type III, all EBNA proteins are detectable; this is observed in naïve B cells of the tonsil during acute infection and in immunodeficiency-related lymphoproliferative disorders (2).

EBV has a highly restricted host range. Although during acute EBV infection, as many as 10% of all circulating B cells, representing 50% or more of all memory B cells, may be infected, in a healthy individual remote from infection, only about 1 to 50 B cells per million are infected (9). During lytic replication, more than 70 proteins are expressed, including the virus capsid antigens (VCA) and the early antigens (EA) used in diagnostics. This occurs primarily in epithelial cells and in B cells as they undergo plasmacytoid differentiation.

EPIDEMIOLOGY AND TRANSMISSION

Virtually everyone becomes infected with EBV at some time during their life. In lower socioeconomic strata and developing countries, EBV infection occurs mainly during the first years of life and is usually subclinical. However, in developed countries, particularly in upper socioeconomic strata, only about 60% are infected before puberty. In these settings, infection often occurs in adolescence and early adult life, resulting in an infectious mononucleosis (IM) syndrome or atypical symptomatic infection in 23 to 89% of cases (10). As a result, by the age of 20 years ~90% of individuals are seropositive, and by the age of 40 almost 100% of the population have seroconverted.

Transmission of EBV occurs primary through saliva exchange (3). Some investigators also believe that sexual

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contact may be important (11, 12); however, recent evidence suggests that deep kissing associated with sexual activity is the more relevant transmission route (10). Contrary to previous beliefs, recent data generated using nucleic acid detection techniques (NAT) suggest that EBV shedding in saliva is continuous, persistent, and rapid in all previously infected subjects (3, 13). Therefore, use of saliva for the diagnosis of acute infections is discouraged. Although infection via blood products is theoretically possible, the importance of blood products as a source of EBV infection is uncertain, particularly in settings where blood products are universally leukodepleted.

Among immunosuppressed transplant recipients, latently EBV-infected cells transmitted with the donor organ or hematopoietic stem cells from seropositive donors to seronegative recipients are the most important source of infection in the early posttransplantation period.

EBV strains may be classified into types 1 and 2 (sometimes referred to as types A and B, respectively) based on the polymorphism of their EBNA genes. Prototypic viruses differ mainly in their nucleotide sequences for EBNA 2 and differ to a lower extent in other EBNA proteins not routinely used in diagnostics. Since current enzyme immunoassays (EIAs) predominantly use EBNA 1 antigen, strain differences might not be relevant for routine diagnostics. Both EBV types have a worldwide distribution, though they predominate in different geographical areas. Dual infections with the two types are not uncommon. In immunosuppressed individuals, coinfection with multiple strains of EBV has also been demonstrated (14).

CLINICAL SIGNIFICANCE

EBV is associated with a variety of disease states in immunocompetent and immunosuppressed individuals. Longitudinal studies with African children with acute EBV infection suggest that asymptomatic infection at a very early age (before 6 months) may be related to higher and more persistent viral loads in peripheral blood because of age-related immune immaturity; a relationship of early infection to risk for Burkitt's lymphoma has been postulated (15). Passive maternal antibody may provide protection against infection very early in life, although the duration of that protection is uncertain (15).

IM is believed to be an immunopathologic disease with symptoms resulting from an exaggerated T cell response of a more mature immune system to a self-limited lymphoproliferative process and perhaps high viral loads (10). In this model, peak viral loads antecede clinical symptoms. In young adults with acute symptomatic EBV infection, EBV DNA (possibly representing virions) is rapidly cleared from plasma within a week of symptom onset, but EBV DNA in whole blood (perhaps representing episomal DNA in latently infected B cells) takes up to 200 days to clear to levels seen in healthy carriers (10, 16, 17). *In vitro*, acyclovir, ganciclovir, and foscarnet inhibit EBV replication (18–20). However, because IM appears to be an immune-mediated rather than a directly virally mediated disease, treatment of IM with antiviral agents results in termination of viral shedding but has no or only small effects on symptoms (21–24). A meta-analysis of five randomized controlled trials showed no significant benefit associated with acyclovir use for IM treatment (22). Antiviral therapy is therefore not recommended for treatment of IM. Steroids may be useful in severe complicated disease, including tonsillar enlargement threatening airway obstruction, autoimmune hemolytic anemia, severe thrombocytopenia, and aplastic anemia. Steroids have also been used by some investigators to treat EBV-associated neurologic and cardiac disease. Uncomplicated IM is generally treated with supportive treatment only. Trauma to the spleen is avoided, and acetaminophen and/or anti-inflammatory agents are used for pain and fever relief.

Symptoms vary from mild to severe, and recent data suggest that the initial viral inoculum, viral type (type 1 versus 2), and human leukocyte antigen (HLA) class 1 polymorphisms may be risk factors for symptomatic disease (25–27). The classical symptom triad of IM is fever, sore throat, and lymphadenopathy; symptoms and signs such as pharyngitis, tonsillitis, hepatitis, splenomegaly, and lymphocytosis with atypical lymphocytes are often present. Although rash in a variety of forms is seen in approximately 5% of IM patients, a pruritic maculopapular rash occurs almost universally in IM patients treated with ampicillin or amoxicillin. This antibiotic-associated rash is not predictive of future drug intolerance. The time between infection and symptoms is relatively long, at 30 to 50 days (28), and clinical disease generally persists for 1 to 4 weeks. However, protracted illness and postinfectious fatigue and/or depression for up to a year are not uncommon (29). Atypical presentations in which hematologic features such as anemia, including autoimmune hemolytic anemia thrombocytopenia, or neurologic conditions such as meningoencephalitis, radiculitis, or mononeuritis predominate can occur. These presentations may be the only symptoms or signs of acute EBV infection. When visceral organs are affected, almost any symptom resulting from inflammation of the affected organ may be seen. Death due to IM is rare but has been described; it occurs most often as a result of splenic rupture, airway obstruction, or neurologic complications. A severe and sometimes fatal complication of primary infection is hemophagocytic lymphohistiocytosis, a hemophagocytic syndrome characterized by exaggerated macrophage activation and phagocytosis caused by virally driven T cells. Extremely high levels of EBV DNA are observed in both peripheral blood mononuclear cells (PBMC) and sera of these patients (6, 30, 31). Atypical presentations, including acute cholestatic hepatitis sometimes misdiagnosed as acute cholecystitis, are common in older subjects (>40 years) with acute EBV infection (32). EBV is often reactivated in healthy individuals, but reactivation has not been clearly associated with symptoms. It should also be noted that primary EBV infections cause only approximately 50 to 90% of mononucleosis syndromes (33). Other infectious agents, such as acute cytomegalovirus, adenovirus, *Toxoplasma gondii*, and human immunodeficiency virus (HIV), as well as lymphoma and autoimmune diseases can cause symptoms and signs similar to those seen in EBV-associated IM.

Chronic active EBV infection is a rare, severe (often fatal) EBV-associated condition most often described to occur in Japanese children; it is believed to be a precursor state for T/NK lymphoma (34). It is characterized by a clonal expansion of EBV-infected T cells and NK cells and a high viral load in both PBMC and serum and plasma. The EBV DNA in the latter is believed to be free DNA rather than virion associated. Patients often show atypical patterns of EA antibodies (6, 35–37). There is no proven role for acyclovir or ganciclovir therapy in this condition. Although chemotherapy and immunomodulatory therapy have been attempted as treatment, most cases are chemotherapy resistant. Hematopoietic stem cell transplantation (HSCT) is currently the preferred treatment option (37).

EBV is associated with a variety of tumors primarily of lymphoid and epithelial origin, not related to obvious immunosuppression. The EBV genome has been found in tumor cells in >95% of endemic Burkitt lymphomas and NPC, <5 to 70% of cases of Hodgkin's disease (depending on the subtype), 2 to 16% of gastric adenocarcinomas, 40 to >90% of T/NK lymphomas (depending on the subtype), and 40 to 95% of lymphoepithelioma-like carcinomas. There is no proven role for antiviral agents in the management of these malignancies; standard cancer treatment is used for management. Adoptive immunotherapy targeting specific EBV proteins expressed in some EBV-associated malignancies has been successfully used for some patients and is being explored further in clinical trials (38, 39).

X-linked lymphoproliferative syndrome is a rare hereditary immunodeficiency primarily involving the inability of T cells and NK cells to engage B cells (40, 41). Affected boys become symptomatic after exposure to EBV because of EBV's exquisite tropism for B cells, developing acute fatal IM or lymphoproliferative disorders and/or dysgammaglobulinemia. Most patients have germ line mutations in the SH2D1A gene encoding the adaptor molecule signaling lymphocytic activation molecule (SLAM)-associated protein; a subset of patients carry mutations in the X-linked inhibitor of apoptosis gene (XIAP) (42). Untreated, the disease is often lethal, and patients benefit from stem cell transplantation (43). The role of antiviral therapy early during acute EBV infection in these patients is uncertain; treatment with anti-CD20 antibody has been advocated by some investigators.

In immunocompromised patients, both primary infection and EBV reactivation can result in lymphoproliferative disorders. Posttransplant lymphoproliferative disorders (PTLD) represent a spectrum of disorders varying from benign polyclonal proliferations to true malignancies containing clonal chromosomal abnormalities. They are classified based on tissue pathology using a World Health Organization (WHO) lymphoma classification system of immunodeficiency-related lymphoproliferative disorders (44). PTLD are often extranodal, can be restricted to the allograft after solid-organ transplantation (SOT), and, unlike lymphoma in immunocompetent patients, can involve the brain in isolation or as part of multisystem disease. Principal risk factors are (i) EBV primary infection after transplantation in the setting of SOT and (ii) severe T cell depletion (e.g., after administration of T cell antibodies) in both SOT and HSCT settings (45–47). Although >90% of early (first posttransplant year) PTLD lesions after SOT have demonstrable EBV, >50% of late PTLD is EBV negative (48). The role of EBV in the pathogenesis of these late EBV-negative PTLD is uncertain. In many centers, EBV viral load measurements in peripheral blood have become standard of care aiding in the prevention, diagnosis, and management of PTLD (49–52).

EBV-related lymphomas are also common malignancies seen in HIV-infected and AIDS patients. Burkitt's lymphoma, lymphomas of the central nervous system (CNS), and smooth muscle tumors are strongly associated with EBV (~95%) (53–55); the last are also observed in transplant recipients (56). Oral hairy leukoplakia, a white nonmalignant lesion of the lateral borders of the tongue observed in HIV patients and transplant recipients, is associated with lytic EBV infection and is responsive to antiviral therapy (57).

After routine implementation of EBV DNA measurement in peripheral blood, a number of investigators observed that some SOT recipients experiencing primary EBV infection or PTLD have sustained elevation of EBV viral load after asymptomatic infection or resolution of EBV disease or PTLD. These asymptomatic patients show a chronic viral load phenotype. Although a study in pediatric thoracic SOT suggests that 45% of patients with this phenotype developed late-onset EBV-positive PTLD at a median follow-up of 7 years (58), risk appears, in part, to be organ specific, with intermediate risks observed after intestinal SOT (59) and low risk after liver SOT from the same center (60). However, even among recipients of specific allograft types, such as pediatric liver transplant recipients, reported long-term risks differ among centers (60, 61). Additional studies are required to determine allograft-specific long-term risks and the usefulness of ongoing viral load monitoring in this setting. This chronic load phenotype has also been observed in children and adults with HIV infection. Specific EBV "set points" for quantitative load in individual patients as well as in EBV infection of NK and T cells have been observed in this setting (62, 63). The pathogenesis of this EBV infection state is unknown.

The prevention and management of PTLD are complex, require a multidisciplinary team that includes transplant physicians, infectious disease specialists, virologists, and hematologists/oncologists, and have been reviewed elsewhere (52, 64). Approaches used include immune reconstitution by lowering immunosuppression and adoptive immunotherapy, the reduction of tumor burden by surgery or radiotherapy, and the use of chemotherapeutic agents, particularly anti-CD20 therapy (64, 65) and cytotoxic chemotherapy (52, 64).

Although antiviral drugs such as acyclovir/valacyclovir and ganciclovir/valganciclovir with or without intravenous immunoglobulin (IVIG) have been used to treat PTLD, there is no evidence to suggest that these agents impact clinical outcome. PTLD lesions, when clinically apparent, contain almost exclusively latently infected cells. Some investigators have proposed a novel oncolytic strategy of combining antiviral agents targeting the lytic EBV cycle with drugs, such as arginine butyrate and bortezomib, that induce EBV lytic replication in latently infected PTLD cells. This approach is currently being explored in research settings (52). On theoretical grounds, antiviral drugs and IVIG might have greater efficacy in preventing or modifying the course of primary or reactivation EBV infection early after SOT and HSCT in patients at high risk of donor- or community-acquired EBV infection. However, the use of prophylactic antiviral agents in these settings remains controversial. Although many SOT centers give antiviral agents, most often valganciclovir or ganciclovir with or without IVIG, to EBV-mismatched patients (seropositive donor and seronegative recipient) during the initial months after transplantation, the benefit of this approach has not been proven.

Reconstitution of the EBV-specific immune response is key to the management of PTLD and often initially involves reduction of immunosuppression (66). Adoptive immunotherapy using EBV-specific cytotoxic T cell therapies (using autologous and allogeneic T cells) has demonstrated efficacy in both disease prevention and treatment in pilot studies and small clinical trials, but regulatory issues, cost, and logistics currently restrict this approach to the research setting (67).

There is no approved vaccine against EBV. Studies of two different prototype vaccines showed that these vaccines failed to prevent EBV infection but were effective in partly preventing symptomatic disease (68–70).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

For the rare circumstance where oropharyngeal viral excretion is studied, 5 to 10 ml of throat gargle, collected in serum-free tissue culture medium or Hanks' balanced salt solution, can be used. Approximately 5 to 10 ml of heparinized or EDTA blood is required either for cell culture and detection of EBV-transformed B cells or for antigen detection in B cells by immunostaining or immunofluorescent *in situ* hybridization studies (see below). Blood specimens should be processed as soon as possible, although refrigeration for up to 24 h is acceptable for antigen detection. Fresh biopsy samples or thin cryosections (5 μm) are preferable to formalin-fixed material for antigen detection in tissues.

NAT are currently most frequently used to detect EBV in (i) blood, (ii) cerebrospinal fluid (CSF), and (iii) biopsy samples. The choice of sample matrix depends on the EBV-associated disease of interest and the clinical setting in which the test is being used (Table 1). For example, investigators have proposed sampling of PBMC in chronic active EBV infection (30), CSF in AIDS-related primary CNS lymphoma (71), and whole blood or plasma after transplantation (71–73). Plasma but not whole blood has been recommended as the preferred sampling matrix in IM (71–74) and in diseases in which malignant cells do not necessarily circulate in blood, such as NPC (75), nasal T/NK cell lymphoma (76), and Hodgkin's lymphoma (77).

The optimal sample matrix for monitoring transplant recipients has not been definitely determined. There is currently no agreement on whether leukocytes, whole blood (in EDTA; heparin may inhibit the PCR), or serum and plasma should be used for EBV DNA detection for PTLD prevention and treatment monitoring. EBV DNA detection in leukocytes and whole blood has high sensitivity but lower specificity for PTLD risk prediction or disease detection, whereas the opposite holds true for cell-free specimens like serum or plasma. When serially monitoring patients at high risk to detect early infection or reactivation events, a sensitive but less specific matrix like whole blood may be preferable. In contrast, when used as a diagnostic test (single sample) for symptomatic patients in a population with a low prevalence of disease, a cell-free sample matrix is likely to be advantageous. For serial monitoring of individual patients, it is particularly important that the sample matrix is not varied. The sample quantity required varies, but it is advisable to provide at least 1 ml. Source blood specimens for serum or plasma or PBMC should be processed as soon as possible. If transport time is less than 24 h, samples may be kept at room temperature until separated. Separated materials can thereafter be stored at 4 to 7°C for a few days or frozen. Whole blood can be stored for a few days at room temperature or frozen without further processing. For long-term storage, freezing at −70°C is recommended. For detection of EBV in peripheral blood lymphocytes by DNA hybridization, 5 to 10 ml of heparin/EDTA blood is required.

Quantitative NAT (QNAT) of CSF has been used for the diagnosis of EBV-associated CNS disease, particularly lymphoma. CSF without additives should be sent, processed, and stored as described above for blood specimens being tested for EBV DNA by NAT; at least 0.5 ml should be submitted for testing when possible.

Tissues (e.g., nasopharyngeal brushings for NPC) and biopsy samples to be examined for viral nucleic acids or antigens should be collected and refrigerated in saline or balanced salt solutions. Fresh or frozen samples are preferred. For patients with NPC, endoscope-guided nasopharyngeal brushings are an important sample matrix. A brush covered by a plastic catheter should be used to prevent contamination by cells from non-nasopharyngeal sites. The brush tip could be placed in transport medium and transferred immediately to the laboratory or stored at −80°C (78).

For antibody assays detecting heterophile antibody whole blood, serum or capillary fingertip blood may be used for most point-of-care tests. For EBV-specific tests, 50 μl or even less of acute-phase serum may be sufficient; however, it is advisable to provide 1 to 5 ml. Convalescent-phase serum collected 1 to 2 months after symptom onset may occasionally be required in cases with ambiguous results on initial testing. Plasma can be used, but serum is preferable. Serum can be stored at 5°C for several months. For long-term storage, freezing at −20°C or −70°C is recommended.

TABLE 1 Materials and methods to be used for prevention, diagnosis, and monitoring of EBV disease and EBV-associated malignancies[a]

Suspected EBV-associated condition and clinical setting	Validated method	Suitable specimen	Approximate value for clinical significance[b]
IM	Serology	Serum	
Chronic active EBV infection	QNAT	Mononuclear cells[c] Plasma or serum	$\sim10^{2.5}$ gEq/μg of DNA $\sim10^3$–10^4 gEq/ml
Diagnosis of EBV presence in PTLD, other lymphomas (including CNS lymphoma in HIV-positive patients), and all other EBV-associated tumors	*In situ* hybridization of antigen detection	Biopsy tissue	Positive tumor cells detected
Prevention of PTLD using preemptive therapy in high-risk patients	QNAT	Plasma Whole blood	$\sim10^2$–10^4 gEq/ml 10^3–10^5 gEq/ml
Screening for NPC in high-risk populations and monitoring response to therapy	QNAT	Plasma or serum Nasopharyngeal brushings (BARF 1 RNA)	$\sim10^2$ gEq/ml Not defined
	Serology[d]	EBV-specific IgA	Not defined
Diagnosis of XLP	Genetic analysis	Host cells	Specific associated gene mutations

[a] gEq, genome equivalents; XLP, X-linked lymphoproliferative syndrome.

[b] Approximate level of EBV viral load of clinical significance is based on data reported, most often from single centers, using a variety of nonstandardized assays. Until result harmonization is documented and all assays are calibrated to the international reference standard, interinstitutional result comparison remains problematic.

[c] Disease is most often due to clonal expansion of EBV-infected T or NK cells.

[d] Less useful than NAT screening.

DIRECT DETECTION

Electron microscopy, virus isolation, and antigen detection by immunohistochemistry are primarily research tools. EBV DNA detection by molecular methods is the most common and often only method used for routine direct detection of EBV in tissue and other specimens in most clinical diagnostic laboratories.

Microscopy

Electron microscopy is not an appropriate diagnostic technique for EBV diagnosis, since most EBV-associated disease is associated with latent infection and virions are rarely present in sufficient quantity to be detected in either infected tissues or other sample matrices such as plasma, cells, or CSF.

EBV Antigen Detection by Immunohistochemistry and EBER by *In Situ* Hybridization

Antibodies specific to a wide range of EBV antigens have been used to detect EBV in tissue biopsy samples using immunohistochemical techniques. Studies using antibodies targeting EBNA 1, EBNA 2, LMP 1, LMP 2, BamHI H right-frame 1 protein (BHRF 1), BamHI Z left-frame 1 protein (BZLF 1), and BamHI M right-frame 1 protein (BMRF 1) (7, 79–82) have been described previously. EBNA 1 is the only antigen expressed in all EBV-infected cells expressing EBV proteins (cells with latency states other than latency state 0). However, the sensitivity of immunohistochemistry techniques for the detection of EBV-infected cells using currently available EBNA 1 antibodies is lower than that observed using *in situ* hybridization targeting EBER (7). EBER is present in high copy numbers in EBV-infected cells regardless of latency state. This has made its detection using *in situ* hybridization techniques ideal and the “gold standard” for detecting EBV-infected cells in tissues and tumors (83). Despite this, some authors report having documented EBER-negative EBV-infected cells (84).

The cellular tropism of EBV in the peripheral blood of patients with high persistent EBV viral loads is also being studied using assays that combine immunofluorescent staining for cell surface proteins (cell phenotyping) with fluorescent or *in situ* hybridization using nucleic acid probes for EBER (85) or other targets (86). Detection can be performed on slides by microscopy or in suspension by flow cytometry. Preliminary studies report interesting observations of EBV infection in cells other than memory B cells in these patients (85, 86). These assays are not yet available in routine diagnostic laboratories.

Nucleic Acid Amplification Techniques

Molecular techniques are important tools for the detection of EBV in clinical specimens other than tissues. The choice of method and sample matrix is dependent upon the clinical question being addressed (Table 1).

Clinical diagnostic assays for direct detection of EBV DNA can be performed on a wide range of clinical specimens following DNA extraction. A variety of primers, probes, and techniques have been used. In general, quantitative viral load assessment is superior to qualitative detection. Although commercial assays are becoming increasingly available, many laboratories continue to use laboratory-developed tests (87, 88).

Assessment of viral load can be broken down into two basic strategies, based upon the genomic region being targeted. (i) The conserved BamHI W region, coding for a long internal repeat sequence of EBNA 1, is present in multiple copies (7 to 11 copies) in EBV-infected cells. NAT targeting this region have maximal sensitivity in clinical samples. (ii) The genes for LMP 1 or thymidine kinase or other parts of the EBNA 1 gene are often targeted for quantitative PCRs since these are single-copy regions (89). Some studies comparing assays using various EBV genome regions as NAT targets in parallel observed target-specific quantitative differences in results obtained for EBV viral load upon testing the same sample (90–93).

Gene targets that result in small amplicons may be more useful when measuring viral DNA in plasma samples, since EBV DNA in plasma is often free DNA, i.e., not encapsidated, and so may be highly fragmented (94).

Quantitative real-time PCR is the most popular currently used technology for measuring EBV viral load in patients at risk for EBV-associated disorders (88, 94, 95). Proficiency panel studies have demonstrated significant variability (4 $\log_{10}$) in both qualitative and quantitative reporting of EBV viral load results among laboratories, limiting the validity of interinstitutional result comparison (96). Proficiency panel results in both Europe (Quality Control for Molecular Diagnostics) and North America (College of American Pathologists [CAP]) have illustrated that for analytes assayed using NAT and reported quantitatively, interlaboratory variability is significantly reduced when an international reference standard is available and commercial assays calibrated to this standard are used. In October 2011, the WHO approved the first international standard for EBV (a whole-virus preparation of EBV B95-8 with a potency of 5×10^6 IU/ml) created by the National Institute for Biological Standards and Controls (United Kingdom). Quantitative standards traceable to this international standard should be used in all EBV NAT assays. For accurate quantitation, the standards should be extracted together with the clinical samples, in a matrix resembling the clinical sample. They should not be diluted in buffer, although the international reference standard is reconstituted in nuclease-free water. It is hoped that the international reference standard will improve result harmonization. However, until this impact has been validated, interinstitutional result comparison requires formal cross-referencing of assays between institutions. Recent analysis of CAP proficiency testing results for EBV DNA suggests that calibrators are not the only issue preventing result harmonization. Commercial reagents and gene targets used also contribute to result variability (97). For more details on preferred sample matrix and detection methods, see Table 1.

Results should be reported in international units per milliliter. Normalization of viral load by using cell number or micrograms of DNA is generally unnecessary in routine diagnostics when testing samples of whole blood or CSF. Recent studies showed a close correlation between results reported in copies per milliliter and those reported as copies per microgram of DNA when testing whole blood. Similar dynamic trending in patients using both reporting formats was also observed, suggesting that normalization to cell number or genomic DNA in cellular specimens may also be unnecessary (98, 99). There is a strong correlation between viral load measured in PBMC, B cells, and whole blood. The correlation coefficient between results reported for plasma and other blood compartments (whole blood, PBMC, or B cells) is lower. Results from biopsy samples should be reported in international units per microgram of DNA; when coamplification of a cellular single-copy gene is performed, results should be reported as international units per number of cells.

The precision of EBV NAT assays is such that changes in values should be at least 3-fold (0.5 $\log_{10}$ copies/ml) to represent biologically important changes in viral replication. QNAT variability is greatest for low viral loads; thus, for viral load values at or near the limit of quantification, QNAT viral load changes may need to be greater than 5-fold (0.7 $\log_{10}$ copies/ml) to be considered significant.

Appropriate controls for extraction, contamination, and inhibition should always be included in the assays. Participation in external quality control programs is a mandatory requirement for all clinical diagnostic laboratories (for examples, see http://www.cap.org, http://www.QCMD.org, or http://www.instandev.de).

ISOLATION AND IDENTIFICATION PROCEDURES

EBV isolation is currently not routinely performed in diagnostic laboratories. Freshly fractionated human cord blood lymphocytes are inoculated with cell-free, filtered saliva or throat gargle specimens and monitored for 4 weeks (100). Individual viral isolates can be characterized by molecular techniques on the basis of polymorphism of the EBNA 1 and EBNA 3 genes. In addition, the sizes of many of the EBNA proteins (EBNA 1 to 6) are strain dependent. Each isolate induces a distinctive banding profile in Western blotting, thus allowing the fingerprinting or "EBNo-typing" of each virus (14). Direct sequencing of EBV isolates using next-generation deep-sequencing techniques is also being explored for strain typing directly from clinical samples (U. Allen, P. Hu, S. Pereira, J. Beyen, D. Ho, N. Khodai-Booran, T. Nalpathamkalam, and S. Read, presented at the International Congress on Oncogenic Herpesviruses and Associated Diseases, Philadelphia, PA, 1 to 4 August 2012). EBV strain typing and strain polymorphism studies may be important for improving our understanding of the epidemiology, biology, and immunology of EBV and to study potential associations between strains and disease states. However, presently there are no data to allow clear prediction of a patient's clinical course or treatment outcome based on EBV strain polymorphisms (reviewed in reference 102). EBV genotyping is therefore not useful in clinical practice and is currently not performed in diagnostic laboratories.

SEROLOGIC TESTS

Heterophile Antibodies

Heterophile antibodies are not EBV specific. The test relies on the detection of antibodies reacting with erythrocytes of nonhuman animals that develop as a result of polyclonal B cell stimulation occurring in the setting of IM. Historically, sheep erythrocytes were used (Paul-Bunnell test) as assay targets, but these assays lacked sensitivity (103). Later, horse erythrocytes were used, and to improve specificity, the Davidsohn differential test was added. This test discriminates between the EBV heterophile response and the heterophile response seen in serum sickness and rheumatic diseases (targets the Forssman antigen). To exclude agglutination resulting from heterophile antibodies against the non-EBV Forssman antigen, in the Davidsohn differential test, these non-EBV-related heterophile antibodies are first removed by adsorption of serum with guinea pig kidney cells that express Forssman antigen. As an additional control, serum is also adsorbed with bovine erythrocytes not expressing the IM antigen. When the two adsorbed sera are then mixed with sheep or horse erythrocytes, stronger agglutination in the guinea pig-adsorbed serum indicates a positive result.

Latex agglutination tests using erythrocyte antigens are currently used for heterophile antibody detection and show a high degree of sensitivity (103). Although they are all intended for laboratory and point-of-care use, the agglutination assays may not be suitable for use by persons without laboratory training. Evaluation of a true positive agglutination result requires experience, since spontaneous agglutination will inevitably develop after some time. Immunochromatographic methods may therefore be easier to read and perform better than agglutination assays in point-of-care settings (103).

EBV-Specific Antibodies

Serologic testing using EBV-specific assays remains the method of choice for the diagnosis or exclusion of EBV infection (Table 2) (104, 105). The tests differ in (i) methods, (ii) antigens, and (iii) antibody isotypes tested for. Although immunofluorescence assays (IFAs) and blot techniques are more reliable, EIAs and chemiluminescence assays (CLIAs) are used most frequently. At present, flow cytometry-based assays are used in some laboratories to detect EBV antibodies to different antigens (e.g., VCA, EA, and EBNA 1) at the same time, in a multiplex approach. In the case of unclear results, any of these methods may be supplemented by avidity testing. Traditionally, antibodies to three antigen complexes have been measured, including VCA, EA, and EBNA. In addition, different immunoglobulin isotypes (IgG, IgM, and sometimes IgA) can be detected. The large numbers of assays can lead to complex antibody patterns, making results difficult to interpret. When possible, kits from the same manufacturer should be used for detecting different markers in an individual patient. The antibody profile in a patient with suspected mononucleosis is presented in Fig. 1.

Immunofluorescence is still the gold standard for EBV serology, but it is labor-intensive and interpretation is subjective. On the whole, IFAs have fairly uniform performance characteristics, principally because suppliers use the same cell substrates. The performance of EIAs is much more variable, due to the plethora of antigen preparations used in the different kits. They range from cell extracts to recombinant or fusion proteins and synthetic peptides (104). This means that reference criteria established for interpretation of IFAs may not apply to all EIAs, even when the antigens are referred to by the same name. For example, EBNA is a complex of several large, native proteins detected in Raji cells by anticomplement immunofluorescence (ACIF); in EIAs, the same designation may be given to a single oligopeptide derived from EBNA 1 or an EBNA 1 recombinant protein. Recombinant proteins and peptides, however, are easier to standardize than cell-grown antigens, and assays using synthetic antigens will likely be more widely used in the future.

Recently, flow cytometry-based microparticle assays have become more popular since they allow parallel detection of different antigens. In theory, up to 100 uniquely identifiable fluorescently labeled microspheres can be detected with this technique, each particle coated with a different antigen (106–108). This approach combines the advantages of a complete antibody profile, like that of a blot, with a turnaround time of 1 to 2 h as well a random-access feature. In addition to EBV-specific antigens, some assays also include heterophile antibodies or non-EBV antigens. However, specialized instruments are necessary for signal detection (e.g., xMAP system; Luminex, Austin, TX).

TABLE 2 Overview of commercial products for EBV diagnosis[a]

Method	No. of products	Differences between kits	Advantage(s)	Limitations
Heterophile antibodies				
Slide agglutination tests	~40 kits	Mostly latex particles: rarely bovine, horse, or sheep erythrocytes	Very rapid (<15 min), inexpensive	Measures heterophile antibodies; can be positive for 1 yr after IM
Immunochoromatographic strip tests			Very rapid (<15 min), inexpensive, easier to read than agglutination tests	Not specific for EBV; can be positive with lymphoma, acute HIV, or other infectious agents
EBV-specific antibodies				
Immunochromatographic strip or paddle tests	~2 kits	Different antigens	Rapid (<25 min)	Depending on the kit, the spectrum of antigens may not be sufficient
EIA: ELISA	~15 kits	Different antigens and antigen preparations	Might be combined with avidity testing for atypical results	Turnaround time of ~2–3 h; interpretation schema of some manufacturers include diagnoses that are not clinically relevant
EIA: dot technique	1 kit		Combined with CMV, *Toxoplasma gondii*	Turnaround time of ~2–3 h
EIA: CLIA	~2 kits	Different antigens and antigen preparations	Rapid (<1 h); random-access avidity testing commercially available	Specific instruments needed
Flow cytometry based, with microparticles	~3 kits	Combined with CMV or with heterophile antibodies	Rapid (<1 h); reaction to specific antigens similar to blot assay	Specific instruments needed; variable quality of antigens
Immunofluorescence	3 kits	With EBNA (ACIF) or without	Gold standard, high specificity, might be combined with avidity testing	EBNA ACIF detects not only IgG against EBNA 1 but also IgG against EBNA 2; must be combined with a non-IFA method to detect only EBNA 1 IgG; labor-intensive; turnaround time of ~2–3 h
Immunoblotting	~10 kits	Western blot or line blots	Line blots are easier to read; excellent as confirmation for atypical results; can be combined with avidity testing	Expensive, labor-intensive; turnaround time of ~2–3 h
NAT				
Real-time PCR	~10 kits	Different gene targets; different amplicon lengths	High sensitivity and specificity	NAT is not standardized; different kits are not comparable; calibration to the international standard should be performed, expensive

[a] ELISA, enzyme-linked immunosorbent assay; CMV, cytomegalovirus.

Moreover, rapid tests using immunochromatography (e.g., on strips) have been developed for EBV, enabling a fast diagnosis (less than 5 min up to ~20 min) from a single sample. These tests are particularly suited to point-of-care use (109).

Avidity testing, measuring the strength of antibody-antigen binding, is available for all methods, not only for EIA. It can be applied to differentiate between a primary infection (low avidity) and a past infection (high avidity). Avidity is calculated as the percentage of signal loss resulting from pretreatment of the serum with urea compared with a regular assay. These assays are used more often in Europe than in North America.

The main VCA is a 150-kDa protein, but the p18 and p23 proteins are also included in the VCA complex of proteins expressed in P3HR1 cells, and all three are used for serology. VCA EIAs using antigens from infected cells have been available for a long time, and assays using affinity-purified antigen still perform better than EIAs using recombinant or synthetic antigens (110). EIAs and line blots using recombinant VCA proteins (p23–p18) and peptide p18 reportedly give mixed results compared with IFA (110–113).

Anti-EA/D (diffuse distribution in cells) and anti-EA/R (restricted to cytoplasm) are antibodies traditionally measured with Raji cells activated to enter the lytic phase by phorbol ester or sodium butyrate. Ethanol fixation eliminates the EA/R complex; cells fixed in ethanol are used for studies of EA/D antibodies. Currently, separation of EA antibodies is rarely used for diagnostic applications. Tests using cell-derived and recombinant EA proteins are available but often lack both specificity and sensitivity (103, 114). Because of their high heterogeneity with respect to specific protein targeted and consequently their low comparability, EA assays are only rarely useful for the diagnosis of acute EBV infection. Furthermore, EA antibodies are present in a significant proportion of healthy blood donors and may thus be more problematic than helpful for diagnosis of acute EBV infection (115).

ACIF detecting not only EBNA 1 but also EBNA 2 is no longer used in routine diagnostics, since commercial EIAs

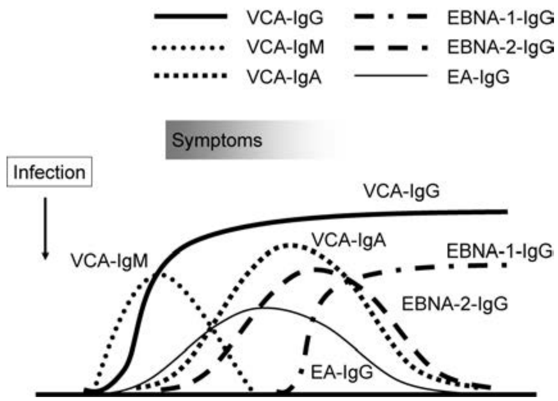

FIGURE 1 Development of antibodies to EBV antigens following primary infection. While there is marked interpatient and interlaboratory variation in titers, the typical relative development of titers by antibody class and antigen specificity is given. doi:10.1128/9781555817381.ch101.f1

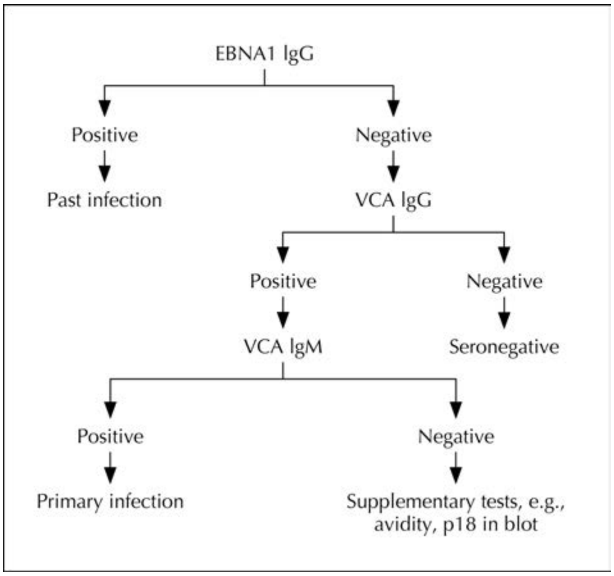

FIGURE 2 Diagnostic algorithm and interpretation scheme based on EBNA 1 used with microparticle multiplex assays or rapid tests with random access in Europe. Diagnostic procedures and interpretation may start with EBNA 1 IgG. If this parameter is positive, a past infection is proven; if EBNA 1 IgG is negative, a negative VCA IgG results in the diagnosis of seronegative and a positive VCA IgM in the diagnosis of a primary infection. If VCA IgG is positive and the other parameters are negative, supplementary tests should be applied, e.g., avidity testing or blotting using p18 antigen. doi:10.1128/9781555817381.ch101.f2

containing cell-derived EBNA or, even better, recombinant EBNA 1 (p72) are available and demonstrate high sensitivity (≥95%).

For the serologic diagnosis of EBV infection in the United States, EBV antibody panels of two, three, or four markers are commonly performed using EIA; IFA is less frequently used. Antibody panels can include VCA IgG and VCA IgM only; VCA IgG, VCA IgM, and EBNA IgG; or the last 3 antibodies plus antibody to EA. If an atypical pattern is observed (Table 3), retesting options include repeating the EIA, testing by IFA, or repeat testing of a second follow-up blood sample in 1 to 2 weeks.

An alternative algorithm frequently used in Europe for serologic diagnosis is provided in Fig. 2. In this cost-effective approach, stepwise analysis and data interpretation feature testing for EBNA in serum as the key initial step. This schema is feasible for assays with short incubation times and random access (e.g., microparticle multiplex assays). EBNA 1 IgG antibodies normally appear between 6 weeks and 7 months after onset of IM and should be negative in acute IM. They are maintained for life and are therefore a good marker of prior infection. However, in about 5 to 10% of patients, EBNA 1 IgG may be present in low titers or even undetectable (116). Increasing age, severe immunosuppression, or rheumatic disorders are often a reason for reduced or loss of EBNA 1 IgG titers. Diagnosis of primary EBV infections should not rely solely on the detection of VCA IgM, since both false-positive and false-negative results are possible. False positives result mainly from the presence of rheumatoid factor cross-reacting with other herpesvirus infections and antinuclear factors (117). False negatives may result from specimen collection in the window period prior to antibody development or insufficiently sensitive assays. False-negative results may also occur when the

TABLE 3 Typical EBV serological profiles using the most frequently employed antigens and Ig isotypes[a]

Condition	Antigen /Ig isotype					
	VCA IgG	VCA IgM	VCA IgA	EA/D IgG	EA/R IgG	EBNA Ig
Susceptible/no previous exposure	−	−	−	−	−	−
Primary infection	++	+++	+	+	+/−	−
Past infection	+	−	+/−	−	+/−	+
Inconclusive: primary or past infection[b]	+	+	+/−	+/−	+/−	+
Inconclusive: primary or past infection[c]	+	−	+/−	+/−	+/−	−
Chronic active EBV infection	+++	+	++	++	+	+/−
NPC	+++	−	+++	+++	+	+++
X-linked lymphoproliferative syndrome	+/−	−	−	−	−	−

[a] +/−, antibodies absent or present; +, antibodies present; ++, antibodies present in elevated titers; +++, antibodies present in strongly elevated titers. Atypical patterns include VCA IgM positive only, EBNA positive only, and VCA IgM and EBNA positive but VCA IgG negative. Atypical patterns merit repeat testing, testing of a follow-up sample, or testing by alternate methods.

[b] Primary infection with early detection of EBNA 1 IgG or past infection with a prolonged detection of VCA IgM. The antibody titer might help to distinguish primary from past infection; e.g., a high IgM titer combined with a very low EBNA 1 IgG might be an primary infection, whereas a past infection might be more likely if EBNA 1 IgG is present at a high titer and VCA IgM has a low positive value. Additional methods are available to distinguish the two infections (see text).

[c] Primary infection without VCA IgM or past infection with loss of EBNA 1 IgG. Depending on the time since exposure, VCA IgM may no longer be detectable in patients with symptoms of IM. Some patients with past infection lose EBNA 1 IgG over time.

specimen is collected late during a period after IgM has disappeared.

In rare cases, immunoblotting including VCA p23, VCA p18, and EBNA 1 p72 may be performed (118). VCA p18 may be important since antibodies of the IgM class are detectable quite early during infection, whereas IgG antibodies are produced late (similar to EBNA 1 IgG) (104, 119). If EBNA 1 IgG and p18 IgG are negative, an avidity test for VCA IgG can be informative (118).

In the setting of NPC, different antibody isotypes (IgG and IgA) targeting a great variety of antigens, e.g., VCA and LMP 2 (120), LMP 1 (121), thymidine kinase (79), BamHI Z-encoded EBV replication activator (ZEBRA) (122), EBNA 1 (123, 124), zta (123), or EA (125), have been studied for disease diagnosis and management.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Antibody assays are the method of choice for diagnosing acute EBV infection in immunocompetent hosts. NAT is discouraged in this setting, since EBV DNA may also be detected in healthy seropositive individuals (17, 126, 127). Recent studies suggest that EBV DNA is cleared rapidly from plasma (within 15 days of IM) (13) but persists significantly longer when measured in whole blood, with clearance from this matrix occurring in most subjects within 200 days (128). Detection of infection in young children (<12 months) in the presence of maternal antibody using serologic techniques is problematic. The use of NAT to document infection in this setting may be useful and requires further study (15).

Heterophile antibodies are a marker of IM but not necessarily of acute EBV infection. High levels of heterophile antibodies are seen during the first month of IM, normally followed by a rapid decrease. Low but persisting heterophile antibody titers can be found after primary EBV for up to 1 year, which can be misleading, and more importantly, heterophile antibodies can sometimes be found in an acute HIV infection, lymphoma, or infection with other infectious agents (e.g., cytomegalovirus, rubella virus, or *Toxoplasma gondii*) (129, 130). EBV-specific serology should always be performed in settings where the interpretation of a positive heterophile result is uncertain. False-negative heterophile antibody results (15 to 20%) are the rule among young children (103, 116, 131) but also occur for adolescents and adults, particularly when atypical clinical presentations are present. Thus, a negative heterophile antibody test should be supplemented with EBV-specific serology when EBV infection is suspected.

Because peak EBV viral load antecedes the onset of IM symptoms, almost all IM patients have high titers of IgG to various lytic-phase EBV antigens (VCA and EA) but lack antibodies to the EBNA antigens (for details, see Fig. 1). The patient's EBV status can generally be ascertained from a single serum sample by measuring VCA IgG, VCA IgM, and EBNA 1 IgG. In upwards of 90 to 95% of cases, the antibody profile is sufficiently distinct to determine whether the patient (i) is still susceptible to EBV, (ii) has a current primary infection, or (iii) has a past infection (Table 3). The use of terminology such as "recent," "convalescent," or "reactivated" infection, as suggested by some manufacturers in result reporting, is discouraged, since as these diagnoses correspond to infection states in healthy individuals they are of limited clinical relevance when testing is performed for symptomatic patients. The exact antibody titers and the time needed to develop the complete antibody profile of past infection vary widely between individuals and do not correlate with severity of disease (128). Thus, quantitative measurement of antibodies is of limited usefulness in routine diagnostics (132).

In NPC serology, specifically VCA IgA, EA IgA, and EBV DNA in plasma or serum are established diagnostic and prognostic markers and are also used for screening in high-risk patients in areas of endemicity. Recently, data to support the important role of EBV viral load measurement in nasopharyngeal brushings for NPC case detection and management have been reported (78). EBV DNA in this setting acts as a tumor marker in plasma and exists as free nonencapsidated DNA (84). Viral load and serological markers are elevated in patients prior to the tumor becoming clinically apparent, during disease, and during recurrence. Reductions in viral load or antibody titers are a marker of effective therapy. In a recent analysis of long-term survival in patients treated for NPC, a higher relapse rate, a worse relapse-free survival rate, and poorer overall survival rate were observed in patients with higher pretreatment or persistently detectable posttreatment plasma viral loads (133).Viral load measurement is superior to serology in its prognostic value, especially for tumor recurrence (78, 125, 134). For example, DNA becomes rapidly undetectable within a few hours of tumor surgery (135), whereas serology follows the slower kinetics of antibody production and half-life.

In the setting of transplantation, EBV serostatus determination is required for risk stratification. Patients are considered seronegative if negative for both anti-VCA IgG and anti-EBNA IgG. Serostatus determination is problematic with young children and recently transfused patients because of passive antibody. In these settings, donors are considered seropositive and recipients seronegative for risk stratification purposes.

High viral load states often antedate symptoms and signs of EBV-associated PTLD occurring early after transplantation. Routine posttransplantation monitoring of EBV viral load has been recommended for PTLD prevention in high-risk settings such as when the donor is seropositive and the recipient is seronegative at the time of SOT (50, 52). Monitoring is combined with preemptive interventions that include reduction in immunosuppression with or without antiviral therapy after SOT (136, 137) and rituximab (anti-CD20) therapy after HSCT (138). Reductions in the incidence of early EBV-positive PTLD have been reported by centers using this approach. However, quantitative viral loads that could be used as trigger points for intervention remain undefined at a global level because of lack of assay standardization and few natural history studies with sufficiently large patient populations. Trigger points may also vary by specimen type and patient group. Along with the absolute viral load, the kinetics of viral load increase may play an important role in defining risk.

In high-risk asymptomatic SOT recipients being serially monitored, the use of EBV viral load as a diagnostic test (i.e., levels above a specific quantitative threshold being diagnostic of PTLD) has good sensitivity for detecting EBV-positive PTLD but misses EBV-negative PTLD and some cases of localized and donor-derived PTLD. However, it has poor specificity, resulting in good negative (greater than 90%) but poor positive (as low as 28% and not greater than 65%) predictive values in these populations. Monitoring over the first year after transplantation after SOT and for the first 6 months after HSCT in high-risk populations is recommended in international guidelines (50, 52, 72, 73). There are insufficient data to support routine monitoring beyond this period. Data to support EBV viral load monitoring in populations at low risk of PTLD, such as adult SOT patients seropositive before transplantation, are lacking. EBV DNAemia is frequently detected in EBV-seropositive patients after transplantation, and quantitative EBV levels have been proposed as a marker of global immunosuppression in this setting. However, significant further study and validation are required before EBV viral load can be used to determine a patient's global state of immunosuppression (136, 139–141).

Formal evaluation of EBV NAT assays in peripheral blood as an adjunct diagnostic tool when used as a one-time test for patients presenting with symptoms or signs (usually mass lesions) and no history of recent or previous monitoring has not been carried out for populations at high risk for PTLD after SOT. In low-risk adult SOT recipients EBV seropositive before transplantation being investigated for signs and symptoms compatible with PTLD, high EBV QNAT lacked sensitivity, missing all cases of EBV-negative PTLD and some cases of localized EBV-positive PTLD, but was highly specific for EBV-positive PTLD (91). However, this study, carried out in an oncology practice, may have a referral bias and not reflect the broader use of these assays after SOT. Monitoring in plasma may have better specificity than whole-blood monitoring when used in this setting.

Data on the use of EBV NAT assays with peripheral blood to monitor PTLD treatment response and predict PTLD relapse are limited. A short-term fall and clearance of viral load coincident with clinical and histologic regression in response to interventions such as reduction of immunosuppression (142) and adoptive immunotherapy (143) have been observed in PTLD patients and patients with high viral load receiving preemptive therapy. In contrast, when rituximab was used, viral load measured in cellular blood components often fell dramatically and remained low even when disease progressed and relapsed (144, 145). In pediatric SOT patients, particularly those experiencing primary infection, asymptomatic intermittent or persistent viral load rebound occurs frequently with no short-term consequences (142). Adult PTLD patients have been observed to relapse in the presence of a persistently low viral load (144). There are some preliminary data to suggest that plasma may be preferable to whole blood when used to assess treatment response and predict relapse.

Testing of viral load in the CNS is often used to assist in the diagnosis of CNS lymphoma, particularly in immunodeficient populations, based on the observation that CSF EBV viral loads in patients with CNS lymphoma are higher than those seen in peripheral blood or may be detectable only in CSF (125, 146). However, the detection of EBV DNA in CSF has a poor positive predictive value for CNS lymphoma since it is has poor specificity (147–152). Use of quantitative levels may improve diagnostic specificity (150). Tissue diagnosis remains the gold standard for the diagnosis of CNS lymphoma, with the presence of EBV documented using immunohistochemistry or *in situ* hybridization in biopsy tissue. Although EBV detection in CSF is also used to diagnose EBV CNS disease in the setting of acute EBV infection in immunocompetent hosts, the sensitivity and specificity of both the qualitative and quantitative tests in this setting are difficult to determine and have not been extensively studied. Studies of this type are difficult to perform since brain biopsy and tissue confirmation of CNS involvement are seldom obtained; a gold standard for the diagnosis of EBV neurologic involvement during acute infection is lacking. The prevalence and quantitative levels of CSF EBV DNA observed in patients with acute EBV infection but without neurologic symptoms are also unknown since CSF is rarely collected in this setting. Low-level EBV DNA was detected in the CSF of patients without typical clinical symptoms in one study (147). At the present time, EBV neurologic disease associated with acute infection is a diagnosis of exclusion. The detection of EBV DNA in CSF can support but does not definitively confirm this diagnosis, particularly when at high levels relative to peripheral blood, in a patient with serologic evidence of acute EBV infection and self-limited neurologic symptoms or signs.

Patients with X-linked lymphoproliferative syndrome typically have high viral loads and do not develop EBNA antibodies. The definitive diagnosis of this condition requires genetic studies to detect specific associated hereditary mutations.

FUTURE PERSPECTIVES

Adjunctive laboratory testing may improve the specificity of high viral load as a predictor of PTLD. The best studied and most promising are assays measuring EBV-specific T cell responses using concomitant EBV-specific T cell enzyme-linked immunospot assay and tetramer assays (151–155). Although data suggest that the specificity and positive predictive value of EBV viral load can be significantly improved using these assays, they are complex, costly, and difficult to implement in a routine diagnostic laboratory (50, 52). Although further validation of their usefulness is required, some cytokine genetic polymorphisms have been correlated with development of PTLD in gamma interferon, interleukin-10, and transforming growth factor β genes.

A particularly interesting source of new EBV diagnostics involves the study of host and viral microRNAs (miRNAs), a class of recently identified small noncoding RNAs that are involved in gene regulation by inhibition of protein translation or mRNA degradation (156). Research with miRNAs has been particularly groundbreaking in hematologic malignancies. Several studies have confirmed that miRNAs play important roles in B cell differentiation and lymphoma pathogenesis and are prognostic for clinical outcome (157, 158). EBV encodes 44 mature miRNAs in two gene clusters, BHRF 1 and BART (BamHI A rightward transcripts), and viral and human miRNAs interact with each other in host gene regulation (159, 160). An advantage to the study of miRNAs is that due to their small size and molecular stability, they can be studied in formalin-fixed paraffin-embedded tissue used for diagnostic purposes and have been detected in plasma samples (161). Human and EBV miRNA profiles are being extensively studied in a number of EBV-associated malignancies to understand pathogenesis, develop new therapeutic targets, and tailor therapy and as prognostic markers (101, 162–165).

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Human Herpesviruses 6, 7, and 8

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The viruses discussed in this chapter are human herpesviruses 6A and 6B (HHV-6A and HHV-6B), human herpesvirus 7 (HHV-7), and human herpesvirus 8 (HHV-8; also called Kaposi's sarcoma [KS]-associated herpesvirus). These viruses cause diseases that are clinically significant primarily in small children and in immunocompromised patients (Table 1).

HHV-6

Taxonomy

HHV-6A, HHV-6B, and HHV-7 represent virus species in the *Roseolovirus* genus of the betaherpesvirus subfamily (1). Roseoloviruses share many features of their genomic architecture and genetic content, the ability to replicate and establish latent infections in lymphocytes, associations with febrile rash illnesses in young children and with a variety of neurologic disorders, and the ability to act as opportunistic pathogens in immunocompromised patients. For convenience, we frequently refer to HHV-6A and HHV-6B collectively as HHV-6.

Description of the Agent

HHV-6 virions consist of four concentrically arranged major components: a double-stranded DNA genome (160 to 170 kb) within the core of an icosahedral capsid surrounded by a proteinaceous tegument, all of which is surrounded by a lipid bilayer envelope that is studded with a variety of viral and cellular proteins. The viral genomes carry approximately 100 unique genes.

Epidemiology and Transmission

Epidemiology

Collectively, HHV-6A and HHV-6B are highly prevalent (>90% seroprevalence) (2). Most individuals become infected by the age of 2 to 3 years (3). In the United States, Japan, and Europe, HHV-6B is the main source of nearly universal early childhood HHV-6 infections, and HHV-6A prevalence in these areas may exceed 50% by adulthood. In Zambia, HHV-6A was detected in >85% of asymptomatic infants, suggesting significant international differences in the epidemiology of the HHV-6 variants (4).

Tissue Distribution

The cell surface receptor for HHV-6A is CD46, and that for HHV-6B is CD134 (5). HHV-6B DNA has been detected in as many as 90% of peripheral blood mononuclear cell (PBMC) specimens, >80% of brains, >90% of tonsils (in epithelial cells of tonsillar crypts), and 40 to 90% of oral fluid and nasal mucus specimens; it has been detected less frequently in skin, lungs, endomyocardial biopsy specimens, goblet cells and histiocytes of the large intestine, cervical fluids, and ocular aqueous humor. During primary infection in young children in the United States, HHV-6A was found by PCR in 2.5% of PBMC and 17% of cerebrospinal fluid (CSF) specimens, while HHV-6B was found in 99% of PBMC and 86% of CSF specimens (some specimens were coinfected) (6). The median salivary viral level of HHV-6 increased from approximately 10^3 copies of HHV-6/ml at 1 week postinfection to 10^5 copies of HHV-6/ml by 8 weeks postinfection (3). HHV-6A DNA has been detected in over half of lung and skin specimens, mostly in conjunction with HHV-6B. HHV-6 can use the olfactory pathway for entry into the central nervous system (7). Among adult brains, 28% were positive for HHV-6A and 75% for HHV-6B (some were dually positive) (8). For bone marrow transplant recipients, HHV-6A and HHV-6B were both detected in plasma, while HHV-6B was the variant detected in PBMC (9).

Latency, Persistence, and Transmission

Other than during the acute phase of roseola, HHV-6 can seldom be detected by culture, even though its DNA can be detected intermittently in PBMC from most children by PCR, and transcripts can occasionally be detected in asymptomatic children (~1% of specimens) (10). This suggests that a true latent infection is established in PBMC. Latent HHV-6 has been detected in monocytes, their CD34$^+$ bone marrow progenitors, and PBMC-derived dendritic cells. HHV-6 DNA is more frequently detected in salivary glands than in saliva, in contrast with HHV-7, which is frequently detected in both the gland and the fluid. HHV-6B primary infection typically occurs within a matter of months of the waning of maternal antibody, with transmission likely being via saliva (11). Breast milk is not a significant vehicle for transmission. Little is known of HHV-6A transmission.

A small percentage of individuals (0.5 to 1%) harbor germ line chromosomally integrated HHV-6A or HHV-6B genomes (ciHHV-6) at a chromosomal telomere, so all of their cells are positive for viral DNA (12), which may be a source for the occasionally detected persistence of high

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TABLE 1 Disease associations for HHV-6, HHV-7, and HHV-8

Virus	Age at primary infection	Principal disease associations		
		Primary infection	Immunocompromised patients	Other
HHV-6A	Unknown	Occasional roseola or febrile illness	Disseminated infections, HCMV-like and HCMV-associated disease, pneumonitis, graft loss	HT
HHV-6B	Before 2 yr	Mostly roseola, plus related febrile rash illnesses; febrile seizures and febrile status epilepticus	PALE and epileptic encephalopathy following PALE, delirium and declines in cognitive functioning, mortality following HSCT, disseminated infections, HCMV-like and HCMV-associated disease, pneumonitis, graft loss	DRESS/DIHS, myocarditis
HHV-7	>50% by 2 yr	Occasional roseola, febrile status epilepticus		
HHV-8	During childhood in Africa; postadolescence in the United States and Europe	Unknown	KS, MCD, PEL, acute bone marrow failure in transplant recipients	

levels of HHV-6 DNA in serum (13) as well as for congenital transmission of HHV-6 (14, 15).

Clinical Significance

While infections with HHV-6 are generally mild or subclinical in immunocompetent individuals, they can be severe in immunocompromised persons. Many of the more severe presentations have neurologic components.

Primary Infection

HHV-6 primary infection causes roseola (also called roseola infantum, sixth disease, and exanthem subitum) in approximately a quarter of children. While HHV-6B is the predominant etiologic agent of roseola, rare cases of HHV-6A-associated roseola or febrile illness have been reported. About 90% of children are symptomatic at the time of primary infection, with specifically associated symptoms including fever (58%), fussiness (70%), rhinorrhea (66%), diarrhea (26%), rash (31%), and roseola (24%) (3).

Classic roseola symptoms include an abrupt rise in temperature (39 to 40°C), which persists for 2 to 5 days. Fever abatement coincides with onset of a maculopapular rash (only rarely vesicular) (Fig. 1) which resolves in 1 to 3 days. Initial appearance of the rash is on the neck, behind the ears, and on the back, followed by spread to the rest of the body, usually excluding the face and distal extremities. The illness lasts 2 to 7 days, usually with no sequelae. Other clinical symptoms may include palpebral edema and suboccipital, postauricular, and cervical lymphadenopathy prior to rash onset.

Primary HHV-6 infection may also present as fever without rash or rash without fever. Less common but more severe forms of primary HHV-6 infection may include fever of >40°C, respiratory tract distress, tympanic inflammation, diarrhea, and convulsions. Although seizures are rare for HHV-6 primary infections, such infections, with or without clinical roseola, account for a significant proportion of febrile seizures in young children (16, 17). Other serious but rare complications reported for HHV-6 primary infections include hepatosplenomegaly, Gianatti-Crosti syndrome (an acrodermatitis of childhood that has also been associated with hepatitis B virus and Epstein-Barr virus [EBV] infections), bulging fontanels, aseptic meningitis, encephalitis (perhaps 1% of meningitis and encephalitis cases) (16), seizures and poor neurologic outcomes following congenital infection, and disseminated fatal infection.

Primary HHV-6 infection in adults is rare but can be severe. Clinical presentation in these cases may include a mononucleosis-like illness, prolonged lymphadenopathy, and fulminant hepatitis.

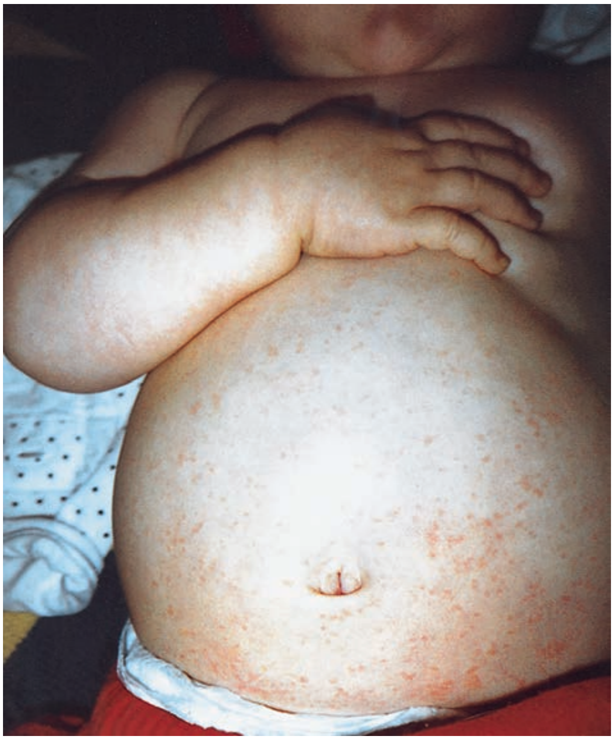

FIGURE 1 Child with roseola during primary HHV-6B infection. Photo courtesy of Stephen Dewhurst, University of Rochester. Previously published in reference 106. doi:10.1128/9781555817381.ch102.f1

Immunocompromised Patients

In immunocompromised patients, such as transplant recipients or AIDS patients, HHV-6 can reactivate, sometimes causing disease. HHV-6 reactivation along with human cytomegalovirus (HCMV) reactivation can potentially exacerbate the severity of disease (18).

Transplant Recipients

HHV-6 reactivation occurs in approximately half of hematopoietic stem cell transplant (HSCT) recipients and a third of solid organ transplant (SOT) recipients (2, 19, 20). There appears to be a temporal pattern of HHV-6, HHV-7, and HCMV reactivation for SOT recipients, with the median times posttransplantation being 20 days, 27 days, and 36 days, respectively (2). Coinfections with these viruses do not affect the HCMV response to ganciclovir or valganciclovir (21).

HHV-6 is a consistent, low- to moderate-frequency contributor to posttransplant illness that can sometimes be severe, and its activity might be both a marker of and contributor to immune suppression (22). In HSCT and stem cell transplant (SCT) recipients, HHV-6 has been associated with encephalitis/encephalopathy, pneumonitis, delayed engraftment, erythematous papular rash, fever, CMV-like disease, graft-versus-host disease, seizures, hepatitis, diarrhea, and bone marrow suppression (reviewed in references 2, 23, and 24). In SOT patients, HHV-6 has been associated with encephalitis, pneumonitis, graft-versus-host disease, fungal disease, and bone marrow suppression. HHV-6B is detected more frequently than HHV-6A in transplant patients. However, HHV-6A viremia was more frequent than HHV-6B viremia in Hungarian renal transplant recipients (25). In one study, HHV-6A partitioned into plasma and HHV-6B into both plasma and circulating lymphocytes (9), suggesting that studies focused on one or the other of these compartments may have missed part of the story.

ciHHV-6 may contribute to clinical outcomes in transplant patients, but further study is required (26).

HIV-Infected Patients

Although HHV-6 infects and replicates in CD4$^+$ T cells and there is evidence for possible pathogenic interactions, no specific role has been established for HHV-6 in AIDS pathogenesis.

Infections in the Brain

HHV-6 can grow in neuronal cells, and neurological symptoms are clearly associated with primary infection. HHV-6 encephalitis occurs during primary infection and reactivation, with slightly different disease characteristics (27). HHV-6 activity is associated with post-HSCT acute limbic encephalitis (PALE) (20), epileptic encephalopathy following PALE (28), and delirium and cognitive decline (29).

The involvement of HHV-6 in multiple sclerosis is not conclusive (30). However, the more precisely linked the specimens have been to diseased tissue (e.g., microdissected lesions versus cerebrospinal fluid versus serum), the stronger the associations have been.

There are conflicting data with respect to the association of HHV-6 with progressive multifocal leukoencephalopathy (31, 32).

Other

DIHS

Exposure to various drugs occasionally results in a severe and sometimes fatal reaction variously known as drug-induced hypersensitivity syndrome (DIHS), drug rash with eosinophilia and systemic symptoms (DRESS), and anticonvulsant hypersensitivity syndrome (33). HHV-6 reactivations have been identified in a number of DRESS cases, but it remains to be seen whether HHV-6 contributes to either initiation or exacerbation of the events.

Rosai-Dorfman Disease

Rosai-Dorfman disease (histiocytosis with massive lymphadenopathy) patients have had increased HHV-6 antibody titers, HHV-6 DNA detected by *in situ* hybridization, and strong immunohistochemical (IHC) staining for HHV-6 antigen in abnormal histiocytes.

HT

Hashimoto's thyroiditis (HT) is an autoimmune disease mainly affecting women. A statistically significantly higher prevalence and viral load of HHV-6A in thyroid fine-needle aspirates was found for HT patients than for controls, along with higher natural killer cell activity and T-cell responses to HHV-6-infected thyroid cells (34). In addition, natural killer cells from HT patients had increased activity toward HHV-6-infected thyroid cells, and HT patients showed enhanced T-cell responses to HHV-6 antigen compared with controls.

Cardiomyopathy

HHV-6B antigens and DNA have been detected in damaged cardiac tissues of patients with acute and chronic cardiomyopathies (35).

Other Diseases

There is no conclusive evidence for causal associations between HHV-6 and malignancy or chronic fatigue syndrome.

Therapy

Antiviral treatment of HHV-6 has been reviewed in depth (2). No antivirals are licensed for treatment of HHV-6, but approaches such as those for HCMV have been used. In a study of HHV-6 and HHV-7 coinfections, antiviral therapy for HCMV had no discernible effect on HHV-6 or HHV-7 DNA loads (21). The clinical circumstances in which antiviral intervention for HHV-6 might be appropriate include HHV-6 reactivation in immunocompromised individuals (20). It is important to distinguish ciHHV-6 from HHV-6 reactivation for consideration of antiviral therapy in patients with clinical symptoms compatible with HHV-6 activity (12). Artesunate has anti-HHV-6 activity *in vitro* and was reported to effectively treat a case of HHV-6B-associated myocarditis (35).

Collection, Transport, and Storage of Specimens

Saliva specimens can be collected for virus detection by various methods, including filter paper strips (36), oral swabs, throat swabs, and expectorated saliva collection. Plasma should be separated from cells shortly after collection to avoid false-positive results for individuals with ciHHV-6 due to release of cellular DNA. Serum, plasma, and cells intended for PCR can be shipped frozen.

Direct Examination

Direct detection of HHV-6 can be done by IHC (37, 38), *in situ* hybridization, and *in situ* PCR (39, 40) (Tables 2 through 4). While those methods have their niches, the most common method of direct detection is PCR. The sensitivity of PCR is of particular value for the detection

TABLE 2 Diagnostic methods for HHV-6, HHV-7, and HHV-8[a]

Virus	Method	Approach	Advantage(s)	Limitation(s)	Clinical availability	Key reference(s)
HHV-6	Virus isolation	Cocultivate PBMC with stimulated primary CBL	Gold standard for active infection	Slow, need for ongoing access to umbilical cord blood	Not practical in clinical labs	47–49
	Antigen detection	Use of HHV-6A and -6B variant-common and variant-specific MAbs against p41/p38, gp116/54/64, gp82/105, and 101K for IHC and culture confirmation	Specific, readily available reagents, precise localization in tissues	Specialized interpretation required; lower throughput than that of PCR	MAbs are commercially available	50
	Serology (major antigen, p100/101K)	IgM and IgG by neutralization, immunoblotting, IFA, ACIF, ELISA		Single IgG-positive results are uninformative; need paired acute- and convalescent-phase specimens to document disease-linked seroconversion	Kits and reagents are commercially available	51
		Antibody avidity	Clear identification of primary infections	Clinical significance of IgM in individuals of >3 years of age is questionable	Avidity test is not commercially available	
	PCR	Qualitative and quantitative amplification from PBMC, plasma/serum, and CSF		Optimal target not determined; assays not standardized with respect to viral gene target and specimen type; clinically relevant quantitation threshold not standardized; not able to differentiate active infections from chromosomally integrated high-level HHV-6 expression	Practical for clinical labs; assay and testing are commercially available	Numerous methods reported in the literature (57)
		RT-PCR amplification from PBMC to detect active infections	Confirms active infection	Not standardized	No commercially available kit	105
		Herpesvirus multiplex	Convenient for the lab	Clinicians may not want testing done for multiple herpesviruses because of interpretation and clinical utility issues		41, 42
HHV-7	Virus isolation	Cocultivation of PBMC with stimulated primary CBL; inoculation of CBL with saliva from healthy and ill patients	Gold standard for active infections	Not practical in clinical labs	Rare	48
	Antigen detection	Several MAbs available from research laboratories, including a MAb against pp85	Useful for IHC and culture confirmation	MAbs no longer commercially available	Rare	70
	Serology (major antigen, pp85)	IgM and IgG NT, immunoblotting, IFA, ELISA		Uncertain reliability of IgM for identifying recent infections; need paired acute- and convalescent-phase specimens to document disease-linked seroconversion	Commercially available	51
		Antibody avidity	Identification of primary infections			

(Continued on next page)

TABLE 2 Diagnostic methods for HHV-6, HHV-7, and HHV-8[a] *(Continued)*

Virus	Method	Approach	Advantage(s)	Limitation(s)	Clinical availability	Key reference(s)
	PCR	Qualitative and quantitative amplification from PBMC or plasma/serum			Commercially available testing	71
		Herpesvirus multiplex	Convenient	Clinicians may not want testing done for multiple herpesviruses because of interpretation and clinical utility issues	Commercially available kit	41, 42
HHV-8	Virus isolation	Not available				
	Antigen detection	MAbs against K8.1, ORF59, and LANA, for IHC	Identification of HHV-8 in tissues from suspected KS, PEL, and MCD cases; distinguishes KS from clinical mimickers	Lower throughput than that of PCR	MAbs are commercially available	96
	Serology (major lytic antigens, ORF65 and K8.1; major latent antigen, LANA-1)	IgG IFA based on unstimulated (latent) or stimulated (lytic) PEL cells	Identification of HHV-8-infected individuals	Not specific for a particular disease	Reagents, kits, and testing are commercially available	99, 100
		ELISAs based on synthetic peptides, purified virions, and expressed proteins				99, 100
		Neutralization assay				102
		Immunoprecipitation with chemiluminescence detection				101
	PCR	Qualitative and quantitative	Verification of presence of HHV-8 in tissues from suspected KS, PEL, and MCD cases	PCR from blood is insensitive for detecting infection in absence of disease	Commercially available reagents and testing	104
		Herpesvirus multiplex		Clinicians may not want testing done for multiple herpesviruses because of interpretation and clinical utility issues	Commercially available kit	41

[a] ACIF, anticomplement immunofluorescence; IHC, immunohistochemistry; IFA, immunofluorescence assay; ELISA, enzyme-linked immunosorbent assay; KS, Kaposi's sarcoma; LANA, latency-associated nuclear antigen; MAb, monoclonal antibody; MCD, multicentric Castleman's disease; NT, neutralization test; ORF, open reading frame; PBMC, peripheral blood mononuclear cells; PEL, primary effusion lymphoma; RT-PCR, reverse transcriptase PCR. No FDA-approved tests are available for these viruses.

TABLE 3 Commercial sources of reagents and assays[a]

Supplier	HHV-6			HHV-7			HHV-8		
	Serology	IHC	PCR	Serology	IHC	PCR	Serology	IHC	PCR
Advanced Biotechnologies	ELISA	MAb	DNA, qDNA	IFA		qDNA	IFA, ELISA	MAb	DNA, qDNA
bioMérieux			qPCR			Qualitative PCR			Qualitative PCR
Fisher Scientific	IFA, ELISA	MAb	qPCR, qDNA			qDNA			
Panbio	ELISA (IgG and IgM)								
Abcam, Antibodies-online, Genway, Lifespan Biosciences, Millipore, Santa Cruz Biotechnology		MAb						MAb	
AbD Serotec, Meridian Life Sciences, Raybiotech		MAb							

[a] DNA, purified DNA; qDNA, DNA for use as a quantitative reference; qPCR, kit for quantitative PCR.

of HHV-6, given that even during periods of viral activity, viral DNA can be at a relatively low concentration relative to cellular DNA. A multitude of PCR strategies have been published that utilize numerous HHV-6 genomic targets and, in some instances, inclusion in multiplex assays (41, 42). Although it is not necessarily clinically informative, HHV-6A and HHV-6B can readily be distinguished by numerous methods. Gene targets have not been developed or evaluated systematically, and commercial assays do not specify the target gene.

Reverse transcriptase PCR for detection of HHV-6 mRNA can be useful for specific detection of active HHV-6 replication (43).

Quantitative PCR (from whole blood or PBMC) is particularly useful for the determination of baseline viral load levels in transplant patients, with significant increases above baseline indicating active infection. While it is often used for HCMV and EBV PCR testing, plasma is not as sensitive as whole blood or PBMC for HHV-6 testing.

We suggest the use of quantitative real-time assays that incorporate a coamplified positive control and allow the HHV-6 variants to be distinguished. Important variables that need to be carefully standardized and then incorporated into the sensitivity calculation and interpretation are the amount of material initially lysed to prepare the template (e.g., volume of fluid or number of cells) and the amount of this material that is included in each reaction mixture (44, 45). Sources of reagents and the assays are summarized in Table 3, and current sources for commercial PCR testing are summarized in Table 4. Thorough evaluations of HHV-6 PCR assays are essential (41), as are ongoing comparisons and proficiency testing to improve the standardization of quantitative assays (46). This is challenging given the lack of an international standard for calibration and comparison of assays.

Isolation Procedures

HHV-6 can be isolated from patient PBMC by cocultivation with stimulated human umbilical cord blood lymphocytes (47–49), but virus culture is not used for routine clinical diagnostics.

Identification

HHV-6 virions have typical herpesvirus morphology by electron microscopy, i.e., approximately 200-nm spherical enveloped virions containing a 90- to 110-nm icosahedral capsid with an electron-dense core surrounded by an amorphous tegument (1). Numerous monoclonal antibodies (MAbs) are available (both commercially and from research laboratories) that react with HHV-6A and/or HHV-6B (50).

Typing Systems

HHV-6A and HHV-6B can be typed through the use of species-specific MAbs and species-specific PCR assays (Table 2). For purposes of patient management and treatment, differentiation of HHV-6A and HHV-6B is not essential, but use of assays that discriminate between HHV-6A and HHV-6B serves the purpose of better understanding the clinical manifestations of these viruses. Typing systems are typically based on primer binding regions that are highly conserved between HHV-6A and HHV-6B, with an intervening region that can be targeted by probes specific for one or the other virus.

Serologic Tests

Numerous serological methods for detecting HHV-6 antibodies have been described, and commercial reagents are

TABLE 4 Some commercial sources for HHV-6, HHV-7, and HHV-8 testing[a]

Manufacturer	URL	Serology			PCR		
		HHV-6	HHV-7	HHV-8	HHV-6	HHV-7	HHV-8
ARUP Laboratories	www.aruplab.com	IgG, IgM			Q		q, Q
Focus Diagnostics	www.focusdx.com	IgG, IgM	IgG, IgM	IgG	q, Q	Q	q, Q
Labcorp	www.labcorp.com	IgG, IgM			q, Q		
Medical Diagnostic Laboratories	www.mdlab.com	IgG			q	q	q
Quest Diagnostics	www.questdiagnostics.com	IgG, IgM	IgG, IgM	IgG	q, Q	Q	q
University of Washington	www.depts.washington.edu/labweb/Divisions/Viro/index.htm	IgG			q, Q, ci		q, Q
Viracor	www.viracor.com				Q	Q	Q

[a] ci, ciHHV-6; Q, quantitative PCR; q, qualitative PCR.

available. These serological methods are useful for diagnosis of primary infections but are of very limited utility for identifying HHV-6 reactivations. Recent versus past infections can be discriminated through the use of immunofluorescence-based antibody avidity tests (51). Maturation from low- to high-avidity HHV-6 antibody following primary HHV-6 infection takes approximately 5 months. Antibody avidity testing can also be used to differentiate between HHV-6 and HHV-7 primary infections. Neutralizing antibodies typically become detectable 3 to 8 days following the onset of fever during primary infection.

Specificity issues for HHV-6 serological methods involve cross-reactivity between HHV-6A, HHV-6B, HHV-7, and CMV (23, 51). A recently described assay appears to distinguish between HHV-6A and HHV-6B antibodies (52). The simultaneous rise in antibodies to both HHV-6 and HCMV seen in some patients may be due to various combinations of simultaneous activity of both viruses and responses to cross-reactive antigens. There is some cross-reactivity between HHV-6 and HHV-7 antibody responses; adsorption methods and antibody avidity tests can be used for differentiation (51).

Antiviral Susceptibilities

The highest *in vitro* antiviral sensitivities and selectivities have consistently been seen for foscarnet, cidofovir, and ganciclovir. Mutations in the HHV-6 U69-encoded protein kinase or the HHV-6 DNA polymerase gene (U38) can cause HHV-6 resistance to these drugs (53, 54). HHV-6 antiviral susceptibility testing is not generally available, but molecular genotyping can be accomplished by PCR-based methods (55). Ganciclovir-resistant HHV-6 has been reported in posttransplantation cases, but it is rare (56).

Evaluation, Interpretation, and Reporting of Results

Laboratory diagnosis of HHV-6 is challenging given the many biological and molecular similarities among HHV-6A, HHV-6B, and HHV-7, the association between CMV and HHV-6 reactivation, the need to differentiate between primary and reactivation diseases, and the issue of chromosomal integration of HHV-6 (12, 51). In the absence of a simple “gold standard,” clinical virology laboratory methods for these viruses must be evaluated thoroughly in the relevant clinical context (e.g., febrile and/or rash illness in children under 3 years of age and specific forms of disease in immunocompromised individuals, such as transplantation patients) to assess sensitivity, specificity, and positive and negative predictive values.

For routine testing, serology has value only for the diagnosis of primary HHV-6 infections in children under 3 years of age with symptoms characteristic of roseola. In such situations, (i) IgM-positive serology, (ii) low-avidity IgG serology, or (iii) IgG seroconversion or a significant rise in titer between acute- and convalescent-phase sera can be considered confirmatory for primary HHV-6 infections (51). Quantitative PCR-based approaches can be used for detection of both primary infections and reactivation of HHV-6.

HHV-6 PCR diagnostics are confounded by the presence of ciHHV-6 in 1% of individuals, which results in exceptionally high HHV-6 DNA loads in their PBMC (~1 copy per cell equivalent of DNA). Individuals with ciHHV-6 can harbor 5 $\log_{10}$ HHV-6 genomes/ml in serum and >6 $\log_{10}$ HHV-6 genomes/ml in whole blood, whereas much lower viral loads are seen in non-ciHHV-6 active and reactivated HHV-6 scenarios (57, 58). Quantitative PCR-based assays can help to differentiate between latent or reactivated HHV-6 and ciHHV-6. HHV-6 DNA levels of >5.5 $\log_{10}$ genomes/ml in whole blood should be evaluated further for ciHHV-6 (12). Droplet digital PCR is well suited for this purpose because it enables precise ratiometric comparison between HHV-6 and cellular genomes, with ratios of 1.0 (most common), 2.0, and sometimes 3.0 indicating ciHHV-6 (59). Whole blood or PBMC are recommended for quantitative PCR because HHV-6 DNA in plasmas of individuals with ciHHV-6 may be a product of lysed PBMC rather than circulating HHV-6 virus indicative of active infection (60).

Confirmation of HHV-6 in encephalitis cases requires detection of HHV-6 in the CSF and low levels of viral DNA in serum or whole blood. Simultaneous detection of HHV-6 in CSF and high levels of viral DNA in serum or whole blood is indicative of ciHHV-6 and is not diagnostic for HHV-6 encephalitis (51).

Clinical testing for HHV-6 should be limited to circumstances for which there has been a well-characterized association between the disease and HHV-6 infection and when the results of a given test will be diagnostically informative. Such scenarios include primary infection in children under 3 years of age, primary infection or reactivation in immunocompromised individuals, such as AIDS patients or transplantation patients, mononucleosis-like syndromes, and meningitis/encephalitis cases. Ljungman et al. (61) provided useful recommendations for HHV-6 diagnostics post-SCT, including for HHV-6 encephalitis. It is still relevant that in some parts of the world HHV-6 has been identified as the cause of rash illness incorrectly diagnosed as measles virus infection. The possibility of primary HHV-6 or HHV-

7 infection should be considered when febrile and/or neurologic illness occurs shortly following routine immunizations; such illness may be incorrectly attributed to the vaccine (51). Further studies are needed to improve our understanding of the associations between HHV-6 and disease and to improve the quality of HHV-6 diagnostic testing.

HHV-7

Taxonomy and Description of the Agent

Because of the relative similarities of its nucleotide sequences, genomic architecture, and biology to those of HHV-6A and HHV-6B, HHV-7 is included as a member of the *Roseolovirus* genus of the betaherpesvirus subfamily (1). HHV-7 virions consist of four concentrically arranged major components: a double-stranded DNA genome (145 kb) contained in the core of an icosahedral capsid surrounded by a proteinaceous tegument, all of which is surrounded by a lipid bilayer envelope that is studded with a variety of viral and probably some cellular proteins. HHV-7 genomes carry approximately 100 unique genes.

Epidemiology and Transmission

Epidemiology

HHV-7 seroprevalences in healthy adults in Europe, Japan, and the United States range from 60 to 90%. Following waning of maternal antibody, HHV-7 seroprevalence rapidly increases to over 50% by 2 years of age and then progresses toward the adult level over the remaining years of childhood.

Tissue Distribution

Although the *in vitro* host range of HHV-7 is restricted to lymphocytes, the virus has a broad cellular host range *in vivo*. One of its cellular receptors is the lymphocyte antigen, CD4; other receptors must be involved in the infection of other cell types. HHV-7 lytic antigens have been detected in salivary gland acini, lungs, skin, and mammary glands and, more sporadically, in the liver, kidneys, and tonsils (62). HHV-7 DNA has been detected by PCR in PBMC, salivary glands, gastric mucosa, skin, cervical swabs, and bronchoalveolar lavage samples. The virus has seldom been detected in brain tissues. Infectious HHV-7 has occasionally been isolated from PBMC during roseola and is easily isolated from the saliva of healthy adults. The ability to detect HHV-7 DNA in uncultured lymphocytes by PCR, along with the inability to detect infectious virus in the absence of T-cell activation, suggests that the virus is latent in PBMC. Macrophages represent another potential reservoir (63).

Transmission

HHV-7 can be cultured from the saliva of ~75% of healthy adults, with viral loads frequently exceeding 6 $\log_{10}$ genomes per ml. While the most plausible route of transmission is via saliva, HHV-7 has also been detected in breast milk, urine, and cervical secretions. In contrast to the case of HHV-6, congenital transmission of HHV-7 has not been documented (64).

Clinical Significance

Primary Infection

HHV-7 causes perhaps 5% of roseola cases, as well as other febrile illnesses in children. In one small study, HHV-7 appeared to cause milder roseola than that with HHV-6 in terms of mean and maximum fever, fever duration, and duration of rash. However, other studies suggested an equal or higher rate of central nervous system complications during HHV-7 primary infections than during HHV-6 infections (16). HHV-7 neurologic involvement includes hemiplegia and febrile seizures, including prolonged seizures that manifest as febrile status epilepticus (17).

Immunocompromised Patients

HHV-7 becomes latent in lymphocytes following primary infection and can reactivate in immunocompromised hosts, such as transplant recipients and AIDS patients. In bone marrow, liver, and renal transplant patients, simultaneous CMV and HHV-7 activities have sometimes been associated with clinical events (see corresponding HHV-6 section above), and a case of fatal HHV-7-associated encephalitis has been reported, but overall, HHV-7 infections are not frequent contributors to posttransplantation illness.

Like HIV, HHV-7 uses CD4 as its major cellular receptor. *In vitro*, HHV-7 can inhibit HIV infection, possibly due to receptor interference. There is no clear evidence of a role for HHV-7 in AIDS progression.

Other

HHV-7 DNA was detected more frequently and at higher loads in affected tissues from nonimmunocompromised patients with interstitial pneumonia (65, 66). Further study will be required to fully evaluate the significance of this observation.

Data for an association between HHV-7 and pityriasis rosea are inconclusive (67, 68).

HHV-7 DNA was detected in CSF from a neurosarcoidosis patient, in a lymph node from a Kikuchi's disease patient, and in Grave's disease patients, with uncertain clinical significance.

Therapy

HHV-7 antiviral sensitivities are similar to those for HHV-6 and CMV, with ganciclovir, foscarnet, and cidofovir being much more inhibitory than acyclovir (69). Drug-resistant HHV-7 has not been identified, but given the widespread use of ganciclovir for therapy of CMV and "CMV disease," it will likely occasionally emerge. Therapeutic regimens have not been defined; for transplant recipients, they may ultimately be linked to strategies for dealing generically with betaherpesvirus activity.

Collection, Transport, and Storage of Specimens

The methods described above for HHV-6 are appropriate.

Direct Examination

By electron microscopy, HHV-7 virions have appearances typical of herpesviruses. MAbs are available upon request from some research laboratories (70). The most widely used qualitative PCR primer set for HHV-7 was described by Berneman et al. (71); others have also been described, but their performances have not been compared. Real-time PCR methods for HHV-7 have been described (42, 72), including multiplex assays for detection of multiple herpesviruses (41, 42) (Tables 2 to 4).

Isolation Procedures

HHV-7 isolation can be accomplished by using methods similar to those for HHV-6. HHV-7 isolates initially propagated in primary cell culture or previously adapted

HHV-7 strains (e.g., strain SB) can be grown in SupT1 cells in a manner similar to propagation of HHV-6 in continuous cells. Although culture of HHV-7 is possible, it is not of practical utility for clinical diagnostics (48).

Identification

The PCR methods described above provide sensitive, specific, and rapid ways to unambiguously identify the virus. MAbs can be useful for IHC analysis of tissues.

Typing Systems

Biologically meaningful subtypes of HHV-7 have not been identified.

Serologic Tests

HHV-7 serologic assays include neutralization tests, immunoblotting, immunoprecipitation, immunofluorescence assay (IFA) (using commercially available reagents), and enzyme-linked immunosorbent assay (ELISA) (reviewed in reference 70) (Tables 2 to 4). In one comparison of IFA, immunoblots, and ELISA, the overall performances of the three assays were quite similar, with a small sensitivity advantage for the ELISA and a specificity advantage for the immunoblot assay (73). As described above, antibody avidity testing for both HHV-6 and HHV-7 allows differentiation between HHV-6 and HHV-7 primary infections as well as recent and past HHV-7 infections (51).

Antiviral Susceptibilities

Antiviral susceptibilities have been evaluated by measuring inhibition of viral replication by immunofluorescence, by use of antigen slot blots, and by inhibition of virus-induced cytotoxicity (69), but these tests are not available for routine clinical application.

Evaluation, Interpretation, and Reporting of Results

There are no circumstances that warrant routine single-agent monitoring for HHV-7 activity, but its inclusion in multiplex assays may prove to be of value for monitoring children with suspected encephalitis and/or febrile convulsions (especially in association with recent vaccination) (16) and for monitoring transplant recipients. As for HHV-6, there is no universal “gold standard” for diagnosing HHV-7 infection. The main issues for HHV-7 diagnostic testing are distinguishing HHV-6 from HHV-7, differentiation of primary HHV-7 infection from reactivation, and distinguishing latent and active infections. Differentiating HHV-6 from HHV-7 can be done both by molecular diagnostics (e.g., PCR) and through the use of specific antibodies.

HHV-8

Taxonomy

HHV-8 is a member of the *Rhadinovirus* genus of the gammaherpesvirus subfamily. Gammaherpesviruses, which include EBV, are characterized by being able to replicate and establish latency in lymphoblastoid cells.

Description of the Agent

HHV-8 virions consist of four concentrically arranged major components: a double-stranded DNA genome of 165 kb that is contained in the core of an icosahedral capsid surrounded by a proteinaceous tegument, all of which is surrounded by a lipid bilayer envelope that is studded with virally encoded proteins and some cellular proteins. HHV-8 genomes encode ~80 proteins and 12 microRNAs (74).

Epidemiology and Transmission

Epidemiology

HHV-8 seroprevalences range from <10% in most of Europe, the United States, and Japan to >50% in much of Africa (75). Seroprevalences are ~30% to 50% in men who have sex with men (MSM) in the United States and Europe. At least four major HHV-8 genotypes are in global circulation (76).

Tissue Distribution

In the absence of KS, HHV-8 DNA or antigens are seldom detected in seropositive immunocompetent individuals, most frequently in the saliva of HIV-negative MSM (77). In HIV-positive MSM, viral DNA can be detected in essentially all KS lesions, >50% of saliva or oral fluid specimens, ~30% of PBMC specimens, ~10% of semen specimens, and less frequently in other types of specimens. The virus is capable of replicating in many cell types, including CD19-positive B cells in AIDS patients, placental tissues (78), and circulating endothelial progenitors during classic KS (79).

Latency and Persistence

Evidence for persistence of HHV-8 in immunocompetent individuals comes from seropositive organ transplant recipients who subsequently developed KS (80). The virus is relatively quiet in immunocompetent individuals, inducing low antibody titers, producing low viral loads, and infrequently causing disease. *In vivo*, the virus can establish latency in $CD34^+$ and $CD19^+$ cells. HHV-8 latency has been studied extensively in cell culture systems that employ primary effusion lymphoma (PEL) cells, in which most cells harbor the virus in a latent state. Lytic replication can be induced by agents such as phorbol esters or the HHV-8 ORF50 lytic inducer gene. A subset of HHV-8 genes are transcribed during latency, including the ORF73 gene (encoding the major latency-associated nuclear antigen 1 [LANA-1]), v-cyclin, and the highly expressed K12 gene (74).

Transmission

Most HHV-8 transmission is probably via oral fluids but may also occur via breast milk and semen. In Africa, the virus is typically acquired in a maternally linked manner early in life. Although not the major route, HHV-8 seropositivity is linked to sexual risk factors among adolescents with high-risk sexual behaviors and among MSM (81, 82). The virus can be transmitted via injection drug use and by blood transfusion (83, 84).

Clinical Significance

Primary Infection

Little is known of primary HHV-8 infection. HHV-8 DNA has been detected in febrile children in areas where the virus is endemic. Among HIV-negative MSM, diarrhea, fatigue, localized rash, and lymphadenopathy were sometimes noted in association with primary infection (85). In immunocompromised patients, HHV-8 primary infection was associated with KS, fever, arthralgia, lymphadenopathy, splenomegaly, and cytopenia (86, 87).

KS

All four major forms of KS are associated with HHV-8: (i) African endemic KS, which primarily affects children; (ii)

Mediterranean or classic KS, which affects older men from Mediterranean Europe who have no known immunologic dysfunction; (iii) transplant-associated or idiopathic KS, which affects approximately 1% of SOT recipients in the United States; and (iv) AIDS-associated KS, which is the most aggressive form of KS. The importance of immune deficiency in KS is indicated by the >1,000-fold higher KS incidence in HHV-8-seropositive individuals who are immunocompromised. KS incidence has declined but has not been eliminated since the advent of highly active antiretroviral therapy.

KS is a reactive angioproliferative disease characterized by reddish brown plaque or nodular lesions on the skin of the trunk or the extremities, in the oral cavity, and on internal organs. The disease can be disfiguring, disabling, and life-threatening and has elevated mortality in HIV-infected patients when accompanied by immune reconstitution inflammatory syndrome. KS lesions are characterized by networks of vascular slits, spindle-shaped cells of endothelial origin, extravasated red blood cells, and purplish deposits of hemosiderin. HHV-8 is present in the spindle cells, lytically in a small percentage and latently in the remainder.

PEL

PELs are a rare (approximately 3% of AIDS-related non-Hodgkin's lymphomas) but frequently aggressive subset of body cavity-based lymphomas with a median survival of <6 months (88, 89); they are most common in HIV-infected young or middle-aged men. PELs are lymphomatous effusions in body cavities, occasionally disseminate beyond the cavity of origin, and have cell morphologies between those of large-cell immunoblastic lymphoma and anaplastic lymphoma. PEL cells express CD45 and associated antigens and have clonal immunoglobulin rearrangements suggesting a B-cell origin, but they seldom express B-cell antigens. PEL cells harbor HHV-8 in every cell, express LANA-1, and are frequently infected with EBV.

MCD

Multicentric Castleman's disease (MCD) is a multicentric angiofollicular expansion of plasmablastic cells in germinal centers of lymph nodes (90, 91). Systemic manifestations include fever, fatigue, recurrent lymphadenopathy, hepatosplenomegaly, bone marrow plasmacytosis, and polyclonal hypergammaglobulinemia. HHV-8-associated MCD occurs predominantly in HIV-infected individuals and has a poor prognosis. HHV-8 is present in scattered B cells in the mantle zone of germinal centers, interleukin-6 is activated, and EBV coinfection is uncommon.

Disease in Transplant Recipients

Organ transplant recipients are at risk for developing KS (92), with the risk being highest in regions with a high HHV-8 seroprevalence. In most cases, the disease is from reactivated prior infection, but graft-derived infections are common. In renal transplant recipients, parenchymal KS infiltration has led directly to marrow aplasia with plasmacytosis, fever, splenomegaly, cytopenia, marrow failure, graft failure, and disseminated KS (93). PELs have been reported following cardiac transplants, as has donor-derived fatal disseminated KS in liver transplant recipients.

Other Diseases

Multiple HHV-8-associated cellular proliferations can occur simultaneously in the same individual (e.g., KS plus MCD plus PEL), even in the same lymph node. HHV-8 has been associated with several other diseases, including angioimmunoblastic lymphoproliferative disease, oral plasmablastic lymphomas, prostate cancer in some populations, and some cutaneous disease.

Therapy

An important form of KS therapy in immunocompromised patients is immune reconstitution, i.e., highly active antiretroviral therapy for patients infected with HIV and tapering or modifying of immunosuppressive regimens (for example, by changing from calcineurin inhibitors to inhibitors of mammalian target of rapamycin) for organ transplant recipients. When such approaches are not possible or are ineffective, surgical removal, cytotoxic and tissue-destructive regimens, and immune enhancement by alpha interferon can be attempted (93–95). In cell culture, HHV-8 is exquisitely sensitive to cidofovir and is also sensitive to ganciclovir and foscarnet. Although oral valganciclovir can suppress oral shedding of the virus, the therapeutic effects of these agents have been equivocal in small trials. Nonetheless, in large retrospective analyses, foscarnet or ganciclovir used for controlling other herpesvirus infections has been associated with a lower KS risk, possibly due to these agents being more effective for prevention than for treatment of KS.

PEL and MCD are rare, and there have been no controlled therapeutic trials. Anti-CD20 MAb (rituximab), valganciclovir, and high-dose zidovudine therapies have improved survival for MCD patients (90). PEL is refractory to conventional systemic cytotoxic chemotherapy, but responses have been seen to bortezomib, rituximab, and combined treatment with pleurodesis and bleomycin (74, 89).

Collection, Transport, and Storage of Specimens

Conventional methods can be used for collection and storage of serum or plasma for serology and for preparation of lymphocyte DNA for PCR. Oral fluids can be collected for PCR by the methods described above for HHV-6. Biopsy specimens are used for pathologic analyses that may include hematoxylin-and-eosin-stained cytopathic examination, PCR on total DNA extracted from a section, *in situ* PCR, or IHC. Standard methods can be used for the preparation of such specimens.

Direct Detection

KS can readily be distinguished from its mimickers, such as benign vasculoproliferative lesions and tumors that have a prominent spindle cell component, by IHC employing a MAb to HHV-8 LANA-1 (encoded by ORF73) and by heat-based antigen retrieval methods (96) (Tables 2 to 4).

Numerous systems have been described for quantitative PCR, which is used for epidemiology, monitoring of virus loads, and diagnosis of unusual cases and has possible applications in clinical trials (Table 2). Because of the low frequency of circulating HHV-8-infected lymphocytes and the low viral copy number in latently infected cells, important variables for PCR amplification from blood specimens include the number of cells in the initial lysis sample and the total quantity of resulting DNA included in the PCR mixture (44). Plasma and serum are both suitable for evaluating viremia in patients with HHV-8-associated lymphoproliferative diseases (97). A kit for simultaneous detection and quantitation of HCMV, HHV-6, HHV-7, and HHV-8 (Argene 69-100B; bioMérieux, Verniolle, France) had a sensitivity of 96% in comparison to in-house assays (41).

Isolation Procedures

Reliable systems have not been developed for primary culture of HHV-8 from KS lesions. Ascitic fluids from PEL patients have been used to derive PEL cell lines.

Identification

HHV-8 is readily identified by PCR, hybridization with nucleic acid probes that target abundant transcripts, and use of MAbs. By electron microscopy, HHV-8 virions have a morphology similar to that of other herpesviruses (Table 2).

Typing Systems

HHV-8 genotyping is based predominantly on the sequence of the highly variable K1 gene sequence (76). HHV-8 genotypes have been associated with the global migration of the virus in association with human migration but have not been associated specifically with any of the major forms of the disease or conclusively associated with pathogenic variation.

Serologic Tests

Serologic testing is the best way to identify HHV-8-infected individuals from single specimens. It has potential application in matching organ donors and recipients in areas where HHV-8 is endemic, as well as for enrolling HHV-8-seropositive individuals in studies of KS prevention.

Latent versus Lytic Antigens

EBV and HHV-8 share the following two biologic properties relevant to their serodiagnosis: (i) *in vivo*, they maintain their latent state in circulating lymphocytes; and (ii) *in vitro*, they are propagated in cell lines in which the virus is present in a latent form in >95% of cells but in which the lytic cycle can be induced by treatment with an inducer, such as a phorbol ester. In addition to LANA-1, useful serologic antigens include the latently expressed viral cyclin, encoded by ORF72, and the lytically expressed antigens encoded by ORF65 and K8.1.

Serologic Assays

IFAs can be sensitive and specific but have a low throughput. ELISAs can have a high throughput but have not been as sensitive for detecting serologic responses in immunocompetent individuals.

The two main varieties of IFA in use for detection of HHV-8 antibodies are based on (i) uninduced PEL cell lines that target LANA-1 and (ii) lytically induced PEL cell lines. LANA-1 fluorescence is restricted to the nucleus, while lytic fluorescence is predominantly cytoplasmic. Most laboratories use serum dilutions in the range of 1:40 to 1:100 for IFA. In addition to PEL cell lines that express complex mixtures of HHV-8 antigens, IFAs have been developed based on single defined antigens expressed via recombinant Semliki Forest viruses, as well as multiple defined antigens expressed via baculoviruses (98). The most widely used HHV-8 ELISAs are based on purified virions, purified bacterially expressed ORF65, and synthetic peptides based on ORF65 and K8.1 sequences (99, 100). These assays enable high-throughput and objective evaluation of individual specimens relative to a defined cutoff but are less sensitive than lytic IFA. A system has been described that employs transfection of mammalian cells with luciferase-linked antigens, with detection based on chemiluminescence from immunoprecipitates (101). A neutralizing antibody assay based on inhibiting infectivity of a recombinant HHV-8 strain that has been engineered to express green fluorescent protein in infected cells was used to demonstrate the potential importance of neutralizing antibody titers in preventing KS (102). Little attention has been paid to serologic methods for identifying and discriminating recent from past infections, such as detection of IgM or antibody avidity.

Sensitivity, Specificity, and Interassay Agreement

Establishing assay cutoffs and assessing the specificity and sensitivity of HHV-8 serologic assays have been complicated by several issues that revolve around the problem of unambiguously identifying individuals who are truly positive or negative for the virus. Healthy blood donors are often defined as HHV-8 negative, although some of them are likely to be positive for the virus, and KS patients are frequently regarded as truly positive, even though some may be seronegative. Most tests identify >95% of specimens from KS patients as being positive, and most identify >90% of European and U.S. blood donors as being negative. In-house assays have often performed well in comparison to commercial tests, and there has been little agreement about which blood donor specimens are positive (100, 103).

Antiviral Susceptibilities

Antiviral susceptibilities of HHV-8 are commonly measured as a function of the extent of DNA replication inhibition, for example, through the use of quantitative PCR (104).

Evaluation, Interpretation, and Reporting of Results

We anticipate an expanded role for clinical microbiology and pathology laboratories in the management of HHV-8-related illness. Many individuals at risk for HHV-8 infection and associated disease are under ongoing clinical scrutiny as part of managing their HIV infection, and our understanding of the utility of various virologic and immunologic markers as prognostic indicators is expanding. In addition, consideration is being given to whether donors and recipients should be matched with respect to HHV-8 status or whether the HHV-8 status of transplant recipients should be monitored to allow for heightened surveillance for KS and other HHV-8-associated diseases.

KS

While KS is often diagnosed on the basis of clinical or histologic presentation, as mentioned above, LANA-1-based IHC is important for establishing a definitive diagnosis of KS. An area of active research is identification of markers for progression to KS. The major risk identified thus far is being dually positive for HHV-8 and HIV.

PEL and MCD

PCR can be useful for initial screening of suspected cases. More definitive diagnosis requires analysis of biopsy specimens or cell smears with a method such as virus-specific IHC (74).

FUTURE DIRECTIONS

HHV-6, HHV-7, and HHV-8 cause clinically relevant infections that require accurate clinical and laboratory diagnosis. However, this need is generally confined to restricted populations, such as organ transplant recipients or individuals infected with HIV, and these infections occur at frequencies that have limited the development of convenient licensed commercial assays. Thus, we anticipate continued use of in-house assays that are available only from reference laboratories or in research settings. In such environments, standardization can be improved through the use of shared reference materials and interlaboratory comparisons. Multiplex PCR systems that include testing for these viruses are coming into wider use. This places an additional responsibility on clinical virologists to ensure that test interpretation is done in the context of what is known of the biology of these viruses.

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Adenoviruses

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In 1953, Rowe and colleagues described an "adenoid degeneration agent" that induced spontaneous deterioration of tissue cultures prepared from adenoids of children (1). In 1954, Hilleman and Werner cultivated a similar agent from military recruits with respiratory illnesses and called it RI-67 (2). The two viruses were subsequently shown to be related, and in 1956 the term "adenovirus" (AdV) was proposed to acknowledge their initial source. Epidemiologic studies soon identified the virus as a major cause of acute respiratory disease (ARD) and ocular and gastrointestinal disease. In the mid-1960s, adenoviruses were found to cause tumors in rodents, prompting studies that revealed fundamental processes of molecular biology but failed to link the virus convincingly to human cancer. More recently, adenoviruses have generated considerable interest as vectors for gene delivery and as emerging human pathogens.

TAXONOMY

Human adenoviruses belong to the *Adenoviridae* family, *Mastadenovirus* genus. Seven species (A through G) have been recognized by the International Committee on Taxonomy of Viruses (ICTV) based on immunologic, biologic, and genetic properties (3) (Table 1). Species B is further subdivided into species B1 and B2. Within each species, there are many serotypes, which are defined by neutralization and hemagglutination (HA) assays using specific antisera. To date, 51 serotypes are widely accepted, while it is still controversial whether AdV serotype 52 is a simian or human strain (4). Recently, additional new "putative types" have been described by means of sequencing and genomic data analysis. Some of these new types (AdV 53, 55, and 56) are intertypic recombinants (5). Whether the latter represent truly new viruses that should be given their own type designations or rather simply reflect evolutionary recombination without clinical relevance remains unclear. The use of phylogenetic analysis as the sole means of classifying new strains remains controversial and has been an intensive area of taxonomy discussion among adenovirologists.

Within a given serotype, molecular variants can be defined by restriction enzyme analysis. These variants are called "genome types," which is different from genotypes. Genome types result from variations in DNA but do not result in different serologic properties (6). Genome types are designed with lowercase letters after the numbered serotype (e.g., adenovirus serotype 7h or 7p). The reference prototype is denoted by the letter "p." Intraspecies recombination resulting in intermediate strains with hexon sequences of one serotype and fiber sequences of another serotype has been described (7).

DESCRIPTION OF THE AGENT

Adenoviruses are large, nonenveloped, icosahedral viruses 70 to 90 nm in diameter (Fig. 1). Each particle consists of a single, linear, double-stranded DNA molecule of about 36 kb that encodes approximately 40 genes. The DNA is covalently attached to a terminal protein at both 5′ ends and encased by core proteins. A viral protease is also present. Seven structural proteins form the capsid. The major capsid proteins are the hexon, penton base, and fiber. The capsid is formed primarily by 252 capsomeres consisting of the 240 hexons that form the 20 triangular faces and 12 pentons, one at each of the 12 vertices. Each penton consists of a base and a fiber, a rod-like projection of variable length with a terminal knob which interacts with cellular receptors. The hexon has antigenic sites common to all human adenoviruses, but these sites reside within the capsid, so neutralizing antibodies are not induced. Hexons also contain the ε determinant, which induces serotype-specific neutralizing antibodies. The fiber has mostly serotype-specific antigenic determinants and some species specificity. The knob region of the fiber includes the γ determinant, which is responsible for HA *in vitro*.

Productive infection begins by attachment of the viral fiber knobs to cell surface molecules, such as the coxsackie adenovirus receptor, although most species B viruses and certain species D viruses use CD46 or sialic acid. The specificity of this interaction is an important determinant of tissue tropism. Following secondary interaction of capsids with integrins, particles are internalized by endocytosis and are transported to the nucleus, where viral transcription and DNA replication initiate. Viral structural proteins synthesized in the cytoplasm likewise migrate to the nucleus, where the particles assemble and aggregate, forming large crystalline arrays (Fig. 2A). Productive infection results in 10,000 to over a million viral particles per cell, only 1 to 5% of which are infectious.

Latent infection is not well understood. Early studies showed latency mostly associated with species C adenoviruses in the mucosal T lymphocytes of tonsils and adenoids of asymptomatic young children. More-recent work has

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TABLE 1 Properties of the 51 human adenovirus serotypes by species characteristics[a]

AdV species	Serotype(s)	Oncogenic potential	% G+C	HA[b]		Fiber length (nm)
				Rhesus	Rat	
A	12, 18, 31	High	47–49	–	±	28–31
B1	3, 7, 16, 21, 50	Weak	50–52	+	–	9–11
B2	11, 14, 34, 35	Weak	50–52	+	–	9–11
C	1, 2, 5, 6	None	57–59	–	±	23–31
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51	None	57–60	±	+	12–13
E	4	None	58	–	±	17
F	40, 41	None	52	–	±	~29

[a] Modified from reference 105 with permission of the publisher.

[b] –, absent HA; ±, partial HA; +, complete HA.

shown a wider spectrum of viral species and has demonstrated viral genomic material in gastrointestinal-associated lymphoid cells (8). Similar reservoirs may exist elsewhere in the body. The highest quantities of viral DNA are detectable in the tissues of young children, suggesting that virus stores may decline with age (9). In contrast, little or no viral DNA is detectable in the circulating white blood cells of healthy individuals (10, 11).

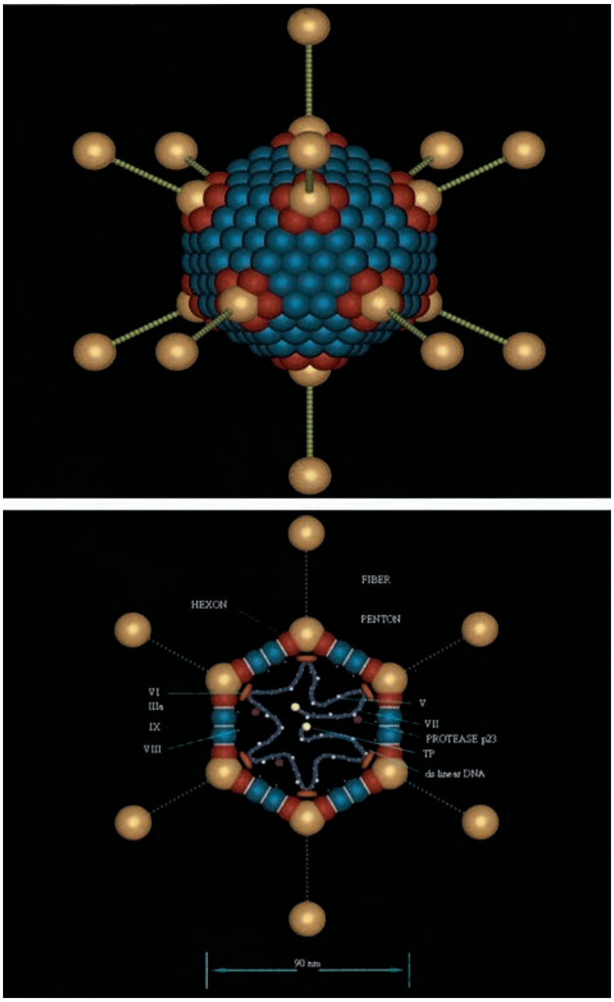

FIGURE 1 Model of an adenovirus particle. (Top) Exterior; (bottom) interior. doi:10.1128/9781555817381.ch103.f1

EPIDEMIOLOGY AND TRANSMISSION

Adenovirus infections are common and ubiquitous. About half of the known serotypes cause disease. Adenoviruses are responsible for 1 to 5% of respiratory infections overall but induce 2 to 24% of all respiratory infections and 5 to 15% of all acute diarrheal illnesses in children and 30 to 70% of all respiratory illnesses in unvaccinated new military recruits.

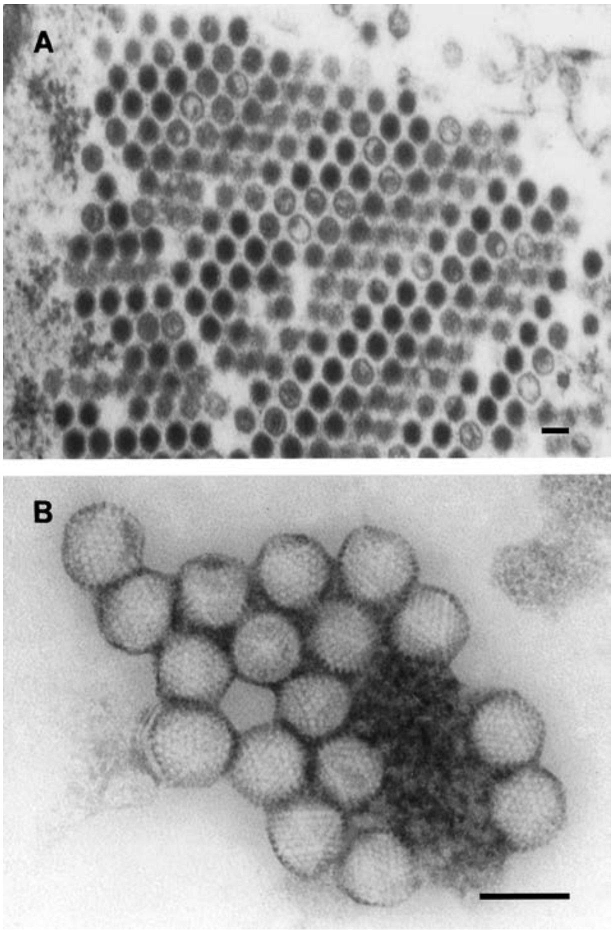

FIGURE 2 Ultrastructure of adenovirus particles. (A) Transmission EM of a hepatocyte nucleus containing complete (dark) and empty (clear) adenovirus particles; (B) direct EM of a cluster of adenovirus particles in stool from a child with diarrhea. Bar = 100 nm. doi:10.1128/9781555817381.ch103.f2

Most infections occur in the first few years of life, and about half are asymptomatic. By the age of 10, most children have been infected with one or more serotypes. Coinfection with different serotypes and different species is documented, especially when different body sites are sampled (12). The incidence of infection is highest in crowded closed settings, such as day care centers, boarding schools, geriatric facilities, military training camps, and hospitals. Intrafamilial infections are common. There is no obvious difference in susceptibility by gender or ethnic group. Infections occur worldwide, with some differences in serotypes associated with various syndromes in other parts of the world.

Adenovirus infections can be epidemic, endemic, or sporadic, with the pattern of circulation, specific syndrome, and severity varying by serotype, population, and route of exposure. Sporadic infections occur year-round. Respiratory infections are most often associated with species B, C, and E, with serotypes 1, 2, 5, and 6 causing endemic infections and serotypes 4, 7, 14, and 21 causing small epidemics mostly in winter to early summer. A survey of U.S. isolates conducted in 2004 to 2006 identified the most common serotypes in civilians as 3, 2, 1, and 5, with an increasing amount of serotype 21 (13). Epidemics of severe respiratory disease in U.S. military recruits typically occur in the winter and spring. Historically, serotypes 4 and 7 were the most common cause. Serotype 4 now predominates, with lesser amounts of species B serotypes 7 and 21 detected. The emerging serotype 14 was also identified (13, 14). Serotype 14 has also caused clusters of severe disease and fatalities among civilians in several states. A novel genome type (serotype 14a) is the apparent cause (15). Large epidemics of keratoconjunctivitis are caused mostly by serotypes 8, 9, and 37. Smaller outbreaks of serotypes 3, 4, and 7 occur in the summertime and are associated with contaminated swimming pool water. Adenovirus-associated gastroenteritis is caused primarily by serotypes 40 and 41, which are also known as the “enteric adenoviruses.” These infections are endemic globally and occur year-round.

Adenovirus infections in persons with primary or acquired immunodeficiencies are increasingly reported (16). Severe infections are reported for individuals with congenital deficiencies, but the most frequent and severe infections are associated with hematopoietic cell transplantation (HCT) and solid-organ transplantation (SOT). HCT recipients have the highest incidence of infection (up to 30%) and highest morality rates (exceeding 80% in some studies) (17). Risk factors for serious disease in this population include young age, allogeneic transplantation, T-cell depletion, graft mismatch, cord blood transplantation, and severe graft-versus-host disease (18). Infections with species A to F (mostly species C and A) are described, and mixed or sequential infections with different serotypes are common (19). Data for SOT recipients are more limited (20), but this group generally experiences fewer and milder infections than HCT recipients. The lowest incidence is for renal transplant patients, who tend to acquire species B adenoviruses of serotypes 7, 11, 34, and 35. Adenovirus infections can be problematic in lung, small bowel, or liver transplant recipients and lead to graft dysfunction (or loss) and fatalities. In the preantiviral era, mortality rates as high as 53% were reported for young liver transplant patients with adenovirus disease. Most infections are with species C viruses, and serotypes 1, 2, and 5 are the most common. Transplant patients can acquire adenovirus as a result of endogenous reactivation of the virus from recipient or donor tissue, as well as from the community or hospital (21). Transplantation of adenovirus-positive donor tissue into a negative recipient confers a higher risk than if both parties are either negative or positive. Adenoviruses once caused many serious infections in human immunodeficiency virus/AIDS patients, mostly with species D serotypes rarely identified or problematic in other settings (22). The introduction of highly active antiretroviral therapy has dramatically reduced the incidence of such infections in this population.

Transmission is primarily by the respiratory or fecal-oral route. Airborne transmission occurs by small droplets and, to a lesser extent, large-droplet aerosols. Fecal-oral spread probably accounts for the majority of enteric adenovirus infections. Adenoviruses are also spread by contaminated fomites, fingers, and liquids, such as ophthalmic solutions and sewage. Preceding eye trauma facilitates infection and the spread of keratoconjunctivitis in environments with high levels of airborne particulates. The infection’s spread among new military recruits may involve direct contact as well as aerosol exposure via ventilation systems. Stress and fatigue may be contributory host factors in this setting. The mean incubation period for most respiratory tract infections is 5.6 days, and that for gastroenteritis is 3 to 10 days (23).

Transmission is facilitated by adenoviral resistance to chemical decontaminants and physical treatments. Stability of particles in gastric secretions, bile, and pancreatic proteases also permits passage through the stomach, followed by replication in the intestine. The prolonged period of virus shedding from various body sites aids transmission as well. Length of shedding varies by serotype, body site, patient age, and immune status. In general, nonenteric adenoviruses are shed for several days by adults with upper respiratory tract infections, for a few weeks after ocular infections, and for 3 to 6 weeks from the throat or stool of children with respiratory or generalized illness. Infected children may excrete the virus initially from the respiratory tract and later from the gastrointestinal tract. Excretion in stool can be intermittent and prolonged for 18 months or longer after recovery (24) (although the possibility of serial reinfection cannot be ruled out in all cases). In contrast, enteric adenoviruses are shed for only a few days after recovery. Immunocompromised individuals shed adenovirus longer than immunocompetent individuals.

CLINICAL SIGNIFICANCE

The spectrum of adenovirus-associated disease is broad due to the many serotypes and their tissue tropisms (Table 2). Clinical manifestations also depend on the age and immune status of the infected person. Most infections affect the respiratory tract, eye, and gastrointestinal tract, with lesser involvement of the urinary tract, heart, central nervous system, liver, pancreas, and genital tract. Solid-organ involvement and disseminated disease are seen primarily in immunocompromised patients.

Most respiratory tract infections occur early in life and are self-limited and mild. Usual signs and symptoms are fever, nasal congestion, coryza, pharyngitis, cervical adenopathy, and cough, with or without otitis media. An exudative tonsillitis clinically indistinguishable from infection with group A streptococcus has been described. A pertussis-like syndrome has been reported, although adenoviruses are more likely to be copathogens or to reactivate in this syndrome than to be a significant cause. The role of adenoviruses in asthma remains controversial (25). Adenoviral lower respiratory tract infections, such as bronchitis, bronchiolitis, croup, and pneumonia, can be severe and sometimes fatal, particularly in young children less than 2 years of age. Long-term sequelae of these lower respiratory tract

TABLE 2 Adenovirus diseases, associated serotypes, hosts, and suitable specimens[a]

Disease	Associated serotype(s)		Frequent hosts	Specimen(s)
	Frequent	Infrequent		
URI	1–3, 5, 7	4, 6, 11, 14, 15, 18, 21, 29, 31	Infants, children	NP aspirate or swab, throat swab
LRI	3, 4, 7, 21	1, 2, 5, 7, 8, 11, 35, 14	Infants, children, IP	NP aspirate or swab, BAL fluid, lung tissue
Pertussis syndrome	5	1, 2, 3, 12, 19	Children	Throat swab
ARD	4, 7	2, 3, 5, 8, 11, 14, 21, 35	Military recruits	Throat swab, BAL fluid, lung tissue, NP aspirate or swab
Acute conjunctivitis	1–4, 7	6, 9, 10, 11, 15–17, 19, 20, 22, 37	Children	Conjunctival swab or scraping
Acute hemorrhagic conjunctivitis	11	2–8, 14, 15, 19, 37	Children	Conjunctival swab or scraping
Pharyngoconjunctival fever	3, 4, 7	1, 2, 5, 6, 8, 11–17, 19–21, 29, 37	Children	NP aspirate or swab, throat swab, conjunctival swab or scraping
Epidemic keratoconjunctivitis	8, 9, 37	2, 3, 4, 5, 7, 10, 11, 13–17, 19, 21, 23, 29	Individuals of any age	Conjunctival swab or scraping
Gastroenteritis	40, 41	1, 2, 3, 5, 7, 12–18, 21, 25, 26, 29, 31, 52	Children	Stool
Hemorrhagic cystitis	11	7, 21, 34, 35	Children, IP	Urine
Hepatitis	1–3, 5, 7	4, 31	Infants, children, IP	Liver tissue, blood
Myocarditis	7, 21		Children	Heart tissue, blood
Meningoencephalitis	7	1–3, 5, 6, 11, 12, 26, 32	Children, IP	Brain tissue, CSF
Sexually transmitted disease	2, 37	1, 4, 5, 7, 9, 11, 18, 19, 31, 49	Teens, adults	Lesion swab
Disseminated disease	1, 2, 5, 11, 34, 35	3, 6, 7, 14, 21, 29, 31, 32, 37–39, 43, 45	IP, newborns	Blood, BAL fluid, urine, involved tissue

[a] Modified from reference 105 with permission of the publisher. ARD, acute respiratory disease; BAL, bronchoalveolar; IP, immunocompromised persons; NP, nasopharyngeal; LRI, lower respiratory tract illness; URI, upper respiratory tract illness.

infections are frequent in some populations (26). Higher mortality is reported for serotypes 5 and 21 and other genome types, such as 4a and 7h, and when extrapulmonary manifestations occur (13, 27).

Frequent outbreaks of adenovirus-associated ARD in young military recruits were noted beginning in 1953. Symptoms included febrile cold-like illness, pharyngitis with tonsillitis, bronchitis, and pneumonia. Hospitalization rates were as high as 50%, and some fatalities occurred. Live, oral adenovirus vaccines directed against serotypes 4 and 7, the most common serotypes involved at the time, were introduced for this population in 1980 and significantly reduced disease. In 1996, vaccine production lapsed, and the problem recurred. Although the current outbreaks in military facilities are not as severe as in the prevaccine era, they continue be problematic and involve a broader array of serotypes (13). In particular, serotype 14 has been associated with numerous outbreaks in recent years among both military recruits and the general population. Interestingly, serotype 4 has been an uncommon cause of respiratory disease in civilians. Reintroduction of vaccine (again to serotypes 4 and 7) to the military occurred in 2011.

Ocular adenovirus infections are common. The most frequent manifestation is acute follicular conjunctivitis, which is usually superficial and resolves without consequence in a few weeks. Pharyngoconjunctival fever is a follicular conjunctivitis accompanied by upper respiratory tract symptoms, fever, and occasionally lymphadenopathy, pharyngitis, and malaise. Epidemic keratoconjunctivitis is a more serious infection that starts with conjunctivitis but progresses to painful edema of the eyelids, sometimes followed by corneal erosions and infiltrates. Symptoms may resolve in 2 weeks, although reduced vision, photophobia, and foreign-body sensation may persist for months to years. Epidemics of acute hemorrhagic conjunctivitis similar to those caused by enterovirus are also described.

The enteric adenoviruses 40 and 41 are a common cause of viral gastroenteritis in children less than 2 years old (Table 2). Diarrhea is usually watery and nonbloody, lacks fecal leukocytosis, and lasts a mean of 10 days, which is somewhat longer than diarrhea due to rotavirus (28). Mild fever, vomiting, and abdominal pain can occur, and respiratory symptoms are sometimes present. Most immunocompetent patients recover uneventfully, although infants with ileostomies or colostomies can have prolonged symptoms, and rare fatalities have been reported for immunocompetent patients. Gastrointestinal syndromes associated with nonenteric adenovirus infections include intussusception, acute mesenteric lymphadenitis, and appendicitis.

Clinical manifestations of adenovirus in immunocompromised patients depend on the individual's underlying disease or transplanted organ, patient age, and serotype involved (16). Infections in transplant patients can involve the graft or other organ systems. Presentations in HCT patients most often include hemorrhagic cystitis, pneumonia, hepatitis, enteritis, and disseminated disease. Fatalities are most frequent in patients with pneumonia or disseminated disease. In liver transplant patients, infections are most often associated with jaundice, hepatomegaly, and hepatitis, with pneumonia and diarrhea in some patients. Enterocolitis, with occasional spread to the liver, occurs in intestinal transplant recipients. The major clinical presentation in renal transplant patients is acute hemorrhagic cystitis and, to a lesser extent, pneumonia. However, recent work has reported systemic involvement with graft dysfunction (29). Infections of the graft in lung transplant recipients can result in necrotizing pneumonitis, leading to respiratory failure and progressive graft loss. Bronchiolitis obliterans can be a late consequence. In the post-highly active antiretroviral therapy era, clinical manifestations in patients with human immunodeficiency virus/AIDS are uncommon until the immune system deteriorates. AIDS patients have presented with adenovirus-associated pneumonia, meningoencephalitis, and hepatitis as well as generalized disease. Symptoms of hemorrhagic cystitis in immunocompromised patients can be especially severe and may signal the start of disseminated disease (30).

Less common clinical manifestations include exanthems, neonatal disease, which can be severe and frequently fatal, neurologic manifestations, such as meningoencephalitis and encephalitis, acute myocarditis in otherwise healthy persons or associated with graft rejection in cardiac transplant patients, macrophage activation syndrome, and genitourinary disease, including genital lesions and urethritis. The role of adenoviruses in fetal demise remains unclear. Adenovirus type 36 has recently been linked to obesity (31), although this association remains controversial. Some adenovirus infections resemble Kawasaki disease, but adenovirus has also been described concomitantly with Kawasaki disease, and so one should be cautious when making these diagnoses (32).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Adenoviruses are best detected from affected sites early in the course of illness. Suitable specimens vary with the clinical syndrome and test requirements (Table 2). Collection of cell-rich specimens usually results in the highest sensitivity. Flocked nylon swabs (Copan Diagnostics, Murrieta, CA) have shown excellent yield compared to those from cotton swabs or nasopharyngeal aspirates for the detection of respiratory viruses, including adenovirus (33–35). When a deep-seated infection is suspected and tissue from the affected site is unavailable, collection from multiple sites (e.g., respiratory, stool, and urine) or blood is recommended.

Recovery is best if specimens are kept cold (2 to 8°C), transported, and processed as described in earlier chapters. Suitable viral transport media can be laboratory prepared or purchased from commercial sources. Some commercial formulations, such as MicroTest multimicrobe medium M4RT (Remel, Lenexa, KS) or universal transport medium UTM (Copan), preserve infectivity for prolonged periods at room temperature and are also suitable for detection of viral antigen or DNA (36). If plasma or serum samples are being tested, they should be separated within several hours of collection. Specimens, virus isolates, or DNA extracts can be frozen at −70°C indefinitely, with minimal loss of activity. Long-term storage in self-defrosting freezers and repeated freeze-thaw cycles ultimately reduce infectivity and degrade adenovirus DNA. Such conditions are not recommended for storage of any clinical specimens.

Adenoviruses are highly resistant to inactivation by chemical and physical treatment. Most serotypes are stable for a week at 36°C, for several weeks at room temperature, and for several months at 4°C. Infectivity is retained for several weeks on paper or in saline and for over a month on nonporous surfaces. Strict adherence to conventional safety practices, such as the use of personal protective equipment and biologic safety cabinets, disinfection of work surfaces, and hand washing, minimizes laboratory infections. Avoid hand-to-eye or hand-to-mouth contact due to the affinity of adenoviruses for mucosal and ocular tissue. Treatments that eliminate infectivity include a 1:10 dilution of household bleach (0.5% sodium hypochlorite) for 30 min, heating surfaces to 56°C for 30 min or 60°C for 2 min, and autoclaving. Serotype 4 is especially heat resistant. Alcohol-based hand gels can destroy infectivity, although several minutes of contact may be required for some products. Variable disinfection is achieved with povidone-iodine, formaldehyde, and UV light. Viral DNA can be detected long after loss of infectivity.

DIRECT DETECTION

Microscopy

Adenovirus-infected cells can be visualized by light microscopy as "smudge cells" in hematoxylin-and-eosin- or Wright-Giemsa-stained tissues, fluid sediments, or cultures. Smudge cells are large late-stage-infected cells containing solitary, central, basophilic nuclear inclusions composed of adenoviral particles (Fig. 2A). Other types of inclusions have been described as well (37). Adenoviral inclusions can be mistaken for those of cytomegalovirus, herpes simplex virus, or polyomavirus. Further identification by immunohistochemistry or *in situ* hybridization is recommended to avoid misdiagnosis (38, 39). The characteristic morphology of adenovirus particles permits their detection by electron microscopy (EM) without need for further identification. Particles, often in large crystalline arrays, can be visualized in the infected cells by transmission EM (Fig. 2A). The large quantity of virus (10^6 to 10^9 particles/ml) in the stools of children with acute diarrhea (Fig. 2B) also permits detection by direct EM (40). The sensitivity of direct EM can be increased by ultracentrifugation, by immunoelectron microscopy using antihexon antibodies, or by a special ultracentrifuge rotor (Airfuge; Beckman Coulter, Inc., Fullerton, CA) that permits concentration of virus onto grids prior to examination (41). Few clinical laboratories have access to EM facilities, so other methods are typically used.

Antigen Detection

Antigen detection can be used for the rapid detection of adenoviruses in respiratory, ocular, and gastrointestinal tract specimens. Sensitivity is often poor, particularly when adults or immunocompromised patients are tested, and particularly compared to the sensitivities of molecular methods (42). However, some series focused on detection of adenoviral conjunctivitis have shown improved sensitivity with more recently available assays (43). Most antigen assays target conserved regions of the adenovirus hexon protein and utilize monoclonal antibodies (MAbs). Immunofluorescence (IF), enzyme immunoassay (EIA), and lateral-flow immunochromatography (IC) are the most common formats.

IF can be used for definitive identification of culture isolates and for primary detection of respiratory tract infections. Suitable specimens for direct detection are washes, aspirates, or swabs containing exfoliated ciliated columnar epithelial cells from the posterior nasopharynx or midturbinate. Touch preparations of fresh tissue or frozen or formalin-fixed tissue sections can be tested as well. However, these may not be approved applications for a given commercial reagent and as such would require further validation prior to routine clinical use. General techniques for IF are described in chapter 80. Positive cells usually display con-

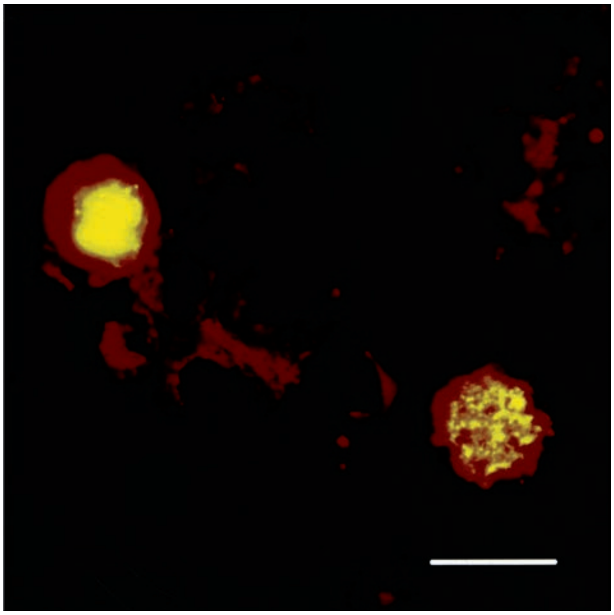

FIGURE 3 Adenovirus antigen demonstrated in two cells of a nasopharyngeal aspirate by direct IF using a rhodamine-conjugated antibody (yellow). Bar, 40 μm.
doi:10.1128/9781555817381.ch103.f3

densed nuclear or granular cytoplasmic staining (Fig. 3). Although indirect IF is highly sensitive and specific, direct IF can provide satisfactory and rapid results (44). Labeled MAbs directed solely against adenovirus or pools of MAbs labeled with different fluorochromes directed against adenoviruses and other respiratory viruses can be used. Pools may be most economical for laboratories with a low prevalence of adenovirus. The reported sensitivity of IF for adenovirus detection in respiratory specimens is 40 to 60% of that of culture, which is lower than the detection rate of most other respiratory viruses. A sensitivity higher (60 to 80%) than that of culture is achievable using cytocentrifugation (44) and specimens from pediatric patients. The specificity of IF for adenovirus is excellent (>99%) in most studies.

Antigen detection can also be performed by EIA, IC, or latex agglutination (LA). These technologies are fully described in other chapters. Two types of EIA are available, a serotype 40- and 41-specific EIA and a generic EIA to detect all serotypes (45, 46). Both assays utilize microtiter plates and adenovirus-specific MAbs as capture and detector reagents. This format is most economical for large laboratories which usually test in batch mode. The sensitivity of the serotype 40/41 assay compared to that of 293 cell culture or EM is >90% in most studies, although sensitivity can be lower with some variants (47). Specificity is >97%. The generic EIA detects species F and other species in a single reaction. Some have shown high sensitivity (>95%) when stool is tested, but the sensitivity is only 65 to 75% when other specimen types are tested. Urine may give the lowest value, and occasional false-positive reactions occur (45, 48), although others have had better results. Other formats used less frequently include time-resolved fluoroimmunoassay and radioimmunoassay.

The IC format is attractive for smaller laboratories because tests can be run individually, and results are usually available in less than 30 min. The SAS rapid adenovirus test (SA Scientific, San Antonio, TX) is a commercial assay for testing of eye swabs, nasopharyngeal secretions, and fecal material. Initial reports suggested good sensitivity (84 to 95%) compared to those of culture and PCR on nasopharyngeal specimens, but sensitivity was only 55% when a broader array of specimen types was evaluated (42, 49). Performance is best with specimens from young children. The RPS adenovirus detector (Rapid Pathogen Screening Inc., South Williamsport, PA) is available for testing of eye swabs. The sensitivity and specificity were 89% and 94%, respectively, compared to those for PCR in a recent study (50). The assay is insensitive if swabs are placed in viral transport medium prior to testing (51). A generic LA assay for stool specimens is available but infrequently used. Its sensitivity is only moderate, and false positives have been reported with control latex particles coated with non-adenovirus-reactive antibodies (52).

Nucleic Acid Detection

Nucleic acid detection has become commonplace for sensitive and rapid viral detection and quantification. Although this methodology was initially used primarily for specimens (for example, blood) that were not amenable to antigen detection or culture, more recently, molecular amplification has become a primary detection modality for respiratory tract specimens. In fact, adenovirus has been detected by PCR or similar methods in virtually all specimen types, so the appropriate specimen depends largely on the associated disease (Table 2). Amplification is necessary for detection in most sample types, although viral DNA can be directly detected in some stools by gel electrophoresis and viral DNA can be demonstrated without PCR in infected tissues by *in situ* hybridization.

Both endpoint PCR and real-time amplification methods are in current use. Degenerate or nondegenerate primers and probes for the hexon or fiber gene or the viral-associated (VA) RNA I and II regions are usually selected due to the extensive homology of these regions among serotypes. Assays that utilize multiple primer and probe sets are preferred by many laboratories for uniform detection or quantification of all serotypes (53–58). Over the years, a variety of laboratory-developed tests (LDTs) have been described in the literature, their development increasingly facilitated by the availability of well-defined control material and reference strains of a number of serotypes of adenovirus. Commercial reagents were initially limited to those labeled for research use only (RUO) and analyte-specific reagents (ASRs). More recently, tests that are cleared or approved for *in vitro* diagnostics (Table 3) have been marketed (59–61). While these have largely been approved only for detection of respiratory tract viruses, their availability represents a marked advance in our ability to rapidly and accurately detect adenoviral infections.

Detection formats described for endpoint PCR methods have included gel electrophoresis, Southern blotting or liquid hybridization and capture onto microtiter plates, and fluidic or solid-phase microarrays (62). Conventional assays that detect all adenoviruses as well as multiplex PCRs for adenoviruses, herpesviruses, and other respiratory viruses have been reported (12, 63, 64). Increasingly, widely multiplexed methods that detect a full range of respiratory viruses have been developed and marketed (59–61). The sensitivities of such systems for adenovirus and the spectra of viral serotypes detected are widely varied among platforms and test versions. Early versions of some systems have shown marked weakness in the detection of adenovirus, compared to other pathogens. While the trend has been for manufacturers to ameliorate such issues in subsequent test versions,

TABLE 3 Commercial products available in the United States for *in vitro* diagnostic use in the detection of adenovirus infections

Manufacturer	Test name	Format
Luminex Molecular Diagnostics, Inc.	xTAG respiratory viral panel (RVP)	Broad respiratory panel
BioFire Diagnostics, Inc.	FilmArray respiratory panel (RP)	Broad respiratory panel
GenMark Diagnostics	eSensor respiratory viral panel (RVP)	Broad respiratory panel
Hologic Gen-Probe, Inc.	Prodesse ProAdeno+ assay	Adenovirus-only testing
bioMérieux, Inc.	Adenovirus r-gene (US)	Adenovirus-only testing

this weakness points to the need for critically assessing the sensitivity of such tests prior to implementation. Real-time PCR is most often used when targeting adenovirus alone, rather than in a panel approach, as it is more rapid and less prone to contamination than endpoint PCR and can provide quantitative results. Many real-time LDTs have been described (41, 53–55, 57, 58). In almost all reports, irrespective of the specific method used, the sensitivity of PCR has approached or exceeded that of antigen detection or culture (65). The major application of quantitative real-time PCR is the detection and quantification of adenoviremia to predict current or incipient localized or disseminated adenovirus disease in immunocompromised patients (66, 67). Quantitative tests have increasingly been applied to other sample types, such as stool, in an effort to improve the clinical predictive value of positive results (68). Detection and quantification of viremia can be performed using plasma, peripheral blood mononuclear cells (PBMCs), or whole blood. No significant differences in qualitative sensitivity have been observed among these specimen types, although viral load values were slightly higher for whole blood and plasma than for PBMCs in one recent report (69).

Detection of all adenoviral serotypes and genome types represents a tremendous challenge in test design. It is therefore incumbent on both the developer and the user of such tests to verify the sensitivity of a given assay for the whole range of adenoviral species and serotypes of clinical importance in the population being tested. Clinical specificity should also be determined whenever possible because adenovirus DNA can be found in some specimens from healthy control subjects. On average, fewer than 2% of specimens from healthy individuals contain detectable adenovirus DNA, although values can vary significantly depending on the patient population and specimen type (10, 11, 57, 70). Somewhat higher detection rates have been reported for chronic conditions (such as asthma), for tonsil and adenoid tissue, and for gastrointestinal biopsy specimens with negative pathology (9, 25, 71). Each laboratory should therefore define the clinical significance of positive PCR results in its patient population and sample types. Where quantitative tests are used, quantitative colinearity should be assessed across detectable viral types to ensure accuracy when a non-type-specific calibration curve is used.

Many transplant centers now assess viremia with such quantitative tests on a weekly basis for several months after transplantation or when symptoms appear. Routine screening has become especially commonplace for pediatric HCT patients. Screening of asymptomatic patients can be used to trigger preemptive therapy (18). In patients with diagnosed adenoviral infection, changing viral load values can be used to assess the clinical response to antivirals and predict outcomes (72). Due to differences in assay characteristics and patient populations and the lack of universal calibration standards, there is also no single threshold value of viremia that can be recommended to initiate antiviral treatment or preemptive therapy in these patients. Some experts follow *in vivo* dynamic changes by repeat testing in 2 or 3 days after a single low viral load to identify patients with a rapid increase before initiating treatment.

ISOLATION PROCEDURES

Although historically considered the reference standard, viral isolation is now used with decreasing frequency. Viral culture is slower than antigen or DNA assays and, with some specimen types, can be less sensitive than PCR, yet it remains useful to detect serotypes that might be missed by direct methods and yields infectious progeny for further identification and serotyping. Most specimens are suitable for culture (Table 2). Exceptions are blood and cerebrospinal fluid (CSF), which may contain insufficient virus to be cultivated or may contain virus neutralized by antibodies. For these specimens, DNA amplification is typically preferred.

Adenoviruses grow best in human epithelial cells. Primary human embryo or neonatal kidney cells are the most sensitive but are rarely available, so human epithelial cell lines, such as A549, Hep2, or sometimes HeLa or KB, are used. Human fibroblasts and nonhuman cells, such as Vero and primary monkey kidney cells that contain endogenous simian virus 40, may support adenovirus replication, but the yield is low. Enteric adenoviruses are often termed "fastidious" because they are noncultivatable or grow slowly in routine cell lines. Cultivation of enteric adenoviruses is most successful in Graham 293 cells (28).

Adenoviruses can be isolated by conventional tube culture or shell vial centrifugation culture (SVCC). These methods are detailed in earlier chapters. Although SVCC is popular because it produces rapid results, tube culture remains useful (among culture techniques) to detect low concentrations of virus or slow-growing serotypes, particularly of species A or D, which may not be evident for 3 or 4 weeks. Holding tubes for 2 weeks is customary for routine diagnostic work. If tubes are held for longer periods, cells should be subpassaged at least once to maintain the health of the monolayer. Typical cytopathic effects (CPE) consist of grape-like aggregates of swollen, refractile cells (Fig. 4B). Aggregates may not develop with species D, and a web-like flattening is described for serotypes 3 and 7 (73). CPE is usually evident in 2 to 7 days in A549 cells but may be less characteristic and slower to appear in other cell types. As infection progresses, cells can become highly granular and detach completely from the surface. Most infectious virus remains cell associated so that high-titer preparations can be produced by several freeze-thaw cycles of harvested cells. CPE that develops within hours after inoculation with concentrated preparations is known as "lysis from without" and is due to a free penton base. It can be prevented by using a lower concentration of the inoculum.

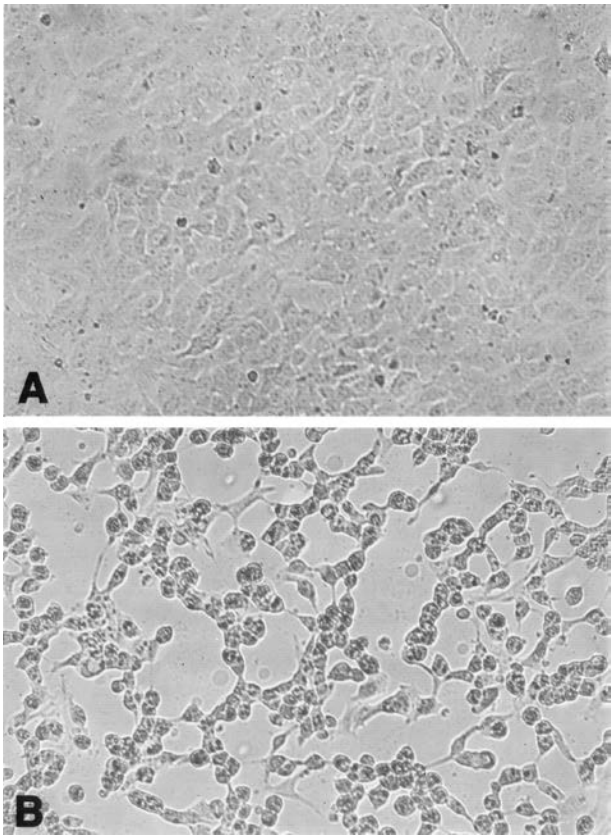

FIGURE 4 Adenovirus CPE in A549 cells. (A) Uninfected cells; (B) advanced adenovirus CPE. Magnification, ×100. doi:10.1128/9781555817381.ch103.f4

A significant proportion of culture-positive specimens can be detected in A549 or Hep2 SVCC after 1 to 5 days of incubation and should be confirmed using fluorophore-conjugated MAbs directed against the adenovirus hexon protein. Often the MAb preparations used to identify viral antigen in direct specimens can be used in SVCC. Maximum detection of fluorescent foci requires at least 30 min of centrifugation at 700 × *g* (20). The sensitivity of SVCC compared to that of tube culture under these conditions is approximately 50% at 24 h, increases somewhat at 2 days, and can approach 100% by 5 days (45, 74–77). Similar results can be achieved using 24-well microtiter plates. Lower detection rates are reported if human fibroblast or monkey cells are used (78). Dexamethasone, which enhances the identification of some viruses in SVCC, does not improve adenovirus detection in SVCC (79). Recovery of adenoviruses from respiratory sources in R-Mix (Diagnostic Hybrids Inc., Athens, OH), a mixture of A549 and mink lung cells, is lower than that for parainfluenza or influenza viruses (80). Enteric adenoviruses can be detected in SVCC if appropriate cells lines are used (81), but other approaches are more sensitive and convenient.

IDENTIFICATION

Culture isolates should be definitively identified before a report is generated. For diagnostic work, genus-specific identification is usually sufficient. Most immunologic or molecular methods for direct detection of adenoviruses are suitable for this purpose. Identification by IF is most often used for culture isolates because the technique is simple and rapid. For IF, cells that appear to contain adenovirus should be harvested when at least 20% of the monolayer shows CPE. Other methods that can be used for identification are LA, IC, or PCR. On occasion, CPE-positive variants or new serotypes that cannot be identified by these methods emerge. Under these circumstances, EM of infected cells, HA of the isolate, or DNA sequence-based methods may aid identification.

TYPING SYSTEMS

Typing of adenoviruses into species or serotypes is used primarily for epidemiology or studies of pathogenesis or to reveal the cause of an unusual or especially severe infection. Traditional and molecular typing methods are available. Traditional typing requires a viral isolate and detects serologically recognized differences in fiber and hexon epitopes. Molecular typing can be performed with isolates or directly on PCR-positive specimens. The most valuable regions for sequencing are in the fiber or hexon genes, but whole-genome sequencing has been used as well. Typing by serology or molecular methods is usually in agreement, although discrepant results can be obtained on occasion. The two approaches are different in that serology measures a phenotypic property, whereas genotyping queries DNA.

Traditional typing is performed by provisionally determining the species of an isolate by HA (Table 1) or, if the isolate is from stools, by a species F (serotype 40/41) EIA. Serotyping by HA inhibition (HAI) or serum neutralization (SN) is then performed, utilizing specific antisera that define the serotypes of that species. HAI is the easier assay, but SN is the primary arbiter of serotype. Modified SN procedures are preferred for speed and simplicity over the conventional 7-day test with human epithelial cells (or 293 cells for stool isolates). Intersecting pools of antibody mixtures reduce the number of individual reactions. Modified SN tests include a 3-day test with monkey kidney cells and a 5-day microneutralization test with Vero cells (105). Interpretation of traditional typing is not always straightforward, especially with intermediate strains, but it remains the gold standard to validate new systems and classify novel viruses.

Molecular typing has increasingly replaced serology as the method of choice for routine strain typing and epidemiologic studies. Direct methods use extracted viral DNA from an isolate or specimen. Restriction endonuclease analysis (REA) is the classic direct typing method (82), but some genome types in current circulation do not retain the cleavage patterns of the prototypes, so interpretation can be challenging. REA is still used, however, for presumptive identification of new serotypes, for identification of new genome types associated with severe disease, or to confirm results obtained by other means. Single-stranded confirmation polymorphism and heteroduplex mobility assays are other direct approaches. Sequence-based typing is rapid and highly accurate. Some assays can be performed directly with clinical material, but isolates are often preferred because of their larger amount of viral DNA. Amplification is then performed by generic or multiplex PCR, or sometimes by nested PCR, to obtain sufficient DNA for analysis (83). Methods of analysis can include measurement of product length, REA, DNA sequencing, REA followed by DNA sequencing, or mass spectrometry (84–87). In addition, probe-based methods include reverse line blotting, in-well hybridization, type-specific real-time PCR, and solid-phase microarrays (88–90). Common regions for sequence interrogation are hypervariable regions 1 to 6 or 7 of the hexon gene or the fiber gene, although other regions, such as E3, can be analyzed. The presence of intermediate strains or coinfections can often be detected by sequence analysis (91). Sequence analysis of polymorphisms in long nucleotide repeats (microsatellites) is proposed to track strains (92). Some molecular typing systems identify only the species or serotypes recovered from a single body site, whereas others are more comprehensive (62, 83, 93).

SEROLOGIC TESTS

Most primary adenovirus infections (in immunocompetent patients) are accompanied by a diagnostic rise in virus-specific immunoglobulin G (IgG), a less-predictable IgA response, and an IgM response in 20 to 50% of cases. Neutralizing antibodies can persist a decade or more in a relatively undiminished titer, probably maintained by periodic reinfection, reactivation, and heterotypic anamnestic antibody responses. The IgG response can be delayed in many children for months after infection and may not appear in immunocompromised individuals. An IgM response occasionally occurs when virus reactivates.

In recent years, serodiagnosis of adenovirus infections has largely been superseded by other methods of virus detection. Its current use is for epidemiologic investigations, to confirm associations between virus detection and unusual clinical outcome, and to study the immune response. The value of serology for patient care is limited due to the retrospective nature of demonstrating seroconversion, false-negative results due to the delay of the IgG response in children, the insensitivity of IgM assays, persistent infections, and the lack of antibody responses in immunocompromised individuals. False positives also occur due to heterotypic responses or late antibody rises in children unrelated to their current problem.

Serology is typically performed by documenting at least a 4-fold rise in virus-specific IgG (seroconversion) or by detecting an IgM response in the right setting. For clinical work, it is usually sufficient to know that adenovirus caused the infection, so a genus-specific EIA to detect IgG or IgM antibodies against the hexon protein is adequate. Formats with a viral antigen preparation or a "capture" immunoglobulin on the solid phase are commercially available and sensitive. Serotype-specific antibody tests are infrequently used for diagnosis but can pinpoint serotypes responsible for a cluster of infections when other specimens are unavailable. HAI and SN are the serotype-specific assays of choice. SN is the standard, with the 3-day monkey kidney test or microneutralization preferred. Complement fixation, indirect IF, LA, and radioimmunoassay are rarely used. Assays utilizing genetically modified cells and fluorescent reporter molecules as surrogates for SN activity are increasingly used for serosurveys (94).

Treatment

Antivirals have been used to treat some adenovirus-associated clinical entities. Several antivirals, including ganciclovir, ribavirin, and cidofovir, as well as the antiretroviral drugs zalcitabine, alovudine, and stavudine, have *in vitro* activities against adenovirus. Ribavirin was initially reported to have *in vitro* activity only against species C serotypes but is now said to be active against most isolates of species A, B, and D and all isolates of species C (95). *In vitro* studies suggest that cidofovir may be effective against severe lower respiratory tract infection caused by the emerging adenovirus serotype 14a, whereas ribavirin may not (96).

The most common drugs utilized in patient care have been ganciclovir, ribavirin, or cidofovir. Ganciclovir has only a moderate effect *in vivo* and is no longer recommended for clinical use. Ribavirin has been used with somewhat greater success, but many failures and fatalities are reported. Ribavirin may be somewhat effective against hemorrhagic cystitis, probably due to the high drug concentrations achievable in urine.

The mainstay of antiviral treatment to date has been cidofovir, an acyclic nucleoside phosphonate analogue and broad-spectrum antiviral agent. All adenovirus serotypes are susceptible to cidofovir *in vitro*. Although resistance can develop with serial passage, little resistance has so far been detected in isolates from cidofovir-treated patients (95). Despite its significant side effects of nephrotoxicity, myelosuppression, and uveitis, cidofovir is increasingly used to treat a variety of clinical presentations, mostly in immunocompromised patients. In HCT patients, efficacies up to 98% are reported, and regimens with acceptable levels of toxicity have been developed (97). Some transplant centers now advocate weekly surveillance of viremia for their high-risk patients in the first few months posttransplantation, followed by preemptive cidofovir treatment if results are positive or rising. This strategy still does not completely eliminate adenovirus disease, however, and some pediatric patients clear their viremia spontaneously without treatment (97). A clinical algorithm that recommends treatment only of HCT patients at high risk (as defined by host factors, transplant type, and viremia) was evaluated in a small retrospective series (98). All six low-risk patients cleared their adenovirus infection without treatment, whereas 1 of 10 high-risk patients died. Several other retrospective and prospective studies have since been published, without a clear consensus. However, recent guidelines from the European Conference on Infections in Leukemia include recommendations for preemptive therapy with cidofovir in high-risk patients (18).

A lipid conjugate of cidofovir, Brincidofovir (CMX001-hexadecyloxypropyl cidifovir) has shown promise in early studies for the treatment of adenoviral infections (99) and is currently in advanced-stage clinical trials. Brincidofovir can be administered orally, with good absorption, and reaches higher intracellular concentrations than cidofovir. It has shown potent *in vitro* antiviral activity against a wide range of double-stranded DNA viruses, and evidence points to less toxicity than that of cidofovir.

Other promising strategies include reduction of immunosuppressive therapy or immunotherapy using donor lymphocyte infusion (100). Both approaches, with or without concomitantly administered antivirals, have improved outcomes for HCT patients in some studies. To avoid inducing graft-versus-host disease with donor lymphocyte infusion, adenovirus-specific cytotoxic lymphocytes have been produced *in vitro*. A strategy to expand naive cord blood T cells *in vitro* with efficacy against adenovirus, Epstein-Barr virus, and cytomegalovirus infections has shown promise (101). Combination approaches using intravenous immunoglobulin, antivirals, and reduced immunosuppression have also met with some success.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The clinical spectrum of adenoviral disease is broad, so laboratory testing is usually required for accurate diagnosis.

Selection of tests should be guided by the patient's symptoms and immune status, the interval between disease onset and specimen collection, and laboratory expertise. In the last few years, testing has shifted primarily toward molecular methods, such that most adenovirus infections can now be detected within hours when automated "sample-to-answer" systems are used or within 1 to 2 days when quantitative LDT-based assays are used. The use of culture (centrifugation or tube culture) continues to diminish. Antigen testing has found limited application, and serologic testing rarely provides clinically actionable data. Testing can be aimed at systemic detection in blood samples (primarily in immunocompromised patients) or at detection in sites of end-organ involvement. In the latter instance, histopathology and adjunctive immunohistochemical and *in situ* hybridization continue to play an important role.

Interpretation of positive results can be challenging since many adenovirus infections are asymptomatic or due to reactivation of endogenous virus. Accurate interpretation should consider patient and specimen characteristics, test method, and, under some circumstances, the viral serotype or quantity detected. This requires close communication between the laboratory and the clinician. In general, detection of virus from the involved organ or in large quantities in a patient with an illness that is epidemiologically associated with adenovirus is evidence that adenovirus is the actual cause of the disease.

PCR-based methodology has now displaced culture and antigen detection as the method of choice for rapid detection of adenoviral respiratory tract infection. These assays typically have a high degree of sensitivity, but like antigen tests, one can expect higher rates of detection in samples from children, as virus is typically shed at higher titers and for longer periods in children than in adults. Caution is advised in the selection of test methods, as the complex phylogeny of adenoviruses can present a daunting assay design challenge. A given test, though claiming broad detection capabilities, may have significantly reduced sensitivity for specific adenoviral serotypes or species. Nonetheless, both sensitivity and speed of detection have markedly improved over time, and the rapid reporting of such results can have a positive impact on patient and fiscal outcomes (102, 103). The sensitivity advantage of PCR over those of other methods is such that confirmatory reflex testing for samples testing negative by other methods should be considered in critically ill or high-risk populations. Although typically utilized for immunocompromised patients, assessment of viremia may be a useful adjunct for the diagnosis of severe adenoviral disease in immunocompetent children (71). The exquisite sensitivity of PCR can also present challenges, however. Low viral DNA loads can be detected in specimens from asymptomatic subjects. Results of tissue PCR may be particularly hard to interpret in the absence of correlative viral inclusions that are positive by immunohistochemistry or *in situ* hybridization. Furthermore, the clinical significance of continued shedding of adenovirus after resolution of symptomatology is an unresolved issue.

Adenovirus gastroenteritis in immunocompetent patients is best detected by a serotype 40/41-specific immunoassay or PCR of stools. Interpretation is usually straightforward, because these infections are usually mild and self-limited in such hosts, with viral shedding infrequent past the symptomatic period. In contrast, nonenteric adenoviruses can be detected in stools of asymptomatic immunocompetent and immunocompromised patients for prolonged periods. In the latter group, severe or fatal gastrointestinal disease can result, and detection in the stool can presage disseminated infection. High viral loads in excess of 10^6 genome copies/ml of stool have been shown to be predictive of subsequent viremia in allogeneic stem cell transplant recipients (104). Detection of nonenteric adenoviruses in stools of immunocompromised patients may be achieved by EIA, PCR, or culture. Viral load is typically determined by PCR. It should be noted that many assays are designed to detect only enteric adenovirus (types 40/41), and any assay should be validated for detection across all species or serotypes of presumed import in a given patient population. Detection of adenovirus in the stools of transplant recipients does not rule out graft-versus-host disease or graft rejection.

Detection of adenovirus in urine has also been associated with hemorrhagic cystitis. Among immunocompetent patients, this picture is typically seen in pediatric patients, with a male predominance, in association with adenovirus serotypes 11 and 21. In HCT patients, hemorrhagic cystitis may be severe and might be the initial site of severe disease or dissemination (30). However, as in other organ systems, adenovirus may be shed asymptomatically in the urine, and the predictive value of routine testing for this sample type is unclear, with some investigators failing to show an association between adenoviruria and hemorrhagic cystitis in HSCT patients.

In contrast, ocular adenoviral detection by EIA, IC, culture, or PCR may be considered diagnostic. Adenovirus encephalitis and meningitis occur infrequently, so studies comparing traditional and newer methods of detection are lacking, although PCR of CSF is likely to be the optimum approach.

Diagnosis of adenovirus disease in immunocompromised patients is challenging due to the many copathogens, the high rates of asymptomatic virus shedding, and the protean manifestations of adenovirus infections in this population. HCT and SOT recipients are at highest risk of severe disease and are the usual focus of testing. Some centers utilize surveillance cultures or PCR studies of multiple body sites to detect virus in temporal association with the onset of new symptoms. Involvement is then classified as infection, probable disease, or definite disease, depending on the number of virus-positive sites, symptoms, or histologic confirmation. Detection of adenovirus viremia by PCR is increasingly used instead of culture for surveillance. Viremia is usually present during severe or fatal adenovirus infection and often precedes the development of disease, although not all PCR-positive patients become symptomatic. Values then decline following successful treatment. Therefore, many centers now monitor weekly viremia in high-risk transplant patients, treat preemptively based on single high-positivity results or dynamic increases from low baselines, and reevaluate viremia to assess response to treatment. Similar approaches may be useful to assess the clinical effectiveness of new treatment modalities for adenoviral disease. However, caution is warranted in adapting universal or consensus breakpoints for institution of therapy. The lack of international quantitative standards, together with wide variations in methodology, and the potential for inaccuracies in quantification across different serotypes for individual assays mean that treatment thresholds must continue to be verified based on the test in use and the population being assessed in any given institution.

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Human Papillomaviruses

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TAXONOMY

Papillomaviruses (PVs) are small (55 nm in diameter), nonenveloped DNA viruses. PVs are classified according to nucleic acid (NA) sequence homology into genus, species, type, subtype, and variant (1). Members of the same genus share at least 60% NA sequence homology. Species are defined by sequence homology of 60 to 70%, with types in the same species having 71 to 89% homology. Subtypes are rare, exhibiting 90 to 98% homology, while variants are more common and show >98% homology. The genus and species groupings based on genotypic variation reflect key phenotypic differences, including species specificity, epithelial tropism (e.g., cutaneous versus mucosal), and oncogenic potential. Human PVs (HPVs) are clustered in five genera: *Alphapapillomavirus*, *Betapapillomavirus*, *Gammapapillomavirus*, *Mupapillomavirus*, and *Nupapillomavirus*. The majority of identified HPVs with clinical significance are found in the genus *Alphapapillomavirus*, which includes types infecting the genital and nongenital mucosae and genital cutaneous surfaces as well as the genotypes associated with human cancers. Table 1 summarizes the clinical manifestations of the genotypes, including oncogenic potential, in the alpha species group. The beta, gamma, mu, and nu types tend to infect nongenital epithelium.

DESCRIPTION OF THE AGENT

PVs contain a circular (or episomal) double-stranded genome and an icosahedral capsid structure consisting of 72 pentamers. The ~8,000-bp viral DNA has eight open reading frames encoding six early (E) proteins and two late structural capsid proteins, L1 and L2. L1 serves as a ligand for virion attachment to the basal cell layer of the epithelium. DNA transcription is controlled by the long control region (also known as the upstream regulatory region), which contains binding sites for many human transcription factors and regulatory elements, including steroid hormones reviewed in reference 2. Viral gene expression is dependent on the differentiation state of the infected epithelium, with early gene expression occurring predominantly in the suprabasal epithelial cells, while late gene expression is limited to the terminally differentiated keratinocytes. The precise mechanisms of differentiation-dependent viral gene expression have not been elucidated, though recent data suggest a potential role for epigenetic mechanisms, including viral DNA methylation (3–5). The early viral proteins have pleiotropic roles which generally involve viral replication and transcription (E1 and E2) and an interaction with key host regulator proteins (E6 and E7) resulting in the loss of a major tumor suppressor function. Specifically, the E6 protein from high-risk (HR) genotypes interacts with the human E6-associated protein (E6AP) to target p53 for ubiquitination and proteosomal degradation (6, 7), thereby impairing appropriate cell cycle arrest in response to genomic damage. The E7 protein from HR genotypes binds with pRB (the retinoblastoma protein) (8, 9) to uncouple cyclin-dependent cell division, allowing for dysregulated cell cycle progression. In self-limiting HPV infections, the expression of E6/E7 is tightly regulated and limited to the middle layers of the epithelium to facilitate viral replication in quiescent cells but is downregulated upon terminal differentiation. The dedifferentiation of the epithelium characteristic of high-grade neoplasia is associated with dysregulated expression of E6/E7 in the upper layers of the epithelium (10). Dysregulated E6/E7 expression can occur via loss of E2 regulatory protein expression due to integration of the HPV DNA into the host genome. While integration occurs in high-grade lesions and cancers, it is not a necessary prerequisite and varies by genotype (11). Data remain conflicting as to whether integration is a cause or a consequence of the tumorigenic process (12, 13).

EPIDEMIOLOGY AND TRANSMISSION

PVs are strictly species specific, with no animal reservoir. HPVs have been shown to infect both cutaneous and mucosal epithelia of the skin, oral cavity, conjunctiva, anus, and lower genital tracts of men and women, with no systemic, or blood-borne, phase. Consequently, transmission occurs via direct epithelium-to-epithelium contact and not exposure to bodily fluids, as in the cases of hepatitis and human immunodeficiency virus (HIV) infections. Infection is thought to occur only in basal epithelial cells, which are presumably accessed through microtrauma incurred during sexual activity through both penetrative and nonpenetrative epithelial abrasive contact. Infection of the skin with the *Betapapillomavirus* species is thought to be nearly ubiquitous (reviewed in reference 14).

Transmission of anogenital HPV in both men and women is largely and predominantly sexual (15, 16); however, some evidence also supports the possibility of autoinoculation between sites and digital/fomite transmission (17, 18). Condoms, which do not cover external epithelial sites harboring

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TABLE 1 Human alphapapillomavirus genotype diversity and clinical manifestations[a]

Species (common use)	Species (ICTV)	HPV genotype(s)	Cancer risk	Common epithelial type of infection and clinical manifestations
1	HPV-32	32, 42	Low	Mucosal
2	HPV-10	3, 10, 28, 29, 77, 78, 94, 117	Low	Cutaneous > mucosal
3	HPV-61	61, 62, 72, 81, 83, 89, 84, 86, 87, 102, 114	Low	Mucosal
4	HPV-2	2, 27, 57	Low	Cutaneous warts of skin; genital lesions of children
5	HPV-26	26, 51, 69, 82	Low and high	Mucosal
6	HPV-53	30, 53, 56, 66	Low and high	Mucosal
7	HPV-18	18, 45, 39, 59, 68, 70, 85, 97	High	Mucosal; HPV-18 is the second-most-common type in invasive cervical cancers, particularly AdCa
8	HPV-7	7, 40, 43, 91	Low	Mucosal and cutaneous; HPV-7 causes butcher's warts; often found in HIV-positive patients
9	HPV-16	16, 31, 33, 35, 52, 58, 67	High	HPV-16 is the most common type in invasive cancers
10	HPV-6	6, 11, 13, 44, 74	Low	Common in benign genital warts; associated with recurrent respiratory papillomatosis; HPV-6 may be associated with verrucous carcinoma
11	HPV-34	34, 73	Possibly high	Mucosal
13	MMPV1	54	Low	Mucosal
14	HPV-90	16, 71, 90	Low	Mucosal

[a] HPVs were recently reclassified by the International Committee on Taxonomy of Viruses (ICTV). Both the new classification (species, ICTV) and the historical classification (species, common use) are represented. MMPV1, *Macaca mulatta* PV 1. Species are listed by type; e.g., HPV-32 is HPV type 32.

HPV, offer partial protection from infection, particularly with consistent and correct use (19–21).

Sexually active individuals are exposed to and can acquire any one or more of the >40 HPV types known to infect the anogenital tract. Concurrent, multiple-type HPV infections at the same anogenital site are common, particularly among young individuals and those with a compromised immune system (e.g., HIV-positive individuals and organ transplant recipients). Having multiple HPV infections may be a marker of high-sexual-risk behavior or a marker of a woman's immune status. While multiple infections may be a marker of compromised immune response to HPV infection and thus increased risk of neoplastic progression, associations between multiple concurrent HPV infections and the risk of cervical intraepithelial neoplasia of grade 2 or higher (CIN2+) have been inconsistently reported (22).

Because of an inability to differentiate new from reactivated infections, per sex act and per partnership transmission rates have been difficult to estimate (15, 16, 23). Transmission rates observed in the HPV Infection and Transmission among Couples through Heterosexual Activity study were 3 to 4/person-month (16), which corresponds to a per partnership transmission probability of 0.20 (95% confidence interval [CI], 0.16 to 0.24), lower than previous model estimates derived from natural history data suggesting 40% transmission in a single sex act (24). Because of the difficulty in the conduct and interpretation of transmission studies, estimates for per partnership or per sex act HPV transmission rates vary from 20 to 40%. It is estimated that at least 80% of sexually active adults will have acquired at least one anogenital HPV infection in their lifetimes (25), further emphasizing the ease of HPV transmission. Studies of college-age men and women observed a 24-month cumulative incidence of any HPV infection of 38.8% in women (26) and 62.4% in men (17). Importantly, nearly 50% of newly sexually active women were found to acquire HPV within 3 years of sexual debut with their first male sexual partner (27). These data highlight the difficulty of any risk stratification to select individuals at high risk of HPV infection.

The average duration of HPV infection in women is 6 to 12 months (28), with approximately 90% of women becoming HPV DNA negative within 24 months of infection. In a recent systematic review, the median duration of any HPV detection was 9.8 months, and HR HPV (9.3 months) persisted longer than low-risk (LR) HPV (8.4 months); HPV type 16 (HPV-16) (12.4 months) persisted longer than HPV-18 (9.8 months) (28). From a natural history perspective, it is not clear whether the loss of HPV DNA detection represents viral eradication, reduction of viral load below detectable limits, or establishment of viral latency (23), though suppression of HPV viral load to undetectable levels and reemergence of replicative virus upon immunosuppression have been well documented for other animal PVs (29). From a clinical standpoint, however, persistently detectable HR-HPV DNA confers a very high risk of progression to cervical precancer (30, 31).

Approximately two-thirds of women with detectable HPV DNA and up to 36% of men with detectable HPV DNA are found to develop a natural type-specific antibody response to infection (32, 33). Recent data have shown that women having naturally produced HPV serum antibodies are likely at a lower risk of future acquisition of HPV infection (34), but the same does not necessarily appear to be true for men (35, 36). These results in women do support the hypothesis that prior HPV infection induces type-specific immunity and is generally consistent with epidemiological data which do not fully support repeated reinfections from dually infected partners. Further studies are indicated for men.

The prevalence and natural history of penile HPV in men are becoming more understood (37, 38). A large international cohort examining male genital HPV infection (combined exfoliated glans/coronal sulcus, shaft, and scrotum epithelial samples) reported an overall HPV prevalence of 65.1% for any type of infection and a prevalence of 29.7% for carcinogenic HPV infection (39). These estimates did not differ significantly by age, in contrast to age-specific patterns of HPV infection observed in women from the same countries. Several studies have recently been published on the natural history of penile HPV infections in men (40). Among 1,159 men enrolled in the HPV Infection in Men (HIM) study from Brazil, Mexico, and the United States, HPV incidence was estimated at 38.4 per 1,000 person-months (40). Studies of the natural histories of men have consistently shown that rates of HPV clearance appear to be higher among men than among women and lower among HIV-seropositive participants than HIV-seronegative ones (41). Smaller studies of incident HPV infection in young men have similarly observed a higher risk than that for women of the same age, with a 2-year cumulative incidence of 62.4%, which was significantly associated with a new sex partner in the previous 8 months and a history of cigarette smoking (17). Circumcised men appear more likely to clear penile HPV infection (42), and data from randomized controlled trials in Uganda, South Africa, and Kenya have demonstrated that adult male circumcision reduced the point prevalence, incidence, and persistence of HPV infection at the glans/coronal sulcus (43–45).

In contrast, relatively few studies are yet available concerning the natural history of anal HPV infections, particularly among women (37). In a cohort study of young women in San Francisco, CA, HPV-16 was less likely to clear than other HPV infections, although the majority of anal HPV infections cleared within 3 years (37). Some studies of anal HPV infection have been conducted among men, particularly among men at higher risk of anal neoplasia, i.e., men having sex with men (MSM), and HIV-seropositive men (38, 46–48). Relatively few oral HPV natural history studies have been conducted, though it is clear that HR-HPV infection (particularly with HPV-16) is associated with a subset of head and neck squamous cell cancers (49). The prevalence of oral HPV infection in the general population is substantially lower than the genital HPV prevalence in men and women (50). Risk factors for oral HPV infection include increasing age (unlike with female cervical HPV infection, for which young age is a risk factor), male gender, HIV infection, iatrogenic immunosuppression, a history of a sexually transmitted infection, and a number of oral sex partners (25). The HIM study found that among over 1,500 men aged 18 to 73 years, 4.4% acquired an oral HPV infection within the first year (51). Risk factors for acquisition of oral HPV included smoking and not being married or cohabiting. The median duration of oral HPV infections was 6.9 months for overall HPV, 6.3 months for high-risk HPV, and 7.3 months for HPV-16. Another study of over 400 HIV-seropositive participants found that HPV infection was less likely to occur or be persistent in the oral than in the anal canal (52).

CLINICAL SIGNIFICANCE

The vast majority of HPV infections at all sites are subclinical and asymptomatic. These infections are characteristically noninflammatory; therefore, most individuals who acquire HPV never know that they have been infected. Because the viruses utilize natural cell death in terminally differentiated keratinocytes to complete their viral life cycle, no tissue destruction or ulceration results from HPV infections. However, despite the abundance of subclinical and self-limited infections, both HR- and LR-HPV infections can cause clinical disease in their hosts.

Clinical Manifestations of Female Anogenital Infection

LR-HPV infection of the anus, cervix, vagina, and vulva can result in benign warts caused predominantly by HPV types 6 and 11. All LR-HPV types can also result in the diagnosis of low-grade squamous intraepithelial lesions (LSILs) on Pap smears. These lesions are characterized by high nucleus/cytoplasm ratios and the presence of koilocytotic atypia (KA). KA is defined as the presence of nuclear atypia as well as a clearly defined perinuclear halo in cervical epithelial cells (Fig. 1). KA is considered the hallmark of HPV infection.

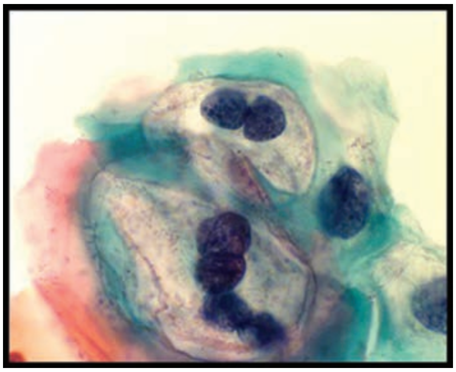

FIGURE 1 Photograph of KA. LSILs on Pap smears are characterized by high nucleus/cytoplasm ratios and the presence of KA, which is defined as the presence of nuclear atypia as well as a clearly defined perinuclear halo in cervical epithelial cells. Photograph courtesy of Patricia Wasserman, Division of Cytopathology, Department of Pathology and Laboratory Medicine, North Shore-LIJ Health System, Lake Success, NY. doi:10.1128/9781555817381.ch104.f1

HR-HPV infection can cause both high-grade squamous intraepithelial lesions (HSILs) and invasive cancer at all female anogenital sites, though HR-HPV cancers occur at a much higher frequency at the cervix than at the other sites. An HSIL is considered a true cancer precursor lesion, in contrast to a low-grade SIL (LSIL), which is generally thought to represent nothing more than viral infection. HSILs are differentiated from LSILs by diminished differentiation, with basaloid cells with a high nucleus/cytoplasm ratio present close to the epithelial surface. HSILs have been found to contain a higher frequency of genomic abnormalities and are therefore at high risk of progression to invasive cancer. Cervical cancers occur predominantly at the transformation zone, or squamocolumnar junction. The most common treatment for preinvasive neoplasia (CIN2 or CIN3) is the loop electrosurgical excision procedure (LEEP) or cold knife cone, which removes the entire cervical transformation zone. Other treatments include laser therapy or cryotherapy; however, LEEP and cone procedures have the advantage of presenting intact tissue specimens for histopathological review for determining lesion margins and have been shown to result in lower recurrence rates (53). A residual presence of HR HPV following excisional therapy is a predictor of disease recurrence (54).

Clinical Manifestations of Male Anogenital Infection

HPV infection in the anogenital tracts of men is more likely to remain undetected, since routine screening for detection of subclinical lesions is not performed in men. Benign warts and flat lesions can occur in all areas of the male lower genital tract, and HR-HPV infection may rarely result in the development of anal and penile cancers. Men who have sex with men and HIV-positive men are at a particularly high risk of anal cancer, and anal HPV infection in these men is nearly ubiquitous (55).

Clinical Manifestations of Male and Female Oral Infection

Oral infection is also rarely symptomatic. A very rare syndrome, recurrent respiratory papillomatosis, is caused by infection with LR-HPV types that are typically found in genital warts (e.g., HPV types 6 and 11). The juvenile-onset form of recurrent respiratory papillomatosis likely is the result of laryngeal HPV acquired from the birth canal of the infected mother. HR HPV, particularly type 16, is also associated with a subset of head and neck cancers, especially cancers of the larynx and oropharynx. Diagnosis of HPV in head and neck tumors yields important prognostic information, as the survival rate for patients with HPV-positive tumors is significantly higher than that for patients with HPV-negative tumors (56).

Primary Prevention

Primary prevention of up to 90% of genital warts and 70% of invasive cervical cancers is now theoretically possible with the introduction of a highly efficacious vaccine. Gardasil (Merck, West Point, PA) targets HPV types 6, 11, 16, and 18 and prevents external genital lesions and CIN associated with these HPV types in both men (57) and women (58, 59). An alternative vaccine, Cervarix (GlaxoSmithKline, Research Triangle Park, NC), targets HPV types 16 and 18 and has demonstrated a similar efficacy (60). Both are approved in the United States for use in females aged 9 to 26 years, and Gardasil is approved for prevention of genital warts in males aged 9 to 26 years. Both vaccines are based on a recombinant construct of the major capsid protein which is expressed and self-assembled in yeast or insect cell systems into a virus-like particle, which is both morphologically and immunologically indistinguishable from the native virus. The virus-like particle, however, carries no viral nucleic acid and therefore confers no risk of inadvertent HPV infection in vaccine recipients. While these vaccines are nearly 100% efficacious in preventing lesions due to the vaccine types, they offer absolutely no therapeutic benefit to women already exposed to or infected with these types. Therefore, Gardasil and Cervarix are most beneficial when offered prior to sexual debut.

Recommendations on Cervical Cancer Screening

The United States Preventive Services Task Force (USPSTF) released new cervical cancer screening recommendations in March 2012 (Table 2) (61). The most important changes from USPSTF's previous recommendations include the following. The USPSTF recommends screening for cervical cancer in women ages 21 to 65 years with cytology (Pap smear) every 3 years or, for women ages 30 to 65 years who want to lengthen the screening interval, screening with a combination of cytology and HPV testing (detection of HPV DNA or HPV E6/E7 mRNA) every 5 years. The USPSTF recommends against screening for cervical cancer in women younger than 21 years. There are important policy implications associated with the USPSTF recommendations. The USPSTF states that their A- or B-rated recommendations are "relevant for implementing the Affordable Care Act." This is the first time that HPV cotesting has been included in federal recommendations, thus potentially facilitating coverage for millions of women in the United States. The American Cancer Society (ACS), the American Society for Colposcopy and Cervical Pathology (ASCCP), and the American Society of Clinical Pathology (ASCP) have published screening guidelines for the prevention and early detection of cervical cancer (62). The ACS-ASCCP-ASCP guidelines are consistent with the USPSTF recommendations and will have an immediate impact on clinical practice in order to improve cervical cancer screening in the United States. The full description of the guidelines is available through the *Journal of Lower Genital Tract Disease* (63). The most significant changes include the following. For women 21 to 29 years of age, screening with cytology alone every 3 years is recommended. HPV testing should not be used to screen women in this age group. Women ages 30 to 65 years should be screened with cytology and HPV testing ("cotesting") every 5 years (preferred) or cytology alone every 3 years (acceptable).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Sample collection, transport, and storage recommendations vary based on the purpose and method of HPV testing. Laboratories should refer to manufacturers' validated recommendations for commercial assays. Collection of serum follows standard procedures and therefore is not detailed here.

HPV Screening Applications

The use of liquid-based cytology (LBC) facilitates a single sample collection that allows for both Pap smear cytology and HPV testing/genotyping from the same vial. The Hybrid Capture 2 (hc2) test (Qiagen, Gaithersburg, MD) (64), Cervista HPV HR test (Hologic, Madison, WI) (65), cobas HPV test (Roche Molecular Systems, Inc., Pleasanton, CA) (66), and Aptima HPV assay (Hologic, San Diego, CA) (67) have U.S. Food and Drug Administration (FDA) approval for testing from residual ThinPrep PreservCyt LBC

TABLE 2 Clinical indications for appropriate use of HPV tests[a]

Indication	Age restriction
Primary cervical cancer screening	25 yr and older[b]
Routine cervical cancer screening in conjunction with cervical cytology (dual testing or cotesting)	30 yr and older
Initial triage management of women with a cytologic result of ASC-US	21 yr and older
Initial triage management of postmenopausal women with cytologic result of LSIL	Postmenopausal
Postcolposcopy management of women of any age with initial cytologic result of AGC or ASC-H (when initial workup does not identify a high-grade lesion)	None
Postcolposcopy management of women 21 yr and older with initial cytologic results of ASC-US or LSIL (when initial colposcopy does not identify a high-grade lesion)	21 yr and older
Posttreatment surveillance	None

[a] Adapted from reference 68. ASC-US, atypical squamous cells of undetermined significance; AGC, atypical glandular cells; ASC-H, atypical squamous cells, cannot exclude a high-grade SIL (squamous intraepithelial lesion).

[b] FDA approval for the Roche cobas assay was obtained in April 2014.

samples (Hologic, Bedford, MA). Exfoliated cell samples are collected from the endo- and ectocervix using a cervical brush or spatula-endocervical brush combination and rinsed into the LBC media. Additionally, Qiagen supplies a specimen transport medium (STM) tube with both brush and Dacron swab collection devices that are approved for use with the hc2 assay. Specimens are transported at room temperature and stored at 2 to 30°C until tested. The validated stabilities of STM and the residual ThinPrep sample vary by manufacturer (Table 3).

The use of non-FDA-cleared collection and transport media, including alternative LBC media, such as BD SurePath (Becton, Dickinson-TriPath, Burlington, NC), viral transport medium (M4), Tris-EDTA buffer, and phosphate-buffered saline, would require rigorous validation before these alternatives are considered comparable to manufacturer-determined performance standards established for the FDA-approved collection and transport media (68, 69). Additionally, alternative processing methods, such as glacial acetic acid treatment of grossly bloody LBC specimens to remove excess blood and mucus, thereby facilitating improved cytological analysis, require comprehensive validation (70).

HPV tests from oral, penile, and anal swab samples are not standard clinical tests, and none of the commercial HPV tests are FDA approved for testing samples from these anatomical sites. Penile and anal swab samples are collected using a saline-moistened Dacron swab, can be placed in the same buffers (e.g., ThinPrep LBC medium and STM) as cervical swabs, and likely have similar levels of stability. Exfoliated oral cells are best sampled using a 30-s oral rinse and gargle with saline or Scope mouthwash. These samples should be stored at −80°C.

TABLE 3 FDA-approved molecular assays for the detection and/or genotyping of high-risk HPV DNA[a]

Test (references)	Method(s) and/or platform(s)	Specimen type (amt)	HPV targets	Internal control	Genotyping capabilities	Storage conditions
Aptima HPV assay by Hologic/Gen-Probe Inc., San Diego, CA (67, 84, 85, 87–89, 106–114, 119)	TMA fully automated on Tigris and Panther	PreservCyt (1 ml in Aptima STT)	E6/E7 mRNA of HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, and 58	None	Reflex to HPV-16, -18, -45	PreservCyt: up to 105 days at 2–30°C prior to transfer to STT STT: 2–30°C up to 60 days PreservCyt or STT: up to 24 mo at −20°C
Cervista HPV HR test by Hologic, Madison, WI (65, 87, 89, 98–100, 117, 118, 140)	Invader Chemistry manual or fully automated on HTA system	PreservCyt (2 ml)	DNA of HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68	Human histone 2 gene	Reflex to HPV-16, -18 assay	PreservCyt: up to 18 wk at 20–30°C
cobas 4800 HPV test system by Roche Molecular Diagnostics, Pleasanton, CA (66, 70, 84, 87, 89, 101–105, 113, 114, 120, 136, 141)	Real-time PCR (fully automated on cobas 4800)	PreservCyt (1 ml)	DNA of HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68	β-Globin gene	Included for HPV-16, -18	PreservCyt: up to 6 mo at 2–30°C
hc2 HPV test by Qiagen GmbH, Hilden, Germany (64, 87)	Hybrid Capture manual or Rapid Capture system	STM, brush PreservCyt (4 ml)	DNA of HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68; DNA of LR types 6, 11, 42, 43, and 44	None	*digene* HPV PS test for types 16, 18, and 45 (not FDA approved)	STM: 2 wk at room temp, up to 3 wk at 2–8°C, up to 12 wk at −20°C PreservCyt: 12 wk at 2–30°C
hc2 HR HPV by Qiagen GmbH, Hilden, Germany (64, 84, 87, 89, 94–97, 99, 104, 105, 108, 109, 111–114, 132, 136, 147, 166)	Hybrid Capture manual or Rapid Capture system	STM, brush PreservCyt (4 ml)	DNA of HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68	None	*digene* HPV PS test for types 16, 18, and 45 (not FDA approved)	STM: 2 wk at RT, up to 3 wk at 2–8°C, up to 12 wk at −20°C PreservCyt: 12 wk at 2–30°C

[a] Abbreviations: TMA, transcription-mediated amplification; HR, high risk; HTA, high-throughput automation; STT, specimen transfer tube.

Self-Collection for HPV Infection

In order to increase access to cervical cancer screening among harder-to-reach populations and those who may be reluctant to obtain a physician-based Pap smear, a number of recent research studies have focused on the validity, feasibility, and acceptability of self-collected samples for cervical cancer screening. Self-collection refers to a technique by which women use a collection brush to obtain cervical and vaginal (cervico-vaginal) cell samples that are later tested for HPV infection and potentially other biomarkers. The self-collection tool is present in a kit with simple instructions. Several recent studies have investigated the use of self-collected samples for HPV screening (71–73). Research suggests that self-collection methods show promise in populations with limited resources and low cervical cancer screening uptake (71, 72). Investigations of acceptance of self-collection for detection of HPV infection in both developed and less developed countries have found that self-collection is well accepted (66% to 94% acceptance) (71, 74, 75). A recent systematic literature review and meta-analysis examined the performance of self-collection to detect high-grade cervical neoplasia of CIN2 or worse (CIN2+) (73, 75). HPV testing by self-collection was shown to be at least as sensitive as cytology for high-grade CIN2+ detection. Most studies found HPV testing via self-collection to be as sensitive for detection of CIN2+ as physician-based sampling, especially when more analytically sensitive PCR-based tests were used. In terms of specificity, HPV testing by self-collection has been shown to be less specific than cytology and as specific as HPV testing by physician collection. Additionally, a prototype Trovagene HPV test (San Diego, CA), developed for use with urine samples, has demonstrated sensitivity estimates for detection of CIN2+ comparable to those of the Roche Linear Array (Roche Molecular Systems, Pleasanton, CA) (76). Trovagene HPV assay specificity was lower, presumably due to the inclusion of LR-HPV types 53 and 70, which will not be included in the commercial version of the assay. A home self-collection method has the potential to overcome many barriers to traditional screening and may help motivate women overdue for screening to follow up with a health care provider-collected Pap test (77).

HPV Diagnostic Applications

HPV testing from diagnostic tissue specimens is not routine in cervical cancer screening applications, as it is assumed that all CINs are HPV positive. However, it has been found to be useful in the differentiation of primary and metastatic tumors and in HPV type attribution in vaccine trials. In contrast, HPV testing from oropharyngeal cancers provides important prognostic information, as survival is significantly improved in HPV-positive cancers (56). Fresh biopsy tissue or resected tissue snap-frozen in liquid nitrogen is ideal and allows for flexibility in detection of DNA, mRNA, and protein. Paraffin-embedded tissue can be used, although the risk of DNA degradation, and especially RNA degradation, with increasing time in fixation is high. Fixation with 10% buffered neutral formalin is recommended for future PCR testing.

DIRECT DETECTION

Microscopy

Direct visualization of HPV infectious particles is not possible using standard light microscopy. The morphological cellular changes characteristic of HPV infection and associated neoplasia are the basis for standard cervical cytology and are classified based on the Bethesda system (78). Conventional cytology, in which exfoliated cells are smeared onto a glass slide and fixed, has been criticized as suffering from inaccurate readings resulting from obscuring blood and inflammatory cells and poor fixation or air drying. LBC alternatives were developed to prevent these problems and offer the added benefit of residual material for DNA- and RNA-based tests. A large randomized trial compared conventional cytology and LBC and found a modest reduction in smear inadequacy using LBC methods (79) but no benefit in disease detected or positive predictive value (PPV) (80).

Antigen Detection

At present, immunohistochemical detection of HPV proteins has not been validated for clinically relevant diagnostic applications. However, immunohistochemical or immunocytochemical detection of cellular proteins, such as p16^{INK} tumor suppressor protein, MCM2, and Ki-67, have been evaluated as molecular markers of anal cancer precursors (81) and CIN2+ (82–85) (Table 4). Of these, p16 immunohistochemistry is recommended as an adjunctive test to increase accuracy and reproducibility in specific cases of diagnostic uncertainty (86). p16 ^{INK} is a good biomarker of HR-HPV infection and oncogene expression, since there is measurable upregulation of p16 ^{INK} expression in cells expressing HPV E7. p16 staining is also used for differentiating HPV-positive and HPV-negative oropharyngeal samples.

NA-Based Detection and Typing Methods

HPV is not culturable, and therefore, the detection and genotyping of HPV are predominantly dependent on molecular tests that target either HPV DNA or mRNA (87–89). DNA-based tests screen for the absence or presence of the HPV genome (integrated or episomal), while the RNA-based tests screen for the expression of E6/E7 mRNA genes. Although DNA-based tests are highly sensitive for the detection of HPV, clinical specificity is low due to the frequent and transient nature of most HPV infections. Therefore, E6/E7 mRNA tests have been developed and evaluated as an alternative test method to increase the clinical specificity of HPV testing and thereby improve the positive predictive value of the result (88). Fewer positive tests in women without disease would improve the management of women with cervical screening abnormalities and theoretically decrease the burden of overtreatment.

All four FDA-approved tests are applicable for the clinical indications for HPV testing listed in Table 2. Currently, only the Roche cobas assay is approved for primary screening in the United States. Standardized protocols are provided with the assay; therefore, details are omitted here. While modifications to these protocols may be warranted, laboratories should understand that performance standards are inextricably linked to the use of the validated protocol. Deviations from the recommended protocol run the risk of compromising performance. Therefore, if deviations are warranted, parallel comparisons are essential to confirm that the deviations do not affect the established performance standards. Reference documents that discuss how to appropriately validate and assess an HR-HPV assay have been published (90–92).

The number of additional commercially available assays for HPV detection has increased dramatically in recent years, many of which are Conformité Européenne (CE) marked for clinical use in Europe. In this section, only assays designed to detect the presence or absence of HR-HPV

TABLE 4 *In situ* hybridization tests for HPV detection

Test (reference[s])	Method(s)	HPV target(s)/specimen type(s)
CINtec p16, mtm laboratories AG, Heidelberg, Germany (82–84, 112, 113)	Immunohistochemistry assay with protein, qualitative	p16/slides prepared from formalin-fixed, paraffin-embedded cervical biopsy specimens
CINtec Plus, mtm laboratories AG, Heidelberg, Germany (81, 85)	Immunocytochemistry assay with protein, qualitative	p16, Ki-67/cervical cytology preparations
GenPoint HPV detection system, Dako, Glostrup, Denmark (87, 129)	Tyramide signal *in situ* amplification, DNA probes, Dako Hybridizer, Autostainer Plus	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68/histology and cytology specimens
Inform HPV II Family 6 probe, Ventana Medical Systems, Tucson, Arizona; Roche Diagnostics GmbH, Mannheim, Germany (87, 128)	Immunochemistry assay with DNA, qualitative	LR types 6 and 11/cervical cytology preparations, paraffin-embedded tissue sections
Inform HPV III Family 16 probe (B), Ventana Medical Systems, Tucson, Arizona; Roche Diagnostics GmbH, Mannheim, Germany (87, 130)	Immunochemistry assay with DNA, qualitative	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66/cervical cytology preparations, paraffin-embedded tissue sections
OncoTect E6/E7 mRNA, IncellDx, Menlo Park, CA (87, 131)	Immunophenotyping fluorescence and flow cytometry with mRNA, quantitative; (i) mRNA overexpression inside each cell, (ii) percentage of cells overexpressing mRNA	E6/E7 mRNA/PreservCyt and SurePath liquid-based cytology
ZytoFast HPV probes, ZytoVision GmbH, Bremerhaven, Germany (87)	Immunochemistry assay with multiple probe pools	LR types 6 and 11; HR types 16 and 18; HR types 31 and 33; HR types 16, 18, 31, 33, and 35; LR and HR types 6, 11, 16, 18, 31, 33, and 35; HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 82; LR and HR types 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 82/cervical cytology preparations, paraffin-embedded tissue sections

infection are discussed, with preference given to those having FDA approval at press time (Table 3). Other non-FDA-approved commercial assays for HPV screening and genotyping are listed in Tables 5 and 6.

NA Isolation and Purification

The appropriate method for HPV NA isolation is a function of the sample type (e.g., fresh tissue, formalin-fixed paraffin-embedded [FFPE] tissue, and exfoliated cells), the collection medium (e.g., STM, PreservCyt LBC medium, or SurePath LBC medium), the type of NA target (e.g., mRNA or DNA), the HPV detection assay used, and the reason for testing.

Fresh Frozen Tissue

NA purification from human tissue samples requires standard methods using organic solvents (e.g., phenol, chloroform, or isoamyl alcohol). Generally, extraction with phenol, chloroform, or isoamyl alcohol and ethanol precipitation can be used. Utilization of commercially available phase-separating microcentrifuge tubes (e.g., Qiagen MaXtract) may help in reproducible recovery of HPV DNA by minimizing operator error. If mRNA is the target of the HPV assay, NA isolation should begin with a standard TRIzol method.

FFPE Tissue

HPV DNA tests are typically performed on one to three 10-μm sections of FFPE tissue. Extreme caution must be used in preparation of sections to avoid specimen-to-specimen contamination, particularly for use in PCR assays. Precautions include the use of disposable microtome blades, thorough cleaning of the microtome between specimens with 70% ethanol, and changing of gloves between each specimen. HPV-negative tissues (e.g., fallopian tubes) should be sectioned periodically as a negative control. Sections are deparaffinized with octane and digested with a buffer containing proteinase K and a nonionic detergent. Large sections can be further purified with organic extraction and ethanol precipitation. DNA from very small tissue biopsy specimens can be lost during organic purification; HPV amplification directly from a crude lysate (after it is heated at 95°C for 10 min to inactivate the protease) has been demonstrated to be sufficient (93).

Exfoliated Cells

LBC samples are likely to be the most frequently encountered sample type for HPV testing in clinical laboratories. Manufacturers of most commercial HPV assays provide validated methods for DNA/RNA purification. Most samples in transport media do not require organic purification, and NA extraction is based on precipitation methods. A sample conversion step, provided by the manufacturer, is required for LBC samples to render them compatible with the hc2 test (64). The Cervista test recommends purification of DNA from PreservCyt samples using the Genfind DNA extraction kit (Hologic) (65). NA extraction using the

TABLE 5 Non-FDA-approved DNA/mRNA-based assays for the detection and limited genotyping of high-risk HPV DNA[a]

Test (reference[s])	Method and platform(s)	Specimen type(s) (amt)	HPV targets	Internal control	HPV genotyping capabilities
DNA-based assays					
Abbott Real*Time* HPV assay, Abbott Molecular GmbH & Co., Wiesbaden, Germany (84, 87, 89, 113, 114, 132–138)	Real-time PCR, m2000*sp*, m2000*rt*	PreservCyt (0.4–0.6 ml)	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68	β-Globin gene	HR types 16 and 18
AdvanSure real-time PCR HPV test, LG Life Science, Seoul, South Korea (138)	Real-time PCR	Cervical/vaginal swab, PreservCyt, SurePath	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 61, 62, 66, 67, 68, and 72; LR types 3, 6, 10, 11, 27, 32, 34, 40, 42, 43, 44, 53, 55, 69, and 73; UR types 26, 54, 57, 70, 71, 74, 81, and 84	Not specified	HR types 16 and 18
BD Onclarity HPV test, BD Diagnostics, Sparks, MD (84, 113, 114)	Real-time PCR, BD Viper LT system	PreservCyt (2 ml)	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68	Human histone 2 gene	HR types 16, 18, 31, 45, 51, 52, and 59; HR groups 33, 58, 35, 39, 68, 56, 59, and 66
*care*HPV test, Qiagen, Gaithersburg, MD (87, 89, 139)	Signal amplification chemiluminescent detection	*care*Path cervical sampler and collection medium	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68	None	None
Cepheid Xpert HPV assay, Cepheid, Sunnyvale, CA	Real-time PCR, Cepheid GeneXpert	PreservCyt (1 ml)	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68	Sample adequacy control	HR 16 and 18/45
Seeplex HPV-4 ACE, Seegene Inc., Seoul, South Korea (87, 140, 141)	Dual-priming oligonucleotide multiplex PCR with auto capillary electrophoresis LabChip DX	Residual PreservCyt cervical swabs	HR types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82; LR types 16 and 11	Amplification and hybridization	HR types 16 and/or 18
Trovagene HPV test, Trovagene, San Diego, CA (76)	PCR, capillary electrophoresis (Genewiz, South Plainfield, NJ)	Urine (1 ml)	Prototype assay: HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68; LR types 53 and 70 Commercial assay: HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68	NA	None
E6/E7 mRNA-based assays					
NucliSENS EasyQ HPV, bioMérieux, Marcy l'Etoile, France (87–89, 142, 143)	NASBA, NucliSENS easyQ	PreservCyt (5 ml)	E6/E7 mRNA types 16, 18, 31, 33, and 45	U1A mRNA	HR types 16, 18, 31, 33, and 45
PreTect HPV-Proofer, NorChip, Klokkarstua, Norway (68, 84, 87–89, 112–114, 144–148)	NASBA	PreTect TM media, PreservCyt (5 ml)	E6/E7 mRNA types 16, 18, 31, 33, and 45	U1A mRNA	HR types 16, 18, 31, 33, and 45

[a]Abbreviations: HR, high risk, LR, low risk; UR, unspecified risk; NASBA, nucleic acid sequence-based amplification.

TABLE 6 Non-FDA-approved DNA-based HPV detection and high-level genotyping assays

Test (reference[s])	Method(s)	HPV targets
AdvanSure HPV GenoBlot assay, LG Life Science, Seoul, South Korea (149, 150)	Real-time PCR blotting	HR types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 69, 70, 73, and 82; LR types 6, 11, 32, 34, 40, 42, 43, 44, 54, 55, 57, 61, 62, 81, and 83
CLART HPV2, Genomica, Madrid, Spain (87, 89, 151)	PCR, low-density microarray platform, CLART (clinical array technology); IC, CFRT	HR types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, and 82; LR types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and 89; UR types 62, 71, 73, 83, 84, and 85
EasyChip HPV blot chip, HPV blot kit, King Car, Taiwan (87, 152)	PCR, reverse dot blot assay; IC, GAPDH gene	HR types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 61, 62, 66, 67, 68, and 82; LR types 6, 11, 30, 32, 42, 43, 44, 54, 55, 69, and 74; UR types, 70, 71, 72, 81, 83, 84, and 85
GeneFinder liquid beads microarray, Innomeditech, Seoul, South Korea (138)	PCR, liquid beads microarray method detection using Luminex analyzer (Luminex, Austin, Texas)	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 61, 62, 66, 67, 68, and 72; LR types 6, 10, 11, 30, 32, 34, 40, 42, 43, 44, 53, 55, 69, and 73; UR types 26, 54, 57, 70, 71, 81, 83, 84, 86, 90, and 97
GeneTrack HPV DNA chip, Genomictree, Daejeon, South Korea (87)	PCR, chip microarray	HR types 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 54, 56, 58, 59, 66, 68, and 70; LR types 6, 11, 40, 42, 43, 44, 62, 67, 69, 71, and 72
GenoArray, Hybribio Ltd., Hong Kong (87, 153, 154)	PCR, flowthrough hybridization with immobilized genotype-specific probes	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68; PHR types 53 and 66; UR types 6, 11, 42, 43, 44, and 81
GenoFlow, DiagCor Bioscience, Inc., Hong Kong (155, 156)	PCR, reverse line blot; IC, yes (not specified)	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 81, and 82; LR types 6, 11, 26, 40, 42, 43, 44, 54, 55, 57, 61, 70, 71, 72, and 84
GG HPV chip, Goodgene, Seoul, South Korea (87)	PCR, DNA chip; IC, β-globin	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 52, 56, 58, 59, 66, 67, 68, 69, 73, and 82; LR types 6, 11, 26, 32, 34, 40, 42, 43, 44, 54, 55, 61, 62, 70, and 72; UR types 81, 83, 84, 90, and 91
HPV direct-flow chip, Master Diagnóstica, Granada, Spain (157)	PCR, automatic reverse dot blot hybridization using e-BRID system (Master Diagnóstica); IC, β-globin	HR types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82MM4; LR types 6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 67, 69, 70, 71, 72, 81, 84, and 89
HybriMax, Hybribio Ltd., Chaozhou, China (158)	PCR, flowthrough hybridization technique	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68; LR types 6, 11, 42, 43, and 44; UR types 53 and CP8304
Infiniti HPV assay, AutoGenomics, Vista, CA (87)	PCR, BioFilmChip microarray	HR types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 69, 73, and 82; LR types 6, 11, 30, 34, 70, and 85
Infiniti HPV-Quad, AutoGenomics, Vista, CA (159)	PCR, BioFilmChip microarray	HR types 16, 18, 31, 33, 45, 35, 68, 39, 56, 58, 52, 59, and 51; LR types 6 and 11
Infiniti HPV-HR Quad, AutoGenomics, Vista, CA	PCR, BioFilmChip microarray	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68
INNO-LiPA, Innogenetics/Fujirebio Europe, Ghent, Belgium (87, 89, 135, 160, 161)	PCR, reverse line blotting; IC, HLA-DPB1 gene	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82; LR types 6, 11, 40, 43, 44, 54, 69, 71, 70, and 74
Linear Array, Roche Molecular Systems, Branchburg, NJ (76, 89, 97, 104, 112, 120, 151, 155, 156, 162)	PCR, reverse line blotting; IC, β-globin gene	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, and 82; LR types 6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 8482v, and 89
MassArray, Sequenom, Inc., San Diego, CA (121, 122)	Multiplex PCR using MALDI-TOF MS	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 73
Multiplex HPV genotyping kit, Progen/Multimetrix, Heidelberg, Germany	PCR, liquid bead microarray method detection using Luminex analyzer; IC, β-globin gene	6, 11, 16, 18, 26, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73 and 82

(Continued on next page)

TABLE 6 Non-FDA-approved DNA-based HPV detection and high-level genotyping assays *(Continued)*

Test (reference[s])	Method(s)	HPV targets
PANArray, Panagene Inc., Daejeon, South Korea (87, 138)	PCR, genotyping chip; IC, β-globin gene	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68; LR types 6, 11, 32, 34, 40, 42, 43, 44, 53, 54, 55, 62, 69, and 73; UR types 26, 70, 81, 83, other
PapilloCheck, Greiner Bio-One GmbH, Frickenhausen, Germany (87, 89, 163–165)	PCR hybridization to PapilloChip DNA chip; IC, ADAT1 gene	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82; LR types 6, 11, 40, 42, 43, and 44
PapType HPV test, Genera Biosystems Ltd., Melbourne, Australia (166, 167)	PCR, flow cytometry AmpaSand bead array; IC, alkaline myosin light-chain protein gene	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68; LR types 6 and 11
REBA HPV-ID, M&D, Wonju, South Korea (87, 168, 169)	One-tube nested PCR and reverse blot hybridization assay	HR types 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68; LR types 6, 11, 42, 43, 44, 70, 72, 81, 84, and 87
Seeplex HPV 6, Seegene Inc., Seoul, South Korea	Dual-primer oligonucleotide-based multiplex PCR, auto capillary electrophoresis	HR types 16, 18, 31, and 45; LR types 6 and 11
Seeplex HPV-18 ASE, Seegene Inc., Seoul, South Korea	Dual-primer oligonucleotide-based multiplex PCR, auto sequencer electrophoresis	HR types 16, 18, 31, 33, 35, 45, 51, 56, 58, 59, 66, 67, and 70; LR types 6, 11, 42, 43, and 44

[a]Abbreviations; HR, high risk, LR, low risk; UR, undetermined risk; IC, internal control; CFRT, cystic fibrosis transmembrane conductance regulator; ADAT1, adenosine deaminase tRNA-specific 1 gene; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cobas HPV test (66) and the Aptima HPV assay (67) are included in the automated test procedures. When possible, the recommended protocol should be utilized to ensure that test performance is consistent with that validated by the manufacturers.

Alternative Methods

Numerous NA isolation systems are commercially available, several with high-throughput biorobotic capabilities. Laboratory validation of these methods against the manufacturer's method is necessary before claims of performance equivalent to that reported in the assay trials can be made (90–93). Sample transport medium composition will determine what level of purification is required, particularly for enzyme-dependent assays. Additionally, test sensitivity will reflect the volume of the original sample used for purification, any concentration or dilution steps, and the fraction of purified material used in the final assay. To claim performance comparable with that of an alternative procedure, the final proportion of sample tested should be similar to that used in the manufacturer's performance validation. Finally, it is critical to note that the validation of NA purification methods has been conducted primarily using uterine cervix samples and that comparable performance when other specimen types are used cannot be guaranteed.

FDA-Approved Molecular Assays for Detection of HPV

hc2 Tests

The Hybrid Capture 2 (hc2) HPV tests (Qiagen, GmbH, Hilden, Germany) are based on hybrid capture signal amplification technology, where full-length RNA probes against 13 HR-HPV genotypes (hc2 HPV and hc2 HR-HPV tests) and 5 LR-HPV types (hc2 HPV test) are hybridized to denatured target DNA (Table 3) (64). HPV RNA/DNA hybrids are detected using a sandwich enzyme-linked immunosorbent assay (ELISA)-type reaction. Hybrids are initially captured onto a solid-surface microwell by anti-RNA/DNA hybrid antibodies, and signal amplification is achieved by the binding of multiple-enzyme-labeled anti-RNA/DNA hybrid antibodies to the captured target hybrid. Addition of substrate produces light, which is measured by a manufacturer-supplied luminometer. The readout of the assay is in relative light units (RLU), and patient sample results are compared to values for the internal positive-control calibrator. For samples collected in STM, the sample RLU/positive-control RLU ratio of ≥1.0 is considered positive for the detection of the HR-HPV types included in the assay. LBC samples with a ratio of >2.5 are considered positive. Samples with RLU ratios between 1.0 and 2.5 are recommended for repeat testing, though reproducibility will be inherently poor due to stochastic sampling error in samples with low concentrations of HPV DNA. A positive result is indicative of ≥5,000 HPV copies/ml in the original sample. hc2 can be performed manually or using the automated Rapid Capture system (Qiagen).

The hc2 test has been extensively evaluated in multiple population-based screening studies and randomized clinical trials (94) and has been shown to have a very high sensitivity (pooled sensitivity = 99.4% [94]). While the hc2 test targets only 13 HR-HPV types, the probe pool cross-reacts with several LR-HPV genotypes (95, 96) and HR type 66. The cross-reactivity affects analytic specificity (97) but has only a modest impact on the clinical specificity and may increase the sensitivity compared to those of less cross-reactive tests, specifically if cases are defined as CIN2 or more severe (97). The hc2 test does not contain an internal control for specimen adequacy, increasing the risk for false-negative results. However, the rate of false negatives in cervical samples tested by assays which include a human DNA control is usually reported as <1%. In FDA clinical trials, hc2 results were compared to those of colposcopy and consensus histology. hc2 sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the detection of CIN2+ were 93%, 61.1%, 17.2%, and 99.9%, respectively (64).

Cervista HR-HPV Test

The Cervista HR-HPV test (Hologic, Madison, WI) detects but does not distinguish the DNA from 14 HR-HPV types (Table 3) (65). The Cervista HR-HPV test is based on signal amplification with the use of Invader chemistry. The test can be performed manually or using the Cervista high-throughput automation (HTA) platform (Hologic). The test includes an internal control for human histone DNA. In clinical trial studies for FDA approval, results obtained with the Cervista HR-HPV test were compared to those of colposcopy and consensus histology for the detection of CIN2+ and CIN3+. Assay sensitivity, specificity, PPV, and NPV for the detection of CIN2+ were 92.8%, 44.2%, 8.3%, and 99.1%, respectively, and for the detection of CIN3+ were 100%, 43.0%, 2.9%, and 100%, respectively (65, 98). Analytical positive percent agreement (PPA) and negative percent agreement (NPA) with the results of a composite comparator (hc2 testing and PCR sequencing) for a population with atypical squamous cells of undetermined significance (ASC-US) were 95.5% and 89.4%, respectively, and for a population with cytology results negative for intraepithelial lesion or malignancy in women 30 years or older (NILM 30+) were 94.4% and 84.2%, respectively. Several additional studies have demonstrated that the analytic performance and clinical performance are comparable to those of hc2 (99, 100).

cobas HPV Test

The cobas HPV test detects the DNA of 14 HR-HPV types, with specific identification of HPV-16 and HPV-18, and an internal cellular control in a single analysis (Table 3) (66, 101). Specimen preparation is automated with the use of the cobas ×480 instrument. The cobas 4800 system utilizes real-time PCR to amplify a sequence of approximately 200 nucleotides (nt) within the polymorphic L1 region and a 330-nt region of the human β-globin gene. One fluorescent dye is used to detect the amplicons from 12 HR-HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), while HPV-16, HPV-18, and β-globin signals are each detected with their own dedicated fluorescent dyes. In clinical trial studies for FDA approval, results obtained with the cobas assay were compared to those of colposcopy and consensus histology for the detection of CIN2+ and CIN3+ (66, 102, 103). Assay sensitivity, specificity, PPV, and NPV for the detection of CIN2+ were 90.0%, 70.5%, 14.0%, and 99.2%, respectively, and for the detection of CIN3+ were 93.5%, 69.3%, 8.4%, and 99.7%, respectively (66). Analytical PPA and NPA with a composite comparator (hc2 testing and PCR sequencing) for an ASC-US population were 97.8% and 95.7%, respectively, and for a population with a cytology NILM 30+ were 96.3% and 82.6%, respectively (104, 105).

Aptima HPV Assay

The Aptima HPV assay (Hologic/Gen-Probe, San Diego, CA) detects E6/E7 mRNA from 14 HR types (Table 3) (67). The assay is fully automated on the Tigris and Panther platforms (Hologic). The three-step test process takes place in a single-tube format. E6/E7 mRNA is isolated using an HPV-specific target capture process, the mRNA is amplified by transcription-mediated amplification (TMA), and detection of the amplification products is performed using the hybridization protection assay (HPA). The assay incorporates an internal control to monitor nucleic acid capture, amplification, and detection. The internal control and target mRNA are distinguished using a dual-kinetics assay. In clinical trial studies for FDA approval, results obtained with the Aptima HPV assay were compared to those of colposcopy and consensus histology for the detection of CIN2+ and CIN3+ (106). Assay sensitivity, specificity, PPV, and NPV for the detection of CIN2+ were 86.8%, 62.9%, 20.1%, and 97.8%, respectively, and for the detection of CIN3+ were 90.2%, 60.2%, 9.4%, and 99.3%, respectively (67). Analytical PPA and NPA with the results of a composite comparator (hc2 testing and reverse transcription [RT]-PCR sequencing) for an ASC-US population were 97.8% and 199%, respectively, and for a population with cytology NILM 30+ results were 93.2% and 80.8%, respectively. Overall, the Aptima HPV assay demonstrated sensitivity comparable to those of DNA-based assays with slightly more specificity for detection of clinically relevant disease (107–114).

Comparison of HPV Assays for the Detection of CIN2+ and CIN3+

Numerous clinical studies have compared the performances of the various HPV assays to that of hc2 in a variety of clinical populations and in regions of varying disease prevalence. Detailed review is beyond the scope of this chapter. However, two large studies compared the performances of multiple tests (FDA approved and for research use only [in the United States]) in women with abnormal cytology (112, 113) and in one study of a screening population (112). The Predictors 1 study compared the performances of 7 tests (hc2, Amplicor [Roche], PreTect HPV-Proofer [NorChip, Klokkarstua, Norway], Aptima HPV assay, Linear Array [Roche], clinical arrays [Genomica, Madrid, Spain], and CINtec p16 immunocytochemistry) for the detection of high-grade CIN in a population referred to colposcopy because of abnormal cytology results (112). In total, 953 women participated in the study, and testing was performed using specimens collected in PreservCyt transport medium. The Predictors 2 study evaluated seven tests (hc2, cobas HPV, PreTect HPV-Proofer, Aptima HPV, Abbott Real*Time* HPV [Abbott Molecular GmbH & Co., Wiesbaden, Germany], BD HPV [BD Diagnostics, Sparks, MD, USA], and CINtec p16) using samples in PreservCyt transport medium collected from 1,099 women referred for colposcopy (113). A recent study by Cuzick et al. compared the performances of six HPV tests, including four DNA-based tests (Abbott Real*Time*, hc2, cobas HPV, BD HPV) and two RNA-based detection assays (Aptima and PreTect HPV-Proofer), in a screening population (114). Residual PreservCyt samples were tested according to each manufacturer's protocol. Each study evaluated the clinical sensitivity, clinical specificity, PPV, and NPV of each assay for the identification of high-grade CIN. Overall, in all three studies, the mRNA-based Aptima assay and the DNA-based assays, with the exception of Genomica clinical arrays, demonstrated high sensitivity for the detection of CIN2+ and CIN3+. In all three studies, the PreTect HPV-Proofer assay demonstrated significantly lower clinical sensitivity, since the assay detects only E6/E7 mRNA of 5 of the most common 14 HR-HPV types associated with CIN2+. Conversely, the PreTect HPV-Proofer demonstrated the highest specificity and PPV, followed by the CINtec p16 and Aptima assays. Using the data from the Predictors 1 and 2 studies, Mesher et al. (84) evaluated triage testing for the detection of CIN2+ and CIN3+ in women with borderline or single mildly dyskaryotic cytology smears. Four HPV DNA assays (hc2, Abbott Real*Time* PCR, BD HPV test, cobas 4800) and one HPV RNA assay (Aptima) had sensitivities of 99% to 100% for the detection of CIN3+ and despite a lower specificity could reduce referrals to colposcopy by 20 to 30% (84). Specificity could be improved by another 20 to 30% if only women positive for HPV-16 or p16^{INK4a} were referred for colposcopy, although sensitivity for CIN2+ was slightly decreased. Less sensitive triage tests, such as PreTect HPV-Proofer and CINtec p16, could reduce the number of unnecessary referrals further by 15 to 25% but miss 15 to 30% of CIN3+ women. These studies confirm that both the DNA-based and the Aptima RNA-based assay can provide high sensitivity for detection of high-grade disease and that the use of RNA-based assays and CINtec p16 can improve the specificity of the HPV testing.

FDA-Approved HPV Genotyping Systems

One of the most characteristic features of HPV infection is the multiplicity of genotypes known to infect the anogenital tract and oral cavity. Complete genotyping has been incredibly useful as a research tool to evaluate the natural history of all genotypes in epidemiological studies; however, the clinical utility is limited. Early natural history studies of HPV utilized quite successfully laboratory-developed versions of the now commercially available assays, as well as alternative primer systems, such as MY09/11 and GP5+/6+. Because of the high degree of similarity among the HPV genotypes, assays utilizing target-specific probes are likely to be more specific than those based on SYBR green dye intercalation. Performance comparable to that of the commercial tests has been demonstrated in only a few highly experienced laboratories. Due to the complexity of HPV genotyping, the use of laboratory-developed assays is strongly discouraged unless there is adequate demonstration of quality control for each genotype targeted.

Direct sequencing of consensus primer PCR products is limited in utility to samples with a single type of infection. Discrimination of HPV sequences from infections with multiple HPV types, which are common, is not possible by standard sequencing technologies. However, deep sequencing, next-generation sequencing, and also mass spectrometry have the potential to provide the means for more-comprehensive genotyping.

Presently, the American Society for Colposcopy and Cervical Pathology provides recommendations for specifically identifying only HPV-16 and HPV-18 (Fig. 2) (115). The utility of discriminating HPV-16 and HPV-18 stems from long-term population-based screening studies which have found that HPV type 16 or 18 detection confers a significant increased risk for progression to CIN2+ within the 2 to 3 years subsequent to detection compared to risks after detection of the other carcinogenic HPV types (116). In addition, HPV types 16, 18, and also type 45 account for the majority of adenocarcinomas (AdCa), which are located internally and often missed by cytologic screening, whereas HPV DNA is frequently detected years prior to the AdCa diagnosis. These data led to the current guidelines for reflex HPV type 16/18 genotyping of HR-HPV DNA test results among cytologically normal women (Fig. 2).

The Cervista HPV genotyping assay (Hologic) identifies and differentiates HPV-16 and -18 (98, 117, 118). The Aptima HPV genotyping assay (Hologic) detects HPV-16, -18, and -45 and differentiates type 16 from types 18 and 45 (119). Both assays are FDA approved for clinical use as a reflex test for samples HR-HPV positive by the Cervista HR-HPV test and the Aptima HPV assay, respectively. The cobas 4800 system tests simultaneously for a pool of the 12 HR-HPV types and separately for HPV-16 and HPV-18 (66, 101, 102, 120).

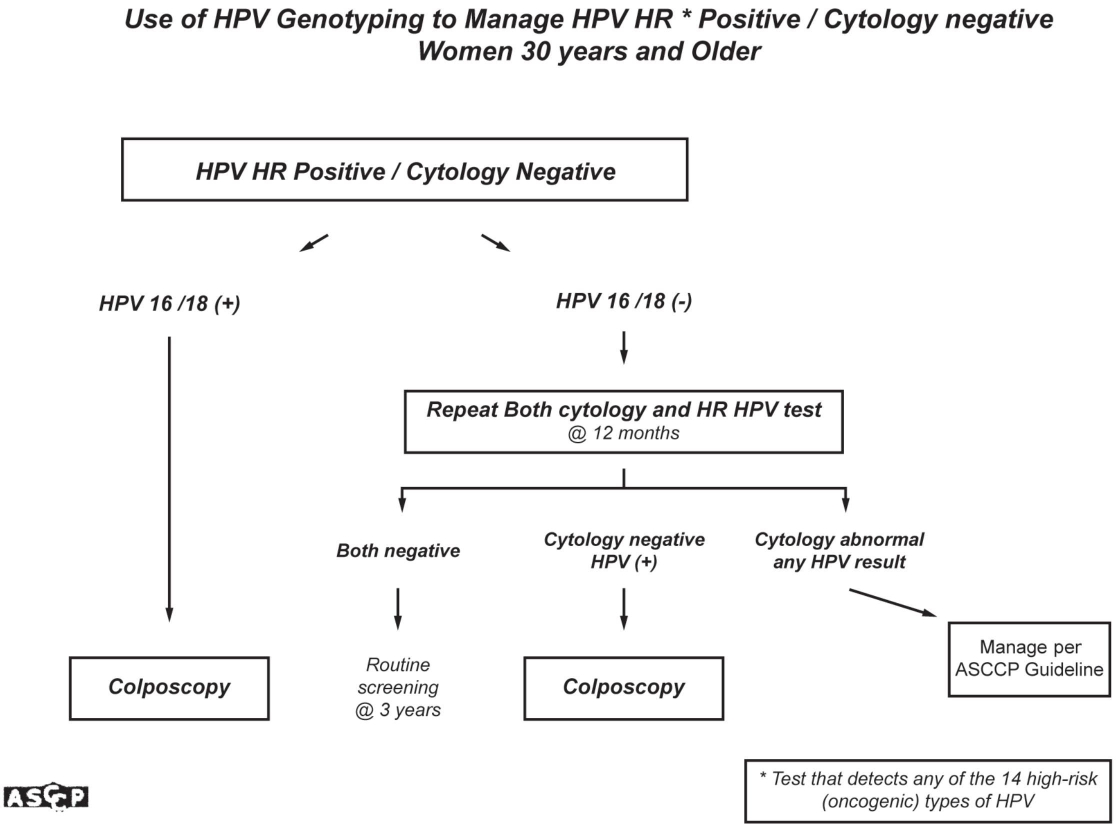
Use of HPV Genotyping to Manage HPV HR * Positive / Cytology negative Women 30 years and Older
HPV HR Positive / Cytology Negative
HPV 16 /18 (+)
HPV 16 /18 (-)
Repeat Both cytology and HR HPV test
@ 12 months
Both negative
Cytology negative HPV (+)
Cytology abnormal any HPV result
Colposcopy
Routine screening @ 3 years
Colposcopy
Manage per ASCCP Guideline
* Test that detects any of the 14 high-risk (oncogenic) types of HPV
ASCCP

FIGURE 2 American Society for Colposcopy and Cervical Pathology 2006 consensus conference recommendations for HPV type 16 or 18 detection (from http://www.asccp.org/pdfs/consensus/clinical_update_20090408.pdf). doi:10.1128/9781555817381.ch104.f2

Research-Use-Only HPV Genotyping Assays

Numerous research-use-only genotyping systems that utilize a variety of methodologies (Table 6) are available for the detection of HR HPV, LR HPV, and types of yet-undetermined significance. The systems are generally based on consensus L1 or E1 PCR amplification of a broad spectrum of HPV genotypes in a single reaction mixture using a pooled HPV primer cocktail and genotype discrimination by reverse hybridization of labeled PCR products to a spectrum of strip-immobilized type-specific probes (e.g. Linear Array [Roche], INNO-LiPA, [Innogenetics/Fujirebio Europe, Ghent, Belgium]), DNA arrays (e.g. PapilloCheck [Greiner Bio-One GmbH, Frickenhausen, Germany]), bead-based arrays (e.g. GeneFinder [Innomeditech, Seoul, South Korea]), and multiplex PCR using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (PCR-MS) (e.g., MassARRAY [Sequenom, San Diego, CA]) (121, 122). Many include an internal control that targets a human housekeeping gene, such as β-globin.

Some type-specific differences in amplification efficiency have been noted for several tests. Because ~30% of HPV-positive women will have two or more genotypes present in a single sample, assays that detect a wide range of genotypes may provide more-realistic type-specific prevalence estimates for population-based surveys and post-HPV vaccine surveillance. Additionally, the selection of a genotyping test is dependent on the source material. For example, the large fragment amplified by the Linear Array test (450 bp) prohibits its utility for HPV typing from fragmented DNA, such as that found in FFPE tissues. The small fragments amplified using the INNO-LiPA SPF10 primers are ideally suited for typing from the fixed tissues.

Alternative Molecular Markers and Methylation of Viral DNA

Testing for HPV infection is very sensitive, although not very specific for the detection of high-grade cervical neoplasia, compared with cytology. New research has been focused on examining the role of human gene methylation as a biomarker for the triage of women testing positive for HR-HPV infection. A recent literature review in 2012 provided evidence of the potential utility of the detection of methylated viral DNA to ascertain a woman's increased risk of high-grade cervical intraepithelial neoplasia of grade 2 or higher (CIN2+) and to differentiate HR-HPV-positive women with and without clinical CIN2+ (123). A recent study in Tanzania found that a 5% increase in DNA methylation of paternally expressed gene 3 (PEG3) was associated with a 1.6-fold-higher risk of invasive cervical cancer. Overall, data have been relatively consistent in showing a higher risk of CIN2+ among women with methylation of CpG sites of the L1 gene and less consistent in showing methylation of CpG sites in the upstream regulatory region (123). Predictors 1 and 2 analyzed DNA methylation levels in the L1 and L2 regions of HPV-16, HPV-18, and HPV-31 and in the EPB4L3 and DPYS human genes among 1,711 women and 582 CIN2/3 cases (124, 125). There will be an emerging literature on the role of epigenetics as potential diagnostic tools that merit further observation.

ISOLATION PROCEDURES

HPV is not culturable, and therefore, the detection and genotyping of HPV are predominantly dependent on molecular tests that target either HPV DNA or mRNA.

SEROLOGIC TESTS

Serologic detection of HPV antibodies is a marker of cumulative exposure and thus cannot determine the site of infection or time of infection. Research-use-only assays demonstrated limited sensitivity, detecting antibodies in only ~60% of women with DNA from the same type of HPV (126). Therefore, the utility of serologic detection of HPV in clinical practice is extremely limited. With the availability of type-specific prophylactic HPV vaccines that lack therapeutic benefit, both patients and some providers may wish to use antibody testing to evaluate susceptibility prior to making a choice to vaccinate. Because of the described problems with HPV serology, particularly in the context of low-titer antibodies produced during natural infection, this practice is strongly discouraged.

There are currently no commercially available serologic tests for the detection of HPV-specific antibodies. However, the use of prophylactic HPV vaccines has precipitated standardization of serologic assays for immunogenicity testing by the vaccine manufacturers. The relative strengths and weaknesses of the three most common assays used to measure serum HPV antibodies (i.e., ELISA, competitive Luminex immunoassay, and neutralization) have been recently reviewed (127).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The goal of HPV testing is to identify individuals at high risk of CIN2+ lesions as part of either routine cervical cancer screening or posttreatment management for prevention of recurrent disease. At present, the four FDA-approved assays have been widely applied, showing reproducibly high sensitivity and negative predictive value when used for the detection and treatment of CIN2+ disease in cervical cancer screening. Detection of the presence or absence of a pool of HR-HPV genotypes is sufficient for the currently approved clinical indications (61, 62, 63). The exception is the possible added positive predictive value in identifying the presence of HPV types 16, 18, and 45 from other HR-HPV types. Broad-spectrum genotyping assays are useful primarily for research purposes, including postvaccination genotype distribution surveillance. The detection and reporting of LR-HPV genotypes have no clinical value. Laboratorians should encourage following the current testing guidelines with respect to patient age, cotesting, reflex testing, and testing frequency.

The Future of HPV Testing

The question that remains is "can HPV testing replace cytology as a primary cervical screening method for the detection of CIN2+ and CIN3+ in women 30 years and older?" In 2009, international guidelines for HPV DNA test requirements for primary cervical cancer screening in women 30 years and older stated that candidate HPV tests must offer an optimal balance between clinical sensitivity (not less than 90% of the clinical sensitivity of hc2) and specificity (not less than 98% of the specificity of hc2) for CIN2+ or higher CIN (90). Assays must be robust and highly reliable and should display inter- and intralaboratory reproducibility. A recent study by Ronco et al. using follow-up data from four European randomized controlled trials determined that HPV-based screening provided 60 to 70% greater protection against cervical cancer than cytology (128). Additionally, the data confirmed that screening intervals can be extended to at least 5 years. In April 2014, based on the data from the ATHENA HPV study and a recommendation from the U.S. FDA advisory panel, the cobas HPV test, not cytology, was approved as a primary screening tool for cervical cancer in women 25 years and older.

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Human Polyomaviruses

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TAXONOMY

Because of the discovery of a significant number of new human polyomaviruses (HPyVs), the taxonomy of these viruses has been in flux since the last edition of this *Manual*. The polyomaviruses were formerly members of the *Papovaviridae* family but have been reclassified into the family *Polyomaviridae*, which has a single genus, *Polyomavirus*. The *Polyomaviridae* Study Group has proposed that the family now be divided into three genera, i.e., *Orthopolyomavirus*, *Wukipolyomavirus*, and *Avipolyomavirus*, with mammalian and human viruses found in the first two genera and bird viruses in the third (1). The recognized human species *BK polyomavirus* (BKPyV), *JC polyomavirus* (JCPyV), *Merkel cell polyomavirus* (MCPyV), *Human polyomavirus 9* (HPyV9), and *Trichodysplasia spinulosa-associated polyomavirus* (TSPyV) would reside in the genus *Orthopolyomavirus*, along with the type species for the genus, the primate virus *Simian virus 40*. The proposed genus *Wukipolyomavirus* would contain the human type species *KI polyomavirus* (KIPyV) and the HPyVs *WU polyomavirus* (WUPyV), *Human polyomavirus 6* (HPyV6), and *Human polyomavirus 7* (HPyV7). Characteristics of other recently discovered HPyVs, including *Malawi polyomavirus* (MWPyV) (2), *St. Louis polyomavirus* (STLPyV) (3), and *Human polyomavirus 12* (HPyV12) (4), suggest that recombination events have generated these viruses, as well as some of the other HPyVs, making their placement in the proposed taxonomy problematic (3). It is likely that changes to the proposed polyomavirus taxonomy will be forthcoming.

Because some of the novel HPyVs were discovered nearly simultaneously in more than one laboratory, different names for what appear to be the same virus, or strains of the same virus, can be found in the literature. For example, the novel viruses MxPyV and HPyV10 (5, 6) appear to be the same species as MWPyV (2), with current usage favoring the name "MWPyV," as it was the first reported. Additionally, in the previous edition of this *Manual*, *Lymphotropic polyomavirus* (LPyV), a strain of *African green monkey polyomavirus*, was listed here because antibodies to that virus were reported in humans, suggesting infection by LPyV or a closely related virus. It now appears that HPyV9 is likely the closely related virus responsible for those findings (7).

From a diagnostic perspective, further subclassification of polyomaviruses is primarily important for BKPyV. BKPyV was originally divided into six subtypes based on VP1 gene sequences. However, whole-genome sequence data support the existence of four subtypes (I to IV), with two subtypes reclassified as subgroups of subtype I (8). Subtypes vary in prevalence and geographic distribution (9). Subtype I is most prevalent and is found worldwide; subtype IV is second most prevalent and is found mostly in Asia and Europe. Subtypes II and III are uncommon and are found throughout the world. Subtype-dependent quantification bias has been observed and can contribute to interassay differences in quantification (see "Nucleic Acid Amplification Methods" below).

DESCRIPTION OF THE AGENTS

The polyomaviruses are small (40- to 45-nm diameter), icosahedral, nonenveloped viruses with a circular double-stranded DNA genome. The size of the genome is relatively small, with all of the human viruses, including the newly identified members and SV40, falling within the range of ~4,900 to ~5,400 bp. JCPyV, BKPyV, and SV40 have been extensively characterized. The newly characterized viruses have been less well studied.

The genome is organized into an early region that is transcribed prior to DNA replication and a late region transcribed after DNA replication. The noncoding control region (NCCR) separates the early and late regions and includes the origin of replication (10).

Early region transcripts code for the large and small T antigens, nonstructural proteins that regulate viral replication, control of viral transcription, induction of host cell division, and transformation. Late gene transcripts code for viral capsid proteins VP1, VP2, and VP3, of which VP1 is the major capsid protein, comprising ~80% of the capsid and being the only surface-exposed protein (10). The late regions of JCPyV, BKPyV, and SV40 also code for a fourth late protein termed the agnoprotein, the function of which is incompletely understood, but which appears to be a nonstructural protein involved in viral assembly and release from infected cells (10).

The genomic organization of the newly identified polymaviruses appears to be similar to those previously described, with open reading frames identified for the T antigens and VP1, VP2, and VP3 (2–4, 11). These viruses appear to differ from JCPyV and BKPyV in that there is no agnoprotein open reading frame (2–4, 11).

The NCCRs of the human viruses BKPyV and JCPyV are hypervariable sequences of ~300 to ~500 bp in length. The NCCR contains the origin of replication and promoters

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for early and late gene transcription. The variable nature of the NCCR is apparent when sequences of this region are compared between viruses obtained from different anatomical sites. The NCCR sequence from kidney and urine isolates is referred to as the archetype. Viruses with archetype NCCR sequences do not replicate well in cell cultures and may be the infectious form of the virus.

EPIDEMIOLOGY AND TRANSMISSION

Both BKPyV and JCPyV appear to infect the majority of humans by adulthood. Seroepidemiology studies reveal that 50% of children acquire anti-BKPyV antibodies by the age of 3 years, with the 50% prevalence for JCPyV being reached by age 6 years (12). For all age groups, the seroprevalence for BKPyV ranges from 55 to 85%, with similar numbers for JCPyV, although there is more variability in the age of seroconversion for JCPyV relative to BKPyV (13). The mechanism of BKPyV and JCPyV transmission is largely speculative, in part because primary infection does not appear to cause clinical illness, although there are reports of signs and symptoms such as fever or respiratory illness accompanying seroconversion (13). Respiratory and oral transmission has been hypothesized, since JCPyV can infect tonsillar cells (14) and has been detected in tonsillar tissue (15). However, studies of oral and respiratory specimens have failed to detect BKPyV and JCPyV (16, 17). Uro-oral transmission has been hypothesized, and circumstantial evidence consists of intermittent BKPyV and JCPyV excretion in urine of immunocompetent and immunocompromised individuals (13, 18), detection of BKPyV and JCPyV in sewage samples (19–21), and the stability of these nonenveloped viruses. Even less is known about the epidemiology and transmission of the newly identified HPyVs. WUPyV and KIPyV are found in respiratory secretions and are widely distributed around the globe (22–30). WUPyV has been detected in respiratory secretions of a 1-day-old infant, raising the possibility of *in utero* or intrapartum infection (28). Studies have also detected WUPyV in small numbers of serum and stool specimens (31, 32). MCPyV is also found in respiratory specimens (33, 34), and seroprevalences of 50 and 80% in children and adults have been documented, suggesting that infection is common (35).

Epidemiological studies of the more recently identified HPyVs are in their infancy, with initial seroepidemiological studies suggesting that infection with these viruses is also common, with seroprevalence low in children and rising in adulthood to 35 to 75% depending on the virus (36). Caution must be exercised in the interpretation of these findings, as cross-reactions are possible among the polyomaviruses and it is likely that unidentified HPyVs exist that could confound the interpretation of seroprevalence studies (36).

Humans were exposed to the nonhuman primate polyomavirus SV40 between 1955 and 1963 when some incompletely formalin-inactivated lots of polio vaccine were found to be contaminated. Monkey kidney cell cultures used to prepare the vaccine were found to be the source of contamination. About 200 cases of paralytic polio occurred due to the presence of infectious poliovirus in the vaccine. Although ~100 million Americans were vaccinated during this period, it is not known how many of the lots contained infectious SV40 or how many people became infected with SV40 as the result of this accident (37).

CLINICAL SIGNIFICANCE

Polyomaviruses cause a number of diseases in different patient populations (Table 1). Progressive multifocal leukoencephalopathy (PML) is a rare disease of the central nervous system (CNS) caused by infection and destruction of myelin-producing oligodendrocytes by JCPyV. Focal demyelination in white matter can be visualized by neuroimaging studies such as magnetic resonance (MR) imaging (Fig. 1). Neurological signs and symptoms include gait or other motor disturbances and cognitive abnormalities (38). Prognosis is poor, with long-term survival defined as >12 months (10). Prior to the development of highly active antiretroviral therapy (HAART), PML was primarily seen in severely immunocompromised HIV-infected individuals. With the advent of HAART, PML has become less common in the HIV-infected patient population but has now appeared in patients treated with immunomodulatory agents, in particular multiple sclerosis patients treated with natalizumab. PML is still uncommon in this group (incidence, ~2/1,000 patients treated), but three risk factors are recognized: duration of natalizumab treatment, prior use of immunosuppressives, and positive JCPyV antibody status (39, 40). Prior to effective anti-HIV therapy, mortality from PML was nearly 100%, but it has now fallen to about 50%. Mortality for PML induced by immunomodulatory agents is lower, although there can be severe neurological sequelae (41). Other CNS diseases such as cerebral atrophy and granule cell neuronopathy appear to be rare forms of JCPyV infection (41).

BKPyV has tropism for uroepithelial cells and can cause a spectrum of presentations. Asymptomatic shedding can occur in urine of normal individuals (especially during pregnancy and in the elderly) and immunocompromised patients. Polyomavirus-associated nephropathy (PVAN) occurs in immunocompromised patients and is the result of BKPyV replication in, and the destruction of, renal tubular epithelial cells. It occurs most commonly in renal transplant recipients (42). BKPyV has also been described as a rare cause of nephropathy in other settings of immunocompromise such as HIV, hematologic malignancy, hematopoietic stem cell transplant, transplant of organs other than kidney, and congenital immunodeficiency (43).

In PVAN, increasing levels of virus are initially found in urine, followed by viremia and then development of the disease (44). Without clinical intervention, PVAN can result in renal graft dysfunction and, ultimately, graft loss (45). In a prospective study of 78 renal transplant recipients, the probability of developing PVAN was 8% (46). Renal transplant patients may have a propensity to develop PVAN due to the use of more-aggressive immunosuppression regimens (45), BKPyV acquisition from the donor kidney, or HLA-mediated predisposition (47, 48). PVAN is treated by reducing immunosuppression; certain antiviral compounds have also been used (44, 49, 50). JCPyV has also been reported to rarely cause PVAN (51, 52).

Other pathological conditions associated with BKPyV include hematuria, ureteral stenosis, and hemorrhagic cystitis. Hemorrhagic cystitis is due to BKPyV infection of the bladder epithelium. It occurs most commonly in the setting of stem cell transplantation and is seen less frequently in other immunocompromised states, including HIV infection. Allogeneic stem cell recipients are at particular risk, and disease occurs several weeks after transplant, differentiating it from chemotherapy-associated disease, which generally occurs immediately. Hemorrhagic cystitis is associated with significant morbidity but is rarely fatal (53). The risk of developing the disease has been shown to correlate with increased levels of BKPyV viruria (54) and viremia (55).

TABLE 1 Diseases associated with HPyV

Virus(es)	Disease association	Comment(s) (reference[s])
BKPyV	PVAN	Seen primarily in kidney transplant recipients (42); occurs rarely in other immunocompromised hosts (HIV, hematologic malignancy, hematopoietic stem cell transplant, transplant of organs other than kidney, and congenital immunodeficiency)
	Hemorrhagic cystitis	Observed primarily in BMT recipients, with a prevalence of 10–25% in this population (53)
	Rare associations	CNS infections and pneumonia have been reported (43)
JCPyV	PML	Observed in patients with compromised cellular immunity (HIV/AIDS, malignancy, and immunomodulatory therapies used to treat multiple sclerosis and psoriasis (161–164)
	PVAN	Associated with rare cases of BKPyV-negative PVAN (51, 52)
	Rare associations	Other CNS diseases such as cerebral atrophy and granule cell neuronopathy have been reported (41)
WUPyV and KIPyV	No firm disease association	Detected in respiratory secretions, stool specimens, and blood (22–28, 31, 32, 56)
MCPyV	Merkel cell carcinoma	Associated with 75–88% of Merkel cell carcinoma tumors (58, 165, 166); MCPyV has also been detected in respiratory specimens, suggesting a mode of acquisition (33, 34)
HPyV6 and HPyV7	No firm disease association	Detected on the skin of 11–14% of the healthy individuals tested (61)
TSPyV	Trichodysplasia spinulosa	TSPyV has been associated with trichodysplasia spinulosa from various geographic locations (65, 67, 167–169), although viral DNA has also been rarely detected in normal tissues (65)
HPyV9	No firm disease association	Initially identified in the serum of a kidney transplant patient and subsequently found in rare specimens from other immunocompromised patients. The close relationship of HPyV9 to the nonhuman primate virus LPyV may explain the occurrence of anti-LPyV antibodies in human sera (7)
MWPyV	No firm disease association	Initially identified in the stool from a Malawian child with diarrhea and subsequently in stools from the United States (2). May be the same viral species as those initially reported as MxPyV from stool (6) and as HPyV10 from warts from an immunocompromised patient (5)
STLPyV	No firm disease association	Initially identified in the stool from a Malawian child and subsequently in ~1% of stools tested from the United States and Gambia (3)
HPyV12	No firm disease association	Initially identified in liver tissue from a cancer patient and subsequently in other liver specimens and rare colon, rectum, and stool specimens from similar patients (4)
SV40	No firm disease association	Primate virus found to contaminate polio vaccine (37, 69–71)

Treatment is supportive, although the use of select antiviral drugs has been attempted (53). Rarer examples of BKPyV diseases in immunocompromised patients, including HIV infection and posttransplant immunosuppression, include disseminated disease, CNS infections, retinitis, colitis, vasculitis, and pneumonia (43).

Attempts to link the newly identified HPyVs with specific human diseases are in their infancy. KIPyV and WUPyV have not been firmly associated with human disease (56). WUPyV DNA and antigens were found in a 2-year-old bone marrow transplant (BMT) patient who died of multiorgan disease (57); a definitive link between virus infection and disease awaits further study.

Merkel cell carcinoma is a rare, highly aggressive human neuroepithelial tumor of the skin noted for its high mortality. In 2008, using molecular viral discovery techniques, a novel polyomavirus, subsequently named MCPyV, was identified in specimens from this cancer (58) (Table 1). Subsequent studies have demonstrated MCPyV in ~80% of Merkel cell carcinoma samples examined (59, 60), although MCPyV has also been detected in skin samples from healthy individuals (60), with one report indicating that MCPyV is chronically shed from the skin of healthy individuals as intact virions, suggesting a mode of transmission (61). The fact that MCPyV infection appears to be ubiquitous and Merkel cell carcinoma is uncommon suggests that other factors, such as immune suppression, sun exposure, female sex, and age >60 years, contribute to the development of this cancer (60, 62).

Trichodysplasia spinulosa is a rare skin disease that occurs only in immunocompromised individuals (60). In 2010, a novel HPyV, subsequently named TSPyV, was identified in specimens from a heart transplant recipient with the disease (63), confirming an earlier report of polyomavirus-like particles seen in electron micrographs (64). High viral loads and the presence of viral proteins in trichodysplasia spinulosa lesions suggest that active TSPyV replication is taking place in diseased tissues (65). As with some of the other HPyVs, TSPyV infection appears to be ubiquitous, with seroepidemiological studies indicating a low seroprevalence in children that rises to ~70% in adults (66, 67). Currently, there is no clear clinical significance that can be ascribed to the remaining novel HPyVs (Table 1), although it is intriguing that many HPyVs, including the

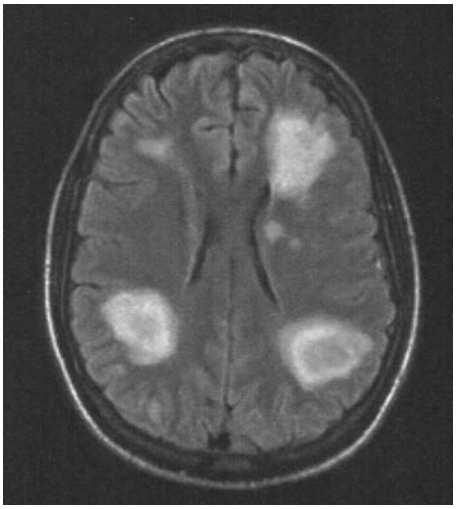

FIGURE 1 MR image showing the brain of a patient with lesions of advanced PML. Lesions are seen bilaterally in frontal lobes and in parieto-occipital subcortical regions. Note that the cortex is spared, with lesions primarily in white matter, consistent with the pathology of demyelination of white matter tracts (fluid-attenuated inversion recovery T2-weighted MR). Reprinted with permission of Lippincott Williams & Wilkins from reference 160. doi:10.1128/9781555817381.ch105.f1

newly identified agents, have been detected in specimens from immunocompromised individuals, including specimens not usually associated with those viruses, such as the detection of TSPyV from nasopharyngeal and stool specimens (68).

Lastly, because of the known ability of polyomaviruses to cause cancer, there have long been concerns that these viruses could be a potential cause of some human cancers. Over the years, there have been many attempts to link SV40 with human cancers, with some of the attempts being reported as successful. However, there has been an inability to reproduce the results of some studies linking particular cancers to this virus due to factors such as contamination with plasmids and serological cross-reactions between SV40 and BKPyV and JCPyV (37). Although debatable, the general consensus today is that, other than the recent case of MCPyV, there is currently no convincing evidence that either human or animal polyomaviruses are a significant cause of human cancers. Readers interested in this subject are referred to several excellent reviews on this topic (37, 69–71).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Although systematic studies of clinical specimens have not been reported for polyomaviruses, as one would expect for these nonenveloped DNA viruses, polyomaviruses appear to be quite stable. A study of JCPyV stability in sewage at 20°C found a t_{90} value (time required for a reduction of 90% of the viral concentration as measured by quantitative PCR) of 64 days and a t_{99} of 127 days, with a greater t_{90} value obtained when the virus was suspended in phosphate-buffered saline (111 days) (72). These data suggest that standard guidelines for the collection, transport, and storage of specimens for viral diagnostics (see chapter 79) should be sufficient for polyomaviruses.

Specimens commonly submitted for the detection of polyomaviruses include blood, cerebrospinal fluid (CSF), urine, respiratory specimens, and tissue. Polyomaviruses can be recovered from peripheral blood mononuclear cells, usually for research purposes. Guidelines for collection and transport of specimens are generally those that apply to molecular testing (see chapter 79 and reference 73). When available, manufacturers' directions for collection, transport, and storage of specimens should be followed. Since nucleic acid detection methods are the primary means by which polyomaviruses are detected in clinical specimens, extracted DNA should be stored in tightly sealed low-binding plastic tubes to prevent evaporation and binding of DNA to the walls of the tubes (73). DNA can be stored in Tris-EDTA buffer for up to 1 year at 2 to 8°C, at −20°C for up to 7 years, or at −70°C or lower for at least 7 years (73).

DIRECT EXAMINATION

Microscopy

Histologic assessment of renal biopsy material is the gold standard for the diagnosis of PVAN (44). PVAN is a tubulointerstitial nephritis that resembles the pathology associated with rejection (42, 74–76). In contrast to rejection, characteristic basophilic, intranuclear viral inclusions are observed in PVAN (Fig. 2) (42, 74, 76). At least two tissue biopsy specimens should be examined prior to ruling out PVAN due to its patchy nature (44, 75). Papanicolaou stain of urine sediment from patients with PVAN reveals abnormal inclusion-bearing cells referred to as "decoy cells" due to their resemblance to malignant cancer cells (Fig. 2) (77).

Antemortem direct microscopic examination of tissue is performed less commonly for PML than for PVAN due to the risks involved in obtaining brain biopsy material. In brain biopsy specimens, PML appears as foci of demyelination containing macrophages, enlarged oligodendrocytes with basophilic or eosinophilic nuclear inclusions, and enlarged bizarre astrocytes with pleomorphic nuclei, typically in the subcortical white matter (Fig. 3) (78, 79).

Electron microscopy (EM) can be used in the diagnosis of PVAN and PML. Intracellularly, polyomaviruses appear as 40-nm-diameter virions packed in paracrystalline arrays or so-called "stick and ball" or "spaghetti and meatballs" structures (Fig. 2 and 3) (80–82). Extracellular BKPyV virions are found by EM in aggregates called "Haufen" in urine of BMT patients (83) and PVAN patients (84). EM may be useful in the diagnosis of Merkel cell carcinoma, as polyomavirus particles have been observed in some cases (85). EM has also been used to demonstrate typical polyomavirus virions in tissue from trichodysplasia spinulosa patients (65).

Antigen Detection

Immunohistochemical staining of tissue sections using antibodies reactive with polyomavirus antigens has been employed as an adjunct to histopathological examination of tissue for the diagnosis of PVAN (42, 81, 86, 87) and PML (82, 88) (Fig. 2 and 3). In the case of PVAN, the demonstration of polyomavirus antigen can help in determining whether PVAN or rejection is the cause of renal pathology. Commercially available antibodies raised against the SV40 large T antigen are most commonly used for this purpose (see http://www.biocompare.com for suppliers). These antibodies are cross-reactive with JCPyV and BKPyV antigens and can be used to detect polyomaviruses but cannot be used to definitively identify which virus is present.

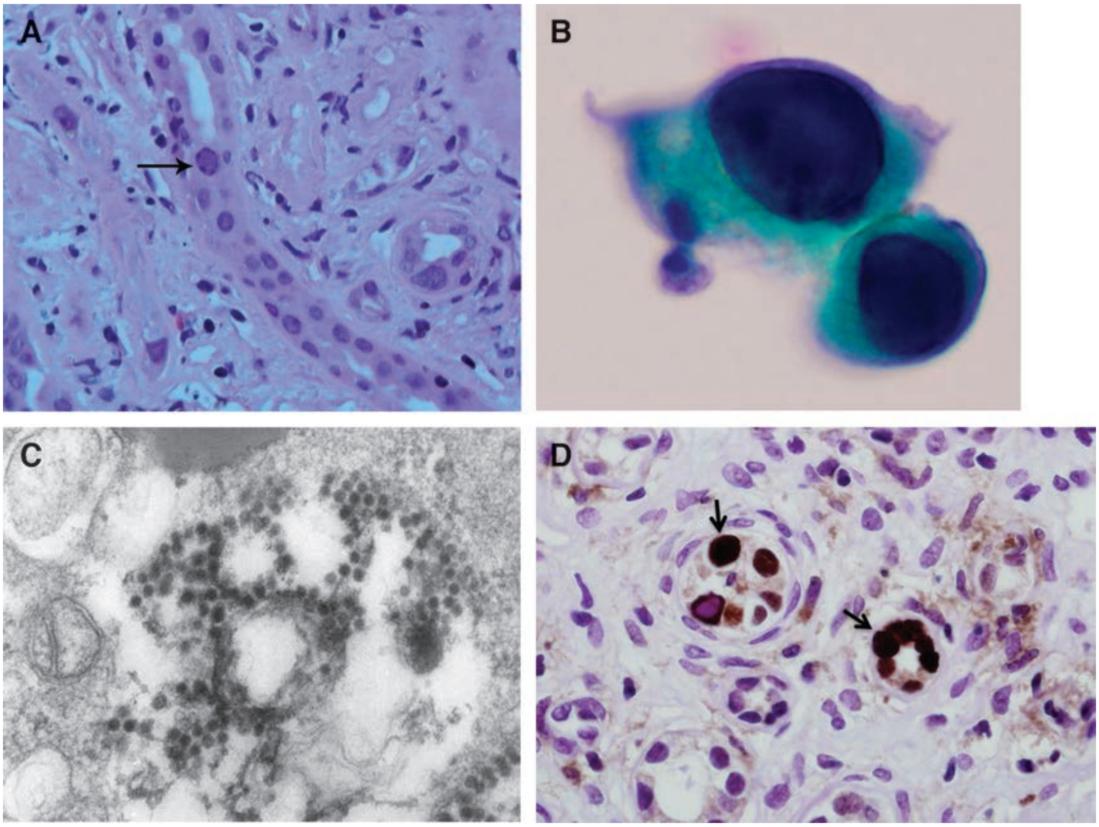

FIGURE 2 PVAN pathology. (A) Photomicrograph of hematoxylin and eosin-stained renal biopsy specimen showing nuclear inclusion (arrow) in epithelial cell in collecting duct (magnification, ×200). (B) Photomicrograph of Papanicolaou-stained decoy cells from the urine of a patient with PVAN demonstrating the typical enlarged basophilic nuclei (magnification, ×400). (C) Electron micrograph of a renal biopsy specimen showing arrays of typical 40- to 45-nm-diameter polyomavirus virions (magnification, ×12,000). (D) Photomicrograph of an immunostained renal biopsy specimen showing staining of homogenous type 1 nuclear inclusions (arrows) in epithelial cells of distal tubes. Images courtesy of Helen Liapis, Washington University.
doi:10.1128/9781555817381.ch105.f2

JCPyV- and BKPyV-specific monoclonal antibodies against respective VP1 proteins are available and can be used for this purpose. For trichodysplasia spinulosa, a lab-developed, noncommercial immunofluorescence assay has been used to detect VP1 proteins of TSPyV in affected tissues (65).

Nucleic Acid Detection

ISH

In situ hybridization (ISH) using probes specific for BKPyV or JCPyV is an adjunct histopathologic method sometimes employed for the laboratory diagnosis of PVAN and PML (Fig. 3) (49, 86, 89–91). The addition of a PCR step may increase sensitivity (90). In the case of PVAN, as is the case for immunohistochemical staining, ISH can aid in discriminating PVAN from rejection by, in this case, localizing the presence of BKPyV nucleic acid to the site of pathology. Biotin- and digoxigenin-labeled BKPyV and JCPyV probes suitable for ISH are commercially available (Enzo Life Sciences, Farmingdale, NY).

Southern Blotting

Although Southern blotting is primarily a research tool, the monoclonal integration of MCPyV in Merkel cell tumors can be demonstrated in genomic DNA extracted from Merkel cell tumors using standard Southern blotting techniques followed by hybridization with MCPyV probes (58, 92).

NAATs

Although there are analyte-specific reagents and research use only reagents, there are currently no Food and Drug Administration (FDA)-approved assays for the detection or quantification of polyomaviruses. Laboratories are therefore left to develop their own tests and confront various issues, including selecting suitable extraction methods, controlling for various biochemical steps in testing, and choosing an amplification method and reagents. Information specific to polyomavirus nucleic acid amplification tests (NAATs) addressing these issues is provided below.

Template Extraction

The ideal performance characteristics for BKPyV and JCPyV NAATs are different. Monitoring for PVAN is usually performed with quantitative NAATs for BKPyV that must be reproducible (yielding results with low coefficients of variation) but not necessarily exquisitely sensitive, as low virus concentrations are not considered significant in most instances. In contrast, extremely sensitive assays for the detection of JCPyV in CSF are desirable for use in the

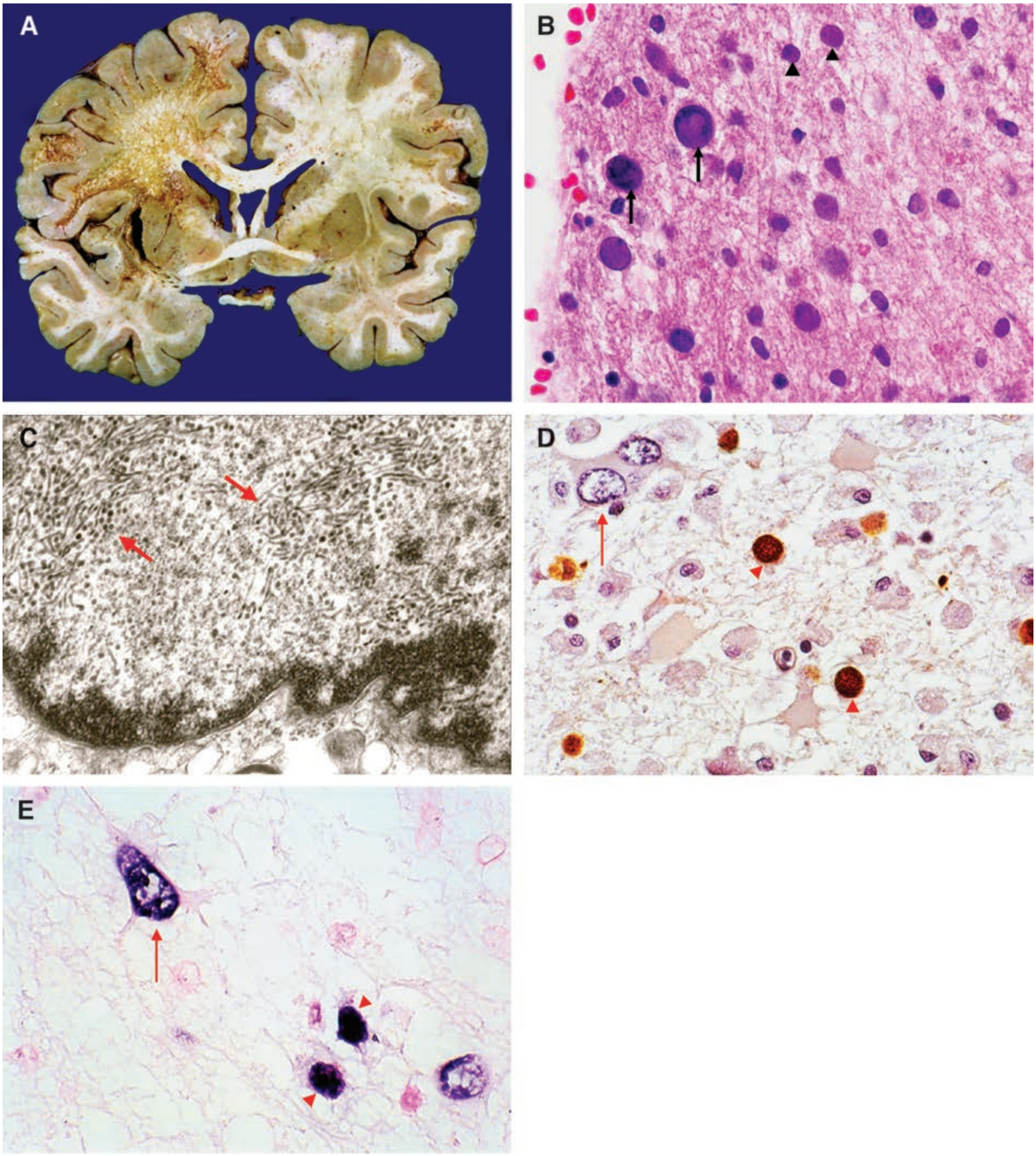

FIGURE 3 PML pathology (A) Gross section of the brain from a patient with PML demonstrating asymmetric focal patches of involvement mostly confined to the white matter. (B) Photomicrograph of hematoxylin and eosin-stained section of brain from a patient with PML showing plum-colored oligodendroglial nuclei (arrows), some with marginated chromatin and inclusions. Infected oligodendrocytes are markedly enlarged compared to more normal-sized oligodendroglia (arrowheads) (magnification, ×400). (C) Electron micrograph of brain from a patient with PML showing the "stick and ball" or "spaghetti and meatballs" (arrows) appearance of JCPyV in an oligodendrocyte (magnification, ×58,000). (D) Photomicrograph of an immunostained brain section demonstrating JCPyV proteins in enlarged immunoreactive oligodendroglial nuclei (arrowhead) but little involvement of atypical astrocytes (arrow) (magnification, ×600). (E) Photomicrograph of ISH using a labeled JCPyV probe showing a positive signal in oligodendrocytes (arrowheads) and an atypical labeled astrocyte (arrow) (magnification, ×1,000). Images courtesy of Robert Schmidt, Washington University. doi:10.1128/9781555817381.ch105.f3

diagnosis of PML. The nucleic acid extraction method should be selected to help meet these different performance criteria.

There exist in the peer-reviewed literature several evaluations of extraction methods that examine the ability of different methods to extract and purify BKPyV DNA from clinical specimens (93–97). In general, there were minimal differences between the automated and manual methods examined for BKPyV-containing specimens, although one study noted a difference in the ability to remove PCR inhibitors from urine between two automated platforms (98) and differences were observed in the relative amounts of hands-on or turnaround time for the different methods.

Cross-contamination between specimens due to the extremely high levels of viruria that can occur is a major concern in BKPyV DNA extraction. No evidence of cross-contamination was observed with an automated extractor, suggesting that instrumentation is a viable option (99). Extraction may not be necessary if small sample volumes can be used in NAATs. Two microliters of unprocessed urine added to a 20-μl PCR reaction produced qualitative and quantitative BKPyV results that were not significantly different from those with samples extracted using a manual spin column method (100). A larger sample volume (5 μl) resulted in inhibition.

Internal Controls

Internal controls are particularly important when testing urine specimens for polyomaviruses. Such controls can be used to detect, but cannot distinguish between, problems that arise during extraction, such as incomplete elimination of amplification inhibitors (the primary concern with urine specimens) or poor nucleic acid extraction efficiency. Chemically modified noninfectious BKPyV virions that can be used as extraction controls are commercially available (ZeptoMetrix, Buffalo, NY). A seven-member inhibition panel consisting of quantified substances recognized as being NAAT inhibitors of reactions is also available (AcroMetrix, Benicia, CA) and can be used during test validation to assess assay robustness in the presence of known inhibitors.

Positive Controls and Standards

All NAAT reactions require the use of positive and negative controls, while quantitative NAATs require an additional set of quantitated standards for use in constructing a standard curve. There are several options available for laboratories seeking controls and standards for use in NAATs for polyomaviruses. There are no commercially available materials for the newly identified polyomaviruses. For these viruses, laboratories can either identify positive specimens that can be used to clone viral DNA and produce controls and standards or contact researchers in the field for materials. BKPyV and JCPyV control materials are commercially available (whole virus and plasmids encoding entire virus genomes [American Type Culture Collection, Manassas, VA], quantified viral DNA [Advanced Biotechnologies, Inc., Columbia, MD], and quantitated inactivated BKPyV virions [ZeptoMetrix]). There are currently no international reference quantitation standards for any of the HPyVs.

Nucleic Acid Amplification Methods

Conventional laboratory methods for viral diagnosis such as cell culture, rapid antigen detection, and serology have significant drawbacks for the laboratory diagnosis of HPyVs, while other methods such as immunohistochemical stains, EM, and ISH are traditionally not performed in microbiology laboratories. Therefore, for the majority of diagnostic microbiology laboratories, nucleic acid amplification will be the method of choice for the detection and/or quantitation of HPyVs in clinical specimens.

PCR has been used almost exclusively as a NAAT for the amplification of HPyVs from clinical specimens, although alternative amplification formats have been described. PCR methods can be classified as having either conventional or real-time formats. With conventional PCR assays, oligonucleotide primers amplify a specific DNA sequence, the products of which are then resolved by agarose gel electrophoresis, Southern hybridization, or microplate hybridization. In the case of real-time PCR, the assay usually includes some type of dye-labeled oligonucleotide that emits a characteristic fluorescence upon binding to the specifically amplified amplicon. Although both conventional and real-time PCR methods are capable of producing qualitative or quantitative results, real-time methods are much better suited for performing quantitative assays through the use of quantitation standards and instrument software. This feature, along with other advantages such as rapidity of results, less hands-on time, and a closed system less prone to contamination issues, has made real-time PCR the method of choice for many laboratories.

PCR assays detecting BKPyV and/or JCPyV using different formats have been described in the literature, including conventional assays with either gel detection (101, 102) or microplate colorimetric hybridization detection (103) of amplified products. Real-time PCR assays performed on the Roche LightCycler platform (104–107) as well as TaqMan probe-based assays (108–110) have also been described. A novel NAAT employing loop-mediated isothermal amplification has also been described for BKPyV (111). In the United States at this time, there are still relatively few commercial NAAT products available for the detection of BKPyV and JCPyV (Table 2).

NAATs for BKPyV and JCPyV are challenging to design due in part to species-specific sequence variation. A recent study of seven different assays employing two different calibrators reported significant variability in quantification related to the calibrators employed and, more importantly, to primer and probe designs. The most important cause of error was nucleotide mismatch between viruses and amplification/detection oligonucleotides, particularly in the case of BKPyV subtypes III and IV (112). An assay using two modified primer/probe sets was better able to accurately quantify all subtypes detected in the study. Another study found significant nucleotide mismatches in up to 31% of the BKPyV strains when primers and probes from five published real-time PCR assays were aligned against 716 sequences, with subtypes IVa, IVb, and IVc being most problematic (113). Another report detailed the use of primers and probes with degenerate bases to improve detection of BKPyV sequence variants (114). Sequence variability may also affect JCPyV NAATs. False-negative NAAT results (proved by amplification of an alternate target) have been reported in an individual with progressive CNS illness and polyomavirus-like particles by EM in brain biopsy material (115). Attention to this issue in the assay design phase is important; broadly reactive real-time PCR reagents and less-variable target sequences have been described (106, 112–114). Additionally, laboratories should align their primers and probes against new sequences at regular intervals to ensure detection of these viruses.

PCR methods have also been published for the newly identified HPyVs, although due to the novelty of these

TABLE 2 Commercial nucleic acid amplification products available in the United States for the detection of JCPyV and BKPyV

Virus(es)	Product (manufacturer)	Comment(s) (regulatory status)[a]
BKPyV	BK Virus r-gene Primers/Probe (bioMérieux, Durham, NC)	Primer/probes for 5′ nuclease real-time assay targeting a sequence in the large T antigen (RUO)
	BK Virus R-gene Quantification Kit (bioMérieux)	Kit for the quantification of BKPyV utilizing 5′ nuclease real-time assay. Includes extraction, inhibition, sensitivity, and negative controls (RUO)
	BKV Primer Pair (Focus Diagnostics, Cypress, CA)	Primers for a real-time assay utilizing a FAM-labeled forward primer amplifying a segment of the VP2 gene (ASR)
	MGB Alert BK Virus Primers and Probe (ELITechGroup, Princeton, NJ)	Separate set of primers and MGB probe, capable of performing a melt curve analysis, targeting a sequence in the VP1 region (ASR)
	MultiCode BK Virus Primers (Luminex, Madison, WI)	Set of primers targeting VP3; combined with use of synthetic bases, allows for amplification and real-time detection (ASR)
JCPyV	JC Virus r-gene Primers/Probe (bioMérieux)	Primer/probes for 5′ nuclease real-time assay targeting a sequence in the large T antigen (RUO)
	MultiCode JC Virus Primers (Luminex)	Set of primers targeting VP3; combined with use of synthetic bases, allows for amplification and real-time detection (ASR)
JCPyV/BKPyV	JC/BK Consensus (bioMérieux)	Amplification and differentiation of JCPyV and BKPyV through PCR and product hybridization to probes in a microwell plate (RUO)

[a] ASR, analyte-specific reagent; FAM, 6-carboxyfluorescein amidite; MGB, minor groove binder; RUO, research use only.

agents relative to JCPyV and BKPyV, much less information is available regarding sequence variation. Conventional PCR methods are available (24, 27, 116), and real-time PCR assays employing TaqMan probes (28, 31, 117, 118) have been reported for WUPyV and KIPyV. Conventional PCR assays (58) and real-time PCR assays employing TaqMan probes have also been published (33, 34) for MCPyV. PCR assays for the more recently discovered HPyVs are also available, including HPyV6 and -7 (61), TSPyV (63, 65), MWPyV (2, 6), STLPyV (3), HPyV9 (7, 119), and HPyV12 (4).

ISOLATION AND CULTURE PROCEDURES

JCPyV and BKPyV can be isolated and propagated in cell culture (120–122), although currently there is no role for these methods in the laboratory diagnosis of HPyV infections. JCPyV demonstrates restricted host range (human cells only) and can be grown in primary embryonic cells, human brain-derived cells (particularly astrocytes and oligodendrocyte precursors), and the permanent cell line SVG. BKPyV has an expanded host range compared to JCPyV and can grow in similar primary cell cultures, primary human embryonic kidney and lung cells, human foreskin fibroblasts, and continuous cell lines (HeLa and monkey cell lines such as CV-1 and Vero). The cytopathic effect of both viruses includes cellular translucency, nuclear enlargement, and gradual loss of the monolayer. Successful propagation often requires weeks and multiple rounds of blind passage. Most virus remains cell associated.

SEROLOGIC TESTS

Serologic tests for HPyV are primarily investigational tools with little role in the routine diagnosis of HPyV disease. The majority of assays are laboratory developed. For JCPyV and BKPyV, two types of serologic tests have been described, including hemagglutination inhibition (HAI) and enzyme immunoassays (EIAs) employing either crude antigens or the use of virus-like particles (VLPs). Detection of an antibody response by HAI is based on the capacity of specific antiviral antibodies to inhibit the agglutination of human erythrocytes mediated by the viral structural VP1 proteins of JCPyV and BKPyV (122). The development of EIAs for the detection of antibodies to both BKPyV and JCPyV was made possible when engineered cell lines capable of producing adequate amounts of JCPyV antigen were developed (123). A comparison of EIA and HAI demonstrated that antibody titers measured by the two assay formats were statistically significantly correlated, although EIA titers were higher (124). VLPs have also been used as antigens in EIAs for the detection of antibodies. VLPs are empty particles that retain the full antigenicity of intact virions. They form spontaneously when viral capsid proteins are expressed in certain systems and are preferred as antigen sources for EIAs because they contain native conformational epitopes that may be missing when other forms of viral antigens are employed (125).

Serologic tests for HPyV are used primarily for seroepidemiological studies and not for diagnosis of infection, although a study has demonstrated a correlation between kidney donor BKPyV antibody titers and infection in the transplant recipient (47). An exception to this is the FDA-approved, commercially available Stratify JCV test offered by Focus Diagnostics (Cypress, CA). The purpose of the assay is to determine the JCPyV serostatus of patients undergoing natalizumab therapy as part of a strategy to determine the risk for the development of PML (126–128).

Despite the fact that JCPyV and BKPyV are closely related, it appears that the antigenic epitopes responsible for the human antibody response are not cross-reactive and antibody tests can discriminate between infection by these two viruses (124). In contrast, it appears that seroreactivity to SV40 is due largely to cross-reactivity with BKPyV (125, 129, 130). The identification of novel HPyVs in recent years has complicated polyomavirus serology and highlighted issues associated with the interpretation of some polyomavirus serologic assays. Further complicating matters, it appears that human sera contain antibodies that can react with animal polyomaviruses, suggesting possible transmission from animal to human. This and the likelihood that more HPyVs will be discovered suggest that caution should be employed when interpreting the results of some HPyV assays. These and other issues concerning HPyV serology are covered in depth in a recent review (36).

ANTIVIRAL SUSCEPTIBILITIES

For PML, a number of antiviral agents have been examined, including cidofovir, an acyclic nucleoside phosphonate; topotecan, an inhibitor of DNA topoisomerase I; leflunomide, an immunosuppressive agent with antiviral properties; vidarabine (ara-A) and cytarabine (ara-C), synthetic nucleosides that inhibit viral DNA polymerases; and alpha interferon (131). Following evidence that JCPyV uses the serotonin 2A receptor to infect cells in the CNS, interest has been shown in investigating drugs, such as risperidone, that bind to this receptor and are approved for treatment of certain neuropsychiatric diseases (132), although another study reported that infection of cells by JCPyV was independent of the serotonin receptor (133). A study that examined a collection of 2,000 currently approved drugs found evidence that the antimalarial drug mefloquine had activity against JCPyV *in vitro* (134). Unfortunately, a clinical trial of mefloquine treatment for PML was stopped early when interim data indicated that the drug offered no benefit (135). For some of these agents, antiviral activity against JCPyV has been demonstrated *in vitro*, and encouraging results have at times been reported in case reports. However, studies have been hampered by the fact that PML is a rare disease and the majority of patients are HIV infected and receiving potentially confounding anti-HIV therapy, making it difficult to evaluate the effect of the drug being studied. Unfortunately, most clinical studies of antiviral treatments for PML have failed to demonstrate a consistent benefit (136–140) and there are no currently FDA-approved drugs for the treatment of this devastating disease. Current approaches to therapy include optimal HAART for HIV-infected patients, with the aim of increasing CD4 cell counts and decreasing HIV RNA level (38). For non-HIV-infected individuals, reversal or amelioration of the immunosuppressed state ultimately responsible for the disease is recommended (136). While not an antiviral therapy, removal of immunomodulatory agents such as natalizumab can be achieved with plasma exchange. This in turn can trigger an immune reconstitution inflammatory syndrome, a condition that, while itself dangerous, can improve survival, although sequelae are common (41).

Many of the same agents studied as possible antiviral treatments for PML have also been investigated for the treatment of BKPyV PVAN and, less commonly, hemorrhagic cystitis. In addition to these compounds, fluoroquinolone antibiotics and intravenous immunoglobulin have also been studied. Similar to the situation with the development of antiviral drugs for the treatment of PML, there are currently no FDA-approved agents for the treatment of BKPyV diseases. The lack of antivirals can be attributed to several factors, including the need for prospective randomized controlled studies, the nephrotoxicity exhibited by a number of the drugs, and the confounding variable of the near universal approach of improving immune function in transplant recipients showing signs of BKPyV disease by some form of reduction in the level of immunosuppression. A review of the literature on antiviral treatment of PVAN found a total of only 184 patients in 27 published studies examining the use of cidofovir, 189 patients in 18 studies of the use of leflunomide, and 14 patients in 2 studies of the use of fluoroquinolones, with the authors concluding that there was no consensus on the use of antivirals for PVAN (141). Currently, there is an ongoing phase 1/2 study of the use of nucleoside analogs for treating BKPyV infection (142), but until this or potential future studies can demonstrate a benefit for a particular antiviral, the mainstay of current therapy for PVAN is the reduction of immunosuppression, although in cases where a patient is in danger of losing a graft to PVAN, a course of an antiviral may be of use (143–145).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The current trend in laboratory testing for PVAN is to monitor levels of the BKPyV in urine and blood and to reduce immunosuppression when viruria or viremia reaches levels that predict progression to disease. A management algorithm published in 2005 for monitoring BKPyV viruria and viremia in renal transplant recipients recommended that patients be screened for viruria every 3 months for the first 2 years after the transplant; if viruria is detected, then a quantitative NAAT should be employed for monitoring of urine or plasma, with sustained high levels of viruria ($>10^7$ copies/ml) or viremia ($>10^4$ copies/ml) triggering a renal biopsy (86). Testing for BKPyV viruria has a high negative predictive value and a variable positive predictive value for the development of PVAN, with sustained high levels of viruria ($>10^7$ copies/ml) having a positive predictive value of up to 67% (145). Sustained high levels of BKPyV viruria are frequently followed by the appearance of viremia. The majority of individuals with PVAN have been found to have BKPyV viremia, with the positive predictive value for viremia being ~60% for the development of PVAN (145). A recent review indicates that these guidelines have yet to be superseded and goes into more detail than is possible here on other testing modalities, such as the detection of BKPyV mRNA, which appears to have no advantages over the detection of viral DNA (142).

These recommendations are helpful in directing NAAT use and interpretation of NAAT results. However, implementing them is complicated by interlaboratory variability in BKPyV quantification due to the lack of internationally recognized standards that can serve as calibrators and by the use of assays that may not optimally detect and quantify all BKPyV strains. For example, in 2008, the College of American Pathologists sent four proficiency samples to laboratories performing quantitative BKPyV assays to compare results among the different assays. All 48 laboratories (38 using user-developed assays and 10 using commercial tests) that tested a sample that did not contain BKPyV DNA reported a negative result. When BKPyV-positive specimens were tested, the results were less encouraging. The sample producing the most variation was tested by 41 laboratories with the following results: mean, 700,946 copies/ml; standard deviation (SD), 1,729,692 copies/ml; coefficient of variation (CV), 246.8%; and range, 129 to 10,400,000 copies/ml. For 35 laboratories that also reported log-transformed data on this specimen, the results were as follows: mean, 4.806; SD, 1.231; and range, 1.02 to 6.51. The BKPyV-positive specimen with the lowest CV produced the following results: mean, 277,288 copies/ml; SD, 284,990; CV, 102.8%; and range, 7,800 to 945,000 copies/ml. (All data used with the permission of the College of American Pathologists.) Obviously, there is a significant amount of interlaboratory variation in quantitative BKPyV results.

Until the problems of assay variation are resolved, laboratories should work toward the consistent intralaboratory performance of their own assays. Careful coordination between physicians and the laboratory can result in the establishment of in-house viruria and viremia thresholds for adjusting immunosuppression regimens. The additional effort this requires is clearly worthwhile, since successful implementation of testing algorithms has been shown to significantly reduce PVAN incidence (143, 146).

To draw accurate conclusions regarding the course of infection, patients should be monitored at one site if possible. Sequential measurements by different laboratories are difficult to interpret given the likelihood of interlaboratory differences in BKPyV quantification. The laboratory should educate physicians and transplant coordinators about this issue.

In cases where PVAN is suspected and BKPyV titers are negative or low, the possibility of a false negative should be considered, due potentially to sequence mismatches between the virus and real-time PCR primers or probe. Alternatively, it may be possible that JCPyV is responsible, in which case it may be best to try to identify this virus either in biopsy material by histological methods or in plasma. Shedding of this virus is common in the urine and may not indicate the presence of a causative organism. Unfortunately, guidelines for interpreting JCPyV levels in peripheral blood are not available.

Quantitative NAATs of urine from BMT recipients to either predict the development of or diagnose hemorrhagic cystitis may be useful. High levels of viruria ($>1 \times 10^9$/ml) can be seen in hemorrhagic cystitis due to BKPyV (54, 147); a negative result suggests another cause of this condition. The interpretation of BKPyV viruria is complicated by the fact that BMT recipients frequently excrete BKPyV in their urine and by the lack of guidelines for thresholds that can be applied to predicting or diagnosing disease. Studies have yielded variable data. One report suggested that viruria of 10^9 to 10^{10} or more copies/ml or an increase of $\geq 3 \log_{10}$ relative to baseline levels may help implicate BKPyV as the cause (53). Another found that levels of BKPyV viremia of $>10^4$ copies/ml were associated with hemorrhagic cystitis (53); however, this relationship was not observed in a similar study (54).

Serology and culture have little role in the diagnosis of PML. A majority of individuals are seropositive for JCPyV by adulthood; culture requires special cells and prolonged incubation and is insensitive. Therefore, the preferred diagnostic test for PML is a JCPyV-specific NAAT. The detection of JCPyV DNA in CSF is distinctly abnormal. In combination with appropriate imaging studies and patient history, it is strongly suggestive of PML. JCPyV NAAT sensitivities in CSF range from 42 to 100%, with most between 70 and 80%; specificities range from 92 to 100% (148). False-negative JCPyV PCR results in patients with PML, possibly due to mismatches between primers and probes and target sequences (115, 149), have been observed. Additionally, decreased PCR sensitivity has been reported in the HAART era (89.5% pre-HAART versus 57.5% HAART, with the negative predictive value falling from 98 to 89%) (150). These findings underscore the fact that a negative JCPyV result cannot rule out the presence of PML. It may be advantageous to retest CSF with a different NAAT if the initial result is negative in a presumptive case of PML. It should be noted that in patients with natalizumab-induced PML who are treated with plasma exchange to remove the drug, JCPyV DNA has been reported to persist in the CSF for periods ranging from months to years (151).

Because of the devastating nature of PML, laboratory testing methods have been explored to either help identify patients at increased risk for developing PML or to provide a prognosis for those patients who have already been diagnosed. A single qualitative test for JCPyV DNA in urine and blood does not appear to be useful for the identification of patients at risk of PML, as demonstrated by studies of individuals with HIV and those with immunosuppression unrelated to HIV (including immunomodulatory therapy with natalizumab) (152, 153), and is therefore not recommended. Persistent viruria and rising urine viral loads have been found to be associated with the development of PML in HIV-positive patients (154). However, a large study that tested >12,000 urine and blood specimens from 1,397 natalizumab-treated multiple sclerosis patients for the presence of JCPyV DNA reported the following: (i) the prevalence of JCPyV viremia was very low in treated patients (<1%) and similar to that seen in untreated healthy controls; (ii) the detection of JCPyV viremia did not correlate with the development of PML; and (iii) the prevalence of JCPyV viruria was high (~25%) at both baseline and after 48 weeks of treatment, leading the authors to conclude that detection of viremia or viruria was not useful in determining the risk of development of PML in this population (155).

Quantitative PCR for the determination of the JCPyV load in the CSF of PML patients has been reported to be potentially useful as a prognostic measure for disease progression. However, because of small study sizes and the lack of standardized quantitative JCPyV NAATs, it has been difficult to formulate guidelines for threshold CSF viral loads. Different reports have indicated, roughly, that JCPyV CSF viral loads of between 10^4 and 10^5 copies/ml separate those PML patients whose disease progresses quickly from those whose illness is stable or progressing more slowly (153, 156–158).

The commercial Stratify JCV offered by Focus Diagnostics is used to determine the JCPyV serostatus of multiple sclerosis patients undergoing natalizumab therapy. Data in support of using this test to determine risk for development of PML come from a study that revealed that 54% of 831 multiple sclerosis patients were seropositive for JCPyV and that the 17 patients who subsequently developed PML were all JCPyV seropositive (126). In the same study, it was reported that the JCPyV false-seronegative rate was 2.5%, as determined by the detection of viruria in 5 of 204 seronegative patients. However, another study of natalizumab-treated patients found a much higher false-seronegative rate of 35% in 17 patients with JCPyV viremia, suggesting that, while the determination of JCPyV serostatus may be useful in helping to evaluate the risk of development of PML, other measures of risk need to be developed and employed to supplement serology and that JCPyV serology needs to be interpreted with caution (159).

Laboratory testing for the newly identified HPyVs is considered investigational at this time, so it is not possible to offer meaningful interpretations for test results for these agents.

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Parvovirus B19 and Bocaviruses

KEVIN E. BROWN

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TAXONOMY

Parvoviruses are small (~22-nm-diameter), nonenveloped, icosahedral viruses with a linear, single-stranded DNA genome. They take their name from *parvum*, the Latin for "small," and *Parvoviridae* are among the smallest known DNA-containing viruses that infect mammalian cells. The *Parvoviridae* are divided into two subfamilies, *Parvovirinae* and *Densovirinae*, on the basis of their ability to infect vertebrate or invertebrate cells, respectively. The vertebrate viruses (*Parvovirinae*) are currently subdivided into five genera on the basis of their number of open reading frames (ORFs), their transcription map, their ability to replicate efficiently either autonomously or with helper virus, and their sequence homology. The eight genera are *Protoparvovirus*, *Dependoparvovirus*, *Erythroparvovirus*, *Bocaparvovirus*, *Aveparvovirus*, *Copiparvovirus*, *Tetraparvovirus*, and *Amdovirus* (1).

At least four different parvoviruses are known to infect humans. Parvovirus B19 (B19V) is the best characterized and is classified as the type member of the *Erythrovirus* genus. The other human viruses are the human bocaviruses (*Bocaparvovirus*); parvovirus 4 (PARV4) (*Tetraparvovirus*), and the nonpathogenic adeno-associated viruses (*Dependoparvovirus*).

PARVOVIRUS B19

Description of the Agent

B19V has the typical features of a member of the *Parvoviridae*: the virions are nonenveloped, 15 to 28 nm in diameter, and show icosahedral symmetry (Fig. 1). The virions hemagglutinate, and this is through the primary viral receptor, blood group P, also known as globoside (2–4).

The genome consists of a single strand of DNA of 5,596 nucleotides, with long inverted terminal repeat sequences at each end. The genome has two large ORFs, with the left ORF encoding the nonstructural (NS) protein and the right ORF encoding the two capsid proteins, VP1 and VP2, by alternative splicing.

The B19V virion consists of 60 copies of the capsid proteins, of which 95% are VP2, with 5% or less of the larger VP1 protein (5). VP2 capsid proteins self-assemble to form virus-like particles (VLPs) in the absence of B19V DNA; VP1 is not required for capsid formation (6, 7). Although three different genotypes have been described, there is serologic cross-reactivity, and thus only one serotype.

In 1985, the virus was officially recognized as a member of the *Parvoviridae*, and the International Committee on Taxonomy of Viruses recommended the name B19V to prevent confusion with other viruses (e.g., human papillomavirus). It is classified as a member of the *Erythrovirus* genus with the name parvovirus B19 (official abbreviation, B19V) (1).

Epidemiology and Transmission

B19V is a common infection in humans, and although there is some variation in different countries (8), 50% of 15-year-olds and 80% of the elderly have detectable IgG (9, 10). Infections in temperate climates are more common in late winter, spring, and early summer months. Rates of infection may also increase every 3 to 5 years, and this is reflected by corresponding increases in the major clinical manifestations of B19V infection, mainly transient aplastic crisis (TAC) and erythema infectiosum (11).

The virus can be readily transmitted by close contact through the respiratory route. In one study, the secondary attack rate from symptomatic patients to susceptible (IgG-negative) household contacts was ~50%. (12). The highest secondary attack rates and also seroprevalence and annual seroconversion rates are seen among workers with close contact with young children, e.g., day care providers and school personnel (10, 13).

Infectious virus can also be found in serum, and infection can be transmitted by blood and blood products. Although ~1% of blood donations have low-level B19V DNA detectable (14), reports of transmission of B19V infection by individual units of blood or platelets are rare. However, transmission from pooled products is more common, and recommendations in Europe and North America now require all plasma pools for fractionation to be screened for high-titer parvovirus DNA to try to minimize the transmission of B19V by blood products.

Currently there is no vaccine for B19V, although B19V VLPs expressed in either insect or yeast cells are highly immunogenic (15, 16), and the results of the first phase 1 trial looked very promising. However, a more recent, larger trial was stopped due to the development of unexplained skin rashes in three recipients (17).

Clinical Significance

B19V primarily infects erythroid progenitor cells, inducing cell death through apoptosis and cessation of red cell production. Thus, the presentation of infection depends on the significance of the drop in red cell production and/or the immune response to infection, and unlike many virus

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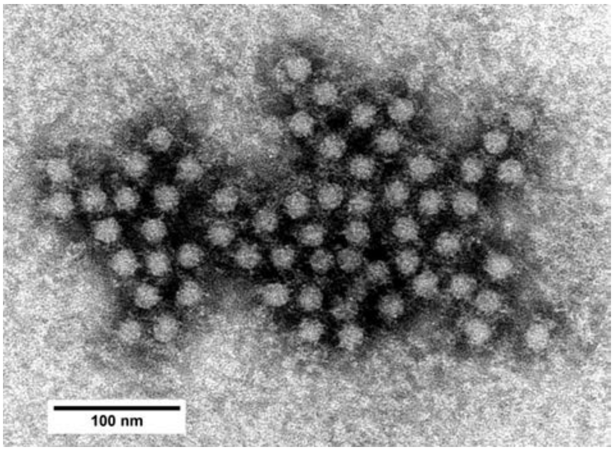

FIGURE 1 Immune electron microscopy image of B19V, showing typical 22-nm-diameter particles clumped together with a polyclonal human serum. Image courtesy of Hazel Appleton. doi:10.1128/9781555817381.ch106.f1

infections, the disease manifestation of infection with B19V varies widely with the immunologic and hematologic status of the host (Table 1).

Healthy Individuals—Normal Immune Response

Although a significant number of infections are asymptomatic, the major manifestation of B19V infection is erythema infectiosum, also known as fifth disease or slapped-cheek disease due to the characteristic facial rash. After a 2- to 5-day nonspecific prodromal illness (fever, chills, and myalgia) (Fig. 2A), the classic slapped-cheek rash appears, followed 1 to 4 days later by an erythematous maculopapular exanthema on the trunk and limbs. This rash is almost certainly immune mediated, and the timing correlates to detection of an antibody response. As the rash on the trunk and limbs fades, it takes on a typical lacy appearance. There may be great variation in the appearance of the rash, and rarely B19V may present as a petechial rash or papulo-purpuric “gloves and socks” syndrome (although this is more often at the viremic stage of the infection, and not primarily immune mediated). The classic slapped-cheek rash is more common in children than adults, and the second-stage eruption may vary from a very faint erythema that is easily missed to a florid exanthema and may be transient or recurrent over 1 to 3 weeks.

In children, B19V infection is usually mild and of short duration. However, in adults and especially in women, there may be a symmetrical arthropathy primarily affecting the small joints of the hands and feet in ~50% of patients (18). Joint symptoms often last 1 to 3 weeks, although in 20% of affected women, arthralgia or frank arthritis may persist or recur for >2 months or even years. In the absence of a history of rash, the symptoms may be mistaken for acute rheumatoid arthritis, especially as B19V infection can be associated with transient rheumatoid factor production (19). It has been postulated that B19V is involved in the initiation and perpetuation of rheumatoid arthritis, leading to joint lesions (20), but these results have not been reproducible by other groups. In contrast, B19V DNA is frequently found in synovial tissue of patients with rheumatoid arthritis, chronic arthropathy, and control subjects, and it seems unlikely that B19V plays a role in classic erosive rheumatoid arthritis. The association between B19V and juvenile rheumatic disease is more convincing (21), but whether B19V is the cause of the disease or one of many potential triggers is less clear.

Patients with Increased Erythropoiesis

TAC, the abrupt cessation of red cell production (Fig. 2B), due to B19V has been described in a wide range of patients with underlying hemolytic disorders, including hereditary spherocytosis, thalassemia, and red cell enzymopathies such as pyruvate kinase deficiency and autoimmune hemolytic anemia (22). Even in hematologically normal individuals, acute anemia or a drop in red cell count has been described (23, 24). Other blood lineages may also be affected, and there may be varying degrees of neutropenia, thrombocytopenia, and transient pancytopenia (22). Rarely the patient may present with a petechial or purpuric rash, and some cases of idiopathic thrombocytopenia purpura (25) and Henoch-Schönlein purpura (26) have also been linked to B19V infection.

Although it is a self-limiting disease, patients with aplastic crisis can be severely ill, with dyspnea, lassitude, and even confusion due to the worsening anemia. Congestive heart failure and severe bone marrow necrosis may develop (27, 28), and the illness can be fatal (29). TAC is readily treated by blood transfusion.

TABLE 1 Clinical diseases associated with parvovirus B19 infection and methods of diagnosis

Host(s)	Disease presentation	IgM	IgG	PCR	Quantitative PCR
Healthy children	Fifth disease	Positive	Positive	Positive	$>10^4$ IU[a]/ml
Healthy adults (often women)	Polyarthropathy syndrome	Positive within 3 mo of onset	Positive	Positive	$>10^4$ IU/ml
Healthy children	Petechial or purpuric rash	Negative/positive	Negative/positive	Positive	Maybe $>10^{12}$ IU/ml, but rapidly decreases
Patients with increased erythropoiesis	TAC	Negative/positive	Negative/positive	Positive	Often $>10^{12}$ IU/ml, but rapidly decreases
Immunodeficient or immunocompetent patients	Persistent anemia/ pure red cell aplasia	Negative/weakly positive	Negative/weakly positive	Positive	Often $>10^{12}$ IU/ml, but should be $>10^6$ in the absence of treatment
Fetus (<20 wk)	Hydrops fetalis/ congenital anemia	Negative/positive	Positive	Positive amniotic fluid or tissue	NA[b]

[a] 1 IU = ~1 genome.
[b] NA, not applicable.

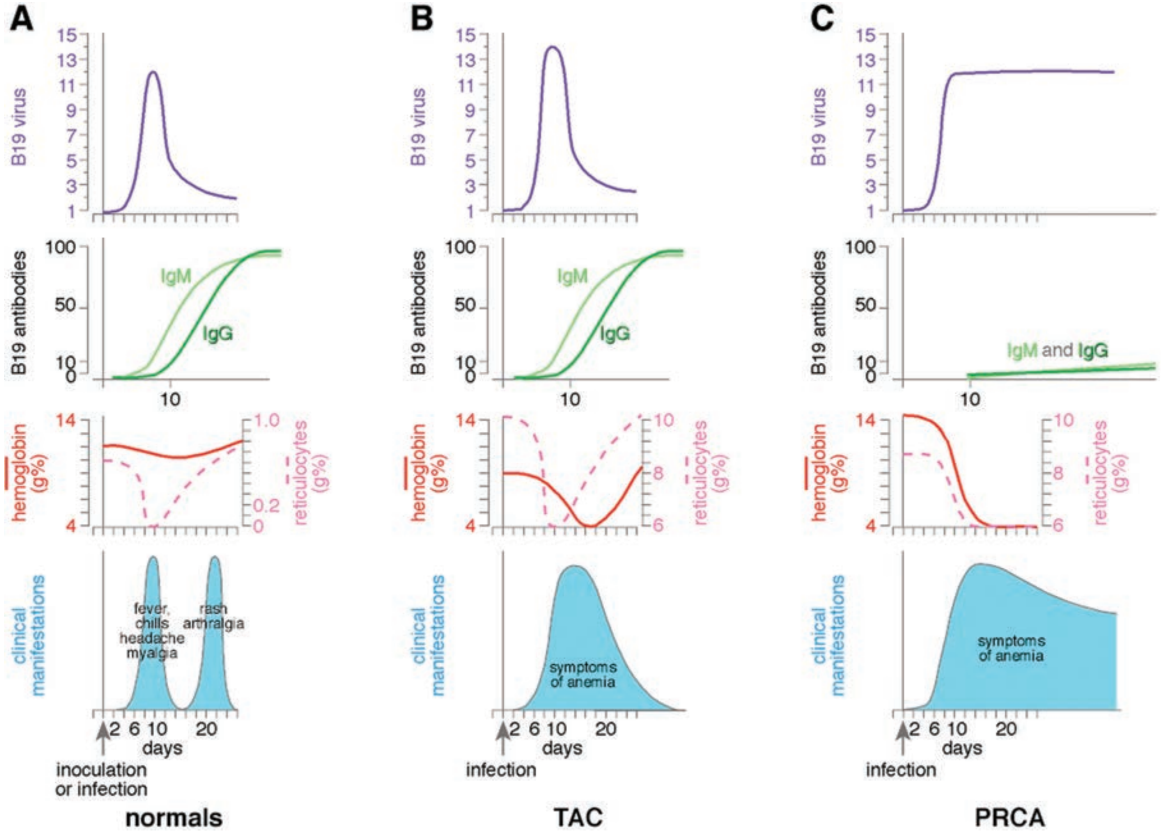

FIGURE 2 Time course of B19V infections in (A) healthy individuals, (B) patients with transient aplastic crisis, and (C) immunosuppressed patients. PRCA, pure red cell aplasia. Reprinted with permission of Massachusetts Medical Society from reference 115.
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Infection during Pregnancy and Congenital Infection

B19V is a known cause of fetal hydrops and miscarriage. Studies have estimated an increased fetal loss of 9% in women with confirmed B19V infection in the first 20 weeks of pregnancy (30–32). Many cases of B19V-induced hydrops fetalis are now treated with intrauterine blood transfusion.

Rare cases of congenital anemia after a history of maternal B19V exposure have been reported (33). In these cases, the virus load is generally low and the anemia does not respond to immunoglobulin therapy. The B19V infection may mimic Diamond-Blackfan anemia (34), and the role of *in utero* B19V infection in inducing constitutional bone marrow failure such as that in Diamond-Blackfan anemia is still not clear.

Immunosuppressed and Immunocompromised Patients

In patients with a compromised immune system, there may be a failure to induce a neutralizing antibody response, leading to chronic infection of erythroid progenitors and, as a consequence, prolonged failure of red cell production and development of chronic anemia (Fig 2C). Persistent B19V infection resulting in pure red cell aplasia has been reported in a wide variety of immunosuppressed patients, ranging from patients with congenital immunodeficiency, AIDS, and lymphoproliferative disorders to transplant patients (35). The stereotypical presentation is persistent anemia rather than the immune-mediated symptoms of rash or arthropathy. Often there is a pure red cell aplasia, but invariably other lineages may also be affected. Treatment is by reduction of the immunosuppression, if this is feasible, or more often, administration of immunoglobulin (36).

Other Presentations

B19V infection has been associated with a wide range of other manifestations including vasculitis, hepatitis, myocarditis (and cardiomyopathies), glomerulonephritis, Kawasaki disease, and virus-associated hemophagocytic syndrome.

Treatment

Treatment for all presentations of B19V infection is symptomatic only. None of the currently available antivirals is known to have antiparvovirus activity. B19V does not encode either a DNA polymerase or viral proteases, so targets for antivirals are limited.

Collection, Transport, and Storage of Specimens

Due to its small DNA genome and lack of a lipid envelope, B19V is resistant to most types of physical inactivation and is heat stable. Therefore, no special precautions are needed for transportation and storage of most clinical specimens, although repeated freeze-thaw cycles should be prevented where possible. Serum (or plasma) samples should be obtained if possible, and permit measurement of both viral load and B19V-specific IgM and IgG levels. Additional samples may include cerebrospinal fluid (for investigation of neurologic infection), amniotic fluid (investigations in pregnancy), and bone marrow samples (hematologic disease), but these should always be in addition to serum samples where possible, as interpretation may be difficult without a concurrent serum sample. For investigation of fetal deaths, fetal tissues can be used, either frozen or fixed, although frozen material is preferable for DNA analysis. Samples can usually be transported at room temperature, with the exception of tissue samples (and especially fetal liver samples), which, if not fixed, should be frozen as soon as possible after collection and kept frozen until processed (due to the large amount of proteases and DNase activity often present in such samples).

Direct Examination

Microscopy

Infected cells are characterized both *in vivo* and *in vitro* by the presence of giant pronormoblasts, or "lantern cells" (37). These are early erythroid cells, 25 to 32 μm in diameter, with cytoplasmic vacuolization, immature chromatin, and large eosinophilic nuclear inclusion bodies (Fig. 3). Electron microscopy of such cells shows that the inclusion is made up of large viral arrays, which can be confirmed by monoclonal antibody staining. These cells can be found in the bone marrow of infected individuals at the time of their peak viremia, and occasionally in the peripheral circulation (38). Although they have been said to be pathognomonic of B19V infection, similar cells can be seen in the bone marrow of patients with other infections, including HIV, and so should not be used for diagnosis of B19V infection without other confirmation.

When seen as a large viral array within a cell, B19V can be readily identified. However, often the infected cells do not have inclusions, and in these cases, electron microscopy cannot always distinguish intracellular virus from ribosomes, limiting its practicalities apart from as a research tool.

High viral loads (>10^9 IU/ml) can be detected in serum samples (after concentration) by electron microscopy, in which the characteristic 22-nm-diameter, icosahedral virions can be seen (Fig. 1). Immune electron microscopy using polyclonal serum to clump the virions together can increase the sensitivity and make the virions easier to identify.

Antigen Detection

Antigen detection, generally using monoclonal antibodies, can be used with standard histochemistry (Fig. 4) to identify infected cells in blood samples, tissues, or cell culture. It is relatively insensitive and can be useful for identifying infected cells in cases where the significance of low levels of B19V DNA is unknown. Its role in clinical microbiology is limited.

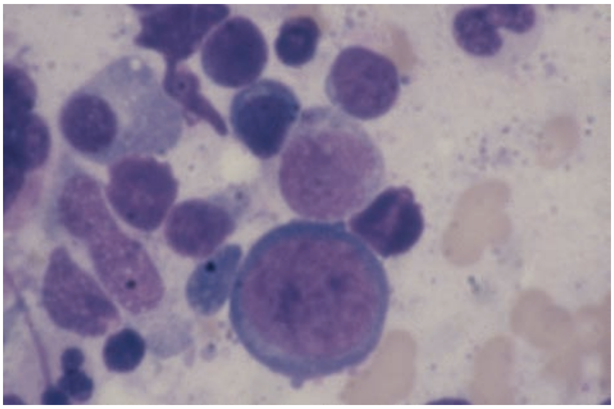

FIGURE 3 Giant pronormoblast (lantern cell) typical of B19V infection. doi:10.1128/9781555817381.ch106.f3

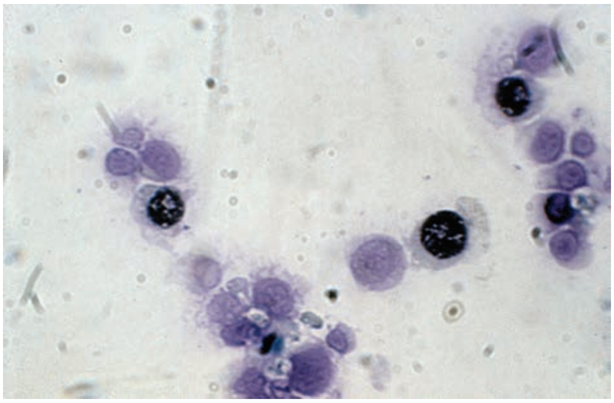

FIGURE 4 Immunohistochemistry of infected erythroid progenitor cells from human fetal liver, stained with anticapsid antibody. Note the typical intranuclear localization of capsid protein. doi:10.1128/9781555817381.ch106.f4

B19V antigen-based assays are relatively insensitive (>10^6 virus particles/ml), but antigen detection can be used for detecting high concentrations of virus in serum or plasma settings and has been suggested as a screening method for detecting high-titer virus in blood and plasma samples (39). The Japanese Red Cross has also used a modified hemagglutination assay for detection of high-titer virus in plasma samples (receptor-mediated hemagglutination) (40).

Nucleic Acid Detection

Detection of B19V DNA is the preferred method for identifying B19V in samples. Although *in situ* hybridization can be used to identify B19V DNA within specific cells, and direct hybridization was used for many years to detect B19V DNA in serum samples, more commonly these days DNA detection is by PCR. Due to the exquisite sensitivity of PCR, B19V DNA can then remain detectable for months or even years at low levels even following complete recovery, and thus quantitative PCR is required to distinguish recent infection from previous infection with the virus. All assays should be calibrated against the World Health Organization (WHO) B19V nucleic acid amplification technology standard (41) and the results reported as international units per milliliter.

A large number of different PCR primers (and probes) have been described in the literature (42–44), and commercial assays are available from a number of different companies, including Abbott Diagnostics (Abbott Park, IL), altona Diagnostics (Hamburg, Germany), bioMérieux (Marcy l'Etoile, France), Qiagen (Hilden, Germany), Fast-track Diagnostics (Sliema, Malta), Focus Diagnostics (Cypress, CA), Ingenetix (Vienna, Austria), and Roche Diagnostics (Indianapolis, IN). When choosing an assay, primers should be designed from the more conserved parts of the virus genome, such as the region in NS where there is a second ORF (45), and should be chosen to specifically detect all three genotypes. Although the genome is relatively conserved, a single nucleotide difference may markedly alter the sensitivity of the assay (46). There is now a 1st WHO International Reference Panel available comprising the different genotypes so that assays can be checked to make sure that all three genotypes are detectable in an assay (47).

Detection of viral RNA can be very useful for confirming the presence of replicating virus and active infection. Often this is achieved through amplification across a spliced junction, so that there is distinction between the spliced RNA and viral DNA (48). This generally is used only as a research tool.

Isolation Procedures

B19V, like all autonomous parvoviruses, is dependent on mitotically active cells for its own replication. B19V also has a very narrow target cell range and can only be efficiently propagated in human erythroid progenitor cells. For erythroid progenitors from bone marrow, susceptibility to B19V increases with differentiation; the pluripotent stem cell appears to be spared and the main target cells are CD36-positive erythroid colony-forming cells and erythroblasts (49, 50). Thus, B19V cannot be grown in standard tissue culture.

A number of semipermissive cell lines have been described, including UT7/Epo (51, 52) and KU812Ep6 cells (53). However, more efficient replication is obtained using primary cultures from bone marrow or fetal liver (37, 54). Methods for enhancing erythroid differentiation have been developed, so that efficient B19V replication can be achieved in the research laboratory (44). Routine culture is not recommended for investigation of B19V infection.

Typing Systems

B19V is now recognized to have three different genotypes, with ~10% variability at the DNA level between genotypes (45, 55, 56). Most of the B19V identified is genotype 1 (55), the original B19V genotype, which is distributed worldwide. Genotype 3 seems to be the predominant B19V genotype in Ghana, representing >90% of the sequences identified (57). Genotype 2 has been identified primarily in tissues of older patients (born before 1973), suggesting that it may have circulated more frequently prior to the 1970s (58), although blood donations containing high-titer genotype 2 are occasionally identified. Genotype 2 and 3 sequences have been identified in blood and tissues from many different parts of the world (59–61), suggesting a more widespread distribution than originally assumed.

Despite the differences in the DNA sequences, the capsid protein sequence is conserved between the different genotypes, and there is evidence for both serological cross-reactivity and cross-neutralization (62). There is no evidence that the different genotypes show any differences in virological or disease characteristics (63, 64).

Serologic Tests

Serologic surveys were originally performed using the relatively insensitive counterimmune electrophoresis but were superseded first by radioimmunoassays and now almost universally by either enzyme or chemiluminesence immunoassays. Due to the inability to grow B19V in standard cell culture systems, early serology assays were based on the use of synthetic peptides (65) or fusion proteins in *Escherichia coli* (66) as antigen. However, the epitopes presented by these products do not accurately reproduce the epitopes of the native capsids, and the sensitivity and specificity were disappointing. The production of B19V capsid proteins as VLPs using baculovirus (67, 68) or yeast (69) expression systems appears to have overcome these problems, and results based on these antigens show good correlation with assays based on native virus. The antigens are relatively easy to mass-produce and are noninfectious.

Immunoassays using VLPs as antigen are generally both sensitive and specific. Although B19V IgM can be detected by both IgM capture assays and indirect assays, capture assays appear to be more sensitive and less prone to false-positive results due to rheumatoid factor. In contrast, IgG assays are probably more sensitive in an indirect (or sandwich) format, especially for seroprevalence studies. Commercial immunoassays for both B19V IgM and IgG are available from a number of different companies, including Biokit (Barcelona, Spain), DiaSorin (previously Biotrin) (Saluggia, Italy), DRG International (Springfield, NJ), Euroimmun (Lubeck, Germany), Focus Diagnostics, Mikrogen (Neuried, Germany), MyBioSource (San Diego, CA), and Virion/Serion (Wurzburg, Germany), but several of these are designated for research use only, and in the United States only the DiaSorin assays are approved by the Food and Drug Administration for diagnostic use.

Antibody to virus is usually present by day 7 of illness (aplastic crisis) or a day after the onset of erythema infectiosum rash, and probably is lifelong thereafter, although some waning of antibody has been suggested (70).

In immunocompetent individuals, the early antibody response is to the major capsid protein, VP2, but as the immune response matures, reactivity to the minor capsid protein, VP1, dominates (71, 72). Sera from patients with persistent B19V infection typically have antibody to VP2 but not to VP1 (73). Thus, a number of other formats for assays have been described, including epitope-specific assays, Western blot, and avidity tests for B19V infection (71, 74). Apart from a commercial immunoblot assay (Mikrogen), these assays are all in-house assays and are not available outside research laboratories. In specialist settings, and in combination with B19V DNA viral load, epitope-specific assays may be useful, especially for confirming the timing of recent infection, e.g., during pregnancy (75).

Although assays to detect neutralizing antibodies have been described (48), they are very time-consuming and not used outside the research setting.

Evaluation, Interpretation, and Reporting of Results

The diagnosis of B19V infection is very dependent on the host characteristics and the presentation of the illness, with serology and/or quantitative PCR being the most appropriate assay depending on the circumstances (Table 1; Fig. 2).

For otherwise healthy children or adults presenting with the immune-mediated rash illness consistent with B19V (i.e., slapped-cheek or rash of erythema infectiosum), appropriate testing is for the detection of B19V IgM, as the high-titer viremic stage correlates with the time of the prodrome (Fig. 2A). The IgM remains detectable for several months following infection. B19V IgG is also detectable within a day of onset of the rash, and then remains detectable lifelong. Patients with fifth disease do have detectable B19V DNA in blood, with titers of $>10^4$ IU/ml. At 2 to 3 months following infection, B19V DNA titers fall to $<10^4$ IU/ml, and low-level B19V DNA may remain detectable in blood and tissues for the rest of life. False-positive B19 IgM serology can occur with other acute rash infections, including measles and rubella, and may mislead clinicians. In addition, B19V infection may be associated with production of rheumatoid factor and produce false-positive serology in other assays. Measurement of the B19V viral load can then be useful in determining the specificity of the IgM result.

Patients with increased red cell turnover and presenting with TAC often have between 10^8 and 10^{14} genome copies/ml (IU/ml) of virus DNA detectable in their blood

(Fig. 2B), and diagnosis should be by quantitative PCR. IgM and IgG are not initially detectable, and the diagnosis can be missed if these assays are not combined with detection of B19V DNA. Assays for detection of B19V antigen in serum or plasma are not recommended, as they can give false negatives due to immune complex formation.

Similar high viral loads and undetectable IgM (and IgG) in the serum at the time of presentation are also seen in the rare patients who present with a petechial or purpuric rash. In these patients, B19V infection cannot be excluded on the basis of a negative IgM test alone.

Immunosuppressed or immunocompromised patients with persistent parvovirus infection often have a low or absent antibody response but high titers (>10^6 IU/ml) of B19V DNA in blood or serum (Fig. 2C). Diagnosis should be made using quantitative PCR.

B19V infection in the fetus is usually suspected following confirmation of maternal infection. However, infection of the fetus follows maternal infection, and it can be that the mother's B19V IgM response is negative at the time of the fetal hydrops. In these cases, B19V IgG avidity (if available) or B19V DNA titer may be useful in determining the time of maternal infection (75).

As low levels of B19V DNA remain detectable in serum or tissues of otherwise healthy, immunocompetent individuals lifelong following an acute infection, the finding of low-titer B19V DNA alone should not be interpreted as an indicator of recent infection or the causal agent of pathology.

HUMAN BOCAVIRUSES

Human bocavirus (HBoV) was discovered in 2005 as part of a virus discovery program to identify the causes of lower respiratory tract infections in children (76). This respiratory virus is now classified as HBoV1. Related viruses have subsequently been detected in fecal samples: HBoV2 to -4 (77–79).

Description of the Agent

HBoV1 has the typical structure of a member of the *Parvoviridae*. The full-length genome of HBoV1 is 5,543 nucleotides, with dissimilar hairpin sequences at the 5′ and 3′ ends. The genome has three large ORFs (as seen in other members of the *Bocavirus* genus) encoding the nonstructural protein (NS1), the capsid proteins (VP1 and VP2), and a second nonstructural protein (NP1) (80).

The major capsid protein, VP2, has been expressed in insect cells, and it self-assembles to form VLPs (Fig. 5) that are the basis of most serology assays. The coding sequences for the other HBoVs (HBoV2 [77], HBoV3 [81], and HBoV4 [79]) have been determined. The different HBoV species show between 10 and 30% divergence, with increased genetic variation and evidence for recombination (79).

Epidemiology and Transmission

HBoVs have a worldwide distribution and have been identified in all countries that have looked for it. HBoV1 is found predominantly in respiratory secretions, including in 2 to 20% of samples from children with upper or lower respiratory tract disease (82). Although HBoV1 DNA can be detected throughout the year, primary infection occurs predominantly in the winter and spring months (76, 83, 84), as for other respiratory infections. Based on serologic studies using HBoV1 as antigen, most, if not all, individuals are infected in early childhood before the age of 6 (85).

HBoV2, HBoV3, and HBoV4 are identified predominantly in fecal samples, both in patients (children and adults) with gastroenteritis and in healthy controls (79, 82). HBoV2 appears to be the most commonly identified species, followed by HBoV3 and then HBoV4 (82, 85). This is also reflected in the seroepidemiology, with increased seroprevalence in the order HBoV1 > HBoV2 > HBoV3 > HBoV4.

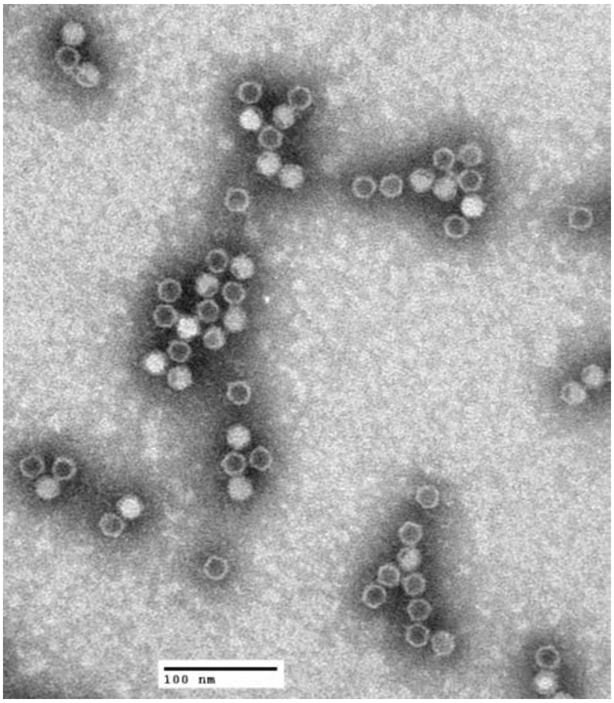

FIGURE 5 Bocavirus VLPs expressed in insect cells.
doi:10.1128/9781555817381.ch106.f5

HBoV1 appears to be transmitted predominantly by the respiratory route, although HBoV1 DNA has been detected in urine and fecal samples (86, 87), suggesting that it may also be spread by the fecal-oral route. HBoV2–4 are found mainly in fecal samples and appear to be spread by the fecal route.

Clinical Significance

Although HBoV1 DNA is commonly found in respiratory secretions of hospitalized children with respiratory symptoms (82), in many cases HBoV1 is found with other pathogens, raising questions as to whether it is the main cause of symptoms. It is now clear that there can be prolonged persistence of bocavirus DNA in respiratory tract (and fecal) samples, and if a stricter definition for diagnosing HBoV1 infection is used (high viral load in respiratory secretions, DNA in serum, and/or a serologic response), several groups have shown that HBoV1 infection is associated with both upper and lower respiratory tract infection and specifically with wheezing (88–90).

Similar criteria for the diagnosis of infection due to the fecal bocaviruses have not been identified, and although HBoV2–4 can be found in patients with acute gastroenteritis, in controlled studies they are found in healthy controls at similar rates (91, 92).

Direct Examination

Microscopy and Antigen Detection

Although parvovirus-like particles had been observed in fecal samples by electron microscopy, it is only with the identification of the nucleic acid sequence that their true identity has been confirmed. Microscopy and antigen detection do not have a role in the diagnosis and management of bocavirus infection.

Nucleic Acid Detection

The mainstay of bocavirus diagnosis is by nucleic acid detection, preferably by quantitative PCR. A number of different primers and probes have been described (93–103), but there have been virtually no studies comparing the relative specificity or sensitivity of the different assays. The NS1 and NP1 regions are the most conserved regions of the virus, and therefore the preferred targets. Commercial assays have also been produced (by, e.g., bioMérieux, Fast-track Diagnostics, and Gentaur [Kampenhout, Belgium]), often as part of a multiplex combined with other respiratory targets.

Isolation Procedures

HBoV1 can be grown in human airway epithelia (104), and cells in the respiratory tract are presumed to be the main site of replication during infection. Although HBov1 can be grown *in vitro* in well-differentiated airway epithelia cells (105, 106), replication is relatively inefficient, and this is a research tool only. For clinical purposes, virus detection is generally by identification and quantitation of viral DNA by PCR.

Typing Systems

At least four different HBoVs have been described, and they can be readily distinguished based on the DNA using specific primers (95). The viruses are also serologically distinct, although antigenic differences are used more to characterize the serologic response than to identify the viruses (107).

Serologic Tests

As with B19V, several groups have expressed the major capsid protein of HBoV capsids in insect cells (Fig. 5) and developed serologic assays to detect both IgM and IgG (108). In addition, bocavirus IgG avidity assays have been described (109). However, most assays probably measure cross-reacting antibodies to any of the four HBoVs, and there have only been few studies to try to distinguish between the antibody responses (107). None of these assays is commercially available.

Evaluation, Interpretation, and Reporting of Results

It is now recognized that diagnosis of HBoV infection should not be based on detection of bocavirus DNA in respiratory or fecal samples alone, because of the persistence of DNA at these sites. For HBoV1, detection should be based on the detection of viral DNA in the serum and evidence by serology of recent infection. This is generally by evidence of IgG seroconversion or by detection of IgM or low-avidity antibody. If serum is not available for serology and PCR, then high-titer ($>10^4$ genome copies/ml) HBoV1 DNA in respiratory secretions should be used (82).

Similar viral load criteria have not been developed for HBoV2–4 infections.

HUMAN PARVOVIRUS 4

PARV4 was also discovered in 2005 as part of a virus discovery program looking for new viruses in plasma samples using sequence-independent single primer amplification. The initial sample was from a daily-injecting intravenous drug user with signs of acute viral infection (110).

So far, 5,268 nucleotides of the sequence have been identified, and although this encodes the full-length genome, the terminal inverted terminal repeat sequences are incomplete (110). The genome has two large ORFs but a very different transcription profile from other parvoviruses; it is now classified in the new genus *Tetraparvovirus*.

The PARV4 sequences can be divided into three main groups or genotypes. Genotypes 1 and 2 are found predominantly in North America and Europe and genotype 3 in Africa, and although there may be differences in transmission (this has not been confirmed), it is not known if there any other differences in virology or pathogenicity between the genotypes.

Testing of pooled plasma products from Europe and North America showed that PARV4 DNA can be readily detected in plasma pools (4 to 5%), with viral loads varying from <100 copies/ml to 4×10^6 copies/ml (111). The prevalence may be significantly higher in other parts of the world.

PARV4 has not been grown in culture, but several groups have expressed capsid protein in insect cells. Serologic studies suggest that infection is unusual in the general population but have observed higher seroprevalence in those who participate in needle-sharing activities or receive blood products (112) and in some parts of Africa (113). Although the main route of transmission in Europe and North America appears to be parenteral, transmission via the fecal-oral route cannot be dismissed.

Very little information is available on the clinical features of acute infection with PARV4, with only very limited studies on cohorts at high risk of acquiring infection through parenteral exposure (114).

Diagnosis is generally by the detection of PARV4 viral DNA by PCR. Patients with acute infection appear to have transient high levels of PARV4 DNA in serum. However, the duration of the high-level viremia before the development of an IgM and IgG response is not known. No commercial assays are available for PARV4, and testing for PARV4 is currently a research tool only.

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Poxviruses

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TAXONOMY

All poxviruses described in this chapter belong to the family *Poxviridae* and subfamily *Chordopoxvirinae* (see chapter 78). The genera and species of the viruses discussed in this chapter are shown in Table 1. DNA-based assays, including DNA sequencing, are the most precise methods for poxvirus genus, species, strain, and variant identification and differentiation. The G+C contents of orthopoxviruses, yatapoxviruses, molluscum contagiosum virus (MCV), and parapoxviruses are ~33, ~32, ~60, and ~63%, respectively.

DESCRIPTION OF THE AGENTS

Virions are large and brick shaped (orthopoxviruses, yatapoxviruses, and molluscipoxvirus) or ovoid (parapoxviruses). Virions range in length from 220 to 450 nm and in width and depth from 140 to 260 nm. The appearances of virions under an electron microscope vary somewhat with sample preparation. By cryoelectron microscopy, in unstained, unfixed vitrified specimens, vaccinia virus appears as smooth, rounded rectangles; a uniform core is surrounded by a 30-nm-thick membrane. In conventional negatively stained thin sections, the core appears dumbbell shaped and is surrounded by a complex series of membranes. Lateral bodies of undefined function occupy the space between the outer membrane and the bar of the dumbbell. This feature may be a dehydration effect of negative staining.

Virus particles contain about half of the ~200 potential virus genome-encoded proteins. Virions consist of structural proteins and enzymes, including a virtually complete RNA polymerase system for primary transcription of viral genes (1). The genome is a 130- to 375-kbp (depending on the genus) double-stranded DNA molecule that is encapsidated in a nucleoprotein complex (nucleosome) inside the core. The genome is covalently closed at each end, and its ends are hairpin-like telomeres. Complete genome DNA sequences have been reported for several different poxviruses. GenBank entries are compiled at a dedicated website (http://www.poxvirus.org).

During virus replication (1), virion morphogenesis begins in the cytoplasm in areas known as cytoplasmic viral factories, where cellular organelles are largely absent. Thin-section electron microscopy observations of cells early after infection show crescent-shaped membrane structures, which progress to ovoid structures, called immature virions, which enclose a dense nucleoprotein complex. Primary transcription precedes the production of the crescents (cup-shaped in three dimensions) and the immature virions. Brick-shaped, membrane-covered mature virions (MVs; also known as intracellular MVs [IMVs]) form as the viral core condenses. These are features that aid the electron microscopist in the identification of poxviruses in clinical materials (2).

A small portion of MVs may be further transported on microtubules from the viral factory and processed to acquire a bilayer tegument (envelope) of Golgi intermediate compartment membrane that contains specific viral proteins. The intracellular enveloped MV then moves along cellular microtubules to the cell surface, where the outermost membrane fuses with the cellular membrane to reveal a cell-associated enveloped virus on the cell surface. The cell-associated enveloped virus can prompt actin polymerization behind the virion, which may facilitate cell-to-cell infection with virus. Enveloped virions can also exit the cell to spread more distantly.

A virus receptor has not been identified. Enveloped and nonenveloped forms attach to cells differently; however, studies suggest that the nonenveloped particle (IMV) is the particle that enters, via fusion, into the host cell. A complex of viral proteins is believed to act as a fusion complex. The common result of entry is uncoating of the particle, release of viral contents into the cell, and initiation of virus-controlled transcription of early-class proteins. Reviews of virion entry, morphogenesis, and exiting processes have been published (3–6).

EPIDEMIOLOGY AND TRANSMISSION

All current, naturally occurring poxviruses that infect humans are sporadic zoonotic agents, except the *Molluscipoxvirus* species *Molluscum contagiosum virus*, which is transmitted strictly between humans. The zoonotic poxviruses include members of the genera *Orthopoxvirus* (monkeypox virus, cowpox virus, and the vaccinia virus subspecies [including buffalopox virus]), *Parapoxvirus* (orf, pseudocowpox, sealpox, and papulosa stomatitis viruses), and *Yatapoxvirus* (tanapox virus [TPV], Yaba monkey tumor virus [YMTV], and Yaba-like disease virus [YLDV]). Orf virus and MCV infections are the most common poxvirus infections worldwide. These dermatologic lesions often can be readily identified, and laboratory confirmation of clinical diagnosis is often not utilized (7–9).

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TABLE 1 Taxonomy of poxviruses that infect humans

Genus	Species
Orthopoxvirus	*Variola virus*, *Vaccinia virus*, *Cowpox virus*, *Monkeypox virus*
Parapoxvirus	*Orf virus*, *Pseudocowpox virus*,[a] *Bovine papular stomatitis virus*, *Sealpox virus*
Yatapoxvirus	*Tanapox virus*, *Yaba-like disease virus*, *Yaba monkey tumor virus*
Molluscipoxvirus	*Molluscum contagiosum virus*

[a] Causes milker's nodule in humans.

Orthopoxviruses

Variola Virus

Variola virus, the cause of smallpox, had a strict human host range and no known animal reservoir. The virus was most often transmitted between humans by large-droplet respiratory particles inhaled by susceptible persons who had close, face-to-face contact with an infectious person. It was spread less commonly by aerosol, by direct contact with the rash lesion, or by sloughed crust material from a scab (10).

Monkeypox Virus

Human monkeypox was first reported in 1970 in the Democratic Republic of the Congo (DRC). Since 1970, the disease has been seen in Liberia, the Ivory Coast, Sierra Leone, Nigeria, Benin, Cameroon, and Gabon, but most cases have been in the DRC. In the 1980s, serosurveys and virologic investigations in the DRC by the World Health Organization (WHO) indicated that (i) monkeys are sporadically infected, as are humans; (ii) three-fourths of cases, mainly those in children <15 years of age, were from animal contact; (iii) the protective efficacy of vaccinia vaccination is ~85%; (iv) monkeypox virus has a broad host range, including squirrels (*Funisciurus* spp. and *Heliosciurus* spp.); and (v) human monkeypox has a secondary attack rate of 9% among unvaccinated contacts within households (i.e., it is much less transmissible than smallpox). In an outbreak in the DRC, ~250 serosubstantiated cases of monkeypox occurred among 0.5 million people in 78 villages from February 1996 to October 1997. Unlike those in the earlier outbreak, about three-fourths of the cases appeared to result from human-to-human transmission; however, the secondary attack rate of 8% among unvaccinated contacts within households appeared to be about the same as that found in the 1981-to-1986 surveillance (11–14). More-recent case series have been reported from the DRC (15, 16), and disease with a sustained chain of human-to-human transmission was reported in the Republic of the Congo in 2003 (17).

The emergence of monkeypox in the United States provides another example of the ability of this zoonotic disease to exploit new ecologic niches. The North American prairie dog, diseased after exposure to an infected West African rodent(s), subsequently infected and caused illness in U.S. human populations (18). The characterization of human disease in the United States, caused by a West African monkeypox virus variant, and its comparison with that classically described in the Congo Basin in the 1980s, enabled clinical and epidemiologic descriptions of distinct monkeypox viral diseases (19, 20).

Vaccinia Virus

Certain strains of vaccinia virus were used for human vaccination to globally eradicate smallpox (10, 21). Since the early 1980s, vaccinia and certain other poxviruses have been used as recombinant vectors for the expression of a variety of proteins, including vaccine immunogens. Infection can be transmitted to laboratory workers by accidental exposure, and significant pathology has been observed in unvaccinated individuals (22). Vaccination is therefore currently recommended for personnel working with live, replicative orthopoxviruses, including vaccinia virus.

The origin of vaccinia virus is uncertain. (For insights on the origins of vaccinia and cowpox viruses, see reference 23). Vaccinia virus infections are not generally regarded as naturally occurring, although vaccinee-to-cattle and cattle-to-human transmissions occurred on farms during the smallpox eradication campaign. Sporadic outbreaks of infection caused by the vaccinia virus subspecies *Buffalopox virus* that involve transmission between milking buffalo, cattle, and people have been reported, mainly in India but also in Egypt, Bangladesh, Pakistan, and Indonesia. Vaccinia-like lesions have been observed on the animals' teats and the milkers' hands; milk is infectious. Biological data and limited DNA analyses of isolates from an outbreak in India in 1985 suggest that buffalopox virus may be derived from vaccinia virus strains transmitted from humans to livestock during the smallpox vaccination era (24).

Quite interestingly, multiple distinct vaccinia viruses, possibly related to the vaccine strain used during smallpox eradication in Brazil, were described in cattle and their farm worker handlers in rural Rio de Janeiro (25, 26) as well as various locales within the Minas Gerais state (25, 27–29). Historic collections, when reevaluated with additional techniques, suggest that vaccinia-like viruses were previously isolated in the 1960s and 1970s (30). Inadvertent exposure to a vaccinia virus-vectored recombinant rabies virus vaccine dispersed to control rabies in wildlife has resulted in at least two instances of human infection; in both cases the bait was encountered via the family dog (31, 32).

Cowpox Virus

Cowpox, sometimes a rare occupational infection of humans, can be acquired by contact with infected cows. Other animals, e.g., infected rats, pet cats, and zoo and circus elephants, have more often been sources of the disease. *Cowpox virus* is a rather diverse species and has been isolated from humans and a variety of animals in Europe and adjoining regions of Asia (33, 34). An outbreak of human disease in Europe was associated with contact with pet rats (35). A serosurvey of wild animals in Great Britain found orthopoxvirus antibodies in a portion of bank and field voles and wood mice, which is consistent with small rodents being reservoir hosts (36).

Yatapoxviruses

The epidemiology and natural history of yatapoxviruses are poorly understood. YMTV and YLDV infections have occurred in animal handlers (37); however, TPV is the main naturally occurring human pathogen in the *Yatapoxvirus* genus (38). Tanapox is an endemic zoonosis of equatorial Africa that is thought to be transmitted mechanically to humans by biting insects, especially during the rainy season (39). Reports have demonstrated that travelers to regions where the disease is endemic can be infected (40, 41).

Parapoxviruses

Many different parapoxvirus diseases occur in humans (38, 42), generally as occupational infections: milker's nodule (in dairy cattle, the disease is termed pseudocowpox or paravaccinia), orf (in sheep and goats, the disease has been referred to as orf, contagious ecthyma, contagious pustular dermatitis, contagious pustular stomatitis, and sore mouth), and papulosa stomatitis (in calves and beef cattle, the disease is termed bovine papular stomatitis). Parapoxvirus infections are transmitted to humans by direct contact with infected livestock through abraded skin on the hands and fingers, and ocular autoinoculation sometimes occurs (38, 42). Sealpox virus infections have been transmitted to humans from pinnipeds (43).

Molluscipoxvirus

MCV is the sole member of the genus *Molluscipoxvirus*. MCV appears to have a human-restricted host range, and it does not grow readily in culture. Molluscum contagiosum occurs worldwide. In children, it is transmitted by direct skin contact, and sexual transmission occurs in adults (38).

CLINICAL SIGNIFICANCE

Orthopoxviruses

A global commission of the WHO declared smallpox eradicated in December 1979, and the declaration was sanctioned by the World Health Assembly in May 1980 (10). Human monkeypox, an emerging zoonotic smallpox-like disease caused by monkeypox virus, with recurrent (and likely endemic) disease in the Congo Basin countries of Africa, is now regarded as the most serious naturally occurring human poxvirus infection (11, 15, 19, 44). The emergence of monkeypox virus as a human pathogen in the United States in 2003 is a classic example of a pathogen's exploitation of new ecologic niches and hosts.

Variola Virus

Variola major virus strains produced "variola major," a syndrome consisting of a severe prodrome, fever, prostration, and a rash. Toxemia or other forms of systemic shock led to case fatality rates of up to 30%, with secondary attack rates of 30 to 80% among unvaccinated contacts within households. Variola minor virus strains (alastrim, amass, and kaffir viruses) produced "variola minor," a less severe infection with case fatality rates of <1%, although secondary attack rates among unvaccinated contacts were as high as those observed with variola major virus infections. DNA and biological data have indicated that alastrim variola minor viruses obtained from Europe and South America are similar to each other but distinct from the so-called African variola minor viruses, which appear to be variola major virus variants (7). Epidemiologically, the disease syndromes were discriminated by case fatality rates; current sequence data indicate that the genetic distinctions between strains causing major or minor disease manifestations are multiple and varied. The last naturally occurring smallpox case occurred in Somalia in October 1977, although a fatal laboratory-associated infection with variola major virus occurred at the University of Birmingham, Birmingham, United Kingdom, in August 1978 (10).

Naturally acquired variola virus infection caused a systemic febrile rash illness. For ordinary smallpox, the most common clinical presentation, after an asymptomatic incubation period of 10 to 14 days (range, 7 to 17 days), was fever, quickly rising to about 103°F, sometimes with dermal petechiae. Associated constitutional symptoms included backache, headache, vomiting, and prostration. Within a day or two after incubation, a systemic rash appeared that was characteristically centrifugally distributed (i.e., lesions were present in greater numbers on the oral mucosa, face, and extremities than on the trunk). Initially, the rash lesions appeared macular and then papular, enlarging and progressing to a vesicle by day 4 to 5 and a pustule by day 7; lesions were encrusted and scabby by day 14 and sloughed off. Skin lesions were deep seated and were in the same stage of development on any one area of the body. Milder and more severe forms of the rash were also documented. Less-severe manifestations (modified smallpox or variola sine eruptione) occurred in some vaccinated individuals, whereas hemorrhagic or flat-pox types of smallpox occurred in patients with impaired immune responses.

Variola major smallpox was differentiated into four main clinical types: (i) ordinary smallpox (~90% of cases) produced viremia, fever, prostration, and rash; (ii) modified smallpox (5% of cases) produced a mild prodrome with few skin lesions in previously vaccinated people; (iii) flat smallpox (5% of cases) produced slowly developing focal lesions with generalized infection and an ~50% fatality rate; and (iv) hemorrhagic smallpox (<1% of cases) induced bleeding into the skin and the mucous membranes and was invariably fatal within a week of onset. A discrete type of the ordinary form resulted from alastrim variola minor infection (10).

Prior to its eradication, smallpox as a clinical entity was relatively easy to recognize, but other exanthematous illnesses were mistaken for this disease (10). For example, the rash of severe chicken pox, caused by varicella-zoster virus, was often misdiagnosed as that of smallpox. However, chicken pox produces a centripetally distributed rash and rarely appears on the palms and soles. In addition, in the case of chicken pox, prodromal fever and systemic manifestations are mild, if present at all. Chicken pox lesions are superficial in nature, and lesions in different developmental stages may be present on the same area of the body. Other diseases confused with vesicular-stage smallpox included monkeypox, generalized vaccinia, disseminated herpes zoster, disseminated herpes simplex virus infection, drug reactions (eruptions), erythema multiforme, enteroviral infections, scabies, insect bites, impetigo, and molluscum contagiosum. Diseases confused with hemorrhagic smallpox included acute leukemia, meningococcemia, and idiopathic thrombocytopenic purpura. The Centers for Disease Control and Prevention (CDC), in collaboration with numerous professional organizations, has developed an algorithm for evaluating patients for smallpox. The algorithm and additional information are available at http://www.bt.cdc.gov/agent/smallpox/ and at www.bt.cdc.gov/EmContact/index.asp. Experience with the algorithm has been summarized previously (45).

Monkeypox Virus

Reviews of human monkeypox infection are available (11, 44). Monkeypox was first recognized by Von Magnus in Copenhagen in 1958 as an exanthem of primates in captivity. Later, the disease was seen in other captive animals, including primates in zoos and animal import centers.

The clinical appearance of human monkeypox, typified by the Congo Basin variant, is much like that of smallpox, with fever, a centrifugally distributed vesiculopustular rash (appearing also on the palms and soles), respiratory distress, and, in some cases, death from systemic shock. Like variola virus, monkeypox virus appears to enter through skin abrasions or the mucosa of the upper respiratory tract, where it produces an enanthem and cough. During the primary viremia, the virus then migrates to regional lymph nodes, and during secondary viremia, it is disseminated throughout the body and the skin rash appears. During the prodrome, lymphadenopathy (generally inguinal) with fever and headache is common. Individual skin lesions develop through stages of macule, papule, vesicle, and pustule. Sequelae involve secondary infections, permanent scarring and pitting at the sites of the lesions, and sometimes alopecia and corneal opacities. Acute illness in the United States in 2003, caused by the "West African" variant, appeared generally more mild (19, 20); genomic sequence analyses and comparative epidemiologic and clinical data support the existence of two distinct clades of monkeypox virus. Additional information on clinical manifestations of disease (46) is also available.

Vaccinia Virus

Humans have historically encountered vaccinia virus most commonly in the form of smallpox vaccine (now called vaccinia vaccine), a live-virus preparation that is cross-protective against other orthopoxvirus infections. The most recent recommendations of the Advisory Committee on Immunization Practices (ACIP) on vaccinia vaccination are available at http://www.cdc.gov/mmwr//preview/mmwr html/rr5010a1.htm (47). The ACIP recommends vaccination as a safeguard for laboratory and health care workers who are at high risk of orthopoxvirus infection. In the United States, the CDC Drug Service provides the vaccine after CDC approval of a formal request for this purpose by the administering physician. Vaccinia immunoglobulin is available to treat possible postvaccination complications, which can be severe.

Vaccination is done by using a multiple-puncture technique that causes a local lesion, which develops and recedes in a distinctive manner in primary vaccinees during a 3-week period. At the site of percutaneous vaccination, a papule forms within 2 to 5 days, and the lesion reaches maximum size (~1 cm in diameter) by 8 to 10 days postvaccination after evolving through vesicle and pustule stages; an areola may encircle the site. The pustule dries into a scab, which usually separates by 14 to 21 days after vaccination. In some vaccinated children, fevers with temperatures as high as about 100°F have occurred, though these have been uncommon in adults, and a regional lymphadenopathy has been observed.

Because of an increased risk for serious adverse events, such as eczema vaccinatum or vaccinia necrosum, the ACIP has stated that the vaccine is contraindicated for persons with eczema or immunocompromising conditions. The possible complications of vaccinia vaccination are described in the ACIP report (47). Despite attempts to prescreen potential vaccinees for contraindications, instances of generalized vaccinia rash, which may arise 10 to 14 days postvaccination, continue to be reported (48). On a clinical basis alone, it is often difficult to distinguish between generalized vaccinia, which represents virus presumably spread hematogenously, and a form of erythema multiforme, which may be immunologically mediated. Laboratory identification of virus within the disseminated rash may differentiate these conditions (48).

Cowpox Virus

In humans, cowpox lesions occur mainly on the fingers, with reddening and swelling. Autoinoculation of other parts of the body may occur, and severe systemic infections have been reported. Skin lesions are likened to those from a primary vaccinia vaccination. The site becomes papular, and a vesicle develops in 4 to 5 days. Healing takes about 3 weeks (33).

Yatapoxviruses

The three members of the genus *Yatapoxvirus*, TPV, YLDV of monkeys, and YMTV, are serologically related (37). DNA maps of TPV and YLDV are extremely similar, suggesting that they are the same agent. However, these DNA maps are markedly different from YMTV DNA maps, even though the DNA from the three viruses cross-hybridizes extensively (49).

TPV and YLDV infections in humans consist of a brief fever, followed by development of firm, elevated, round, maculopapular nodules that become necrotic and are distinct from the vesiculopustular lesions of orthopoxvirus infections. Generally, few lesions develop, and these occur primarily on the skin of the upper arms, face, neck, or trunk (50). Symptoms that occur prior to the appearance of lesions include fever, backache, and headache. Lesions umbilicate without pustulation during recovery from infection. They usually heal in 2 to 4 weeks. YMTV produces epidermal histiocytomas, tumor-like masses of histiocytic polygonal mononuclear cell infiltrates that advance to suppurative inflammation.

Parapoxviruses

Milker's nodule occurs as a reddened hemispheric papule that matures into a purplish, smooth, firm nodule varying up to 2 cm in diameter. The lesions usually are not painful and can persist for about 6 weeks. Human orf virus infection is usually found on the fingers, hands, and arms but may also be found on the face and neck. Fever and swelling of draining lymph nodes may be present, and the lesions often ulcerate and are painful. Autoinoculation of the eye may lead to serious sequelae (8). Contact with (e.g., skinning of) certain wild animals, including deer, reindeer, chamois, and Japanese serow, has also been a source of human parapoxvirus infection. Technicians handling gray seals have contracted sealpox virus (42, 43).

Molluscipoxvirus

In children and teenagers, molluscum contagiosum lesions generally appear on the trunk, limbs (except the palms and soles), and face, where there may be ocular involvement. Infection is usually transmitted by direct skin contact. When MCV infection is transmitted sexually among teenagers and adults, the lesions are mostly on the lower abdomen, pubis, inner thighs, and genitalia. Lesions are pearly, flesh-colored, raised, firm, umbilicated nodules, ~5 mm in diameter. The lesions tend to disseminate by autoinoculation. Prior to highly active antiretroviral therapy, MCV was an opportunistic pathogen in ~15% of patients with AIDS in the United States. Restriction endonuclease mapping of isolates suggests that there are at least three MCV subtypes (9). Two predominant MCV subtypes, MCV1 and MCV2, have been detected in a limited number of samples examined by restriction pattern and base sequence analyses, but no correlation of subtype with disease syndrome or geographic distribution has been confirmed (8, 9). A rapid PCR and restriction fragment length polymorphism analysis using skin lesion material has been described previously for differentiating the MCV subtypes (51).

Antiviral Therapy

Currently there are no drugs approved for use in the treatment of poxvirus infections; this is an area of active research and development (52). The experimental drug ST-246, an inhibitor of poxvirus egress, appears to be effective against orthopoxviruses including variola virus and monkeypox virus infections of nonhuman primates (53), and has been used investigationally in the treatment of human smallpox (vaccinia) vaccine adverse events (54). The DNA polymerase inhibitor cidofovir and its orally bioavailable derivative CMX-001 similarly have *in vitro* and *in vivo* data to support anti-orthopoxvirus activity and have been used as investigational agents in the treatment of vaccine adverse events (55). Vaccinia immune globulin is licensed for use as a treatment for severe adverse events associated with vaccination and can be obtained from the CDC.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

A suspected case of smallpox should be immediately reported to the appropriate local or state health department. After review by the health department, the case should be immediately reported to the CDC if the diagnosis of smallpox is still suspected. Current international recommendations advise that work with variola virus be done using WHO-sanctioned biosafety level 4 laboratories. Two WHO collaborating centers (WHOCCs) currently have the capability to handle smallpox specimens: one at the CDC in Atlanta and the other at the State Center for Virology and Biotechnology, Koltsovo, Russia. The WHOCC at the CDC also has containment facilities appropriate to work with monkeypox virus and other exotic poxviruses (e.g., TPV). Generally, clinical specimens suspected of containing other poxviruses (e.g., parapoxviruses and MCV) can be tested by experienced local staff using biosafety level 2 containment facilities and equipment. Additionally, laboratories testing samples from laboratory workers with potential occupational exposures to vaccinia virus may wish to consider vaccination of staff, in addition to the use of biosafety level 3 containment facilities, equipment, and work practices.

Suitable specimens for laboratory testing of most suspected poxvirus infections are at least two to four scabs and/or material from vesicular lesions. Scabs can be separated from the underlying intact skin with a scalpel or a 26-gauge needle, and each specimen should be stored in a separate container to avoid cross-contamination. Coexistent infectious rash illnesses, including simultaneous chicken pox and monkeypox infections, have been noted (14).

Lesions should be sampled so that both the vesicle fluid and the overlying skin are collected. Once the overlying skin is lifted off and placed in a specimen container, the base of the vesicle should be vigorously swabbed with a wooden applicator or polyester or cotton swab. The viscous material can be applied onto a clean glass microscope slide and air dried. A "touch prep" can be prepared by pressing a clean slide onto the opened lesion by using a gradual pressing motion. If available, three electron microscope grids can be applied in succession (shiny side to the unroofed vesicle) to the lesion by using minimal, moderate, and moderate pressure. The glass slides and electron microscope grids should be allowed to air dry for about 10 min and then placed in a slide holder or a grid carrier box for transport to a laboratory.

Alternative lesion sampling processes, including storing material on appropriate filter paper types, are being evaluated. Sample storage in transport medium (as done, for example, with herpesviruses) is discouraged, since specimen dilution decreases the sensitivity of direct evaluation by electron microscopy. Specific recommendations for electron microscopy sampling and specimen processing can be found on the Internet (http://www.bt.cdc.gov/agent/smallpox/lab-testing/pdf/em-rash-protocol.pdf). A biopsy of lesions may also provide material suitable for direct viral evaluation or immunohistochemistry. A 3- to 4-mm punch biopsy specimen can be made, and the specimen can be bisected, with half placed in formalin for immunohistochemical testing and the remainder placed in a specimen collection container. Blood and throat swabs obtained from suspected smallpox patients during the prodromal febrile phase and early in the rash phase were also a potential source of virus during the smallpox era (56).

Patient serum can also be obtained for serology to substantiate viral infection diagnoses or to infer a retrospective diagnosis. Paired acute- and convalescent-phase serum specimens can be of great value for diagnosis of infection. In this case, serum should be obtained as early as possible in the disease course and then 3 to 4 weeks later.

Most virus-containing specimens should be stored frozen at −20°C or on dry ice until samples reach their transport destination. Storage at standard refrigeration temperatures is acceptable for <7 days. Electron microscopy grids and formalin-fixed tissues should be kept at room temperature.

CLINICAL UTILITY OF LABORATORY TESTS FOR POXVIRUS DIAGNOSIS

Poxvirus infections can often be distinguished by the appearances of rashes and associated dermatopathologies (57). In addition, multiple different clinical laboratory tests can be useful for identifying and differentiating poxviruses, including electron microscopy, antigenic testing, nucleic acid detection, determination of virus growth features, and serology. The utility of these test methods for the diagnosis of poxvirus infections is shown in Table 2.

DIRECT DETECTION

Microscopy

Electron microscopy is a first-line method for laboratory diagnosis of poxvirus infections. Negative-stain electron microscopy of lesion samples was widely used during the smallpox eradication era. Because the clinical diagnosis of

TABLE 2 Diagnostic tests for poxviruses[a]

Virus(es)	HP	EM	HA	NAT	Isolation	Serology
Orthopoxviruses	X	X	X	X	X^b	X
Parapoxviruses	X	X		X	X^c	X
Yatapoxviruses	X	X		X	X^c	X
MCV	X	X		X		

[a] Abbreviations: EM, electron microscopy; HA, hemagglutination; HP, histopathology; NAT, nucleic acid test. X's indicate the utility of the tests for the specified viruses.

[b] Pock formation on CAM and tissue culture isolation are useful.

[c] Isolation in tissue culture only; viruses do not produce pocks on CAM.

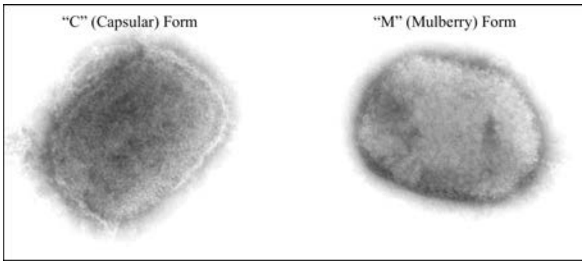

FIGURE 1 A negative-stain electron micrograph demonstrates the two forms of the brick-shaped monkeypox virus from a cell culture. The surfaces of M (mulberry) virions are covered with short, whorled filaments, while C (capsular)-form virions penetrated by stain present as a sharply defined, dense core surrounded by several laminated zones of differing densities. Image 3945 from the CDC Public Health Image Library; courtesy of C. Goldsmith, I. Damon, and S. Zaki.
doi:10.1128/9781555817381.ch107.f1

poxvirus infection is now infrequent, electron microscopy observations may provide one of the first clues into the cause of an unknown rash illness. Although electron microscopy can distinguish between orthopoxvirus and parapoxvirus morphologies, it cannot differentiate between species; selected serologic, biologic, and DNA-based assays must be used.

Orthopoxviruses have a distinctive brick-shaped, knobby morphology when examined with sodium phosphotungstate or other heavy-metal negative stains (Fig. 1). Parapoxviruses appear ovoid with a spiraling criss-cross surface. The relative sensitivity of the negative-stain method was ~95% for detecting variola virus in smallpox lesions and ~75% for detecting vaccinia virus (58, 59). Sensitivity may be improved by directly pressing a prepared grid into the base of an unroofed skin lesion, as described above (60). Descriptions of methods for negative-stain evaluation and pictures of negative-stained particles are available on the Internet (http://www.bt.cdc.gov/agent/smallpox/lab-testing/pdf/em-rash-protocol.pdf) and elsewhere (59, 61).

Poxviruses produce inclusions that have characteristic appearances when stained with May-Grunwald Giemsa and hematoxylin-eosin stains. Perinuclear basophilic or B-type cytoplasmic inclusions (virus factories or viroplasm) are observed in cells infected with any of the poxviruses and represent sites of virus replication. Certain species (e.g., the *Orthopoxvirus Cowpox virus*) produce acidophilic inclusions or A-type inclusions. Depending on the strain, A-type inclusions may (V^+) or may not (V^-) contain virions.

Antigen Detection

Orthopoxvirus is the only genus whose members produce a hemagglutinin (HA) antigen, which is detectable by hemadsorption or hemagglutination assays using chicken erythrocytes that are pretested to be suitable for such tests. Orthopoxvirus antigen can also be rapidly detected by the Orthopox BioThreat Alert assay (Tetracore, Rockville, MD), which uses a lateral flow system to rapidly detect the presence of virus in clinical specimens. It has been shown to recognize vaccinia and monkeypox viruses at levels that may be found in rash lesions (62). Direct detection of poxvirus antigens in clinical specimens is not routinely performed in many laboratories as a diagnostic assay.

Nucleic Acid Detection

PCR analysis is used by the WHOCC at the CDC to detect poxviruses DNA in samples. A recent development is the validation of a pan-poxvirus PCR assay, which can screen specimens for the presence of poxviruses other than avipoxviruses (63). Multiple single-gene PCR assays, followed by restriction fragment length polymorphism analysis or sequence analysis of the amplicon, permits species identification of orthopoxviruses. A number of different targets are used, including the HA gene, which is unique for the genus *Orthopoxvirus*; the gene for the B cytokine response modifier (CrmB, one of several different tumor necrosis factor receptor homologs produced by orthopoxviruses); and the gene for the A-type inclusion body protein. In these assays, DNA that is present in any orthopoxvirus is amplified (Table 3). The amplicon is digested with the appropriate restriction endonuclease, and digested fragments are electrophoresed. Fragment sizes are compared to reference restriction fragment length polymorphism profiles to discriminate species. Conventional PCR tests for other poxvirus genera have also been reported (64). Other nucleic acid diagnostic approaches include random amplified polymorphic DNA fragment length polymorphism for orthopoxvirus species and strain discrimination (65, 66). Microchips have also been utilized to identify poxvirus species by discernment of PCR-amplified, fluorescence-labeled DNA fragments by hybridization to orthopoxvirus species-specific immobilized DNA.

The high sensitivity of and rapid results from real-time PCR assays make them attractive for laboratory clinical diagnostic use (67). In the United States, many national reference laboratories, as well as the Laboratory Response Network laboratories, use this format of nucleic acid testing for rapid response to diagnose suspected orthopoxvirus infections and/or to rule out smallpox infections. The use of a probe or probes in this type of assay allows for the specificity; however, the extreme sensitivity of the assays can lead to false-positive contaminants from specimen carryover. Many of these methods for detection of orthopoxviruses and other poxviruses are summarized in Table 4. These types of assays are performed primarily at specific reference centers, including the CDC Poxvirus Program/WHOCC for Smallpox and Other Poxvirus Infections.

TABLE 3 Conventional PCR assays for orthopoxvirus detection

Target	Primers (amplicon size [bp])	Detection method[a]	Reference(s)
HA	Old World viruses: EACP1/EACP2 (900)	TaqI	82
	New World viruses: NACP1/NACP2 (600)	RsaI	
CrmB	VL2N/VL33 (vaccinia, 1,200; monkeypox, variola, cowpox, 1,300)	NlaIII[b]	83
A-type inclusion body protein	ATI-low-1/ATI-up-1 (1,500–1,700)	BglII or XbaI	84, 85

[a] Amplicons are digested with specified restriction enzymes to distinguish different viruses.
[b] To distinguish monkeypox, variola, and cowpox viruses.

TABLE 4 Real-time PCR assays for poxvirus detection[a]

Genus; species	Genetic target[b]	Platform or method	Comments	Limitations	Limit of detection	Reference
Orthopoxvirus, *Parapoxvirus*, and *Molluscipoxvirus*	Assay 1: E_ORF_C (*Orthopoxvirus*) (FAM) Assay 2: orf envelope protein (*Parapoxvirus*) (VIC) Assay 3: MC001R (*Molluscipoxvirus*) (FAM and VIC)	TaqMan	Multiplex assay that detects which genus of poxvirus is causing the infection based on multicolor profile and includes a control for cellular DNA		<10 copies	86
Orthopoxvirus	HA/A56R	LightCycler with hybridization probes	Melting curve analysis differentiates variola virus from other orthopoxviruses	Several cowpox and camelpox virus strains have melting temperatures identical to that of variola virus	5–10 copies	87
Orthopoxvirus; *Variola virus*, *Monkeypox virus*, *Cowpox virus*, and *Vaccinia virus*	Assay 1: A38R (VAR) (FAM) Assay 2: B7R (MPX) (TAMRA) Assay 3: D11L (CWX) (JOE) Assay 4: B10R (VAC) (Cy5)	TaqMan	Multiplex assay that detects which species of orthopoxvirus is causing the infection based on multicolor profile		ND	65
Orthopoxvirus; *Variola virus*	HA/A56R	TaqMan	Variola virus-specific probe cleavage		25 copies	88
Orthopoxvirus; *Variola virus*	HA/A56R	LightCycler with hybridization probes	Melting curve analysis differentiates variola virus from other orthopoxviruses	Several cowpox virus strains have melting temperature identical to that of variola virus	2.74–9.88 copies	89
Orthopoxvirus; *Variola virus*	Assay 1: Rpo 18 Assay 2: VETF Assay 3: A13L (VAR) Assay 4: A13L (nVAR-OPX)	LightCycler with hybridization probes	Melting curve analysis differentiates variola virus from other orthopoxviruses		ND	90
Orthopoxvirus; *Variola virus*	Assay 1: B10R Assay 2: B9R Assay 3: HA/A56R	TaqMan		Assays 1 and 2: some cowpox virus strains are amplified Assay 3 is identical to the assay described in reference 88, with a slightly shortened probe	12–25 copies	91
Orthopoxvirus; *Variola virus*	14kD/A27L	LightCycler with hybridization probes	Melting curve analysis differentiates variola virus from other orthopoxviruses		>12 copies	92
Orthopoxvirus; *Variola virus*	*crmB*	LightCycler with hybridization probes	Melting curve analysis differentiates variola virus from other orthopoxviruses	Specific identification of variola virus has to be performed by restriction enzyme analysis of PCR amplicons	5 copies	93
Orthopoxvirus; *Variola virus*	*crmB*	Two TaqMan probes	One probe is variola virus specific		10–100 copies	94
Orthopoxvirus; *Monkeypox virus*	Assay 1: B5R (MPX) Assay 2: E9L (nVAR-OPX)		Monkeypox virus-specific assay (MGB probe) Orthopoxvirus-non-variola detection assay (TaqMan)	MGB probe performs optimally only in the iCycler iQ and SmartCycler platforms	10 ng 2 pg–20 fg	95

(Continued on next page)

TABLE 4 *(Continued)*

Genus; species	Genetic target[b]	Platform or method	Comments	Limitations	Limit of detection	Reference
Orthopoxvirus; Monkeypox virus	Assay 1: G2R (MPX) Assay 2: G2R (MPX-West African) Assay 3: C3L (MPX-Congo Basin)	TaqMan			3.5 copies	96
Orthopoxvirus; Variola virus	14kD/A27L	TaqMan	Two probes; one probe is variola virus specific		25 copies	97
Orthopoxvirus; Variola virus	HA/A56R	TaqMan	Two probes; one probe is variola virus specific		50–100 copies	98
Orthopoxvirus; Variola virus	HA/A56R	LightCycler with hybridization probes	Melting curve analysis differentiates variola virus from other orthopoxviruses		13–1,300 copies	99
Orthopoxvirus; Vaccinia virus	B8R	TaqMan			10–10^6 copies	100
Orthopoxvirus; Cowpox virus	D11L	TaqMan			20 copies	101
Orthopoxvirus; Cowpox virus	A55R	TaqMan		Certain cowpox viruses have this target deleted	ND	102
Yatapoxvirus	101 nt of PstIL fragment	TaqMan		Detects YLDV and TPX, not YMTV	8 copies	103
Parapoxvirus; Orf virus	B2L	TaqMan			10–10^6 copies	104
Parapoxvirus; Orf virus	RNA polymerase	TaqMan	Two different primer/probe sets: one specific for *Parapoxvirus* genus; one specific for *Orf virus* species		ND	105
Parapoxvirus; Orf virus	DNA polymerase	TaqMan		Not tested for specificity against other parapoxviruses	15 copies	106
Parapoxvirus	B2L	TaqMan			4.7 copies	56
Molluscum contagiosum virus	P43K MC080R	TaqMan	Pyrosequencing of the p43K product differentiates MCV1 and MCV2		10 copies	107

[a] Abbreviations: CWX, *Cowpox virus*; FAM, 6-carboxyfluorescein; MGB, minor groove binder; MPX, *Monkeypox virus*; ND, not determined; nVAR-OPX, non-variola *Orthopoxvirus*; VAC, *Vaccinia virus*; VAR, *Variola virus*.

[b] Orthopoxvirus assays use the target's Vaccinia-Copenhagen nomenclature.

ISOLATION AND IDENTIFICATION

Orthopoxviruses can be grown in a variety of established cell culture lines, including Vero, BS-C-1, CV-1, LLCMK-2 monkey kidney cells, human embryonic lung fibroblast cells, HeLa cells, chicken embryo fibroblast cells, and MRC-5 human diploid fibroblast cells, as well as in fetal rhesus monkey kidney (FHRK-4) cells. Cytopathic effects (CPEs) appear as cell rounding with long cytoplasmic extensions (Fig. 2 and 3). In some cases, syncytium formation can also be seen; in Fig. 3B, this is seen in the monkeypox virus-induced CPE. The timing of CPEs is dependent on the infectious inoculum. Most laboratories confirm the presence of a specific orthopoxvirus via PCR (see above). Methods for growing and discriminating the morphologies of orthopoxviruses on the chorioallantoic membranes (CAMs) of 12-day-old chicken embryos have been described previously (58, 59, 68, 69). Orthopoxviruses are the only human poxviruses that produce pocks (68, 69) on the CAMs of fertile chicken eggs; pock morphology is useful for biologic species and variant differentiation. Parapoxviruses, yatapoxviruses, and MCV do not form pocks on the CAM, although avipoxviruses, leporipoxviruses, and capripoxviruses do so. Poxvirus genera can usually be identified and differentiated by virus neutralization testing with hyperimmune reference sera (24, 38, 58, 59, 70). However, it can be difficult to identify the infecting species since poxviruses are antigenically closely related within a given genus.

SEROLOGIC TESTS

Orthopoxviruses

When virus-containing clinical specimens are not available, antibody detection may be the only way to define the etiology of the disease. Serologic methods currently used to detect antibodies against human orthopoxviruses include enzyme-linked immunosorbent assays (ELISAs), the virus neutralization test, Western blotting, and hemagglutination inhibition. Various protocols for poxvirus serologic testing used at the CDC are detailed elsewhere (39).

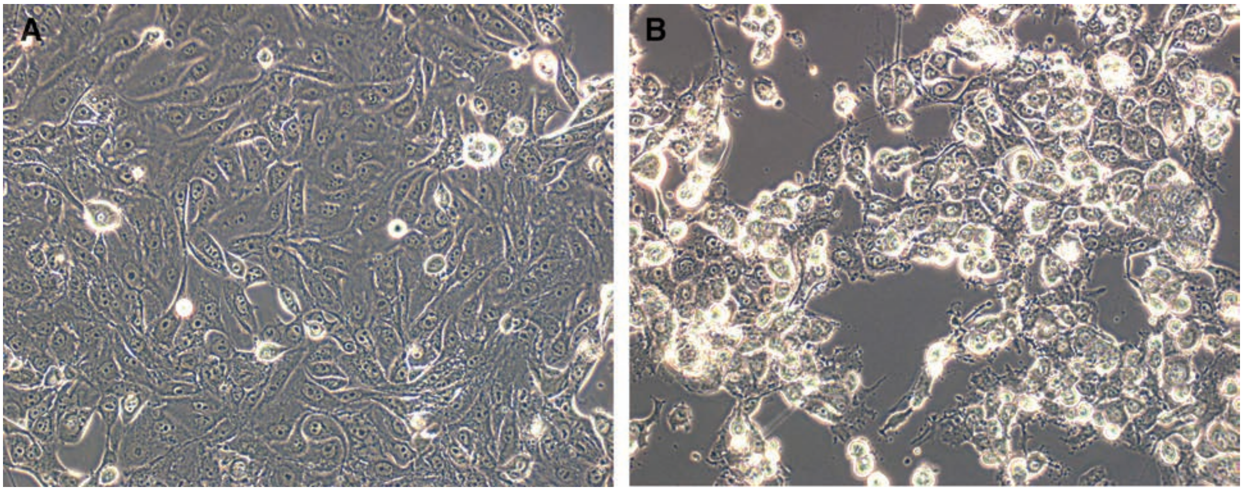

FIGURE 2 Vaccinia virus ("Dryvax") CPE in FHRK-4 cells. (A) Early CPE; (B) mature CPE. Courtesy of V. Olson. doi:10.1128/9781555817381.ch107.f2

The description of an orthopoxvirus IgM assay offers great promise to enhance investigations of orthopoxvirus infection outbreaks, often semiretrospective in nature (39). This technique offers the advantage of measuring recent infection or illness with an orthopoxvirus. It is useful for evaluating disease incidence in epidemiologic surveillance studies. For example, during the 2003 U.S. monkeypox outbreak, the IgM capture assay demonstrated ~95% sensitivity and ~95% specificity for epidemiologically linked and laboratory test-confirmed patients when sera were obtained between days 4 and 56 post-rash onset. A low-grade response, termed "equivocal," awaits further research. This assay was also used to detect anti-orthopoxvirus IgM in the cerebrospinal fluid of an encephalitic patient with monkeypox (71). A peptide-based ELISA for the identification of monkeypox virus-specific antibodies has been reported (72, 73). It was capable of differentiating between monkeypox virus and vaccinia virus. However, this ELISA is known to cross-react with variola virus and, due to sequence conservation across the orthopoxviruses, is also likely to cross-react with many strains of cowpox viruses. It remains an investigational tool rather than a clinical test since its clinical utility has not been further established. A monkeypox virus-specific antibody has been isolated, and characterization of the epitope has provided insight into the design of peptides to be used in a monkeypox-specific serologic assay (74, 75).

In the current state of bioterrorism response awareness, tests to evaluate residual protection from previous vaccination are being requested. It is important to note that there is no one routine immunologic test that defines a person's degree of protection against a poxvirus infection. Protection is genetically defined and requires a concert of cell-mediated and humoral immune responses. Studies (76) suggest that humoral responses may be the critical component of recovery from and survival of a systemic orthopoxvirus infection. The presence of neutralizing antibodies generally indicates recovery from an infection, not always protracted protection from future infection. Neutralizing antibodies against variola, monkeypox, cowpox, or vaccinia viruses may be detectable as early as 6 days after infection or vaccination. Neutralizing antibodies have been detected as long as 20 years

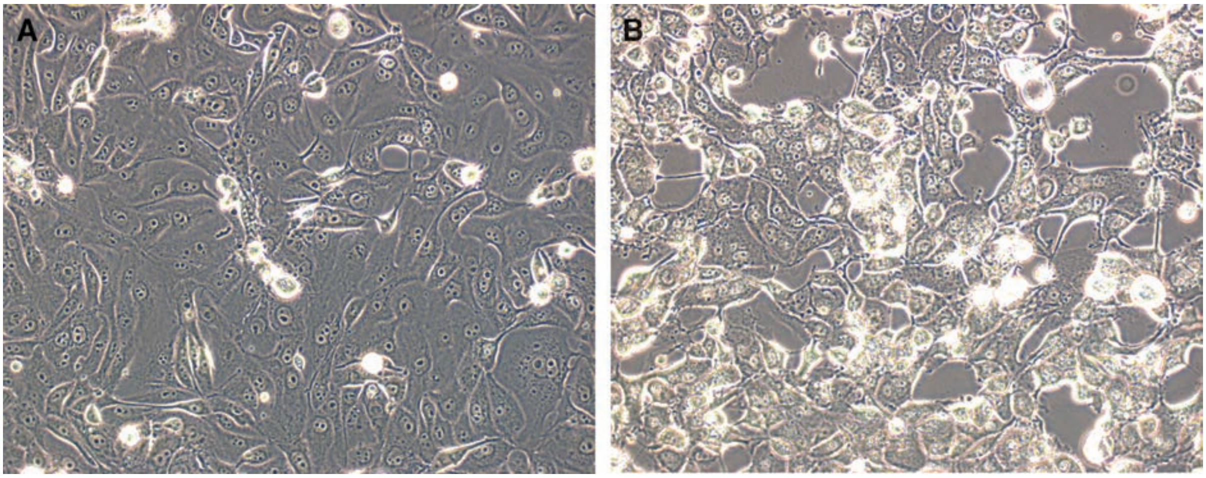

FIGURE 3 Monkeypox virus (Congo Basin clade, v79-I-005) CPE in FRHK-4 cells. (A) Early CPE; (B) mature CPE with syncytia. Courtesy of V. Olson. doi:10.1128/9781555817381.ch107.f3

after vaccinia vaccination or natural infection with other human orthopoxviruses.

The orthopoxvirus neutralization test has been traditionally performed by looking for a reduction in plaque number. This was improved upon using a green fluorescent protein-tagged vaccinia virus and measuring viral entry into tissue culture cells (77, 78). In the virus neutralization assay, a 4-fold rise in antibody titer between serum samples drawn during the acute and convalescent phases is usually considered diagnostic of poxvirus infection. When only one serum specimen is available from one phase of infection, confirmation of a clinical diagnosis may be difficult to impossible. Because orthopoxviruses are closely related, serum cross-absorption tests, such as those performed in immunofluorescent-antibody or immunodiffusion methods, have been used with variable success with patient and animal sera. Orthopoxvirus antigen cross-absorption assays have been performed using hyperimmune animal sera and have been utilized in serosurveys for animal or human monkeypox infection (58, 59). False-positive results should be ruled out by using appropriate control sets of sera of known provenance.

The Western blot assay is performed essentially as described by Towbin et al. (79) and uses various antigens, including purified virus and sometimes the concentrate of culture fluid from infected cells maintained under medium that contains 1% or no serum supplement. Few laboratories are using this method, as reliable standardization has not been achieved.

Pseudocowpox, Orf, Tanapox, and Molluscum Contagiosum Viruses

Serologic methods used to help confirm parapoxvirus infections (milker's nodule and orf) have included ELISAs (80) and Western blot assays that use various antigen preparations. Serologic testing for TPV infection by standard ELISA (80) with antigens obtained from concentrates of infected cell culture, by an indirect immunofluorescent-antibody test, and by neutralization test has been moderately effective. Optimally, sera should be collected at the time of actual disease and 3 to 5 weeks or later after the presumed onset date. Because MCV cannot be readily grown in culture, no routine serologic test is available. Molluscum contagiosum is readily diagnosed clinically, often with the aid of electron microscopy and histopathologic testing performed by a local diagnostic facility.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

For confirmation of an infectious agent, cell culture or other mechanism for demonstrating viable virus should be regarded as the gold standard. Absent readily available or feasible tissue culture methods, the use of multiple diagnostic assays or techniques improves the specificity of a diagnosis. Nucleic acid amplification tests, while sensitive, can result in false-positive results. A proficiency survey of nucleic acid amplification tests performed by 33 labs spanning three geographic areas (Europe, Australasia, and the United States) found a substantial rate of false-positive results (~12%), highlighting the need for sound molecular practices, which becomes even more critical when testing for potential biothreat agents (81). The use of multiple nucleic acid tests, with different detection targets, can improve the specificity of a diagnosis. Electron microscopy can be used to evaluate generically for the presence of a poxvirus and can be used to infer viable agent if multiple virus forms are present but cannot be used to make a specific genus diagnosis except in the case of parapoxvirus infections. Serologic assays are available at a few reference laboratories worldwide but only rarely can be used to make a specific species diagnosis. Histochemistry, combined with immunologic analysis, can be used to identify poxvirus genera in a few reference centers worldwide. A combination of nucleic acid amplification, growth of agent in culture, serology, electron microscopy, and/or immunohistochemistry techniques improves the sensitivity and specificity of a diagnosis.

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Hepatitis B and D Viruses*

REBECCA T. HORVAT AND RYAN TAYLOR

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HEPATITIS B VIRUS

Introduction

Epidemic hepatitis was first described in the 5th century B.C. The earliest documented blood-borne outbreak of hepatitis occurred in Bremen, Germany, in 1883 among shipyard workers who received a smallpox vaccine stabilized with human serum. In 1950, Bauer and Wyman referred to viral hepatitis as either infectious hepatitis (hepatitis A) or serum hepatitis (hepatitis B) based on the different epidemiologic characteristics of the diseases (1). This terminology was adapted by the World Health Organization (WHO) in 1973 to distinguish the two agents of hepatitis.

Subsequently, Blumberg and colleagues in 1965 discovered the "Australia" antigen, now known as hepatitis B virus (HBV) surface antigen (HBsAg), in blood specimens from Australian aborigines (2, 3). A few years later, in 1968, Prince (103) and Okochi and Murakami (104) isolated the Australia antigen in patients with HBV infection, which led to the first HBV vaccine in 1981. There is now abundant information on the natural history of HBV, including the pathogenesis and epidemiology. This has led to more accurate diagnostic assays as well as successful antiviral treatment and safe and effective vaccines. Due to the tremendous burden of this disease, laboratory tests used to diagnose and monitor disease as well as the effectiveness of the vaccine are keys to the control of this disease.

Taxonomy

HBV is an enveloped DNA virus in the *Hepadnaviridae* family and has a narrow host range. HBV infects only species closely related to humans, such as gibbons and several monkey species (4). As with other viruses in this family, the HBV genome is partially double stranded. It has a circular DNA molecule that replicates via an RNA intermediate. The replication of HBV DNA by reverse transcription is unique for human DNA viruses (4).

Description of the Agent

HBV is a 42-nm, partially double-stranded DNA virus that replicates in the nucleus of the host cell. HBV-infected cells have no cytopathic features because the virus causes little damage to the host cell (5). The virion is composed of HBsAg embedded in a lipid membrane surrounding a viral nucleocapsid core. The complete virus particle is known as the Dane particle, after its discoverer (6). The viral nucleocapsid core is surrounded by a specific viral core protein (HBcAg) and encloses a single molecule of partially double-stranded DNA, hepatitis B e antigen (HBeAg), and a DNA-dependent polymerase (Fig. 1A). The terminology used to describe the various antigens and antibodies associated with HBV as they appear at different stages of infection is given in Table 1.

HBV particles found in the sera of patients with active HBV infection reveal three distinct morphologic entities in varying proportions (Fig. 1A and B). The most abundant forms (by a factor of 10^4 to 10^6) are the small, pleomorphic, spherical, noninfectious particles (17 to 25 nm in diameter). These noninfectious particles have a low buoyant density, reflecting the presence of lipids from the host cell membrane (7). Less numerous are the tubular or filamentous forms, which have diameters similar to those of the small particles. The third and least numerous particle is the complete HBV virion, with a diameter of approximately 42 to 47 nm (6). At present there is no cell culture system that supports the growth of HBV.

The HBV genome is approximately 3,200 bases and contains overlapping genes (Fig. 1C). There are four open reading frames (ORFs) in the complete, minus strand. These genes encode the structural proteins (HBsAg and HBcAg), replicative proteins (polymerase and X protein), and regulatory proteins. The genome is compact, and most sequences are essential for productive infection (4).

After binding to hepatocytes, the HBV virion is taken up and uncoated. The partially double-stranded, relaxed circular DNA is converted by the host polymerase to a covalently closed circular DNA (cccDNA) template in the cell nucleus. The cccDNA form is used as a template for transcription of the pregenomic RNA (pgRNA) and mRNA (4). The pgRNA is transcribed from the cccDNA in the nucleus and then moves into the cytoplasm of the host cell. In the cytoplasm, the pgRNA serves as the template for the HBV reverse transcriptase enzyme as well as the core protein. At the same time, the polymerase converts the pgRNA to a new circular DNA molecule. Early in infection, some newly synthesized genomes from the cytoplasm circulate back to the nucleus to build up and maintain the pool of cccDNA (8).

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*This chapter contains information presented by Rebecca T. Horvat and Gary E. Tegtmeier in chapter 106 of the 10th edition of this *Manual*.

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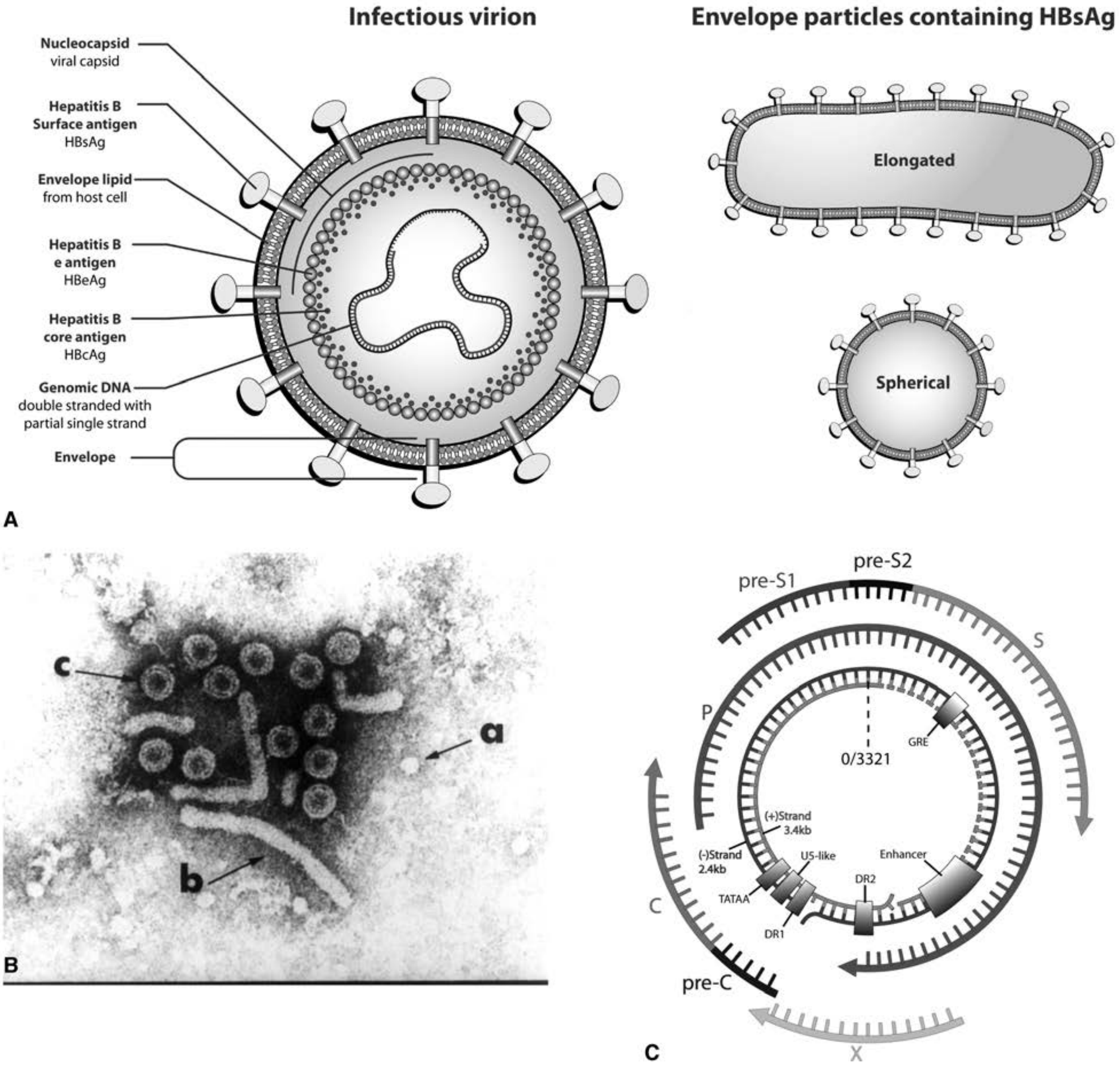
Infectious virion
Envelope particles containing HBsAg
Nucleocapsid viral capsid
Hepatitis B Surface antigen HBsAg
Envelope lipid from host cell
Hepatitis B e antigen HBeAg
Hepatitis B core antigen HBcAg
Genomic DNA double stranded with partial single strand
Envelope
Elongated
Spherical
A
c
a
b
B
pre-S1
pre-S2
S
P
GRE
0/3321
(+)Strand 3.4kb
(-)Strand 2.4kb
U5-like
Enhancer
TATAA
DR2
DR1
C
pre-C
X
C

FIGURE 1 (A) The intact infectious HBV virion and the empty particles are shown. The two HBV particles (right) comprised of HBsAg are shown as elongated or tubular particle and as spherical particles. These particles vastly outnumber the virions. (Designed by the University of Kansas Graphic Design Department, 2009.) (B) Electron micrograph of serum showing the presence of three distinct morphologic entities: 17- to 25-nm-diameter pleomorphic, spherical particles (a); tubular or filamentous forms with diameters similar to those of the small particles (b); and 42- to 47-nm-diameter double-shelled spherical particles representing the HBV virion (Dane particle) (c). Magnification, ×10^5. (C) Diagrammatic representation of HBV coding regions. The functioning genome is a double-stranded circular DNA molecule, shown in the middle. RNA transcripts (arrows) are generated using both the plus-strand [(+)Strand] and minus-strand [(−)Strand] DNA templates. The largest transcript codes for the viral polymerase shown around the genome as the P transcript. The transcript for surface antigen (S) is produced as three separate transcripts, pre-S1, pre-S2, and S. The core protein is translated from the C transcript. HBeAg is encoded within the HB core gene. The transactivating protein is coded by the X transcript. (Designed by the University of Kansas Graphic Design Department, 2009.) doi:10.1128/9781555817381.ch108.f1

TABLE 1 HBV markers in different stages of infection and convalescence[a]

Stage of infection	Molecular marker: HBV DNA	Protein antigen markers		HBV-specific antibody markers			
		HBsAg	HBeAg	Anti-HBc		Anti-HBe	Anti-HBs
				IgM	Total		
Susceptible	–	–	–	–	–	–	–
Early incubation	+	–	–	–	–	–	–
Late incubation	+	+	–/+	–	–	–	–
Acute infection	+	+	+	+	+	–	–
Recent infection[b]	–/+	–	–	++	+	+	+++
Remote infection[c]	– or very low	–	–	–	+	+/–	+
Remote infection or chronic infection with HBsAg mutation[c]	–/+	–	–	–	+	+/–	–
HBsAg-negative acute infection	– ($<10^3$ IU/ml)	–	–	+	+	–	–
HbsAg variant infection	–/+	–	–/+	+/–	+	–	–
Immune active carrier	++ ($>10^5$ IU/ml)	+	–/+	–/+	+++	–	–
Inactive HBsAg carrier	– ($<10^3$ IU/ml)	+	–	–	+	+	–
Immune tolerant carrier	+++	+	+	–	+	–	–
Vaccination response	–	–	–	–	–	–	+

[a] –, not detected; +, detected; –/+, sometimes detected; ++, detected at high levels; +++, detected at very high levels.

[b] This description can be applied to early convalescence and to individuals that remain HBV DNA positive for prolonged periods in the absence of HBsAg.

[c] Remote infection can be applied to individuals with anti-HBc in the absence of other serologic markers, including DNA. These patients may or may not have anti-HBs. There is evidence that these patients may reactivate HBV during immunosuppression. HBsAg, complex antigen found on surface of HBV and on 20-nm-diameter particles and tubular forms; HBcAg, antigen associated with 27-nm-diameter core of HBV; HBeAg, protein that results from the proteolytic cleavage of the precore/core protein by cellular proteases and secreted as soluble protein in serum.

The HBV genome is highly efficient in that every nucleotide in the genome is in at least one coding region. More than half of the genome is transcribed in more than one ORF. Four viral mRNA transcripts are translated into HBV proteins. These viral mRNAs are transcribed from different promoter regions on the cccDNA template (Fig. 1C). The longest mRNA acts as the template for genome replication (pgRNA) and also the translation of precore, core (HBcAg), and polymerase proteins (4). The second transcript encodes the pre-S1 protein (39 kb), pre-S2 protein (33 kDa), and S protein (24 kDa; also known as HBsAg). A third transcript encodes the pre-S2 protein and the S protein. The smallest mRNA codes for the X protein, which is responsible for transactivating transcription. Mature virions are produced by transcription of RNA into a circular DNA molecule. The long RNA transcript and the polymerase protein are packaged into mature core particles, and the reverse transcriptase enzyme synthesizes a new viral DNA genome. These particles are then transported to the HBsAg in the endoplasmic reticulum (ER) of the host cell and exported from the cell. The unused HBsAg is also released from infected cells as spherical and filamentous particles devoid of HBV genomes or core proteins. Thus, these HBsAg particles are used as a diagnostic marker in serum to detect active viral replication (5, 9).

The envelope proteins of HBsAg are made up of three polypeptides encoded by the S/pre-S region of the HBV genome (Fig. 1C). The major protein is the smallest peptide. It is encoded by the S region and is a glycosylated polypeptide. The middle protein, encoded by the S and the pre-S2 regions, has an additional glycosylation site. The large S protein consists of 389 to 400 amino acids encoded by the pre-S1, pre-S2, and S regions of the genome. The production of HBsAg exceeds what is needed for virion production, and this excess antigen circulates in the blood of infected individuals as spherical and tubular particles and can be detected in clinical assays to detect active HBV infection (Fig. 1A and B) (10). This antigen may persist in the serum for variable periods after initial infection and in some patients can be as high as 10^{13} per ml.

Two additional HBV-specific proteins play a key role in diagnostic testing. HBcAg and HBeAg have different antigenic specificities, and both can be distinguished from HBsAg. The HBcAg is a polypeptide encoded by the C gene of HBV and is translated from the pregenomic mRNA (Fig. 1C). The precore sequence within the C gene contains the start codon for HBeAg translation (10). Because of the different start codons the two proteins, HBcAg and HBeAg, are antigenically unrelated. HBeAg lacks the viral DNA-binding domain found on HBcAg. The first 29 amino acid residues of the HBeAg are encoded in the precore region of the C gene. This part of the HBeAg directs the protein to the host ER. In the ER this "homing" portion of the protein is cleaved off, and HBeAg is released from the host cell to the blood. In the blood HBeAg is a soluble protein or is bound to albumin, α-1-antitrypsin, or immunoglobulin. It is a dependable marker for the presence of intact virions indicating high infectivity. In some HBV strains, a mutation at the end of the precore region of the C gene results in a stop codon which prevents the translation of HBeAg. These precore mutants contribute to the pathogenesis of chronic HBV disease, leading to acute exacerbation of disease (11, 12). Other HBV gene mutations have been observed in the core, core promoter, envelope, and polymerase regions. The envelope protein variants become relevant, as some of them lead to antigenic changes that are not recognized by the HBV vaccine. These viruses are called escape HBV mutants, and infected patients do not have detectable antibody to HBsAg and do not respond to hepatitis B immunoglobulin (HBIG) therapy (13–15).

Epidemiology and Transmission

According to the WHO, HBV is 50 to 100 times more infectious than HIV-1 (http://www.who.int/csr/disease/hepatitis/whocdscsrlyo20022/en/index3.html#transmission). HBV infections are prevalent around the world and represent a global public health problem. The WHO estimates that 2 billion people are infected with HBV, and approximately 600,000 people die each year as a result of acute or chronic HBV (16, 17).

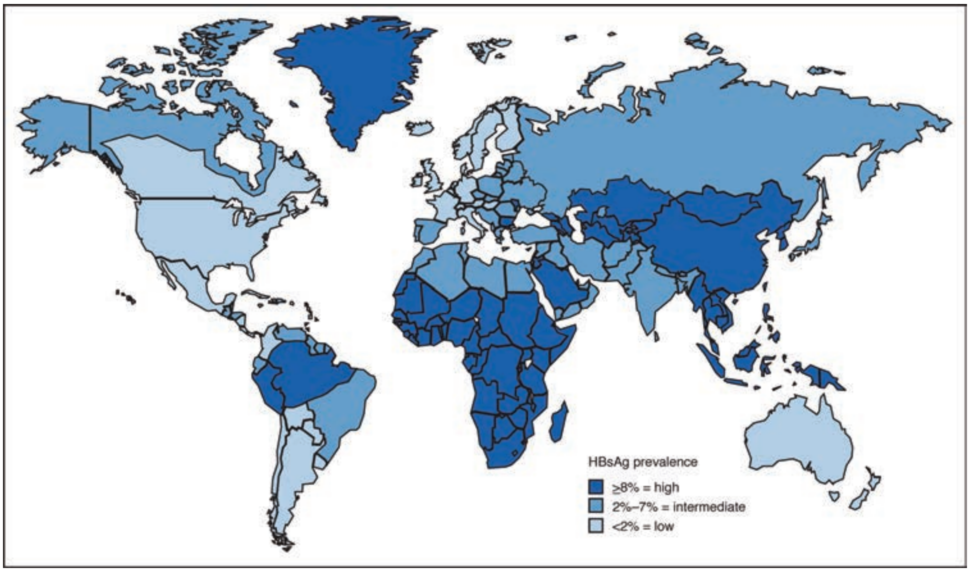

FIGURE 2 Worldwide distribution of chronic hepatitis B (CDC).
doi:10.1128/9781555817381.ch108.f2

The majority of these HBV-infected individuals live in Asia or Africa (Fig. 2). Approximately one-fourth of adults who were infected with HBV as children experience serious complications from liver cirrhosis and/or hepatocellular carcinoma (HCC) linked to chronic HBV (18). The Centers for Disease Control and Prevention (CDC) estimates that 800,000 to 1.4 million individuals in the United States are chronically infected with HBV despite a decline of new infections during the last 20 years (http://www.cdc.gov/hepatitis/Statistics/2010Surveillance/Commentary.htm, accessed February 2015). In the United States, most infections are in adults, in whom the risk of chronic infection is lower. In contrast, outside the United States, perinatal exposure is more common and leads to chronic HBV infections (18, 19).

The most common modes of HBV transmission are vertical transmission (mother to child perinatally), early childhood infections from close contact with infected individuals, sexual activity (both heterosexual and male homosexual), and injection drug use or other physical contact with infected body fluids (occupational exposure, contaminated blood products, etc.) (4). HBV is not transmitted by casual activities such as talking, hand holding, or hugging, by ingestion of food or water, or from a cough or sneeze.

HBV causes chronic hepatitis, cirrhosis, and HCC worldwide. In most countries HBV infections are reportable to the public health authorities. HBV is a public health concern that can be addressed by accurate diagnostic detection, infection control, and vaccinations (5).

Clinical Significance

HBV infects hepatocytes, leading to either an acute infection that resolves or a chronic infection lasting years. In infected individuals, subclinical hepatitis presents as a mild disease without symptoms or jaundice. It is not uncommon for many HBV-infected people to be asymptomatic early in disease. Nevertheless, some patients may have vague symptoms such as abdominal pain, nausea without jaundice (anicteric hepatitis), or nausea with jaundice (icteric hepatitis). HBV infections can result in the complete recovery of the patient, fulminant hepatitis with mortality, or a chronic viral infection. Three phases of chronic HBV disease are recognized: (i) the immune tolerant phase, (ii) the immune clearance phase, and (iii) the inactive carrier phase (20).

The incubation period of acute HBV infection ranges from 6 weeks to 6 months. During this time the infection can be symptomatic or asymptomatic. Newborns infected with HBV usually have chronic, asymptomatic infections, while older children and adults are typically symptomatic after a primary infection (4, 9, 21).

The symptoms of acute HBV infection are often mild but sometimes include physical signs such as jaundice, dark urine, clay-colored stools, and hepatomegaly (4). Some patients experience weight loss, right upper quadrant pain, and a tender, enlarged liver (21). Acute HBV infections are usually self-limited, and most patients recover completely after specific antibodies (anti-HBs) clear the virus (22).

The disease outcome of acute HBV is age dependent, and most patients with acute disease are adults. Acute liver damage is caused by the host immune response to HBV-infected hepatocytes (4). This results in massive necrosis, which can lead to permanent damage to the liver. Mortality associated with fulminant hepatic failure is high without liver transplantation. After transplantation, HBV reinfection of the "new" liver is common, resulting in injury to the new liver in some patients. HBIG and/or antiviral therapy can prevent this outcome (20). Pathologic features of acute HBV include both degenerative and regenerative liver parenchymal changes that lead to lobular disarray. Cytotoxic T lymphocytes (CTLs) play a role in facilitating viral clearance during acute infection as well as the pathogenesis of hepatocellular injury (23).

Patients with perinatally acquired HBV are usually immune tolerant to HBV antigens. This explains the absence of severe liver disease despite high levels of virus (24). Patients who continue to have detectable HBsAg or detectable HBV DNA in serum for at least 6 months after infection are considered to have chronic HBV infections (20). Chronic carriers can remain positive for HBsAg indefinitely, although some HBsAg-positive patients spontaneously convert to HBsAg negative after the appearance of anti-HBs. Many of these patients continue to have detectable HBV DNA (4, 5). Symptoms of chronic HBV are generally nonspecific and may be unrecognized for years.

The National Institutes of Health (NIH) sponsored a consensus conference in 2008 on HBV infections (20). The conference defined three phases of chronic HBV infection, the immune tolerant phase, the immune active phase, and the inactive carrier phase, as mentioned above. The immune tolerant phase usually occurs in children who are infected with HBV early in life. It is characterized by high levels of HBV DNA with little damage or inflammation in the liver (Table 1). This stage can last for a few years to several decades (4, 5, 20).

Most HBV-infected individuals progress to the immune active phase, in which a liver biopsy shows inflammation with fibrosis, thus the term "active." This pathology results from a persistent immune response to the HBV proteins on infected hepatocytes. The last phase of chronic HBV infection is the inactive carrier phase, characterized by less inflammation as determined by liver biopsy and normal liver enzyme levels. These patients have a lower risk for HCC (9, 22).

Chronic HBV infection is associated with an increased risk for HCC (5, 9, 22). During HBV infection, the viral DNA randomly integrates into the hepatocyte genome. Over time this results in high levels of integrated viral DNA in host cells that can persist for years. Since the viral DNA integrates randomly, the number of integration sites also increases over time (20). Some of this randomly integrated viral DNA can activate cellular proto-oncogenes or suppresses the regulation of gene expression (25). In addition, some HBV proteins, such as the X protein and the truncated pre-S/S protein, are potent transactivators of cellular genes (26). Likewise, several other factors have been associated with the development of HCC, such as smoking, alcohol consumption, infections with other hepatotropic viruses (hepatitis C), and mycotoxins (aflatoxin).

Safe and effective vaccines against HBV have been available since 1981. The efficacy of HBV vaccination has been proven worldwide and is monitored by the WHO (17). The complete HBV vaccination series is protective in >95% of infants, children, and young adults (17, 22). Vaccine-induced protection lasts at least 20 years and may be lifelong (22). Currently there are two vaccines that contain only HBsAg and three different combination vaccines that contain HBsAg along with antigens to other pathogens. The immune responses induced by vaccines made by different manufacturers are not significantly different. Individuals with a known or suspected exposure to HBV should be given HBV vaccine as soon as possible after the exposure, in addition to HBIG.

Chronic HBV infections should be treated with antiviral drugs. The primary goal of treating chronic HBV is to suppress viral replication and slow the progression of liver damage (4). At present, seven therapeutic agents have been approved for use in the United States by the FDA. These drugs are classified into two categories, the interferons and nucleoside/nucleotide analogs. The first nucleoside analog approved for treating chronic HBV infection was lamivudine (27). More recently, several new agents have become available for the treatment of chronic HBV. These include adefovir, entecavir, tenofovir, emtricitabine, and telbivudine (20, 28, 29).

Collection, Transport, and Storage of Specimens

HBV infection is diagnosed by serologic and molecular markers detected in serum or plasma. All FDA-approved assays have specific specimen requirements defined in their package inserts. These requirements state the specimen types acceptable as well as describe the processing and storage of the specimen. In general, HBV antigens and antibodies are stable at room temperature for days, can be stored at 4°C for months, and can be frozen at −20 to −70°C for years. Although HBV markers are stable in serum stored at −70°C, repetitive freezing and thawing can lead to their degradation. The use of hemolyzed samples should be avoided due to the potential of these specimens to interfere with the detection signals used in immunoassays.

Plasma recovered from blood collected in tubes containing EDTA (lavender-top tubes) or in tubes containing anticoagulant citrate dextrose (ACD; yellow-top tubes) is used for nucleic acid analysis. Plasma should be separated from red cells within 6 h and stored at 4°C until tested. EDTA stabilizes the viral nucleic acid, which can then be stored at 4°C for up to 5 days without significantly affecting the results (30). For long-term storage, plasma should be kept at −70°C. Heparinized plasma is unacceptable for most nucleic acid analysis because heparin interferes with *Taq* polymerase-mediated PCR (30, 31). There is evidence that HBV DNA is stable and detectable without a loss of sensitivity after many freeze-thaw cycles (32, 33). Useful information about many diagnostic tests can be found by consulting the FDA website and the specific year of test approval (http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/DeviceApprovalsandClearances/Recently-ApprovedDevices/default.htm, accessed February 2015).

The HBV virion is very hardy and remains infectious for at least 7 days outside the host (30). Thus, spills or splashes should be cleaned using absorbent material and disinfected with an appropriate disinfectant. Decontamination should be carried out while wearing gloves. Laboratory personnel should regard all specimens as potentially dangerous (34). The Occupational Safety and Health Administration (OSHA) standards for occupational exposure to blood-borne pathogens (35) are designed to protect employees exposed to blood and other potentially infectious materials. OSHA mandates that all employees whose job requirements put them at risk for blood-borne pathogens be offered HBV vaccine at no cost. OSHA standards and additional safety recommendations can be found in the literature.

Direct Detection

The diagnosis of HBV uses a combination of tests. These assays detect specific HBV proteins, nucleic acid, and specific antibodies in plasma or serum (Table 1).

Microscopy

Microscopic detection of HBV does not play a role in the diagnosis of disease. However, a liver biopsy is often used to assess the extent of histologic involvement as well as the response to therapy. Histologic examination is useful for distinguishing among acute viral hepatitis, chronic hepatitis, and cirrhosis.

Antigen

Several HBV-specific proteins can be detected in patient specimens during infection. These are a marker of active viral replication. The presence of HBsAg and/or HBeAg in serum occurs during primary infection and during chronic HBV infection (Table 1). HBsAg is located on the outer surface of the HBV particles, while HBeAg is translated from the precore mRNA of HBcAg (Fig. 1C). The function of HBeAg and its role in disease have not been clearly identified; however, the detection of this protein in serum indicates high viral replication. Both HBsAg and HBeAg are made in large excess by infected host cells and can be detected in serum during active infection (4).

Diagnostic assays with high sensitivity and specificity are available to detect these HBV antigens. Table 1 lists the assays used to determine the stage of HBV disease. HBV antigens are detected using solid-phase assays based on capture with a monoclonal antibody and then detected with a second antibody attached to a signal. These assays use microparticles with different compositions and sizes and are performed on automated instruments (36, 37). Antigen capture and detection reagents are specific for the major immunodominant region of HBsAg. Current detection methods use enzyme reactions, chemiluminescence, or fluorescence polarization to detect specific antigens (Table 2).

HBsAg

The detection of HBsAg in serum plays a central role in establishing the diagnosis of HBV infection. Each HBsAg assay is approved by the FDA either for diagnostic use only, for testing donors of blood, organs, and tissue only, or for both applications. The "Name and Intended Use" section of the package insert should be consulted to determine what sample types have been approved for use with each HBsAg test (Table 2).

The presence of HBsAg in the serum indicates that the patient is highly infectious. Patients who resolve an acute infection eventually produce anti-HBs (see "Serologic Tests" below). However, when HBsAg is present, anti-HBs can be negative in diagnostic tests because the antibody is bound to the HBsAg.

For all commercially available diagnostic assays, any specimens nonreactive for HBsAg are considered negative and do not require further testing. In contrast, specimens reactive for HBsAg are often repeated to verify positive results. These repeatedly HBsAg-positive results may be confirmed by a neutralization assay provided by the manufacturer consistent with FDA approval protocols (Table 2). If the HBsAg-reactive serum is neutralized by the anti-HBs, then the specimen is considered positive for HBsAg. Conversely, if the anti-HBs does not neutralize the HBsAg, then the HBsAg test must be considered nonconfirmed and a new specimen should be requested and/or a recommendation that the patient be tested for other markers of HBV infection such as IgM anti-HBc or total anti-HBc should be made.

All HBsAg assays are capable of detecting subnanogram amounts of protein with no loss of specificity (38, 39). For diagnostic applications, this level of sensitivity is sufficient to detect the HBsAg in the sera of individuals with actively replicating HBV. However, a recent concern is that some assays cannot detect variants of HBsAg that have mutations within the major antigenic region of the protein. These mutant HBsAg can be missed by some diagnostic assays (14, 40, 41), so initial testing should include the detection of antibodies to both HBsAg as well as HB core.

The major antigenic determinant on the HBsAg is designated the "a" determinant. This antigenic site is formed by a conformational structure containing a disulfide bond that results in a specific three-dimensional epitope. The region between amino acids 124 and 147 is found within the major hydrophilic loop of the protein (42, 43). There is a concern that current diagnostic assays do not detect HBsAg with alterations within this major antigenic epitope, since some HBsAg assays use monoclonal antibodies that capture the HBsAg using this immunodominant epitope. These HBV strains are known as "escape mutants." The first escape mutant was described to occur in a child born to an HBV-positive mother who transmitted HBV to the child despite vaccination and HBIG (44). This virus had a substitution in HBsAg at amino acid position 145 of arginine for glycine. A single amino acid change altered the antigenic portion of the protein such that vaccine-induced antibody no longer recognized the antigen. This allowed the altered virus to persist in the infant. Subsequently, the patient remained positive for HBV DNA and HBsAg (with mutation) for longer than 12 years. Since that time, a number of other substitution mutants within the "a" determinant region of HBsAg have been recognized (45, 46). Recent studies have evaluated HBsAg assays to determine their ability to detect well-defined HBsAg mutants and have found that some mutations in the HBsAg may be missed by diagnostic assays (10, 40, 44, 45, 47).

In response to the concern that blood donors with an HBsAg mutant are not be identified by HBV antigen assays, most countries screen blood donors for anti-HBc in addition to screening for HBsAg. Blood donors in the United States are also tested for HBV DNA (48). Individuals with positive tests for HBV DNA and anti-HBc and/or patients with positive results for HBeAg and/or HBV DNA but negative for HBsAg could be infected with an escape mutant (49).

HBeAg

The detection of HBeAg in serum is a sign of rapid viral replication usually associated with high HBV DNA levels. HBeAg-positive patients are highly infectious. However, some HBV strains do not make the HBeAg due to a precore mutation. Patients infected with these mutant strains may have high HBV DNA levels in the absence of detectable HBeAg (42). Several commercial assays are available for the detection of HBeAg in serum and use principles similar to those of the HBsAg detection system; i.e., initially positive specimens should be retested and confirmed with neutralization (Table 2).

Nucleic Acid Detection

Molecular assays to quantitate HBV DNA are used for the initial evaluation of HBV infections and the monitoring of patients with chronic infections during treatment. In addition, as mentioned above, blood donors are routinely screened for HBV DNA using qualitative tests to identify donors in the early stage of HBV infection.

A number of quantitative assays have been developed to detect and monitor HBV DNA levels in infected patients. Monitoring of HBV DNA levels provides information on the effectiveness of antiviral treatment and can indicate when a change in the antiviral regimen is needed. Several assays are available for these purposes and use a variety of different methods (38, 50). Most of the available assays have a lower limit of detection, between 5 and 50 copies/ml, and can quantify levels up to 1 million copies/ml. This wide range of quantitation allows for monitoring HBV DNA early after infection and identifying HBV infections resistant to antiviral therapy (Table 2) (51). Recent studies have shown that the persistent suppression of HBV DNA is a primary measure of therapeutic success (20, 52, 53). A high level of HBV DNA following resolution of clinical hepatitis indicates a failure to control viral replication (4).

Commercially available HBV DNA assays differ in their limits of detection, dynamic ranges, and methods used to measure DNA levels (Table 3). An international HBV DNA standard was established in 2001 by the WHO in response to the need to standardize HBV DNA quantification (32, 54). The WHO standard virus preparation is a high-titer genotype A preparation (code 97/746) which has been assigned a potency of 10^6 IU/ml. The standard has established that 1 IU of HBV is equivalent to 5.4 genome

TABLE 2 Commercial systems for serologic testing of HBV antigens and antibodies

Name of assay (manufacturer)	Detection method[a]	Type(s) of specimen	Reportable range[b]	Approved use(s)[c]
HBsAg[d]				
Architect HBsAg (Abbott Laboratories)	CMIA	Serum, plasma	Qualitative	A
AxSYM HBsAg (Abbott Laboratories)	MEIA	Serum, plasma	Qualitative	A
Prism HBsAg (Abbott Laboratories)	CIA	Serum, plasma (also ACD)	Qualitative	A
Advia Centaur HBsAg (Siemens Healthcare Diagnostics)	CEIA	Serum, plasma	Qualitative	A
Immulite 2000 (Siemens Healthcare Diagnostics)	CIA	Serum, plasma	Qualitative	A
Genetic Systems HBsAg 3.0 (Bio-Rad Laboratories)	EIA	Serum, plasma (also ACD)	Qualitative	A
ETI-MAK-2 Plus DiaSorin	EIA	Serum, plasma	Qualitative	A
Vitros HBsAg (Ortho-Clinical Diagnostics)	CEIA	Serum, plasma	Qualitative	A
Elecsys HBsAg, Cobas HBsAg (Roche Diagnostics)	ECL	Serum, plasma (lithium heparin not validated)	Qualitative	A
HBeAg[d]				
ETI-EBK Plus (DiaSorin)	EIA	Serum, plasma	Qualitative	A
Elecsys HBeAg (Roche Diagnostics)	ECL-EIA	Serum, plasma	Qualitative	A
ADVIA Centaur HBeAg (Siemens Healthcare Diagnostics)	EIA	Serum, plasma	Qualitative	A
Vitros HBeAg (Ortho-Clinical Diagnostics)	CIA	Serum	Qualitative	A
Total anti-HBs				
Architect AUSAB (Abbott Laboratories)	CMIA	Serum, plasma[e]	Quantitative (range: 8 to >1,000 mIU/ml)	A, D
AxSYM AUSAB (Abbott Laboratories)	MEIA	Serum, plasma (Na heparin only)	>12 mIU/ml (reactive), <8 mIU/ml (nonreactive),[f] 8–12 mIU/ml (indeterminate)	A, D
Advia Centaur (Siemens Healthcare Diagnostics)	CMIA	Serum, plasma[e] (note: Na and Li heparin plasma have high background)	Qualitative; <1, negative; ≥1, positive; ≥0.75 to <1.25, retest	A, D
Monolisa Anti-HBs (Bio-Rad Laboratories)	EIA	Serum, EDTA, or citrated plasma	Qualitative and quantitative; LOD = 4.14 mIU/ml; results of signal/assay cutoff of >1 are positive	A, D
Immulite 2000 (Siemens Healthcare Diagnostics)	CEIA	Serum, EDTA, or heparinized plasma	Qualitative; positive, >10 mIU/ml; negative, <10 mIU/ml	A, D
ETI-AB-AUK Plus (DiaSorin)	EIA	Serum, plasma	Qualitative only; positive, >10 mIU/ml; negative, <10 mIU/ml	A, D
Vitros Anti-HBs (Ortho-Clinical Diagnostics)	CIA	Serum	>12 mIU/ml; positive; <5 mIU/ml, negative; values between >5 and <12 mIU/ml, indeterminate	A, D
Monolisa, HBsAg 3.0 (Bio-Rad Laboratories)	EIA	Serum, plasma[e] (also ACD), cadaveric serum	Qualitative cutoff = mean OD of 10-mIU/ml calibrator	B, D
Prism HBsAg (Abbott Laboratories)	CIA	Serum, plasma[e] (also ACD) (do not use heparin)	Semiquantitative	C

(Continued on next page)

TABLE 2 Commercial systems for serologic testing of HBV antigens and antibodies *(Continued)*

Name of assay (manufacturer)	Detection method[a]	Type(s) of specimen	Reportable range[b]	Approved use(s)[c]
Total anti-HBc (IgM and IgG)				
Architect Core (Abbott Laboratories)	CMIA	Serum, plasma[e]	Qualitative (nonreactive, gray zone,[f] reactive)	A
AxSYM Core 2.0 (Abbott Laboratories)	EIA	Serum, plasma[e]	Qualitative (nonreactive, gray zone,[f] reactive)	A
Prism HBc assay (Abbott Laboratories)	CIA	Serum, plasma[e] (also ACD) (do not use heparin)	Qualitative (nonreactive, reactive) (note that reactive specimens must be retested in duplicate)	C
Advia Centaur HBc (Siemens Healthcare Diagnostics)	CMIA	Serum, plasma[e]	Qualitative (nonreactive, reactive)	A
Immulite 2000 Anti-HBc (Siemens Healthcare Diagnostics)	CMIA	Serum, plasma[e]	Qualitative (negative, positive)	A
ETI-AB-Corek Plus (DiaSorin)	EIA	Serum, plasma[e]	Qualitative (negative, equivocal,[f] positive)	A
Monolisa Anti-HBc	EIA	Serum, plasma[e]	Qualitative (nonreactive, borderline,[f] reactive)	A
Vitros Anti-HBc (Ortho-Clinical Diagnostics)	CIA	Serum, plasma[e]	Qualitative (negative, indeterminate,[f] reactive)	A
Anti-HBc IgM				
Architect Core-M (Abbott Laboratories)	CMIA	Serum, plasma[e]	Qualitative (nonreactive, gray zone,[f] reactive)	A
AxSYM Core-M (Abbott Laboratories)	MEIA	Serum, plasma	Qualitative (nonreactive, gray zone,[f] reactive)	A
Advia Centaur HBc IgM (Siemens Healthcare Diagnostics)	CMIA	Serum, plasma	Qualitative (nonreactive, gray zone, reactive)	A
Immulite 2000 HBc IgM (Siemens Healthcare Diagnostics)	CEIA	Serum, plasma	Qualitative (negative, equivocal, positive)	A
Monolisa HBc IgM (Bio-Rad Laboratories)	EIA	Serum, plasma	Qualitative (nonreactive, borderline, reactive)	A
Vitros Anti-HBc IgM (Ortho-Clinical Diagnostics)	CEIA	Serum, plasma	Qualitative (nonreactive, indeterminate, reactive)	A
Anti-HBe				
ETI-AB-EBK Plus (anti-HBe) (DiaSorin)	EIA	Serum, plasma[e]	Qualitative (negative, equivocal, positive)	A

[a] CMIA, chemiluminescent microparticle immunoassay; CIA, chemiluminescent immunoassay; ELFA, enzyme-linked fluorescent assay; ECL, electrochemiluminescence; MEIA, microparticle EIA.

[b] All information on methods is derived from FDA submissions and manufacturers' information when available.

[c] A, diagnostic use only; not for use in evaluation of blood, blood products, or tissue or blood donors; B, diagnostic and screening of blood, blood products, and/or tissue or blood donors; C, screening of blood, blood products, and/or tissue or blood donors only; D, evaluation of postvaccination response.

[d] All assays require positive results to be repeated and confirmed with a separate confirmation assay specific for each kit.

[e] Serum includes specimens collected in serum separator tubes; plasma includes collections in potassium EDTA, sodium citrate, sodium heparin, lithium heparin, and/or plasma separator tubes unless otherwise stated for a specific test.

[f] All samples with reactive and/or gray zone/indeterminate results are retested in duplicate before reporting results. See package insert for specific instructions.

TABLE 3 Molecular assays used to detect HBV nucleic acid

Assay name	Method	Quantitative range[a]	Sensitivity[a]	Genotype detection[a]
COBAS TaqMan HBV[b,c]	Target DNA amplification with HBV-specific primers	20–170,000,000 IU/ml 1 IU = 5.82 copies	4–10 IU/ml	A–G; several precore mutations
artus HBV PCR[b,c]	Various real-time PCR instruments (Roto-Gene and Lightcycler)[c]	10 to 10^9 IU/ml (Roto-Gene) 10 to 10^{10} IU/ml (Lightcycler) 1 IU = 6 copies	3.8 IU/ml (Roto-Gene) 5.8 IU/ml (Lightcycler)	A–H
Abbott real-time PCR[b]	Amplification of target gene within a segment of the surface gene	10 to 10^9 IU/ml 1 IU = 3.41 copies	10–15 IU/ml	A–H and several polymerase gene variants
HBV trender[b]	Amplification and detection using Scorpion probe/primer Smart system Cepheid; Mx3000P/3005P, iCycler iQ/iQ5, Rotor-Gene 3000/6000, ABI 730	1×10^8 to 3×10^8 IU/ml 1 IU = 7.0 copies	3.3 IU/ml	A–H

[a] Data from manufacturers' literature and websites.
[b] CE-IVD, European Conformity In Vitro Diagnostic Medical Devices.
[c] FDA-IVD, Food and Drug Administration In Vitro Diagnostic Product.

equivalents (54). The WHO standard has allowed results for different HBV DNA assays to be reported in international units per milliliter.

However, despite the availability of this standard, the various quantitative assays usually have different conversion factors, demonstrating their variability (Table 3). Thus, the best practice is the consistent monitoring of patients using the same manufacturer's assay in the same laboratory (51).

Some of the variability of HBV DNA quantification may occur during collection and processing of the specimen. Most assays use serum or plasma (EDTA or ACD) as the specimen of choice. Regardless of the collection tube, the specimen should be separated from the clotted blood within 4 to 6 h after collection. As with all FDA-approved diagnostic assays, the package insert should be followed for specimen type, processing, and storage. Repeated thawing and freezing (>2 times) should be avoided, as this may reduce assay sensitivity.

Most commercial assays quantitate HBV DNA and cover a 7- to 8-$\log_{10}$ range, which permits the accurate evaluation of HBV levels above a million down to very low levels that occur during treatment or in inactive carriers. Several studies have shown that a reduction of 2 $\log_{10}$ in HBV DNA levels in the first 6 months of antiviral therapy indicates treatment efficacy (38, 51, 53).

Many of these assays use oligonucleotide primers that recognize a conserved sequence within the HBV precore/core gene (51). The assays quantitate HBV DNA using a series of standards containing a known amount of the target nucleic acid. These standards provide the standardization curve used to correlate the results from each patient sample. The viral load is determined by computerized analysis of individual results, which are compared to the standard curve (51, 53).

Isolation Procedures

Although HBV can infect hepatocytes *in vitro*, culture of HBV is not used as a diagnostic test.

Identification

The methods for identification of HBV infection use a combination of molecular, antigenic, and serologic methods described in "Direct Detection" above and "Serologic Tests" below.

Typing Systems

Antigenic variation occurs naturally in HBV due to genetic heterogeneity. These various genetic differences are used to classify HBV into eight distinct genotypes, designated A through H (Table 4). They are distinguished by a genetic divergence of 8% or more within the complete nucleotide sequence (4, 22). There are substantial data that have correlated HBV genotypes with disease outcome. The most common genotypes associated with chronic HBV infections are B and C. Patients with genotype B are more likely to spontaneously convert to anti-HBeAg and thus have less severe liver damage. Genotype C is more common in Asia and has been associated with a high risk of progression to cirrhosis. However, in European studies, genotype D has been shown to be more likely to be associated with active liver disease than other genotypes (42). It is noted that most of these patients have acquired these infections in childhood and had more exposure to HBV than other patients evaluated. It is not clear yet how the variances in HBV genotypes affect clinical outcomes. It is possible that the differences are associated with the viral expression of immune epitopes or the loss of critical control over viral replication such as precore and core promoter mutations. Thus, in spite of the intriguing studies on HBV genotypes, the role of HBV genotyping in predicting clinical and therapeutic outcomes has not been firmly established. However, with additional studies, the value of HBV genotyping to determine specific treatment will likely become apparent (18, 25, 43).

Serologic Tests

Serologic tests for HBV-specific antibodies are used to determine the stage of HBV disease and to establish immunity after HBV vaccination. As the host mounts an immune response, the first antibodies to appear are IgM specific for HBc (IgM anti-HBc); this is followed by the appearance of total anti-HBc, anti-HBe, and finally anti-HBs (24). Several commercial assays are available for HBV serologic testing (Table 2).

IgM Anti-HBc

IgM anti-HBc persists for several weeks to months after an initial infection. The detection of IgM anti-HBc indicates an infection of less than 6 months' duration (Table 1). During this stage of disease, the patient's liver enzymes may be elevated. A negative IgM anti-HBc excludes a recent,

TABLE 4 HBV genotypes and geographic circulation[a]

Genotype	Subtypes and serotypes	Genome size (nucleotides)	Geographic connection	Disease relationship
A	Subtypes A1, A2, A3; serotypes adw2, ayw1	3,221	North-Western Europe, Spain, Poland, USA, Central Africa, India, Brazil	HCC and cirrhosis in older patients; core promoter mutations (A1762T, G1764A) detected
B	Subtypes B1–B6; serotypes adw2, ayw1	3,215	Southeast Asia, Taiwan, Japan, Indonesia, China,	HCC and cirrhosis in both younger and older patients; precore mutations (G1896A) detected
C	Subtypes C1–C4; serotypes adw2, adrq+, adrq−, ayr	3,215	Asia, Indonesia, India, Australia, USA, Brazil	Higher risk of HCC and cirrhosis than with genotype B; precore mutations (G1896A) and core promoter mutations (A1762T, G1764A) detected
D	Subtypes D1–D4; serotypes ayw2, ayw3	3,182	Mediterranean area, Middle East, India, Spain, USA, Brazil (generally found worldwide)	Chronic HBV, cirrhosis in older patients; precore mutations (G1896A) and core promoter mutations (A1762T, G1764A) detected
E	Subtypes unknown; serotype ayw4	3,212	West Africa	Unknown
F	Subtypes D1–D4; serotypes adw4q−, adw2, ayw4	3,215	Central and South America, Bolivia, Venezuela, Polynesia, Alaska	HCC in young children and adults
G	Subtypes unknown; serotype adw2	3,248	Australia, France, Germany, USA	Almost always is a coinfection with other HBV types
H	Subtypes unknown; serotype adw4	3,215	Central and South America, USA	Unknown

[a] Information derived from references 38, 76, and 102.

acute infection but does not rule out chronic infection (22, 55). The presence of IgM anti-HBc identifies patients who are acutely infected; such patients usually have high levels of HBV DNA (55).

Total Anti-HBc

A negative anti-HBc test indicates that a person does not have a history of infection with HBV. A positive result can indicate either an acute infection in which the patient is also HBsAg positive and IgM anti-HBc positive or a resolved (HBsAg-negative) or chronic (HBsAg-positive) HBV infection (Table 1) (4, 55). Total anti-HBc antibodies remain after IgM anti-HBc disappears and can be detected for many years. These antibodies persist longer than anti-HBs. Thus, total anti-HBc is the best marker for documenting prior exposure to HBV infection. Vaccines do not include HBc; thus, vaccination induces only anti-HBs. Therefore, anti-HBc is not present in vaccinated individuals unless they were infected with HBV prior to vaccination (17, 56). Individuals positive for antibody to HBc without any other serologic evidence of HBV infections should be considered infected with HBV (55). This serologic pattern is consistent with a remote, past HBV infection that has resolved and in the case of which the viral DNA levels are at times negative or very low (4, 22).

Commercial kits for detection of total anti-HBc are available and use a variety of different methods and instrumentation (Table 2). All assays use recombinant HBc antigen for capture of antibody. In competitive tests, if anti-HBc is present in the sample, it competes with a known amount of added labeled human anti-HBc. Samples containing anti-HBc lead to signal suppression that is proportional over a limited range to the amount of anti-HBc present. Values generated by positive and negative kit controls are required to calculate assay cutoff and to establish validity. Each assay should be calibrated on a regular basis using the calibrators provided in each kit. All anti-HBc assays have a test algorithm that involves testing initially reactive specimens in duplicate in an independent run. If one or both duplicates are reactive, the sample can be reported as positive.

Anti-HBs

A negative result for anti-HBs in the absence of any other HBV specific antigen or antibody indicates that a person has not been infected with HBV, nor has the individual been vaccinated with HBV. A positive result is consistent with immunity to HBV due to an infection or from effective vaccination (Table 1).

Commercial assays for anti-HBs are solid-phase tests based on the sandwich principle. These assays provide a quantitative result to assist in determination of adequate immunity after vaccination. An initially reactive result for anti-HBs requires repeat testing in duplicate in an independent run. If one or both duplicates are reactive, the sample is positive. Anti-HBs quantitation panels are commercially available and should be used when validating anti-HBs assays.

Anti-HBs is a key serologic marker for both vaccine-induced immunity and immunity due to infection. As HBV vaccination has become more widespread, this serologic marker is used to monitor vaccine success. Both the WHO and CDC recognize a level of >10 mIU/ml of anti-HBs as an indication of protective immunity. However, it has been demonstrated that levels of anti-HBs determined by one commercial assay system cannot be compared with those detected with a different system despite the use of international standards (57).

Postvaccination testing for the presence of anti-HBs is not recommended for infants, children, or most adults. The CDC lists exceptions to this rule, which are infants born to mothers who are HBsAg positive, immunocompromised patients, dialysis patients, and to confirm successful vaccination in health care workers to prevent the transmission of HBV in the health care setting (58, 59).

HBV escape mutants in which the HBsAg has mutated can result in HBV infections without detectable anti-HBsAg (42). Conformational changes in the major antigenic determinant of HBsAg result in reduced detection of the anti-HBsAg by diagnostic assays.

Anti-HBe

A positive test for anti-HBe indicates the resolution of acute infection and is associated with a decrease in viral replication (Table 1). During acute infection, these antibodies are bound to the HBe antigen; the antibody will not be detected until the HBeAg levels decrease. This decrease is associated with a reduction of virus and thus the resolution of acute infection.

Patients who have recovered from acute HBV infection have detectable anti-HBe, anti-HBc, and anti-HBs. Interestingly, patients infected with HBeAg-negative strains of HBV still have anti-HBe (22).

Competitive binding is the basis for most anti-HBe assays. To detect anti-HBe, the patient sample and a measured amount of recombinant HBeAg are added simultaneously to the assay. If anti-HBe is present in the patient's sample, it competes with solid-phase anti-HBe for the limited number of binding sites on the added HBeAg. Positive and negative control values are used in validity calculations.

Test algorithms for HBeAg and anti-HBe kits vary. Initially reactive samples in the anti-HBe assays are often retested. If the repeat test is reactive, the sample is reported as positive. The enzyme immunoassays (EIAs) have indeterminate or gray zones around the assay cutoffs. If the samples repeatedly yield values in this zone, additional testing should be performed with a new specimen.

Antiviral Susceptibility Testing

Therapy for HBV usually requires long-term treatment with nucleoside or nucleotide analogs. A disadvantage of long-term therapy is the subsequent development of antiviral resistance. As noted above, HBV replicates through an RNA intermediate. The HBV RNA-dependent DNA polymerase is not precise during rapid replication cycles and does not correctly proofread the final copies, leading to frequent errors. Some of these errors create resistant mutants, which are selected in the presence of antiviral agents (38, 60). Over time the HBV strains with antiviral resistance become the major viral species (61, 62). The current recommendation is that a patient who has a 1-$\log_{10}$ increase from the lowest HBV DNA level should be evaluated for the development of antiviral resistance (28, 38, 52, 63).

Recently the nomenclature of HBV antiviral resistance was standardized in order to track nucleotide changes associated with drug resistance and to recognize new mutations (Table 5) (38). Antiviral resistance mutations can be detected by molecular methods that recognize known mutations associated with resistance (11, 38, 62, 64). Methods used to detect these mutations are available; however, the interpretation of results is not always straightforward. Some mutations predict resistance to multiple drugs. A single mutation at A181T is associated with resistance to lamivudine, adefovir, tenofovir, and telbivudine (11, 65) (Table 5). In other situations genetic sequence changes may not confer resistance when present alone but do contribute to the resistance when additional mutations are present. For example, lamivudine resistance does not occur due to a single L180M mutation, but with the addition of a second mutation, such as A181T, resistance to lamivudine occurs. Thus, the detection of an L180M, A181T double mutation, which alters the position of a critical residue in the nucleotide binding pocket, is required for resistance to be apparent (28, 29, 52). An additional concern is that patients treated with multiple drugs will have a combination of sequence changes that represent drug resistance mutations in addition to mutations that occur in order to balance the fitness of the HBV strains selected out after antiviral therapy (46). Many of these single nucleotide changes have no recognized phenotype and may represent the random genetic changes found in viruses that use a reverse transcriptase during replication (28).

Commercial assays are available to detect mutations associated with antiviral resistance in HBV. One assay employs biotinylated amplicons that hybridize to membrane-bound oligonucleotides that represent mutation sequences (INNO-LiPA HBV; Innogenetics). The first generation of this assay detects several lamivudine resistance mutations at amino acids 180, 204, and 207 (66, 67). The second-generation assay detects additional resistance sequences, including those associated with lamivudine resistance at amino acids 80, 173, 180, and 204, as well as changes at amino acids 181 and 236 associated with adefovir resistance (47, 68, 69). This version detects HBV resistance mutations in specimens that contain both wild-type HBV and mutant virus. Some disadvantages with these assays are the facts that the reading of the reaction lines on each strip can be difficult (up to 34 lines per strip), faint bands can be problematic to interpret, and at times the test results show no bands in viral preparations with known mutations (66, 68, 70).

A second assay (Trugene HBV genotyping kit; Siemens Medical Solutions Diagnostics) is available as a research-use-only assay. This system detects antiviral mutations by direct sequencing from specimens, including specimens with low HBV levels (68, 71). Comparison between the sequence assay and the hybridization assay shows a high concordance (68, 70).

A number of individual laboratory-developed assays have been used to detect antiviral mutations in HBV. These

TABLE 5 Antiviral agents and HBV mutations associated with resistance[a]

Antiviral agent	Description of drug	Mutation(s) associated with resistance
IFN-α	Immune modulation	None known
Pegylated IFN-α2a	Immune modulation	
Lamivudine	Nucleoside analog (cytidine)	L180M + M204V/I/S, A181V/T, S202G/I
Telbivudine	Nucleoside analog (dTTP)	M204I, A181T/V
Entecavir	Nucleoside analog (2-deoxyguanosine)	T184S/C/G/A/I/L/F/M, S202G/C/I, M250V/I/L, M204V/I
Emtricitabine	Nucleoside analog (cytidine)	
Adefovir	Nucleotide analog (dATP)	A181V/T, N236T
Tenofovir	Nucleotide analog (dATP)	A194T, N236T, A181V/T

[a] Resistance is defined as virologic breakthrough confirmed by an increase on two consecutive occasions with HBV DNA levels that increased by >1 $\log_{10}$ copies/ml during therapy. Data are derived from references 28, 52, 62, and 102.

assays use a variety of different techniques for the detection of known resistance mutations in HBV (38, 72). One of these methods is to use direct DNA sequencing to detect such mutations. The advantage of DNA sequencing is that it is accurate and mutations can be identified in any part of the HBV genome (55). However, a serious disadvantage is that these methods lack sensitivity and detect mutants present in 10 to 30% of the viral population. This level of detection does not always consistently identify resistance mutants, which make up at least 10% of the virus population (73). Thus, this limits its use clinically. Additionally, testing by this method can be labor-intensive and the reagents can be costly.

However, newer technology, such as ultra-deep pyrosequencing, is now being used to detect minor viral populations that could emerge during antiviral treatment. These HBV variants are present in the patient in low numbers and can have random genetic changes that lead to antiviral drug resistance. During treatment, the HBV variants can be monitored to determine when the patient needs a different anti-HBV treatment (74, 75).

The HBV viral level is often used for monitoring and detecting the development of resistance to antiviral therapy. Resistance to antiviral agents is defined clinically by a 1-log increase in serum HBV DNA levels during antiviral therapy (53). The disadvantage of using the HBV DNA levels to detect antiviral resistance is that other factors, such as compliance and drug metabolism, may also be responsible for increasing HBV DNA levels. Therefore, the combined use of a genotyping assay for mutation detection and quantitative detection of viremia with a reproducible and sensitive assay is warranted for optimal monitoring of HBV antiviral therapy (11, 50).

Phenotypic testing is used to detect HBV antiviral resistance in research and development laboratories. These methods detect drug resistance based on the use of molecular or cellular techniques or using animal models (38, 53) and are not always applicable for clinical use. These tests are labor-intensive and required long testing periods.

Evaluation, Interpretation, and Reporting of Results

HBV infection leads to several different disease presentations, from acute hepatitis with fulminant hepatic failure to chronic hepatitis leading to cirrhosis and/or HCC (4, 22, 76). The initial assessment for suspected viral hepatitis should include laboratory tests that measure serum transaminases, direct and total bilirubin, albumin and total protein, a complete blood count, coagulation tests, and alpha fetoprotein. The specific laboratory tests to detect and monitor HBV infection are a mix of viral antigen detection, molecular measurements of HBV DNA, and serologic markers. The tests to diagnose new HBV infection are anti-HBc IgM, HBsAg, and HBV DNA (Table 1).

During acute HBV disease, IgM anti-HBc is usually positive when serum transaminases are elevated. At this phase, HBsAg and/or HBeAg may be detectable. In a typical acute HBV infection, HBsAg can be detected 2 to 4 weeks before the liver enzyme levels become abnormal and before symptoms appear; thus, when the patient presents with clinical signs, HBsAg may not be detected. Additionally, HBV DNA can be detected 3 to 4 weeks before HBsAg, long before the onset of symptoms; thus, it is usually not tested until serologic markers of infection are detected (56, 76). The levels of IgM anti-HBc eventually decline as the disease resolves or becomes chronic. Most patients with an active HBV infection which resolves have detectable anti-HBs shortly after the disappearance of HBsAg. Figure 3 illustrates a typical serologic pattern that occurs in patients with an acute HBV infection. Chronic HBV stages can be distinguished using a combination of laboratory tests and clinical signs (Table 1 and Fig. 4).

The immune tolerant phase usually occurs when the patient acquires HBV infection at birth or during early childhood. Infection in this case is associated with a high level of viral production and the presence of HBeAg. These markers indicate a high rate of viral replication (4). There is an absence of liver disease despite high levels of HBV replication. This is a consequence of immune tolerance; however, the underlying pathology is poorly understood (4, 20, 38).

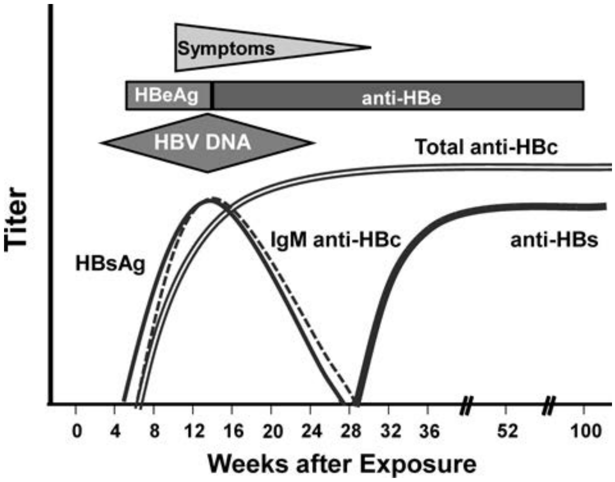

FIGURE 3 Typical sequence of serologic markers in patients with acute HBV infection with resolution of symptoms.
doi:10.1128/9781555817381.ch108.f3

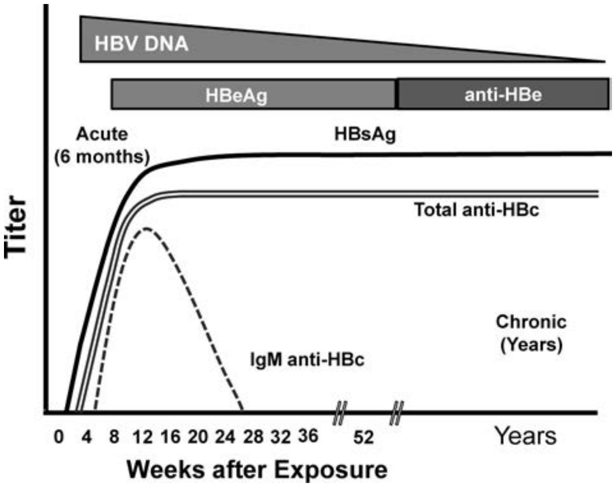

FIGURE 4 Typical sequence of serologic markers in patients with HBV infection that progresses to chronicity. In patients with chronic HBV infection, both HBsAg and IgG anti-HBc remain persistently detectable, generally for life. HBeAg is variably present in these patients.
doi:10.1128/9781555817381.ch108.f4

As the host's immune response matures, the patient often moves to the immune active phase (also referred to as HBeAg-positive chronic hepatitis phase), during which HBV-specific epitopes are recognized by the host immune system, leading to immune-mediated injury in the liver. Individuals who acquired HBV perinatally often transition from the immune tolerant phase to the HBeAg-positive chronic hepatitis phase between 20 and 30 years of age (4, 77). The liver biopsy in this stage shows active inflammation accompanied by fibrosis. Patients who remain HBeAg positive have a higher risk of progressing to liver disease due to the induction of a chronically active immune response by high rates of HBV replication (22, 55). Such patients have high HBV DNA levels and increased levels of serum transaminases. However, as these individuals develop anti-HBe, they revert to HBeAg negativity and move to the inactive carrier phase (Table 1) (21, 55). The transition from the immune tolerant phase is often not recognized, since patients with HBeAg-positive, chronic hepatitis often remain asymptomatic (55).

The inactive carrier phase is characterized by the seroconversion to anti-HBe, and patients alternate between low and undetectable levels of HBV DNA. The seroconversion to anti-HBe is associated with a decrease in liver damage and the normalization of serum transaminase levels. Mild hepatitis may be noted on biopsy (55). Many patients remain in this phase for years. Patients in the inactive carrier phase have detectable HBV DNA in serum, at intermittent or low levels, and usually have normal serum transaminase levels.

A portion of inactive HBsAg carriers (about one-third) develop chronic hepatitis which recurs in the absence of HBeAg in their sera (HBeAg-negative chronic hepatitis). These patients are infected with an HBV variant that cannot express HBeAg due to mutations in the precore or core promoter regions of the HBV core gene (42, 44). Patients with chronic hepatitis that are HBeAg negative are more likely to have more advanced liver disease in spite of lower serum HBV DNA levels (42, 76, 78). HBsAg-positive patients can transmit HBV sexually, percutaneously, or perinatally. Individuals with detectable HBeAg pose the highest risk of transmitting HBV to others.

Patients who are negative for HBsAg, anti-HBc, and HBV DNA are not infected with HBV. In some individuals the presence of anti-HBc alone may be the only evidence of an active, occult HBV infection of remote origin. Patients infected with an HBsAg escape mutant test negative for HBsAg but are positive when tested for anti-HBc and HBV DNA (20, 44).

Anti-HBs without anti-HBc develops in individuals who receive hepatitis B vaccine (which contains only HBsAg), and anti-HBs levels of ≥10 mIU/ml are considered protective (80). Due to the prevalence of vaccinated individuals, the detection of anti-HBc is used to evaluate past or current infection with HBV and identify individuals who should receive the HBV vaccination. Passive transfer of anti-HBs or anti-HBc may be observed in neonates of mothers with current or past HBV infections (56). However, passive-antibody levels decline gradually over 3 to 6 months, while levels of antibody induced by infection are stable over many years (79, 80). Since blood donations are tested for HBsAg and total anti-HBc, passive transfer of these HBV markers following blood transfusions is unlikely.

Most individuals vaccinated for HBV have detectable levels of anti-HBs; however, some vaccinated people test negative due to waning levels of anti-HBs. They usually respond to a challenge dose of HBsAg vaccine with an anamnestic response in approximately 2 weeks (81). Studies of vaccinated individuals who no longer have detectable anti-HBs show that infection can occur but is blunted by the anamnestic anti-HBs response such that liver damage is minimal and symptoms do not occur (81). In contrast, an HBsAg-negative carrier may fail to produce detectable levels of anti-HBs after vaccination (82). Individuals who do not have a detectable response to the first series of HBV vaccine should be given a second three-dose vaccine series (19, 59). Individuals who do not respond to the second vaccination series should be considered susceptible to HBV infection and should be given HBV immunoglobulin prophylaxis after any known exposure to HBV-positive body fluid. It is important to note that some HBV vaccine nonresponders are chronically infected with HBV. Thus, individuals who do not have detectable anti-HBs after six doses of vaccine should be tested for HBsAg (34, 60).

Testing for the HBV genotype is usually not required except for selected patients from regions around the world that have variability in HBV genotypes (Table 4). Testing for mutations associated with antiviral resistance is also not useful during the initial evaluation of patients (20, 38, 83). The "gold standard" for assessing inflammatory activity (grade) and degree of fibrosis (stage) is the liver biopsy, which is a useful baseline for future follow-up (55).

Molecular assays are used to determine HBV DNA levels and contribute to establishing the stage of disease in newly diagnosed patients. They are also used to monitor patients on antiviral therapy. The reduction of HBV DNA during antiviral therapy is a measure of treatment response and predicts histologic improvement. Increasing HBV DNA levels are associated with chronic liver disease, cirrhosis, and possibly death (22, 50, 63). The WHO standard for HBV DNA has helped standardization of HBV DNA assays, and results are now reported in international units per milliliter (32, 84).

Antiviral therapy is given to patients to prevent progression of liver disease (4, 28, 38). During the course of therapy, treatment response is monitored using biochemical, virologic, serologic, and histologic results. Currently the most accurate monitor of virologic activity is the HBV DNA level using an assay with a wide dynamic range (28, 38, 50, 55). The loss of HBsAg, seroconversion to anti-HBs, and long-lasting suppression of HBV DNA indicate a successful response to therapy (50, 63). Patients who appear to have suppressed HBV DNA levels are monitored periodically because relapse due to antiviral resistance is possible. The most reliable measure of a successful long-term treatment response is the sustained suppression of HBV DNA (38, 50, 55, 63).

When HBV DNA levels increase by 1 $\log_{10}$ in a patient taking antiviral treatment, it may indicate antiviral resistance (38, 55, 63). It is not recommended that resistance testing be performed before starting therapy even if the patient has a very high viral level (20, 50). Resistance detection is useful only after a patient has been treated for several months and fails to show an HBV DNA reduction of at least 1 $\log_{10}$. Such patients are classified as primary nonresponders. They should be tested for the presence of resistant mutants to assist in selecting a new treatment regimen (20, 50). For successfully treated patients who have a virologic breakthrough, antiviral resistance testing should also be performed to determine selection of resistant viral strains (11, 25, 52, 61).

HEPATITIS D VIRUS

Introduction

Hepatitis D virus (HDV) is a defective RNA virus that requires the presence of HBV for its replication. HDV has a single-stranded, circular, negative-sense RNA genome that is completely nonhomologous with HBV DNA. The agent was first described in 1977 as occurring in Italian patients with chronic HBV who developed episodes of serious acute disease (85).

Taxomony

HDV is unable to replicate without the presence of HBV, which is the host virus. Thus, it is a subviral particle rather than a true virus. The HDV particle is similar to those of plant subviral agents (86). However, there are some major differences between the plant viroids and HDV. The plant viroids do not encode a specific protein and do not utilize a host or helper virus as HDV does with HBV (87, 88).

Description of the Agent

HDV is generally spherical, with an average diameter ranging from 36 to 43 nm, which is slightly smaller than that of the HBV particle (88). The 1.7-kb RNA genome of HDV encodes only a single protein, the HDV nucleocapsid protein, known as the hepatitis delta antigen (HDAg). It is nonglycosylated and is produced in two forms: a short peptide consisting of a 195-amino-acid 24-kDa protein and a larger peptide consisting of a 214-amino-acid 27-kDa protein (89, 90). The protein cannot assemble into viral particles without the presence of HBsAg (88, 89). The HDV RNA genome is surrounded by HDAg, which is then surrounded by HBsAg. Consequently, HDAg is undetectable on the complete HDV particle. In spite of this, infected individuals produce antibody to the HDV antigen (anti-HDV). However, anti-HDV does not neutralize this particle, whereas anti-HBs does have neutralizing activity (88).

The HDV genome displays a high degree of complementarity, which creates a three-dimensional, double-stranded, stable structure that resists dry heat for long periods. The extensive internal complementarity of the HDV genome results in a rod-like structure much like that of plant RNA satellite viruses. The manner by which this virus replicates is unusual. The replication of the HDV genome uses the host RNA polymerase II rather than an HDV- or HBV-encoded RNA polymerase (88, 89). The only enzymatic activity inherent to HDV is mediated by RNA elements termed ribozymes which cleave the newly synthesized, circular RNA genomes, producing linear molecules (92). After that cleavage, replication of the HDV genome occurs via a rolling-circle mechanism with self-cleavage. Synthesis of the HDV particles suppresses HBV viral production. A unique, obligatory relationship exists between HDV and HBV (88).

Epidemiology and Transmission

Worldwide, it is estimated that approximately 15 to 20 million people are HDV carriers (85, 93). Several areas of the world have a high prevalence of HDV-infected individuals, including countries bordering the Mediterranean, the Middle East, Central Asia, West Africa, the Amazon Basin, and the South Pacific Islands (85). In these areas of endemicity, HDV appears to be transmitted by close person-to-person spread, such as household contact. Additionally, many individuals have acquired HDV through exposure to blood-contaminated needles and blood products. Persistent epidemics of severe acute HDV have been observed in these populations, which have high HBV carrier rates (85, 94).

Clinical Significance

HDV can be transmitted only to individuals who are infected with HBV already or when both viral agents are transmitted together. A coinfection occurs when a naive individual is infected simultaneously with both viruses; coinfection occurs in only 2% of the cases (Fig. 5) (88). A superinfection occurs when an individual chronically infected with HBV is infected with HDV (Fig. 6) (88). Superinfection occurs in more than 90% of infected patients (85, 94). Acute HDV superinfection has a greater risk of fulminant hepatitis and liver failure than HBV infection alone. Likewise, chronic HDV infection is associated with more rapidly progressing liver damage than infection with HBV alone (94).

Rates of fulminant hepatitis can be as high as 5% in patients with HBV and HDV coinfection (85, 88). A biphasic clinical course is sometimes observed during coinfection. HDV infection does not increase the rate of chronicity of acute HBV but may convert an asymptomatic or mild, chronic HBV infection into a rapidly progressive, fulminant or severe disease (87, 95, 96). Limited success has been achieved in treating chronic HDV patients with gamma interferon (IFN-γ). High-dose, long-term therapy is required, and relapses are common after therapy is stopped (83, 95, 97, 98). Patients with HDV who have hepatic decompensation are candidates for liver transplantation and in general have had favorable outcomes (96). Treatments with antiviral agents known to reduce HBV titers have been studied. However, it appears that therapy with IFN-α in combination with either ribavirin or lamivudine is not useful in treating chronic HDV infections (95, 97). Successful vaccination for HBV also prevents HDV infection, since HDV cannot replicate in the absence of a concurrent HBV infection.

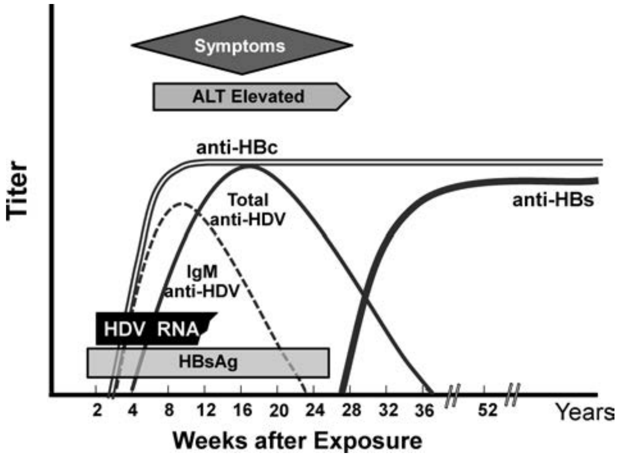

FIGURE 5 Serologic course of HDV infection, with resolution when the virus is acquired as a coinfection with HBV. doi:10.1128/9781555817381.ch108.f5

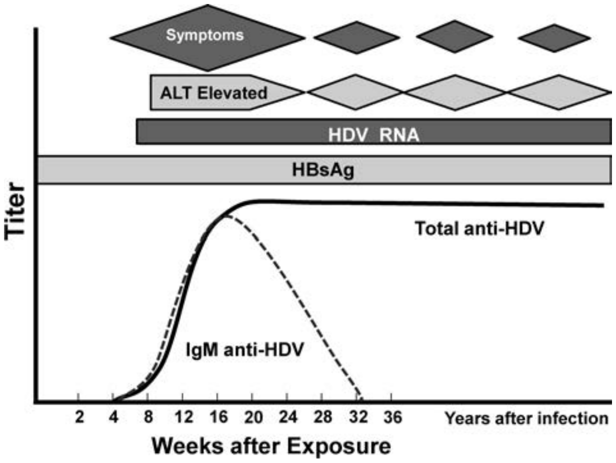

FIGURE 6 Serologic course of HDV infection when the virus is acquired as a superinfection with HBV. Symptoms and alanine aminotransferase (ALT) levels are shown to indicate the intermittent nature of symptoms and liver involvement. doi:10.1128/9781555817381.ch108.f6

Collection, Transport, and Storage of Specimens

HDV infection is diagnosed by serologic and molecular markers using serum or plasma and is suspected only for those patients who are HBV infected from countries in which HDV is prevalent.

Direct Detection

The laboratory diagnosis of HDV depends on the detection of specific antibodies to HDV, HDAg, and/or HDV RNA. HDV infections may also be diagnosed by detecting HDV RNA directly in liver tissue (88, 99). The most current method for the detection of HDV RNA is reverse transcription and amplification (88, 91).

Typing

Based on sequence similarity, the HDV genomes have been classified into three genotypes. Genotype I is the most common and is found worldwide. Genotype II has been identified primarily in Japan and Taiwan, while genotype III is typically found in South America. It is unclear if these differences in genome play a role in disease presentation (99, 100).

Serologic Tests

The diagnosis of HDV infection relies on the detection of total anti-HDV using commercial or laboratory-developed EIAs. A positive result for anti-HDV may indicate either acute or chronic HDV infection. IgM anti-HDV can be detected during either a coinfection or a superinfection. All of the serologic and molecular assays are available from reference laboratories.

Evaluation, Interpretation, and Reporting of Results

Diagnosis of a coinfection with HBV and HDV is based on the detection of HBsAg, HBeAg, HBV DNA, and IgM anti-HBc in a serum sample that is positive for HDAg, anti-HDV IgM, and HDV RNA (Fig. 5). Anti-HDV appears during acute infection and usually disappears with resolution. A superinfection occurs when HDV infects a patient with chronic HBV, a circumstance usually leading to chronic HDV infection. The diagnosis of HDV superinfection is made when anti-HDV is found simultaneously with HBsAg and anti-HBc in the absence of IgM anti-HBc. Anti-HDV is present indefinitely in patients with chronic HDV infection (99, 100) (Fig. 6).

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Transmissible Spongiform Encephalopathies

MARKUS GLATZEL AND ADRIANO AGUZZI

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TAXONOMY

Prions

According to the generally accepted prion hypothesis, the infectious agent causing transmissible spongiform encephalopathies (TSEs) now referred to as prion diseases is the "prion" (for proteinaceous infectious particle). An essential component of prions is the scrapie-like prion protein (PrP^{Sc}), an abnormally folded, protease-resistant, beta-sheet-rich isoform of a normal cellular prion protein (PrP^{C}) (1). According to this theory, which is consistent with a large body of experimental data and has not been disproven to date, the prion does not contain any informational nucleic acids, and its infectivity propagates simply by "autocatalytic" conformational conversion of PrP^{C} into disease-associated PrP^{Sc} (2). Importantly, it has been possible to generate synthetic prions, consisting entirely of recombinantly expressed PrP^{C}, which is misfolded to a PrP^{Sc}-like state by physicochemical treatment (3). The size of the minimal infectious unit of PrP^{Sc} is still a matter of debate. Oligomeric PrP^{Sc}, consisting of 14 to 28 PrP^{Sc} molecules, seems to harbor the highest converting activity (4).

DESCRIPTION OF THE AGENT

As outlined above, the data arguing in favor of PrP^{Sc} representing an essential component of infectious prions are overwhelming. PrP^{C} is encoded by the *PRNP* gene on the short (p) arm of chromosome 20. Mutations in *PRNP* lead to genetic forms of prion disease (see below and Table 2). A polymorphism of codon 129 of *PRNP* (encoding either methionine or valine) is both a susceptibility factor for the development of a prion disease (see genetic testing) and a disease modifier (see "Epidemiology and Transmission" below) (5). The three-dimensional structure of PrP^{C} has been solved by structural biology (6). Similar analyses have not been possible for PrP^{Sc}. Studies employing low-resolution biophysical techniques yielded conflicting data. The following structures have been proposed for PrP^{Sc}: (i) a left-handed β-helix, (ii) a spiraling core of extended β-sheets, or (iii) a stack of parallel β-sheets (7).

One of the defining features of PrP^{Sc} is its relative resistance to digestion by proteinase K (see "Direct Examination" below). Recent data indicate that proteinase K-sensitive forms of infectious prions may exist (8). These data are further supported by novel studies showing that proteinase K-sensitive PrP^{Sc} may also contribute to prion infectivity (9, 10).

EPIDEMIOLOGY AND TRANSMISSION

Human prion diseases manifest as sporadic, genetic, and acquired disorders. They are referred to as sporadic Creutzfeldt-Jakob disease (sCJD), genetic CJD (gCJD), iatrogenic CJD (iCJD), and variant CJD (vCJD). For historical reasons, kuru, a prion disease endemic to tribal regions of Papua New Guinea in the latter half of the last century, deserves to be mentioned here. This disease, which was caused by endocannibalistic funeral practices of the Fore people, appears to have come to an end with the last case reported in 2005 (11).

Sporadic CJD

sCJD is a rapidly progressive dementia, usually leading to death within 6 months of disease onset (12). The cause of sCJD remains enigmatic. To date, no obvious risk factors have been identified (13). Because of the short mean duration of this disease, incidence and mortality rates for sCJD are similar; thus, mortality rates are routinely used to describe the epidemiology of this disease (14). Mortality rates are fairly constant, both over time and between countries, oscillating around 1.5 cases per million per year with the highest incidences in patients between the ages of 65 and 75 years (http://www.eurocjd.ed.ac.uk/allcjd.htm). Recently, a novel type of sCJD has been described. This entity, termed variably protease-sensitive prionopathy (VPSPr), is characterized by typical clinical signs and absence of bona fide protease-resistant PrP^{Sc} (Table 1) (8).

Genetic CJD

gCJD can be subdivided into three phenotypes: hereditary Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). The mode of inheritance in all of these diseases, which cosegregate with mutations in *PRNP*, is autosomal dominant (15). Some mutations within *PRNP* (Y145stop, Y226stop, Q227stop) cause distinct disease phenotypes, i.e., an angiopathy with deposition of prion protein in vessels of the central nervous system (CNS) referred to as cerebral amyloid angiopathy (PrP-CAA) (16). Incidences of gCJD vary

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TABLE 1 Human prion diseases: clinical, diagnostic, and neuropathological features[a]

	Clinical features			Diagnostic tests							Postmortem neuropathological examination	
				CSF					Genetics			
Human prion disease	Age at onset (yr)	Mean disease duration (range)	Leading clinical symptoms	CSF 14-3-3	Tau	EEG	MRI	Biopsy	Codon 129	*PRNP* mutation	Histopathological features	Biochemical tests
Sporadic CJD	60–70	6 mo (1–35 mo)	Progressive dementia and neurological signs: (e.g., myoclonus, cerebellar ataxia, visual problems, extrapyramidal symptoms)	Positive in >90%	Positive in 86%	PSWC 60–70%	Brain atrophy hyperintensities in basal ganglia and/or cortical 67%	Brain	MM, 70%; MV, 14%; VV, 16%	N/O	Spongiform changes, neuronal loss, astrogliosis, PrP deposition (various patterns)	PrP^{Sc} typing (WB)
Inherited CJD												
Genetic CJD	50–60	6 mo (2–41 mo)	Clinical symptoms similar to sCJD	Positive in 70–90%	Positive in 72%	PSWC 75%	Similar to sCJD			Over 25 disease-associated mutations, e.g., E200K	Similar to sCJD	
GSS	50–60	5–6 yr (3 mo–13 yr)	Cerebellar dysfunction (ataxia, nystagmus, dysarthria)	Usually negative	NA	Nonspecific	Normal or nonspecific cerebral or cerebellar atrophy			P102L (plus 11 less common mutations)	Spongiform changes, neuronal loss, astrogliosis, Prp deposition (multicentric plaques)	
FFI	50 (20–63)	13–15 mo (6–42 mo)	Insomnia, autonomic dysfunction	Negative	NA	Nonspecific	Normal or nonspecific cerebral or cerebellar atrophy		M (on the mutated allele)	D178N	Involvement of thalami	
Acquired CJD												
Variant CJD	26 (12–74)	14 mo (6–24 mo)	Early psychiatric symptoms (depression, anxiety, social withdrawal), dysesthesia, later neurological deficits and cognitive decline	Positive in 77%	Positive in 90%	Nonspecific, no PSWC	Hyperintensities in the posterior thalamus ("pulvinar sign") 78%	Tonsil	MM 100%	N/O	Spongiform changes, neuronal loss, astrogliosis, Prp deposition (florid plaques)	
Iatrogenic CJD	—[b]	Similar to sCJD	Clinical symptoms similar to sCJD	Positive in 77%	NA	Similar to sCJD	Similar to sCJD		MM 57% MV 20% VV 23%	N/O	Similar to sCJD	

[a] Abbreviations: PSWC, periodic sharp wave complexes; GSS, Gerstmann-Sträussler-Scheinker syndrome; FFI, fatal familial insomnia; N/O, not observed; NA, not assessed; WB, Western blotting.

[b] —, age at onset depending on iatrogenic exposure; incubation period, 1 to 30 years.

from country to country, with Slovakia reaching the highest incidences at more than 1 case per million (17, 18).

Variant CJD

vCJD, a relatively new member of human prion diseases, was first reported in 1996 (19). Biochemical, neuropathological, epidemiological, and transmission studies indicate that vCJD represents the transmission of bovine spongiform encephalopathy (BSE) prions to humans (20). The incidence of vCJD in the United Kingdom rose each year from 1996 to 2001, evoking fears of a large upcoming epidemic (21). Since 2001, the incidence of vCJD in the United Kingdom has dropped; however, other countries that were presumably exposed to BSE prions, such as France and Spain, have reported a significant number of vCJD cases (http://www.eurocjd.ed.ac.uk). Predictions on the future of the vCJD epidemic indicate that the total number of vCJD victims will be limited (22). vCJD has a distinct clinicopathological profile including young age of onset (median age at death, 29 years) and long disease duration (Table 1; see also Fig. 1 and 2) (19).

Iatrogenic CJD

iCJD is caused by accidental prion exposure during medical or neurosurgical procedures such as implantation of human dura mater or treatment with human cadaveric pituitary extracts or blood transfusion (23). Iatrogenic CJD is rare, with fewer than 300 published cases (23). The majority of cases were caused by implantation of dura mater and injection of pituitary growth hormone. Recent epidemiological data confirm the observation that iCJD affects mainly individuals younger than 39 years (14).

Prion Mechanisms in Other Dementias

Clinical manifestation of prion diseases requires spread of infectious prions from the periphery to the CNS, in the case of iatrogenic and variant CJD, and spread of prions within the CNS in the case of all forms of human prion disease. The mechanism by which prions spread is referred to as nucleated seeding, a process that is thought to include formation of small PrP^{Sc} seeds that can act as templates to facilitate further protein aggregation. Similar mechanisms have been described for other cerebral proteopathies such as Alzheimer's, Parkinson's, or Lou Gehrig's disease (24, 25).

CLINICAL SIGNIFICANCE

Initial symptoms of sCJD include rapid cognitive decline, sleep disturbances, and behavioral abnormalities. As the disease progresses, other clinical features such as extrapyramidal symptoms (i.e., akinesia, which is the inability to initiate movement) and pyramidal symptoms (i.e., loss of fine motor skills), ataxia, and visual disturbances appear and patients usually develop myoclonus (involuntary twitching of a muscle) (12). Terminally affected sCJD patients typically develop a state of akinetic mutism prior to death. The disease course is usually short, the mean duration of the illness being 4 to 5 months (Table 1). VPSPr is characterized by psychiatric signs, speech deficit, and cognitive decline in the absence of rapid course or myoclonus (8).

The clinical presentation of gCJD varies with the underlying mutation. Some mutations present with a clinical picture that is similar to that of sCJD (Table 2). The age at onset tends to be younger and the disease duration longer than that for sCJD. FFI and GSS represent exceptions. FFI has a unique clinical course that is characterized by profound disruption of the normal sleep-wake cycle, insomnia, and sympathetic overactivity such as accelerated heart rate or perspiration, whereas GSS presents with a progressive cerebellar ataxia (15).

In iCJD, the site of prion exposure seems to dictate the incubation time from exposure to onset of prion disease-related symptoms. Direct intracerebral exposure to prions and implantation of prion-contaminated dura, for example, are associated with short incubation periods (16 to 28 months), whereas exposure to prions at sites outside the central nervous system results in long incubation times ranging from 5 to 30 years (23). Furthermore, there is evidence that the route of prion exposure influences the clinical presentation. Dura mater or growth hormone-related cases of iCJD present with a predominantly ataxic phenotype, whereas dementia was the initial symptom in cases in which prions were directly introduced into the CNS.

The fact that vCJD carries a distinct clinical profile has facilitated the formulation of diagnostic criteria. vCJD victims are much younger than sCJD patients (median age at death, 29 years). Furthermore, initial features and illness duration are relatively specific, with initial psychiatric symptoms and median illness duration of 14 months (Table 1). It has been hard to estimate incubation times for vCJD due to the fact that the exact time points of prion exposure are not defined.

The diagnosis of human prion diseases is based on the evaluation of clinical signs and auxiliary examinations (26). Electroencephalography (EEG) has historically been used to substantiate the diagnosis of a human prion disease. The usefulness of EEG has been questioned due to its limited sensitivity, which ranges between 0 and 73% for sCJD (27). Recent advances in neuroimaging, especially in magnetic resonance imaging (MRI), have revealed that different human prion diseases have specific patterns. For vCJD, the "pulvinar sign," a high T2 MRI signal in the posterior thalamus, seems to be pathognomonic (28). For sCJD, a high T2 MRI signal in the striatum as well as a high MRI signal in the cortex in fluid-attenuated inversion recovery sequences (a pulse sequence used in brain imaging to suppress cerebrospinal fluid (CSF) effects on the image) are classical findings (28).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Safety and Security

Human prions are classified as biosafety level 2 (BSL2) or BSL3 depending on the source of the infectious material and the level of infectivity of the source specimen. Unfixed samples of brain or spinal cord, as well as other tissues such as lymphoid tissue specimen from vCJD-diseased individuals known to contain high amounts of infectious prions, should be handled at BSL3 (29). In clinical laboratories, personal protective clothing such as disposable gowns, gloves, and barrier protection for mucous membranes (eye protection or full face visor) is recommended when working with potentially contaminated specimens. In addition, strict adherence to standard working procedures aimed at minimizing the chance of penetrating injuries is essential. Prions are not inactivated by formalin. A procedure recommended by the College of American Pathologists (http//:www.cap.org) for safe handling of tissues is adequate formalin fixation, followed by agitation in a sufficient amount of formic acid (95 to 100% [vol/vol]; the sample should be entirely immersed in formic acid) for 1 hour, and subsequent formalin fixation for 2 days prior to embedding. Formic acid inactivates formalin-treated prions

TABLE 2 Disease-causing mutations in *PRNP*[a]

Mutations that cause GSS	Mutations that cause a disease that is clinically similar to sCJD	Mutations that cause FFI	Mutations that cause nonclassifiable neuropsychiatric symptoms
P102L (codon129V)	P105S	D178N (codon129M)	G114V
P105K (codon129V)	V120I		Q160stop (codon129M)
A117V (codon129V)	G124S		N171S
G131V (codon129M)	I138M		T183A
Y145stop (codon129M)	D178N (codon129V)		H187R
H187R (codon129V)	V180I		
F198S (codon129V)	V180I + M232R		
D202N (codon129V)	T188R		
Q121P	T188A		
Q217R (codon129M)	T188K		
M232T	E196K		
192-bp insertion	E200K		
	V203I		
	R208H		
	E211Q		
	Y226stop		
	Q227stop		
	M232R		
	P238S		
	24-, 48-, 96-, 120-, 144-, 168-, 216-bp insertions		

[a] Abbreviations: GSS, Gerstmann-Sträussler-Scheinker syndrome; FFI, fatal familial insomnia.

but has minimal effect on the quality of histology. Disposable histologic equipment should be used whenever possible.

CSF may contain detectable amounts of infectious prions and must be handled with caution. Analysis of samples from confirmed CJD patients should not be performed in automated equipment, and any materials coming in contact with CSF should be incinerated or decontaminated according to one of the methods listed below (see also http://www.advisorybodies.doh.gov.uk/acdp/tseguidance/). Blood and bone marrow are presumed to contain low levels of infectivity (http://www.advisorybodies.doh.gov.uk/acdp/tseguidance/) and can be handled safely under BSL2 conditions by adhering to universal precautions for prevention of transmission of bloodborne pathogens. These specimens can be tested in automated analyzers found throughout clinical laboratories if instruments are enclosed and can contain spillage and if waste can be disposed of safely. Maintenance and emergency procedures that protect the user from exposure should be outlined in laboratory standard operating procedures and implemented. Manual processing (specimen decanting, for example) should be performed inside a negative-pressure laminar flow hood in a contained environment.

Prions can be substantially but not completely inactivated by physical exposure to steam or dry heat at high temperatures. Disposable laboratory equipment should be used whenever possible. Potentially contaminated laboratory waste should be autoclaved (134 to 137°C, 20 minutes) and then incinerated (http://www.advisorybodies.doh.gov.uk/acdp/tseguidance/). Chemical exposure to high concentrations of either sodium hypochlorite (>5.25% solution, freshly prepared) or sodium hydroxide (1 N solution) for 1 hour is effective against prions and can be used to disinfect spills. Reusable laboratory material should be immersed in a freshly prepared >5.25% solution of sodium hypochlorite or a 1 N solution of sodium hydroxide for 1 hour and then rinsed with water before being packaged for autoclave sterilization at 134°C for at least 20 min (http://www.advisorybodies.doh.gov.uk/acdp/tseguidance/). The guidelines for reprocessing of instruments may vary between countries; therefore, readers should also refer to their country's specific guidelines.

Specimen Collection

Whole blood may be used for isolation of DNA for genetic analysis (exclusion of gCJD). Blood can be shipped at room temperature. Currently, an approved blood-based screening test for human prion diseases is not available. CSF is routinely used to monitor the elevation of nonspecific neuronal injury markers. CSF should be shipped at 4°C. Tissue may be used for histopathology and biochemical examination. Brain biopsies are the only way to confirm the diagnosis of sCJD in living patients. Biopsies of muscle and olfactory mucosa have been suggested but lack validation (30, 31). Biopsies of lymphatic tissue are useful only in order to confirm the suspicion of vCJD (32). Tissue should be fixed in formalin for histologic assessment as described above and snap-frozen for Western blotting. Formalin-fixed material may be shipped at room temperature, whereas snap-frozen material must be shipped on dry ice.

Shipping

Shipping of infectious material must comply with the "Recommendations of the United Nations Committee of Experts on the Transport of Dangerous Goods." In these guidelines, human prions are listed in category 6 (Toxic and Infectious Substances), division 2 (Infectious Substances). The code numbers UN 2900 apply for this type of pathogen. Certified shipping containers must be used.

DIRECT EXAMINATION

Microscopy

Histological examination of brain biopsy specimens is a diagnostically valid premortem test. Routine hematoxylin

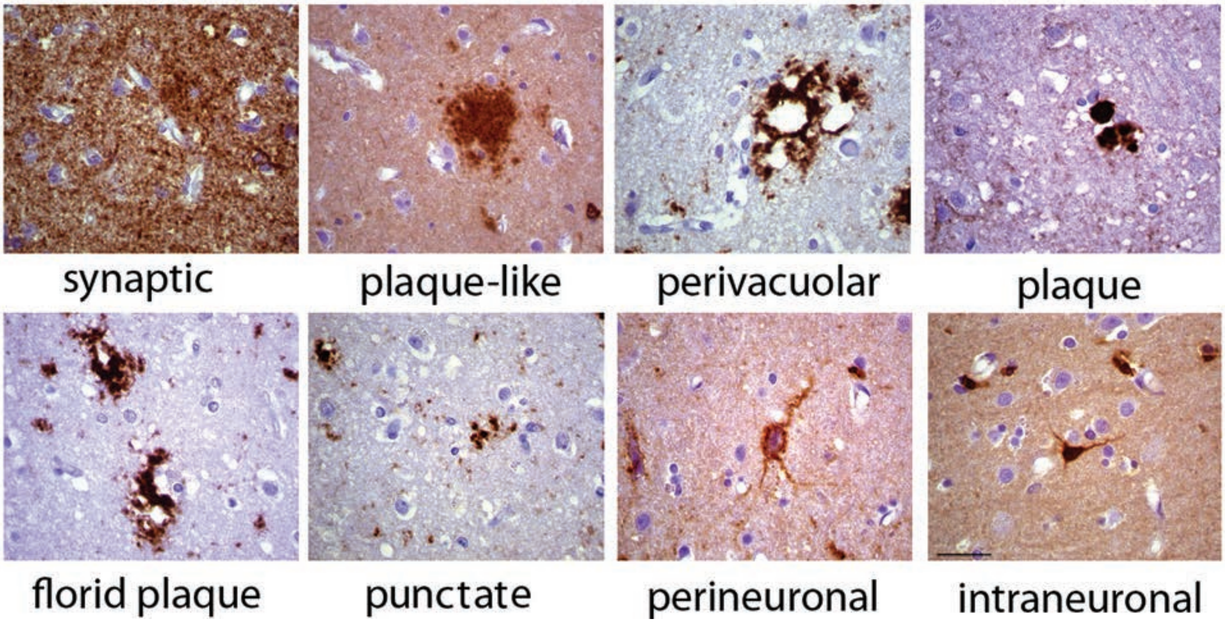

FIGURE 1 Deposition patterns of PrP^{Sc} in the brain. Shown are examples of specific deposition patterns of PrP^{Sc}. These become apparent upon immunolabeling with antibodies directed against the prion protein. The nomenclature of deposition patterns is indicated below histology. doi:10.1128/9781555817381.ch109.f1

and eosin staining is used to interpret the vacuolization patterns, whereas immunohistochemical demonstration of PrP is necessary in order to determine PrP deposition patterns (Fig. 1). Postmortem, defined regions within the central nervous system (cerebellum and thalamus) and nonneuronal tissues can be sampled to demonstrate distinct PrP deposition patterns (33). Histologically, prion diseases are characterized by spongiform changes, astrogliosis, and neuronal loss. These changes are most evident in the cerebral cortex and in the cerebellum. The prominent cerebellar involvement is typical for prion diseases and clearly separates this group of diseases from other dementing illnesses such as Alzheimer's disease or diffuse Lewy body disease (34). In vCJD and in about 10 to 20 percent of sCJD patients, prion plaques are a prominent feature and may be demonstrated by Congo red staining or immunohistochemistry (Fig. 1). Types of prion deposits are indicative for the type of prion disease (e.g., florid plaques surrounded by spongiform changes can be found only in vCJD [Fig. 1]). Birefringence under polarized light, a feature of proteins assuming β-pleated sheet conformation, usually a characteristic for amyloid plaques, may be absent due to formic acid pretreatment of fixed tissue.

Antigen Detection

Western blotting is performed on unfixed tissue originating from the central nervous system, usually in research settings. Biosafety measures have to be taken and material handled at BSL3. Generally, this test is undertaken with postmortem material. In rare cases, a brain biopsy specimen may be used for this test.

The basis of biochemical characterization of PrP^{Sc} resides in the relative resistance of PrP^{Sc} to proteolytic degradation. Whereas PrP^{C} is entirely digested by proteinase K, identical treatment of PrP^{Sc} leads to removal of a variable number of N-terminal amino acids. Western blotting of digested PrP^{Sc} reveals three distinct bands, corresponding to di-, mono-, and unglycosylated forms (34). The molecular classification of PrP^{Sc} takes three parameters into account: molecular weight of unglycosylated PrP^{Sc}, the relative amounts of PrP^{Sc} di-, mono-, and unglycosylated forms, and the status of a *PRNP* polymorphism at codon 129, where either methionine (M) or valine (V) can be encoded (see "Nucleic Acid Detection: Genetic Testing" below). Thus, defined PrP^{Sc} types correlate with distinct disease phenotypes. The resulting information is then used to establish the "type" of PrP^{Sc} according to proposed schemes (35, 36) (Fig. 2). Depending on the exact conditions under which the protease digestion and the Western blotting procedure are performed, between 3 and 6 different PrP^{Sc} types can be distinguished. Distinct PrP^{Sc} types are thought to represent the molecular correlates of distinct prion strains. The fact that the PrP^{Sc} types found in vCJD patients and in BSE-diseased cattle are identical is one of the main arguments supporting the theory that BSE prions are responsible for the vCJD epidemic in humans (20). Novel anti-PrP antibodies are able to discriminate PrP^{Sc} types and have thus facilitated diagnostic procedures (37).

In addition to Western blotting, sandwich enzyme-linked immunosorbent assays (ELISAs) are available to detect partially proteinase K-resistant PrP^{Sc}. Although these tests do not give exact information on PrP^{Sc} types, recent studies support their use in a diagnostic setting for human prion diseases (38).

Nucleic Acid Detection: Genetic Testing

For genetic testing, DNA is routinely extracted from whole blood. The extraction procedure significantly reduces the already low titers of infectious prions in blood if isolation protocols using protein denaturing steps are employed. The entire *PRNP* open reading frame may be amplified for sequencing using PCR.

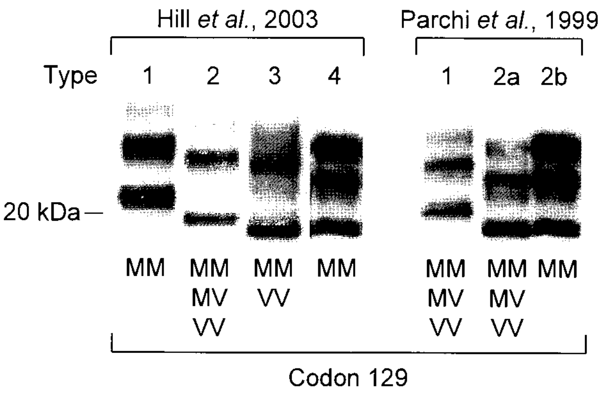

FIGURE 2 Western blot analysis of PrP^{Sc}. Depicted are PrP^{Sc} types according to two proposed schemes (36, 45), which discriminate PrP^{Sc} types based on the mobility of the unglycosylated band of PrP^{Sc} and the signal intensity of PrP^{Sc} di-, mono-, and unglycosylated forms and the genotype of codon 129 on *PRNP* (M, methionine; V, valine). One scheme (36, 45) differentiates 4 principal PrP^{Sc} types (types 1 to 4). Three principal PrP^{Sc} types (1, 2a, and 2b) are proposed in the second scheme (45). doi:10.1128/9781555817381.ch109.f2

Sequencing of *PRNP* allows for the exclusion of gCJD (39). More than 30 disease-causing insertional or point mutations in *PRNP* have been identified to date (Table 2).

In addition, there are several *PRNP* polymorphisms, one of which (codon 129M/V) has a disease-modifying function (40). Homozygosity for methionine at codon 129 constitutes a risk factor for the development of prion disease: methionine homozygotes are overrepresented among sCJD patients, and all definite vCJD patients for which codon 129 status was tested were homozygous for methionine at codon 129. These analyses are available at most academic human genetic institutions.

CSF Analysis

To date, there are no serologic screening or confirmatory tests for prion diseases. Rather, several studies have evaluated the usefulness of surrogate markers, such as 14-3-3 and Tau protein, that are found in CSF as a consequence of neuronal death (41). These proteins are ubiquitously expressed. The 14-3-3 protein participates in protein kinase signaling and is involved in neuronal migration. Tau is a phosphoprotein that promotes assembly and stability of axons by binding to microtubules. Elevation of these proteins is nonspecific and can be seen in other conditions such as encephalitis, cerebral infarction, and paraneoplastic neurological disorders. In studies on large cohorts of patients with suspected prion disease, 14-3-3 has a better sensitivity than Tau (95% compared to 86%) but Tau has better specificity (28% for 14-3-3 and 67% for Tau) (42). While 14-3-3 is routinely tested using a Western blot assay, Tau may be measured in an ELISA format (41). Other CSF markers used to monitor or diagnose CJD such as S100, a marker protein for cells derived from the neural crest or neuron-specific enolase (NSE), perform worse than 14-3-3 and Tau (26). For CSF analyses, blood contamination can lead to false-positive results. Repeat testing improves sensitivity considerably. Novel test methods such as real-time quaking-induced conversion, a technology able to amplify PrP^{Sc} *in vitro*, have been propagated, yet large studies using this test have not been performed (43). Measurement of the serine proteinase inhibitor α_1-antichymotrypsin in urine has been put forward as a sensitive and easily assessable biomarker for sCJD, yet follow-up studies are lacking (44).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The diagnosis of human prion disorders can be difficult to establish but is best approached through careful consideration of clinical presentation and other clinical studies such as neuroimaging and, in certain patients, brain biopsy with histological assessment. Detection of spongiform changes and deposition of PrP^{Sc} in affected areas can secure the diagnosis of prion disorders. The absence of these changes (for example, due to sampling bias) cannot be taken as an exclusion criterion of these disorders. Less invasive tests such as detection of surrogate markers (Tau, 14-3-3) in CSF can be helpful when interpreted in the context of clinical and neuroimaging data. Genetic testing can be performed to establish the diagnosis of genetic CJD. The constellation of tests that can be helpful in the diagnosis of each of these disorders is shown in Table 2. All testing other than detection of surrogate markers in the CSF, sequencing of *PRNP*, biochemical detection of PrP^{Sc}, and neuropathology is usually performed on a research basis.

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section V

ANTIVIRAL AGENTS AND SUSCEPTIBILITY TEST METHODS

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Antiviral Agents

AIMEE C. HODOWANEC, KENNETH D. THOMPSON, AND NELL S. LURAIN

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The use of antiviral agents for the treatment of viral diseases continues to expand. Most of the agents currently approved by the Food and Drug Administration (FDA) are active against one or more of the following viruses: human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2); hepatitis viruses B and C (HBV and HCV); the human herpesviruses; and influenza A and B viruses. This chapter is organized according to these virus groups with cross-referencing for agents with activity against more than one group of viruses. The major targets of these agents are viral replication enzymes, proteases, and entry/exit pathways (1–7). In a few cases, approved drugs for the above families of viruses have also been used to treat viruses in other families. The expanded spectrum of drug usage is discussed under the individual drug sections.

AGENTS AGAINST HIV-1 AND HIV-2

There are now five classes of antiviral agents for treatment of HIV-1: (i) nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs/NtRTIs); (ii) nonnucleoside reverse transcriptase inhibitors (NNRTIs); (iii) protease inhibitors (PIs); (iv) entry/fusion inhibitors; and (v) integrase strand transfer inhibitors (INSTIs). Current information on each drug is available through the AIDSinfo website (http://AIDSinfo.nih.gov), which has separate guidelines for use of approved antiretroviral agents in (i) adolescents and adults, (ii) pediatric patients, and (iii) pregnant HIV-1-infected women (8–10). These guidelines describe the agents along with dosage, adverse effects, and drug interactions. Working groups for each of these patient populations regularly update the guidelines. Additional information can be obtained from the package inserts available from the pharmaceutical company websites. Changes in recommended drug doses as well as observed adverse effects and drug interactions occur frequently, making it necessary to consult the most up-to-date sources.

Antiretroviral agents are administered in combinations of different drug classes termed combined antiretroviral therapy (cART) to maximize efficacy and to minimize the induction of drug resistance. cART is now generally regarded as any combination regimen designed to achieve the goal of complete virus suppression. These regimens comprise a minimum of three drugs, which are usually NNRTI based (2 NRTIs and/or NtRTIs plus 1 NNRTI), PI-based (2 NRTIs and/or NtRTIs plus 1 or more PIs), or more recently INSTI based (2 NRTIs and/or NtRTIs plus an INSTI) (9).

There are currently 26 approved antiretroviral drugs (1) with numerous possible combinations for treatment regimens. Recommended regimens for adults and adolescents are given in the guidelines (9) for treatment-naive and treatment-experienced patients. The large number of drugs creates a tremendous potential for drug interactions among the different classes as well as interactions with other types of drugs prescribed for conditions associated with HIV infection. Close monitoring of these complex interactions is required to avoid detrimental changes in drug levels and/or toxicity.

Table 1 summarizes the structure, mechanism of action, and major adverse effects of the individual drugs and drug combinations approved by the FDA. The drug interactions described below for each drug are only highlights of potential interactions. Frequent updates and more-comprehensive information can be obtained from the AIDSinfo website listed above.

Nucleoside and/or Nucleotide Reverse Transcriptase Inhibitors

The NRTI-NtRTI class of drugs is not active as administered but must be phosphorylated by cellular kinases to the nucleoside triphosphate form, which lacks a 3-prime hydroxyl group for DNA chain elongation. The NRTIs require triphosphorylation, while the NtRTIs require only diphosphorylation (1). These antiviral agents act as competitive inhibitors of the viral reverse transcriptase (RT), which results in chain termination. They are active against both the HIV-1 and HIV-2 RTs, and they are used as dual-combination backbones in regimens with NNRTIs, PIs, and INSTIs (9). Several of them also are active against the HBV DNA polymerase, which has reverse transcriptase activity (see "Agents against Hepatitis B Virus" below) (11). Lactic acidosis with hepatic steatosis is a rare but very serious adverse effect associated with all members of this class. These toxic effects of NRTIs and NtRTIs appear to be the result of inhibition of the mitochondrial DNA polymerase γ (12, 13).

Abacavir

Pharmacology

The oral bioavailability of abacavir (ABC) is 83%. The plasma half-life is 1.5 h, and the intracellular half-life is 12

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TABLE 1 Antiviral agents for HIV therapy

Antiviral agent (abbreviation)	Trade name (pharmaceutical company[a])	Mechanism of action/route of administration	Major adverse effects[b]
Nucleoside or nucleotide reverse transcriptase inhibitors (NRTI-NtRTIs)			
Abacavir (ABC)	Ziagen (GSK)	Converted to triphosphate analogue of dGTP by cellular kinases, competitive inhibitor of reverse transcriptase (RT), viral DNA chain terminator; administered orally.	Hypersensitivity reaction associated with HLA-B*570, lactic acidosis and severe hepatomegaly with steatosis
Didanosine (ddI)	Videx (BMS)	Converted to dideoxy triphosphate analogue of dATP by cellular kinases. Activity and administration similar to ABC.	Pancreatitis, peripheral neuropathy, nausea, diarrhea, lactic acidosis and severe hepatomegaly with steatosis
Emtricitabine (FTC)	Emtriva (Gilead)	Converted to triphosphate analogue of dCTP by cellular kinases. Activity and administration similar to ABC.	Minimal toxicity, skin hyperpigmentation, lactic acidosis and severe hepatomegaly with steatosis, posttreatment exacerbation of hepatitis B coinfection
Lamivudine (3TC)	Epivir (GSK)	Converted to triphosphate analogue of dCTP by cellular kinases. Activity and administration similar to ABC.	Minimal toxicity, lactic acidosis and severe hepatomegaly with steatosis, posttreatment exacerbation of hepatitis B coinfection
Stavudine (d4T)	Zerit (BMS)	Converted to triphosphate analogue of dTTP by cellular kinases. Activity and administration similar to ABC.	Peripheral neuropathy, lipodystrophy; motor weakness, lactic acidosis, and severe hepatomegaly with steatosis
Tenofovir disoproxil fumarate (TDF)	Viread (Gilead)	Diester hydrolysis required for conversion to tenofovir, monophosphate analogue requires diphosphorylation by cellular kinases. Activity and administration similar to ABC.	Asthenia, headache, GI symptoms, lactic acidosis and severe hepatomegaly with steatosis, posttreatment exacerbation of hepatitis B coinfection
Zidovudine (AZT or ZDV)	Retrovir (GSK)	Converted to triphosphate analogue of dTTP by cellular kinases. Activity and administration similar to ABC.	Bone marrow suppression, GI symptoms, headache, insomnia, lactic acidosis, and severe hepatomegaly with steatosis
Nucleoside or nucleotide reverse transcriptase inhibitors (NRTI-NtRTI) combined formulations			
Abacavir (ABC) + lamivudine (3TC)	Epzicom (GSK)	See individual NRTIs above.	See individual NRTIs above.
Abacavir (ABC) + zidovudine (AZT) + lamivudine (3TC)	Trizivirr (GSK)	See individual NRTIs above.	See individual NRTIs above.
Emtricitabine (FTC) + tenofovir (TDF) + efavirenz (EFV)	Atripla (Gilead and BMS)	See individual NTRIs-NtRTIs above.	See individual NTRIs-NtRTIs above.
Tenofovir (TDF) + emtricitabine (FTC)	Truvada (Gilead)	See individual NTRIs-NtRTIs above.	See individual NTRIs-NtRTIs above.
Nonnucleoside reverse transcriptase inhibitors (NNRTIs)			
Efavirenz (EFV)	Sustiva (BMS)	Noncompetitive inhibitor binds to HIV-1 RT close to catalytic site, disrupts normal polymerization function. Administered orally.	Skin rash (Stevens-Johnson syndrome), psychiatric symptoms, central nervous system symptoms (e.g. dizziness, insomnia, confusion), elevated transaminases, teratogenic
Etravirine (ETR)	Intelence (Tibotec)	Activity and administration similar to EFV	Skin rash (Stevens-Johnson syndrome), GI symptoms
Nevirapine (NVP)	Viramune (BI)	Activity and administration similar to EFV	Severe hepatotoxicity, skin rashes (Stevens-Johnson syndrome)
Rilpivirine (RPV)	Edurant (Tibotec)	Activity and administration similar to EFV	Rash, depression, headache, insomnia, hepatotoxicity

(Continued on next page)

TABLE 1 *(Continued)*

Antiviral agent (abbreviation)	Trade name (pharmaceutical company[a])	Mechanism of action/route of administration	Major adverse effects[b]
Protease inhibitors			
Atazanavir (ATV)	Reyataz (BMS)	Peptidomimetic protease. Binds competitively to active site of HIV protease, prevents cleavage of viral polyprotein precursors, produces immature, noninfectious viral particles. Administered orally.	Indirect hyperbilirubinemia, prolonged PR interval, hyperglycemia; fat redistribution; increased bleeding episodes with hemophilia, nephrolithiasis
Darunavir (DRV)	Prezista (Tibotec)	Nonpeptidic protease. Inhibits protease dimerization. Prevents cleavage of viral polyprotein. Administered orally.	Skin rash (Stevens-Johnson syndrome), hepatotoxicity, hyperglycemia, fat redistribution, GI symptoms, elevated transaminase, increased bleeding episodes with hemophilia, nephrolithiasis
Fosamprenavir (FPV)	Lexiva (GSK)	Converted to amprenavir by cellular phosphatases. Activity and administration similar to ATV.	Skin rash, GI symptoms, headache, hyperlipidemia, fat redistribution, elevated transaminases, hyperglycemia, increased bleeding episodes with hemophilia
Indinavir (IDV)	Crixivan (Merck)	Activity and administration similar to ATV	Nephrolithiasis/urolithiasis, GI symptoms, indirect hyperbilirubinemia, hyperlipidemia, hemolytic anemia, headache, hyperglycemia, fat redistribution, increased bleeding episodes with hemophilia
Lopinavir (LPV) + ritonavir (RTV)	Kaletra (Abbott)	Activity and administration similar to ATV	GI symptoms, asthenia, hyperlipidemia, elevated transaminase, hyperglycemia, hyperlipidemia, fat redistribution, elevated transaminases, increased bleeding episodes with hemophilia
Nelfinavir (NFV)	Viracept (Pfizer)	Activity and administration similar to ATV	Diarrhea, hyperlipidemia, hyperglycemia, fat redistribution, elevated transaminases, increased bleeding episodes with hemophilia
Ritonavir (RTV)	Norvir (Abbott)	Activity and administration similar to ATV	Severe GI symptoms, circumoral paresthesias, hyperlipidemia, hepatitis, asthenia, taste disturbance, hyperglycemia, fat redistribution, increased bleeding episodes with hemophilia
Saquinavir (SQV)	Invirase (Roche)	Activity and administration similar to ATV	GI symptoms, hyperlipidemia, elevated transaminase, headache, hyperglycemia, hyperlipidemia, fat redistribution, increased bleeding episodes with hemophilia
Tipranavir (TPV)	Aptivus (BI)	Nonpeptidic protease. Activity and administration similar to DRV	Hepatotoxicity, hyperglycemia, sulfa allergy skin rash, hyperlipidemia, fat redistribution, increased bleeding episodes with hemophilia, rare intracranial hemorrhage
Entry inhibitors			
Enfuvirtide (T20)	Fuzeon (Roche)	Binds to first heptad repeat in gp41, prevents conformational changes required for fusion of viral and cellular membranes. Administered by injection.	Local injection site reactions, pneumonia, hypersensitivity reactions
Maraviroc (MVC)	Selzentry (Pfizer)	CCR5 coreceptor antagonist. Allosteric binding to CCR5 alters conformation, prevents gp120 binding. Administered orally.	Upper respiratory infections, cough, pyrexia, rash, dizziness

(Continued on next page)

TABLE 1 Antiviral agents for HIV therapy *(Continued)*

Antiviral agent (abbreviation)	Trade name (pharmaceutical company[a])	Mechanism of action/route of administration	Major adverse effects[b]
Integrase strand transfer inhibitors			
Dolutegravir (DTG)	Tivicay (Viiv/ GSK)	Prevents formation of covalent bond between unintegrated HIV DNA and host DNA, preventing formation of provirus. Administered orally.	Headache, insomnia, fatigue, elevated AST/ ALT, elevated CPK
Elvitegravir (EVG) + cobicistat (COBI) + TDF + FTC	Stribild (Gilead)	EVG prevents formation of covalent bond between unintegrated HIV DNA and host DNA, preventing formation of provirus. Requires pharmacologic boosting. Administered orally. Cobicistat: pharmacokinetic enhancer, inhibits CYP3A4. TDF and FTC: see protease inhibitors above.	Coformulation EG-COBI-TDF-FTC: GI symptoms, renal impairment, decreased bone density
Raltegravir (RAL)	Isentress (Merck)	Prevents formation of covalent bond between unintegrated HIV DNA and host DNA, preventing formation of provirus. Administered orally.	Headache, GI symptoms, asthenia, fatigue, pyrexia, CPK elevation

[a] Pharmaceutical companies: Abbott Laboratories, North Chicago, IL; BI, Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT; BMS, Bristol-Meyers Squibb, Princeton, NJ; Gilead Sciences, Foster City, CA; GSK, GlaxoSmithKline, Research Triangle Park, NC; Merck & Co., Whitehouse Station, NJ; Pfizer, New York, NY; Roche Pharmaceuticals, Nutley, NJ; Tibotec Therapeutics, Division of Ortho Biotech Products, L.P., Raritan, NJ.

[b] Abbreviations: GI, gastrointestinal (symptoms include nausea, vomiting, and diarrhea); AST, aspartate aminotransferase; ALT, alanine aminotransferase; CPK, creatine phosphokinase.

to 26 h. ABC can be administered with or without food. It is metabolized by alcohol dehydrogenase and glucuronyltransferase, and 82% of the metabolites are excreted by the kidneys. Placental passage has been demonstrated in animal studies (10). ABC penetration of the central nervous system (CNS) is adequate to inhibit HIV replication (14). ABC is recommended as an alternative drug in dual NRTI backbones. Although several studies suggested that ABC is associated with an increased risk of cardiovascular disease, more-recent data have failed to show an association (15–17). The guidelines recommend using caution when prescribing ABC in patients with high risk for cardiovascular disease. ABC is contraindicated as an alternative drug in dual NRTI/NtRTI backbones in combination with an NNRTI or PI in patients who are positive for the HLA-B*5701 major histocompatibility complex class I (MHC-I) allele, which is associated with a hypersensitivity reaction to the drug (9, 18). Combination formulations of 2 and 3 NRTIs and/or NtRTIs containing ABC are commercially available (Table 1).

Drug Interactions

ABC decreases the level of methadone. Ethanol increases the concentration of ABC in plasma through common metabolic pathways (9).

Didanosine

Pharmacology

The oral bioavailability of didanosine (ddI) is 30 to 40%. The serum half-life is 1.5 h, and the intracellular half-life is >20 h. It should be administered without food. One-half of the drug is excreted by the kidney. There is low penetration of the CNS, but ddI has been shown to cross the human placenta (10). ddI is no longer recommended for use in treatment-naive patients (9).

Drug Interactions

Administration of ddI with either stavudine (d4T) or tenovir disoproxil fumarate (TDF) can increase the rate and severity of toxicities associated with each individual drug. Ganciclovir (GCV), valganciclovir (val-GCV), ribavirin (RBV), and allopurinol also increase ddI exposure, leading to increased ddI toxicity (9, 19, 20).

Emtricitabine

Pharmacology

The oral bioavailability of emtricitabine (FTC) is 93%. The plasma half-life is 10 h, and the intracellular half-life is >20 h. FTC can be administered with or without food. It is excreted mostly unchanged (86%) by the kidneys, and the remainder is eliminated in the feces. It has intermediate penetration of cells of the CNS (21), but it is not known whether FTC crosses the human placenta (10). FTC is recommended as a preferred drug in combination with tenofovir (TDF) in NNRTI-based, PI-based, or INSTI-based regimens for treatment-naive patients. Coadministration with lamivudine (3TC) is not recommended, because both drugs have similar resistance patterns and there is no therapeutic advantage for the combination (9).

Drug Interactions

No significant interactions with other antiretroviral agents have been reported (9, 22).

Lamivudine

Pharmacology

The oral bioavailability of lamivudine (3TC) is 86%. The serum half-life is 5 to 7 h, and the intracellular half-life is 18 to 22 h. The drug can be administered with or without food, and 71% is excreted by the kidney. 3TC crosses the human placenta (10) and has intermediate penetration of the CNS (21). 3TC is recommended in alternative dual-NRTI regimens with ABC, combined with either an NNRTI, PI, or INSTI for treatment-naive patients (9). Coadministration of 3TC with FTC is not recommended (see " Emtricitabine" above).

Drug Interactions

3TC is actively excreted by the kidney by the organic cationic transport system; therefore, possible interactions should be considered with other drugs such as trimethoprim-sulfamethoxazole, which use the same pathway (9).

Stavudine

Pharmacology

The oral bioavailability of stavudine (d4T) is 86%. The serum half-life is 1.0 h, and the intracellular half-life is 7.5 h. d4T can be administered with or without food. Fifty percent of the drug is excreted by the kidneys. Placental passage occurs in animals, and d4T has intermediate penetrance of the CNS (10, 23). d4T is no longer recommended for use in treatment-naive patients (9).

Drug Interactions

d4T combined with ddI can increase the rate and severity of toxicities associated with each individual drug. Zidovudine (ZDV) and RBV inhibit the phosphorylation of d4T (24, 25).

Tenofovir Disoproxil Fumarate

Pharmacology

The oral bioavailability of tenofovir disoproxil fumarate (TDF) is 25% without food and 39% with a high-fat meal, although the drug is administered without regard to meals. The serum half-life is 17 h, and the intracellular half-life is >60 h. The drug is excreted mostly unchanged (70 to 80%) by the kidneys. TDF has been shown to cross the placenta in animal studies, but it has low penetrance of the CNS (10, 21). It is less likely than other NRTIs-NtRTIs to be associated with mitochondrial toxicity; however, renal dysfunction and decreased bone mineral density have been reported with TDF use. TDF is recommended as a preferred NRTI-NtRTI in dual-combination backbones with FTC (9, 26). Tenofovir alafenamide (TAF) is a prodrug of tenofovir currently in clinical trials. TAF achieves higher intracellular levels and lower levels of tenofovir in plasma than does TDF. It is hoped that TAF will be associated with improved antiviral efficacy and reduced toxicity compared to TDF (27).

Drug Interactions

TDF increases the concentration of ddI in plasma, leading to increased toxicity (19, 20). There may be increased toxicity associated with coadministration of GCV, valGCV, acyclovir (ACV), or cidofovir (CDV) (9).

Zidovudine

Pharmacology

Zidovudine (ZDV, AZT) was the first agent used for antiretroviral therapy (28). The oral bioavailability is 60%, the serum half-life is 1.1 h, and the intracellular half-life is 7 h. ZDV can be administered without regard to meals. It is metabolized to the glucuronide form, which is excreted by the kidneys.

ZDV crosses the blood-brain barrier to achieve effective concentrations in the CNS (21) and also crosses the placenta. ZDV with 3TC is a preferred dual-NRTI backbone for combination regimens in pregnant women (10). It can be given intravenously to pregnant women during labor to prevent maternal-fetal transmission if the mother has ≥400 copies/ml of HIV or if the HIV viral load is unknown near the time of delivery. Intrapartum ZDV is no longer recommended for HIV-infected mothers who achieve virologic control on cART. ZDV can be administered orally to the child at birth (10, 29). For adults and adolescents, ZDV can be given with 3TC as dual-NRTI backbone with NNRTI-based and PI-based regimens. However, this is no longer considered a preferred or alternative regimen, as it requires twice-daily dosing and has greater associated toxicity than TDF/FTC or ABC/3TC (9).

Drug Interactions

ZDV inhibits the phosphorylation of d4T by thymidine kinase (24). RBV inhibits phosphorylation of ZDV (25). GCV and alpha interferon (IFN-α) may enhance the hematologic toxicity associated with ZDV (30, 31).

NRTI/NtRTI Combination Formulations

There are seven fixed-dose combinations involving NRTIs and NtRTIs, which are available as commercial formulations for convenience of administration: ABC-3TC-ZDV (Trizivir); ABC-3TC (Epzicom); FTC-TDF (Truvada); 3TC-ZDV (Combivir); FTC-TDF-EFV (Atripla); FTC-RPV-TDF (Complera); and FTC-elvitegravir (EVG)-cobicistat (COBI)-TDF (Stribild). Clinical trials have shown the triple combination ABC-3TC-ZDV to be equivalent to PI-based regimens but inferior to NNRTI-based regimens in reducing HIV RNA load in plasma below detectable levels (9, 32). Therefore, ABC-3TC-ZDV is no longer recommended for initial therapy. The dual combinations are used as NRTI-NtRTI backbones in combination with an NNRTI, PI, or INSTI in triple- or quadruple-drug therapy. The triple coformulation FTC-TDF-EFV is a preferred initial regimen, while FTC-RPV-TDF and FTC-EVG-COBI-TDF are considered alternative regimens (9).

Nonnucleoside Reverse Transcriptase Inhibitors

Drugs in the NRTI class do not require intracellular anabolism for activation. There is no common structure; however, they bind noncompetitively to the HIV-1 RT close to the catalytic site. Disruption of DNA polymerization activity leads to premature DNA chain termination. The HIV-2 RT is resistant to this class of drugs (1).

There are currently four available NNRTIs: nevirapine, efavirenz, etravirine, and rilpivirine. All are metabolized by the cytochrome P450 (CYP450) system, which also metabolizes the PIs (see below) and other drugs used to treat conditions associated with HIV infection. The common pathway can lead to serious interactions, which either induce or inhibit individual drug metabolism.

NNRTIs are often preferred for first-line therapeutic regimens with two NRTIs and/or NtRTIs for the following reasons: (i) there is a low incidence of gastrointestinal (GI) symptoms; (ii) NNRTIs have a long half-life that tolerates missed doses; and (iii) use of NNRTIs saves PIs for future regimens. The disadvantages of the NNRTIs are (i) the relatively low number of mutations required to confer cross-resistance to many of the drugs in this class; (ii) side effects related to the central nervous system; and (iii) the teratogenic effects associated with the preferred NNRTI, efavirenz (EFV) (9, 33).

Efavirenz

Pharmacology

The oral bioavailability of efavirenz (EFV) is undetermined. The serum half-life is 40 to 55 h. The drug should be administered without food. EFV is 99.5% protein bound in the plasma, mainly to albumin. CNS penetration is intermediate (21), but EFV has been shown to cross the placenta in animals (10). EFV is metabolized by CYP3A4 and CYP2B6 and is an inducer and inhibitor of CYP3A4. Glucuronidated metabolites are excreted in the urine (14 to 34%) and eliminated in the feces (16 to 61%). EFV is the preferred NNRTI in triple regimens with 2 NRTIs or NtRTIs, except in pregnant women, because teratogenic effects have been observed in cynomolgus monkeys during the first trimester of pregnancy (10).

Drug Interactions

Dose modifications may be necessary for potential drug interactions between EFV and the following: indinavir (IDV), lopinavir-ritonavir (LPV-r), fosamprenavir (FPV), nelfinavir (NFV), saquinavir (SQV), clarithromycin, rifabutin, rifampin, simvastatin, lovastatin, methadone, itraconazole, anticonvulsants, and oral contraceptives (9, 25). Contraindicated drugs are rifapentine, cisapride, midazolam, triazolam, ergot derivatives, St. John's wort, voriconazole, and, in treatment-experienced patients, atazanavir (ATV).

Etravirine

Pharmacology

The oral bioavailability of etravirine (ETR) is unknown. The serum half-life is 41 h ± 21 h. Drug levels are reduced under fasting conditions; therefore, ETR should be taken with meals. ETR is 99.9% protein bound in plasma, mainly to albumin. It is not known whether ETR penetrates the CNS or crosses the placenta. ETR is metabolized by CYP3A4, 2C9, and 2C19. It induces CYP3A4 and inhibits 2C9 and 2C19. ETR is eliminated in the feces (93.7%) and excreted in the urine (1.2%) (34). ETR has not been studied in large trials of treatment-naive patients and therefore is not yet recommended for treatment in this population. ETR is reported to be active against HIV-1 strains that are resistant to other NNRTIs, including HIV-1 group O (35); therefore, it is currently used in regimens for treatment-experienced patients who have failed therapy (34).

Drug Interactions

Dose modifications may be required for the following: LPV-r, SQV, antiarrhythmics, dexamethasone, erectile dysfunction drugs, warfarin, lipid-lowering drugs, diazepam, voriconazole, ketoconazole, and itraconazole. ETR should not be coadministered with the following drugs: EFV, NVP, ATV, FPV, tipranavir (TPV), hormonal contraceptives, St. John's wort, clarithromycin, rifampin, rifapentine, rifabutin (if coadministered with RTV-boosted PI), and phenobarbital (9, 34).

Nevirapine

Pharmacology

The oral bioavailability of nevirapine (NVP) is > 90%, and the serum half-life is 25 to 30 h. NVP is 60% protein bound. Penetration into the CNS is high; the concentration in the CSF is 45% of the concentration in plasma (21). Nevirapine can be administered with or without food. It is metabolized in the liver by CYP450 isoenzymes and is a CYP3A4 inducer. Glucuronidated metabolites are excreted in the urine (80%) and feces (10%). NVP is known to cross the human placenta (10). It has been used in resource-limited regions as a single oral agent in an intrapartum/newborn prophylaxis regimen to prevent maternal-to-child transmission (10, 36). NVP has been associated with serious hepatic events, particularly among patients with high baseline CD4$^+$ T cell counts. NVP is no longer considered a preferred or alternative agent for initial therapy. However, in certain circumstances NVP may be considered in women with CD4$^+$ T cell counts of ≤250 cells/mm^3 or in males with counts of ≤400 cells/mm^3 in the absence of moderate to severe hepatic impairment (Child-Pugh class B or C) (9).

Drug Interactions

NVP reduces the concentrations in plasma of IDV, SQV, oral contraceptives, fluconazole, ketoconazole, clarithromycin, and methadone (25). Coadministration of ATV, ETR, rifampin, rifapentine, or St. John's wort with NVP is contraindicated (9).

Rilpivirine

Pharmacology

The oral bioavailability of rilpivirine (RPV) is unknown, and the serum half-life is 50 h. It is not known whether RPV penetrates the CNS or crosses the placenta. RPV should be administered with food. It is a CYP3A4 substrate (9). RPV in combination with TDF-FTC or ABC-3TC is an alternative regimen for treatment-naive patients. However, RPV use is not recommended in patients with a pretreatment HIV viral load of >100,000 copies/ml, as it has been associated with virologic failure in these patients. In addition, patients with CD4$^+$ T cell counts of <200 cells/mm^3 are more likely to experience virologic failure when treated with an RPV-based regimen (9).

Protease Inhibitors

Protease inhibitors, like the NNRTIs, require no intracellular anabolism for antiviral activity. The target is the HIV-encoded protease, which is required for posttranslational processing of the precursor gag polyprotein (37). Most PIs are peptidomimetic, because they mimic the peptide bond normally cleaved by the protease (1). TPV and darunavir (DRV) are nonpeptidic molecules that are reported to inhibit protease dimerization as well as normal enzymatic activity (38). Inhibition of this activity by PIs results in production of immature noninfectious viral particles. The relative activity of PIs against the HIV-1 versus HIV-2 protease varies among the drugs and is dependent on the amino acid sequences of the target binding sites (6).

PIs are commonly used in cART regimens in combination with NRTI and/or NtRTIs for maximum antiretroviral activity and to minimize the development of resistance. PI-based regimens introduced initially led to treatment failure related to the limited bioavailability, frequent dosing, and toxicity. There are several characteristics of these drugs that lead to these treatment-related problems. They are highly bound to plasma protein, mainly alpha-1 acid glycoprotein (AAG) (39). The low concentration of unbound drug is responsible for the therapeutic activity as well as toxicity. PIs are substrates for P glycoprotein (P-gp) and multidrug resistance-associated protein (MRP). These are efflux transporters, which enhance elimination of the drugs from cells in the intestine, liver, and kidneys and reduce intracellular drug concentrations (40). All of the PIs are metabolized in the intestine and liver by enzymes of the CYP450 system (41), mainly by CYP3A, CYP2C9, and CYP2C19. An individual PI can induce and/or inhibit specific CYP450 isoenzymes, which can enhance or reduce its own metabolism or that of other PIs. As noted above, the CYP450 system metabolizes the NNRTIs and numerous other drugs that may be used for conditions associated with HIV infection. Thus, the choice of treatment regimens is complicated by multiple potential drug-drug interactions, which may enhance toxicity and/or require dose modifications of coadministered drugs (9).

Although most PIs are inhibitors of CYP3A4, ritonavir (RTV) is the most inhibitory. For this reason, RTV is used in boosting regimens to improve the pharmacokinetic profile of a second PI (40, 42). Subtherapeutic concentrations of RTV increase the systemic exposure of a second PI by reducing the rate of metabolism and increasing the half-life (41), which lowers dosing requirements and food effects for the second drug. An example is LPV, which alone has very little bioavailability and a very short half-life but in combination with RTV is used therapeutically both in treatment-naive patients and in salvage therapy (40, 42, 43). The effect of RTV on the pharmacokinetics of other PIs varies as a result of differences in interaction with components of the CYP450 system that determine bioavailability (40). Specific recommendations are described below for each drug.

Atazanavir

Pharmacology

Atazanavir (ATV) is an azapeptide PI that differs structurally from other peptidomimetic PIs. The bioavailability is undetermined, and the serum half-life is 7 h. The bioavailability, however, is increased by administration with food. ATV is 86% protein bound and penetrates the CNS (44). It is metabolized in the liver by CYP3A4, and it is also an inhibitor of this enzyme. The metabolites are eliminated in the feces (79%) and urine (13%). ATV crosses the placenta at minimal levels (10).

ATV has the advantage of once-daily dosing and a low pill burden, and the drug exposure can be increased by boosting with RTV (45). ATV boosted with RTV is a preferred PI for use in regimens with 2 NRTIs or NtRTIs. TDF or ddI combined with 3TC is not recommended in regimens with unboosted ATV (9).

Drug Interactions

Drugs that may require dose modifications or cautious use with ATV include antifungal agents, rifabutin, clarithromycin, oral contraceptives, atorvastatin, the anticonvulsants carbamazepine, phenobarbital, and phenytoin, methadone (RTV boosted), erectile dysfunction agents, H2 receptor antagonists, proton pump inhibitors, antacids, and buffered medications. Drugs that are contraindicated for coadministration with ATV include IDV, NVP, ETR, EFV (in treatment-experienced patients), the antihistamines astemizole and terfenadine, the calcium channel blocker bepridil, simvastatin, lovastatin, rifampin, rifapentine, cisapride, proton pump inhibitors, pimozide, midazolam, triazolam, ergot derivatives, St. John's wort, and irinotecan (9).

Darunavir

Pharmacology

The bioavailability of darunavir (DRV) is 37% alone and 82% when boosted with RTV, and the serum half-life is 15 h when boosted. It should be administered with food. The plasma protein binding is 95%, mainly to AAG. DRV is metabolized in the liver by CYP3A4, for which it is an inhibitor. It is eliminated in the feces (79.5%) and the urine (13.9%). It is not known whether it crosses the placenta (10). DRV boosted with RTV is recommended in preferred PI-based regimens with 2 NRTIs or NtRTIs for treatment-naive patients (9).

Drug Interactions

Drugs that may require dose modifications are the antidepressants paroxetine and sertraline, erectile dysfunction drugs, methadone (RTV boosted), atorvastatin, and rosuvastatin. Drugs that are contraindicated are LPV-r, SQV, TDF, lovastatin, simvastatin, midazolin, triazolam, ergot derivatives, St. John's wort, rifampin, rifapentine, astemizole, terfenadine, cisapride, pimozide, carbamazepine, phenobarbital, phenytoin, and fluticasone.

Fosamprenavir

Pharmacology

Fosamprenavir (FPV) is a prodrug with no antiviral activity, which must be converted to amprenavir (APV) by cellular phosphatases (46). The bioavailability of APV is undetermined, and the serum half-life is 7.7 h. It can be administered with or without food. The plasma protein binding is 90%. APV is metabolized in the liver by CYP3A4, for which it is an inhibitor and inducer (47). It is eliminated in the feces (75%) and urine (14%). It is not known whether APV crosses the placenta (10). FPV boosted with RTV has high penetrance in the CNS (21). However, it is not recommended for treatment-naive patients (9).

Drug Interactions

Drugs that may require dose modifications or cautious use with FPV include erectile dysfunction drugs, atorvastatin, and methadone. Drugs that are contraindicated for coadministration with FPV include ETR, DLV, TPV, simvastatin, lovastatin, rifampin, rifapentine, cisapride, pimozide, midazolam, triazolam, antihistamines, ergot derivatives, St. John's wort, and oral contraceptives (9, 25).

Indinavir

Pharmacology

The bioavailability of indinavir (IDV) is 65%, and the serum half-life is 1.5 to 2.0 h. IDV should be administered with low-caloric, low-fat food. It is 60% plasma protein bound, mainly to AAG (39). IDV is an inhibitor of CYP3A4. The majority of the drug (83%) is eliminated as metabolites in the feces. There is minimal passage of IDV across the placenta (10), but RTV-boosted IDV penetrates the CNS (21, 48).

RTV-boosted or unboosted IDV is not recommended as a component of PI-based regimens for treatment-naive patients, because of inconvenient dosing (unboosted) and the adverse complication of nephrolithiasis (RTV boosted) (9).

Drug Interactions

Coadministered drugs that may require dose modifications or cautious use include antifungal agents, anticonvulsants, calcium channel blockers, atorvastatin, erectile dysfunction drugs, methadone (RTV boosted), and vitamin C, especially in grapefruit juice. Drugs that are contraindicated for coadministration with IDV include ATV, TPV, amiodarone, simvastatin, lovastatin, rifampin, rifapentine, ergot derivatives, midazolam, triazolam, cisapride, pimozide, and St. John's wort (9, 25).

Lopinavir-Ritonavir

Pharmacology

LPV is administered only in combination with low-dose RTV (LPV-r), and the combined formulation (trade name, Kaletra) is commercially available. The bioavailability of LPV-r is undetermined, and the half-life is 5 to 6 h. The oral tablet formulation can be taken with or without food; the oral solution should be taken with food of moderate fat content. The plasma protein binding is 99%, mainly to AAG. LPV-r inhibits CYP3A4 and to a lesser extent CYP2D6. It is eliminated mainly in the feces (82.6%) and urine (10.4%) as metabolites. LPV crosses the placenta (10). LPV-r has high penetration of the CNS (21) and is an alternative drug in PI-based regimens with 2 NRTIs and/or NtRTIs for treatment-naive patients (9).

Drug Interactions

Drugs that may require dose modifications when coadministered with LPV-r include erectile dysfunction drugs, rosuvastatin, atorvastatin, calcium channel blockers, and methadone. Drugs that are contraindicated for coadministration include DRV, FPV, TPV, simvastatin, lovastatin, oral contraceptives, midazolam, triazolam, flecainide, propafenone, rifampin, rifapentine, astemizole, terfenadine, cisapride, pimozide, ergot derivatives, fluticasone, and St. John's wort (9, 25).

Nelfinavir

Pharmacology

The bioavailability of nelfinavir (NFV) is 20 to 80%, and the serum half-life is 3.5 to 5 h. NFV shows the greatest accumulation in cells of all the other PIs; however, the protein binding is >98% (39). It should be administered with food. NFV is both an inhibitor and inducer of CYP3A4 (47). The majority of the drug (87%) is eliminated in the feces. There is minimal placental passage (10) and low penetration of the CNS (21). NFV is not recommended in PI-based regimens with 2 NRTIs and/or NtRTIs for treatment-naive patients because of lower antiretroviral efficacy (9). Boosting with RTV does not affect exposure.

Drug Interactions

Drugs that require dose modifications or cautious use include rifabutin, atorvastatin, anticonvulsants, methadone, and erectile dysfunction agents. Drugs that are contraindicated for coadministration with NFV include TPV, the antiarrhythmics amiodarone and quinidine, simvastatin, lovastatin, rifampin, rifapentine, cisapride, pimozide, midazolam, triazolam, astemizole, terfenadine, ergot derivatives, St. John's wort, proton pump inhibitors, and oral contraceptives (9, 25).

Ritonavir

Pharmacology

The oral bioavailability of ritonavir (RTV) is undetermined, and the serum half-life is 3 to 5 h. RTV should be administered with food. It is 98% plasma protein bound and is metabolized by CYP3A. The major metabolite is isopropylthiazole, which has the same antiviral activity as the parent drug. RTV is eliminated in the feces (86.4%) and urine (11.3%) (9). Passage across the placenta is minimal (10).

The main role of RTV in current HIV therapeutics is to enhance the pharmacokinetics of a second PI (42), because RTV is such a strong inhibitor of CYP3A4. Low-dose RTV is a pharmacoenhancer of IDV, FPV, SQV, LPV, ATV, TPV, and DRV. RTV alone in PI-based regimens is not recommended because of gastrointestinal intolerance (9). RTV-boosted PIs are recommended in combination with 2 NRTIs and/or NtRTIs in PI-based regimens for treatment-naive and treatment-experienced patients (9, 42).

Drug Interactions

In general, RTV is a very strong inhibitor of CYP3A4 and has numerous potential drug interactions requiring close monitoring (9, 25). Coadministered drugs that may require dose modifications or cautious use include ketoconazole, itraconazole, rifampin, rifabutin, clarithromycin, atorvastatin, pravastatin, carbamazepine, clonazepam, ethosuximide, methadone, erectile dysfunction drugs, atovaquone, quinine, rosuvastatin, desipramine, trazodone, and theophylline. Drugs that are contraindicated for coadministration with RTV include ETR, bepridil, amiodarone, flecainide, propafenone, quinidine, simvastatin, lovastatin, rifapentine, cisapride, pimozide, midazolam, triazolam, ergot derivatives, oral contraceptives, and St. John's wort.

Saquinavir

Pharmacology

The oral bioavailability of saquinavir (SQV) is approximately 4%. The serum half-life is 1 to 2 h. SQV is a CYP3A4 inhibitor. It should be administered with food. SQV is 97% bound to plasma proteins and is eliminated mainly in the feces (81%) (9). There is minimal passage of SQV across the placenta (10) and very low penetrance of the CNS (21). SQV RTV-boosted and unboosted regimens with 2 NRTIs and/or NtRTIs are not recommended for treatment-naive patients (9).

Drug Interactions

Coadministered drugs or foods that require dose modifications or cautious use include antifungal agents, dihydropyridine, diltiazem, atorvastatin, rosuvastatin, anticonvulsants, methadone, erectile dysfunction agents, proton pump inhibitors, and grapefruit juice. Drugs that are contraindicated for coadministration with SQV include TPV, DRV, amiodarone, bepridil, flecainide, propafenone, quinidine, astemizole, terfenadine, fluticasone, simvastatin, lovastatin, rifampin, rifapentine, cisapride, pimozide, oral contraceptives, midazolam, triazolam, ergot derivatives, St. John's wort, garlic supplements, and dexamethasone (9, 25).

Tipranavir

Pharmacology

Tipranavir (TPV) is a nonpeptidic protease inhibitor (49). The oral bioavailability is undetermined, and the half-life is 6 h. It can be administered with or without food. TPV is >99.9% protein bound in plasma to both albumin and AAG. Metabolism is mainly through CYP3A4. TPV is eliminated in the feces (82.3%) and urine (4.4%). It is not known whether TPV crosses the placenta (10), and penetration of the CNS is low (21). TPV requires coadministration with RTV to reach effective levels in plasma (5, 41). TPV is not recommended for use in PI-based regimens for treatment-naive patients. The current indicated use is in patients who are highly treatment experienced or who are infected with virus strains resistant to multiple protease inhibitors.

Drug Interactions

Coadministration of TPV with the following drugs may require dose modification: rosuvastatin, atorvastatin, methadone, and erectile dysfunction agents. Coadministration of the following drugs is contraindicated: ATV, ETR, FPV, LPN, NFV, SQV, bepridil, amiodarone, flecainide, propafenone, quinidine, rifampin, rifapentine, lovastatin, simvastatin, midazolam, triazolam, ergot derivatives, cisapride, pimozide, astemizole, terfenadine, oral contraceptives, St. John's wort, and fluticasone (5, 9, 41).

Entry Inhibitors

Newer classes of antiretroviral agents that target the entry of HIV into the host cell have been developed. Enfuvirtide (T20), a fusion inhibitor, was the first of these drugs to be approved. It is a linear synthetic peptide of 36 L-amino acids that binds to the first heptad repeat in the gp41 subunit of the HIV-1 envelope glycoprotein. The sequence of the peptide was derived from that of HIV-1_{LAI}, a subtype B strain (50). The binding prevents conformational changes that are required for fusion between the virus envelope and the cell membrane (2). Entry is inhibited, thereby preventing infection of the target cell.

Maraviroc (MVC), a CCR5 antagonist, is a second drug targeting viral entry. The use of this drug is dependent on the prior determination of the viral tropism, because only virus strains utilizing the CCR5 coreceptor (R5) are susceptible. The rationale for this antiviral target is that coreceptor tropism of primary HIV-1 infection is most commonly CCR5, and the switch to CXCR4 or dual tropism occurs much later in the course of infection. Allosteric binding of MVC to the CCR5 coreceptor results in a conformational change, which inhibits HIV-1 gp120 binding and viral entry into the target cell (51).

Enfuvirtide

Pharmacology

The bioavailability of enfuvirtide (T20) by subcutaneous injection is 84% (2), and the serum half-life is 3.8 h. T20 is 92% protein bound in plasma. It is assumed that the metabolism of the drug produces the constituent amino acids, which enter the amino acid pool in the body and are recycled. It is not active against HIV-2, but there are recent data suggesting that it is active against HIV-1 non-B subtypes and possibly group O as well (52). Limited data indicate that T20 does not cross the placenta (10) and that it does not penetrate the CNS (70). T20 is not recommended for use in NNRTI- or PI-based regimens in treatment-naive patients, because there are no data from clinical trials, and it requires injection for delivery. It is presently used in salvage therapy regimens for treatment-experienced patients who have not responded to their current antiretroviral therapy (10, 53).

Drug Interactions

There is no evidence that T20 induces or inhibits any of the CYP450 isoenzymes; therefore, it is unlikely to interact with any of the drugs that are metabolized by the CYP450 system. No significant interactions with other antiretroviral drugs have been identified (2).

Maraviroc

Pharmacology

Maraviroc (MVC) prevents HIV-1 binding of R5 strains to the CCR5 coreceptor but has no activity against X4 strains. The bioavailability is 33%, and the serum half-life is 14 to 18 h. It is 76% protein bound in the plasma to both albumin and AAG. It can be administered with or without food. MVC is metabolized by CYP3A4 and eliminated in the feces (76%) and urine (20%). It is not known whether it crosses the placenta (10). Although MVC may be used in combination with 2 NRTI and/or NtRTIs in treatment-naive patients known to have CCR5-tropic virus, it is not considered a preferred or alternative agent because of its twice-daily dosing schedule and need for tropism testing (9, 54).

Drug Interactions

Coadministration of MVC with the following drugs may require dose modification: itraconazole, ketoconazole, voriconazole, carbamazepine, phenobarbital, phenytoin, clarithromycin, rifabutin, rifampin, EFV, and all PIs except TPV. Coadministration with rifapentine and St. John's wort is contraindicated.

Integrase Strand Transfer Inhibitors

Integrase strand transfer inhibitors, a class of antiretroviral drugs, target the HIV-1 integrase enzyme that mediates transfer of the reverse-transcribed HIV-1 DNA into the host chromosome. The activity of this enzyme includes 3-prime processing of the reverse transcribed DNA to generate hydroxyls at the 3-prime ends of both strands followed by strand transfer that joins viral and host DNA. The integrase inhibitors that are approved are recommended for use in INSTI-based regimens for treatment-naive patients (1, 4).

Raltegravir

Pharmacology

Raltegravir (RAL) is the first approved INSTI. It is reportedly active against HIV-1 group O isolates (35) as well as HIV-1 group M and HIV-2 (4). The bioavailability has not been established, and the serum half-life is 9 h. It is 83% protein bound in plasma. RAL crosses the placenta (10). It can be administered with or without food. It is eliminated in the feces (51%) and urine (32%). Clearance is by UDP-glucuronosyltransferase (UGTA1) glucuronidation. RAL with TDF-FTC is now recommended as an INSTI-based preferred regimen for treatment-naive patients (4, 9, 55, 56).

Drug Interactions

Coadministration of the following drugs may require dose modification: rifampin, phenytoin, phenobarbital, ATZ, TPV, EFV, ETR, and TDF. RAL is not an inducer or inhibitor of cytochrome P450 enzymes; therefore, it does not affect the pharmacokinetics of most of the drugs that interact with the other classes of antiretroviral agents (4).

Elvitegravir

Pharmacology

Currently, elvitegravir (EVG) is approved only in coformulation with other antivirals plus cobicistat (EVG-COBI-TDF-FTC; trade name, Stribild). It achieves therapeutic concentrations only when combined with a pharmacologic booster (COBI). EVG has a serum half-life of 13 h. It is 99% protein bound in plasma (57). EVG should be taken with food. CSF and placental penetration levels are unknown. The combination pill EVG-COBI-TDF-FTC is recommended as an alternative regimen in treatment-naive patients (9).

COBI was developed for use with EVG, because it has no anti-HIV activity, but like RTV, it is a strong inhibitor of CYP3A4. The result is higher concentrations of EVG at lower doses (58). COBI also interacts with intestinal transport proteins to increase absorption of other anti-HIV drugs, including atazanavir and darunavir (59).

Drug Interactions

EVG is primarily metabolized by the cytochrome P450 pathway and therefore interacts with other drugs that utilize this pathway. Because EVG is available only as a coformulated tablet, data regarding interactions of EVG are lacking. EVG-COBI-TDF-FTC may increase levels of selective serotonin reuptake inhibitors, tricyclic antidepressants, itraconazole, posaconazole, and voriconazole. EVG-COBI-TDF-FTC administration should be separated from antacid administration by more than 2 hours. Coadministration of rifabutin, rifampin, rifapentine, lovastatin, or simvastatin is contraindicated (9).

Dolutegravir

Pharmacology

Dolutegravir (DTG), the newest INSTI, was FDA approved in August 2013. It is approved for use in both HIV treatment-naive and treatment-experienced patients. It has been shown to have little cross-resistance with the other available INSTIs, RAL and EVG (60). DTG has a 14-hour half-life and can therefore be administered once a day in select patients. Twice-daily dosing is recommended in patients with known/suspected INSTI resistance and when coadministered with EFV, FPV-RTV, TPV-RTV, or rifampin (61). DTG can be administered with or without food (62). It is eliminated in the feces (53%) and urine (31%).

Drug Interactions

Coadministration with the following drugs may require dose modification: EFV, FPV-RTV, TPV-RTV, or rifampin. The following drugs should not be coadministered with DTG: carbamazepine, phenytoin, NVP, phenobarbital, and St. John's wort.

AGENTS AGAINST HEPATITIS C VIRUS

Until recently, the only available antiviral regimens with the potential to achieve a sustained virologic response (SVR) against chronic HCV infection were those combining an α-interferon with ribavirin (RBV) (63) (Table 2). Unfortunately, these regimens achieve an SVR in only 40 to 50% of more-difficult-to-treat subsets of HCV-infected patients (64). There are many factors that impact the virologic response to HCV therapy. One of the most important factors is the HCV genotype. Of the six HCV genotypes, genotypes 2 and 3 are the most IFN-responsive, while the rates of SVR are lower for genotypes 1, 4, 5, and 6 (65–67). Other factors associated with poor virologic response include HIV coinfection, African American race, and cirrhosis (64). Single nucleotide polymorphisms (SNPs) near the IL28B gene are also predictive of response to IFN-α-containing regimens. The C/T polymorphism of reference SNP 12979860 is most commonly reported (68). The highest rates of response are associated with the C/C allele; the lowest response rates are associated with the T/T allele. Response rates for the C/T allele are marginally better than those for T/T.

In 2011, two PIs, telaprevir and boceprevir, were approved by the FDA for use in combination with pegylated IFN-α and ribavirin for the treatment of HCV infection. These agents were the first direct-acting antiviral agents (DAAs) approved for the treatment of HCV, and they resulted in a dramatic improvement in SVR rates. However, in the short time since their approval, telaprevir and boceprevir have already been replaced by new DAAs with less frequent dosing requirements, fewer side effects, and fewer drug interactions (and will therefore not be reviewed in detail here). New DAAs are being combined in highly effective, well-tolerated, IFN-free regimens. Given the rapidly changing landscape of HCV treatment, please visit www.hcvguidelines.org for the most current information (69).

Interferon-Ribavirin

Pegylated interferon-α 2a and 2b

The IFN-α agents are produced through recombinant DNA technology from cloned human leukocyte interferon genes expressed in *Escherichia coli*. The pegylated interferons (PEG-IFN) are covalent conjugates of standard IFN-α with a single-branched (IFN-α 2a) or straight (IFN-α 2b) polyethylene glycol (PEG) chain. The molecular masses are 60,000 (2a) and 31,000 (2b) Da. Pegylation reduces clearance of the drug, which in turn prolongs the half-life compared to standard IFN (71).

Pharmacology

Both PEG-IFN 2a and 2b are administered by subcutaneous injection. For HCV antiviral therapy, PEG-IFN 2a and 2b are always administered with RBV. PEG-IFN has decreased renal clearance and an extended half-life of 160 h (2a) or 40 h (2b), which allows for once-weekly dosing (72). PEG-IFN is eliminated in part (30%) by the kidneys.

Drug Interactions

The potential interaction of PEG-IFN with drugs metabolized by CYP2C8/9 (e.g., warfarin, phenytoin) and CYP2D6 (e.g., flecainide) may require dose modification. PEG-IFN may also affect the metabolism of theophylline and methadone.

Ribavirin

Pharmacology

The bioavailability of ribavirin (RBV) is reported to be 52% (73) and is increased by a high-fat meal; therefore, RBV should be administered with food. The half-life in plasma is 120 to 170 h, and the drug may persist in other body compartments for up to 6 months. The pathway for elimination has not been determined. RBV appears not to be a substrate for the CYP450 isoenzymes. RBV is used as standard therapy, always in combination with other antiviral agents, for the treatment of HCV. RBV monotherapy is not effective against HCV infection.

RBV has been used as monotherapy to treat other RNA viruses, including respiratory syncytial virus (RSV), Lassa fever virus, influenza virus, parainfluenza virus, and hantavirus; however, there are no conclusive data demonstrating RBV treatment efficacy (74–81). An aerosolized formulation of RBV (Virazole; Valeant Pharmaceuticals, Costa Mesa, CA) has been approved for treatment of

TABLE 2 Antiviral agents for HCV therapy

Antiviral agent (abbreviation)	Trade name (pharmaceutical company[a])	Mechanism of action/route of administration	Major adverse effects
Pegylated alpha 2a interferon (PEG-IFN-α 2a)	Pegasys (Roche)	Binds to cell receptors, inducer of innate immune response, stimulation of IFN response genes; inhibition of viral replication in infected cells, broad biological effects on noninfected cells. Administered by subcutaneous injection.	Fever, myalgia, headache, fatigue, neuropsychiatric disorders, infection, cytopenia, cerebral vascular disorders, hypersensitivity, autoimmune disorders, pancreatitis
Pegylated alpha 2b interferon (PEG-IFN-α 2b)	Peg-Intron (Merck)	Same as Peg-IFN-α 2a	Same as Peg-IFN-α 2a
Ribavirin (RBV)	Copegus (Roche); Rebetol (Merck)	Mechanism not established; administered orally.	Anemia,[b] myocardial infarction, teratogenic, hypersensitivity, impairment of pulmonary function, GI symptoms[c]
Simeprevir (SMV)	Olysio (Janssen)	Binds to NS3 active site, preventing viral replication. Administered orally.	Photosensitivity, rash, hyperbilirubinemia
Sofosbuvir (SOF)	Sovaldi (Gilead)	A nucleotide analogue inhibitor of HCV NS5B polymerase. Inhibits viral RNA synthesis. Administered orally.	Fatigue, insomnia, headache, GI symptoms[b]
Telaprevir (TVR)	Incivek (Vertex)	NS3/4A protease inhibitor. Binds to NS3 active site, preventing viral replication. Administered orally.	GI symptoms,[c] rash, pruritis, anemia
Ledipasvir (LDV)/ sofosbuvir (SOF)	Harvoni (Gilead)	NS5A inhibitor (LDV) and a nucleotide analogue inhibitor of HCV NS5B polymerase (SOF). Administered orally.	Fatigue, insomnia, headache, GI symptoms[c]
Ombitasvir, paritaprevir, ritonavir, and dasabuvir	Viekira Pak (AbbVie)	NS5A inhibitor (ombitasvir), NS3/4A protease inhibitor (paritaprevir), and RNA polymerase inhibitor (dasabuvir). RTV is an HIV protease inhibitor without anti-HCV activity	Fatigue, rash, GI symptoms[c]

[a] Pharmaceutical companies: AbbVie, North Chicago, IL; Gilead, Foster City, CA; Janssen Pharmaceutica, Beerse, Belgium; Merck & Co., Inc, Whitehouse Station, NJ; Roche Pharmaceuticals, Nutley, NJ.

[b] Ribavirin can cause anemia via hemolysis or decreased red cell production.

[c] GI (gastrointestinal) symptoms include nausea, vomiting, and diarrhea.

hospitalized infants and young children with severe RSV lower respiratory tract infections.

Drug Interactions

Coadministration of ddI or d4T with RBV is contraindicated. ZDV plus RBV is linked to higher rates of anemia (9, 82).

Protease Inhibitors

The HCV PIs are inhibitors of the nonstructural 3/4A HCV protease enzyme. Although these agents exhibit potent anti-HCV activity when administered as monotherapy, they exhibit a low barrier to resistance and therefore must be given in combination with other anti-HCV agents. In addition, there are several mutations associated with decreased PI susceptibility.

Simeprevir

Pharmacology

Simeprevir (SMV) is administered orally. It has a longer half-life (41 hours in HCV-infected subjects) than the other approved PIs, allowing for once-daily dosing. It is eliminated in the feces by biliary excretion (U.S. National Library of Medicine [http://dailymed.nlm.nih.gov]).

Drug Interactions

SMV is metabolized by CYP3A. Levels of the following drugs and drug classes may be increased when coadministered with SMV: antiarrhythmics, calcium channel blockers, statins, and benzodiazapines. Coadministration of SMV with azoles, HIV-PIs, EFV, NVP, and rifampin is not recommended (see U.S. NLM website above).

Polymerase Inhibitors

The NS5B RNA-dependent RNA polymerase represents another target for anti-HCV therapy. Drugs in this class include nucleoside or nucleotide analogue inhibitors (NIs) and nonnucleoside inhibitors (NNIs). In 2013, sofosbuvir, a nucleotide inhibitor, was the first agent in this class to be FDA approved. The availability of this agent is a remarkable advancement in HCV therapy, as sofosbuvir has pangenotypic activity, has limited drug-drug interactions, and is associated with high response rates even when used in short treatment courses (85). Sofosbuvir is not recommended as monotherapy.

Sofosbuvir

Pharmacology

Sofosbuvir (SOF) is a prodrug that is converted to an active metabolite following oral administration. It can be taken with or without food. SOF is primarily eliminated by the kidneys (85; http://dailymed.nlm.nih.gov).

Drug Interactions

SOF is a substrate of P-gp and should therefore not be coadministered with potent P-gp inducers. However, as metabolism of SOF does not involve P450 CYP3A/4, it has few clinically significant drug-drug interactions (85).

NS5A Inhibitors

NS5A is a nonstructural protein that is integral to HCV viral replication and assembly (83). There are currently two agents approved for the treatment of HCV that target this protein: ledipasvir and ombitasvir. Both of these drugs are available only in combination with other anti-HCV agents. Please see "HCV Combination Therapies" below for additional information on these agents.

New HCV Combination Therapies

Ledipasvir-Sofosbuvir

This fixed-dose combination (FDC) was approved in 2014 for the treatment of genotype 1 chronic HCV infection. It was the first combination pill to be approved for HCV treatment and was the first IFN-free regimen to be approved for HCV treatment. Ledipasvir is an inhibitor of the HCV NS5A protein (necessary for HCV replication), and sofosbuvir is a polymerase inhibitor.

Pharmacology

This FDC is administered by mouth once daily. Sofosbuvir is primarily excreted in the urine, ledipasvir in the feces. It is not recommended for use in patients with end-stage renal disease or a creatinine clearance of <30 ml/min (84).

Drug Interactions

Both ledipasvir and sofosbuvir are substrates of P-gp. This FDC should therefore not be coadministered with strong inducers of P-gp and monitoring is recommended when coadministering with P-gp inhibitors and substrates. Increases in gastric pH may decrease the concentration of ledipasvir. Administration of this FDC should therefore be separated from antacid administration (84).

Ombitasvir, Paritaprevir, Ritonavir, and Dasabuvir

This is a copackaged product consisting of ombitasvir, paritaprevir, and ritonavir as a FDC and dasabuvir as an individual tablet. Ombitasvir is an NS5A inhibitor, paritaprevir is an NS3/4A protease inhibitor, and dasabuvir is an HCV RNA polymerase inhibitor. This product is approved for the treatment of genotype 1 HCV infection. In some patients (those with cirrhosis or genotype 1a infection), it is recommended that RBV be added to the regimen (86).

Pharmacology

Please see the prescribing information for the pharmacologic properties of the components of this regimen (86).

Drug Interactions

Due to the inclusion of ritonavir in this regimen, there are significant interactions with CYP3A4 inducers and inhibitors. Please see the prescribing information for the drug interactions of the components of this regimen (86).

AGENTS AGAINST HEPATITIS B VIRUS

There are two major classes of drugs available to treat HBV: nucleoside or nucleotide analogues and interferons. Because a large percentage of patients are coinfected with HIV, these agents are categorized by whether they have activity against both viruses or only HBV. Of the drugs that are specifically approved for HBV, PEG-IFN 2a or 2b and telbivudine (LdT) are active only against HBV, while 3TC, TDF, adefovir (ADV), and entecavir (ETV) are active against both HBV and HIV (Table 3). Though neither ADV nor ETV is currently recommended for the treatment of HIV, use of these agents should be avoided in HIV/HBV-coinfected patients who are not on a suppressive antiretroviral regimen in order to prevent the development of HIV resistance. FTC is approved only for HIV, but it has been shown to have activity against HBV (9, 88). The common target for antiviral drugs active against both viruses is the reverse transcriptase function of the HIV and HBV replication enzymes (11, 89, 90).

Chronic HBV infection plays an important role in the morbidity and mortality of HIV-infected patients (91). The strategy for selecting antiviral therapy regimens for coinfected patients is based on the need to treat one or both viruses. If only HIV requires treatment, drugs with activity against both HIV and HBV, such as 3TC or TDF, should be withheld. If only HBV needs to be treated, drugs without HIV activity, such as PEG-IFN 2a/2b or LdT, can be used. However, it is recommended that all patients with HIV and HBV coinfection be started on cART regardless of CD4 count, as this may slow the progression of liver disease (9). cART regimens with dual NRTIs and/or NtRTIs, such as TDF with 3TC or FTC, that suppress replication of both viruses are preferred (9, 91). 3TC monotherapy rapidly selects for HBV resistance (92); therefore, combination therapy with one NRTI (3TC or FTC) and one NtRTI (TDF or ADV) is required. PEG-IFN can be used to treat HBV infection, but the side effects limit the length of therapy, and the response appears to be lower in HIV-HBV-coinfected patients (91). It should be noted, however, that PEG-IFN is the only drug that can eliminate covalently closed circular (ccc) HBV DNA to cure chronic HBV infection (93).

Of note, there are 8 different HBV genotypes (A to H), each with certain geographic predilections (94). There is evidence that genotype impacts IFN responsiveness. In particular, in the treatment of hepatitis B e antigen-positive chronic hepatitis B, greater rates of hepatitis B e antigen seroconversion have been observed for genotype A than for genotype D and for genotype B than for genotype C (95). A correlation between genotype and treatment response to other anti-HBV therapies has not been established.

Nucleoside/Nucleotide Analogues

Adefovir Dipivoxil

Pharmacology

Adefovir-dipivoxil is a diester prodrug that is converted to the active drug ADV. ADV-dipivoxil is administered without regard to food, and the bioavailability is 59%. The half-life of ADV is 7.5 h, and it is excreted by the kidneys. There are no data for placental passage of the drug.

ADV was originally developed as an antiretroviral drug; however, the high dose required for HIV therapy is associated with nephrotoxicity (11). A much lower dosage is effective against HBV (90, 91). ADV is effective for treatment of chronic HBV infection. The rate of viral load decline is slower, but development of drug resistance is delayed compared to that seen with other NRTIs and NtRTIs (96) that are active against HBV. The primary role of ADV is in the treatment of 3TC-resistant HBV

TABLE 3 Antiviral agents for HBV therapy

Antiviral agent (abbreviation)	Trade name (pharmaceutical company[a])	Mechanism of action/route of administration	Major adverse effects
Adefovir dipovoxil (ADV)	Hepsera (Gilead)	Prodrug converted to the nucleotide monophosphate analogue adefovir. Requires diphosphorylation by cellular kinases. Inhibitor of HBV (RT), viral DNA chain terminator. Administered orally.	Headache, asthenia, GI symptoms, severe acute exacerbations of hepatitis B on discontinuation of treatment, nephrotoxicity, lactic acidosis and severe hepatomegaly with steatosis
Entecavir (ETV)	Baraclude (BMS)	Inhibitor of HBV DNA polymerase (RT) functions: priming, reverse transcription, positive-strand DNA synthesis. Administered orally.	Headache, fatigue, dizziness, severe acute exacerbations of hepatitis B on discontinuation of treatment, lactic acidosis and severe hepatomegaly with steatosis
Lamivudine (3TC)	See HIV antivirals, Table 1.	Inhibitor of HBV DNA polymerase	Note: additional adverse reaction for HBV-infected patients, severe acute exacerbations of hepatitis B on discontinuation of treatment
Telbivudine (LdT)	Tyzeka (Novartis)	Same as 3TC	Fatigue, increased creatine kinase, headache, myopathy, cough, GI symptoms, severe acute exacerbations of hepatitis B on discontinuation of treatment, lactic acidosis, and severe hepatomegaly with steatosis
Tenofovir (TDF)	See HIV antivirals, Table 1.	Inhibitor of HBV DNA polymerase	Note: additional adverse reaction for HBV-infected patients, severe acute exacerbations of hepatitis B on discontinuation of treatment
Pegylated alpha 2a/2b interferon (PEG-IFN-α 2a/2b)	See HCV antivirals, Table 2.		

[a] Pharmaceutical companies: BMS, Bristol-Meyers Squibb, Princeton, NJ; Gilead Sciences, Foster City, CA; Novartis Pharmaceuticals Corporation, East Hanover, NJ.

infection (97). However, TDF and entecavir have largely replaced ADV for this indication.

Drug Interactions

ADV is not a substrate, inhibitor, or inducer of any of the CYP450 isoenzymes. There is no interaction with 3TC, ETV, or TDF. It is possible that drugs that reduce renal function or compete for active tubular secretion could increase the concentration of ADV and/or the coadministered drug in serum.

Emtricitabine

Pharmacology

See the discussion on HIV antiviral agents above and Table 1. Emtricitabine (FTC) is approved for antiviral therapy in HIV-infected patients. It has activity against HBV but is not licensed for HBV antiviral therapy. FTC and 3TC are biochemically similar and appear to be interchangeable for potential use in treatment of HIV-HBV-coinfected patients. However, they also share the same HBV resistance mutations; therefore, combined therapy with these two drugs is not recommended (11, 91). In addition, as with 3TC, severe acute exacerbations of HBV can occur once therapy is discontinued (9).

Drug Interactions

See the discussion on HIV antiviral agents above.

Entecavir

Pharmacology

The bioavailability of entecavir (ETV) is approximately 100%, and the half-life is 24 h. ETV should be administered without food. It is excreted by the kidney (62 to 73%) mainly as unmetabolized drug. ETV maintains activity against lamivudine-resistant HBV, but a higher dose is recommended for patients with lamivudine-resistant HBV infection (91). ETV has shown low activity against HIV; however, there is evidence that resistance mutations are selected. For this reason, it is recommended that ETV only be used in HIV-coinfected patients if they are receiving effective antiretroviral therapy (90, 91, 98).

Drug Interactions

ETV is not a substrate, inhibitor, or inducer of any of the CYP450 isoenzymes. There is no interaction with 3TC, ADV, or TDF. It is possible that drugs that reduce renal function or compete for active tubular secretion could increase the concentration of ETV and/or the coadministered drug in serum.

Lamivudine (3TC)

Pharmacology

See the discussion on HIV antiviral agents above as well as Table 1. Lamivudine (3TC) was the first nucleoside analogue that was approved for chronic HBV infection. Because it has activity against both HIV and HBV, it has been effective in reducing loads of both viruses in plasma as part of cART regimens. However, HBV-specific drug resistance mutations are selected over long-term therapy at a higher rate (20% per year) in coinfected patients than in those that are HIV negative (90). Selection of HBV drug resistance mutations eventually decreases efficacy for treatment of chronic hepatitis. Discontinuation of 3TC in HBV-infected patients can produce severe flare-ups of hepatitis, which are usually self-limited but have been fatal in a few cases. Another common problem is the rebound viremia that occurs when therapy is terminated (9). This is thought to be derived from the viral cccDNA, which is not affected by nucleoside or nucleotide therapy and remains in the infected hepatocytes (11). For coinfected patients, recent recommendations suggest using combination dual NTRI-NtRTI therapy that includes TDF to reduce the rate of selection of HBV 3TC-resistant strains (99).

Drug Interactions

See the discussion on HIV antiviral agents above.

Telbivudine

Pharmacology

The bioavailability of telbivudine (LdT) is 68%, and it can be administered with or without food. The half-life is 40 to 50 h, and the drug is excreted mainly by the kidneys. LdT has a relatively low genetic barrier to resistance; therefore, it is not recommended as a first-line drug for treatment of chronic HBV (100). Hepatitis exacerbations have also been reported upon discontinuation of LdT.

Drug Interactions

LdT does not alter the pharmacokinetics of other nucleoside or nucleotide analogues used in the treatment of HBV (e.g., 3TC, ADV, or TDF). Coadministration with PEG-IFN 2a may be associated with increased risk of peripheral neuropathy (90).

Tenofovir Disoproxil Fumarate

Pharmacology

See the discussion on HIV antiviral agents above as well as Table 1. Tenofovir disoproxil fumarate (TDF) is approved for treatment of both HBV- and HIV-infected patients. It does not show cross-resistance with HBV 3TC-resistant mutants, and it appears to have a lower potential for selection of resistance mutations. For this reason, TDF and FTC or TDF and 3TC are recommended as dual-nucleoside backbones in therapeutic regimens to reduce the possibility of selection of HBV drug-resistant strains in coinfected patients who are on antiretroviral therapy (9, 90, 91, 99).

Drug Interactions

See the discussion on HIV antiviral drugs above.

PEG-IFN-α2a/2b

See the discussion on HCV antiviral drugs above. In studies of HBV-infected patients who were not coinfected with HIV, combination therapy of PEG-IFN and 3TC did not produce a better response than PEG-IFN alone (101, 102). There is some evidence that HBV genotypes may affect response to treatment (103). Compared to newer anti-HBV therapies, PEG-IFN is associated with more-frequent and more-significant side effects. However, it offers the advantages of a finite treatment course and a lower risk of antiviral resistance. PEG-IFN is contraindicated in patients who (i) are HIV coinfected, (ii) are hypersensitive to PEG-IFN, (iii) have autoimmune hepatitis, or (iv) have hepatic decompensation (91, 104).

AGENTS AGAINST HERPESVIRUSES

Most of the antiviral compounds that are approved to treat the eight human herpesviruses are nucleoside or nucleotide analogues, which inhibit DNA replication. Several of these compounds require phosphorylation by a virus-encoded enzyme as well as cellular kinases for activation. The ultimate target of most of these drugs is the viral DNA polymerase, although other enzymatic steps in DNA synthesis also may be inhibited (105). In addition to the nucleoside and nucleotide analogues, the antiherpesvirus compounds include a pyrophosphate analogue (foscarnet) that targets the viral DNA polymerase directly and an entry inhibitor (docosanol). The structure, mode of action, route of administration, and adverse effects of each drug are summarized in Table 4.

Acyclovir and Valacyclovir

Acyclovir (ACV) was one of the first effective antiviral compounds available clinically and has been in general use for the past 30 years. Valacyclovir (val-ACV), the L-valyl ester prodrug, is rapidly converted to ACV after oral administration (106). ACV is phosphorylated by the viral thymidine kinases (TK) of herpes simplex virus 1 (HSV-1), HSV-2, and varicella-zoster virus (VZV) and by the UL97 kinase of human cytomegalovirus (HCMV) (107).

Spectrum of Activity

ACV and val-ACV are active against HSV-1, HSV-2, VZV, and EBV (108). Of note, ACV and val-ACV are active only against replicating virus. Therefore, their role in the treatment of EBV-associated disease processes, which are primarily driven by latent virus, is limited. (109). In addition, both drugs have some activity against HCMV. Although ACV and val-ACV are not recommended for HCMV treatment, they have been used prophylactically to prevent HCMV disease in some patients following transplantation (110, 111).

Pharmacology

The pharmacokinetics of ACV after oral administration has been evaluated in healthy volunteers and in immunocompromised patients with HSV and VZV infection. The plasma protein binding for val-ACV is 13.5 to 17.9% and for ACV is 22 to 33%. The bioavailability of ACV administered as val-ACV is 54%, while the bioavailability resulting from oral ACV is 12 to 20%. The ACV half-life is 2.5 to 3.3 h in patients with normal renal function but increases to 14 h in patients with end stage renal disease (106). ACV may be administered with or without food.

ACV is excreted by the kidney with inactive metabolites 9-[(carboxymethoxy) methyl] guanine and 8-hydroxy-9-[2-(hydroxyethoxy)methyl]guanine. A dosage adjustment is recommended for patients with reduced renal function (106).

Drug Interactions

There are no clinically significant drug-drug interactions in patients with normal renal function.

TABLE 4 Antiviral agents for herpesviruses

Antiviral agent (abbreviation)	Trade name (pharmaceutical company[a])	Mechanism of action/route of administration	Major adverse effects	Antiviral activity
DNA polymerase inhibitors				
Acyclovir (ACV)	Zovirax (GSK)	Converted to guanosine monophosphate by viral kinase. Converted to triphosphate by cellular kinases. DNA chain terminator. Oral or intravenous formulations.	Minimal toxicity. GI symptoms,[b] headache, nephrotoxicity. Precipitation in renal tubules if exceed maximum solubility.	HSV-1, HSV-2, VZV
Valacyclovir (Val-ACV)	Valtrex (GSK)	L-Valyl ester prodrug of ACV with increased bioavailability. Activity same as ACV.	GI symptoms, headache, dizziness, abdominal pain, nephrotoxicity, thrombotic thrombocytopenia, hemolytic uremic syndrome (high dosage)	HSV-1, HSV-2, VZV, HCMV[c]
Cidofovir (CDV)	Vistide (Gilead)	Cytidine nucleotide analogue. Converted to di- and triphosphate by cellular kinases. DNA chain terminator (2 successive molecules required); intravenous administration with probenecid.	CDV: renal toxicity, decreased intraocular pressure, neutropenia, fever. Probenecid: headache, GI symptoms, rash.	HCMV,[d] HSV-1, HSV-2, VZV
Foscarnet (FOS)	Foscavir (AstraZeneca)	Pyrophosphate analogue. Noncompetitive inhibitor of DNA polymerase pyrophosphate binding site. Intravenous formulation only.	Renal impairment, fever, nausea, anemia, diarrhea, vomiting, headache, seizures, altered serum electrolytes.	HCMV, HSV-1, HSV-2, EBV
Ganciclovir (GCV)	Cytovene (Roche)	Guanosine analogue. Converted to monophosphate by HCMV UL97 kinase or HSV or VZV TK. DNA chain terminator. Oral and intravenous formulations.	Fever, neutropenia, anemia, thrombocytopenia, impaired renal function, diarrhea.	HCMV,[e] HSV-1, HSV-2

(Continued on next page)

TABLE 4 Antiviral agents for herpesviruses *(Continued)*

Antiviral agent (abbreviation)	Trade name (pharmaceutical company[a])	Mechanism of action/route of administration	Major adverse effects	Antiviral treatment
Valganciclovir (Val-GCV)	Valcyte (Roche)	Oral prodrug of GCV with increased bioavailability. Activity same as GCV.	Diarrhea, neutropenia, nausea, headache, and anemia	HCMV[e]
Penciclovir (PCV)	Denavir (Novartis)	Guanosine analogue. Mode of action similar to ACV. Limited DNA chain elongation. Topical formulation only.	Headache and application site reaction no different from placebo	HSV-1[f]
Famciclovir (FCV)	Famvir (Novartis)	Oral prodrug of PCV. Mode of action same as PCV.	Headache, GI symptoms, anorexia	HSV-1, HSV-2, VZV
Trifluridine	Viroptic (Monarch)	Mode of action not established, may inhibit viral DNA synthesis. Ophthalmic aqueous solution for topical use.	Burning on instillation and palpebral edema, punctate keratopathy, hypersensitivity reaction, stromal edema, keratitis sicca, hyperemia, increased ocular pressure.	HSV-1[g]
Drugs with other antiviral mechanisms				
Docosanol	Abreva (GSK)	Prevents HSV entry into cells by inhibition of fusion between HSV envelope and cell membrane. Nonprescription topical cream formulation.	Headache and skin rash	Oral HSV

[a] Pharmaceutical companies: AstraZeneca, Wilmington, DE; BMS, Gilead Sciences, Foster City, CA; GSK, GlaxoSmithKline, Research Triangle Park, NC; Monarch Pharmaceutical, Bristol, TN; Novartis, East Hanover, NJ; Roche Pharmaceuticals, Nutley, NJ.

[b] GI (gastrointestinal) symptoms include nausea, vomiting, and diarrhea.

[c] Valacyclovir used in some transplant settings for HCMV prophylaxis.

[d] Cidofovir also has reported activity against human papillomavirus, polyomavirus, adenovirus, and poxvirus.

[e] Ganciclovir and valganciclovir also have *in vitro* activity against EBV, HHV-6, HHV-7, and HHV-8.

[f] Penciclovir is used to treat herpes labialis but also has activity against HSV2 and VZV.

[g] Trifluridine is used to treat herpes keratitis but also has activity against HSV2 and VZV.

Cidofovir

Cidofovir (CDV) is a nucleotide analogue of deoxycytidine monophosphate, which does not require a virus-encoded enzyme for activation. After phosphorylation by cellular kinases, CDV diphosphate becomes the active nucleotide triphosphate, which inhibits the HCMV DNA polymerase. In HCMV, two successive CDV molecules must be incorporated for complete chain termination (112).

Spectrum of Activity

CDV is active against several herpesviruses, including HCMV, HSV, and VZV (108). CDV also has antiviral activity against poxviruses (113), adenovirus (114), polyomaviruses (115), and human papillomavirus (116, 117).

Pharmacology

CDV must be administered with probenecid (118, 119). Approximately 90% of the CDV dose administered is recovered unchanged in the urine within 24 hours. The half-life is 2.4 to 3.2 h. When CDV is administered with probenecid, the renal clearance of CDV is reduced to a level consistent with creatinine clearance, suggesting that probenecid blocks active renal tubular secretion of CDV (119). *In vitro*, CDV is less than 6% bound to plasma or serum proteins.

Drug Interactions

No clinically significant interactions have been identified for CDV. However, the required administration of probenecid with CDV may produce drug-drug interactions resulting from the potential block of acidic drug transport in the kidney (119).

Foscarnet

Spectrum of Activity

Although foscarnet (FOS) is active against several herpesviruses including HSV, HCMV, VZV, and EBV, it is most commonly used to treat drug-resistant HSV and HCMV.

Pharmacology

Pharmacokinetic data indicate that FOS undergoes negligible metabolism, appears to be distributed widely from the circulation, and is eliminated via the renal route. The available data, however, indicate that the pharmacokinetics of the drug varies among patients and within the individual patient, particularly with regard to plasma FOS levels (120). The FOS terminal half-life determined by urinary excretion is 87.5 ± 41.8 hours, possibly due to release of FOS from bone (121). Approximately 90% of FOS is excreted as unchanged drug in urine. Systemic clearance of FOS decreases and half-life increases with diminishing renal function, which may require FOS dosage modification (122).

Drug Interactions

Because FOS is reported to decrease calcium concentrations in serum, caution is advised for patients receiving agents known to affect calcium levels in serum such as intravenous pentamidine. Renal impairment is a major adverse effect of FOS; therefore, the use of FOS in combination with other potentially nephrotoxic drugs such as aminoglycosides, amphotericin B, and intravenous pentamidine (123) should be avoided.

Ganciclovir and Valganciclovir

Ganciclovir (GCV) was the first effective anti-HCMV drug developed for clinical use. GCV is an acyclic nucleoside analogue of 2′-deoxyguanosine, which requires phosphorylation by a viral kinase to become active. GCV monophosphate is subsequently phosphorylated to the di- and triphosphate forms by cellular kinases (7).

Spectrum of Activity

GCV is active against HCMV as well as HSV-1, HSV-2, VZV, EBV, HHV-6, HHV-7, and HHV-8 (124–127).

Pharmacology

Valganciclovir (val-GCV), the L-valyl ester prodrug of GCV, is rapidly converted to GCV after oral administration (128). The bioavailability of val-GCV is 60.9% compared to 5.6% for the oral formulation of GCV. The half-life of GCV is 4 h in healthy volunteers and 6.5 h in transplant recipients (129, 130). GCV is only 1 to 2% protein bound. Renal excretion of unchanged drug by glomerular filtration and active tubular secretion is the major route of elimination of GCV (91%). Val-GCV should be administered with food.

Drug Interactions

Coadministration of GCV with ddI results in significant increased levels of ddI (20, 127). Coadministration of GCV with ZDV requires dose modifications of both drugs, because of their common adverse hematological effects of neutropenia and anemia. Dosage modifications may also be required with drugs that inhibit renal tubular secretion, such as probenecid. Imipenem-cilastatin should not be administered with GCV (127).

Penciclovir and Famciclovir

Famciclovir (FCV), an oral prodrug, is the diacetyl 6-deoxy analogue of penciclovir (PCV) (131), which undergoes rapid conversion to the active compound, PCV. PCV is available only as a 1% cream for the topical treatment of herpes labialis (132). FCV was developed to improve the bioavailability of the parent compound (133).

Spectrum of Activity

PCV and FCV are active against HSV-1, HSV-2, and VZV (134). Neither of these compounds is active against other human herpesviruses.

Pharmacology

The bioavailability of PCV is 77%, and the half-life is 2 h. It can be given with or without food. PCV is <20% protein bound and is eliminated in the urine (73%) and feces (27%) (135). Although PCV is structurally related to ACV, it has a higher affinity for the HSV TK than ACV. However, ACV triphosphate has a higher affinity for the HSV DNA polymerase than does PCV triphosphate. As a result, the two compounds have similar anti-HSV potencies (136).

Drug Interactions

No clinically significant drug interactions have been identified for PCV.

Trifluridine

Spectrum of Activity

Trifluridine is a fluorinated pyrimidine nucleoside approved for the topical treatment of epithelial keratitis caused by HSV (137). It has activity against HSV-1, HSV-2, and vaccinia virus (138).

Pharmacology

Intraocular penetration of trifluridine occurs after topical instillation into the eye. Decreased corneal integrity or stromal or uveal inflammation may enhance the penetration of trifluridine into the aqueous humor. Systemic absorption following therapeutic dosing with trifluridine appears to be negligible (139).

Drug Interactions

There are no reported drug interactions by the topical route of administration.

n-Docosanol

Spectrum of Activity

n-Docosanol exhibits *in vitro* antiviral activity against several lipid-enveloped viruses including HSV-1, HSV-2, and RSV (140).

Pharmacology

A topical preparation of *n*-docosanol is available without prescription as a 10% cream for the treatment of herpes labialis.

Drug Interactions

There are no reported drug interactions with topical administration.

Other Drugs against Herpesviruses

There are several antiviral agents that are undergoing clinical trials or that are approved for conditions other than antiviral therapy. Maribavir is a novel antiviral agent in the benzimidazole drug class (141). Unlike ganciclovir, which is phosphorylated by the UL97 kinase, maribavir inhibits UL97 kinase activity directly. Importantly, maribavir has also been found to be effective *in vitro* against ganciclovir-resistant strains of HCMV. Maribavir is not associated with nephrotoxicity or hematologic toxicities but has been associated with taste disturbances (142). Unfortunately, phase 3 clinical trials of maribavir for the prevention of CMV disease in stem cell and liver transplant recipients have found maribavir to be inadequate for prevention of CMV disease (143, 144). However, there is speculation that this lack of demonstrable efficacy may be due to inadequate dosing.

Two agents that have antiherpesvirus activity and are currently in phase 3 studies are brincidofovir and letermovir. Brincidofovir is an orally administered lipid conjugate of cidofovir (145). It has activity against all of the herpesviruses, including GCV-resistant CMV and ACV-resistant HSV, as well as polyomaviruses, poxviruses, and adenovirus. Letermovir prevents CMV replication through inhibition of the terminase complex (145).

Two additional drugs that are approved for other medical conditions also have been reported to have antiviral activity against HCMV, although no clinical trials have been conducted. These are leflunomide, which is approved for treatment of rheumatoid arthritis (146, 147), and artesunate, which is an antimalarial agent (148, 149).

AGENTS AGAINST INFLUENZA VIRUSES

The two classes of antiviral agents for the treatment of influenza are M2 protein inhibitors and neuraminidase inhibitors. The M2 inhibitors are active against only type A influenza viruses, while the neuraminidase inhibitors have activity against both type A and type B viruses (150–152). The structure, mode of action, route of administration, and adverse effects of each drug are summarized in Table 5. Recommendations for use of these antivirals for influenza prevention and therapy are available from the Centers for Disease Control and Prevention website (http://www.cdc.gov/flu).

M2 Protein Inhibitors

The virus-encoded M2 protein facilitates the hydrogen ion-mediated dissociation of the matrix protein-ribonucleoprotein (RNP) complex within the endosome and the release of the viral RNP into the cytoplasm of the host cell. The M2 inhibitors block the passage of H^+ ions

TABLE 5 Antiviral agents for influenza virus

Antiviral agent (abbreviation)	Trade name (pharmaceutical company[a])	Mechanism of action/route of administration	Major adverse effects
Amantadine/ rimantadine	Symmetrel/ Flumadine (Endo/Forrest)	Prevents release of nucleic acid by interfering with viral M2 protein. Administered orally.	CNS symptoms,[b] GI symptoms[c]
Oseltamivir	Tamiflu (Roche) (Gilead [licensor])	Sialic acid analogue. Competitive inhibitor of neuraminidase affecting release of influenza virus particles from host cells. Administered orally.	GI symptoms[c] (usually mild), transient neuropsychiatric symptoms[d]
Peramivir	Rapivab (Biocryst)	Same as oseltamivir. Administered intravenously.	GI symptoms[c], leukopenia/neutropenia
Zanamivir	Relenza (GSK)	Same as oseltamivir. Administered by oral inhalation.	Respiratory function deterioration after inhalation

[a] Pharmaceutical companies: Biocryst Pharmaceuticals, Durham, NC; Endo Pharmaceuticals Inc., Chadds Ford, PA; Forest Laboratories, Inc., St. Louis, MO; Roche Laboratories Inc., Nutley, NJ, Licensor: Gilead Sciences, Inc., Foster City, CA; GSK, GlaxoSmithKline, Research Triangle Park, NC.

[b] Central nervous system (CNS) symptoms include confusion, anxiety, insomnia, difficulty concentrating, dizziness, hallucinations, and seizures.

[c] GI (gastrointestinal) symptoms include nausea, vomiting, and anorexia.

[d] Neuropsychiatric symptoms include self-injury and delirium.

through the M2 ion channel, which prevents uncoating of the virus (105, 150, 153, 154).

Amantadine and Rimantadine

The adamantanes differ in their metabolism and adverse effects, but they have similar antiviral activity against influenza A viruses. Neither drug has activity against influenza B viruses. Recent reports indicate that both the seasonal influenza virus, H3N2, and the current pandemic virus, H1N1, have a high incidence of resistance to both drugs (156, 157); therefore, the adamantanes are no longer recommended for influenza prophylaxis and empiric therapy.

Neuraminidase Inhibitors

The influenza virus neuraminidase (NA) is an envelope glycoprotein that cleaves the terminal sialic residues, releasing the virion from the infected cell. The virus-encoded NA allows the influenza virus to spread from cell to cell. Three neuraminidase inhibitors (NAIs) are approved for the treatment of influenza A and B viruses: oseltamivir, zanamivir, and peramivir (158). Of these, oseltamivir is the most widely used. In 2007–2008, a high percentage of seasonal H1N1 influenza virus isolates were resistant to oseltamivir as the result of a single amino acid substitution, but they remained sensitive to zanamivir (157, 159). In 2011, the CDC estimated that greater than 99% of circulating influenza virus strains were susceptible to the NAIs (160, 161).

Oseltamivir

Pharmacology

Oseltamivir phosphate is an ethyl ester prodrug requiring ester hydrolysis for conversion to the active form, oseltamivir carboxylate. After oral administration, oseltamivir phosphate is readily absorbed from the gastrointestinal tract and is extensively converted to oseltamivir carboxylate, predominantly by hepatic esterases (162). At least 75% of an oral dose reaches the systemic circulation as oseltamivir carboxylate. The binding of oseltamivir carboxylate to plasma protein is low. The plasma half-life is 6 to 10 hours. There are fewer side effects if administered with food. Oseltamivir carboxylate is not further metabolized and is eliminated in the urine (161). The efficacy of oseltamivir in preventing naturally occurring influenza illness has been demonstrated in treatment and prophylaxis studies (163–165).

Drug Interactions

Studies of oseltamivir suggest that clinically significant drug interactions are unlikely, because neither the drug nor the metabolite oseltamivir carboxylate is a substrate for the CYP450 isoenzymes or for glucuronyltransferases. The potential exists for interaction with other agents such as probenecid that are excreted in the urine by the same pathways (161). Oseltamivir should not be administered in a time period 2 weeks before and 48 h after administration of live influenza vaccine.

Peramivir

Peramivir was approved in late 2014 for the treatment of uncomplicated influenza.

Pharmacology

Peramivir has poor oral bioavailability and is therefore only available as an intravenous formulation. It is given as a single dose for the treatment of uncomplicated influenza. It is primarily eliminated by the kidneys (86).

Drug Interactions

There are no significant drug interactions (87).

Zanamivir

Zanamivir treatment has been shown to reduce the severity and duration of naturally occurring, uncomplicated influenza illness in adults (166). Zanamivir is administered only to the respiratory tract by oral inhalation using a blister pack (167). The contents of each blister are inhaled using a specially designed breath-activated plastic device for the inhaling powder. This route rapidly provides high local concentrations at the site of delivery. Because of the respiratory route of administration, zanamivir is contraindicated in patients with underlying airway disease such as asthma. As noted above, the H1N1 strains that have become resistant to oseltamivir remain sensitive to zanamivir.

Pharmacology

The absolute oral bioavailability of zanamivir is low, averaging 2%. After intranasal or oral inhaled administration, a median of 10 to 20% of the dose is systemically absorbed, with maximum concentrations in serum generally reached within 1 to 2 hours. The remaining 70 to 80% is left in the oropharynx and is eliminated in the feces. The median serum half-life ranges between 2.5 and 5.5 hours, and the systemically absorbed drug is excreted unchanged in the urine. The low level of absorption of the drug after inhalation produces low concentrations in serum with only modest systemic zanamivir exposure (161).

Drug Interactions

Zanamivir is not metabolized; therefore, there is a very low potential for drug-drug interaction (168).

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Mechanisms of Resistance to Antiviral Agents

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Antiviral drug resistance is mediated most often by mutations in the molecular targets of drug therapy, and the development of drug resistance is the most compelling evidence that an antiviral drug acts specifically by inhibiting the virus rather than its cellular host. Drug-resistant virus subpopulations may exist at low levels in clinical isolates or may arise only during drug exposure. The error-prone polymerase enzymes in RNA viruses render the development of resistance more frequent than in DNA viruses.

Drug-resistant viruses are identified by *in vitro* passage experiments in which wild-type viruses are cultured in increasing concentrations of an inhibitory drug and by *ex vivo* analysis of virus isolates obtained from individuals receiving antiviral therapy. Measuring drug susceptibility involves culturing a fixed inoculum of virus in serial dilutions of an inhibitory drug. The drug concentrations required to inhibit virus replication by 50% (EC_{50}) or 90% (EC_{90}) are the most commonly used measures of susceptibility. Drug susceptibility assays are not designed to determine the drug concentration required to inhibit virus replication *in vivo*. They are instead designed to identify viruses with reduced drug susceptibility relative to wild-type viruses. The clinical significance of reductions in drug susceptibility is determined by studying the treatment response of individuals harboring viruses with reduced susceptibility.

One of the fundamental paradigms of viral drug resistance to emerge in the past 2 decades is that antiviral drug resistance mutations often consist of major mutations that reduce drug susceptibility by themselves and accessory mutations that generally compensate for the decreased fitness associated with many of the major mutations. With a few notable exceptions, major drug resistance mutations do not occur in previously untreated patients, whereas accessory mutations are often polymorphic.

HERPESVIRUSES

All currently licensed systemic drugs for the herpesviruses target the viral DNA polymerase (Table 1). Nucleoside analogs that are selectively phosphorylated by viral enzymes are preferred as initial therapy. Oral bioavailability is improved by the use of prodrugs that are metabolized to the parent drug. Acyclovir (ACV), its prodrug valacyclovir, and famciclovir, the prodrug of penciclovir (PCV), have been used successfully for genital herpes simplex virus (HSV) infections, varicella-zoster virus (VZV) infections, and mucocutaneous HSV infections in immunocompromised hosts. Ganciclovir (GCV) and its prodrug valganciclovir are used to treat cytomegalovirus (CMV) infection. These guanosine analog antivirals are supplemented by foscarnet (phosphonoformate [FOS]), a pyrophosphate analog, and cidofovir (CDV), a cytosine nucleotide analog, which do not depend on prior activation by viral enzymes. FOS and CDV are generally used as second- and third-line drugs when there is a lack of response to initial therapy. An orally bioavailable hexadecyloxypropyl conjugate of CDV (brincidofovir, CMV001) is in clinical trial.

Herpes Simplex Virus

ACV and PCV are initially monophosphorylated by the HSV thymidine kinase (TK) and then converted to triphosphates by cellular enzymes. ACV triphosphate causes chain termination of replicating DNA and formation of a dead-end complex that strongly inhibits the viral DNA polymerase (1). As compared with ACV, PCV triphosphate is formed at higher concentration and persists longer in infected cells but is a less potent inhibitor of the viral DNA polymerase (2). Because the viral TK enzyme is not essential for HSV replication, a wide variety of TK mutations may decrease the phosphorylation of nucleoside analogs, and this is by far the most common mechanism of ACV and PCV resistance (3). TK mutations confer no cross-resistance to drugs that act independently of virally mediated phosphorylation, including FOS and CDV.

Drug-resistant TK mutants are classified as TK negative, usually arising from frameshift or stop mutations that delete important functional domains, or TK low, where the mutation reduces the phosphorylation of both natural nucleosides and antiviral drugs (3–5). TK-altered mutants that selectively reduce the phosphorylation of antiviral nucleosides occur less commonly. Although TK mutations selected by PCV may differ from those selected by ACV, cross-resistance is expected (6, 7). Among clinical isolates, frameshifting nucleotide insertion or deletion mutations at homopolymeric TK loci (e.g., runs of G bases) are among the most common (5). TK amino acid substitutions show some clustering at conserved regions such as ATP and nucleoside binding sites. The effect of specific mutations on TK activity and drug susceptibility can be defined by site-directed mutagenesis of control strains, but many remain uncharacterized because of the tremendous diversity of TK mutations and sequence polymorphisms (3–5, 7). Mixed heterogeneous populations of resistant genotypes evolve in individual subjects (8, 9). Because these factors complicate the genotypic

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TABLE 1 Mechanisms and mutations associated with herpesvirus antiviral drug resistance

Virus	Antiviral agent(s)	Mechanism of resistance	Mutations
HSV	Acyclovir, valcyclovir, penciclovir, famciclovir	Thymidine kinase (TK) and/or DNA polymerase mutations	TK frameshift (commonly at strings of multiple G or C bases) or substitution mutations causing a TK-deficient phenotype and cross-resistance among the four drugs. Less commonly, TK mutations conferring altered substrate specificity or DNA polymerase mutations clustered in conserved regions (e.g., II and III).
	Foscarnet	DNA polymerase mutations	Mutations clustering in regions II, VI, and III (probable pyrophosphate binding regions)
	Cidofovir	DNA polymerase mutations	Mutations in exonuclease and conserved regions II, III, and V (based on limited data from laboratory strains)
VZV	Acyclovir, valcyclovir, penciclovir, famciclovir	TK and/or DNA polymerase mutations	TK frameshift or substitution mutations causing a TK-deficient phenotype and cross-resistance among the four drugs. Less commonly, TK-altered or DNA polymerase mutations
	Foscarnet	DNA polymerase mutations	Mutations clustering in regions II, VI, and III (probable pyrophosphate binding regions)
	Cidofovir	DNA polymerase mutations	No VZV-specific data are available.
CMV	Ganciclovir	UL97 kinase mutations and/or DNA polymerase mutations	Usually UL97 mutations at residues 460, 520, and 590–607, with M460V/I, H520Q, C592G, A594V, L595S, and C603W most common. DNA polymerase mutations are less common (mainly at exonuclease and V regions, e.g., at residues 408, 412, 522, 545, and 987) and usually add to preexisting UL97 mutations.
	Foscarnet	DNA polymerase mutations	Mutations in polymerase catalytic domains involved in pyrophosphate binding and nucleotide recognition (clustered in and between regions II, VI, and III, e.g., at residues 700, 715, 756, 781, 802, and 809). Some may also decrease ganciclovir ± cidofovir susceptibility.
	Cidofovir	DNA polymerase mutations	Exonuclease and V regions with cross-resistance to ganciclovir. Low-grade cross-resistance from some region III foscarnet-associated mutations.

diagnosis of HSV drug resistance, phenotypic resistance assays remain in common use (3).

TK-negative HSV mutants are attenuated in mouse models with respect to virulence, latency, and reactivation potential (10). However, animal studies (11) and clinical case reports (12, 13) show that resistant viruses cause overt disease and recurrent infection. Even isolates with frameshift mutations that are predicted to be TK negative may in fact express some TK activity that enables reactivation from latency (11) through such mechanisms as ribosomal frameshifting (14, 15). Although drug-resistant HSV TK mutants can easily be selected in cell culture, the frequency of isolation of ACV-resistant HSV in clinical practice has remained stable in most reports at <1% in immunocompetent hosts and 7 to 11% in immunocompromised hosts (3, 16). However, ACV-resistant corneal HSV isolates were found in 11 (6.4%) of 173 immunocompetent patients with keratitis (17); this may result from persistence and reactivation of virus, including resistant strains, at sanctuary sites evading normal host immune defenses (4, 9).

DNA polymerase (*pol*) mutations are occasionally responsible for HSV resistance to ACV, PCV, FOS, and CDV (3–5, 7). Because the viral DNA polymerase is an essential gene for replication, mutations are more selective and usually consist of single amino acid changes in functional domains of the enzyme. Mutations conferring ACV or FOS resistance are distributed among several conserved *pol* regions, with some clustering of mutations in regions II, VI, and III (4). Some mutations confer resistance to both ACV and FOS (5, 18). Little is known about the range of CDV-resistance mutations in clinical isolates, but available information suggests that there is little cross-resistance with ACV or FOS (4).

Varicella-Zoster Virus

VZV infections are generally treated with the oral prodrugs valacyclovir or famciclovir, or with intravenous ACV, with outcomes dependent more on the early initiation of adequately potent therapy than on antiviral susceptibility. However, immunocompromised subjects may develop prolonged and recurrent infection, increasing the risk of emergence of resistant strains after extensive antiviral treatment (19, 20).

Mechanisms of VZV drug resistance are analogous to those of HSV. Most ACV-resistant VZV contains TK gene mutations that result in TK deficiency or altered substrate specificity (21). These include stop, frameshift, and substitution mutations (21, 22). Detection of such mutations may be used for genotypic resistance assays (23), since the phenotypic resistance testing of VZV clinical isolates is more limited than for HSV. Although VZV TK appears to have less sequence polymorphism than the HSV TK, uncharacterized amino acid substitutions prevent clear interpretation (23). Alternative drugs for ACV-resistant VZV are FOS (24, 25) and CDV. ACV and FOS resistance maps to codons in conserved functional domains II and III of the VZV DNA polymerase, with some mutations conferring cross-resistance (21).

Cytomegalovirus

CMV lacks a TK, but does have another kinase encoded by the UL97 gene, which is essential for the normal production of normal amounts of infectious virus in cell culture and is a potential antiviral drug target (26). GCV, the standard initial therapy for CMV disease, is monophosphorylated by the UL97 kinase and subsequently converted to the active triphosphate form in infected cells (27). Risk factors for CMV drug resistance are prolonged viral replication (as seen in immunocompromised hosts) coupled with extended antiviral drug exposure, usually a few months or more (28). Genotypic resistance testing for CMV is standard clinical practice because of the unavailability of timely phenotypic testing on clinical isolates. An extensive database of resistance mutations validated by recombinant phenotyping provides a basis for practice guidelines (29).

More than 90% of GCV-resistant CMV isolates contain UL97 mutations that are proven or presumed to impair GCV phosphorylation with relative preservation of biological kinase function (28). UL97 mutations in GCV-resistant CMV isolates are preferentially localized to codons 460, 520, and 590–607, with the seven most common mutations being M460V/I, H520Q, C592G, A594V, L595S, and C603W (28). These specific mutations confer a 7- to 10-fold increased resistance to GCV (drug EC50), except for C592G, which confers 3-fold increase. Various other point mutations and in-frame deletion mutations conferring varying degrees of GCV resistance have been observed, mainly at codons 590–607 (30). UL97 mutation is not known or expected to be involved in resistance to FOS or CDV.

After prolonged exposure to GCV, mutations in the UL54 DNA polymerase (*pol*) gene may be selected, usually adding to preexisting UL97 mutations and contributing to an increased overall level of resistance to the drug (31–33), although the *pol* mutations by themselves typically confer low-grade resistance. Uncommonly, mutation in the *pol* gene may be the initial marker of GCV resistance (28). Cross-resistance to other drugs is expected for GCV-resistant *pol* mutants. Mutations conferring GCV and significant CDV resistance have been reported at *pol* codons 301, 408–413, 501–545 (which corresponds to exonuclease regions), and 978–988 (region V), notably A987G (28).

FOS-resistance mutations in CMV *pol* range widely but often map to amino-terminal, palm, and finger structural features of the enzyme, including conserved sequence regions II (e.g., codons 700 and 715), III (e.g., codons 802 and 809), and VI (e.g., codon 781) and also some nonconserved loci (e.g., codon 756) (28). Most FOS-resistance mutations confer 2- to 5-fold increased EC_{50} values. FOS-resistance mutations may confer low-grade GCV ± CDV cross-resistance (28), but are not preferentially selected after initial treatment with GCV. Such cross-resistance likely involves polymerase residues with roles in recognition of the incoming nucleotide triphosphate and in pyrophosphate exchange. Uncommonly, single *pol* mutations (e.g., A834P or 981-2del) are sufficient to confer significant resistance to all three drugs, GCV, CDV, and FOS (32, 34). Some CMV *pol* mutations, especially in the codon range 578–845, have been associated with a slow growth phenotype (20, 35–37). Despite a fairly extensive database of baseline sequence polymorphisms, there remain many *pol* sequence variants of unclear relevance for drug resistance and that require characterization by recombinant phenotyping (28).

HUMAN IMMUNODEFICIENCY VIRUS

Twenty-seven antiretroviral drugs belonging to six classes have been approved for human immunodeficiency virus type 1 (HIV-1) treatment, including eight nucleoside and one nucleotide reverse transcriptase (RT) inhibitor (NRTI), nine protease inhibitors (PIs), five nonnucleoside RT inhibitors (NNRTIs), three integrase inhibitors (INIs), one fusion inhibitor, and one CCR5 inhibitor (Table 2).

HIV-1 genetic variability results from the high rate of RT enzyme errors, the high rate of virus replication *in vivo*, recombination when viruses with different sequences infect the same cell, and the accumulation of proviral variants during the course of infection (38). The virus population within an individual consists of an ensemble of innumerable related genotypes often called a quasispecies. The selection of drug-resistant variants depends on the extent to which virus replication continues during incompletely suppressive therapy, the ease of acquisition of a particular mutation, and the effect of drug-resistance mutations on drug susceptibility and virus replication.

In previously untreated individuals with drug-susceptible HIV-1, combinations of three drugs from two drug classes lead to prolonged virus suppression and, in most patients, immune reconstitution. Once complete HIV-1 suppression is achieved, it usually persists indefinitely if therapy is not interrupted. However, because antiretroviral therapy does not inhibit proviral HIV-1 DNA, viral eradication is not possible. Recurrent viremia and immunological decline ensue whenever therapy is discontinued, regardless of the previous duration of virologic suppression.

HIV-1 drug resistance may be acquired or transmitted. Drug resistance is acquired in patients in whom ongoing virus replication occurs in the presence of incompletely suppressive therapy. Whereas incompletely suppressive therapy was once a consequence of an insufficient number of active drugs, it is now usually a consequence of treatment interruptions or incomplete adherence. The proportion of HIV-1-infected persons with transmitted drug resistance is about 15% in the United States, 10% in Europe, 7% in South and Central America, and <5% in most parts of sub-Saharan Africa and South and Southeast Asia (39). The presence of either acquired or transmitted drug resistance before starting a new antiretroviral treatment regimen is associated with reduced virological response to that regimen.

NRTI Resistance

Of the eight U.S. Food and Drug Administration (FDA)-approved NRTIs, five are recommended for routine clinical use: abacavir, emtricitabine, lamivudine, tenofovir, and zidovudine. Dideoxycytidine has rarely ever been used. Stavudine and didanosine are being used less often because of their toxicities. NRTIs are usually administered in two-drug combinations together with an NNRTI, PI, INI, or CCR5 inhibitor. All recommended first-line and salvage therapy regimens include one of the cytosine analogs lamivudine or emtricitabine (40).

NRTIs must be triphosphorylated (or diphosphorylated in the case of the nucleotide analog tenofovir) to their active form. The dependence of NRTIs on intracellular phosphorylation complicates the *in vitro* assessment of their activity because phosphorylation occurs at different rates in the highly activated lymphocytes used for susceptibility testing and the wider variety of cells infected *in vivo*, explaining why NRTI resistance levels differ in their dynamic ranges (41, 42). Specifically, clinical isolates from persons failing NRTI therapy may have several hundred-fold reductions in susceptibility to zidovudine, lamivudine, and emtricitabine, but will rarely have more than 5-fold reductions in susceptibility to didanosine, stavudine, and tenofovir. However, slight reductions in susceptibility to this second category of drugs—as low as 1.5-fold—are clinically significant.

There are two biochemical mechanisms of NRTI resistance. One mechanism is mediated by discriminatory mutations that reduce RT affinity for an NRTI, preventing its addition to the growing DNA chain (43). Another mechanism is mediated by mutations that facilitate primer unblocking

TABLE 2 Genetic mechanisms of resistance to antiretroviral agents

Drug class[a]	Mechanism(s)	Drug resistance mutations
Nucleoside/nucleotide RT inhibitors (NRTIs): Abacavir Emtricitabine Lamivudine Tenofovir Zidovudine Didanosine* Stavudine*	Mutations that allow RT to discriminate between NRTIs and naturally occurring nucleoside triphosphates	M184V/I confers resistance to lamivudine and emtricitabine. K65R confers resistance to didanosine, abacavir, tenofovir, stavudine, lamivudine, and emtricitabine, and increases susceptibility to zidovudine. L74V/I confers resistance to didanosine and abacavir. Q151M confers intermediate resistance to tenofovir, lamivudine, and emtricitabine and high-level resistance to the remaining NRTIs. It usually occurs in combination with two or more of the following mutations: A62V, V75I, F77L, F116Y. T69 deletions occur in combination with K65R or Q151M.
	Mutations that promote ATP-dependent hydrolytic removal of chain-terminating nucleotide monophosphates (TAMs)	M41L, D67N, K70R, L210W, T215F/Y, and K219Q/E develop in isolates from persons receiving zidovudine and stavudine but confer clinically significant cross-resistance to each of the other NRTIs except possibly lamivudine and emtricitabine. T69 insertions (most commonly T69SSS) occur with TAMs and in this context cause intermediate lamivudine and emtricitabine resistance and high-level resistance to the remaining NRTIs. D67 deletions occur in combination with T69G and TAMs; these have effects similar to T69 insertions.
Nonnucleoside RT inhibitors (NNRTIs): Efavirenz Etravirine Nevirapine Rilpivirine	Mutations in the NNRTI-binding pocket of RT that interfere with inhibitor binding	Nevirapine: K101E/P, K103N/S, V106A/M, Y181C/I/V, Y188C/H/L, G190A/S/E, F227C, M230L Efavirenz: L100I, K101P, K103N/S, V106A/M, Y188C/H/L, G190A/S/E, F227C, M230L Etravirine: L100I, K101P, Y181C/I/V, G190E, F227C, M230L Rilpivirine: L100I, K101EP, E138A/Q/G/K, Y181C/I/V, Y188L, G190E, F227C, M230L Accessory: A98G, K101H, V108I, V179D/E/F, P225H, F227L, K238T
Protease inhibitors (PIs): Atazanavir Darunavir Lopinavir Fosamprenavir* Indinavir* Nelfinavir* Saquinavir* Tipranavir*	Protease mutations in or near the substrate cleft that interfere with inhibitor binding or compensate for the decreased replication associated with substrate cleft mutations.	Substrate cleft mutations: L23I, D30N, V32I, I47V/A, G48V/M, I50V/L, V82A/F/S/T/M/C, I84V/A/C Flap mutations: M46I/L, F53L, I54V/M/T/L/A/S Mutations at other conserved sites: L10F/R, V11I, K20T, L24I/F, L33F, K43T, Q58E, A71I/L, G73S/C/T/A, T74P, L76V, N88D/S, L89V, L90M Common polymorphic accessory mutations: L10I/V, K20R/I/M, M36I, L63P, A71V/T
Integrase inhibitors (INIs): Raltegravir Elvitegravir Dolutegravir	Mutations in residues surrounding the enzyme's active site	Raltegravir: Q148H/R/K ± G140S/A/C ± E138A/K; N155H ± E92Q; Y143C/H/R Elvitegravir: E92Q ± N155H; Q148H/R/K ± G140S/A/C ± E138A/K; T66A/I/K; S147G Dolutegravir: Q148H/R/K + G140S/A/C ± E138A/K Common accessory mutations: H51Y, L74M, T97A, V151Y, G163R/K, R263K Rare potent mutations: F121Y, P145S, Q146P Mutations selected *in vitro* by dolutegravir: G118R, R263K
Fusion inhibitors*: Enfuvirtide (T20)	Mutations in the first heptad repeat region (HR1) of the gp41 transmembrane protein	Mutations in a highly conserved region between gp41 residues 36–45. G36D/E/V/S, I37V, V38E/A/M/G, Q48H, N42T, N43D/K/S, L44M, L45M. Alone, G36D/E, V38E/A, Q40H, and N43D reduce enfuvirtide susceptibility >10-fold. Two mutations are usually required to reduce susceptibility >100-fold.
CCR5 inhibitors: Maraviroc	Expansion of preexisting CXCR4-tropic variants not detected at the start of therapy	CXCR4-tropic variants: positively charged residues at positions 11 and 25 of the V3 loop of gp120 and several other combinations of mutations primarily, but not exclusively, within the V3 loop are associated with CXCR4 tropism.
	In vitro, and rarely *in vivo*, resistance is caused by gp120 *env* mutations that facilitate gp120 binding to an inhibitor bound CCR5 molecule.	No consistent pattern of gp120 mutations is associated with virus binding to an inhibitor-bound CCR5 receptor.

[a] *, Antiretroviral agents that may have a role in treating some patients but are used infrequently or no longer recommended for routine use.

through the phosphorolytic removal of NRTIs incorporated into the HIV-1 primer chain (reviewed in reference 44). Primer unblocking mutations, because they are selected by the thymidine analog inhibitors zidovudine and stavudine, are also referred to as thymidine analog mutations or "TAMs." The most common TAMs include M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E. A subset of these mutations—M41L, L210W, and T215Y—is associated with greater cross-resistance to didanosine, abacavir, and tenofovir (45–47).

Lamivudine and emtricitabine have a low genetic barrier to resistance in that a single mutation—the discriminatory mutation M184V (or less commonly M184I)—confers >200-fold reduced susceptibility to these drugs. Indeed, M184V is the most common mutation to emerge in patients developing virological failure on a first-line regimen. Although M184V limits the antiviral activity of lamivudine and emtricitabine, they retain efficacy because M184V reduces HIV-1 fitness and increases susceptibility to zidovudine, stavudine, and tenofovir (42).

In patients receiving regimens without thymidine analogs, K65R, L74V/I, and Y115F are the mutations that occur most commonly in combination with M184V (48). K65R reduces susceptibility to tenofovir, abacavir, didanosine, stavudine, lamivudine and emtricitabine and increases susceptibility to zidovudine (49, 50). L74V/I reduces abacavir and didanosine susceptibility. Y115F reduces abacavir and tenofovir susceptibility. K65N and K70E/G/Q are uncommon discriminatory mutations that reduce susceptibility to tenofovir, stavudine, abacavir, and didanosine.

T69SSS and Q151M are multi-NRTI resistance mutations. T69SSS is a double amino insertion at HIV-1 RT position 69, which usually occurs with multiple TAMs, and in this setting it causes intermediate resistance to lamivudine and emtricitabine and high-level resistance to the remaining NRTIs (51). Q151M usually occurs in combination with several otherwise uncommon mutations (A62V, V75I, F77L, and F116Y). It causes intermediate resistance to tenofovir, lamivudine, and emtricitabine and high-level resistance to the remaining NRTIs (52, 53). Amino acid deletions between codons 67 and 69 are also associated with multi-NRTI resistance. Deletions at codon 67 generally occur with T69G and multiple TAMs or Q151M (54). Deletions at codon 69 generally occur with K65R or Q151M (54).

NNRTI Resistance

The NNRTIs inhibit HIV-1 RT allosterically by binding to a hydrophobic pocket about 10 Å from its active site (43). This hydrophobic NNRTI-binding pocket is less well conserved than the enzyme's dNTP-binding active site. Indeed, HIV-2 and HIV-1 group O are intrinsically resistant to most NNRTIs (55) and HIV-1 group M viruses have greater inter-isolate variability in their susceptibility to NNRTIs than to NRTIs (56).

Resistance emerges rapidly when NNRTIs are administered as monotherapy, suggesting that NNRTI resistance can be caused by the selection of preexisting low-abundance populations of mutant viruses. For example, a single dose of nevirapine used to prevent mother-to-child HIV transmission often selects for NNRTI-resistant mutants that can be detected by standard sequencing for several months and can interfere with the subsequent response to an NNRTI-containing treatment regimen (57, 58).

Nevirapine and efavirenz have low genetic barriers to resistance in that a single mutation in the NNRTI binding pocket may result in high-level resistance. Etravirine has a higher genetic barrier to resistance in that two or more mutations are usually required to cause high-level resistance (59). Etravirine's higher genetic barrier to resistance is a result of its ability to rearrange itself, thereby adopting multiple binding modes within the NNRTI binding pocket (60). Although rilpivirine has a similar structure to etravirine, its genetic barrier to clinically significant resistance is lower than that of etravirine because it is administered at a much lower dose.

The most common NNRTI-resistance mutations are L100I, K101E/P, K103N/S, V106A/M, Y181C/I/V, Y188C/H/L, G190A/S/E, and M230L. With the exception of L100I, these mutations cause high-level resistance to nevirapine (61). With the exception of Y181C/I/V, they cause intermediate- or high-level resistance to efavirenz (61). L100I, K101E/P, Y181C/I/V, Y188L, and 230L cause resistance to rilpivirine and etravirine (61). In addition, E138K, a previously unusual mutation that reduces rilpivirine susceptibility by 3-fold, is the most common mutation to emerge in patients with virological failure on a first-line rilpivirine-containing regimen (62).

PI Resistance

There are nine FDA-approved PIs: atazanavir, darunavir, fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, and tipranavir. Ritonavir is now administered solely at subtherapeutic doses to increase or "boost" the tissue concentrations of other PIs via its inhibition of the cytochrome P4503A pathway. Nelfinavir and indinavir, once commonly used PIs, are rarely used because of their poor pharmacokinetic properties when administered alone and their toxicity when administered with ritonavir. Of the remaining six PIs, lopinavir (which is coformulated with ritonavir) and ritonavir-boosted atazanavir and darunavir are used most frequently.

More than 80 nonpolymorphic PI-selected mutations have been reported (63). Most of these reduce susceptibility to one or more PIs (64). Mutations in the substrate cleft, including D30N, V32I, I47V/A, G48V/M, I50V/L, V82A/T/L/F/S, and I84V, reduce PI-binding affinity. Several mutations in the enzyme flap, including M46I/L and I54V/M/L/T/S/A, and the enzyme core, including L33F, L76V, and N88S, also markedly reduce susceptibility. These and other accessory mutations indirectly reshape the substrate cleft or compensate for the decreased kinetics of enzymes with substrate cleft mutations (65, 66). Compensatory mutations at several Gag cleavage sites are also selected during PI treatment (67).

Ritonavir-boosted lopinavir and darunavir have high genetic barriers to resistance, with multiple mutations required before antiviral activity is compromised (68, 69). Lopinavir/r alone and darunavir/r alone are each effective at fully suppressing HIV-1 RNA level below detectable levels for 48 weeks in most patients, and monotherapy with these PIs is a highly effective simplification regimen (70). Viruses from patients with virological failure on an initial boosted-PI-containing regimen rarely contain PI-resistance mutations, suggesting that such virologic failure may often result from nonadherence rather than PI resistance. Indeed, a high proportion of such patients often experience resuppression of virus levels without a change in therapy.

INI Resistance

Following reverse transcription and the generation of double-stranded viral DNA, HIV-1 integrase catalyzes the cleavage of the conserved 3′ dinucleotide CA (3′ processing) and the ligation of the viral 3′-OH ends to the 5′ DNA of host chromosomal DNA (strand transfer). HIV-1 IN is composed of three functional domains: the N-terminal domain (NTD), encompassing amino acids 1 to 50; the catalytic core domain (CCD), which encompasses amino acids 51 to 212 and contains the catalytic triad D64, D116, and E152; and the C-terminal domain (CTD), which encompasses amino acids 213 to 288 and binds host DNA nonspecifically. IN strand transfer inhibitors bind the CCD active site and chelate the divalent metal ions critical for enzymatic function. Raltegravir was licensed in 2008. Elvitegravir was licensed in 2012 as a fixed-dose combination with the CYP3A4 inhibitor cobicistat and the NRTIs tenofovir and emtricitabine. Dolutegravir was licensed in 2013.

There are crystal structures of the CCD plus CTD domains, the CCD plus NTD domains, and the CCD bound to the prototype diketo acid inhibitor 5CITEP (71–73). But the relative conformation of the CCD, NTD, and CTD domains and the tetrameric functional form of HIV-1 IN have been inferred primarily from crystallographic studies of the homologous IN of the prototype foamy virus (74).

The mutations with the greatest effect on reducing INI susceptibility are highly conserved residues in the INI binding site and residues 140 to 149, which form a flexible loop important for catalysis following IN-DNA binding. Q148 interacts with a 5′-terminal cytosine of viral DNA and likely forms a hydrogen bond with most INIs (73, 75). N155 points into the active site and forms a hydrogen bond with D116, one of the three catalytic aspartate residues (76). Y143 is part of the highly flexible active-site loop that participates in DNA and INI binding.

There are three main overlapping genetic pathways to raltegravir resistance: (i) Q148H/R/K ± G140S/A/C ± E138A/K; (ii) N155H ± E92Q; and (iii) Y143C/R ± T97A (77–79). Elvitegravir resistance is caused primarily by the first two mutational pathways and by T66A/I/K and S147G (80). Dolutegravir has a high genetic barrier to resistance. Clinically significant phenotypic resistance to dolutegravir appears to require Q148H/R/K + G140S/A/C plus one or more additional accessory resistance mutations (81, 82). There are also several rare mutations associated with marked reductions in elvitegravir or raltegravir susceptibility, several common accessory INI-resistance mutations, and several mutations of uncertain significance selected by dolutegravir *in vitro* (reviewed in references 79 and 83).

Fusion Inhibitor Resistance

The HIV-1 envelope consists of surface (gp120) and transmembrane (gp41) glycoproteins. gp120 binds to the CD4 receptor and to one of the chemokine coreceptors (CCR5 or CXCR4) on target cells. After gp120-CD4-coreceptor binding, gp41 undergoes a conformational change that promotes fusion of viral and cellular membranes. Two heptad repeat regions (HR1 and HR2) of gp41 form a helical bundle that contains trimers belonging to HR1 and HR2. Enfuvirtide is a highly active synthetic peptide that inhibits fusion by binding to HR1 and preventing its bundling with HR2.

Enfuvirtide-resistant isolates contain either single or double mutations between positions 36 and 45 of gp41 HR1 (84, 85). Single mutants typically have on the order of 10-fold decreased enfuvirtide susceptibility, whereas double mutants typically have on the order of 100-fold decreased susceptibility. Despite the fact that enfuvirtide is one of the most potent inhibitors, the genetic barrier to enfuvirtide resistance is low, with virological rebound emerging rapidly when this drug is not administered with a sufficient number of other active inhibitors (86).

CCR5 Inhibitor Resistance

Maraviroc allosterically inhibits gp120 *env* of CCR5-tropic HIV-1 strains from binding to the seven-transmembrane G protein-coupled CCR5 receptor (87). Whereas HIV-1 gp120 binds to the N terminus and second extracellular loop region of CCR5, maraviroc binds to a pocket formed by the transmembrane helices (87). In patients receiving CCR5 inhibitors, the most common mechanism of virological failure is the expansion of preexisting CXCR4-tropic viruses that are intrinsically resistant to CCR5 inhibitors (88). Positively charged residues at positions 11 and 25 of the V3 loop of gp120 and several less common combinations of mutations primarily but not exclusively within the V3 loop are associated with CXCR4 tropism (89).

During *in vitro* passage experiments CCR5 inhibitor resistance develops by a different mechanism—gp120 mutations that enable HIV-1 to bind to the CCR5/CCR5-inhibitor complex, resulting in a plateau in the maximal percent inhibition at maximal CCR5 inhibitor concentrations (90). However, this mechanism of resistance occurs rarely in patients and it is not associated with a consistent pattern of responsible mutations.

Inter-Subtype Variation

During its spread among humans, group M HIV-1 has evolved into multiple subtypes that differ from one another by 10 to 30% along their genomes (91). HIV-1 proteases and RTs of different subtypes differ by from 10 to 12% of their nucleotides and 5 to 6% of their amino acids. Naturally occurring polymorphisms differ among subtypes, but these polymorphisms are not responsible for clinically significant effects on drug susceptibility (56, 92).

Most data on the genetic mechanisms of HIV-1 drug resistance were based on observations of subtype B viruses. Although the drug-resistance mutations observed in subtype B isolates are the most common ones observed in non-B subtypes (93), many studies have reported differences in the frequency with which specific mutations occur in viruses belonging to different subtypes subjected to the same antiretroviral selection pressures (reviewed in reference 48).

HEPATITIS B VIRUS

Hepatitis B virus (HBV) is a partially double-stranded DNA virus of about 3.2 kb. Following infection, its genome localizes to the nucleus, where it is converted to the covalently closed circular DNA (cccDNA) form that serves as template for transcription of mRNA and genomic RNA. Genomic RNA is then reverse transcribed to viral DNA, and the resulting viral cores either bud into the endoplasmic reticulum and are exported from the cell or return to the nucleus for conversion back to cccDNA. HBV cccDNA can be eliminated by cell turnover but not by drug therapy; therefore, eradication of infection occurs infrequently (94, 95).

HBV replicates at a high rate, producing about 10^{11} virions per day (96). In the absence of therapy, HBV DNA levels are often as high as 10^8 to 10^{10} copies/ml. HBV polymerase, because it has RT activity, is functionally and structurally similar to HIV-1 RT and has an error rate similar to that of other retroviruses (97). However, the overlapping arrangement of open reading frames in the HBV genome limits the viability of many spontaneous mutants (98). For example, the nucleotides encoding the HBV polymerase also encode the HBV envelope in a different reading frame. Therefore, the rate at which mutations become fixed is considerably lower than for HIV-1 (99).

HBV is classified into at least eight genotypes differing from one another by about 10% of their nucleotides. Several small studies suggest that viruses belonging to different genotypes may respond differently to interferon, but there is no evidence that genotype influences the response to nucleoside analogs (100, 101). In 2001, a standardized numbering system for polymerase mutations was established for the RT part of the HBV *pol* gene (102).

Anti-HBV Drug Therapy

There are two forms of interferon and five nucleoside analogs approved for the treatment of chronic HBV infection (Table 3). Alpha interferon was approved in 1992 and pegylated alpha interferon 2a was approved in 2005. Pegylated interferon remains an important treatment option because 1 year of therapy offers the possibility of sustained virological remission (103). Lamivudine (1998), adefovir (2002), entecavir (2005), telbivudine (2006), and tenofovir (2008) have also been approved. The antiretroviral drug emtricitabine, which is similar to lamivudine in its chemical structure and antiviral activity, is also used for HBV treatment because it is coformulated with tenofovir for treating HIV-1.

Both tenofovir and entecavir monotherapies are recommended regimens for initial HBV treatment because they are potent inhibitors and the emergence of drug resistance is exceedingly uncommon in patients receiving either one for initial therapy (103–105). Once the mainstay of NRTI therapy, lamivudine monotherapy is no longer recommended because of the high incidence of drug resistance associated with its use: 15 to 30% of patients treated for 1 year, 40 to 50% treated for 3 years, and 70% treated for 5 years (106). Telbivudine is associated with a 25% incidence of drug resistance at 2 years (107). Adefovir resistance occurs in about 10 and 30% of patients after 2 and 5 years, respectively (108).

HBV RT and NRTI Resistance

Homology modeling suggests that HBV polymerase shares regions similar to the fingers, palm, and thumb configuration of HIV-1 (109, 110). High-level (>1,000-fold) lamivudine resistance is caused by a mutation, M204V/I, which is in the YMDD motif, characteristic of all RTs, and which is analogous to M184V/I, the HIV-1 lamivudine-resistance mutation (111, 112). M204V/I mutations are also frequently accompanied by compensatory mutations, particularly L180M and less commonly V173L and L80I. M204V/I causes high-level cross-resistance to emtricitabine and telbivudine but does not reduce susceptibility to the acyclic nucleotides adefovir and tenofovir (111, 112). Although viruses with M204V/I replicate less well than wild-type viruses, the development of M204V/I is associated with virological and clinical progression (113, 114).

Adefovir and tenofovir retain complete *in vitro* susceptibility to viruses with M204V/I and its associated mutations (115, 116). However, because tenofovir is more potent than adefovir, it is recommended for treating patients with lamivudine resistance (111). Adefovir resistance is caused by the mutations N236T and A181V/T (117–119). Although the levels of reduced susceptibility associated with these mutations—3- to 10-fold—are much lower than the levels of M204V/I-associated lamivudine resistance, these reductions are associated with virologic breakthrough (117). N236T confers intermediate cross-resistance to tenofovir but not to lamivudine, entecavir, or telbivudine (120, 121).

A181V and A181T emerge with lamivudine and, more commonly, adefovir (118, 122, 123). Isolates with these mutations remain susceptible to entecavir and have minimally reduced tenofovir susceptibility. A181T causes a stop codon in the S protein reading frame, potentially allowing for ongoing hepatocellular replication without accompanying viral load rebound (124).

Although entecavir retains considerable antiviral activity against lamivudine-resistant variants, the risk of virological failure and emergence of high-level entecavir resistance is higher when entecavir is used to treat patients with lamivudine-resistance mutations than patients who have not been treated with NRTIs (125–127). High-level etravirine resistance results from the combination of M204V/I plus L180I and one or more of the following mutations: I169T, T184G, S202I, and M250V (111, 121, 128).

The adefovir-selected mutations N236T and A181VT confer partial cross-resistance to tenofovir (111, 121). Although tenofovir often maintains considerable antiviral activity in patients developing virological failure with adefovir, the risk of virological failure is increased in patients harboring adefovir-resistance mutations (129). In such patients, entecavir or tenofovir plus a lamivudine or emtricitabine are recommended (121).

There are anecdotal reports linking I233V to adefovir therapy and reduced adefovir susceptibility (130) and A194T to tenofovir therapy and reduced tenofovir susceptibility (131). However, neither of these mutations appears to be consistently selected by adefovir or tenofovir or to reduce their susceptibility in laboratory clones (132, 133).

HEPATITIS C VIRUS

Hepatitis C virus (HCV) is a positive-sense, single-stranded enveloped virus with a genome of about 9.5 kb. The genome

TABLE 3 Genetic mechanisms of resistance to inhibitors of hepatitis B virus (HBV)

Drug class	Mechanism(s)	Mutations
Interferon	Unknown	Unknown
Nucleoside/nucleotide analogs (NRTIs): L-Nucleosides: Lamivudine Telbivudine Emtricitabine[a] Deoxyguanosine analog: Entecavir Acyclic nucleotides: Adefovir Tenofovir	Mutations that allow RT to discriminate between NRTIs and naturally occurring nucleoside triphosphates. Whether some of these mutations also cause primer unblocking is not known.	M204V/I ± L180M, V173L, L80I: lamivudine, emtricitabine, and telbivudine; partial cross-resistance to entecavir M204VI + L180M ± I169T, T184G, S202I, M250V: entecavir A181V/T: adefovir, lamivudine; partial cross-resistance to tenofovir N236T: adefovir, partial cross-resistance to tenofovir

[a] Emtricitabine (FTC) is not FDA approved for the treatment of HBV. Its activity and cross-resistance profile are similar to 3TC. It is frequently used in combination with tenofovir to treat HIV-coinfected patients.

encodes a single 9.0-kb open reading frame flanked by conserved 5′- and 3′-untranslated regions. The 5′-untranslated region contains the internal ribosomal entry site (IRES), which is necessary for initiating translation. Viral replication occurs in a membrane-associated cytoplasmic replicase complex consisting of the nonstructural proteins NS3 protease, NS4A protease cofactor, NS5B RNA-dependent RNA polymerase (RdRp), and two proteins involved in maintaining the replicase complex: NS4B and NS5A (134). The resulting duplex RNA serves as a template for the synthesis of multiple copies of the positive-strand genome for protein production and packaging.

HCV plasma levels typically range from $10^{4.5}$ to $10^{6.5}$ IU/ml, where 1 IU is about one to five RNA copies depending on the assay used for quantification. HCV has an estimated half-life of about 3 hours and, in the absence of antiviral therapy, up to 10^{12} virions are produced daily (135). Like other RdRp, HCV polymerase has a high error rate, estimated at 10^{-4} substitutions per nucleotide per round of replication (136).

There are seven human HCV genotypes differing from one another by more than 30% of their genomes (137). Many of the genotypes have been subdivided into different subtypes that differ from one another by more than 15% of their genomes (137). HCV exists *in vivo* as an ensemble of viral genomes differing from one another by up to 5 to 10% (138).

HCV persists in up to 70% of untreated infected persons. The lifelong persistence of HCV in the majority of infected persons in the absence of treatment demonstrates its ability to evade the innate and adaptive immune system of its host (139). The absence of a stable intracellular reservoir, however, makes viral eradication possible. Indeed, the absence of detectable plasma virus at 6 months following a treatment regimen constitutes a sustained virological response and usually a virological cure.

Anti-HCV Therapy

Prior to 2011, pegylated interferon plus ribavirin for 6 to 12 months was the only recommended treatment for hepatitis C. This regimen cured about 45% of patients with genotype 1 infections and about 75% of viruses with genotype 2 and 3 infections. In 2011, the PIs telaprevir and boceprevir became the first FDA-approved directly acting agents (DAAs) approved for use in combination with interferon plus ribavirin for treating genotype 1 HCV. Combinations of these PIs with interferon and ribavirin increased the cure rate for genotype 1 to about 70% but were associated with toxicity and a high frequency of PI resistance. In 2013, the PI simeprevir was approved for use in combination with pegylated interferon plus ribavirin for most genotype 1 viruses (140).

In 2013, the nucleotide analog sofosbuvir was approved for use in combination with either ribavirin alone or ribavirin plus pegylated interferon for genotype 1, 2, 3, and 4 viruses (141). Many other DAAs are in phase II and III clinical trials, including PIs, nucleoside/nucleotide (NIs) and nonnucleoside RdRp inhibitors (NNIs), and NS5A inhibitors (Table 4). Proof-of-concept phase II trials have demonstrated that agents targeting host factors required for HCV replication, such as microRNA-122 and cyclophilin A, markedly reduce plasma HCV levels (142, 143). Several interferon-free combinations have demonstrated higher cure rates than those obtained with the currently recommended telaprevir- and boceprevir-based regimens (144–147).

The NIs are usually active against all HCV genotypes and have high genetic barriers to resistance. The PIs, NS5A inhibitors, and particularly NNIs vary in their activity against viruses of different genotype and subtype and have lower genetic barriers to resistance (148). Most initial drug development efforts and clinical trials have targeted genotype 1 viruses. However, telaprevir is also active against genotype 2 viruses (149), and simeprevir is also active against genotype 2 and 4 viruses (150). As more data become available on the frequency of pretherapy drug-resistance mutations in different genotypes and subtypes, genotypic resistance testing will likely also be used to help select DAAs for interferon-free combination regimens.

The development of HCV replication and cell culture assays for different HCV genotypes has been essential for discovering DAAs, quantifying their inhibitory activity, and selecting for drug-resistance mutations and assessing their susceptibility (reviewed in reference 151). Subgenomic replicons have been used to assess the susceptibility of pre- and posttherapy samples by creating recombinant viruses containing patient-derived gene targets (152).

Interferon and Ribavirin

Interferon plus ribavirin remains the backbone of therapy for use in combination with PIs. However, with the approval of the NI sofosbuvir and the presence of many more DAAs in development, most patients will be treated with interferon-free regimens. Ribavirin will likely remain an important component of many regimens because it is active against viruses of multiple genotypes, it does not select for resistance, and it is less expensive than DAAs. Ribavirin interferes with dNTP metabolism by inhibiting cellular inosine monophosphate dehydrogenase and may also directly inhibit HCV RNA polymerase or increase HCV mutagenesis. However, no genetic mechanism for clinically relevant ribavirin resistance has been identified (153, 154).

Protease Inhibitors

The NS3 serine protease comprises the 189 N-terminal amino acids of NS3. NS3 protease forms a heterodimer with the 54-amino-acid NS4A, which together function to cleave four sites in the HCV polypeptide precursor to generate the N termini of NS4A, NS4B, NS5A, and NS5B. HCV protease also appears to take part in host immune evasion by cleaving several critical intracellular immune mediators (reviewed in reference 139). Multiple structures of protease with and without inhibitors have been solved by X-ray crystallography (134, 155). HCV protease contains an H57, D81, and S139 catalytic triad typical of other members of the trypsin family of serine proteases. The substrate-binding pocket contains a large number of residues including F56, V78, D79, Q80, V132, I/L135, K136, F154, K155, A157, C159, and A164 (156). HCV protease is a challenging drug target because it has a shallow substrate-binding pocket, which binds a long peptide substrate with which it forms multiple weak interactions.

Telaprevir and boceprevir are linear peptidomimetics, which bind covalently but reversibly to the protease substrate cleft (134). The newer PIs, including simeprevir (157), are noncovalent binding linear or macrocyclic peptidomimetics. Although the newer PIs have improved pharmacokinetic and toxicity profiles compared with telaprevir and boceprevir, they have overlapping resistance profiles and low genetic barriers to resistance (158, 159).

Most patients with virological failure on a PI-containing regimen develop antiviral resistance (158). The most commonly selected telaprevir- and boceprevir-resistance mutations include V36A/M and T54A/S, which cause low-level resistance, and R155K/T and A156S/T/V, which cause

TABLE 4 Genetic mechanisms of resistance to hepatitis C virus inhibitors

Drug class[a]	Mechanism(s)	Mutations
Interferon	Unknown	Genotype 1 isolates respond less well than genotype 2 or 3 isolates but the molecular basis for this is not known.
Ribavirin	Interferes with dNTP metabolism, inhibits HCV RNA polymerase, and/or increases viral mutagenesis	Uncertain
Protease inhibitors (PIs): Telaprevir Boceprevir Simeprevir Faldaprevir* Vaniprevir* Asunaprevir* Danoprevir* ABT-330*	Mutations in the NS3 protease substrate binding cleft, particularly the P2 and P3 pockets	Telaprevir: V36A/M/L/C, T54A/S, R155K/T, A156S/V/T Boceprevir: V36A/M/L/C, T54A/S, R155K/T, A156S/V/T, D170A Simeprevir: Q80K, R155K/T, A156S/V/T, D168A/V Additional substitutions at the positions listed above
Nucleoside inhibitors (NIs): Sofosbuvir Mericitabine*	Mutations near the active site that inhibit nucleoside analog incorporation	Sofosbuvir: S282T + compensatory NS5B mutations are selected *in vitro* and associated with ~10-fold reduced susceptibility. S282T was detected transiently following sofosbuvir relapse in one genotype 2 virus. L159F is a nonpolymorphic mutation detected in six sofosbuvir relapse subjects with genotype 3a viruses; it is associated with 2-fold reduced sofosbuvir susceptibility in genotype 1a and 1b replicons. Mericitabine: S282T + compensatory NS5B mutations are selected *in vitro* and associated with ~10-fold reduced susceptibility. L159F + L320F, which are associated with reduced mericitabine and sofosbuvir susceptibility and several inconsistent patterns of NS5B mutations, have been observed in patients receiving mericitabine.
NS5A inhibitors: Daclatasvir* Ledipasvir*{L}	Unknown	Daclatasvir and ledipasvir: M28T, Q30E/H/R, L31M/F/V, P32L, Y93C/H/N. The phenotypic effect of these mutations is greater in genotype 1a than 1b viruses. Q30 and Y93H mutations each occur in about 10% of DAA-naive patients with genotype 3 viruses.
Nonnucleoside inhibitors (NNIs) *	NS5B mutations that prevent binding to the four NNI binding domains	Thumb 1 domain:P495S/A/L, P496S/A, V499A Thumb 2 domain: L419V/M, M423T, I482L/V/T, V494A/I Palm 1 domain: H95R, C316Y, M414T, C451R, G554D, G558R Palm 2 domain: L314, C316N/Y, S365T, M414T, Y144H/C

[a] *, DAAs in phase III clinical trials. Telaprevir, boceprevir, and simeprevir are FDA approved for treating genotype 1 HCV. Sofosbuvir is FDA approved for treating genotype 1, 2, 3, and 4 HCV. However, the use of these drugs and other investigational drugs for treating HCV of different genotypes and subtypes may change as more drugs and data become available.

high-level resistance (160, 161). The macrocyclic PIs—including simeprevir and the investigational drugs ABT-450, asunaprevir, danoprevir, and vaniprevir—and the linear PI faldaprevir select for the mutations R155K/T, A156V/T, and D168A/V/T/H (159, 162). R155K occurs most commonly in genotype 1a viruses because this substitution requires a 1-base-pair mutation (AGG → AAG) in genotype 1a viruses and a 2-base-pair mutation (CGG → AAG) in genotype 1b viruses (158, 160). Most emergent PI-resistance mutations reduce viral fitness and are gradually replaced by wild-type variants in the months following treatment discontinuation in patients with PI resistance (163).

Q80K, which reduces simeprevir susceptibility about 10-fold, is a common natural polymorphism in genotype 1a viruses. Viruses with Q80K respond less well to the combination of simeprevir plus interferon/ribavirin than viruses without Q80K (140, 164). Genotypic testing is recommended to exclude the presence of this mutation in patients with genotype 1a viruses treated with simeprevir (140).

NI and NNI RdRp Inhibitors

HCV RdRp is encoded by the 530 N-terminal amino acids of the NS5B gene. A C-terminal extension of NS5B anchors the catalytic domain to the endoplasmic reticulum as part of a larger viral replication complex. HCV RdRp, like other polymerases, contains palm, thumb, and finger subdomains that enclose the RNA template groove and GDD catalytic triad (165, 166). HCV RdRp inhibitors include chain-terminating nucleoside and nucleotide analogs (NIs) and non-nucleoside analogs (NNIs) that target NS5B allosterically. Most HCV NIs differ from HIV-1 and HBV NRTIs in that chain termination is caused largely by steric hindrance rather than the absence of the 3′-hydroxyl group (167).

The active site mutation S282T is the main NI resistance mutation selected *in vitro* by sofosbuvir, mericitabine, and other 2′-modified NIs (167–170). S282T, which usually requires additional NS5B mutations to compensate for its markedly reduced replication, is associated with about 10-fold reduced susceptibility to sofosbuvir and mericitabine (167–169, 171). S282T has been detected transiently in one patient who relapsed 4 weeks after receiving sofosbuvir monotherapy (141).

L159F is a nonpolymorphic mutation which has been detected in six sofosbuvir-relapse subjects with genotype 3a viruses (141) and in one patient receiving mericitabine (169). It reduces sofosbuvir susceptibility by about 2-fold (169). Several additional NS5B mutations at conserved positions have been reported to emerge *in vitro* and in patients receiving an NI. However, these mutations have not been observed consistently or found to alone reduce NI susceptibility (168, 169).

NNIs are chemically diverse compounds belonging to four classes—thumb 1, thumb 2, palm 1, palm 2—based on their allosteric binding sites and drug-resistance mutations (134, 166, 172, 173). NNIs have a low genetic barrier to resistance, and their activity is largely restricted to genotype 1a or 1b viruses (172). Mutations associated with resistance to each of the four allosteric sites have been selected *in vitro* and in patients (158, 173–175). The two palm NNI-binding sites partially overlap and share several drug-resistance mutations. Several NNI-resistance mutations have been reported to occur in previously untreated individuals as dominant variants detected by standard sequencing and as minor variants detected by more sensitive methods (176, 177).

NS5A Inhibitors

NS5A is an ~450-amino-acid membrane-associated phosphoprotein that is an essential part of the HCV replicase complex and an antagonist of endogenous interferon. The structure of the N-terminal domain of NS5A has been crystallized, but how this protein functions is not known (134). Daclatasvir and the subsequently identified NS5A inhibitors emerged from high-throughput cell-based screens targeting nonenzymatic HCV targets (178). Daclatasvir has an EC_{50} ranging from the low picomolar range in genotype 1a and 1b replicons to the low nanomolar range for genotypes 2 through 6 (179). Rapid declines in plasma HCV levels have been reported for patients infected with genotype 1a, 1b, 3a, and 4a viruses. Daclatasvir, and at least two other NS5A inhibitors—ledipasvir and ABT-267—are in phase III trials as key components of highly effective interferon-free DAA combinations (146, 180).

The most commonly occurring daclatasvir-resistance mutations are Q30E/H/R, L31M/V, and Y93H/C/N. Each of these mutations reduces the susceptibility of both daclatasvir and ledipasvir to genotype 1a viruses by more than 100-fold. Genotype 3 viruses may be less susceptible to current NS5A inhibitors because of the natural occurrence of Y93H in 8% of patients and of several Q30 variants in 10% of patients (181).

INFLUENZA VIRUSES

Influenza A and B viruses have segmented minus-strand RNA genomes, each associated with a polymerase complex. The RNA particles are located inside an M1 protein shell that lines the viral lipid membrane. Embedded in the membrane of influenza A and B viruses are two spiked glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and a channel protein.

Sixteen HA and nine NA influenza A antigenic subtypes have been reported in avian species. A subset of these has infected humans. There are two pandemic strains: A(H3N2), which became prevalent in 1968, and A(H1N1)pdm09, which emerged in 2009 (182) and soon replaced the previously circulating seasonal H1N1 strain. There are also two highly pathogenic avian influenza species responsible for human infections: A(H5N1), which has been widely disseminated in water fowl since at least 1997 and has infected persons in China, Southeast Asia, the Middle East, Africa, and Europe (183, 184), and A(H7N9), which has infected more than 142 persons in China since March 2013 (184).

There are two classes of licensed anti-influenza drugs in the U.S.: M2 channel blockers (the adamantane derivatives amantadine and rimantadine) and NA inhibitors (zanamivir and oseltamivir) (Table 5). M2 channel blockers are currently inactive against circulating influenza A viruses and are intrinsically inactive against influenza B viruses (185). NA inhibitors are intrinsically active against most influenza A and B viruses. Zanamivir, which is approved for inhaled use, is also available as an investigational intravenous preparation for emergency use (186). Peramivir is an intravenous NA inhibitor that was available through an Emergency Use Authorization in 2009 during the emergence of A(H1N1)pdm09 (187).

M2 Channel Blockers

M2 is a tetrameric, pH-activated, proton-selective channel that plays a role in virus uncoating (188). Passage of hydrogen ions through the M2 channel into the virion following endocytosis promotes M1 dissociation from the ribonucleoprotein complexes. Amantadine and rimantadine interfere with the penetration of hydrogen ions through the M2 channel, thereby preventing transport of the ribonucleoprotein complex to the nucleus (189).

Cross-resistance to both amantadine and rimantadine results from single amino acid substitutions at position 26, 27, 30, 31 or 34 within the M2 transmembrane domain (190). Most mutations, such as those at positions 30 and 31, inhibit M2 channel binding, whereas others, such as those at position 27, may allow binding without affecting channel function (191). Adamantane resistance develops in about 30% of persons receiving amantadine or rimantadine (192, 193). Amantadine- and rimantadine-resistant variants maintain normal infectivity and virulence in animal models and cause typical disease in humans (190, 192).

Sharp increases in the prevalence of adamantane resistance began in 2002, caused almost entirely by the S31N

TABLE 5 Genetic mechanisms of resistance to influenza virus inhibitors

Drug class	Mechanism(s)	Mutations
M2 channel blockers: Amantadine Rimantadine	M2 transmembrane mutations that block hydrogen ion transfer	Mutations at residues 27, 30, 31, and 34, particularly S31N Influenza B and most circulating lineages of A(H3N2), A(H1N1)pdm09, and the pathogenic avian influenza strains A(H5N1) and A(H7N9) are resistant as a result of S31N.
Neuraminidase (NA) inhibitors (NAI): Oseltamivir Zanamivir	NA mutations in the receptor binding site associated with decreased inhibitor binding	A(H1N1) and A(H1N1)pdm09: oseltamivir: H274Y > Y155H, I222M/R/K, S246N/G, S294S; zanamivir: Q136K, Y155H, I222R A(H3N2): oseltamivir: E119V/I, R292K, N294S; zanamivir: E119I, R292K A(H5N1): oseltamivir: H274Y A(H7N9): oseltamivir: R292K; zanamivir: R292K B: oseltamivir: R152K, R371K > D198N/E, I222T; zanamivir: R152K > D198N/E, I222T, R371K

mutation. By the 2005–2006 season, 92% of H3N2 strains changes in the U.S. were adamantane resistant (194). Although most seasonal H1N1 strains prior to 2009 were susceptible to the adamantanes, A(H1N1)pdm09 and most of the circulating pathogenic avian influenza strains (H5N1 and H7N9) are adamantane resistant.

Neuraminidase Inhibitors (NAIs)

HA binds to sialic (*N*-acetylneuraminic) acid-containing cellular receptors to initiate viral infection. NA cleaves alpha-ketosidic bonds that link terminal sialic acid residues to adjacent oligosaccharide moieties, thus preventing the formation of aggregates between HA and cell surface sialic acids and allowing newly formed viruses to be released from the surface of infected cells (195). NA may also promote viral spread within mucosal secretions in the respiratory tract. Because HA binds to cellular receptors to initiate infection, whereas NA destroys cellular receptors to allow virus release from cells, a balance between these two activities is required for efficient infection (196). Indeed, HA mutations appear to modulate the replication fitness of viruses containing NAI-resistance mutations (197).

The three-dimensional structure of NA from influenza B and from several influenza A subtypes has been solved by X-ray crystallography (195, 198, 199). NA is a homotetramer, containing monomers of about 470 amino acids depending on the type and subtype. NA has a hydrophobic stalk peptide at its N terminus, responsible for membrane anchoring. A globular head contains its active site—a pocket into which sialic acid and substrate analogs bind. The most commonly used numbering system is based on alignment to the influenza A N2 subtype.

Influenza NAs of different A subtypes differ from one another at about 50% of their amino acids, and influenza A differs from influenza B at about 70% of its amino acids. Nonetheless, the folded structure of the polypeptide brings into proximity a number of amino acids that are nearly invariant in all influenza strains. Eight of these strain-invariant amino acids directly contact sialic acid, including R118, D151, R152, R224, E276, R292, R371, and Y406. Ten provide the framework that supports these catalytic residues: E119, R156, W178, S179, D/N198, I222, E227, H274, N294, and E425 (200, 201).

Oseltamivir and zanamivir are sialic acid analogs that competitively inhibit NA. They are active against a wide range of human and avian strains including influenza B and each of the nine identified avian influenza A NA subtypes (185, 201, 202). Although they are intrinsically less active against influenza B than A, oseltamivir and zanamivir have nonetheless demonstrated clinical efficacy at treating influenza B at the doses used in clinical trials (185). Oseltamivir, which is administered orally, is a carbocyclic analog of sialic acid with a bulky side chain necessitating a conformation change in NA to allow binding (199). Because zanamivir is administered as an aerosol, and because its structure is more similar to the natural sialic acid than oseltamivir, zanamivir resistance occurs much less frequently than oseltamivir resistance (202).

The NA mutations responsible for NAI resistance depend on the influenza type (A versus B) and subtype (e.g., N1 versus N2) for influenza A. In addition, specific genetic lineages within an influenza A subtype may differ in their levels of NAI susceptibility and in their predisposition to specific NAI-resistance mutations. Therefore, ongoing genotypic and phenotypic surveillance is required to identify changes in the intrinsic susceptibility of circulating NA variants and the spectrum of mutations selected in patients receiving NAIs.

NAI susceptibility is monitored primarily by genotypic and enzymatic NA inhibition assays (197, 203). Genotypic assays are useful for clinical management and surveillance (197). Enzymatic assays are useful for surveillance and for characterizing novel NA variants. Cell culture assays are possible but must be interpreted cautiously because the cells used for culture have variable concentrations of glycoconjugate receptors and different influenza viruses vary in their dependence on NA activity (204, 205).

During the first few years of its use, oseltamivir resistance was reported to occur in about 1 to 4% of treated adults and in a higher proportion of treated children (206, 207). There was no evidence of naturally occurring oseltamivir resistance, and there were no reports of transmitted resistance (208). In the 2007–2008 season, a significant proportion of worldwide H1N1 infections were caused by a strain containing the oseltamivir-resistant framework mutation H274Y (209, 210). By the 2008–2009 season, >95% of seasonal H1N1 strains in the U.S. were oseltamivir resistant (209).

H274Y reduces oseltamivir susceptibility by several hundred- to several thousand-fold and causes high-level cross-resistance to peramivir but not zanamivir (208). Epidemiologic and molecular phylogenetic data suggest that the fixation of H274Y in H1N1 strains was not a result of selective drug pressure (211). Rather, H274Y evolved in the dominant circulating strain in seasonal 2007–2008 H1N1 viruses or specifically complemented other “permissive” mutations in that clade, possibly R222Q, V234M, and D343N (199, 212). Although *in vitro* cell culture and ferret model experiments showed that viruses with H274Y have greatly diminished replication (213), the H274Y-containing H1N1 viruses that emerged in 2007 retained their transmissibility and pathogenicity (214).

H274Y is the most frequently occurring NAI-resistance mutation in patients receiving oseltamivir (215) and has been reported in a widespread community cluster of A(H1N1)pdm09 in Australia in 2011 (216). Although H5N1 viruses are intrinsically susceptible to oseltamivir and zanamivir (217, 218), virological failure resulting from H274Y appears to occur at a higher frequency among patients receiving oseltamivir than in pre-2007 seasonal H1N1 and in A(H1N1)pdm09 strains (219).

Additional NAI-resistance mutations in A(H1N1) or A(H1N1)pdm09 include Q136K, Y155H, I222M/K/R/V, S246N/G, and N294S (202, 220, 221). I222 and S246 mutations appear to increase the replication fitness of viruses with H274Y and to cause low-level resistance (5- to 30-fold reduced susceptibility) to oseltamivir and zanamivir when they occur alone (220, 221). Q136K and Y155H are uncommon naturally occurring N1 mutations which reduce zanamivir (Q136K) and oseltamivir and zanamivir (Y155H) susceptibility >30-fold (205, 222). Q136K was present at low proportions in primary clinical specimens but arose during cell culture, raising questions as to its clinical significance (222, 223).

Among H3N2 viruses, the catalytic mutations R292K, E119I/V, and N294S have been reported in patients receiving oseltamivir (202, 207, 215, 224). E119I/V reduces oseltamivir susceptibility by more than 30-fold and causes low-level cross-resistance to zanamivir. R292K and N294S cause high-level oseltamivir resistance and lower levels of zanamivir cross-resistance. R292K has also been reported in two H7N9 viruses from patients receiving oseltamivir (225).

Among influenza B viruses, the most commonly reported NAI-resistance mutations include D198E/N and I222T, and R371K. D198N and I222T reduce oseltamivir and zanamivir susceptibility by less than 10-fold (185). R371K, which was identified in a 2004–2008 surveillance study, was associated with >100-fold reduced susceptibility to oseltamivir and about 30-fold reduced susceptibility to zanamivir (210). R152K, which causes high-level resistance to both oseltamivir and zanamivir, has been reported in an immunocompromised patient receiving prolonged therapy with inhaled zanamivir (226).

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Susceptibility Test Methods: Viruses*

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FDA-approved antiviral drugs are available and used for the treatment and management of herpes simplex virus types 1 and 2 (HSV-1, HSV-2), human cytomegalovirus (HCMV), varicella-zoster virus (VZV), human immunodeficiency virus type 1 (HIV-1) (1), hepatitis B virus (HBV), hepatitis C virus (HCV) (2, 3), and influenza virus. When the drugs are used over long periods of time and/or inconsistently, variants may be selected that may become “drug resistant” and are no longer susceptible to therapy (4–11). Since these variants are transmissible, learning if the virus may be drug resistant is a critical step in the treatment strategy (12).

Testing for viral susceptibility is now standard practice for the management of viral infections for optimum patient care. The only FDA-cleared assays available for viral susceptibility testing over the past decade target the HIV-1 protease (Pro) and reverse transcriptase (RT) genes. However, interest in testing for viral susceptibility has increased for other viruses, such as HBV, HCV, and HIV-1, for which there are newly approved antiviral drugs using research use only (RUO) test kits or analyte-specific reagents (ASRs) in laboratory-developed tests (LDTs) (13).

ANTIVIRAL RESISTANCE AND CAUSES OF DRUG FAILURE

Failure to respond to drug therapy can occur for different reasons. More commonly, the virus population being treated is inherently resistant to the drug(s) being given. Alternatively, host biological and sociobehavioral factors (e.g., therapeutic nonadherence) may influence therapeutic success. Viral susceptibility testing can identify resistant populations and guides patient management strategies.

True Antiviral Resistance

Antiviral resistance is expressed as a drop in the efficacy of a drug to inhibit viral replication. Resistance is demonstrated clinically by an increase or no change in amounts of circulating virus in the infected individual despite active treatment. The loss of susceptibility can be measured by *in vitro* testing, which evaluates the phenotypic activity of drug/virus combinations. If the decrease in drug effect is related to specific mutations in the virus genome, affecting function of the protein to which the drug is targeted, then the resistance to that drug may be an inherently transmissible feature of the virus. This genotypic and phenotypic expression provides combined evidence for the loss of drug activity against the virus and is documented during drug development. Independent confirmation of these correlations is made by antiviral testing of viral isolates obtained from participants in clinical drug trials. Diagnostic assays may then be developed that measure the change in response of the virus to a drug and/or identify the presence of the specific viral mutations associated with the observed loss of susceptibility. Interpretations of the data from these assays are used to clinically manage viral infections.

Host Factors

The failure to respond to therapy may occur even if the infecting virus does not harbor any mutations associated with antiviral susceptibility. Host physiologic factors can interfere with a successful clinical response. The genetic background of the host may lessen the effect of the drug where host polymorphisms, such as those identified for the IL28b gene, may affect the activity or bioavailability of pegylated-interferon (peg-IFN)/ribavirin in HCV treatment (14–16). Another nonviral factor is the potential for drug-drug interactions. For example, in early clinical trials of the newer protease inhibitors to treat chronic HCV, pharmacokinetic influences and drug-drug interactions occurred with some HIV-1 protease treatments in HIV/HCV-coinfected individuals (17). Coadministration of the HCV and HIV protease inhibitors could then cause the viral loads of either virus to increase (18). In this scenario, there were no identified mutations associated with antiviral resistance present in the viral genomes.

Patient Sociobehavioral Influence

Individuals undergoing drug treatment may not adhere to their prescribed antiviral regimen for a variety of reasons. For instance, physiologic and psychological side effects associated with antiretroviral drugs and IFN treatment may occur, fueling patient intolerance for the drug. The acute- and long-term symptoms, as well as complexities of the treatment such as pill burden and timing, may initiate problems with adherence (19). Consequently, the patient may choose to suspend treatment of his or her own accord. In addition, consistent access and follow-through of therapy may be difficult for some individuals due to socioeconomic concerns (20). In turn, this would result in treatment gaps which foster the selection or generation of an underlying

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genetically resistant viral population. In this type of patient, the class of drug to which the virus has become resistant is no longer effective for future use (21). Clinical trials and studies are now being conducted that study adherence outcomes (22). Such studies are likely to also include genotypic resistance testing.

CLINICAL INDICATIONS FOR ANTIVIRAL SUSCEPTIBILITY TESTING

It is important to distinguish when the lack of effective response to treatment with an antiviral drug results from true antiviral resistance, i.e., genetic changes in the patient's virus. Clinical resistance to a particular antiviral drug can be misinterpreted as viral resistance. The failure to recognize this difference could result in the inappropriate use of more toxic drugs, leading to higher morbidity and mortality with much higher test costs.

The clinical category and status of the patient are important in determining whether antiviral resistance testing is indicated. Table 1 presents instances for both immunocompetent and immunocompromised patients in whom resistance testing should be considered. In general, sustained or increasing viral load with a worsening clinical condition is a reliable indicator for the presence of emerging drug resistance. If these markers of resistance are present, it may be prudent to directly test the virus for drug resistance. Antiviral susceptibility test methods can be phenotypic or genotypic. The choice will often rely on the specific virus being tested. Interpretation of the testing data may be more complex for those individuals on combination therapy.

TESTING METHODS: PHENOTYPIC ASSAYS

Plaque Reduction Assay and Dye Uptake

The standard method of antiviral susceptibility testing is the plaque reduction assay or PRA as shown in Fig. 1. The PRA test principle relies on the ability of the antiviral agent to inhibit the production of viral plaques at a predetermined drug concentration. A 50% inhibition point (IC_{50}) is then calculated and subsequently reported in micrograms per milliliter or micromolar concentration. A higher IC_{50} of the sample virus compared to that of a wild-type control strain denotes resistance. Depending on the virus, this value may vary for the drug. This method is especially useful for the herpesviruses (e.g., HSV, CMV, and VZV) but is time-consuming, exhibiting an extended turnaround time of about 2 to 3 days for HSV, approximately 7 days for VZV, and about a month for CMV (23–26). There is also the question of method standardization; an approved standard document from the Clinical Laboratory and Standards Institute (CLSI) for HSV has been published (M33A). The use of a phenotypic method such as PRA is still recommended because of the presence of uncharacterized mutations among the herpes viruses that can contribute to antiviral resistance. Table 2 lists the proposed guidelines for antiviral susceptibility results for PRA of herpes group and influenza A viruses.

The dye uptake (DU) assay is based upon the ability of viable cells to incorporate the neutral red vital dye (26). It is used for antiviral susceptibility testing of HSV. An IC_{50} is calculated, similar to the PRA, but measured colorimetrically.

Enzyme Immunoassays

Enzyme immunoassays (EIAs) have also been developed for antiviral susceptibility testing (5–7, 27–29). These methods have been used successfully for both the herpesviruses and influenza type A virus. They compare favorably with the PRA method, but are less labor-intensive and allow for a quantitative measurement. This method measures absorbance as related to viral antigen detection in virus-infected cells and results in the determination of an IC_{50}.

Neuraminidase Inhibition Assay

Two neuraminidase inhibitors, oseltamivir and zanamivir, have been FDA approved for the treatment of both influenza type A and type B virus infection (30). A fluorogenic assay resulting in the generation of an IC_{50} was developed and

TABLE 1 Clinical situations where antiviral resistance testing may be indicated

Clinical category	Clinical status	Indicators of resistance
Immunocompetent host (e.g., influenza or genital HSV)	Culture or molecular testing evidence of viral infection and disease with clinical indications for short-term antiviral therapy	Sustained culture positivity with known therapeutic drug adherence Sustained or increasing viral load by quantitative molecular testing Worsening clinical condition without evidence of infection by other agents Epidemiological evidence of emerging antiviral resistance (e.g., oseltamivir resistance for influenza type A virus)
Immunocompromised host (e.g., immunosuppression due to cancer, transplantation, or AIDS)	Prolonged antiviral drug therapy or combination therapy used for prophylaxis or treatment	Increasing viremia or viral load while on long-term antiviral therapy or a history of failed antiviral therapy Unexplained worsening clinical disease condition Documented cross-resistance known from the use of specific antiviral drugs (e.g., HIV-1 antiretroviral therapy) Suboptimal viral load reductions achieved (e.g., lack of success with a change of HIV-1 therapy even with baseline retesting)

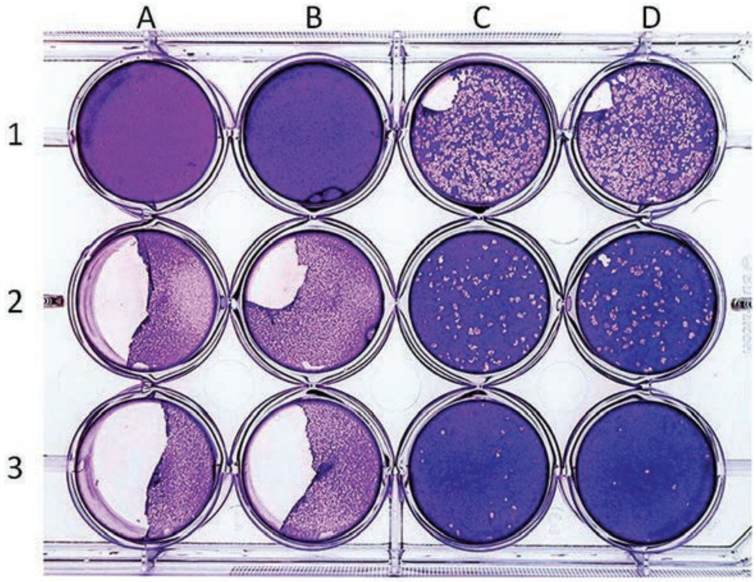

FIGURE 1 Example of a PRA (i.e., phenotypic test) used for determining acyclovir resistance in HSV-1. The reduction in the number of plaques is shown for an acyclovir-sensitive HSV-1 tested in duplicate starting with uninfected cells in the first two wells (1A/1B). Increasing acyclovir concentration is added downward to duplicate wells beginning with 2A/2B to 3A/3B and continuing from the top, right downward (1C/1D to 3C/3D) of the 12-well microtiter plate. The lowest number of plaques appears in wells 3C/3D where the highest concentration of acyclovir is added. doi:10.1128/9781555817381.ch112.f1

has been used successfully to detect antiviral resistance to the two neuraminidase inhibitor drugs (31). The assay was subsequently commercialized and is available under the name NA-Star (Applied Biosystems, Foster City, CA).

Recombinant Virus Assays

Recombinant virus assays (RVAs) for drug susceptibility have been developed for clinical use. These assays monitor phenotypic behavior in the presence of an antiviral drug that can be attributed to specific genes on the virus genome that may contain mutations known to correlate with antiviral resistance. The first clinically useful RVA was used to measure the phenotypic resistance of HIV-1 to protease and RT inhibitors. In the RVA protocol strategy, the gene of interest (e.g., HIV-1 protease or RT) is PCR-amplified directly from virus in the patient specimen. Afterwards, the PCR product is ligated into a retroviral vector where the protease or RT gene was removed. The concept is that the ligation adds back the protease or RT activity associated with the gene from the patient's virus. The vector also has the *env* gene, which codes for the receptor-binding protein, replaced with a reporter gene coding for a light-emitting protein such as luciferase. This vector, which now contains the HIV-1 gene of interest, is then cotransfected into a susceptible cell line along with a different plasmid. However, this plasmid contains a gene to provide a non-HIV receptor-binding protein able to attach to and infect the chosen test cells. After transfection, the newly assembled chimeric virus, called a pseudotype, incorporates the non-HIV receptor-binding protein, the genome from the retroviral vector containing the added HIV-1 protease or RT from the patient virus, and the reporter gene. The pseudotype virus can now infect the cells of choice to start replication for a single round. This RVA strategy allows the activity of the protease or RT derived from patient HIV to be tested in cells in the presence of antiretroviral drugs. Measurements of the replication efficiency of the engineered virus can be monitored by light emission of the luciferase expressed in the cells only during successful replication of the virus. Chimeric virus containing a protease or RT sensitive to the antiviral drug would then not emit light from infected cells, while virus containing a protease or RT resistant to the antiviral drug would emit measurable light.

The RVA is labor-intensive and time-consuming and requires a long turnaround time. RVA utilizes cell culture; however, the use of a standard cell line also provides assay consistency that is not possible with the use of primary cells such as peripheral blood mononuclear cells. An advantage of a recombinant assay compared to conventional culture is that it provides direct evidence of the antiviral activity associated with a specific gene derived from the virus population in a patient specimen. Also, if RVA were linked with genotyping the patient's virus to show the presence of known resistance mutations, a direct correlation between the level of phenotypic resistance could be made to the mutations observed, especially in patients on a complex treatment regimen. This information would allow a more accurate snapshot of HIV-1 resistance and could offer the clinician a more accurate choice of antiretroviral drugs available if a change in treatment is contemplated.

Modifications of the RVA have been made to allow fusion, integrase, and coreceptor usage to be monitored in instances where inhibitors of these HIV-1 activities are candidates for a patient's antiviral therapy. Currently, HIV-1 RVA testing is provided from commercial reference labs. Available RVAs measure HIV-1 protease-reverse transcriptase, fusion, integrase, and coreceptor activities. There is also an LDT variation of this strategy that has been

TABLE 2 Proposed guidelines for antiviral susceptibility results of herpes group and influenza A viruses

Virus	Antiviral agent	Method	IC_{50} denoting resistance	Reference(s)
HSV	Acyclovir	PRA	≥2 µg/ml	26
		DNA hybridization	≥2 µg/ml	9, 180
		DU	≥3 µg/ml	26, 181
	Famciclovir (active metabolite 5 penciclovir)	PRA and DNA hybridization	Definitive breakpoints cannot be established	132, 182
	Foscarnet	PRA	>100 µg/ml	183
	Vidarabine	PRA	≥2-fold increase of IC_{50} compared to control or pretherapy isolate	184
HCMV	Cidofovir	PRA and DNA hybridization	>2 µM	49–51
	Foscarnet	PRA and DNA hybridization	>400 µM	49, 50
			>324 µM	51
	Ganciclovir	PRA and DNA hybridization	≥3–4-fold increase of IC_{50} compared to pretherapy isolate or control strain (~3 µg/ml)	58, 185 186
			>6 µM	50, 174
			>8 µM	51
Influenza A virus	Amantadine/rimantadine	EIA	>0.1 µg/ml	5, 6
Influenza A and B viruses	Oseltamivir and zanamivir	NI assay	>8-fold decrease in NA activity	109, 136
VZV	Acyclovir	PRA and DNA hybridization	≥3–4-fold increase of IC_{50} compared to pretherapy isolate or to control strain	23, 187, 188
	Famciclovir	PRA and DNA hybridization	Definitive breakpoints cannot be established	182
	Foscarnet	Late antigen-reduction assay	300 µM	188

developed for CMV resistance testing where linkage between phenotypic and genotypic data has been made (32).

TESTING METHODS: GENOTYPIC ASSAYS

The gene targets for antiviral genotypic testing and their respective mutations are shown in Tables 3 and 4. There are 37 drugs currently available for treatment of HIV-1, targeting the protease, RT, integrase, viral fusion, and entry. The list of known mutations associated with resistance to these and newly approved inhibitors is updated every 2 years. The 2013 list can be downloaded from the International AIDS Society-USA website (www.iasusa.org/tam/article/update-drug-resistance-mutations-hiv-1-march-2013) or from the Stanford HIV Drug Resistance Database (hivdb.stanford.edu).

Knowledge of the mutations is the basis for genotyping assays used to identify antiviral resistance. Analysis of raw genotyping-sequence data involves subjective input from an operator for final processing and interpretation. This expertise requires specific training and experience in order to correctly identify relevant mutations in the template. The basic flow of a genotypic assay is shown in Fig. 2. Commercial reference laboratories and large medical centers offer these highly complex FDA-approved/cleared or LDT assays. A basic report often consists of a section showing the relevant mutations associated with drug resistance, followed by an interpretation of the mutations. Smaller diagnostic laboratories with the proper training and equipment can also perform antiviral genotypic assays. However, there is a need for strict control over the generation of results and accurate/correct interpretation of the data for any size laboratory.

Genotypic Platforms

Genotypic platforms used for antiviral susceptibility testing may include Sanger sequencing, pyrosequencing, *in vitro* reverse-hybridization line-probe assay (LiPA), and single-nucleotide polymorphism (SNP) analysis. Real-time RT-PCR allelic discrimination is also being evaluated as an LDT targeting single mutations (33, 34). In general, these assays analyze data and yield results more quickly than phenotypic testing, often at a lower cost. Currently, sequencing assays are more frequently used to detect mutations associated with antiviral resistance. They are also capable of identifying novel mutations associated with antiviral resistance. However, most sequencing platforms will require the use of bioinformatics software for accurate and reproducible data interpretation.

Viral genomes with multiple, clustered resistance mutations are mostly analyzed by the use of assays based on Sanger dideoxynucleotide sequencing (35). For example, in the case of HIV-1 or HCV chronic infection, the viral population consists of multiple variants referred to as “quasispecies” (36). Raw data from Sanger sequencing are displayed in chromatograms, which can show the presence of minor populations in the total virus population. The operator should evaluate the chromatograms and proofread the data manually to determine that the quality of the sequence chromatogram produced is of acceptable resolution (Fig. 2) (37). Every consensus sequence developed should include sequencing the PCR-derived template in both the forward and reverse directions in order to ensure sequence accuracy

TABLE 3 Gene targets used for antiviral resistance testing with genotypic methods

Virus		Antiviral agent	Gene target(s)	Gene mutation(s)	References
Herpesviruses	Cytomegalovirus (CMV)	Cidofovir	UL54, UL97	Refer to Table 4	See Table 4
		Foscarnet	UL54, UL97		
		Ganciclovir	UL54, UL97		
		Maribavir	UL97		
		Valganciclovir	UL54, UL97		
	Herpes simplex virus (HSV)	Acyclovir	TK and/or DNA polymerase	P57H, D59P, K62N, R51W, E83K, A175V, Indels, not substitutions	121–133
		Valacyclovir			
		Penciclovir			
		Famciclovir			
		Foscarnet	DNA polymerase	Regions II, III, and VI	
		Cidofovir	DNA polymerase	Regions δC and II	
	Varicella-zoster virus (VZV)	Acyclovir	TK and/or DNA polymerase	Frameshift and substitution mutations	130, 134, 135
		Valacyclovir			
		Penciclovir			
		Famciclovir			
		Foscarnet	DNA polymerase	Regions II, III, and VI	
		Cidofovir	DNA polymerase	Not specific	
Influenza viruses	Influenza type A	Amantadine	M2 protein	26, 27, 30, 31, 34	5, 6, 136–142
		Rimantadine	M2 protein	26, 27, 30, 31, 34	
		Oseltamivir	NA	H274Y, E119V, R292K, N294S	
		Zanamivir	NA	T198I, R152K	
	Influenza type B	Oseltamivir	NA	R152K	
		Zanamivir	NA	R152K	
Hepatitis viruses	Hepatitis B virus (HBV)	Adefovir	Pol RT (B,D)	rtA181TV, rtN236T	143–162
		Entecavir	Pol RT (A,B,C,E)	rtI169T, rtV173L, rtL180M, rtT184G/S/A/I/L, rtM204V/I, rtS202G/C/I, rtM250I/V	
		Lamivudine	Pol rt (A,B,C)	rtL80V/I, rtL180M, rtM204V/I/S/A	
		Telbivudine	Pol rt (A,B,C)	rtL80V/I, rtL180M, rtM204V/I/S/A	
		Tenofovir	Pol rt (B,C,D)	rtL180M, rtM204V, rtN236T	
	Hepatitis C virus (HCV)	Boceprevir	Protease	T54A, V170A, R155K, A156T/V	2, 163–168
		Ribavirin			
		Telaprevir	Protease	V36C, R155K, A156T/V	

by verifying the presence of the identical mutation(s) in both strands of the sequencing template. This process will validate true nucleotide change as opposed to an artifact generated during the sequencing protocol (i.e., breach in the enzyme proofreading or inconsistencies during the preparation of the sequencing sample). Sanger sequencing is useful in monitoring longer contiguous regions of the genome. Good-quality sequencing reactions can yield up to 600 to 700 readable nucleotides per sequencing primer, assuming the template is generated from a clean PCR product. However, Sanger sequencing does not detect a variant in a mixed virus population reproducibly if the minor population is less than 20 to 25%.

The introduction of next-generation sequencing (NGS) (also called massively parallel sequencing or MPS) technologies has the potential to further elucidate gene sequences and detect minor populations between 1 and 5%. This "ultra-deep sequencing" can detect much lower levels of variants in mixed samples because of the enormous number of genomic sequence reads. These valuable sequence data provide a "snapshot" of the population of the infectious agent in the patient. NGS also allows for accurate verification of single-nucleotide variations that might otherwise be misinterpreted using Sanger sequencing. NGS technology is rapidly evolving and will serve as an important diagnostic tool in the management of human infectious disease.

Pyrosequencing is a more recently developed method used for identifying mutations associated with antiviral drug resistance. This method has been widely used to sequence 16S rRNA for bacterial identification. Pyrosequencing is fundamentally different from Sanger sequencing in several ways and is described thoroughly by Metzker (38). Pyrosequencing

TABLE 4 CMV UL54 and UL97 gene targets and the associated mutations conferring antiviral resistance[a]

Gene region and mutation[b]		CMV susceptibility[c]		
		Cidofovir	Foscarnet	Ganciclovir
UL97 (phosphotransferase)	M460V, M460I, H520Q, Del 591-594, A594V, L595S, L595F, Del 595, C603W, C607Y	NA	NA	R
Other mutations at or next to UL97 codons involved in ganciclovir resistance	N510S, A590T, A591D, A591V, C592G, A594T, L595T, L595W, E596G, E596G, N597I, G598V, K599M, Del 600, C603Y, C606D	NA	NA	R
UL54 (DNA polymerase)[d]	N408D, D413E, **L501F**, L501I, T503I, P522S, L545S, G678S, I722V, Y751H, **A809V**, A987G	R	S	R
	F412V, **F412C**, K805Q, K513R, K513E	R	S or R	R
	P522A, G841A	ND	S	R
	D588E, **L802M**	S or R	R	S or R
	T700A	S	R (S)[e]	S
	V715M	S	R	S
	V781I	S	R	R
	T821I	R	R	R

[a] References 47 and 169–179.

[b] Bolded codon denotes resistance confirmed by recombinant virus data.

[c] S, sensitive; R, resistant; NA, UL97 not a target for this drug; ND, phenotype not determined or known at this time.

[d] Most of the UL54 mutants also have UL97 mutations (exceptions may be UL54).

[e] A single foscarnet-sensitive strain was found with this mutation.

has a lower limit of detection for the discrimination of minor variants in a mixed population because the protocol is more clonal, a result of preparing sequencing template that is derived from one single DNA molecule for the assay. Each copy of sequencing template is bound to a solid support, such as beads, to perform the assay. The sequence data are not produced and captured electrophoretically, but are the result of an enzyme-cascade reaction that generates a bioluminescent signal after single addition of each complementary dNTP. The data produced are not visually represented by chromatograms, but are expressed through numerical compilation of the order and strength of the peaks of light which are then converted to represent the sequence of the template by a statistical algorithm. The sequence output is shorter (currently 300 to 400 usable nucleotides), run times are faster, and the assay allows for high-throughput analysis. The testing is more automated, which allows less oversight and manual intervention by the operator than Sanger sequencing. Generally, pyrosequencing is useful for mixed infections and where the mutations are widely spaced throughout the genome and less clustered. Pyrosequencing is not FDA approved/cleared for use in antiviral susceptibility testing at this time. Verification of test performance will be required to adapt this method as an LDT assay in the clinical diagnostic laboratory (39). In addition, operators will be needed who are highly trained on assorted bioinformatics software handling large quantities of data in order to ensure accurate data analysis and results interpretation.

LiPA testing for antiviral susceptibility testing involves PCR-generated biotin-tagged oligonucleotides from the genetic region of interest of the patient's virus. The amplified product is then hybridized to nitrocellulose strip-bound oligonucleotide probes, spatially embedded on this solid matrix. The probes are derived from a reference virus genome and contain single mutations, which correlate to antiviral drug resistance. Both the probes for the reference and the mutation sequences are on the membrane. Each single mutation to be identified is represented by a different probe. The pattern of probe reactivity on the strip (i.e., color reaction produced by an EIA-type process) indicates the mutations present in the patient's viral population. The LiPA assay will only detect known, previously identified mutations specific for the probes included in the test kit (40). LiPA is also not FDA approved/cleared for use in antiviral susceptibility testing at this time.

Real-time PCR and SNP assays have also been developed for antiviral susceptibility testing. They are especially useful for detecting antiviral resistance in influenza type A virus infection with the introduction of anti-neuraminidase inhibitor drugs (41, 42). SNP assays are multiplexed, so that more than one primer/probe pair is added to each reaction. The probes will distinguish, for example, a wild-type virus from one that carries a resistance mutation. The probes for each are labeled with a different fluorescent dye, and detection of the bound dye indicates the presence or absence of the mutation. These assays are mostly LDTs (ASR) and are more likely to be performed by large commercial reference laboratories.

Applications (Genotyping)

Herpesviruses

HCMV

There are currently no FDA-approved/cleared kits/systems or RUO kits to screen for HCMV antiviral drug resistance mutations. Sanger sequencing is currently used to

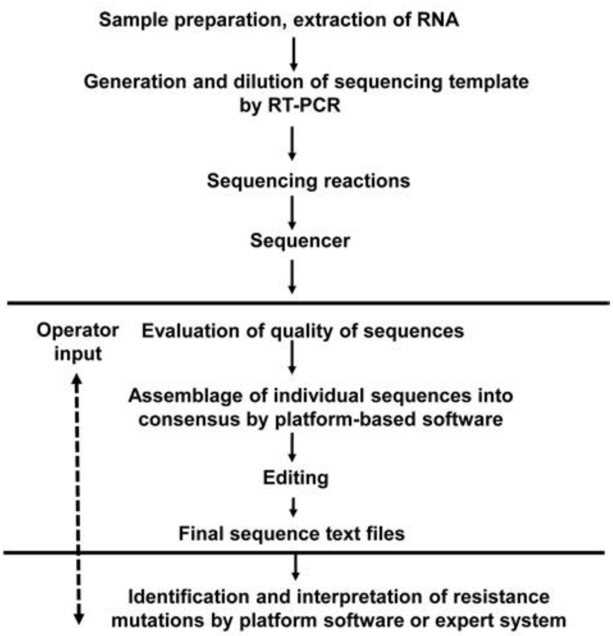
Sample preparation, extraction of RNA
Generation and dilution of sequencing template by RT-PCR
Sequencing reactions
Sequencer
Operator input
Evaluation of quality of sequences
Assemblage of individual sequences into consensus by platform-based software
Editing
Final sequence text files
Identification and interpretation of resistance mutations by platform software or expert system

FIGURE 2 General protocol for sequence-based genotypic assays of plasma samples. The individual steps that are performed in sequence-based genotypic assays are shown. Above the first line are components that are accomplished as a result of specified protocol instructions and machinery. After completion of the block, the subsequent steps are performed with subjective input from the operator within a software program that will accept the chromatograms generated by the sequencer. The operator then chooses the portion of the reference sequence containing the unknown mutations for use in the software of their choice. This is followed by alignment and editing of the sequences to identify these mutations in the patient samples. The most commonly used consensus sequences of HIV-1 strains are either HIV-1 strain HXB2 or HIV-1 consensus B. If the operator is using an in-house genotyping assay for other viruses, the reference sequence used should be based upon the literature or "common practice." doi:10.1128/9781555817381.ch112.f2

screen for mutations in HCMV isolates associated with ganciclovir and other drug resistance. Direct sequencing of HCMV from the blood, cerebrospinal fluid, and culture isolates has been successful in determining drug resistance (43–45). Genotyping offers a much faster turnaround time than phenotyping and is now the test of choice for HCMV antiviral drug resistance for patient samples with a minimum viral load (VL) of 1,000 copies/ml (46).

The UL97 (phosphotransferase) and UL54 (DNA polymerase) genes are well documented targets for numerous mutations associated with HCMV antiviral drug resistance (Table 4) (47). All mutations associated with ganciclovir resistance may be identified by sequencing the entire UL54 gene and the region covering the conserved domains linked to phosphotransferase activity (48–52). However, newly discovered mutations associated with antiviral susceptibility are still being documented, especially in immunocompromised populations (e.g., transplant recipients) (53, 54). UL97 mutations have long been known to be associated with ganciclovir resistance (51, 55, 56). Mutations in UL54 are also known to be linked to resistance in ganciclovir, foscarnet, and cidofovir (codons 300–1,000) (47, 57). Sequencing of both the UL54 and UL97 gene will require multiple primers and generate a relatively large PCR-amplified product, factors that will increase the cost of this assay. It is for this reason that the assay is usually performed by large medical centers or commercial reference laboratories.

HCMV genotyping sequence data from antiviral resistance testing must be carefully interpreted, because some common mutations observed in genotypic assays may not be responsible for phenotypic resistance. For example, a moderate number of the codon changes identified in UL97 are not associated with antiviral drug resistance (50). The laboratory should confirm such mutations with those identified by "recombinant phenotyping" (52) if the literature is not supportive or clinical drug efficacy has not been achieved. (47, 49, 58). In these instances, the aforementioned recombinant assays, which have been developed for HCMV, may be considered (32).

HSV-1 and -2

Currently available antiviral drugs for HSV infection are acyclovir (ACV), penciclovir (PCV), and their prodrugs; valacyclovir and famicyclovir (Table 3). These drugs target the HSV thymidine kinase (TK) and DNA polymerase (*pol*). Drug-resistant isolates frequently develop in HSV-infected immunocompromised patients. The use of antiviral susceptibility testing is especially useful for this patient population (Table 1).

Common target genes for genotypic sequencing assays are the HSV TK (UL23) and the DNA *pol* genes (UL30), which are associated with ACV and PCV drug resistance (59). Many cases of ACV resistance correlate with observed mutations in UL23, especially in areas of photopolymer repeats consisting of guanines or cytosines where hypermutation occurs (60, 61). In contrast, the mutations found in clinical isolates observed in UL30 are scattered over the structural regions II, III, VI, and VII (59).

Antiviral susceptibility testing should be considered when ACV and PCV treatment fails and other reasons for drug failure are ruled out. HSV genotyping is not commonly requested and there are no specific guidelines available to aid in the interpretation of the mutations for drug resistance. Therefore, the resulting mutations are usually compared to the published literature in order to distinguish true antiviral resistance from viral polymorphisms (59).

VZV

In the VZV-infected patient, treatment is usually initiated if the patient is immunocompromised (e.g., HIV-infected, transplant, and cancer patients). Some of the same antiviral drugs used for HSV infection are used for VZV infection and target similar genes (i.e., TK and DNA *pol*) (Table 3). There are fewer studies in the literature on VZV antiviral drug resistance compared to HSV. However, there are supportive data for genotypic testing from a recent phenotypic study (62), which examined cross-reactivity patterns associated with mutations in the VZV TK and DNA *pol* genes. Mutations correlating with drug resistance were mapped for both genes and this information could be used as a source reference for evaluation of mutations found in genotypic assays. However, the phenotypic method has been mostly offered as a commercial reference laboratory test.

HIV

Genotypic screening for mutations associated with HIV resistance is considered the preferred method for monitoring treated, nonresponding patients (http://www.aidsinfo.nih.gov/Guidelines/ accessed 21 September 2009). Regularly updated algorithms released by expert panels are used to interpret mutations detected in genotypic assays. There are currently two FDA-cleared test kits, the TrueGene HIV-1 Genotyping Assay (Siemens Medical Solutions Diagnostics, Tarrytown, NY) and the ViroSeq HIV-1 Genotyping System (Celera/Abbott Diagnostics) (21, 63–67). Both assays employ non-nested PCR amplification of a sequencing template to generate sequence for the entire protease gene. The RT sequence obtained spans codons 40–247 and codons 1–335 for the Trugene and ViroSeq assays, respectively. The assays were validated for HIV-1 subtype B strains, but have been used successfully for genotyping non-subtype B strains, including recombinant HIV-1 strains (64, 66, 68–70), which show mutations similar to those observed for subtype B (71). In recent years, a growing number of clinical laboratories, both commercial and government-supported, have also developed LDT genotyping assays. Genotypic screening for antiretroviral mutations is used for monitoring treatment failures (11, 72, 73) and is recommended for baseline screening of newly diagnosed patients with viral loads of >1,000 copies/ml as well as pregnant women (http://www.aidsinfo.nih.gov/Guidelines). Identification of preexisting HIV mutations will then serve to guide their initial treatment strategy (74, 75) (http://www.aidsinfo.nih.gov/Guidelines).

The drugs currently available to treat HIV-1 infection target the protease, RT, integrase, viral fusion, and entry (http://aidsinfo.nih.gov/drugs; http://www.fda.gov). In the past decade, new HIV-1 drugs for non-Pro-RT targets have been FDA approved. For example, enfuvirtide targets the fusion peptide in gp41 coded for in the *env* gene and is used as an option in heavy treatment-experienced individuals. Resistance mutations for enfuvirtide have been mapped to the ENV first heptad repeat sequence in gp41 (codons 36–43) (76). Raltegravir, approved in 2007, is an inhibitor of the integrase protein coded for in the *pol* gene. Maraviroc, also approved in 2007, is an entry inhibitor based on CCR5 usage and is associated with the V3 loop in the *env* gene (74). Next-generation drugs for all three of these antiretrovirals are available or are being developed. Currently, genotypic assays to identify known antiviral mutations for these drugs are being developed as LDTs. One commercial RUO genotyping kit is available for the detection of integrase-resistance mutations, the ViroSeq HIV-1 Integrase Genotyping system (Celera/Abbott). The system used in this assay is similar to the ViroSeq system for Pro-RT in the extraction method, but uses a combined RT-PCR step to make the sequencing template. The double-stranded sequencing product is detected using an ABI sequencer (ABI/Life Sciences). The resulting chromatograms are then assembled and edited to generate a consensus sequence which spans the entire integrase gene. Software is now available with this system for editing the sequence information similar to the ViroSeqpro-RT system. Previously, sequences were analyzed using SeqScape v2.5 or later (ABI/Life Sciences). There is no official report generated from the data because of the RUO kit designation. The consensus sequence obtained can be examined by the operator to identify the presence of mutations associated with resistance. Afterwards, the consensus sequence can be submitted to other outside websites (e.g., Stanford University HIV-1 drug resistance database or the Geno2Pheno site) to obtain an interpretation for the observed mutations.

There are no RUO or FDA-approved commercially available kits to screen for resistance to maraviroc or other CCR5 inhibitors. The expanded use of maraviroc as a first-line treatment option in drug-naïve individuals carrying virus with a CCR5 tropism (http://www.aidsinfo.nih.gov/Guidelines/, accessed 21 September 2009) requires determination of the baseline coreceptor usage of the virus. Currently, a tropism assay, Trofile, is available commercially to phenotypically determine if the virus employs the CCR5 or CXCR4 coreceptor.

Molecular phenotypic assays are costly and the turnaround time is lengthy. An alternative strategy to determine coreceptor usage is to sequence the V3 loop using Sanger or a pyrosequencing method. Here, the genotypic determination of resistance to maraviroc entails the observation of a coreceptor switch from a CCR5 sequence profile to a mixed coreceptor or solely a CXCR4 profile in the V3 loop. The consensus sequences generated are subsequently submitted to interactive websites employing databases correlating genotype with phenotypic outcome. The analysis infers coreceptor usage using different algorithms to determine the amino acid patterns and net charges of the entire V3 loop or only positions 11 and 25, which predict CCR5 or CXCR4 usage (77–79). Two algorithms frequently used to predict coreceptor tropism are the position-specific scoring matrix (PSSM) (80) and the Geno2Pheno coreceptor algorithm (81). The successful use of this strategy as a clinical tool has been evaluated and reported by several groups (78, 82, 83). Although protocols for sequencing the V3 loop have been circulating for years, development of the various algorithms for analysis, some of which are free Web-based services, has made this an approach feasible and attractive for most LDT users.

Commercially, a tropism assay is also available based on V3 loop sequencing and analysis by either the PSSM and/or Geno2pheno algorithms coupled with a separate heteroduplex assay (84). This is performed using a capillary array on the ABI 37xx sequencer platform.

Hepatitis Virus

HBV. The 5′ nucleoside/nucleotide analogs approved for the management of chronic HBV infection targeting the HBV DNA polymerase/RT are listed in Table 3 (85, 86). Alpha-2b interferon (IFN-α2b) and peg-IFN have been the recommended treatment; however, treatment efficacy is dependent upon the HBV subtype (87, 88) and interferon does not directly target HBV. Since HBV grows poorly in culture, genotyping methods are the tests of choice to monitor resistance. HBV genotypic assays detect known mutations (Table 3) in the polymerase/RT gene. The most common genotypic method for HBV is to directly sequence the polymerase/RT gene in HBV from plasma and determine the resistance-associated mutations. Alternatively, the generation of a PCR product from the specimen can be interrogated using specific probes in a reverse-hybridization assay (86).

There are no FDA-approved/cleared sequencing assay systems for HBV antiviral susceptibility testing. However, there are two RUO kits available for HBV genotyping, which partially sequence the HBV *pol*/RT gene. The Trugene HBV Genotyping kit (Siemens Medical Solutions Diagnostics) generates a consensus sequence of ~1.2 kb in the center of the gene (89). This is the same sequencing platform as the Trugene HIV system and it has internal software to generate a consensus sequence. The other assay is the ViroSeq HBV Genotyping Kit (Celera/Abbott), which targets the HBV *pol*/RT and generates a consensus sequence of ~1.0 kb. This consensus sequence covers an area between bases 130 and 1,161, where known antiviral resistance mutations reside. The sequences are generated using the ABI sequencers 3130/3130XL. There is no associated software with this assay to generate a consensus sequence from the chromatograms, but the use of SeqScape v2.5 or later is recommended for the analysis. The parameters used in the analysis of the HBV sequence in SeqScape are provided with the test kit protocol.

HBV strains possess a small genome of only ~3,200 nucleotides, which varies depending upon the subtype. This feature is used in LDT sequencing assays to detect the HBV genotype and/or for the detection of antiviral drug resistance (90). The ability to consistently detect variants in the HBV population using genotyping assays is usually not achievable unless the minority population is greater than 25% (85, 90). However, the method is capable of detecting all mutations that are known or compensatory, as well as novel mutations that might be associated with antiviral resistance (86). Interpretation of the associated mutations would require a virtual phenotype and the consensus data must be analyzed further by other relational databases such as SeqHepB (Evivar) (91, 92).

A commercial (CE-marked) reverse-hybridization assay, Innogenetics INNO-LiPA HBV DR version 2 (Innogenetics N.V., Ghent, Belgium), also available outside the United States, is capable of detecting known HBV mutations in the *pol*/RT gene. These mutations are associated with resistance to lamivudine, adefovir, emtricitabine, and telbivudine (40, 93) (Table 3). This assay is more sensitive for the detection of minority HBV populations. It is also less labor-intensive than the population sequencing method (85). However, the sensitivity can be affected by neighboring polymorphisms within the sequence (93, 94). The RUO INNO-LiPA HBV genotyping kit (Innogenetics), based on the HBV Sag (hepatitis B virus surface antigen) sequence, can also be used for HBV genotyping (95).

HCV. Treatment of chronic HCV infection was historically limited to pegIFN/ribavirin therapy. Success of this treatment varied and was affected by the virus genotype. Genotype 1 is the least responsive to IFN therapy and the most likely to evolve into a chronic infection (96). Therefore, management of HCV infection should always include virus genotyping (subtyping) to predict the efficacy of pegIFN/ribavrin. Two newly introduced antivirals, boceprevir and telaprivir (Table 3), have been recently FDA approved. They are direct-acting antiviral agents (DAA) which target the nonstructural protein 3/4A (NS3/4A) serine protease of HCV (2, 97).

HCV chronic infection is similar to HIV. However, HCV has the ability to generate a higher number of quasispecies which circulate in the infected individual due to the higher replication error and greater number of new virions produced during each replication cycle. Since the virus is not known to establish itself in reservoirs outside of the liver, the potential exists for curing the chronic infection with the new DAAs (98). There are also many drugs being developed for use as IFN-free treatment (99). Another drug target is NS5B, the viral polymerase gene, where resistance mutations noted during the development phase have been mapped (3, 98). It is possible that genotyping HCV to identify additional mutations related to antiviral resistance will be undertaken in treatment failure cases or to determine if resistance mutations have been transmitted in new infections.

There are currently no FDA-approved/cleared or RUO/ASR kits available to sequence the HCV protease gene. However, there are several LDT assays being developed for clinical use based on sequencing. One study evaluated the performance of 23 laboratories affiliated with the French National Agency for Research on AIDS and Viral Hepatitis (ANRS) Coordinated Action on Hepatitis Virus Resistance to Antiviral Drugs (AC33). Each laboratory used its own LDT to detect resistance mutations associated with protease inhibitors (100). Sequence data from a blinded panel of 12 samples were evaluated for reproducibility, sensitivity, specificity, and accuracy. A common tool to identify mutations was also provided for optional use. The data revealed that only 0.7% of the data produced was incorrect, suggesting that most protocols will provide equivalent data, although the preference would be to develop a more uniform protocol. Pyrosequencing has also been used to detect antiviral resistance mutations (101, 102). In the United States, testing is also commercially available since 2011 through one of the large reference laboratories under HCV GeneSure.

Influenza Virus

Mutations responsible for influenza type A antiviral resistance to amantadine and rimantadine have been mapped to the M2 protein (6) (Table 3). However, these drugs are no longer recommended, because current circulating influenza type A strains show heightened resistance. Current guidelines for the treatment of influenza recommend use of the neuraminidase inhibitors oseltamivir and zanamivir as the drugs of choice against influenza type A (103). Mutations conferring resistance to the neuraminidase inhibitor drugs are located on the neuraminidase gene (NA). Genotypic assays for the determination of anti-neuraminidase resistance have been developed as LTDs. Sequencing assays are also performed to detect other mutations in the NA gene. Although both Sanger and pyrosequencing protocols have been reported in the literature, pyrosequencing is the more widely used method to identify the H275Y mutation in the NA gene (104–107). A pyrosequencing protocol to detect this mutation is available on the Internet (108). Other approaches for the detection of H275Y include a real-time assay (41), a SNP analysis method performed on a sequencer (42), and a real-time allelic discrimination (34), which was adopted by World Health Organization for screening H1N1 isolates. Detection of mutations is more likely to be performed for infected high-risk immunocompromised individuals and for surveillance purposes.

INTERPRETATION OF ANTIVIRAL SUSCEPTIBILITY TESTING

Genotyping

The two FDA-cleared genotypic assays for HIV susceptibility, Trugene (Siemens) in 2001 and ViroSeq (Celera) in 2002, include kit-associated software to analyze the raw data sequences generated from each sample to produce a consensus sequence. In turn, both assays provide guideline-based interpretations of the mutations found in the consensus sequence and generate a formal report. The report helps the physician in choosing the regimen for the patient by indicating the likelihood of antiretroviral drug efficacy. FDA clearance included the software validation and periodic updates of the guidelines for use and interpretation. These systems are considered model protocols for diagnostic genotype testing used for antiviral susceptibility drug testing formats.

Bioinformatics: Virtual Phenotypes

Results generated using RUO or LDT assays are dependent upon bioinformatics sources for providing an interpretation. The laboratory has the option of analyzing the data following sequencing at various steps in the protocol

(Fig. 2). The clinician has the task of determining the significance of the mutations observed based upon the interpretation provided by the laboratory. These assays are mostly represented by the antiviral susceptibility testing for HBV, HCV, and HIV-1 infections.

The clinical laboratory wishing to perform its own sequence-based antiviral susceptibility assays with either an RUO or LDT will need access to analysis software in order to examine raw sequence data. Software for general sequence analysis and for the formation of consensus sequences can be purchased. Access to specialized "expert systems," as explained by Schafer (11), will also be needed to complete the interpretation. These systems are interactive, complex databases that use rules-based algorithms for individual drugs and associated mutations to infer the significance of the data (Table 5). The algorithms are guided by "knowledge"-based systems, which associate primary and secondary mutation(s), phenotypic data, and clinical outcomes.

Another consideration that should be addressed is access to adequate secured computer space or servers for storage of the raw data, software, and analysis results. These cumulative files of sample data analysis can be very extensive. If the laboratories or their institutions are in a position to allocate this computer storage space, it can be very useful for comparison purposes when monitoring a patient over time. There are also commercial entities available at a cost that can provide this service.

Models for the knowledge-based expert systems originated from commercial entities. A system developed early in the highly active antiretroviral therapy (HAART) era was the virtual-phenotype system based on the "Antivirogram" (Virco, now Jannsen Diagnostics). Genotypic sequence data, obtained from a clinical sample sent to this company, were analyzed and interpreted by an algorithm compared against a private database in order to infer a phenotype (11, 109). Their extensive database contained known genotypes which were correlated with phenotypes and/or clinical outcomes. The Monogram Biosciences GeneSure MG test compares an HIV genotype, derived from a submitted specimen, to its own expansive database of linked genotypes and phenotypes. The objective is to identify resistance mutations and predict phenotypic resistance using a proprietary algorithm. Both companies supply an official interpretative report of the analysis.

Consensus sequences can also be submitted through noncommercial interactive websites where interpretative data providing a virtual phenotype are returned to the submitting laboratory (Table 5). These sites are useful but are not regulated or standardized at this time. They employ different algorithms for different virus/drug combinations and allow different levels of operator input (79–81, 91, 92, 110–113). The databases linked with their algorithms connecting genotype and phenotype are also public. The reliability and consistency of the sites have been compared by several investigators (78, 79, 82). Most of these sites can examine consensus sequences to detect relevant mutations linked to resistance, and some can provide interpretations of the significance of the observed mutations with regard to drug efficacy. Some of the sites also provide additional tools for further analyses. All of these sites analyze data derived by Sanger sequencing. Currently, Geno2Pheno is also in the process of optimizing modules that would accept data from pyrosequencing (83, 114–117).

Test Interpretation

There is often minimal or no standardization among the various antiviral testing methods at a technical level, but required proficiency testing will provide some assurance of quality testing. Sequencing assays can be affected by several factors. Assuming the laboratory follows good laboratory practice (GLP), adhering to the stringency of defined use and separation of space to control the possibility of contamination of patient sample is critical. This is especially important when the VL of the patient sample is low. The commercial kits for HIV-1 were optimized for a minimum VL of 1,000 to 2,000 copies/ml of virus in the patient sample, and additional safeguards to reduce the effects of contamination were added within the protocol by using non-nested PCR to produce a sequencing template. Initial required volumes for extraction were between ~200 and 500 µl of, usually, plasma. In situations where the initial starting volumes are much less, as for genotyping of bloodspot samples, or where VL in the patient is much reduced to near undetectable, many LDT assays are incorporating nested PCR into the protocols, an amplification which is vulnerable to contamination. Generation of quality data in these circumstances can still occur if performance of the assay is conducted under rigorous conditions.

The analysis software provided with FDA-cleared diagnostic sequencing assays incorporates algorithms to accurately provide interpretations generated from test results. Updates to the algorithms are also FDA cleared before they are released. Alternatively, non-FDA-cleared assays developed as LDTs rely upon the individual laboratory both to establish test performance and to provide useful interpretations. The sources for such interpretations may come from the literature, actual testing by the laboratory, established Internet sites, or other sources. There is also no standardization or oversight of the interpretation algorithms used, although the inferred clinical significance of the data may be very similar regardless of the algorithm used. Ultimately, the use of these results and the interpretations provided by the laboratory in care of the patient are the responsibility of the primary health care provider.

FUTURE DIRECTIONS AND EMERGING TECHNOLOGIES

Antiviral test methods have dramatically evolved over recent years in parallel with the emerging molecular technologies. These genotypic assays have provided invaluable tools for detecting resistance in viruses that are difficult to culture or cannot even be propagated in culture (e.g., HBV and HCV). This is possible because the gene and the location of the mutations responsible for antiviral resistance are known. In cases where a phenotypic assay can be performed (e.g., HSV), a genotypic assay may provide the same or even more accurate results in far less time. However, in these cases, newly emerging mutations associated with resistance may not be detected by the genotypic assay and phenotypic assay may be more predictive of viral resistance. Consequently, phenotypic assays will remain as very useful laboratory tests until genotypic assays are more predictive of emerging viral resistance.

There is a definite need for more standardization of both phenotypic and genotypic assays. Unfortunately, antiviral assay standardization is very limited. Examples include a CLSI standardized approach reported for HSV plaque assays (118) and approaches at standardizing CMV using the UL97 gene target (119, 120).

The pharmacology industry is actively investigating and developing new antiviral drugs, especially for chronic viral disease agent (e.g., HCV, HBV, and HIV-1). Concurrently, antiviral assays (mostly genotypic) need to be developed

TABLE 5 Bioinformatics-based sites for analysis of resistance from genotyped samples

Sites	User fee	Virus	Genotyped target area	Interactive	Sample submitted	Information provided	Other comments/features
Stanford University HIV-1 Drug Resistance Database (Stanford University, Palo Alto, CA)	None	HIV	Protease-RT Integrase	Yes	FASTA file of consensus sequence	Resistance mutations Polymorphisms Clinical interpretations Subtype	New Web-based link allows individuals and institutions to use site database tools Interactive tools for epidemiologic and treatment observations
Geno2Pheno (Max Planck Institute [part of GENAFOR], Bonn, Germany)	None	HIV	Protease-RT Integrase GAG Coreceptor use	Yes	FASTA file of consensus sequence Clinical data from patient	Resistance mutations Polymorphisms Clinical interpretations Subtype	Most established modules
		HCV	Protease - NS3	Yes	FASTA file of consensus sequence	Resistance mutations Clinical phenotypic interpretations if NS5B genotype known	Submit NS3 and NS5B and data back includes interpreted phenotype based on genotype Should not be sole basis for clinical decisions
			NS5B	Yes	FASTA file of consensus sequence	Genotype, subgenotype	
		HBV	*pol* gene/surface gene	Yes	FASTA file of consensus sequence	*pol* gene mutations *shb* gene mutations Predicted phenotype for 5 antiretroviral drugs	Interpretations require substantial RT sequence to be submitted Should not be sole basis for clinical decisions
PSSM (position-specific scoring matrices) (University of Washington, Seattle, WA)	None	HIV	Coreceptor	No	FASTA of V3 amino acids	Predicts coreceptor usage based on charged V3 loop amino acids	Developed for subtypes B and C Does not predict subtype

(Continued on next page)

TABLE 5 Bioinformatics-based sites for analysis of resistance from genotyped samples *(Continued)*

Sites	User fee	Virus	Genotyped target area	Interactive	Sample submitted	Information provided	Other comments/features
SmartGene Integrated database network-based system IDNS (SmartGene, Lausanne, Switzerland)	Yes	HIV	Protease-RT Integrase GAG Gp41-gp120 (V3)	Yes	Sequence chromatogram files FASTA files	Resistance mutations Polymorphisms Clinical interpretations Subtype	Provides user capability to work with and edit chromatograms within module Network links to sites with different databases and algorithms for clinical interpretation allowing comparisons Site acts as a secured applications and data management center with storage of cumulative data able to be recalled for analysis
	Yes	HCV	NS3 HCV 5′UTR NS5B	Yes	Sequence chromatogram files FASTA files	Resistance mutations Subtype	Same as above
	Yes	Influenza			Sequence chromatogram files FASTA files	Resistance mutations Subtype	Same as above
SeqHepB (Evivar Medical, Melbourne, Australia)	Yes	HBV	*pol* gene (RT)	Yes	FASTA files Patient blood	Resistance mutations Polymorphisms Clinical interpretations Serotype	Linked to *in vitro* phenotypic database

for detecting and monitoring antiviral drug resistance. This is especially important due to the high cost of such antivirals and their long-term use, which is conducive to the development of resistance. One of the ultimate goals is to develop assays that would predict the likelihood of emerging resistance. Such assays may be possible with the newer NGS technologies, which for HIV-1 and CMV can now detect variants at a level of <5%. However, the clinical significance of the presence of such small populations will have to be determined. Also, as previously mentioned, there are logistical concerns with data management and storage, as well as with proficiency and consistency in expertise, which should be addressed in parallel with assay development. In a more immediate timeframe, screening tests for known mutations that can serve as guides for treatment strategies will be needed, which are less costly and allow for wider usage. There may be a trend towards development of more focused LDTs to detect individual mutations associated with resistance, including use of NGS for detection of very low levels of minority resistant variants. Most assays are sequence based, but there are assays using other genotypic platforms. Examples include the real-time allelic discrimination assay used for influenza (107), proposed for use in the detection of M184V in HIV RT (33). It is very likely that antiviral susceptibility testing applications will evolve quickly as new technology is introduced and the characteristics of viral infection and treatment change. Evaluation of the clinical significance of the data acquired from these new technologies will need to keep pace.

Addendum in proof. Trugene (Siemens) was withdrawn from the market as of January 2015.

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MYCOLOGY

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Taxonomy and Classification of Fungi

MARY E. BRANDT AND DAVID W. WARNOCK

113

There are at least 100,000 named species of fungi. However, it has been estimated that the number of undiscovered species ranges from 1 million to more than 10 million, and it has been calculated that about 1,000 to 1,500 new species are described each year (1, 2). Of the named species of fungi, fewer than 500 have commonly been associated with human or animal disease, and no more than 50 are capable of causing infection in otherwise normal individuals. On the other hand, an increasing number of ubiquitous environmental molds are now being implicated as opportunistic pathogens, capable of producing serious or lethal disease in hosts that are immunocompromised or debilitated. These molds are organisms whose natural habitats are in the soil or on plants, wood, compost heaps, or decomposing food. Many are familiar to mycologists, plant pathologists, and food microbiologists, but they present problems for the clinical microbiologist, who often has had no formal training in the identification of fungi. Fungal identification can be challenging and sometimes frustrating because of the importance placed on the morphological characteristics of the organisms, and the need to become familiar with a range of different structures and terms. Indeed, it is fair to state that obscure mycological terms are one of the major factors that discourage many microbiologists from mastering fungal identification. However, investing time to learn the basic structures and principles of taxonomy, classification, and nomenclature can result in the ability to recognize and identify correctly many medically important fungi.

MORPHOLOGICAL CHARACTERISTICS OF THE FUNGI

Fungi form a separate group of eukaryotic organisms that differ from other groups, such as the plants and animals, in several major respects. Fungal cells are encased within a rigid cell wall, mostly composed of chitin, glucan, chitosan, mannan, and glycoproteins in various combinations. These features contrast with the animals, which have no cell walls, and the plants, which have cellulose as the major cell wall component. As in other eukaryotic organisms, fungal cells have a true nucleus with a surrounding membrane, and cell division is accompanied by meiosis or mitosis.

Fungi are heterotrophic (lacking in chlorophyll), and therefore require preformed organic carbon compounds for their nutrition. Fungi live embedded in a food source or medium, and obtain their nourishment by secreting enzymes into the external substrate and by absorbing the released nutrients through their cell wall. Fungi are found throughout nature, performing an essential service in returning to the soil nutrients removed by plants.

Fungi can be multicellular or unicellular. In multicellular organisms, the basic structural unit is a chain of multinucleate, tubular, filament-like cells (termed a hypha). In most multicellular fungi the vegetative stage consists of a mass of branching hyphae, termed a mycelium or thallus. Each individual hypha has a rigid cell wall and increases in length as a result of apical growth with mitotic cell division. In the more primitive fungi, the hyphae remain aseptate (without cross walls). In the more advanced groups, however, the hyphae are divided into compartments or cells by the development of more or less frequent cross walls, termed septa. Such hyphae are referred to as being septate. Fungi that exist in the form of microscopic multicellular mycelium are commonly called molds.

Many fungi that exist in the form of independent single cells propagate by budding out similar cells from their surface. The bud may become detached from the parent cell, or it may remain attached and itself produce another bud. In this way, a chain of cells may be produced. Fungi that do not produce hyphae but just consist of a loose arrangement of budding cells are called yeasts. Under certain conditions, continued elongation of the parent cell before it buds results in a chain of elongated cells, termed a pseudohypha. Unlike a true hypha, the connection between adjacent pseudohyphal cells shows a marked constriction. Yeasts are neither a natural nor a formal taxonomic group, but are a growth form shown in a wide range of unrelated fungi. Many medically important fungi change their growth form as part of the process of tissue invasion. These so-called dimorphic pathogens usually change from a multicellular mold form in the natural environment to a budding, single-celled yeast form in tissue under the influence of temperature as they exhibit thermal dimorphism. *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, and *Sporothrix schenckii* are the best-known examples of this dimorphic change, but many other fungal pathogens show subtle morphological differences between forms found in tissue and in culture.

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Fungi reproduce by means of microscopic propagules called spores or conidia. The term conidium (pl. conidia) is used to describe propagules that result from an asexual process (involving mitosis only). Except for the occasional mutation, asexual conidia are identical to the parent. They are generally short-lived propagules that are produced in enormous numbers to ensure dispersion to new habitats. Many fungi are also capable of sexual reproduction (involving meiosis, preceded by fusion of the nuclei of two cells). Some species are self-fertile (homothallic) and able to form sexual structures between different cells within an individual thallus. Most, however, are heterothallic and do not form their sexual structures unless two compatible isolates come into contact. Once two compatible haploid nuclei have fused, meiosis can occur, and this leads to the production of the sexual spores. In some species the haploid sexual spores are borne singly on specialized generative cells and the whole structure is microscopic in size. In other cases, however, the spores are produced in millions in macroscopic "fruiting bodies" such as mushrooms. Sexual reproduction and its accompanying structures form one scheme for classification of the fungi.

NOMENCLATURE OF FUNGI

The scientific names of fungi are subject to the International Code of Nomenclature for algae, fungi, and plants (ICN) (http://www.iapt-taxon.org), formerly the International Code of Botanical Nomenclature (ICBN), a convention that dates from the time when biologists regarded these organisms as "lower plants." The rules of the ICN must be followed when proposing the name for a new fungal species; otherwise, the name being proposed can be rejected as invalid (3). The main requirements for valid publication are that the name must be in Latin binomial form, and a living culture of the specimen on which the author based his or her original description of the species must be preserved. The previous requirement for a descriptive paragraph in Latin has been removed.

Names of fungi may have to be changed for a number of reasons. Many common and widely distributed species of fungi have been described as new many times and thus have come to have more than one name. In general, the correct name for any species is the earliest name published in line with the requirements of the code of nomenclature. The later names are termed synonyms. To avoid confusion, however, the ICN permits certain exceptions. The most significant of these is when an earlier generic name has been overlooked, a later name is in common use, and a reversion to the earlier name would cause problems (3).

Another reason for changing the name of a fungus is when new research necessitates the transfer of a species from one genus to another or establishes it as the type of a new genus. Such changes are quite in order, but with the provision that the specific epithet should remain unchanged, except for inflection according to the rules of Latin grammar. However often a species is transferred to a new genus, the correct species epithet is always the first one that was applied to that particular organism. As an example, when *Phialophora parasitica* was moved to a new genus, it became *Phaeoacremonium parasiticum*.

If there is one complication of fungal nomenclature that is confusing to many microbiologists, it is the fact that a large number of fungi appear to bear more than one name. This is an apparent departure from the basic principle of biological classification, in which an organism can only have one correct name. As described in the previous section of this chapter, many fungi have an asexual stage (or anamorph) characterized by the production of asexual conidia, and a sexual stage (or teleomorph) characterized by sexual spores (e.g., ascospores, basidiospores). Many fungi propagate asexually and the teleomorph is unknown or only rarely encountered. Because of this, mycologists have often given separate names to the asexual and sexual stages. Often, this is because the anamorph and teleomorph were described and named at different times without the connection between them being recognized. In 2011, the code of nomenclature was modified to apply a policy where a given fungus will have only one name (3). As of 1 January 2013, the system of permitting separate anamorph and teleomorph names ended; it is no longer necessary to provide different names for different morphologies of the same fungus. All legitimate names proposed for a species can serve as the correct name for that species. The name(s) to be widely used will be decided by a Nomenclature Committee for Fungi (3). Various proposals have been made to suggest the name to be retained, and these names should be formalized by 2016. At this time, it is permissible to refer to a fungus by its asexual designation if this is the stage that is usually obtained in culture. For example, *Blastomyces dermatitidis* is the anamorph of the ascomycete *Ajellomyces dermatitidis*. The anamorph is the stage that is ordinarily encountered in culture, and only under certain special conditions is the sexual stage formed.

TAXONOMY AND CLASSIFICATION OF THE FUNGI

The kingdom Fungi is one of the six kingdoms of life (1, 4). It is organized in a hierarchical manner, each rank being named with and recognizable by a particular ending: phylum, -mycota; subphylum, -mycotina; class, -mycetes; order, -ales; family: aceae (5). Each family is composed of a number of genera, and these are divided into species. The kingdom Fungi is currently divided into seven phyla, which include the Ascomycota and Basidiomycota (6). The phylum Zygomycota is no longer accepted due to its polyphyletic nature (6). Pending resolution of their relationships, the organisms that have traditionally been placed in the Zygomycota are at this time divided among the phylum Glomeromycota and four subphyla *incertae sedis*. The subphylum Mucoromycotina has been proposed to accommodate the Mucorales, while the subphylum Entomophthoromycotina has been created for the Entomophthorales (6).

In addition to the true fungi, there are a number of human and animal fungus-like microbes, such as *Lagenidium* spp., *Pythium insidiosum*, and *Rhinosporidium seeberi*, that are now studied by mycologists (see chapter 127). These organisms, while not fungi *sensu stricto*, are "parafungi" or "pseudofungi," protists sharing fungus-like morphological features with the true fungi. *Lagenidium* spp. and *P. insidiosum* are presently classified in the kingdom Straminipila (Stramenopila), phylum Oomycota. *R. seeberi* was placed by Adl et al. (7) in the supergroup Opisthokonta, division Holozoa. Furthermore, molecular studies have established that several organisms, long considered as protists, belong to the kingdom Fungi. These include *Pneumocystis*, now placed in the Ascomycota, and the microsporidians.

Historically, the classification of fungi has largely been based on their morphology, rather than on the physiologi-

TABLE 1 Simplified taxonomic scheme illustrating major groups of the kingdom Fungi in which medically important fungi are classified (modified from reference 12)

Phylum or subphylum	Class	Order	Representative genera
Mucoromycotina		Mucorales	*Lichtheimia*, *Rhizopus*
Entomophthoromycotina		Entomophthorales	*Basidiobolus*, *Conidiobolus*
Basidiomycota	Tremellomycetes	Filobasidiales	*Filobasidium*, *Filobasidiella* (teleomorphs of *Cryptococcus* species)
	Agaricomycetes	Agaricales	*Schizophyllum*
Ascomycota	Pneumocystidomycetes	Pneumocystidales	*Pneumocystis*
	Saccharomycetes	Saccharomycetales	*Issatchenkia*, *Kluyveromyces* (teleomorphs of *Candida* species); *Saccharomyces*
	Eurotiomycetes	Onygenales	*Arthroderma* (teleomorphs of *Microsporum* and *Trichophyton* species); *Ajellomyces* (teleomorphs of *Blastomyces* and *Histoplasma* species)
		Eurotiales	*Emericella*, *Eurotium*, *Neosartorya* (teleomorphs of *Aspergillus* species)
	Sordariomycetes	Hypocreales	*Gibberella*, *Nectria* (teleomorphs of *Fusarium* species)
		Microascales	*Pseudallescheria* (teleomorph of *Scedosporium* species)

cal and biochemical differences that are of such importance in bacterial classification. In recent years, however, the advent of rapid DNA sequencing has ushered in a revolution in fungal taxonomy based on a phylogenetic approach to species recognition (the PSR concept). This relies on comparative analysis of variable nucleic acid characters to define fungal species (8, 9). Under the PSR, a species is defined as a group of organisms that share concordance of multiple gene genealogies (DNA sequences at different genetic loci), rather than organisms that share a common morphology or organisms that can mate with one another. Portions of genes are often used to construct the genealogies. Concordant branches among the gene trees are used to connect species. Although the major application of the PSR, defining species, is beyond the scope of the clinical microbiology laboratory, results obtained by PSR can be applied to medically important fungi. Several new species have been recognized within medically important species formerly defined by morphology. For example, *Coccidioides posadasii* has been separated from *C. immitis* and comprises isolates largely from outside the state of California (10).

The sexual spores and their mode of production have historically formed the main basis for classification of fungi into the Zygomycota, Ascomycota, and Basidiomycota. In some fungi, however, the asexual stage or anamorph has proved so successful as a means of rapid dispersal to new habitats that the sexual stage or teleomorph has disappeared, or at least has not been discovered. Even in the absence of the teleomorph it is now often possible to assign these fungi to the Ascomycota or Basidiomycota on the basis of DNA sequences of the anamorphs (11). In the past, these asexual fungi were classified in an artificial group, the "Fungi Imperfecti" (also termed the "form-division Deuteromycota") and were divided into artificial form classes according to the morphological characteristics of their asexual reproductive structures. There is no longer any separate formal grouping for those fungi that appear to be strictly anamorphic or for which no teleomorph has been discovered. Nonetheless, mycologists continue to employ the asexual reproductive characteristics of molds, at least for routine identification purposes.

A simplified taxonomic scheme illustrating the major groups of medically important fungi is presented in Table 1. These groups are described in the following sections. A simplified key to their identification is provided in Table 2.

Kingdom Fungi

Subphyla Mucoromycotina and Entomophthoromycotina (Formerly Phylum Zygomycota)

The traditional Zygomycota have been divided among the phylum Glomeromycota and four subphyla pending resolution of further taxonomic questions (6). In these groups of lower fungi, the thallus is pauciseptate and consists of wide, hyaline (colorless) branched hyphal elements. The asexual spores (termed sporangiospores) are nonmotile and are often produced inside a closed sac, termed a sporangium, the wall of which ruptures to release them, although in some genera the spores are formed around a vesicle at the tip of the sporangiophore. Sexual reproduction leads to the formation of a single large zygospore with a thickened wall. Most of the medically important species are heterothallic and do not form their sexual structures unless two compatible isolates come into contact.

The subphylum Mucoromycotina contains the order Mucorales, which is the most clinically important, and includes the genera *Lichtheimia* (formerly *Absidia*), *Mucor*, *Rhizomucor*, and *Rhizopus*. The subphylum Entomophthoromycotina contains one order of medical importance, the Entomophthorales. The Entomophthorales includes the genera *Basidiobolus* and *Conidiobolus*, agents of subcutaneous infections.

Phylum Basidiomycota

Most members of this group of higher fungi have a septate, filamentous thallus, but some are typical yeasts. Sexual reproduction leads to the formation of haploid basidiospores on the outside of a generative cell, termed a basidium. Fifteen classes are recognized, but only a few members of this large phylum are of medical importance. The most prominent are the basidiomycetous yeasts with anamorphic stages belonging to the genera *Cryptococcus*, *Malassezia*, and *Trichosporon*. The genus *Cryptococcus*, which contains more than 30 species, has teleomorphs that have been assigned to the genera *Filobasidium* and *Filobasidiella* (12).

TABLE 2 Simplified key to the main groups of medically important fungi

1a	Fungus not culturable on routine media, occurs as cyst-like cells in tissue	Pneumocystidomycetes
1b	Fungus cultivable	2
2a	Colonies consist of budding cells at 30°C	3
2b	Colonies consist of hyphae at 30°C	5
3a	Colonies black	Black yeast
3b	Colonies white, cream, pink, or red	4
4a	Urease test positive	Basidiomycetous yeasts
4b	Urease test negative	Ascomycetous yeasts
5a	Hyphae septate	6
5b	Hyphae nearly aseptate (pauciseptate)	9
6a	Clamp connections present	Filamentous basidiomycetes
6b	Clamp connections absent	7
7a	Fruiting bodies absent	Hyphomycetes
7b	Fruiting bodies present	8
8a	Fruiting bodies containing ascospores in asci	Eurotiomycetes
8b	Fruiting bodies containing conidia	Coelomycetes
9a	Sporulation abundant	Mucormycetes, Entomophthoromycetes
9b	Sporulation absent; zoospores formed in water cultures	Oomycota

In culture, filamentous basidiomycetes often produce fast-growing, nonsporulating (sterile) white colonies with clamp connections. These are hyphal outgrowths which, at cell division, make a connection between the two cells forming a bypass around the septum to allow the migration of a nucleus. The basidia are often produced in macroscopic structures, termed basidiomata or basidiocarps, and the basidiospores are often forcibly discharged. Asexual reproduction is variable; some species produce spores like those of the Ascomycota (see below), but many others are not known to produce spores at all. Most filamentous basidiomycetes are wood-rotting fungi or obligate plant pathogens. The most frequently reported clinically important filamentous basidiomycete is *Schizophyllum commune*.

Phylum Ascomycota

This large phylum contains almost 50% of all named fungal species, and accounts for around 80% of fungi of medical importance (2). Sexual reproduction leads to the development of haploid spores, termed ascospores, which are produced in a sac-like structure termed an ascus. The Ascomycota show a gradual transition from primitive forms that produce single asci to species that produce large structures, termed ascocarps or ascomata, containing numerous asci. Variations in ascus structure are of major importance in the classification of these fungi. Asexual reproduction consists of the production of conidia from a generative or conidiogenous cell. In some species the conidiogenous cell cannot be distinguished from the rest of the mycelium. In others, a special structure is produced that bears one or more conidiogenous cells.

The phylum Ascomycota includes four classes of medical importance: the Pneumocystidomycetes, Saccharomycetes, Eurotiomycetes, and Sordariomycetes. The class Pneumocystidomycetes contains the genus *Pneumocystis*, formerly classified as a member of the kingdom Protozoa but now reassigned to the fungi on the basis of small-subunit rDNA and other gene sequence comparisons (13). The class Saccharomycetes contains the ascomycetous yeasts, while the Eurotiomycetes and the Sordariomycetes contain the filamentous ascomycetes.

The order Saccharomycetales, which belongs to the class Saccharomycetes, is characterized by vegetative yeast cells that proliferate by budding or fission. These fungi do not produce ascomata, the ascus being formed by direct transformation of a budding vegetative cell, by "mother-bud" conjugation, or by conjugation between two independent single cells. Many members of this order have an anamorphic stage belonging to the genus *Candida*. This genus, which consists of around 200 anamorphic species, has teleomorphs in more than 10 different genera, including *Clavispora*, *Debaryomyces*, *Issatchenkia*, *Kluyveromyces*, and *Pichia* (14). Many of these will be renamed as the "one fungus, one name" concept is applied.

In the class Eurotiomycetes, sexual reproduction leads to the formation of ascomata containing asci with ascospores. This class has seven orders that include species pathogenic to humans. Among the more important are the Onygenales, which contains the teleomorphs of the dermatophytes and a number of dimorphic systemic pathogens (including *Histoplasma capsulatum* and *Blastomyces dermatitidis*) and the Eurotiales, which includes the teleomorphs of the anamorphic genera *Aspergillus* and *Penicillium*. In the class Sordariomycetes, the order Sordariales contains many of the teleomorphs of the anamorphic genus *Fusarium*. In addition, the teleomorphs of numerous melanized fungi of medical importance belong to a number of orders in the classes Eurotiomycetes or Sordariomycetes. These include the Chaetothyriales, Microascales, Hypocreales, and Ophiostomatales.

Although most of the septate molds that are isolated in clinical laboratories belong to one of the classes described above, it is unusual to encounter their sexual reproductive structures in routine cultures. The few species that do produce ascomata in relative abundance include *Pseudallescheria boydii* (anamorph: *Scedosporium boydii*) and *Emericella nidulans* (anamorph: *Aspergillus nidulans*). For the most part, however, routine identification of these molds is based on the form and arrangement of the asexual spore-bearing structures and the manner in which the spores are produced (see below).

Kingdom Protozoa

Microsporidians, some of which are obligate parasites of humans, have long been classified with the protozoa. However, phylogenetic analysis has indicated that these organisms belong among the fungi (15), and the phylum Microsporidia within the kingdom Fungi has been created to accommodate them (6). However, parasitologists have been reluctant to part with this group, while mycologists have been just as reluctant to accept it. At this time, these organisms have been classified as true fungi (see chapter 128).

Kingdom Straminipila (formerly Chromista)

Members of the order Pythiales mostly occur in the soil or on plants. Two genera cause infection in mammalian hosts: *Lagenidium* spp. and *Pythium insidiosum* (see chapter 127). Members of the Straminipila have been long regarded as fungi because they develop sparsely septate mycelioid structures similar to those in filamentous fungi. However, these slime-fungus-like microbes are located basal to plants and are closely related to some algal groups; for this reason, they are now placed in the kingdom Straminipila, phylum Oomycota (16).

IDENTIFICATION OF YEASTS

Yeasts are neither a natural nor a formal taxonomic group, but are a growth form found in a wide range of unrelated ascomycetous and basidiomycetous fungi. Their identification relies on a combination of morphological, physiological, and biochemical characteristics. Useful morphological characteristics include the color of the colonies; the size and shape of the cells; the presence of a capsule around the cells; the production of hyphae, pseudohyphae, or arthroconidia; and the production of chlamydospores. Useful biochemical tests include the assimilation and fermentation of sugars and the assimilation of nitrate. Most yeasts of medical importance can be identified using one of the commercial tests systems that are based on sugar assimilation of isolates. However, it is important to remember that microscopic examination of cultures on cornmeal agar is essential to avoid confusion between organisms with identical biochemical profiles (see chapter 115).

The so-called black yeasts are not a formal taxonomic group, but the description is applied to a wide range of unrelated ascomycetous and basidiomycetous fungi that are able to produce melanized budding cells at some stage in their life cycle (17). Because most of these fungi are also able to produce true mycelium, their routine identification is largely based on the morphological characteristics of the asexual spore-bearing structures and the manner in which the spores are produced.

CLASSIFICATION AND IDENTIFICATION OF ANAMORPHIC MOLDS

Most of the septate molds that are isolated in clinical laboratories do not produce their sexual reproductive structures in routine cultures, and their identification is based on the manner in which the asexual spores are produced. Two artificial form classes of anamorphic or "mitosporic" molds are informally recognized, based on the mode of conidium formation. The Hyphomycetes produce their conidia directly on the hyphae or on specialized conidiophores, while the Coelomycetes have more elaborate reproductive structures termed conidiomata. Although these form classes are no longer formally recognized, they continue to offer a useful framework for identification based on morphology.

Form Class Coelomycetes

Three artificial orders are recognized: the Sphaeropsidales, Melanconiales, and Pycnothyriales. In the Sphaeropsidales, the conidia are produced in conidiomata that are either spherical with an apical opening and with conidiogenous cells lining the inner cavity wall (termed pycnidia), or are open and cup-shaped, in which case the conidiogenous cells cover the conidiomatal surface (termed acervuli). A few members of this form class are common pathogens of humans. One of the more frequently encountered species is *Neoscytalidium dimidiatum*, a plant pathogen that can also cause infections of the skin and nails. Until recently, this species was known by the synanamorph name *Scytalidium dimidiatum*.

Form Class Hyphomycetes

The Hyphomycetes contains a large number of septate anamorphic molds of medical importance, including the genera *Aspergillus*, *Blastomyces*, *Cladophialophora*, *Fusarium*, *Histoplasma*, *Microsporum*, *Penicillium*, *Phialophora*, *Scedosporium*, and *Trichophyton*. In addition, numerous Hyphomycetes have been reported as occasional opportunistic pathogens of humans. For this reason, it is important for clinical microbiologists to be able to recognize and correctly identify this group of fungi.

As mentioned earlier, the process of conidiogenesis is of major importance in the identification of these molds. Two basic methods of conidiogenesis can be distinguished: thallic conidiogenesis, in which an existing hyphal cell is converted into one or more conidia; and blastic conidiogenesis, in which conidia are produced as a result of some form of budding process (for a detailed discussion of this topic, see reference 17).

Thallic Conidiogenesis

In this form of conidiogenesis, the conidia are produced from an existing hyphal cell. Arthroconidia, which are derived from the fragmentation of an existing hypha, represent the simplest form of thallic conidiogenesis and have evolved in many different groups of fungi. The first step in the examination of cultures of these molds should be to ascertain whether another spore form is present. If so, identification should be based upon that form. The few molds of medical importance that produce arthroconidia as their sole means of conidiogenesis include *Coccidioides* species.

Aleurioconidia are formed from the side or tip of an existing hypha and, during the initial stage before a septum is laid down, can resemble short hyphal branches. This form of thallic conidiogenesis is characteristic of the dermatophytes (*Epidermophyton*, *Microsporum*, and *Trichophyton* spp.), as well as a number of dimorphic systemic pathogens, including *Blastomyces dermatitidis*, *Histoplasma capsulatum*, and *Paracoccidioides brasiliensis*.

Blastic Conidiogenesis

Many fungi have evolved some form of repeated budding that allows them to produce large numbers of asexual spores from a single conidiogenous cell. There are two basic forms of blastic conidiogenesis: holoblastic development, in which all layers of the wall of the conidiogenous cell swell out to form the conidium; and enteroblastic development, in which the conidium is produced from within the conidiogenous cell, the outer layers of the cell wall breaking open and an inner layer extending through the opening to become the new spore wall. These two forms of blastic conidiogenesis can be further subdivided according to the details of spore development. Almost all molds that produce holoblastic conidia have melanized cell walls and thus are similar in colonial appearance. The morphological characteristics of their conidia and the manner in which the spores are produced serve as the main distinguishing features. Holoblastic conidia range in size from minute unicellular to large thick-walled multicelled conidia. In some species, the first-formed conidium buds to produces a second, and the second produces a third, and so on until a chain of conidia is produced with the youngest at its tip (acropetal). As each spore can produce more than one bud, a branching chain becomes possible (e.g., *Cladophialophora* species). In another group, the conidiogenous cell that produced the first spore then grows past it to produce a second. If this process is repeated, it will result in an elongated conidiogenous cell with numerous lateral single spores along its sides. This is termed sympodial development and is typical of species of *Bipolaris* and *Exserohilum*.

In molds that produce enteroblastic conidia, the wall of the conidium is derived from the inner layer of the wall of the conidiogenous cell. This permits a succession of conidia to be produced from the same point. There are two main forms of enteroblastic conidiogenesis: phialidic, in which the specialized conidiogenous cell from which the conidia are produced is termed a phialide, and annellidic, in which the conidiogenous cell is termed an annellide.

In phialidic conidiogenesis, the first blown-out cell breaks open at its tip and remains as a collarette, from the inside of which conidia are produced in succession. In some species, the collarette is distinct (e.g., *Phialophora* species), but in others it is almost invisible at the tip of the phialide. In some phialidic molds, such as species of *Fusarium* and *Acremonium*, the conidia are not firmly attached to each other and often move aside to accumulate in a wet mass around the phialide. In other phialidic molds, such as species of *Aspergillus* and *Penicillium*, continuous replenishment of the inner wall of the tip of the phialide results in the formation of an unbranched chain of connected conidia, with the youngest at the base (basipetal).

Annellides, like phialides, are conidiogenous cells that produce conidia at their tips in unbranched chains (e.g., *Scopulariopsis* species) or in wet masses (e.g., *Scedosporium* species). In annellidic conidiogenesis the first blown-out cell becomes a conidium, and subsequent conidia are formed by blowing out through the scar of the previous one. Unlike phialides, annellides increase in length each time a new spore is produced. An old annellide that has produced many conidia will have a number of apical scars or annellations at its tip.

IDENTIFICATION OF MOLDS

Most molds can be identified after growth in culture, but the criteria for recognition often differ from the fundamental characteristics that are used as a basis for classification. Macroscopic characteristics, such as colonial form, surface color, pigmentation, and growth rate, are often helpful in mold identification. Although the culture medium, incubation temperature, age of the culture, and amount of inoculum can influence colonial appearance and growth rate, these characteristics remain sufficiently constant to be useful in the process of identification. Molds that fail to sporulate in culture may be impossible to speciate using morphology, and it is therefore important to select culture conditions that favor sporulation. Although molds often grow best on rich media, such as Sabouraud's dextrose agar, overproduction of mycelium often results in loss of sporulation. In such cases, subculture to a low-nutrient medium may help to stimulate sporulation.

POLYPHASIC IDENTIFICATION

A frequent problem with the traditional morphological approach to fungal identification is that nonsporulating organisms cannot be identified or given a taxonomic placement. With comparative DNA sequence analysis, most such isolates can now be identified and classified by applying PSR concepts. Interpretive criteria for identification of fungi using DNA sequencing have been published (18).

Many clinical laboratories today employ DNA sequencing as part of their routine protocol for fungal identification. In circumstances where morphology-based identification is not helpful, an isolate may be a candidate for DNA-based identification. This approach may be useful when an isolate displays atypical morphology, fails to sporulate, requires lengthy incubation or incubation on specialized media in order to sporulate, or if the phenotypic results are nonspecific or confusing. Precise identification of particular isolates may also be necessary as part of outbreak investigations or during other studies of the epidemiologic significance of particular groups of organisms. In these cases, DNA sequencing may be required. A polyphasic approach to fungal identification that combines both morphological and genotypic approaches may be the most useful, practical, and cost-effective way forward for fungal identification at this time (19).

COMMON MYCOLOGICAL TERMS

Acervulus (plural, acervuli): an open or cup-shaped structure on which conidia are formed.

Acropetal: pertaining to a chain of conidia in which new spores are formed at the tip of the chain.

Aleurioconidium (plural, aleurioconidia): a thallic conidium that is formed from the end of an undifferentiated hypha or from a short side branch.

Aleuriospore: *see* Aleurioconidium.

Anamorph: the asexual form of a fungus.

Annellide: a specialized conidiogenous cell from which a succession of spores is produced and which has a column of apical scars at its tip.

Annelloconidium (plural, annelloconidia): a blastic conidium that is formed from an annellide.

Annellospore: *see* Annelloconidium.

Apophysis: the enlargement of a sporangium just below the columella.

Appressorium (plural, appressoria): a swelling on a germ tube or hypha, typical of *Colletotrichum* spp.

Arthroconidium (plural, arthroconidia): a thallic conidium produced as the result of fragmentation of an existing hypha into separate cells.

Arthrospore: *see* Arthroconidium.

Ascocarp: a structure that contains asci.

Ascoma (plural, ascomata): *see* Ascocarp.

Ascospore: a haploid spore produced within an ascus following meiosis.

Ascus (plural, asci): a thin-walled sac containing ascospores, characteristic of the Ascomycota.

Aseptate: without cross walls or septa.

Ballistoconidium: a conidium that is forcibly discharged.

Ballistospore: *see* Ballistoconidium.

Basidiocarp: a structure that produces basidia.

Basidioma (plural, basidiomata): *see* Basidiocarp.

Basidiospore: a haploid spore produced on a basidium following meiosis.

Basidium: a cell upon which basidiospores are produced, characteristic of the Basidiomycota.

Basipetal: a chain of conidia in which new spores are formed at the base of the chain.

Blastic: one of the two basic forms of conidiogenesis in which enlargement of the conidial initial occurs before a delimiting septum is laid down.

Blastoconidium (plural, blastoconidia): a blastic conidium produced by the enlargement of a part of a conidiogenous cell before a septum is laid down.

Blastospore: *see* Blastoconidium.

Catenate: in chains.

Cerebriform: having a convoluted surface (a term used to describe colonies).

Chlamydospore: a resting conidium, formed as a result of the enlargement of an existing hyphal cell.

Clavate: club-like in shape, narrowing towards the base.

Cleistothecium (plural, cleistothecia): a form of closed ascocarp with no predefined opening, which splits open to release the ascospores.

Coelomycete: an artificial taxonomic grouping referring to anamorphic molds that form conidia within a specialized multihyphal structure, such as an acervulus or pycnidium.

Collarette: a cup-shaped structure at the tip of a conidiogenous cell.

Columella (plural, columellae): swollen tip of the sporangiophore projecting into the sporangium in some Mucorales.

Conidiogenesis: the process of conidium formation.

Conidiogenous cell: any cell that produces or becomes a conidium.

Conidioma (plural, conidiomata): a specialized conidia-bearing structure.

Conidiophore: a specialized hypha or cell on which, or as part of which, conidia are produced.

Conidium (plural, conidia): an asexual spore.

Cruciate: cross-shaped (a term used to describe spore septa).

Cuneiform: thinner at one end than at the other (a term used to describe spores).

Dematiaceous: darkly pigmented.

Denticle: a small tooth-like projection on which a spore is borne.

Distoseptate: of or pertaining to spores in which the individual cells are each surrounded by a sac-like wall distinct from the outer wall.

Echinulate: of or pertaining to spores with small, pointed spines.

Endoconidium (plural, endoconidia): a conidium formed inside a hypha.

Enteroblastic: a form of conidiogenesis in which conidia are produced from within a conidiogenous cell.

Euseptate: of or pertaining to spores in which the outer and inner walls of the septum are continuous.

Floccose: having a cotton-like texture (a term used to describe colonies).

Fusiform: having a spindle-like shape (a term used to describe spores).

Geniculate: of or pertaining to an irregular conidiogenous cell formed by some holoblastic molds.

Glabrous: having a wax-like texture (a term used to describe colonies).

Gymnothecium (plural, gymnothecia): an ascocarp in which the asci are distributed within a loose network of hyphae.

Heterothallic: self-sterile; sexual reproduction of a heterothallic fungus cannot take place unless two compatible mating strains are present.

Hilum: a slightly prominent scar at the base of a conidium.

Holoblastic: a form of conidiogenesis in which both the inner and outer walls of the conidiogenous cell swell out to form the conidium.

Homothallic: self-compatible; sexual reproduction of a homothallic fungus can take place within an individual strain.

Hülle cell: a large, thick-walled, sterile cell found in some *Aspergillus* spp.

Hyaline: colorless, transparent, translucent.

Hypha (plural, hyphae): one of the individual filaments that make up the mycelium of a fungus.

Hyphomycete: an artificial taxonomic grouping referring to anamorphic molds that form conidia directly on the hyphae or on specialized conidiophores.

Macroconidium (plural, macroconidia): the larger of two different sizes of conidia produced by a fungus in the same manner.

Meristematic: perpetual increase in biomass in all directions with concordant septum formation.

Merosporangium (plural, merosporangia): a cylindrical outgrowth from the end of a sporangiophore in which a chain-like series of sporangiospores is produced, characteristic of *Syncephalastrum* spp.

Metula (plural, metulae): a conidiophore branch that bears phialides, characteristic of *Aspergillus* and *Penicillium* spp.

Microconidium (plural, microconidia): the smaller of two different sizes of conidia produced by a fungus in the same manner.

Mitosporic: an anamorphic fungus.

Mold: a filamentous fungus.

Moniliaceous: hyaline or lightly colored.

Muriform cell: a thick-walled, darkly pigmented cell found in tissues affected by chromoblastomycosis.

Mycelium: a mass of branching filaments that make up the vegetative growth of a fungus.

Oligokaryotic cell: a cell with several nuclei.

Oospore: a sexual spore produced in the Oomycota.

Ostiole: the opening through which spores are released from an ascocarp or pycnidium.

Pauciseptate: having few septa.

Perithecium (plural, perithecia): a flask-shaped ascocarp with an apical opening (ostiole) through which the ascospores are released.

Phialide: a specialized conidiogenous cell from which a succession of spores is produced.

Pleoanamorphism: term used to describe a fungus that has more than one anamorph.

Pleomorphic: of or pertaining to a nonsporing strain of a fungus.

Pseudohypha (plural, pseudohyphae): a chain of yeast cells that have arisen as a result of budding and that have elongated without becoming detached from each other, forming a hypha-like filament.

Punctate: marked with small spots (a term used to describe colonies).

Pycnidiospore: a conidium formed within a pycnidium.

Pycnidium (plural, pycnidia): a flask-shaped structure with an apical opening (ostiole) inside which conidia are produced.

Pyriform: pear-like in shape.

Rhizoid: a short branching hypha that resembles a root.

Sclerotic body: *see* Muriform cell.

Sclerotium (plural, sclerotia): a firm mass of hyphae, normally having no spores in or on it.

Septate: having cross walls or septa.

Septum (plural, septa): a cross wall in a fungal hypha or spore.

Sessile: not having a stem.

Sporangiole: *see* Sporangiolum.

Sporangiolum (plural, sporangiola): a small sporangium that contains a small number of asexual spores, characteristic of the Mucorales.

Sporangiophore: a specialized hypha upon which a sporangium develops. Sporangiospore: an asexual spore produced in a sporangium, characteristic of the Glomeromycota.

Sporangium (plural, sporangia): a closed sac-like structure containing asexual spores, characteristic of the Glomeromycota. This term has also been used for members of kingdom Straminipila to denote the segmented hyphal structures (and not the vesicles containing zoospores) that give origin to a germ tube that develops terminal vesicles in which biflagellate zoospores are cleaved.

Sporodochium (plural, sporodochia): a specialized structure in which conidia are borne on a compact mass of short conidiophores.

Stroma (plural, stromata): a solid mass of hyphae, sometimes bearing spores on short conidiophores, or having ascocarps or pycnidia embedded in it.

Sympodial: developing a single conidium at successive sites along a lengthening conidiogenous cell.

Synanamorph: any one of two or more anamorphs that have the same teleomorph.

Synnema (plural, synnemata): a compact group of erect and sometimes fused conidiophores bearing conidia at the tip, along the upper portion of the sides, or both.

Teleomorph: the sexual form of a fungus.

Thallic: one of the two basic forms of conidiogenesis in which enlargement of the conidial initial occurs after a delimiting septum has been laid down.

Thallus: the vegetative growth of a fungus.

Vesicle: the swollen tip of the conidiophore in *Aspergillus* spp., or the swollen part of a sporogenous cell in other fungi.

Villose: covered with long hairs (a term used to describe spores).

Yeast: a unicellular, budding fungus.

Zoospore: a motile asexual spore.

Zygospore: a thick-walled sexual spore produced in the Glomeromycota.

The findings and conclusions in this chapter are those of the authors and do not necessarily represent the views of the CDC. The use of product names in this manuscript does not imply their endorsement by the U.S. Department of Health and Human Services.

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Specimen Collection, Transport, and Processing: Mycology

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Successful laboratory diagnosis of fungal infections requires attentiveness on the part of physicians and nurses, proper collection and transport of appropriate specimens, and comprehensive procedures in the laboratory. This chapter offers guidelines for specimen collection and transport, specimen handling, specimen pretreatment and processing in the laboratory, medium selection, and incubation of cultures.

SPECIMEN COLLECTION AND TRANSPORT

As in bacteriology, the goals of a good mycology laboratory are to accurately isolate and identify fungi suspected of causing infection. It is our responsibility to provide the guidelines for proper specimen selection, collection, and transport to the laboratory. Table 1 is a listing of the types of specimens most commonly submitted for fungal culture (1–3) (see also Fig. 1). Once collected properly, all specimens should be transported in leak-proof sterile containers and processed as soon as possible. Anaerobic transport media or anaerobic containers should never be used for fungi. Fungi are quite resilient, but because some fungi can be affected by temperatures above 37°C and below 10°C, transport at room temperature is recommended. Dermatophytes are particularly sensitive to cold temperatures. With the exception of skin, hair, and nails, specimens that contain the normal bacterial biota should be transported as rapidly as possible because bacterial overgrowth can inhibit slower-growing fungi as well as reduce fungal viability. If such specimens cannot be transported to the laboratory within 2 h, they should be stored at 4°C.

As with other infectious diseases, the best specimen for determining the causative agent comes from the active infective site (e.g., cerebrospinal fluid [CSF] for meningitis). For a number of fungal diseases, however, peripheral specimens as well as specimens from the active infective site may also be useful. Table 2 is a listing of the clinical sites associated with recovery of different pathogenic fungi. Laboratories should not hesitate to suggest that peripheral specimens be taken when specific fungal diseases are suspected. Prostate fluid, for example, is an excellent high-yield specimen when endemic mycoses are suspected, but it is a specimen not often submitted to clinical laboratories (4–8).

Fortunately, many of the specimen collection and transport guidelines for mycology are similar to those used in bacteriology. In those occasions where they differ, it is critical to convey that information to physicians and nurses. One such difference is in specimen volume. The volume of material required for fungal cultures usually exceeds that used in bacteriology because several types of specimens (body fluids, respiratory secretions, etc.) need to be concentrated or pretreated prior to plating to maximize recovery of fungi. In general, except for a few specific sites noted in Table 1, specimens submitted on swabs are not optimal for recovering fungi, and this practice should be discouraged.

Mycology laboratories should be encouraged to offer physicians different types of fungal cultures. The choice of media used for primary isolation as well as the length and temperature of incubation can vary with the culture request. In my laboratory, fungal culture choices include a dermatophyte culture for hair, skin, and nail specimens; a rule-out *Candida* culture for vaginal, urine, skin, and throat specimens; a fungal blood culture (lysis-centrifugation culture); and a complete fungal culture. By choosing the culture type, physicians can signal the laboratory when they suspect a specific pathogen, which can often reduce the time that cultures need to be kept in the laboratory.

SPECIMEN HANDLING, PRETREATMENT, AND SAFETY

If specimens that are unacceptable for any reason are received in the lab, they should be rejected and appropriate physicians should be notified. Poor-quality specimens can result in incorrect information, including false-negative results. As required by the Joint Commission (formerly called JCAHO), a requisition must accompany each specimen and must include the following: patient name, age, sex, and location or address, physician name, specific culture site, date and time of specimen collection, name of person who collected the specimen, clinical diagnosis, and any special culture request. In addition, each specimen must have a firmly attached label indicating the patient name, location, physician, and date and time of collection (9).

Pretreatment of several specimen types is necessary to maximize the recovery of fungi (2, 3). While this takes additional time and effort, it allows the lab to make the most out of every specimen submitted, particularly for those that are difficult to obtain from patients. Pretreatment procedures are listed in Table 3 and include centrifugation of urine and sterile body fluids, mincing of nail and tissue

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TABLE 1 Specimen collection and transport guidelines[a]

Specimen type	Collection procedure	Processing procedure	Transport time and temp	Comments
Abscess (drainage, exudate, pus, wound)	Clean surface with 70% alcohol. Collect from active peripheral edge with sterile needle and syringe. If open, use swab system or aspirate.	If thick, pretreat similarly to sputum specimen.	If ≤2 h, RT	Examine for grains or granules and note color if present.
Blood	Disinfect skin with iodine tincture or chlorhexidine prior to obtaining (2). Use maximum volume of blood recommended for the system used.	Manual	If ≤2 h, RT; if longer, RT	All systems for bacteria can recover all yeasts except for *Malassezia* spp. but do not recover molds. Special fungal media for automated systems are best for molds.
		Biphasic (Septi-Chek)	If ≤2 h, RT; if longer, RT	
		Automated (Bactec [BD Diagnostics, Sparks, MD] BacT/Alert [bioMérieux, Durham, NC] VersaTrek [TREK Diagnostic Systems Cleveland, OH])	If ≤2 h, RT; if longer, RT	
		Lysis-centrifugation (manual or Isolator)	If ≤2 h, RT; if longer, RT but process in ≤16 h	Lysis-centrifugation systems are good for recovery of molds, especially those causing endemic mycoses. They give high contamination and false-positive rates.
Bone marrow	Collect aseptically in a heparinized syringe or lysis-centrifugation tube.	Clotted bone marrow is an unacceptable specimen.	If ≤15 min, RT; if longer, RT	Pediatric Isolator tubes are best.
Catheter tip (intravascular)	Remove distal 3 to 5 cm of line tip and place in sterile container.	Method of Maki at al. (39) used for catheter tips but not validated for fungi.	If ≤15 min, RT. If longer, 4°C	Avoid media containing cycloheximide.
Cutaneous specimen (hair, skin, nails)	Disinfect all types with 70% alcohol. Hair: hair root is most important, plucking is best; submit 10 to 12 hairs in sterile dry container or envelope. Skin: scrape with dull edge of a scalpel or glass slide, or vigorously brush in a circular motion with a soft-bristle toothbrush. Nails: clip or scrape with a scalpel. Material under nail should also be scraped. Submit in sterile container or clean, dry paper envelope.	Only the leading edge of a lesion should be sampled, as centers are often nonviable. All specimens should be pressed gently into the agar with a sterile swab; do not streak agar plates. If used, toothbrushes should be pressed gently into agar as well (Fig. 1).	If ≤72 h, RT (very stable). Never refrigerate, as dermatophytes are sensitive to cold.	Select hairs that fluoresce under a Wood's light. Hair and skin can be collected with a soft-bristle toothbrush. For pityriasis versicolor (M. *furfur*), olive oil or a paper disk saturated with olive oil should be placed on the first quadrant of agar plate.
Eye (corneal scraping, vitreous humor)	Corneal scraping: taken by physicians and media/slides inoculated directly.	Corneal: inoculate noninhibitory media in X- or C-shaped motion.	If ≤15 min, RT; if longer, RT	Very little material is usually available. Avoid media with cycloheximide.
	Vitreous humor: needle aspiration.	Vitreous humor: concentrate by centrifugation; use sediment for media and smears.	If ≤15 min, RT; if longer, RT	

(Continued on next page)

TABLE 1 Specimen collection and transport guidelines[a] *(Continued)*

Specimen type	Collection procedure	Processing procedure	Transport time and temp	Comments
Medical devices	Collected surgically. Transport in sterile container.	Use sterile scalpel to collect (by scraping) biofilm or vegetative growth.	If ≤15 min, RT; if longer, 4°C	Avoid media containing cycloheximide. Device material is recovered best by using liquid medium.
Prostate fluid	Have patient empty bladder, and then massage prostate gland to yield fluid.	Inoculate media directly or transport in sterile wide-mouth container.	If ≤15 min, RT; if longer, RT	Fluid should always be examined microscopically. The first urine following massage has a high yield. This fluid is excellent for detection of endemic mycoses.
Respiratory tract, lower (sputum, bronchial aspirate, bronchoalveolar lavage fluid)	Use first morning sputum collected after brushing teeth. Collect brushings and bronchoalveolar lavage fluid surgically. Place all samples in sterile containers. Inoculate media containing antimicrobial agents with and without cycloheximide.	Viscous lower respiratory specimens should be pretreated and centrifuged to concentrate their contents.	If ≤2 h, RT; if longer, 4°C	Saliva or 24-h sputum is an unacceptable specimen. Methods for mycobacteria decontamination are not acceptable.
Respiratory, upper (oral, oropharyngeal, and sinus samples)	Swab oral lesions, avoiding tongue. Use a thin wire or flexible swab for oropharynx. Collect sinus contents surgically.	Use swab transport system for oral and oropharyngeal samples. Place sinus contents in sterile container.	Oral: if ≤2 h, RT; if longer, RT Sinus: if ≤15 min, RT; if longer, RT	Selective and chromogenic media are best for recovery of *Candida*.
Sterile body fluids (CSF and pericardial, peritoneal, and synovial fluids)	Collect as for bacteriology. Concentrate by centrifugation, and use sediment for inoculation. Clots should be ground.	Except CSF, put sterile body fluids in sterile Vacutainer tubes with heparin or in a lysis-centrifugation tube to prevent blood clotting. Except for CSF, blood culture bottles can be used for recovery of yeast.	If ≤15 min, RT; if longer, RT; never refrigerate.	Sterile fluid sediment should always be examined microscopically. With specimen volumes ≤ 2 ml, fluid should be plated directly, using as much fluid on each plate as possible.
Stool	Specimen use should be discouraged.			
Tissue	Collected surgically. A larger volume is needed than for bacteriology.	Use a sterile container; keep moist (with saline drops) to prevent drying. Except with *H. capsulatum*, mincing, not grinding, is critical. Tissue pieces should be pressed into the agar so they are partially embedded. Grind tissue for recovery of *H. capsulatum*.	If ≤15 min, RT; if longer, RT	Tissue biopsy recommended for invasive disease. Examine subcutaneous tissue for granules (see information for abscesses).
Urine	First morning clean catch, suprapubic, or catheterized specimens; 24-h specimens are unacceptable.	Use a sterile container or urine transport system. Concentrate specimens by centrifugation, and use sediment for inoculation.	If ≤2 h, RT; if longer, 4°C; urine transport systems can stay at RT for up to 72 h.	Chromogenic media best for *Candida*. Use sediment for microscopic examination.
Vaginal	Collect as for bacteriology.	Swab transport system or sterile container for washings.	If ≤2 h, RT; if longer, RT	Antibacterial media or chromogenic agars are best for recovery of *Candida*.

[a] Abbreviations: CSF, cerebrospinal fluid; RT, room temperature.

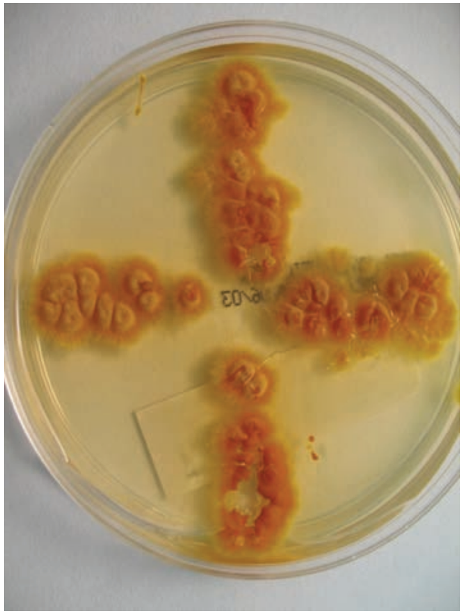

FIGURE 1 Growth of a dermatophyte (*Trichophyton soudanense*) after 4 days of aerobic incubation at 30°C following toothbrush inoculation of Mycosel agar. Courtesy of Marilyn A. Leet, Children's Hospital of Philadelphia, Philadelphia, PA. doi:10.1128/9781555817381.ch114.f1

specimens, lysis and centrifugation of blood or bone marrow received in Isolator tubes (Wampole Laboratories, Cranbury, NJ), and lysis by mucolytic agents, followed by centrifugation, for respiratory secretions. Such procedures release fungi enclosed within cells, concentrate fungal material in the specimen, and help to reduce or eliminate bacteria present in contaminated specimens because of the action of mucolytic agents, such as *N*-acetyl-L-cysteine, 5% oxalic acid, or dithiothreitol (Sputolysin).

All work in mycology should be carried out in a certified type 2 laminar-airflow biosafety cabinet whenever possible. There are different biosafety regulations in Europe and other countries, and for this reason, practices may differ. Biosafety level 2 procedures are recommended for personnel working with clinical specimens that may contain dimorphic fungi as well as other potential pathogenic fungi. Gloves should be worn for processing specimens and cultures. A number of techniques are available for examining clinical specimens microscopically, and these are discussed in chapter 116 in this *Manual*.

SPECIMEN PROCESSING AND CULTURE GUIDELINES

Abscess (Drainage, Exudate, Pus, and Wound Material)

Abscess specimens should be collected from the active peripheral edge of open abscesses or aspirated from closed abscesses by use of a syringe. Abscess, pus, or drainage material should be examined for grains or granules by use of a dissecting microscope. The presence of grains or granules is indicative of a mycetoma. If none are present, the material can be inoculated directly onto media. If the specimen is thick, it should be pretreated similarly to a sputum specimen. If present, grains and granules should be teased out of the specimen and washed in sterile distilled water, sterile saline, or either solution plus antibiotics. The color of the granules should be noted and recorded. A portion should be crushed between two glass slides and examined microscopically for the presence of hyphae. Both true fungal hyphae and bacteria (branching Gram-positive rods) can be observed with

TABLE 2 Common clinical sites for laboratory recovery of pathogenic fungi

Disease	Recovery from specimen[a]														
	Blood	Bone	Bone Marrow	Brain	CSF	Eye	Hair	Nails	Joint fluid	Prostate Fluid	Lower Respiratory Lower	Sinus/nasal Cavity	Skin[b]	Tissue	Urine
Aspergillosis		X		X		X		X			X	X	X	X	X
Blastomycosis		X		X	X				X	X	X	X	X	X	X
Candidiasis	X	X	X	X	X	X		X	X	X	X	X	X	X	X
Chromoblastomycosis				X								X	X	X	
Coccidioidomycosis	X	X	X	X	X				X	X	X	X	X	X	X
Cryptococcosis	X	X	X	X	X	X					X		X	X	X
Fusariosis (hyalohyphomycosis)	X		X	X		X		X	X		X	X	X	X	
Histoplasmosis	X	X	X	X	X	X					X		X	X	X
Mucormycosis/ entomophthoromycosis		X		X		X					X	X	X	X	
Paracoccidioidomycosis	X		X	X	X					X	X	X	X	X	
Penicilliosis/*Talaromyces marneffei*	X	X	X	X					X				X	X	X
Pneumocystosis		X	X								X			X	
Pseudoallescheriosis/scedosporiosis	X		X		X	X					X		X	X	
Sporotrichosis	X	X			X						X	X	X	X	
Trichosporonosis	X	X	X	X	X	X			X	X	X	X	X	X	X

[a] X, recovery of fungus from indicated specimen.
[b] Includes skin and mucous membranes.

TABLE 3 Pretreatment of clinical specimens prior to plating

Specimen	Pretreatment	Comments
Abscess, drainage, pus, granules	Granules should be washed and crushed; other materials should be centrifuged at 2,000 × *g* for 10 min.	Essential for best recovery
Blood, bone marrow	Lysis in Isolator tubes and then centrifugation for 30 min at 3,000 × *g*, using a 35° fixed-angle rotor or swinging bucket	Critical for detection of *H. capsulatum* and other dimorphic fungi
Body fluids	Centrifugation at 2,000 × *g* for 10 min, or membrane filtration	Essential for best recovery with volumes of ≥1 ml; blood clots should be teased apart
Nails	Mince into tiny pieces; push pieces down into agar	Essential for maximum recovery of dermatophytes
Respiratory secretions (bronchoalveolar lavage, sputum)	Lysis with mucolytic agents[a] followed by centrifugation at 2,000 × *g* for 10 min	Critical for *Pneumocystis jirovecii*; improves recovery for other mycoses
Tissue	Mince into tiny pieces or grind in a mortar; push pieces down into agar	Essential for best recovery; for zygomycetes and other molds, mincing is best; for *H. capsulatum*, grinding is best
Urine	Centrifugation at 2,000 × *g* for 10 min	Essential for best recovery, particularly with deep mycoses

[a] *N*-Acetyl-L-cysteine, 5% oxalic acid, or dithiothreitol (Sputolysin).

grains. If branching Gram-positive rods are seen, a modified acid-fast stain should be performed to look for *Nocardia*. Another portion of the grains and granules should be crushed by using sterile technique (sterile glass rod or mortar and pestle) and then inoculated directly onto media (10).

Blood

Fungemia is a major cause of morbidity and mortality in hospitalized patients, with *Candida* species being the major cause (11, 12). Early detection of organisms in the bloodstream is incredibly important because it is an indicator of disseminated disease. As in bacteriology, the volume of blood, the blood-to-broth ratio, and the number of blood cultures are all critical factors, with the volume of blood being the most important variable. For adults, 20 to 30 ml per culture, divided between two bottles, is recommended for the highest recovery rate (13–15) and the shortest time to detection (16). Studies recommend a 5- to 10-fold dilution of blood in broth, and dilutions of <1:5 may result in reduced recovery of organisms (11, 17). For infants and children, total blood volumes based on the weight of the patient are presently recommended (17–19). Unlike bacteremia, fungemia is almost always continuous in patients with infectious endocarditis, so the timing of obtaining a blood culture for fungi is not critical (13). Both iodine tincture and chlorhexidine are effective for skin decontamination prior to obtaining the blood culture (20). In a review of 270 cases of fungal endocarditis in the world literature, the organisms responsible were *Candida albicans* (24%), non-*C. albicans Candida* spp. (28%), *Aspergillus* spp. (24%), *Histoplasma* spp. (6%), and other yeasts and molds (17%) (21). In Europe, if blood cultures are negative in patients with suspected *Candida* endocarditis, then a laboratory diagnosis can often be achieved by *Candida* serology testing.

There are presently a wide variety of manual, biphasic, automated, and continuous monitoring systems for blood cultures, but no single commercial system or culture medium can detect all potential blood pathogens. If manual blood cultures are used, a biphasic system is best for fungi (Septi-Chek; Becton Dickinson Diagnostic Systems, Sparks, MD), and the agar slant should be rewashed with the broth-blood mixture each time the bottles are examined. Many automated and continuous monitoring systems are available, and several have medium modifications to enhance fungal growth. These include the Bactec (BD Diagnostics, Sparks, MD), BacT/Alert 3D (bioMérieux, Durham, NC), and VersaTrek (Trek Diagnostic Systems, Cleveland, OH) blood culture systems. Use of these mycosis bottles ensures the highest sensitivity for detecting fungemia (22). Studies evaluating all of these systems have shown that they can recover all pathogenic yeast species except for *Malassezia* spp. Even without specific fungal media, automated and continuous monitoring systems are able to recover *Candida*, *Cryptococcus*, *Rhodotorula*, and *Trichosporon* spp., with sensitivities equal to or higher than those of manual or lysis-centrifugation methods (13, 16, 23, 24–30). Automated systems with routine bacteriology media are not, however, satisfactory for molds and *Nocardia* spp. (30).

Lysis-centrifugation performed either manually (13) or by using the commercially available Isolator collection system is a more sensitive method for recovery of molds and dimorphic fungi such as *Histoplasma capsulatum* (30–32). Several studies, however, have argued against the routine use of lysis-centrifugation for all fungal blood cultures because of high contamination rates, high false-positivity rates, and equivalent or shorter times to detection of yeasts than those with automated systems (24, 25, 33). If molds are suspected, either a special fungal medium for an automated system, such as Bactec Myco/F lytic medium or BacT/Alert MB, or a lysis-centrifugation system should be considered (34). Blood placed in either adult or pediatric Isolator tubes should be kept at room temperature until it is processed, ideally within 16 h of collection. Sediment from lysis-centrifugation should be streaked onto a variety of enriched media not containing cycloheximide and onto a chocolate agar plate (35). The only yeast species requiring special processing are the *Malassezia* species, which require lipids for growth. This can be achieved by overlaying solid media with a thin layer of olive oil or by adding a paper disk saturated with olive oil to a subculture plate or plates containing sediment from a lysis-centrifugation tube (10, 17, 36). Specialized media such as modified Dixon's, Leeming's, and Ushijima's media can also be used to isolate *Malassezia* species, if available.

Bone Marrow

Bone marrow is most useful for the diagnosis of disseminated candidiasis, cryptococcosis, and histoplasmosis. Approximately 0.5 ml (pediatrics) to 3 ml (adults) should be collected aseptically in a heparinized syringe or pediatric Isolator tube. Because lysis-centrifugation enhances the recovery of *H. capsulatum* and other molds, the use of Isolator tubes is the method of choice for these organisms. Clotted bone marrow is an unacceptable specimen. With the exception of *H. capsulatum*, fungi are rarely seen in bone marrow aspirates from immunocompetent hosts. For immunocompromised patients, however, bone marrow is an excellent specimen, and *Aspergillus* spp., *Candida* spp., *Cryptococcus* spp., *H. capsulatum*, and *Talaromyces marneffei* can all be observed (37). While it is quite clear that microscopic examination of Giemsa-stained bone marrow can be diagnostic, recent data show that compared with other, less-invasive methods, such as blood cultures, bone marrow aspirate cultures are of limited value and should be performed only selectively (38). Bone marrow should not be placed into blood culture bottles because with many continuously monitoring systems, the specimen will quickly register a false-positive result due to CO_2 from massive numbers of white blood cells.

Catheter Tips (Intravascular)

If performed simultaneously with blood cultures, quantitative bacterial and fungal cultures have been advocated to demonstrate catheter tip colonization. Acceptable specimens are intravenous or intra-arterial catheter tips, with the distal 3 to 5 cm of the line tip being submitted to the laboratory for culture. Specimens should be placed in a sterile container and transported and stored at room temperature. The semiquantitative method of Maki et al. (39) is the most common method used in clinical laboratories, where the catheter tip is rolled across the surface of an agar plate four times and cultures yielding ≥15 CFU are considered positive. The semiquantitative method distinguishes infection (≥15 colonies) from contamination and is considered a more specific method for diagnosing catheter-related septicemia than culturing the catheter tip in broth.

Cutaneous Specimens (Hair, Skin, and Nails)

Hair

The hair root is the most important part to culture for detection of fungi, so plucking or pulling rather than cutting hair is recommended. The area should be cleaned with 70% alcohol and allowed to dry. Infected hairs can appear dull, broken, and faded. Hairs that fluoresce under Wood's light should also be selected for culture. Hair should be submitted in a sterile container or clean, dry paper envelope. Hair can also be collected by using a soft-bristle toothbrush and rubbing in a circular motion over margins or patches of alopecia (hair loss) (40).

Skin

The area should be cleaned with 70% alcohol and allowed to dry. The skin should then be scraped with the dull edge of a scalpel or glass slide or vigorously brushed in a circular motion with a soft-bristle toothbrush (40). Only the leading edge of a skin lesion should be sampled, because the centers of lesions are frequently nonviable. Skin can be submitted in a sterile container or clean, dry paper envelope or on a toothbrush.

Nails

Nails should be cleaned with 70% alcohol and then clipped or scraped with a scalpel. If material is present under the leading edge of the nail, it should also be scraped and submitted in a sterile container or paper envelope designed specifically for this purpose. Prior to being plated, nail pieces should be pulverized or minced into tiny pieces by use of a scalpel.

All cutaneous specimens should be inoculated into the agar medium by gently pressing them onto the agar with a sterile swab or scalpel. Distribute the pieces evenly over the agar surface; do not streak the plate with a sterile loop. If a toothbrush is used to collect skin and/or hair specimens, the brush should be pressed gently onto the surface of the agar in four or five places on the plate, leaving an imprint. If organisms are present, growth will occur within the bristle imprint. By nature, cutaneous specimens are usually contaminated with bacteria. For this reason, a plate of inhibitory medium with chloramphenicol and cycloheximide, such as Mycosel, should be used for dermatophytes. Because *Trichosporon* spp., the cause of white piedra, in addition to *Scopulariopsis* and *Fusarium* species, are sensitive to both chloramphenicol and cycloheximide, a noninhibitory medium such as Sabouraud dextrose agar should also be inoculated with nail specimens. For patients suspected of having pityriasis versicolor, caused by *Malassezia furfur*, direct microscopy is normally diagnostic, but a noninhibitory medium such as Sabouraud dextrose agar can be inoculated, and then olive oil or a paper disk saturated with olive oil should be placed on the first quadrant of the plate. *Malassezia furfur* grows best at ≥ 35°C but can grow at 30°C. Cutaneous specimens should never be refrigerated because dermatophytes are sensitive to low temperatures.

Eye Specimens (Corneal Scrapings and Vitreous Humor)

Several types of eye infections require that corneal scrapings and/or vitreous humor be obtained by an ophthalmologist. These include mycotic or fungal keratitis, fungal endophthalmitis, and extension oculomycosis. Mycotic or fungal keratitis is an infection of the cornea. The most common causes are *Acremonium* spp., *Aspergillus* spp., *C. albicans*, *Candida parapsilosis*, *Candida tropicalis*, *Curvularia* spp., and *Fusarium* spp. (41, 42). Fungal endophthalmitis is a late-stage result of hematogenous dissemination of a systemic fungal infection. It can involve many areas of the eye and surrounding tissues. The most common causes are *Aspergillus* spp., *Blastomyces dermatitidis*, *Candida* spp., *C. neoformans*, *Coccidioides* spp., *H. capsulatum*, *Paracoccidioides brasiliensis*, and *Sporothrix schenckii* (41, 42). Extension oculomycosis is a result of rhinocerebral mucormycosis, and like fungal endophthalmitis, it may involve many areas of the eye and surrounding tissues. The diagnosis of these infections requires attempting to demonstrate the organism on a microscopic exam plus positive culture. Corneal scrapings are taken by physicians, and media are inoculated directly by use of a heat-sterilized platinum spatula (43). Very little material is usually obtained because of the risks of corneal thinning or perforation. Physicians should be instructed to first inoculate the specimen directly onto a noninhibitory medium, such as Sabouraud dextrose agar, and then to place some material on a sterile glass slide (in the center) for staining. The scraping should be placed in two or three places on the plate, using an X- or C-shaped motion (3). The inoculated plate should be kept at room temperature and transported immediately to the laboratory. Vitreous, or vitreous humor, is the clear, gelatinous material that fills the space between the lens of the eye and the retina. When taken by physicians, vitreous is often diluted with irrigation fluid. For this reason, it should be concentrated by centrifugation, and the sediment should be used to inoculate media and to make smears. Specimens should be placed onto Sabouraud dextrose agar, inhibitory mold agar, and/or brain heart infusion (BHI) agar with 10% sheep blood and incubated at 30°C. Media containing cycloheximide should be avoided (41, 42, 44).

Medical Devices

A wide variety of medical devices (contact lenses, stents, wound-healing dressings, contraceptives, surgical implants, replacement joints, etc.) may be submitted for fungal culture. Most are collected surgically and should be submitted in a sterile container and transported and stored at room temperature. Each should be examined for vegetative growth and biofilms, and if these are present, they should be scraped from the device by use of a sterile scalpel for direct inoculation of agar media and broth (3). Specimens should be placed onto Sabouraud dextrose agar, inhibitory mold agar, and/or BHI agar with 10% sheep blood and incubated at 30°C. Media containing cycloheximide should be avoided. If biofilm or vegetative growth areas are not obvious, portions of the device should be placed in a broth medium such as BHI broth and incubated at 30°C.

Prostate Fluid

Prostate fluid consists of secretions of the testes, seminal vesicles, prostate, and bulbourethral glands. After the bladder is emptied, the prostate gland is massaged to yield pure prostatic fluid. The prostate is frequently seeded when organisms are present in the bloodstream. A key clinical sign in males with endemic mycoses is the complaint of a history of chronic urinary tract infections but negative urine cultures. Prostatic fluid for such patients is frequently positive when cultured for fungi. The secretions should also be examined microscopically. After the prostate fluid is obtained, the next urine specimen should also be obtained and submitted for culture because this urine has a high yield (4–8).

Lower Respiratory Tract Specimens (Sputum, Bronchial Aspirate, and Bronchoalveolar Lavage Fluid)

After a patient's teeth have been brushed, sputum should be collected as a first morning specimen. Neither saliva nor 24-h sputum specimens are acceptable for fungal culture. Viscous lower respiratory tract specimens should be pretreated before being processed. Lysis with mucolytic agents, such as *N*-acetyl-L-cysteine, 5% oxalic acid, or dithiothreitol (Sputolysin), followed by centrifugation at 2,000 × *g* for 10 min and then plating of the sediment, greatly increases the yield and improves the recovery of many fungi. Sodium hydroxide, which is used to concentrate specimens for detection of mycobacteria, should not be used because it inhibits the growth of many fungi. Unfortunately, centrifugation also increases the number of bacteria in the sediment, and for this reason, media containing antimicrobial agents, with and without cycloheximide, should be used. As in bacteriology, lower respiratory tract specimens should be examined for the presence of blood, pus, or necrotic portions, since these have the highest yields (45, 46). Because *Candida* spp. are the most common yeasts isolated from respiratory specimens of patients with cystic fibrosis, use of a chromogenic medium that is selective and differential for *Candida* is recommended (47). *Scedosporium* is now the second most common mold associated with cystic fibrosis, and use of a *Scedosporium*-selective medium containing the antifungal agents dichloran and benomyl has been proposed to improve isolation of this mold in this patient population (48).

Upper Respiratory Tract Specimens (Oral and Oropharyngeal Specimens)

The mucosal surface of gums, oral lesions, and oropharyngeal specimens submitted for fungal culture are usually screened for candidiasis. When thrush is suspected, lesions should be scraped gently with moist swabs and submitted for microscopy and a rule-out yeast culture. Antibacterial media or chromogenic agars for *Candida* spp. should be inoculated. While culture is not required to make the diagnosis of candidiasis, it can be useful if microscopy is not available in an outpatient setting or if species other than *C. albicans* are suspected. On rare occasions, oral lesions can be seen with histoplasmosis or paracoccidioidomycosis, but they do not resemble those seen with *Candida* spp. If these are suspected, a full fungal culture and microscopic smear should be performed. The use of nasal swabs should be discouraged because contamination from environmental spores in the nasal cavity makes interpretation of culture results difficult. Nasal tissue or sinus washings are better specimens and should be plated on a variety of media containing antibiotics, but not cycloheximide, since significant pathogens recovered from these sites (*Aspergillus* spp.) are sensitive to cycloheximide (49).

Sterile Body Fluids (CSF and Pericardial, Peritoneal, and Synovial Fluids)

With the exception of CSF, sterile body fluids are often placed in sterile Vacutainer tubes with heparin to prevent blood clotting. Lysis-centrifugation Isolator tubes can also be used for this purpose. With specimen volumes of ≥2 ml, these tubes plus CSF lumbar puncture tubes should be centrifuged at 2,000 × *g* for 10 min, and the sediment should be used to inoculate media. The supernatant fluid can be used for serologic tests (44). With specimen volumes of ≤2 ml, the specimen should be plated directly, using as much fluid on each plate as possible. Use of a Cytospin centrifuge to prepare microscopic smears is recommended (50, 51). Because sterile fluids are so rarely culture positive for fungi, many laboratories inoculate medium slants with screw-cap lids rather than plates to avoid questions concerning possible contamination. In some countries, a purity plate is placed in the biosafety cabinet when sterile body fluids or tissues are being processed. Growth on the purity plate would signal a contamination event during processing.

Stool

Submission of stool specimens for routine fungal culture should be discouraged. Many *Candida* spp. are part of the normal stool biota, and anything that disrupts the normal gastrointestinal tract biota, such as diet or use of antibiotics, can yield a predominance of yeast when stool is cultured. Neither colonization with yeast nor a predominance of yeast indicates invasive disease with *Candida*. If invasive disease of the gastrointestinal tract is suspected, a colonoscopy and tissue biopsy should be performed (52, 53).

Tissue

With one exception (*H. capsulatum*), fungi present in tissue are best recovered when the tissue is minced, not ground. For the mucoraceous molds, in particular, mincing is critical for the recovery of organisms. Tissues should be minced by use of a scalpel, and the pieces of tissue should be pressed into the agar so they are partially embedded (54; see Fig. 2). Two to four pieces should be placed onto each piece of medium being inoculated. Further streaking of the plate with sterile loops should not be done. When the medium is inoculated this way, fungi grow out directly from the piece of tissue. Laboratories often question whether growth on agar plates is contamination. When growth comes directly from the tissue piece, it is unquestionably significant growth. A portion of the specimen can be ground for microscopic examination/smears.

When *H. capsulatum* is suspected, the tissue should be ground or homogenized. Because this pathogen is intracellular, organisms need to be released from the cells to be available to grow on media. If needed, a small amount of sterile broth or distilled water can be added to smooth the process of grinding. Subcutaneous tissue should be examined for the presence of granules (as described above, for abscesses). Homogenate or tissue pieces should be inoculated onto enriched media containing antibacterial agents, and for systemic mycoses, enriched media containing both blood and antibacterial agents are best.

Urine

Clean-catch, suprapubic, or catheterized urine specimens should all be obtained as first morning specimens. Large volumes (10 to 50 ml) give the best results and should be centrifuged for maximum recovery, particularly for agents of deep mycoses. Urine should be centrifuged at 2,000 × *g* for 10 min. The sediment should be used for microscopic smears and inoculation of media. Quantification of organisms, as performed in bacteriology, is not useful (36). Twenty-four-hour urine specimens are not acceptable.

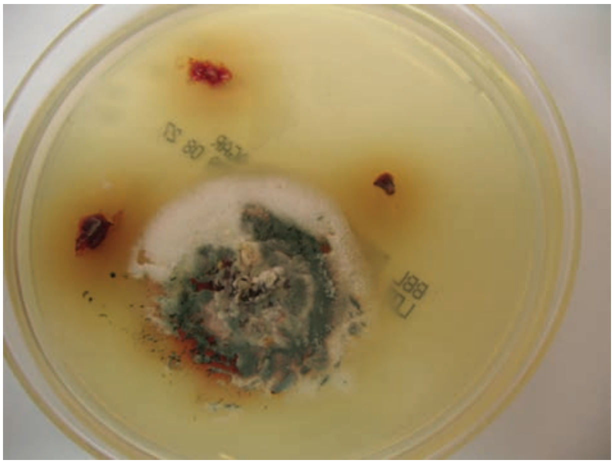

FIGURE 2 Early growth of *Talaromyces marneffei* from tissue piece embedded in Sabouraud dextrose agar with gentamicin incubated aerobically at 30°C. Courtesy of Marilyn A. Leet, Children's Hospital of Philadelphia, Philadelphia, PA. doi:10.1128/9781555817381.ch114.f2

Vaginal Specimens

Vaginal specimens submitted for fungal culture are frequently screened just for vaginal candidiasis. For this reason, having a rule-out *Candida* culture as a culture choice is helpful for clinicians and laboratorians. *Candida* spp. are part of the normal vaginal biota, and their presence alone is not significant. Appropriate clinical symptoms plus a positive microscopic exam or culture are sufficient to diagnose vaginal candidiasis. Culture is not required to confirm vaginal candidiasis, but microscopic exams are not available in all settings, while culture is available. Antibacterial media or chromogenic agars for *Candida* spp. should be inoculated. On rare occasions, vaginal lesions can be seen with histoplasmosis or paracoccidioidomycosis. These lesions do not resemble those seen with *Candida* spp. If these are suspected, a full fungal culture and microscopic smear should be performed.

SELECTION AND INCUBATION OF MEDIA

Table 4 is a listing of various media used for the recovery of fungi from clinical specimens, including primary media, selective and/or differential media, and specialized media. Optimal recovery of fungal pathogens depends upon a number of factors, including the choice of an appropriate specimen(s), collection and transport of a quality specimen, appropriate pretreatment of specimens, the media chosen for inoculation and incubation, a proper incubation temperature, and sufficient time of incubation. A wide variety of media are available for primary isolation, and in many laboratories, the choice is based on personal experience and the technologist's preferences. As with bacteria, a battery of several media needs to be used because no single fungal medium is sufficient for detection of all of the clinically important fungi. Several factors should be considered in selecting a battery of several media, as follows.

- Choices of media should be driven by the type of specimen being processed (1–3). Media that inhibit bacterial growth but allow for the growth of fungi should be used for nonsterile specimens that may contain large numbers of bacteria (BHI or Sabhi medium plus antibiotics or inhibitory mold agar). The eukaryotic protein synthesis inhibitor cycloheximide should be used in one medium choice, with or without antibiotics. The battery for sterile body sites should include one or two media lacking antibiotics plus one medium with antibiotics.
- Cycloheximide is a component in several primary media and suppresses saprophytic fungi, but it is known to inhibit the growth of some *Aspergillus* spp., some *Candida* spp., *Cryptococcus* spp., *Nattrassia mangiferae*, *Pseudallescheria boydii*, *Talaromyces marneffei*, most members of the Mucorales, *Neoscytalidium dimidiatum*, *Scopulariopsis*, *Trichosporon asahii*, and many saprophytic or opportunistic fungi. While cycloheximide prevents fast-growing contaminants from overgrowing slow-growing pathogens such as *H. capsulatum*, it should not be included in all medium choices.
- Traditionally, Sabouraud dextrose agar without antibiotics (SDA) has been used as a primary medium choice. Inhibitory mold agar is a better choice because of its ability to inhibit bacterial growth more effectively than SDA (55).
- Chloramphenicol, with or without other antibiotics, is included in several primary media to suppress the growth of bacteria. While these are excellent medium choices for processing of specimens that contain the bacterial

TABLE 4 Media suggested for the recovery of fungi from clinical specimens

Medium[a]	Properties	Comments
Primary media without antibacterials or antifungals		
BHI	With or without sheep blood	Supports growth of all fungi
Littman oxgall agar	Excellent for primary isolation of fungi and dermatophytes	Supports growth of all fungi; oxgall prevents spreading of colonies
SDA	4% glucose, slightly acid pH	Good for dermatophytes but overgrows with bacteria; poor choice for primary media; C. *glabrata* can grow slowly
SDA, Emmons' modification	2% glucose, near-neutral pH (6.9) is better for fungi	Supports growth of all fungi
Sabhi	With or without sheep blood	Supports growth of all fungi, but designed for dimorphics; blood can inhibit sporulation
Primary media with antibacterials or antifungals		
Any of the above media	Choices of chloramphenicol, ciprofloxacin, gentamicin, penicillin, streptomycin alone or in combination	Inhibit bacteria well; BHI and Sabhi with blood plus antibiotics are best choices for dimorphics
Inhibitory mold agar	Contains chloramphenicol to inhibit bacteria	For isolation of pathogenic fungi other than dermatophytes
Mycosel or Mycobiotic	SAB with chloramphenicol, cycloheximide, and 1% glucose	Inhibits bacteria, but cycloheximide inhibits many pathogenic fungi
Selective/differential media Dermatophyte test medium or dermatophyte identification medium	For diagnosis of dermatophytes using color change of medium as indicator; contains antibiotics to inhibit bacteria	Frequent false-positives with dermatophyte test medium from non-dermatophyte fungi
	Used with chloramphenicol and ammonium hydroxide	Recovery of dimorphics from contaminated specimens
Yeast extract phosphate CHROMagar *Candida* or Albicans ID agar	Selective and differential medium for isolation and presumptive identification of *Candida* spp.; includes chromogenic substrates plus antibacterials	Most bacteria inhibited; excellent for detecting mixed cultures of yeast; number of *Candida* species detected manufacturer dependent
Specialized media		
Corn meal agar	With and without Tween 80	Tween 80 addition results in rapid and abundant chlamydospore formation; supports growth of most fungi but has no bacterial inhibitors
Potato dextrose agar and potato flake agar	Stimulate production of spores and sporulating bodies	Often used for slide cultures; potato flake agar is best for these
Rapid-sporulation agar	Sodium acetate agar	For yeast, induces ascospore production
Scedosporium-selective medium	Contains dichloran and benomyl	May be necessary to detect *Scedosporium* in respiratory specimens from patients with cystic fibrosis
Niger seed or bird seed and esculin base medium	Contain chloramphenicol; for selective isolation and identification of *Cryptococcus* spp.	*Cryptococcus* spp. appear as dark brown colonies

[a] Abbreviations: BHI, brain heart infusion agar; SDA, Sabouraud dextrose agar; Sabhi, Sabouraud dextrose and brain heart infusion agar.

biota, they prevent the growth of *Nocardia* and aerobic actinomycetes. Mycology laboratories that are responsible for culturing these organisms must include other media for these pathogens, such as Sabouraud agar.

- Dimorphic fungi that grow endemically grow best on an enriched medium (BHI or Sabhi medium) with antibiotics and 5 to 10% sheep blood. The sheep blood promotes the growth of dimorphic fungi but inhibits sporulation. Molds recovered on this enriched medium should be subcultured immediately to blood-free enriched medium to promote conidiation and for DNA probe testing.
- Laboratories expecting to frequently isolate and identify *Cryptococcus* spp. should consider esculin-based medium with chloramphenicol and gentamicin for use as a primary medium with CSF, pleural fluid, bone marrow, tissue, and deep respiratory specimens. The dark brown-black pigment produced by *Cryptococcus* spp. on EMB can be used as a presumptive identification (56).

Exceptions to using a battery of media would be for fungal cultures that screen for a specific pathogen, such as a rule-out *Candida* culture, where a chromogenic medium that is selective and differential for yeast can be used, or a rule-out dermatophyte culture, where a single Mycosel or Mycobiotic plate can be used (57). In attempting to isolate *Malassezia* spp., Sabouraud agar with antibiotics should be supplemented with sterile olive oil by adding 0.5 ml of oil to the surface of the plate after inoculation or by dropping an olive-oil-saturated paper disk on the first half of the plate. Alternatively, specialized media, such as modified Dixon's, Leeming's, and Ushijima's media, can also be used, if available.

The container used for fungal media is often debated because of safety issues related to fungi that grow endemically (58). Agar plates are considered more dangerous, are easily contaminated, and dehydrate the fastest, but they offer the largest surface area to work from, and colonies are easier to reach on plates. Plates can be sealed individually or placed in plastic bags to avoid environmental contamination and to provide a layer of safety beyond the use of a laminar-airflow biosafety cabinet. Screw-cap flat-sided bottles and agar slants in tubes both reduce contamination and are safer to use, but both have the disadvantage of a narrow opening through which to access colonies. Most clinical laboratories use plated media, but some compromise by using flat-sided bottles or tubes with those specimen types that are rarely culture positive, such as CSF or other sterile fluids, to avoid later questions concerning possible contaminants.

Inoculated media for fungal culture should be incubated aerobically at 30°C, but if a 30°C incubator is not available, then 25°C can be used. *S. schenckii*, a rare exception, grows faster at 27 to 28°C, but it still grows well at 30°C.

The length of incubation depends on the type of fungal culture requested. Cultures that screen for *Candida* spp. need to be incubated for no longer than 72 h, cultures that screen for dermatophytes need to be incubated for only 8 days, and cultures screening for all pathogens, including fungi that grow endemically, should routinely be held for 4 weeks. A single study suggests that 3 weeks of incubation may be sufficient (59). Culture media should be examined every 2 to 3 days for the first 2 weeks and weekly thereafter.

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Reagents, Stains, and Media: Mycology*

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A variety of stains, media, and reagents are available to the mycology laboratory for the detection, isolation, characterization, and identification of yeasts and moulds. Familiarity with the composition and characteristics of these materials is critical to the diagnostic approach when processing specimens from patients with suspected mycotic diseases.

The direct microscopic examination of properly stained clinical material is rapid and cost-effective and may denote the presumptive identification of the etiologic agent (1–3). Results of direct examination can also guide the laboratory in the selection of media that best support fungal growth *in vitro* (1). Stained preparations made from fungal cultures are essential for definitive identification. It is important to note that when processing patient specimens, universal precautions should be observed and work should be performed in a biosafety hood.

Many media are available for the primary inoculation and cultivation of fungi from clinical specimens. No one specific medium or combination of media is adequate for all specimens. Media should be carefully selected based on specimen type and suspected fungal agents (2–6). If the specimen is from a nonsterile site, it is important to include media that contain inhibitory substances such as chloramphenicol, gentamicin, and/or cycloheximide. Chloramphenicol or gentamicin inhibits most bacterial contaminants, while cycloheximide inhibits most saprobic moulds. Remember that cycloheximide may also inhibit some important opportunistic fungi such as *Fusarium*, *Scopulariopsis*, *Pseudallescheria*, *Trichosporon*, some *Aspergillus* species, *Talaromyces* (*Penicillium*) *marneffei*, mucoraceous fungi, some dematiaceous fungi, and yeasts such as *Cryptococcus* species and some *Candida* species. Therefore, it is necessary to select media both with and without inhibitory agents for the primary inoculation of the specimen. The Clinical and Laboratory Standards Institute (CLSI) Standard M54-A (7) is an excellent source for determining the selection and use of fungal media.

Media can be dispensed into containers such as 25- by 150-mm screw cap tubes or 100-mm-diameter petri dishes. Tubed media (slants/deeps) offer maximum safety and resistance to dehydration and contamination but have a small surface area for inoculation. Petri plates, in contrast to agar tubes, offer the advantage of a large surface area for isolation and dilution of inhibitory substances in the specimens, but they must be poured thick, with at least 25 ml of medium, in order to resist dehydration during extended incubation periods. Because plates are vented, they are more likely to become contaminated during incubation. Plates may be placed in gas-permeable bags or sealed with gas-permeable tape (Shrink Seals; Scientific Device Laboratory, Des Plaines, IL) to offset this disadvantage. The gas-permeable tape also secures the lid of plated media, preventing the accidental removal of the lid outside the biosafety cabinet. Another format, in which media are poured into a flat-bottom flask (Bactiflask [Remel, Thermo Scientific, Waltham, MA]; Mycoflask [BD Diagnostic Systems, Sparks, MD]), provides the combination of an increase in surface area of plated media and the safety of tubed media. Flasked media may be particularly useful when culturing dimorphic pathogens.

Once inoculated, the cultures are incubated at 25 to 30°C. Fungal cultures should be incubated for 4 to 6 weeks before being regarded as negative; however, many cultures can be read as early as 24 h after inoculation. For example, most yeasts are detected within 5 or fewer days, dermatophytes are detected within 1 week, and dematiaceous and dimorphic fungi may require 2 to 4 weeks. Therefore, to account for differences in growth rates, fungal cultures should be examined at regular intervals (e.g., daily during the first week, three times the second week, twice at 3 weeks, and once at 4 weeks) rather than being evaluated once at the end of 4 weeks. While growth may be observed within the first few weeks of incubation, continue to incubate the original plate after subculture to allow for the emergence of slower-growing pathogenic organisms. Manipulation of cultures must be performed inside a certified biological safety cabinet to prevent contamination of the plate and exposure of personnel to potentially hazardous fungi.

Each new lot of medium, whether purchased or prepared in-house, must be subjected to a quality-control protocol that verifies appearance, pH, and performance (7, 8). Both positive and negative control strains need to be included in quality-assurance testing protocols (7, 8). Media for primary isolation should be tested for optimal growth of several fungal pathogens. Selective media should be tested with strains known to be sensitive and resistant to the inhibitory

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agent in the media, while differential media should be evaluated with fungi that produce both positive and negative reactions. Many media are also commercially available as prepared plates or tubes. Although manufacturers perform quality-control testing, clinical laboratories still need to ensure that media meet performance standards. Some widely used commercially prepared media are exempt from routine quality-assurance testing. These media include cornmeal agar, inhibitory mould agar, inhibitory mould agar with gentamicin, soy peptone agar with cycloheximide/chloramphenicol without pH indicators, potato dextrose agar, brain heart infusion agar with 5% sheep blood containing chloramphenicol/gentamicin, Sabouraud's dextrose agar, and Sabouraud's dextrose agar with chloramphenicol/gentamicin (7, 8). Nonexempt media that require specific quality-assurance testing include cornmeal agar with Tween, brain heart infusion agar with 5% sheep blood and cycloheximide/chloramphenicol, bismuth sulfite-glucose-glycine-yeast (BiGGY) agar, birdseed agar, brain heart infusion with 5% sheep blood and penicillin/streptomycin, dermatophyte test medium, and potato flakes agar with or without cycloheximide/chloramphenicol (7, 8).

Unless stated otherwise, the reagents and media listed in this chapter should be prepared by dissolving the components in the stated liquid with a magnetic stirring bar. The standard sterilization technique of autoclaving at 121°C at 15 lb/in^2 for 15 min should be used when needed. However, certain solutions, such as those containing antibiotics or carbohydrates, cannot be autoclaved because they will be denatured. These solutions are sterilized by filtration through a 0.22-μm-pore-size filter; *Candida* chromogenic agars can be heated in a microwave.

Storage of prepared reagents in sterile, airtight, screw-cap containers is recommended. Some reagents require storage in dark containers, and some need to be stored refrigerated (2 to 8°C) instead of at room temperature. Special storage instructions are given when appropriate. Standard safety precautions should be taken when preparing the reagents. Follow the safety guidelines for the chemicals being used, in addition to the laboratory safety protocols.

The stains, media, and reagents listed in this chapter include those commonly used and a few specialized items. For more specific information not included here, refer to the literature cited in the chapter.

REAGENTS

N-Acetyl-L-cysteine (NALC) (0.5%)

NALC (Alpha Tec Systems, Inc.) is a mucolytic agent used for digestion of sputum specimens submitted for detection of *Pneumocystis jirovecii* cysts and/or trophozoites by microscopic examination. This compound can also be used for preparing samples for microscopic examination for a wide range of fungi. Sodium citrate (0.1 M) is included in the mixture to exert a stabilizing effect on the acetyl-L-cysteine. Formulations containing NaOH should be avoided due to adverse effects on fungal organisms.

Dithiothreitol (Sputolysin), 0.0065 M

Dithiothreitol is a mucolytic agent that can be purchased commercially and has been used to prepare sputum specimens for detection of *P. jirovecii*. Equal volumes of sputum and dithiothreitol are mixed and incubated at 35°C. The mixture is periodically mixed vigorously until nearly liquefied (complete liquefaction disperses the cells of *P. jirovecii*, making microscopic detection difficult). As with NALC, dithiothreitol can be useful for preparing samples for microscopic examination of a wide range of fungi. Formulations containing NaOH should be avoided due to adverse effects on fungal organisms.

Potassium hydroxide (KOH)

Wet mounts prepared in 10% KOH are used to distinguish fungi in thick mucoid specimens or in specimens that contain keratinous material such as skin, hair, and nails. The proteinaceous components of the host cells are partially digested, leaving the fungal cell wall intact and more apparent. An aliquot of specimen is added to a drop of 10% or 20% KOH, which can be preserved with 0.1% thimerosal (Sigma Chemical Co.). The slide is held at room temperature for 5 to 30 min after the addition of KOH, depending on the specimen type, to allow digestion to occur. Digestive capabilities can be enhanced with gentle heating or the addition of 40% dimethyl sulfoxide.

Potassium hydroxide (10%) with lactophenol cotton blue (LPCB)

The wet mount with KOH and LPCB is used for the same purpose as the KOH preparation but incorporates LPCB dye (see below). LPCB enhances the visibility of fungi because aniline blue stains the outer cell wall of fungi, and lactic acid serves as an additional clearing agent. The phenol component in LPCB acts as a fungicide.

Sodium hydroxide (10 or 25% with added glycerin)

Solutions of sodium hydroxide may be used as alternatives to potassium hydroxide for the direct microscopic examination of hair, skin, and nails for dermatophyte-mediated infections. Visualization of fungal elements may be enhanced by the addition of glucan-binding fluorescent brighteners such as calcofluor white or Congo red, both of which bind to chitin, a major component of the fungal cell wall.

STAINS

Alcian blue stain

Alcian blue and the more commonly used mucicarmine stain (see below) are mucopolysaccharide stains. These are useful for visualizing the polysaccharide capsule produced by *Cryptococcus* species in histological sections of tissue.

Basic procedure

Deparaffinized sections are stained in Alcian blue (1 g in 100 ml of acetic acid, 3% solution) for 30 min, washed in running tap water, and then rinsed in distilled water. The sections are counterstained in nuclear fast red (0.1 g in 100 ml of aluminum sulfate, 5% solution). After dehydration through 95% and absolute alcohol, the sections are cleared with xylene and mounted in Permount (Fisher Scientific). Capsular polysaccharides stain blue against a pink background.

Ascospore stain

Ascomycetous fungi may produce ascospores when grown on media that promote their formation. Visualization of ascospores can be accomplished with a differential staining procedure consisting of malachite green and safranin. Ascospores stain green, while the vegetative portion of the fungus stains red. The Kinyoun acid-fast stain (see chapter 19) may also be used for visualizing ascospores, as these structures tend to be acid-fast.

Basic procedure

A thin smear of growth is applied to a glass slide and heat fixed. The slide is flooded with malachite green (5 g in 100 ml of distilled water) for 3 min, washed with tap water, decolorized with 95% ethyl alcohol for 30 s, and counterstained with aqueous safranin (5%) for 30 s. The slide is washed with tap water, allowed to air dry, and examined at ×400 to ×1,000 magnification.

Calcofluor white

Calcofluor white and related compounds such as Uvitex 2B and Blankophor are nonspecific, nonimmunological fluorochromes that bind to β1,3 and β1,4 polysaccharides, specifically cellulose and chitin of fungal cell walls. Like the auramine-rhodamine stain, calcofluor white has become commonplace in microbiology laboratories because of the rapidity with which specimens can be observed. The fluorochrome can be mixed with KOH to clear the specimen for easier observation of fungal elements, including *P. jirovecii*. Fungal elements appear bluish white against a dark background when excited with UV or blue-violet radiation. Optimal fluorescence occurs with UV excitation. A barrier filter such as 510, 520, or 530 should be used for eye protection. Organisms impart a green fluorescence (1). Typical *P. jirovecii* cysts are generally 5 to 8 µm in diameter, round, and uniform in size, and they exhibit a characteristic peripheral cyst wall staining with an intense "double-parenthesis-like" structure (9, 10). Yeast cells are differentiated from *P. jirovecii* by budding and intense staining. Care must be used in interpreting the calcofluor white staining results because nonspecific reactions may be observed. Cotton fibers fluoresce strongly and must be differentiated from fungal hyphae. Additionally, tissues such as brain biopsy specimens from patients with tumors may fluoresce and resemble hyphae suggestive of *Aspergillus* or other moulds with branching hyphae.

Basic procedure

Calcofluor white may be purchased from multiple mycological media and chemical suppliers either as premixed or powdered form (see list of suppliers at the end of the chapter). KOH (10%) is mixed in equal proportion with calcofluor white solution (0.1 g of calcofluor white M2R and 0.05 g of Evans blue in 100 ml of water). The specimen is covered with this mixture and a coverslip is applied. Allow the slide to sit for 5 to 10 minutes prior to viewing to permit the tissue to clear and the stain to interact with the fungal elements. Some nail and tissue preparations may require a longer incubation time (up to 30 minutes) for clearing to occur. Alternatively, the slides may be gently warmed on a slide warmer to speed up the clearing process. For optimal results, the slide should be viewed as soon as the tissue has cleared. The preparation is examined using a fluorescent microscope containing appropriate excitation and barrier filters at ×100 to ×400 magnification. Darkly pigmented fungi may stain poorly with calcofluor white due to the pigmentation, which may mask the fluorescence.

Colloidal carbon wet mounts (India ink, nigrosin)

Colloidal carbon wet mounts are used for visualization of encapsulated microorganisms, especially *Cryptococcus* species. The polysaccharide capsule of organisms is refractory to the particles of ink, and capsules appear as clear halos around the organism. Artifacts such as erythrocytes, leukocytes, and talc particles from gloves or bubbles following a myelogram may displace the colloidal suspension and mimic yeast (false positive). These artifacts make it necessary to perform a careful examination of the wet mount for properties consistent with the organisms (e.g., rounded forms with buds of various sizes and double-contoured cell walls). Interpretation can also be hindered if the emulsion with the colloid suspension is too thick, blocking transmission of light.

Basic procedure

Mix equal parts of the patient's cerebrospinal fluid with either commercial India ink or nigrosin on a slide. Add a coverslip and examine at ×100 to ×1,000 magnification. Care must be taken not to contaminate India ink supplied in larger volumes. Limited volume or individual-use dispensers can be purchased through many of the media and reagent distributers.

Fontana-Masson stain

The Fontana-Masson stain was originally developed for demonstrating melanin granules in mammalian tissue. It has a mycological application in detecting dematiaceous (melanin-containing) fungi, and to a lesser extent *Cryptococcus neoformans/gattii*, in histological sections. Fungal elements appear brown to brownish black against a reddish background.

Basic procedure

A silver solution is prepared by adding concentrated ammonium hydroxide to 10% silver nitrate until the precipitated form disappears. Deparaffinized sections of tissue are hydrated and placed in heated silver solution for 30 to 60 min. The slides are then rinsed in distilled water and toned in gold chloride (0.2 g in 100 distilled water) for 10 min followed by fixation in 5% sodium thiosulfate for 5 min. The sections are dehydrated through increasing concentrations of alcohol, cleared in xylene, and mounted with a coverslip.

Giemsa stain

The Giemsa stain is used for the detection of intracellular yeast forms of *Histoplasma capsulatum* in bone marrow and buffy coat specimens. The fungus is usually seen as small oval yeast cells, is often contained within macrophages, stains blue, and has a hyaline halo that represents poorly staining cell wall. The stain can also be used to visualize the trophozoite of *P. jirovecii*.

Basic procedure

A thin smear is prepared on a glass slide and placed in 100% methanol for 1 min. The slide is drained and then flooded with freshly prepared Giemsa stain (stock Giemsa stain diluted 1:10 with phosphate-buffered water). After 5 min, the slide is rinsed with distilled water and air dried. Examine at ×100 to ×400 magnification.

Lactophenol cotton blue

Lactophenol cotton blue is a basic mounting medium for fungi that consists of phenol, lactic acid, glycerol, and aniline (cotton) blue dye. The solution may be filtered to remove precipitated dye and stored at room temperature. It is commonly used for the microscopic examination of fungal cultures by tease or tape preparation. The addition of 10% polyvinyl alcohol (LPCB-PVA) makes an excellent permanent stain or fixative for mounting slide culture preparations (1).

Basic procedure

Concentrated phenol (20% [vol/vol]) is added to a mixture of glycerol (40%), lactic acid (20%), and water (20%), followed by the addition of aniline blue (0.05 g). The solution may be filter-sterilized to remove precipitated dye. A drop is added to a glass slide, and a tease or tape mount is prepared. Add a coverslip and examine at ×100 to ×400 magnification.

Methenamine silver stain

Methenamine silver stains are perhaps the most useful stains for visualizing fungi in tissue. Fungal elements are sharply delineated in black against a pale green or yellow background. They are specialized stains that are more often performed in the histology laboratory than in the microbiology laboratory. Grocott's modification of the Gomori methenamine silver stain is commonly used for the histopathological examination of deparaffinized tissues for fungi. A variation that can be used by counterstaining with the hematoxylin and eosin (H&E) stain displays the silver-stained fungal structures within host tissue reaction.

Basic procedure

Stock methenamine silver nitrate solution is prepared by adding 3% methenamine (3 g in 100 ml of distilled water) to 5% silver nitrate (5 g in 100 ml of distilled water) until a white precipitate forms that clears upon shaking. This solution is then diluted 1:2 with distilled water to which 5 ml of 5% photographic-grade borax is added. Prepared slides are oxidized in a solution of chromic acid (5 g in 100 ml of distilled water), neutralized in sodium bisulfite (1 g in 100 ml of distilled water), placed in the diluted methenamine silver nitrate solution, and heated in an oven to 58 to 60°C until the material turns yellowish brown. After being rinsed vigorously in distilled water, the slides are toned in gold chloride (0.1 g in 100 ml of distilled water). Unreduced silver is removed by placing the slides in a sodium thiosulfate solution (2 g in 100 ml of distilled water) and counterstained in 0.03% light green. Rinse, blot dry, and examine at ×100 to ×400 magnification.

Mucicarmine stain

The mucicarmine stain is useful for differentiating *C. neoformans/gattii* from other fungi of similar size and shape when found in samples of tissue. The mucopolysaccharide in the capsular material of the fungus stains deep rose to red, whereas the other tissue elements stain yellow. *Blastomyces dermatitidis* and *Rhinosporidium seeberi* may also react positively with this stain but are differentiated by the size of the yeast and the intensity of the staining reaction of the mucopolysaccharide capsule of *Cryptococcus*.

Basic procedure

Fixed tissue sections on glass slides are stained first with Weigert's iron hematoxylin and then placed in a solution of mucicarmine (1 g of carmine combined with 0.5 g of anhydrous aluminum chloride in 2 ml of distilled water and then diluted in 100 ml of 50% ethanol) for 30 to 60 min. The slides are rinsed in distilled water and then counterstained in metanil yellow (0.25 g in 100 ml of distilled water). Rinse, blot dry, and examine at ×100 to ×400 magnification.

Periodic acid-Schiff (PAS) stain

The periodic acid-Schiff (PAS) stain is used to detect fungi in clinical specimens, especially yeast cells and hyphae in tissues. Fungi stain a bright pink-magenta or purple against an orange background if picric acid is used as the counterstain, or against a green background if light green is used. The procedure is a multistep method combining hydrolysis and staining. The periodic acid step hydrolyzes the cell wall aldehydes, which are then able to combine with the modified Schiff reagent, coloring the cell wall carbohydrates a bright pink-magenta. The PAS stain is an excellent general stain, because most fungi in clinical material take up the stain. However, the PAS staining procedure is rather involved, requiring several different reagents and time-consuming steps, and it has been replaced in many laboratories by the calcofluor white staining procedure. The PAS stain cannot be used with undigested respiratory secretions, since mucin also stains bright pink-magenta.

Basic procedure

The prepared slide is fixed in formalin-ethanol for 1 min and is then air dried. The slide is then immersed in 5% periodic acid for 5 min, followed by 2 min in basic fuchsin (0.1 g of dye in 5 ml of 95% alcohol-95 ml of H_2O). The slide is rinsed in water and immersed in zinc or sodium hydrosulfite solution for 10 min (1 g of zinc or sodium hydrosulfite in 0.5 g of tartaric acid and 100 ml of H_2O). Rinse in water and counterstain with saturated aqueous picric acid for 2 min or with light green stain (1 g of dye in 0.25 ml of acetic acid and 100 ml of 80% alcohol) for 5 s. Rinse, blot dry, and examine at ×100 to ×400 magnification.

Toluidine blue O

Toluidine blue O is used primarily for the rapid detection of *P. jirovecii* from lung biopsy specimen imprints and bronchoalveolar lavage (BAL) specimens (11). Toluidine blue O stains the cysts of *P. jirovecii* reddish blue or dark purple against a light blue background. The cysts are often clumped and may be punched in, appearing crescent shaped. Trophozoites are not discernible. Although the silver stain, monoclonal antibody, and calcofluor white stains are also used, the toluidine blue O stain is easy and rapid and yields reliable results with appropriate specimens (e.g., BAL specimens).

Basic procedure

After the slide is air dried, place it in the sulfation reagent (45 ml of glacial acetic acid mixed with 15 ml of concentrated sulfuric acid) for 10 min. Rinse in cold water for 5 min, drain, and place in toluidine blue O (0.3 g of dye in 60 ml of H_2O) for 3 min. Rinse in 95% ethanol, followed by absolute ethanol and then xylene. Examine at ×100 to ×1,000 magnification.

MEDIA

Agar slide culture plate

Microscopic morphology of a fungal isolate is best observed using the slide culture method, where an approximately 15- by 15-mm square block of medium is placed on a sterile microscope slide. The desired fungal organism is inoculated on each edge of the block, covered with a sterile 22- by 22-mm coverslip, and incubated in an individual humidified chamber. When sufficient sporulation is present, the coverslip is removed and placed growth-side down on a microscope slide containing a drop of lactophenol cotton blue. Fungal morphology is visualized microscopically at ×200 and ×400 magnification. Commercial sources are available with preformed slide culture setups. Agar and incubation temperature should be selected based on the optimum conditions to induce sporulation for the fungal organism used.

Acetate ascospore agar

Acetate ascospore agar is used for the cultivation of ascosporogenous yeasts such as *Saccharomyces cerevisiae*. A potassium acetate formulation has been shown to be a better sporulation medium than the previously used formulation with sodium acetate. Ascospores produced on this medium are visible microscopically after staining with Kinyoun carbol-fuchsin acid-fast stain.

Antifungal susceptibility testing media

Recent advances in methods for antifungal susceptibility testing have resulted in several media that are now used for this type of testing (see chapter 131). The broth dilution method for yeasts and filamentous fungi described in the Clinical and Laboratory Standards Institute M38-A2 and M27-A3 documents (12, 13) uses RPMI 1640 synthetic medium without sodium bicarbonate and supplemented with L-glutamine. The medium is buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS). Although this medium supports the growth of most fungi, it is not optimal for *Cryptococcus* species (14), nor does it accurately discriminate amphotericin B-resistant *Candida* spp. (15).

The Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST-EUCAST) has described a proposed standard broth microdilution that is based on the M27-A3 broth microdilution reference procedure. Both procedures utilize RPMI 1640 as the primary assay medium, but that used in the EUCAST procedure differs from the CLSI procedure by the incorporation of 2% glucose (16, 17).

Birdseed agar

Birdseed agar is a selective and differential medium used for the isolation of *Cryptococcus* species, especially *C. neoformans* and *C. gattii*, which are unique in that they produce the enzyme phenol oxidase. The breakdown of the substrate (*Guizotia abyssinica* seeds or niger seed) produces melanin, which is absorbed into the yeast wall and imparts a tan to brown pigmentation to the colonies. Colonies of other yeasts are beige or cream in color. Chloramphenicol is the selective agent that inhibits bacteria and some fungi. Creatinine enhances melanization of some strains of *C. neoformans*.

Bismuth sulfite-glucose-glycine yeast (BiGGY) agar

Bismuth sulfite-glucose-glycine yeast agar is a selective and differential medium used for the isolation and differentiation of *Candida* spp. Peptone, glucose, and yeast extract are the nutritive bases. *Candida* species reduce the bismuth sulfite to bismuth sulfide, which results in pigmentation of the yeast colony and, with some species, the surrounding medium. *Candida albicans* appears as brown to black colonies with no pigment diffusion and no sheen, whereas *Candida tropicalis* appears as dark brown colonies with black centers, black pigment diffusion, and sheen. Specific colonial morphologies and growth patterns of the different *Candida* species are also detected. The bismuth sulfite also acts as an inhibitor of bacterial growth, making the medium selective.

Blood-glucose-cysteine agar

A fungal isolate believed to be a thermally dimorphic pathogen can be identified by converting the mould phase to the yeast phase at 37°C. Blood-glucose-cysteine agar medium is used to promote the mould-to-yeast conversion of *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, and *Sporothrix schenckii*. The medium contains tryptose blood agar base, L-cysteine, and defibrinated sheep blood. Penicillin is added to inhibit bacterial contamination.

Brain heart infusion agar (fungal formulation)

Brain heart infusion (BHI) agar with sheep blood is a medium used for the cultivation and isolation of all fungi including fastidious dimorphic fungi. The nutritive base is BHI agar with 10% sheep blood for added enrichment. The antibiotics chloramphenicol and gentamicin are added to make the medium selective by inhibiting bacteria. This medium does not inhibit saprophytic fungi. When attempting to isolate more fastidious moulds, a medium containing cycloheximide should be included to inhibit overgrowth by the saprophytic fungi.

Bromcresol purple-milk solids-glucose medium

Bromcresol purple-milk solids-glucose (Dermatophyte Milk Agar; Hardy Diagnostics) is a differential medium used for the identification of *Trichophyton* species. The medium's differential capacity is based on the type of growth (profuse versus restricted as compared to growth on standard nutrient media) and change in the pH indicator due to the production of alkaline by-products.

Canavanine-glycine-bromthymol blue agar

Canavanine-glycine-bromthymol blue agar is a differential medium for distinguishing *C. neoformans* from *C. gattii*. The medium contains glycine, thiamine, L-canavanine sulfate, and bromthymol blue. A colony of *Cryptococcus* is streaked onto the surface of the agar and incubated at 30°C for 1 to 5 days. *C. gattii* (serotypes B and C) turns the medium cobalt blue, whereas *C. neoformans* var. *grubii* (serotype A)

TABLE 1 Commercial sources of yeast chromogenic agar media

Medium	Manufacturer	*Candida* species identified[a]
Candida diagnostic agar (CDA)	PPR Diagnostics, Ltd	*C. albicans*, *C. kefyr*, and *C. tropicalis*
Candida Chromogenic Agar	Laboratorios CONDA	*C. albicans*, *C. tropicalis*, and *C. krusei*
Candi*Select* 4	Bio-Rad Laboratories	*C. albicans* Presumptive identification: *C. glabrata*, *C. tropicalis*, and *C. krusei*
CHROMagar Candida	CHROMagar Microbiology	*C. albicans*, *C. tropicalis*, and *C. krusei*
BBL CHROMagar	BD Diagnostic Systems	*C. albicans*, *C. tropicalis*, and *C. krusei*
chromID Candida agar	bioMérieux, Inc.	*C. albicans* Presumptive identification: *C. tropicalis*, *C. lusitaniae*, and *C. kefyr*
HardyCHROM Candida	Hardy Diagnostics	*C. albicans*, *C. tropicalis*, and *C. krusei* Presumptive identification: *C. glabrata*
HiCrome Candida differential agar	HiMedia Laboratories	*C. albicans*, *C. krusei*, *C. tropicalis*, and *C. glabrata*
Oxoid Brilliance Candida Agar	Oxoid	*C. albicans*, *C. krusei*, and *C. tropicalis* Presumptive identification: *C. glabrata*, *C. kefyr*, *C. parapsilosis*, *and C. lusitaniae*.

[a] *Candida* species identified as provided by the manufacturer. Species that are presumptively identified require further testing for final identification.

and *C. neoformans* var. *neoformans* (serotype D) leave the medium greenish yellow.

Candida chromogenic media

The introduction of chromogenic media has facilitated the direct and rapid identification of yeasts and is particularly useful for detecting and separating mixed cultures. These media contain chromogenic substrates that are hydrolyzed by species-specific enzymes (e.g., β-*N*-acetylhexosaminidase) and, depending on the medium, a second enzyme, β-glucosidase or phosphatase, resulting in identification of yeasts to the species level based on colonial features and color development (18–20). Commercially available chromogenic media are summarized in Table 1.

A brief description of selected chromogenic agar products that are approved by the U.S. Food and Drug Administration (FDA) for use in U.S. laboratories is provided as an overview of how different yeasts react with chromogenic substrates and the resultant characteristic colony color.

CHROMagar (BBL; BD Diagnostic Systems)

CHROMagar is a differential and selective medium used for the isolation and differentiation of clinically important yeasts. The nutritive base is peptone and glucose. Chloramphenicol makes the medium selective by inhibiting bacteria. The medium is available with and without fluconazole, the former providing the additional selection of fluconazole-resistant yeasts such as *Candida krusei*. A proprietary chromogenic mixture allows the differentiation of many yeast species. For example, *C. albicans* forms yellow-green to blue-green colonies. Colonial morphology as well as distinctive color patterns have been shown to make the presumptive identification of yeast species very reliable (19, 20). The medium has been shown to be more selective than Sabouraud agar and helpful in identifying mixed cultures of yeasts, and it may enhance the rapid assimilation of trehalose by *Candida glabrata* (20). The colonies on the medium should be evaluated at 48 h. Although *C. neoformans* and *Geotrichum* species can grow on this medium, definitive identification requires subculture to a nonselective medium followed by utilization of the appropriate biochemical and morphological characterization tools.

chromID Candida agar (CAN2) (bioMérieux)

Colonies of *C. albicans* produce a blue color following the hydrolysis of a hexosaminidase chromogenic substrate in the presence of an inducer of the enzyme (bioMérieux patent). The hydrolysis of a second substrate (pink color) differentiates mixed cultures and indicates the need for identification of other species of yeast (bioMérieux patent).

Christensen's urea agar

The ability to hydrolyze urea is an important phenotypic characteristic for the presumptive identification of *Cryptococcus*, *Trichosporon*, and *Rhodotorula* spp. Urea hydrolysis also facilitates separation of certain dermatophytes, in particular *T. mentagrophytes* and *T. rubrum*. The medium contains 2% urea with phenol red serving as the indicator.

Cornmeal agar with 1% dextrose

Cornmeal agar with 1% dextrose is used for the cultivation of fungi and the differentiation of *Trichophyton mentagrophytes* from *Trichophyton rubrum* on the basis of pigment production. The replacement of Tween (polysorbate) 80 with dextrose promotes the growth and production of a red pigment by *T. rubrum*.

Cornmeal agar with Tween 80

Cornmeal agar with Tween (polysorbate) 80 is used for the cultivation and differentiation of *Candida* species on the basis of morphological characteristics. Tween 80, a surfactant, is specifically incorporated in lieu of dextrose for the demonstration of pseudohyphae, chlamydospore, and arthrospore formation. Chlamydospore production is best obtained if the yeast inoculum is placed under a coverslip or following subsurface inoculation creating a microaerophilic environment. The basic nutrients for yeast growth are provided by cornmeal infusion.

Czapek-Dox agar

Czapek-Dox agar is a medium used for the differentiation of *Aspergillus* spp. (1, 3, 5). Sucrose is the sole carbon source, with sodium nitrate serving as the sole nitrogen source. Any bacteria or fungi that can utilize sodium nitrate as a nitrogen source can grow on this medium.

Dermatophyte test medium (DTM)

Dermatophyte test medium is used as a screening medium for the recovery, selection, and differentiation of dermatophytes (*Microsporum*, *Trichophyton*, and *Epidermophyton*) from keratinous specimens (hair, skin, and nails). Nitrogenous and carbonaceous compounds are provided by soy peptone. Cycloheximide inhibits saprophytic moulds, chloramphenicol inhibits many Gram-positive bacteria, and gentamicin inhibits Gram-negative bacteria. The morphology and microscopic characteristics are easily identified with this medium. Pigmentation cannot be discerned because of the presence of phenol red indicator. The medium is yellow and turns red with growth of dermatophytes. *Aspergillus* species and other saprophytic fungi can grow and produce pigment on this medium, which accounts for its recommended use BD as a screening medium only.

Inhibitory mould agar

Inhibitory mould agar is a selective and enriched medium that is used for the general cultivation of cycloheximide-sensitive fungi (e.g., *Cryptococcus* and mucoraceous fungi) from contaminated specimens. Casein and animal tissue provide growth nutrients. Yeast extract serves as a source of vitamins. Chloramphenicol inhibits many Gram-positive and Gram-negative bacteria. Gentamicin is another additive that inhibits some Gram-negative bacteria. This is an excellent media for use in the primary cultivation of fungi and has been demonstrated to be more sensitive than the standard Sabouraud dextrose agar (21).

Lactrimel agar (Borelli's medium)

Lactrimel agar (Borelli's medium) is composed of whole-wheat flour, skim milk, and honey, which favors the sporulation of most dermatophytes and the pigment production of *Trichophyton* species. The medium may also be used for the morphological examination of dematiaceous fungi.

Leeming and Notman medium

Leeming and Notman medium is used for the isolation and growth of lipodependent *Malassezia* species. The key components of the medium include ox bile, glycerol monostearate, glycerol, Tween 80, and cows' milk (whole fat) (22). The medium may serve as an alternative to Sabouraud glucose agar, since not all species can grow on this medium (e.g., *M. globosa*, *M. restricta*, and *M. obtusa* require more complex media for their isolation).

Littman oxgall agar

Littman oxgall agar is a selective general-purpose medium used for the isolation of fungi from contaminated specimens. Crystal violet and streptomycin are the selective agents and inhibit bacteria. Oxgall restricts the spreading of fungal colonies. The isolation characteristics of this medium are similar to those of Sabouraud dextrose agar with chloramphenicol and inhibitory mould agar in that it allows the growth of fungi that are sensitive to cycloheximide.

Malt extract (2%) agar

This medium is used for the cultivation of yeasts and moulds. A variety of formulations have been described, but they typically include malt extract with agar and are supplemented with peptone, glucose, maltose, and dextrin and/or glycerol. Malt agar is particularly useful for stimulating the production of macroconidia in *Microsporum canis*.

Mycobiotic or Mycosel agar

Mycobiotic (Remel) and Mycosel (BD Diagnostic Systems) are trade names for a selective medium principally formulated for the isolation of dermatophytes but also used for the isolation of other pathogenic fungi from specimens contaminated with saprophytic fungi and bacteria. The medium consists primarily of peptones from a pancreatic digest of soybean meal and dextrose. The selective agents are cycloheximide and chloramphenicol. Cycloheximide inhibits the faster-growing saprophytic fungi but is also inhibitory to some clinically relevant species. These inhibited fungi include some *Candida* and *Aspergillus* species, mucoraceous fungi, and *C. neoformans*. Chloramphenicol inhibits Gram-negative and Gram-positive organisms.

Niger seed agar

See Birdseed agar.

Potato dextrose agar (PDA)

Potato dextrose agar is a medium used to stimulate conidium production by fungi. The medium also stimulates pigment production in some dermatophytes. This medium is most commonly used with the slide culture technique to view morphological characteristics. Infusions from potatoes and dextrose provide nutrient factors for excellent growth. The incorporation of tartaric acid in the medium lowers the pH, thereby inhibiting bacterial growth.

Potato flake agar

Potato flake agar is a medium useful in the stimulation of conidia by fungi. Its advantages over potato dextrose agar may be preparation and stability. Potato flakes and dextrose provide the nutrient factors that allow excellent growth. The pH is adjusted to 5.6 to enhance growth of fungi and to inhibit bacterial growth. The medium may be made selective by the addition of cycloheximide and chloramphenicol.

Sabouraud brain heart infusion (SABHI) agar

Sabouraud brain heart infusion agar is a general-purpose medium used for the isolation and cultivation of all fungi. The medium is a combination of brain heart infusion agar and Sabouraud dextrose agar. The combined formulation allows for the recovery of most fungi including the yeast phase of dimorphic fungi. The inclusion of sheep blood provides essential growth factors for the more fastidious fungi and enhances the growth of *H. capsulatum*. Selectivity is attained by the addition of chloramphenicol, cycloheximide, penicillin, and/or streptomycin.

Sabouraud dextrose agar

Sabouraud dextrose agar was formulated by Sabouraud for cultivating dermatophytes. The medium consists of pancreatic digest of casein, peptic digest of animal tissue, and dextrose at 4% concentration and buffered to a pH of 5.6. Emmons modified the original formulation by reducing the dextrose concentration to 2% and adjusting the pH nearer to neutrality at 6.9 to 7.0. Antibiotic additives in various combinations include cycloheximide, chloramphenicol, gentamicin, ciprofloxacin, penicillin, and/or streptomycin, which inhibit some fungi and Gram-positive and Gram-negative bacteria to achieve selectivity for this medium. This medium is also available as a broth.

Soil extract agar

Soil extract agar is a medium composed of garden soil, yeast extract, and glucose. The primary use of this medium is to promote sporulation of some saprobic fungi and for mating strains of *B. dermatitidis* (1).

Trichophyton agars 1 to 7

Trichophyton agars are a set of seven media that facilitate the identification of *Trichophyton* species on the basis of their growth factor requirements. The basic ingredients in the media are listed below. Growth in all seven media is then scored on a scale of 1 to 4, and an identification is assigned.

1. Casamino Acids; vitamin-free
2. Casamino Acids plus inositol
3. Casamino Acids plus inositol and thiamine
4. Casamino Acids plus thiamine
5. Casamino Acids plus niacin
6. Ammonium nitrate
7. Ammonium nitrate plus histidine

V8 agar

V8 agar is a medium consisting of dehydrated potato flakes and V8 juice (Campbell Soup Co.; Camden, NJ) that induces early sporulation of some environmental fungi. The naturally low pH makes the medium inhibitory to most bacteria.

Water (tap) agar

This medium is nutritionally deficient (1% water-agar; 1 g of agar, 100 ml of sterile tap water) and promotes the sporulation of dematiaceous fungi, *Apophysomyces elegans*, and *Saksenaea vasiformis* (23). The medium can be supplemented with sterilized carnation leaves for the identification of *Fusarium* spp.(6).

Yeast carbon agar

Yeast carbon agar is a solid medium recommended for use in qualitative procedures for the classification of yeasts according to their ability to assimilate nitrogenous compounds. The yeast carbon base provides amino acids, vitamins, trace elements, and salts that are necessary to support growth. The ability to assimilate nitrogen is tested by the addition of various nitrogen sources such as potassium nitrate.

Yeast extract-phosphate agar with ammonia (Smith's medium)

Yeast extract-phosphate agar with ammonia (Smith's medium) is used for the isolation and sporulation of *H. capsulatum* and *B. dermatitidis* from contaminated specimens. This consists of phosphate buffer (Na_2HPO_4, KH_2PO_4, and distilled water). It should be mixed well with the pH adjusted to 6.0 with 1 N HCl or 1 N NaOH and yeast extract agar solution. Before inoculation of specimens, one drop of ammonium hydroxide is applied to the agar surface and allowed to diffuse into the medium. The combination of ammonium hydroxide and chloramphenicol suppresses bacteria and many moulds and yeasts, thus permitting detection of the slowly growing dimorphic fungi.

APPENDIX

Commercial Manufacturers and Supplies of Fungal Media, Stains, and Reagents

Alpha Tec Systems, Inc.
P.O. Box 5435
Vancouver, WA 98668
800-221-6058
http://www.alphatecsystems.com

BD Diagnostic Systems
7 Loveton Circle
Sparks, MD 21152
800-675-0908
http://www.bd.com

bioMérieux, Inc.
100 Rodolphe St.
Durham, NC 27712
800-682-2666

Bio-Rad Laboratories
5500 East Second St.
Benicia, CA 94510
800-224-6723

CHROMagar
4 Place du 18 Juin 1940
75006 Paris
France
33 1 45 48 05 05
chromagar@chromagar.com
http://chromagar.com/

Fisher Scientific
2000 Park Lane Dr.
Pittsburgh, PA 15275
800-766-7000
http://www.fishersci.com

Fluka Chemika/Biochemika
Industriestrasse 25
9471 Buchs

Switzerland
41 (0) 81 755 25 11
webmaster@sial.com
Flukatec@eurnotes.sial.com

Hardy Diagnostics
1430 West McCoy Lane
Santa Maria, CA 93455
800-266-2222
http://www.hardydiagnostics.com

HiMedia Laboratories Pvt. Ltd.
23 Vadhani Est. LBS Marg
Mumbai 400 086
India
91 22 40951919
info@himedialabs.com
http://www.himedialabs.com

Laboratorios Conda
c/La Forja, 9
28850 Torrejon de Ardoz, Madrid
Spain
Phone: 34 91 761 02 00
Fax: 34 91 656 82 28
http://www.condalab.com

Newcomer Supply Inc.
2505 Parview Road
Middleton, WI 53562-2579
Phone: 800-383-7799
Fax: 608-831-0866
newly@newcomersupply.com

Oxoid Limited
Wade Road
Basingstoke, Hampshire RG24 8PW
United Kingdom
44 (0) 1256 841144
Fax: 44 (0) 1256 814626
Oxoid.info@thermofisher.com

PPR Diagnostics Limited
7 Ivory House
East Smithfield, London E1W 1AT
United Kingdom
Technical: 44 (0) 20 7848 4451
Fax: 44 (0) 1442 866 739
http://www.pprdiag.co.uk

Remel
12076 Santa Fe Dr.
Lenexa, KS 66215
800-255-6730
http://www.remel.com

Scientific Device Laboratory
411 Jarvis Ave.
Des Plaines, IL 60018
847-803-9495
http://www.scientificdevice.com

Sigma-Aldrich Corp
3050 Spruce St.
St. Louis, MO 63103
314-771-5765
http://www.sigmaaldrich.com

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention. Use of trade names and commercial sources is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention.

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General Approaches for Direct Detection and Identification of Fungi*

H. RUTH ASHBEE

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The frequency with which invasive fungal disease occurs has increased markedly over the last decade and the ability to detect and identify the causative fungi rapidly is essential to the successful management of these diseases (1). Culturing the fungus has always been important to determine the etiology of the infection; however, other non−culture-based methods may provide more timely results and offer advantages over culture techniques. Non−culture-based methods reviewed in this chapter include direct microscopic examination, antibody and antigen detection, detection of (1, 3)-β-D-glucan and other fungal metabolites, the use of mass spectrometry, and nucleic acid detection. Although these methods are helpful in clinical use, they have not entirely replaced culture methods and should still be used in conjunction with appropriate culture-based methods (reviewed in chapters 114 and 115). Although cultures from invasive infections are positive only infrequently, when a culture is obtained it allows susceptibility testing and epidemiological comparisons not widely available via non−culture-based methods.

DIRECT MICROSCOPIC EXAMINATION

The ability to detect fungi in clinical material depends on several factors, including the quality of the specimen received into the laboratory. If very small samples are received, few fungi may be present, making detection and identification less reliable. Also, if material is not taken from a representative part of the lesion, fungi may not be present in the part sampled. If very small samples are received, generally culture should take precedence over direct microscopy as culture is more sensitive. The exceptions to this would be dermatological samples where microscopy is diagnostic, and also if mucoraceous moulds are suspected, where microscopy may be positive in the absence of a positive culture. If the sample is large enough, both direct microscopy and culture should be performed, because microscopy is better able to differentiate colonization, tissue invasion, and contamination. Conversely, only nonviable organisms may be present in specimens obtained while the patient is receiving antifungal therapy, and microscopy and molecular methods may be the only means for detecting the etiologic agent. Specimen quantity and quality may be compromised when multiple clinical laboratories process portions of the specimens. Good communication among microbiology services, other pathology services, and the clinician can greatly enhance diagnostic accuracy.

Consideration of the patient's travel history and residence is also important when attempting to detect and identify fungal causes of disease due to the endemicity of certain diseases (Table 1). Lifelong travel history is important in patients who do not normally reside in areas of endemicity, because certain infections (e.g., paracoccidioidomycosis, histoplasmosis) (2) may reactivate decades later. For example, although reported worldwide, most cases of mycetoma come from tropical and subtropical regions around the Tropic of Cancer (3), and specific travel or residence details may improve diagnostic accuracy.

Examination of clinical material before any processing takes place may be very informative and should always be performed. Areas of caseous necrosis, microabscesses, grains, granules, and nodules should all be noted. Grains and granules may indicate mycetoma, granulomas, and caseous necrosis histoplasmosis, while microabscesses are seen with hepatosplenic or renal candidiasis. Grains and granules should be examined for color, shape, size, and consistency as these may be indicative of the etiological agent. For example, *Scedosporium apiospermum*, a common cause of eumycetoma, produces yellowish-white soft grains of 1 or 2 mm diameter, while *Trematosphaeria grisea* produces globose or lobed black grains of 0.5 to 1 mm diameter (3).

Microscopic examination of specimens can be carried out using unfixed specimens or fixed, stained specimens. Obviously, the use of unfixed specimens has the advantages of being quick and relatively simple to carry out, while the use of fixed, stained specimens may highlight features specific to certain fungi and the host response in tissue; however, the latter takes longer to process and thus may delay reporting. Generally, if fixed, stained specimens are being examined, a general stain such as hematoxylin and eosin (H&E) is used for screening. If fungi are suspected after examination of H&E, then stains which are specific for fungal structures (e.g., Gomori methenamine silver [GMS]) can be used, and finally, more specialized stains such as mucicarmine or Fontana-Masson will demonstrate characteristic structures in fungi of interest. A range of

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*This chapter contains some information presented in chapter 114 by Yvonne Shea in the 10th edition of this *Manual.*

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TABLE 1 Characteristic fungal morphology seen by direct examination of clinical specimens

Fungal morphology observed	Organism(s)	Diameter range (µm)	Characteristic features	Geographic distribution
Yeast forms	*Histoplasma capsulatum*	2–5	Small; oval to round budding cells; often found clustered within histiocytes; difficult to detect when present in small numbers; often intracellular (Fig. 23)	1. Worldwide, eastern half of the United States (localized in Ohio and Mississippi River valleys) and throughout Mexico, Central and South America 2. Tropical areas of Africa (Gabon, Uganda, Kenya)
	Sporothrix species	2–6	Small; oval to round to cigar shaped; single or multiple buds present; uncommonly seen in clinical specimens (Fig. 21)	Most often reported from Mexico, Central and South America (Brazil), Asia (Japan). More widespread in temperate and tropical zones.
	Cryptococcus species	4–10	Cells vary in size; usually spherical but may be football shaped; buds usually single and "pinched off"; capsule may or may not be evident; rarely, pseudohyphal forms with or without capsule may be seen (Fig. 7, 15, and 16)	*Cryptococcus neoformans:* Worldwide, especially Europe, Africa, Australia *Cryptococcus gattii:* tropical and subtropical areas, including Australia, America, South Africa, Central America
	Blastomyces dermatitidis	8–15	Cells usually large and spherical, double refractile; buds usually single, but several may remain attached to parent cells; buds connected by broad base (Fig. 22)	Southeast and south-central United States, Great Lakes region, near St. Lawrence River
	Paracoccidioides brasiliensis	5–60	Cell usually large and surrounded by smaller buds around periphery (mariner's wheel appearance); smaller cells (2–5 µm) that resemble *H. capsulatum* may be present; buds have "pinched off" appearance (Fig. 25)	South and Central America
Yeast forms (fission)	*Talaromyces* (*previously Penicillium*) *marneffei*	3	Fission yeast, not budding, elongated, curved with septa visible (Fig. 26)	Southeast Asia and China
Cysts and trophozoites	*Pneumocystis jirovecii*	1–4 4–5	Trophozoites: small pleomorphic forms Cysts: round to cup shaped with as many as 8 intracystic bodies (Fig. 18)	Worldwide
Spherules	*Coccidioides immitis*, *Coccidioides posadasii*	10–200	Spherules vary in size; some contain endospores, others are empty and collapsed; hyphae may be seen in cavitary lesions (Fig. 24)	Southwestern United States, Mexico, Central and South America (*C. posadasii* organisms are non-Californian strains)
Yeast forms and pseudohyphae or true hyphae	*Candida* spp.	3–4 (yeast forms); 5–10 (pseudohyphae)	Cells usually exhibit single budding (Fig. 4, 6); pseudohyphae, when present, are constricted at ends and remain attached like links of sausage (Fig. 4, 5); true hyphae, when present, have parallel walls and are septate	Worldwide
Yeast forms and pseudohyphae	*Malassezia* species	3–8 (yeast forms); 5–10 (pseudohyphae)	Short, curved hyphal elements may be present with round, oval or elongated yeast cells that are round at one end and flattened at point of conidiation (Fig. 8)	Worldwide
Wide pauciseptate hyphae	Mucoraceous moulds	10–30	Hyphae are large, ribbonlike, often fractured or twisted. Occasional septa may be present, branching usually at right angles (Fig. 3, 11, and 12). The Splendore-Hoeppli phenomenon on H&E sections may be seen with *Basidiobolus* and *Conidiobolus* species.	Entomophthorales: worldwide *Basidiobolus ranarum:* tropical areas of Asia, Africa, South America (mostly Brazil), Mexico, Australia (children) *Conidiobolus coronatus* and *C. incongruus*: tropical and subtropical regions of Africa and Southeast Asia (adults)

Hyaline (colorless) septate hyphae	Dermatophytes (skin and nails)	3–15	Hyaline septate hyphae commonly seen; chains of arthroconidia may be present (Fig. 19)	Worldwide
	Dermatophytes (hair)	3–12	Arthroconidia on periphery of hair shaft that produce sheaths indicate ectothrix infection. Arthroconidia formed by fragmentation of hyphae within hair shaft indicate endothrix infection. Long hyphal filaments or channels within hair shaft indicate favus hair infection.	
Hyaline septate hyphae	*Aspergillus* species	4–6	*Aspergillus* spp. are generally septate with consistent diameter throughout; often show repeated dichotomous, 45° angle branching (Fig. 1, 9, 10, 13, and 14)	Worldwide
	Scedosporium species (not eumycetoma), *Fusarium* species, *Paecilomyces* species	3–12	Hyphae are septate, difficult to distinguish from other hyaline moulds. May exhibit less 45° angle and more 90° angle branching.	
Dematiaceous (darkly pigmented) septate hyphae	Dematiaceous fungi	1.5–6	Dematiaceous polymorphous hyphae, budding cells with single septa and chains of swollen rounded cells may be present. Occasionally, aggregates may be present when infection is caused by *Phialophora* or *Exophiala* species (Fig. 2).	*Hortaea werneckii:* subtropical coastal locations *Piedraia hortae:* tropical climates of Central and South America, Southeast Asia, and the South Pacific Islands *Rhinocladiella mackenziei:* Middle East *Cladophialophora bantiana* found in Asia
Sclerotic bodies (muriform cells)	*Cladophialophora carrionii*, *Fonsecaea compacta*, *Fonsecaea pedrosoi*, *Phialophora verrucosa*, *Rhinocladiella aquaspersa*	5–20	Brown, round to pleomorphic, thick-walled cells with transverse septa. Commonly, cells contain two fission plates that form tetrads of cells. Occasionally, branched septate hyphae may be found in addition to sclerotic bodies (Fig. 20).	Occurs worldwide, although most reported cases are from tropical and subtropical regions of the Americas and Africa
Granules (white grain eumycetomas)	*Acremonium* and related spp. (*Sarocladium kiliense*, *Acremonium recifei*)	200–300	White, soft granule; cementlike matrix absent	Asia, North, South and Central America, Oceania, Europe
	Aspergillus nidulans	65–160	White, soft granule; cementlike matrix absent	Africa
	Fusarium spp. (*F. moniliforme*, *F. oxysporum species complex*, *F. solani species complex*)	80–200	White-yellowish color of the grains, edges are entire or lobed; surrounded by an eosinophilic homogeneous material. Hyphae comprising the granules are not embedded in cement.	Europe, South America, Caribbean, Africa, Asia
	Neotestudina rosatii	500–1,000	Round, oval or lobed white grains; compact with cement in the center	West Africa
	Pseudallescheria boydii	1,000–2,000	White-yellow, soft granules composed of hyphae and swollen cells at periphery in cement-like matrix (Fig. 17)	Worldwide, most common in North America

(Continued on next page)

TABLE 1 Characteristic fungal morphology seen by direct examination of clinical specimens *(Continued)*

Fungal morphology observed	Organism(s)	Diameter range (µm)	Characteristic features	Geographic distribution
Granules (black grain eumycetomas)	*Curvularia* spp. (*C. geniculata*, *C. lunata*)	500–1,000	Black, hard grains with cementlike matrix at periphery	Worldwide
	Exophiala jeanselmei	500–1,000	Black, soft granules, vacuolated, cementlike matrix absent, made of dark hyphae and swollen cells	Worldwide
	Falciformespora senegalensis, *Falciformespora tompkinsii*	400–600	Black, soft granules; cement-like matrix; in tissue sections, the central part consists of hyphae, and a black cement-like substance is seen at the periphery	West Africa (specifically Senegal and Mauritania)
	Trematosphaeria grisea	300–600	Black and soft with a brown cementlike material in the periphery of the granules	India, Africa, Central and South America
	Madurella mycetomatis	500–900	Black to brown hard granules of two types: (i) rust, brown, compact, and filled with cementlike matrix and (ii) deep brown, filled with numerous vesicles, 6–14 µm in diameter, cement-like matrix in periphery and central area of light-colored hyphae	India, Africa, and South America
	Medicopsis romeroi, *Biatrospora mackinnonii*	40–100 × 50–160	Black, soft granules composed of polygonal swollen cells at periphery, cementlike matrix	Africa, India, and South America

stains and procedures can be used to detect fungi (Table 2), and the role of histopathological diagnosis has recently been comprehensively reviewed (4). Staining methodology is reviewed in chapter 115.

The Gram stain may be useful in diagnosis, primarily because it is commonly used in most laboratories (hence, laboratory staff are familiar with it) and it may enable differentiation of yeasts from moulds in the initial sample. Although Gram reactions can vary, yeast cells and pseudohyphae generally stain Gram positive and hyphae (septate and aseptate) stain Gram negative. The size and shape of budding yeastlike cells can indicate a presumptive genus identification. *Cryptococcus* yeast cells are generally round and display an amorphous orange-staining material, presumably the capsule.

Familiarity with the appearance of certain fungi in tissue may be helpful when performing direct examination. *Aspergillus* hyphae tend to be of a consistent diameter (4 to 6 µm) and commonly demonstrate 45° branching, with 90° branching less common. *Fusarium* and *Scedosporium* species look very similar to *Aspergillus* species in tissue (5). Sporulation in tissue is rare, but it can be distinctive with certain fungi. For example, aleurioconidia can be indicative of *Aspergillus terreus*. *Fusarium* may produce a combination of hyphae and yeastlike structures, and *Scedosporium* may produce ovoid, pigmented annelloconidia (4). When fungal stains show both unicellular forms and filaments that appear to be hyphae or pseudohyphae, infection with *Fusarium*, *Paecilomyces*, or *Acremonium* species should be considered, although filamentous yeast should also be included in the differential diagnosis (6). Mucoraceous moulds have aseptate or sparsely septate hyphae with wide-angle branching. The hyphae often appear twisted and flattened due to the lack of hyphal support because of the absence of septa (7). The Splendore-Hoeppli phenomenon on H&E sections may be seen with blastomycosis and sporotrichosis as well as with infections caused by *Basidiobolus* and *Conidiobolus* species. This phenomenon is the deposition of amorphous, eosinophilic material in tissue that occurs as a result of the antigen-antibody reaction. Dematiaceous moulds may display multiple forms, such as septate hyphae with parallel walls, rounded forms arranged in chains, or muriform (sclerotic) bodies. The diagnosis of phaeohyphomycosis can be made only if pigmented hyphae are observed. Sometimes pigmentation can be observed by using a traditional KOH preparation; however, care should be taken if these are stained with calcofluor white, because this is not taken up well by melanized hyphae. Some structures can provide definitive diagnosis of the etiologic agent (e.g., spherules of *Coccidioides* spp.). Specimens obtained from patients treated with antifungal drugs may demonstrate atypical structures, such as variations of hyphal size within the same hyphae, lack of septation within the hyphae, or bulb forms within the hyphae or at the hyphal tips.

Many artifacts can be mistaken for fungal structures when performing direct microscopy. Lymphocytes in cerebrospinal fluid (CSF) may be mistaken for *Cryptococcus* species; fibers and debris may be mistaken for hyphae, because they can fluoresce with calcofluor white; and fat droplets may be confused with budding yeastlike cells. Experience reading direct microscopy may overcome these problems, and discussions with colleagues may be necessary where doubt persists. Alternatively, use of another method may help differentiate artifacts from genuine findings.

A positive result on direct microscopy should be reported promptly with as much information as necessary to clarify the result. For example, "Yeast cells seen" is more helpfully reported as "Microscopic appearance may represent commensal species (e.g., *Malassezia*)" in a skin sample if the characteristic broad-based budding associated with this genus is seen. Examples of various characteristic structures seen in direct microscopy of clinical specimens from fungal infections are seen in Fig. 1 to 26.

ANTIBODY DETECTION

Antibody detection may be useful in a range of diseases, particularly when they occur in immunocompetent patients, although it may have limited use in immunocompromised patients. Several immunological techniques have been used to detect antibodies during fungal infections, including immunodiffusion (ID), countercurrent immunoelectrophoresis (CIE), enzyme-linked immunosorbent assay (ELISA), complement-fixation tests (CFTs), and most recently, fluorescent-enzyme immunoassay (FEIA).

Aspergillus Species

Antibodies to *Aspergillus* can be detected in most healthy individuals due to exposure to spores in the air (8), but their role in diagnosing aspergillosis in immunocompromised patients is minimal. In contrast, in patients who are non-neutropenic and develop aspergillosis, antibody detection may be helpful. Various techniques have been used, including countercurrent immunoelectrophoresis and FEIA; these tests mainly measure IgG but often use poorly defined antigen preparations, although recombinant antigens are available from some manufacturers. Antibody detection is helpful in diagnosing allergic bronchopulmonary aspergillosis (ABPA) (9), aspergilloma (10), and chronic cavitary aspergillosis (11). Although background levels of antibody do occur, through careful examination of the performance of tests (e.g., using receiver operator curve analysis), it is possible to define a lower, nonsignificant level and also a level at which antibodies become indicative of disease (12).

An EIA is available commercially for the measurement of *Aspergillus* IgG (Platelia BioRad; Bio-Rad, Hercules, CA), which is CE-marked but not U.S. Food and Drug Administration (FDA) approved. The kit defines antibody levels of <5 U/ml as negative, ≥10 U/ml as positive, and levels in between as intermediate. However, from the data presented by the manufacturer on evaluation of the performance of the kit in patients with cystic fibrosis, it is not clear how the diagnosis of ABPA was made and thus how reliable the figures are for sensitivity and specificity.

Blastomyces Species

Blastomyces yeasts produce two antigens, A antigen and WI-1 antigen, of which only the former is used in commercially available kits. Detection of antibodies to *Blastomyces* can be carried out by immunodiffusion, CFT, or ELISA, and there are commercially available assays or reagents in all these formats. Immunodiffusion may be positive in about 40 to 60% of patients, but the CFT is less helpful, with positivity in less than 20% of patients (13). Using an EIA, antibodies were detected in 83% of patients with blastomycosis, but 47% of specimens from patients with histoplasmosis were also positive (14). Therefore, antibody detection is rarely definitive due to its lack of sensitivity and widespread cross-reactivity with other dimorphic fungi (15).

Candida Species

Detection of antibodies specific to *Candida* is problematic as a diagnostic tool, because many people carry *Candida* as a commensal and thus have low levels of antibodies in the

TABLE 2 Methods and stains available for direct microscopic and histological detection of fungi in clinical specimens

Method	Use	Time required (min)	Color of fungus	Comments
Stains and methods used directly on unfixed tissue or samples				
Potassium hydroxide (KOH)	Clearing and softening of specimen to make fungi more readily visible	5–30	Hyaline moulds and yeasts appear transparent, while dematiaceous moulds may display golden brown hyphae.	Can be used in combination with calcofluor for fluorescence microscopy.
Calcofluor white	Detection of all fungi and cysts of *Pneumocystis jirovecii*	1–2	Depending on which barrier filter is used, fungal elements appear blue-white or bright green against a dark background (Fig. 10 and 12). Cysts have "double parenthesis-like" structure in center.	Requires fluorescence microscope; collagen and swab fibers also fluoresce, fat droplets may look similar to yeast cells. Counterstain minimizes background fluorescence. Melanized fungi may not stain as effectively.
Diff-Quik	Also applied to other specimens, such as BAL fluid, CSF	2–3	Yeast cells and trophozoites appear blue-purple (Fig. 15).	Differentiate from *Leishmania*; *Leishmania* has a kinetoplast, and *Histoplasma* does not.
India ink, nigrosin	Detection of *C. neoformans* in CSF	1	Capsules around yeast cells show as clear halos against a black background (Fig. 16).	When positive in CSF, diagnostic of meningitis. Negative in many cases of meningitis, not reliable
Stains used on heat or alcohol fixed samples or tissues				
Giemsa stain	Primarily used for the examination of bone marrow and peripheral blood smears	15	Detects trophozoite stage of *P. jirovecii*.	Detects intracellular *H. capsulatum* and fission yeast cells of *T. marneffei* (Fig. 26).
Gram stain	Detection of bacteria and fungi	3	Generally, yeast and pseudohyphae stain Gram positive and hyphae (septate and aseptate) appear Gram negative (Fig. 1–7).	*Cryptococcus* species stain weakly in some instances and exhibit only stippling. Often have orange amorphous material around yeast (Fig. 7). Not all fungi are detected.
Toluidine blue	Detection of *P. jirovecii* in respiratory specimens	25	Cyst walls are purple (Fig. 18).	The background and other fungi stain the same color.
Papanicolaou	Cytologic stain used primarily to detect malignant cells	30	Depending on cell type detected, background stains in subtle range of green blue, orange to pink hues. *Candida* stains gold, while other fungi may not stain at all.	Further stains should be performed.
Alcian blue	Detection of *C. neoformans* in CSF	2	Capsule stains blue against a pink background.	Mucopolysaccharide stain, not commonly used; like India ink, does not detect all cases.
Stains used on fixed tissues				
Hematoxylin & eosin (H&E)	General purpose histologic stain	30–60	Fungal cytoplasm is pink and the nuclei are blue. Demonstrates natural pigment of dematiaceous fungi (Fig. 14, 17, and 24). Background tissue is red.	Permits visualization of host tissue response to the fungus. *Aspergillus* spp. and mucoraceous moulds stain well. The Splendore-Hoeppli phenomenon may be seen with some fungi, including *Basidiobolus* and *Sporothrix*.
Periodic Acid-Schiff (PAS)	Detection of fungi	20–25	Fungi stain pink to red purple; nuclei may be blue depending on the counterstain used.	PAS stains glycogen, so other tissue structures can have a similar appearance to yeast cells.
Gomori methenamine silver (GMS)	Detection of fungi, including *Pneumocystis*	5–60	Fungal elements including *Pneumocystis* cysts stain gray to black. Background is green (Fig. 9, 11, 13, and 21).	Often stains fungi too densely to observe structural details. Yeast cells and cysts of *Pneumocystis* may appear similar in size and shape.
Mucicarmine	Stains mucin	60	Stains capsule of *Cryptococcus* pinkish red.	May also stain cell walls of *B. dermatitidis* and *Rhinosporidium seeberi*.
Fontana-Masson (FM)	Detection of melanin of dematiaceous fungi and *C. neoformans*	60	Cell walls black; background pale pink	Many nondematiaceous fungi (some *Aspergillus*, mucoraceous moulds, and *Trichosporon* species) can be FM positive. If hyaline in H&E, examine morphology carefully.

absence of disease (16). The main antigen used is mannan, a cell wall component, and various immunoglobulin classes may be detected, including IgG, IgM, and IgA. Many studies have examined the sensitivity and specificity of antibody detection for diagnosis of invasive candidiasis and found disappointingly low figures (17, 18). Sensitivity as low as 62% and specificity as low as 53% have been reported when antibodies alone are used for diagnosis, and thus most experts now recommend they be used in conjunction with *Candida* mannan antigen detection (18, 19)

Coccidioides Species

Antibodies to *Coccidioides* can be detected by various methods, including ID, CFT, and EIA. Historically, the tube precipitin (20) was also used, which has now been replaced by the ID test using the same antigens. The ID test detects mainly IgM antibodies to a β-glucosidase antigen, which is positive early in disease, while the CFT detects IgG antibodies to a heat-labile chitinase antigen, which is useful in diagnosing acute and chronic disease and can also be used prognostically (21). Early studies indicated that, in primary infection, precipitating antibodies peak by the third week of infection, with approximately 90% of sera positive, but then decline rapidly, with only 10% of sera positive at 5 months. Complement-fixing antibodies are detected in about 50% of sera by 1 month and continue to increase for several months (22).

An EIA is also now available that detects both IgG and IgM and can be used with serum or CSF (Premier Coccidioides EIA; Meridian, Cincinnati, OH). The EIA has higher sensitivity than ID and CFT, but there has been concern about whether the increased sensitivity is associated with decreased specificity (23). One study found no false-positive results (24), while others have found false IgM-positive results in patients (25). This has led to the suggestion that the EIA should be used as a screening tool, with any positive results confirmed by another method (21).

Cryptococcus Species

Antibodies to *Cryptococcus*—including IgG, IgM, and IgA produced against glucuronoxylmannan antigen and also crude antigenic preparations—can be detected in patients with cryptococcosis (26). Such antibodies have been suggested to have prognostic value (27) and can be a marker for reactivation of disease in solid-organ transplant recipients (28). However, their utility in diagnosis is limited because the polysaccharide capsular antigen may inhibit the synthesis of antibodies (29).

Histoplasma capsulatum

Detection of antibodies is an important component in establishing a diagnosis of histoplasmosis. Antibodies appear approximately 4 to 6 weeks after the primary acute infection and decline over a 2- to 5-year period (30). Two tests have been widely used and evaluated, namely ID and CFT.

The antigens used in the tests are extracted from histoplasmin, which is obtained from the mycelial phase of the fungus. Two antigens detected by the tests are the M antigen, which is a catalase (31), and the H antigen, which is a β-glucosidase (32).

The ID test, which is about 80% sensitive, detects precipitating antibodies to the antigens, resulting in the H and M "bands." The M band appears first during infection, can indicate either active or previous infection, and persists for several years. In contrast, the H band occurs during active infection, but is found in only a minority of patients, although it may persist for 1 or 2 years. If both bands are detected, this is considered to be diagnostic (33).

The CFT uses histoplasmin and an antigen produced by the yeast phase to detect complement-fixing antibodies, which appear 3 to 6 weeks after infection. Up to 95% of patients with histoplasmosis have a positive reaction in the CFT, although titers may be relatively low (34). Low titers may represent past infection, but titers of 1:32 or greater or a 4-fold rise in titer indicate active disease (30). Antibodies generally appear first to the yeast antigen and later to histoplasmin and decline over several years in the absence of further exposure (33). The CFT is more sensitive than ID but also subject to more cross-reactivity. Patients with aspergillosis, blastomycosis, candidiasis, coccidioidomycosis, paracoccidioidomycosis, tuberculosis, and other bacterial or viral diseases have been found to have positive reactions in the CFT (35). False-negative results can occur due to the presence of rheumatoid factor or cold agglutinins (36).

ANTIGEN DETECTION

Aspergillus Species

The main antigen used as a diagnostic marker in commercially available kits for invasive aspergillosis (IA) is galactomannan (GM). GM, a carbohydrate molecule with a mannose backbone, is released from the cell wall of *Aspergillus* hyphae during growth *in vivo* but is probably not detectable in patients until angioinvasion has occurred (37). GM can be detected in serum, bronchoalveolar lavage (BAL) fluid, and urine in infected patients. The Platelia *Aspergillus* GM antigen ELISA kit produced by BioRad uses a monoclonal rat antibody (EBA-2) to coat microtiter wells and act as the detector antibody conjugated to peroxidase. The amount of GM in each sample is expressed as a ratio of the sample optical density compared with the cutoff control optical density; GM indices greater than 0.5 are considered positive (Platelia *Aspergillus* Ag Kit insert). Although the manufacturers currently recommend a cutoff of 0.5 for BAL, many laboratories use a cutoff of 1 due to the improved sensitivity of the kit with BAL. The kit (which is CE-marked and FDA approved) is currently approved for the detection of GM in serum and BAL and has been extensively studied in a range of different patient groups; sensitivities and specificities vary widely depending on the setting in which it is used. Studies in patients with hematological malignancies and patients who have undergone hematopoetic stem cell transplantation (HSCT) have generally resulted in higher sensitivity and specificity than patients with solid organ transplantation. For patients with hematological malignancies, sensitivities of up to 100% have been reported in those with proven or probable IA (38), although other groups have found sensitivity of only 17% in proven IA (39). A recent review that analyzed data from 42 studies found that the sensitivity and specificity results varied from 0 to 100% and 50 to 99%, respectively (40). Unsurprisingly, studies that used a higher cutoff reported higher specificity and lower sensitivity, but this difference was not statistically significant. The frequency of testing will also affect the utility of the test, and twice weekly testing may be optimal (41).

Although GM is a useful marker for IA, there are many causes of false positivity. Piperacillin/tazobactam or amoxicillin with clavulanic acid (42), PlasmaLyte (43), and certain foodstuffs, including pasta, vegetables, and milk, are all known to cause false positives. In addition, several other fungal genera are known to be reactive in the kit, including

Penicillium, *Alternaria*, *Rhodotorula*, *Paecilomyces*, *Cryptococcus*, *Blastomyces*, and *Histoplasma*. Detection of GM may be reduced in patients with chronic granulomatous disease (44) and patients treated with mould-active antifungals (45)

As well as being used for diagnosis, GM has been assessed as a prognostic tool. Early studies demonstrated that an increase in the GM index was associated with progression of disease (46) and that patients in whom the GM index remained high had significantly poorer outcomes than those in whom it decreased (47). More recently, it has been shown that the initial level of the GM index in serum and the rate of decay in GM over the first week were predictive of time to mortality. In the first week, if the GM index increased by 1 unit, the risk of time to mortality increased by 25%, whereas a fall of 1 unit decreased the risk by 22% (48).

Although the GM ELISA is the most widely used assay for the diagnosis of IA, a new lateral flow device (LFD) has been developed (Isca Diagnostics, Truro, UK) that uses a monoclonal antibody, JF5, which binds to a protein epitope from an extracellular glycoprotein antigen produced by *A. fumigatus* (8). It was hypothesized that the problems with cross-reactivity would be reduced by targeting a non-GM antigen. The monoclonal antibody is used for both the capture and detection of the antigen in serum or BAL, visualized using colloidal gold, and seen as a line that appears within 10 to 15 minutes of the sample being added to the LFD. Because the antigen is produced only by growing hyphae, any issue due to contamination of samples with *Aspergillus* spores should not produce positive results. This makes the test more useful for testing BAL samples, while it is potentially a more useful marker of invasive disease than other tests that may detect inhaled spores.

A recent comparison of the LFD and GM ELISA in patients after allogeneic stem cell transplantation showed that the negative predictive value of the LFD was high (~92%). If only a single positive sample was used to define positivity, the LFD and ELISA had similar performance; however, if two samples were used for positivity, the LFD performed worse than the ELISA, with a positive predictive value (PPV) of 50% and a sensitivity of 20% (compared with the ELISA PPV of 75% and sensitivity of 30%) (49). Another comparison of the LFD and GM ELISA found that the LFD had a higher sensitivity (82%) and a specificity of 98% (50). Although the LFD has been described as a point-of-care test (8), the requirement to boil serum samples in a pH-buffered EDTA solution and centrifuge them during processing still means that testing must be done in a laboratory, although BAL samples may be processed directly. The kit is CE-marked, and FDA approval is currently being sought.

Blastomyces Species

Diagnosis of blastomycosis is often made by culture, which is ultimately positive in over 80% of patients; however, due to the time taken for the culture to grow, other methods may also be used to give quicker results (51). Currently, only one assay is available to detect *Blastomyces* antigen; provided by MiraVista Diagnostics (MiraVista Diagnostics, Indianapolis, IN), it uses rabbit antibodies raised against whole cells of *B. dermatitidis* in the mould phase (52). The assay can be used for most body fluids and has been evaluated mainly in urine, in which sensitivity is higher than for serum (53). The drawback with the assay is the presence of cross-reactivity in a range of other mycoses, including histoplasmosis, paracoccidioidomycosis, infections with *Talaromyces marneffei*, cryptococcosis, and aspergillosis (http://miravistalabs.com/medical-fungal-infection-testing/antigen-detection/blastomyces-dermatitidis-quantitative-eia-test/). A recent modification of the assay allowed quantification of antigen and increased its sensitivity when used with serum; however, it produced cross-reactivity in 96% of patients with histoplasmosis (54), which is problematic because the endemic areas for histoplasmosis and blastomycosis overlap in the United States. Due to the presence of such high rates of cross-reactivity, the utility of the *Blastomyces* antigen test is limited.

Candida Species

Diagnosis of systemic candidiasis can be problematic because many people carry *Candida* as a commensal, and the presence of *Candida* colonization is often thought to affect the sensitivity and specificity of current diagnostic tests. Commercially available tests usually detect mannan or mannoproteins and include the Platelia *Candida* Ag EIA (Bio-Rad) and the CandTec latex agglutination test (Ramco Laboratories, Stafford, TX). *Candida* mannan is rapidly cleared from the circulation and hence frequent testing is important. A recent meta-analysis has examined the issues of sensitivity, specificity, and the effect of colonization on these parameters, mainly in studies that examined both mannan antigen and antimannan antibody detection (19). One issue with the meta-analysis is the heterogeneity of the studies included, which is reflected in the wide ranges for the results reported. For mannan antigen detection alone, sensitivity ranged from 31 to 100%, with a median of 62% reported for the results. For antimannan antibodies, the figures were

FIGURE 1 (Row 1, left) Gram stain of BAL specimen showing Gram-negative branching septate hyphae of *Aspergillus fumigatus*. Magnification, approximately ×1,000. doi:10.1128/9781555817381.ch116.f1

FIGURE 2 (Row 1, right) Gram stain of skin biopsy with Gram-variable branching hyphae of *Exophiala jeanselmei*. Magnification, approximately ×1,000. doi:10.1128/9781555817381.ch116.f2

FIGURE 3 (Row 2, left) Gram stain of skin biopsy with Gram-negative aseptate hyphae of the *Rhizopus microsporus* group. Magnification, approximately ×1,000. doi:10.1128/9781555817381.ch116.f3

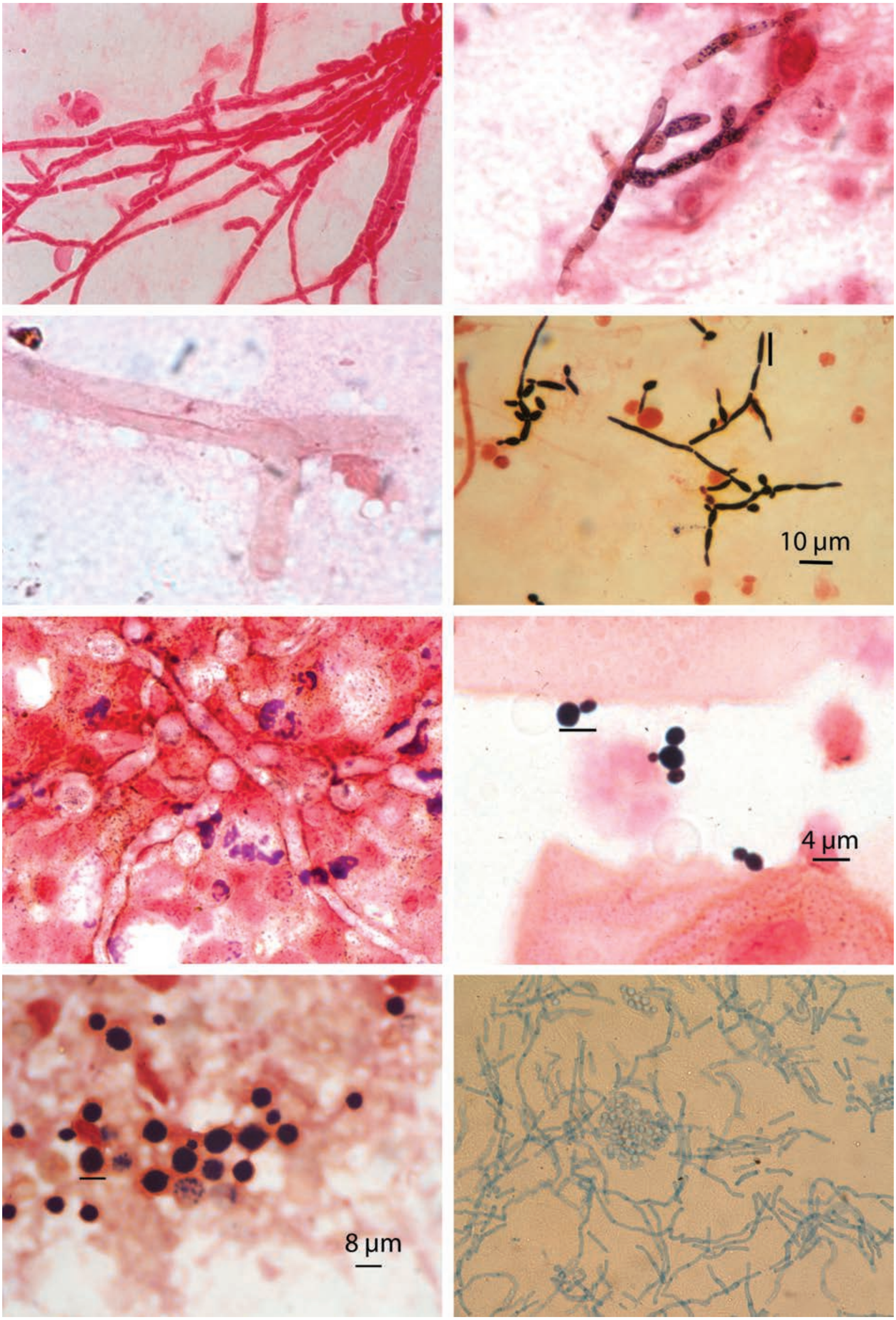
FIGURE 4 (Row 2, right) Gram stain of sputum with *Candida albicans* showing Gram-positive budding yeast cells with pseudohyphae. doi:10.1128/9781555817381.ch116.f4

FIGURE 5 (Row 3, left) Gram stain of spleen demonstrating ghost-like budding yeastlike cells with pseudohyphae. Magnification, approximately ×1,000. doi:10.1128/9781555817381.ch116.f5

FIGURE 6 (Row 3, right) Gram stain of vaginal secretions with Gram-positive budding yeast cells of *Candida glabrata*. doi:10.1128/9781555817381.ch116.f6

FIGURE 7 (Row 4, left) Gram stain of skin abscess with *Cryptococcus neoformans* showing Gram-positive and Gram-variable budding yeast cells of variable size surrounded by amorphous orange halos. doi:10.1128/9781555817381.ch116.f7

FIGURE 8 (Row 4, right) Parker ink stain of skin lesion showing typical yeast cells and short, stubby hyphae of *Malassezia* spp. Magnification, approximately ×400. doi:10.1128/9781555817381.ch116.f8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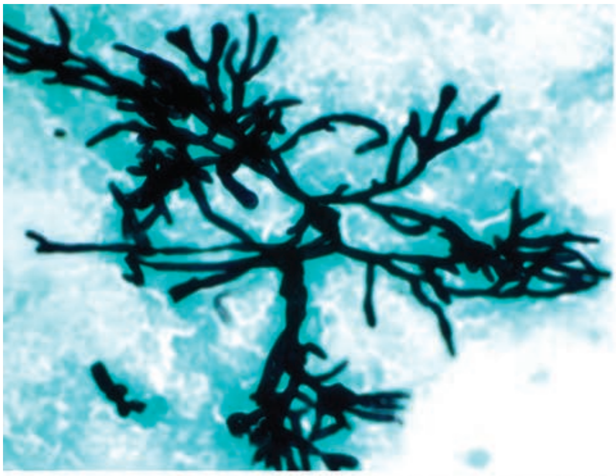
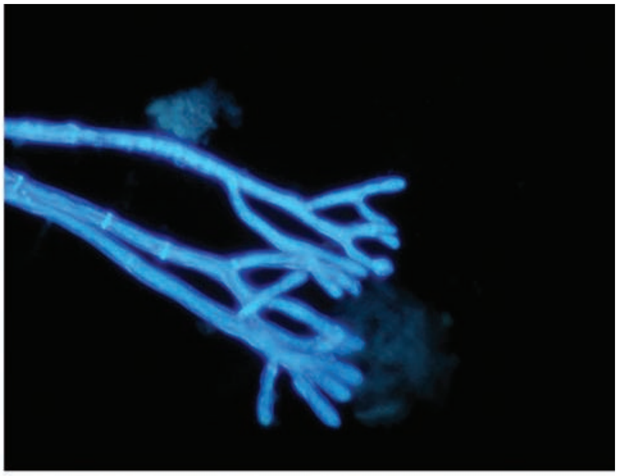
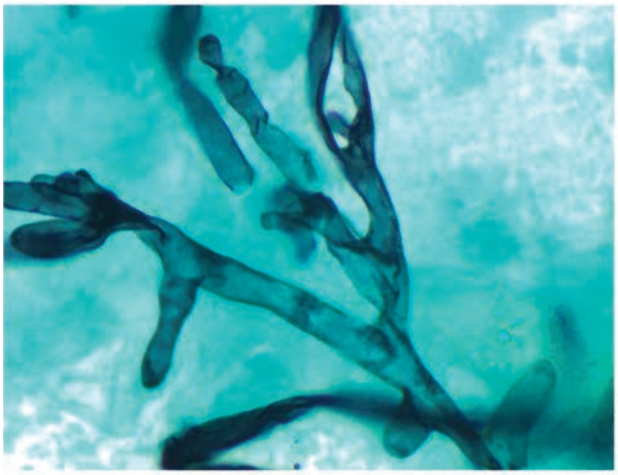
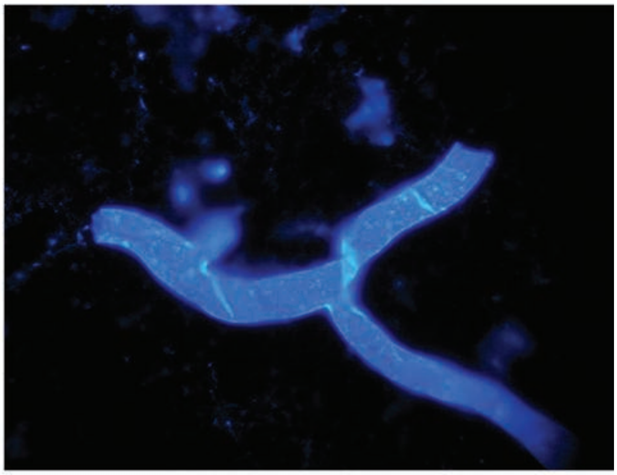
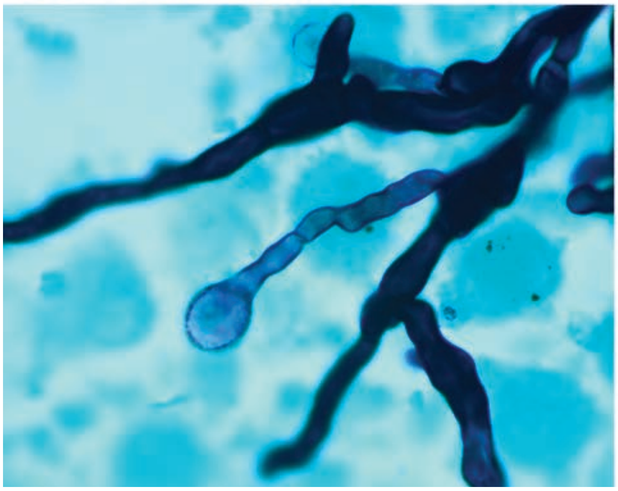










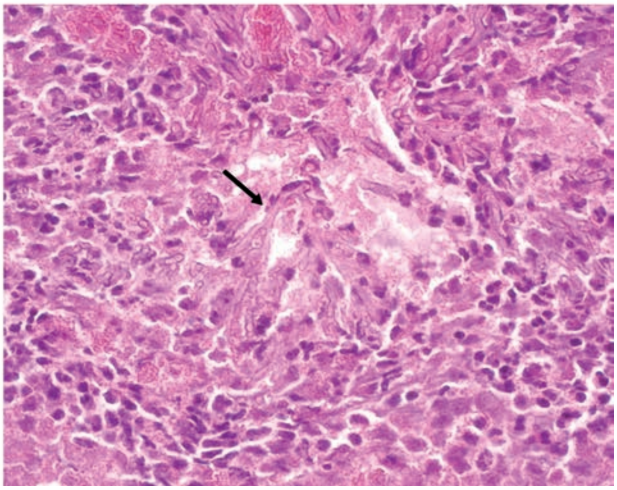


FIGURE 9 (Row 1, left) Gomori methenamine silver (GMS) stain of BAL specimen showing *Aspergillus fumigatus* dichotomously branching septate hyphae. Magnification, approximately ×400. doi:10.1128/9781555817381.ch116.f9

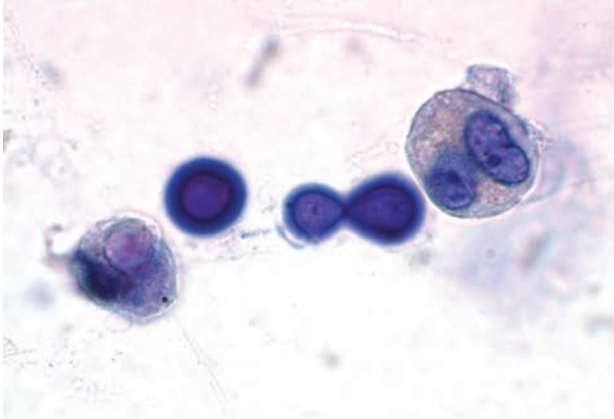
FIGURE 10 (Row 1, right) Calcofluor white stain of BAL specimen showing *Aspergillus fumigatus* dichotomously branching septate hyphae. Magnification, approximately ×500. doi:10.1128/9781555817381.ch116.f10

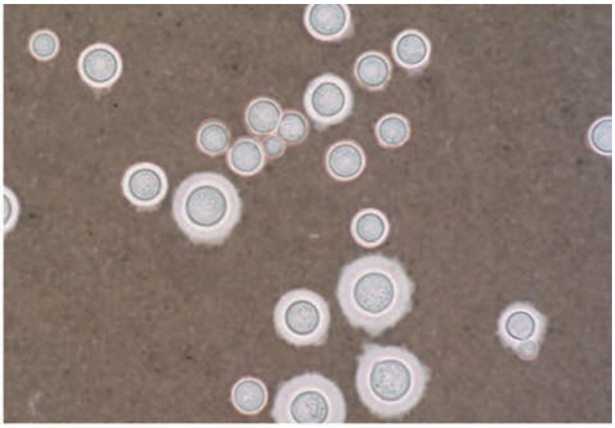
FIGURE 11 (Row 2, left) GMS stain of skin biopsy with *Rhizopus* spp. showing ribbonlike hyphae. Magnification, approximately ×400. doi:10.1128/9781555817381.ch116.f11

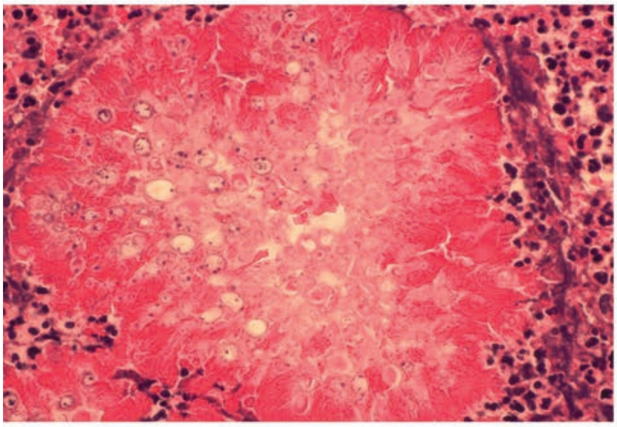
FIGURE 12 (Row 2, right) Calcofluor white stain of *Rhizopus* spp. showing ribbonlike hyphae. Magnification, approximately ×500. doi:10.1128/9781555817381.ch116.f12

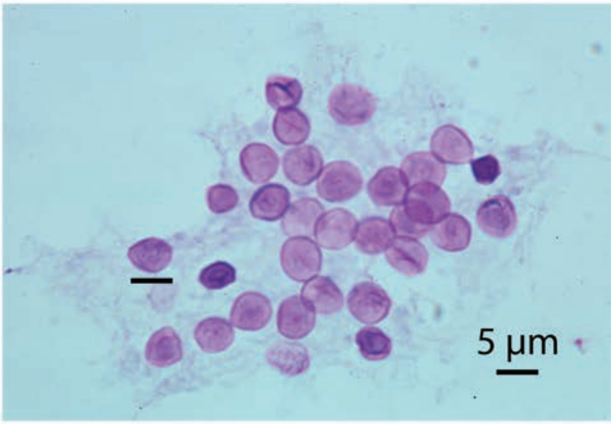
FIGURE 13 (Row 3, left) GMS stain of lung biopsy specimen showing *Aspergillus fumigatus* with bulb formation at hyphal tip. Magnification, approximately ×1,000. doi:10.1128/9781555817381.ch116.f13

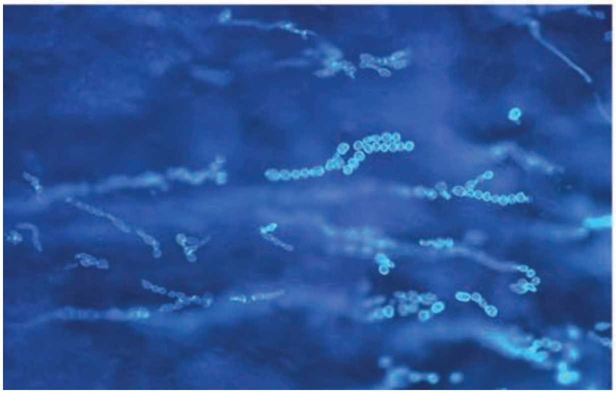
FIGURE 14 (Row 3, right) H&E stain of lung biopsy specimen showing *Aspergillus fumigatus* dichotomously branching septate hyphae (shown at arrow). Magnification, approximately ×400. doi:10.1128/9781555817381.ch116.f14











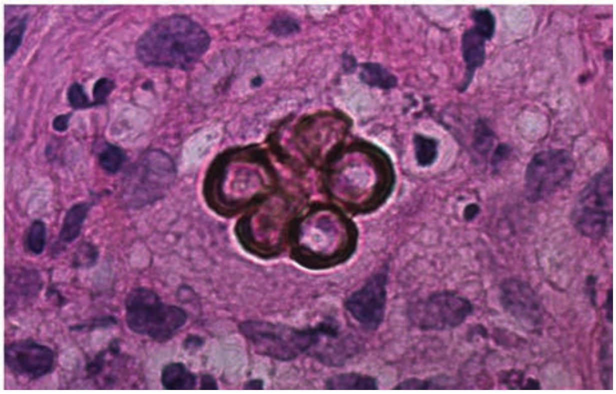


FIGURE 15 (Row 1, left) Diff-Quik stain of CSF with *Cryptococcus neoformans*. Magnification, approximately ×400. doi:10.1128/9781555817381.ch116.f15

FIGURE 16 (Row 1, right) India ink preparation of CSF showing encapsulated budding yeast cell of *Cryptococcus neoformans*. Magnification, approximately ×400. doi:10.1128/9781555817381.ch116.f16

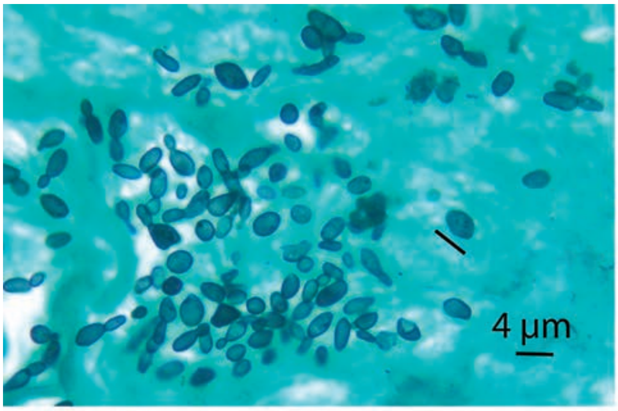
FIGURE 17 (Row 2, left) H&E stain of eumycotic mycetoma granule of *Scedosporium apiospermum*. Magnification, approximately ×400. doi:10.1128/9781555817381.ch116.f17

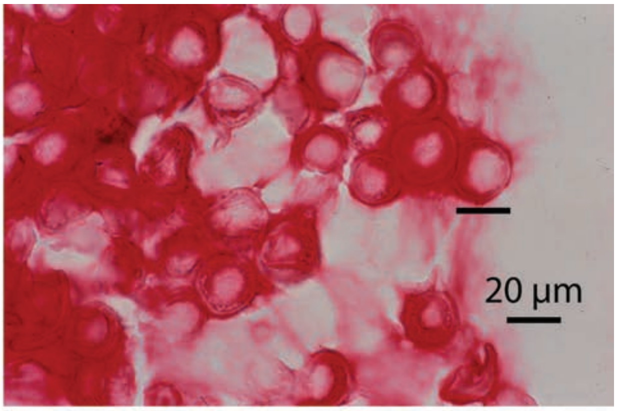
FIGURE 18 (Row 2, right) Toluidine blue stain of BAL specimen showing cysts of *P. jirovecii*. doi:10.1128/9781555817381.ch116.f18

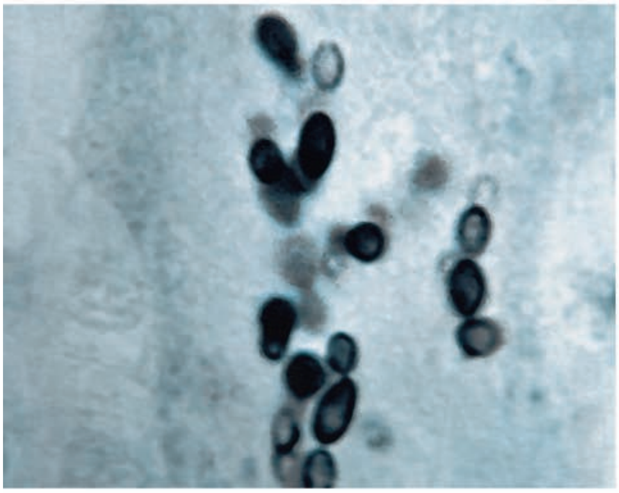
FIGURE 19 (Row 3, left) Calcofluor white stain of toenail specimen showing arthroconidia and hyphae of *Trichophyton rubrum*. Magnification, approximately ×200. doi:10.1128/9781555817381.ch116.f19

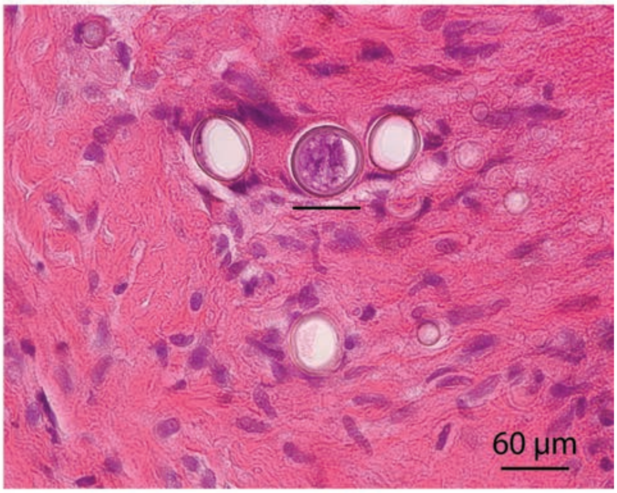
FIGURE 20 (Row 3, right) Muriform (sclerotic) cells (copper penny) in tissue of *Fonsecaea pedrosoi* chromoblastomycosis. Magnification, ×1,000. doi:10.1128/9781555817381.ch116.f20

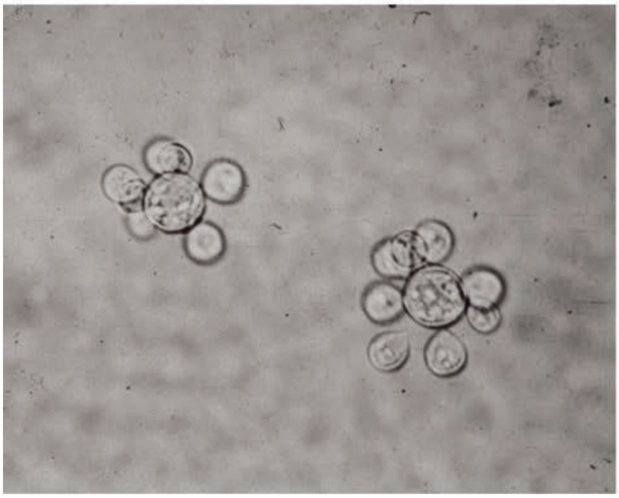
44 to 100%, but when the two approaches were combined, the median sensitivity was 86%. Sensitivity varied for different species of *Candida*, with the best results for *C. albicans*, followed by *C. glabrata* and *C. tropicalis*. Of note, the meta-analysis examined the effect of colonization of patients with *Candida* in four studies and found that neither colonization nor superficial candidiasis was associated with detection of mannan or antimannan antibodies (19). This led the authors to recommend that both mannan and antimannan antibodies should be used for the diagnosis of invasive candi-











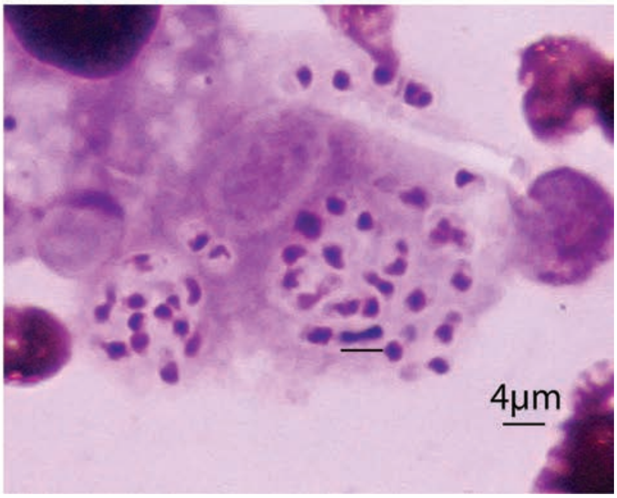


FIGURE 21 (Row 1, left) GMS stain of lymph node showing characteristic cigar-shaped yeast cells of *Sporothrix schenckii*. doi:10.1128/9781555817381.ch116.f21

FIGURE 22 (Row 1, right) Gram stain of abscess material showing large, broad-based, budding yeast cell with thick refractile wall characteristic of *Blastomyces dermatitidis*. doi:10.1128/9781555817381.ch116.f22

FIGURE 23 (Row 2, left) GMS stain of lymph node showing blastoconidia of *H. capsulatum*. Magnification, ×625. doi:10.1128/9781555817381.ch116.f23

FIGURE 24 (Row 2, right) H&E stain of *Coccidioides immitis*. Large, round, thick-walled spherules (10 to 80 µm in diameter) filled with endospores (2 to 5 µm in diameter). Young spherules have a clear center with peripheral cytoplasm and a prominent thick wall. doi:10.1128/9781555817381.ch116.f24

FIGURE 25 (Row 3, left) Bright-field photomicrograph of *Paracoccidioides brasiliensis* showing multiple budding yeast cells resembling mariner's wheels. Magnification, ×1,590. doi:10.1128/9781555817381.ch116.f25

FIGURE 26 (Row 3, right) Wright-Giemsa stain of BAL specimen showing characteristic fission yeast cells of *Talaromyces marneffei*. doi:10.1128/9781555817381.ch116.f26 diasis and that the combined approach was better than use of either technique alone.

Coccidioides Species

Diagnosis of coccidioidomycosis can be achieved using a range of methods, including antibody or antigen detection, or by culture. While culture remains the gold standard, the risks inherent to laboratory personnel dealing with cultures means that use of other methods is to be encouraged. The most fully validated test available for detection of *Coccidioides* antigen is an EIA provided by MiraVista Laboratories (http://miravistalabs.com/medical-fungal-infection-testing/antigen-detection/coccidioides-quantitative-eia-test/). The MiraVista test uses antibodies against *Coccidioides* GM and is suitable for use with urine, serum, plasma, BAL, CSF, and various other body fluids in patients with severe forms of disease (55). Treatment of serum and urine with EDTA to dissociate immune complexes improves the positivity in serum from 28 to 73%, but positivity in urine is only 50% (56). However, as with several other antigen detection assays for the endemic fungi, cross-reactions are common in patients with histoplasmosis or blastomycosis (56).

Cryptococcus Species

Detection of cryptococcal polysaccharide antigen in body fluids is an integral part of the diagnostic process for cryptococcal disease. The antigen detected is glucuronoxylmannan, which is the main component of the capsule and differs structurally among different serotypes. Classically, species of *Cryptococcus* were divided into five serotypes, A, B, C, D, and AD, but recent molecular studies have defined nine types and ongoing work may define further types. Serotype A contains molecular types VNI, VNII, and VNB and is designated *C. neoformans* var. *grubii*. Serotype D contains molecular type VNIV and, with serotype AD hybrids (VNIII), comprises *C. neoformans* var. *neoformans*. Serotypes B and C are *C. gattii* and comprise molecular types VGI, VGII, VGIII, and VGIV (29).

Commercially available kits for detection of cryptococcal antigen, which have both FDA approval and CE marking for serum and CSF, include enzyme immunoassays (Meridian and IMMY [Immuno-Mycologics, Norman, OK]) and latex agglutination assays (BioRad, IMMY, and Meridian). The sensitivity and specificity for the EIA and LA tests are high for both serum and CSF, in which figures of >90% are commonly reported in studies (57, 58). Cryptococcal antigen may also be detected in BAL (59) and urine (60), although these samples are not currently routinely assayed. The presence of high initial CSF titers (≥1:1,024) in patients with HIV infection is known to carry a poor prognosis (61) and, although CSF titers decrease over time, they do so too slowly to be used useful in monitoring therapy (62). Titers obtained from the same sample using different kits may vary, so laboratories should use kits from a single source.

Recently the FDA approved an LFD for use with serum and CSF (IMMY; Immuno-Mycologics). The LFD has been evaluated and shows enhanced sensitivity in serum when compared with both latex agglutination (63) and EIA (64). Several authors have noted that the titers obtained with the LFD are higher and thus are not comparable with results from other kits. Although not approved for use with urine, the LFD also has good sensitivity in this sample type, ranging from 92 to 100% (63, 64). The advantage of the LFD is its lower cost compared with other kits (63) and its ease of use, particularly in resource-poor settings. A recent study demonstrated its superiority in diagnosing the disease in a multicenter evaluation in Africa (65).

Multiple causes of false-positive and false-negative results have been documented in the cryptococcal antigen kits. False-positive results have been attributed to disinfectants and soap (66), agar (67), bacterial infections (68), rheumatoid factor (69), systemic lupus erythematosus (70), and infection with *Geotrichum beigelii* (71), while false-negative results may be caused by low antigen titers (72) or high titers (73). False positives due to high antigen titers are collectively known as the prozone effect; for this reason, it is good practice to test specimens undiluted and at a 1:10 dilution. Modifications of the tests have generally overcome these problems (73, 74).

Histoplasma capsulatum

Histoplasmosis may be diagnosed using different methods, but as with other slow-growing fungi, diagnosis by culture may take several weeks to become positive (30). Other modalities for diagnosis include histopathology, antigen detection, antibody detection, and PCR. There is currently only one commercially available test for *Histoplasma* antigen (MiraVista Diagnostics), which was originally developed in the mid 1980s as a radioimmunoassay but is now an EIA. The assay uses polyclonal rabbit antiserum raised against a polysaccharide antigen from *H. capsulatum* found in various body fluids during infection (75). The sensitivity of the assay varies with patient group, and most experience has been in patients with HIV infection and disseminated disease in whom antigen is found in the urine and serum in 95 and 86% of patients, respectively. In other immunocompromised patients, the test is less sensitive, with 82% sensitivity in urine and 60% in serum (76). It is useful only in the early diagnosis of acute histoplasmosis, within 3 weeks of exposure. As well as disseminated histoplasmosis, the antigen test may also be useful in acute pulmonary disease, in which antigenemia and antigenuria were reported in 68 and 65% of patients, respectively (77). The utility of the test in other forms of histoplasmosis is much lower, with sensitivities of 15% or less for mediastinal or chronic pulmonary disease (76). In addition to serum and urine, the assay has also been evaluated in BAL and CSF. A recent study found that antigen could be detected in BAL from 94% of patients with histoplasmosis and in urine and serum samples from 79 and 65%, respectively, of the same patients (78). Although no large studies have assessed the sensitivity of antigen in CSF, it has been reported to have sensitivities ranging from 38% to 67% in small case series (79).

Antigen titers fall with successful treatment (80) and rise with relapses (81), so the test also has prognostic utility.

One of the major drawbacks with the antigen test is the frequency of false-positive results. Cross-reactivity in the urine test occurs in patients with blastomycosis, paracoccidioidomycosis, and *Talaromyces marneffei* infection. Rabbit antithymocyte globulin can cause false positives in serum, rheumatoid factor, and treatment with rabbit antithymocyte globulin (30).

Although the antigen test from MiraVista is the most widely used and studied, other groups have also developed assays (82, 83). Cloud et al. (83) used commercially available polyclonal antibodies and found good correlations with both positive and negative samples compared with the MiraVista test, but again, the problem of cross-reactivity remained with *Blastomyces*, *Coccidioides*, and *Paracoccidioides*. Although monoclonal antibodies have been produced, overcoming the problem of specificity, they lack sensitivity and thus have not been further developed (84). An assay for urinary *Histoplasma* antigen has been developed by the Centers for Disease Control and Prevention and evaluated specifically for resource-limited settings; it demonstrated a sensitivity of 81% and specificity of 95% during evaluations (85).

The problems of cross-reactivity have led some workers to suggest that the detection of the urinary antigen should not be used as the sole method on which to base a diagnosis of histoplasmosis but that it should be used in conjunction with other methods (30).

(1, 3)-β-D-GLUCAN DETECTION

(1,3)-β-D-Glucan is a polysaccharide present in the cell wall of many fungi, including *Aspergillus*, *Candida*, and *Fusarium*; however, *Cryptococcus*, *Blastomyces*, and the mucoraceous moulds have very little or none. It is found in the blood of patients with invasive fungal infections, and its ability to activate factor G of the horseshoe crab coagulation pathway allows it to be measured and quantified. Several assays are commercially available (86), but the most widely available assay, and the only one with FDA approval and CE marking, is Fungitell, produced by Associates of Cape Cod (Associates of Cape Cod, Inc., Falmouth, MA) (87), which uses the coagulation cascade derived from the *Limulus* horseshoe crab as the detector method.

Early studies of the assay initially used a cutoff of 60 pg/ml (87). However, in a large multicenter study, the sensitivity and specificity were found to be 69.9 and 87.1%, respectively, at 60 pg/ml and 64.4 and 92.4%, respectively, at 80 pg/ml (88); the manufacturers now recommend interpreting <60 pg/ml as negative and ≥80 pg/ml as positive, with an indeterminate region in between (http://www.acciusa.com).

Clinical use of the assay has highlighted several important features. In one study, levels of β-D-glucan varied markedly between and within patients; optimal sensitivity, specificity, and accuracy occurred when two sequential samples had levels of ≥80 pg/ml, and successful clinical outcome did not always result in falls in β-D-glucan levels (89). A large retrospective study of 80 patients with proven and 36 patients with probable invasive fungal disease found that systemic antifungal therapy did not affect the sensitivity of the test. The test was more sensitive in IA than in invasive candidiasis; however, in patients with either hematological malignancy or stem cell transplantation, sensitivity was reduced (90). Optimal sensitivity occurs when patients are tested twice weekly (91). False-positive β-D-glucan results may be due to several factors, such as administration of intravenous immunoglobulins or albumin (90), hemodialysis using cellulose membranes (90), the use of glucan-containing gauzes or swabs (92), bacteremia (93), mucositis or graft-versus-host disease, and the use of various antibiotics (94).

One recent meta-analysis (95) included various β-D-glucan assays (including Fungitell, Glucatell [Associates of Cape Cod, Inc.], Fungitec [Seikagaku Corp, Tokyo, Japan], and Wako BG [Wako Pure Chemicals Co, Osaka, Japan]) and documented a pooled sensitivity of 79.1% and a pooled specificity of 87.7% for the β-D-glucan assays for 365 patients with proven invasive fungal disease; however, the ranges in the individual studies were very wide (sensitivity, 50 to 100%; specificity, 45 to 100%). Interestingly, they found no difference in sensitivity between patients with candidiasis or aspergillosis, in contrast with some single-center studies (90). The authors concluded that the assay has good diagnostic accuracy but that the optimal sampling regimen, criteria to define positive results, and optimal cutoff all required further evaluation, as well as careful consideration of causes of false positivity during routine clinical use.

Recently, β-D-glucan proved useful in diagnosing and monitoring an outbreak of fungal meningitis caused by *Exserohilum rostratum* (96).

β-D-Glucan has also been used as a diagnostic marker for *Pneumocystic jirovecii* in both HIV- positive and HIV-negative patients (97, 98). Sensitivity and specificity in this setting were generally high, with sensitivities of 96.4 to 100% and specificities of 87.8 to 100%, although studies have used widely differing cutoff values to define positives: 23.2 pg/ml (99) and 100 pg/ml (98). Despite successful treatment in HIV patients, the β-D-glucan levels did not decrease and thus the assay could not be used prognostically (99). Two recent meta-analyses have evaluated the test for diagnosis of *P. jirovecii* pneumonia (PCP) and confirmed that it performs better for this indication than for diagnosis of other invasive fungal infections. The diagnostic odds ratio in one analysis for PCP was 102.3, compared with 25.7 for other invasive fungal disease (100). Although the presence of β-D-glucan in patients may indicate the presence of either PCP or another invasive fungal disease, a recent meta-analysis concluded that, because of the risk of false positives in certain patient groups due to their exposure to various procedures and medications, a negative β-D-glucan result might be more helpful in excluding these diagnoses, and a positive result should be treated cautiously (101).

FUNGAL-SPECIFIC METABOLITE DETECTION

Many fungal metabolites, as well as fungal antigens, have been evaluated for their utility in detecting fungi in clinical specimens. Metabolites that have been assessed include D-arabinitol (102), D-mannitol (103), the ratio of D-arabinitol to L-arabinitol (104), gliotoxin (105), and 2-pentylfurane (106). The ratio of D-arabinitol to L-arabinitol in serum has recently been shown to be useful in detecting candidemia in the context of hematological neutropenic patients (18), while 2-pentylfurane has shown promise in diagnosing IA in a breath test (107). The ability to detect these metabolites using mass spectrometry may enable larger studies of their clinical utility (108), but to date, none of these tests is available commercially.

MASS SPECTROMETRY

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry is increasingly used for the identification of both yeast and mould cultures after recovery on solid media (109, 110). However, the requirement to grow the organisms on solid media introduces a delay, and the ability to identify fungi directly in the original clinical sample is desirable. The presence of proteins and hemoglobin in blood cultures can interfere with the spectra produced and confuse the identification obtained (111). Several groups have developed methods for direct detection, but with varying success rates (112, 113), and several of the studies used simulated blood cultures (114, 115), which obviously differ significantly from those obtained in clinical practice. Recently, a large two-center study with 346 positive yeast blood cultures found that direct MALDI-TOF identification yielded correct identifications of 95.9% of *C. albicans* and 86.5% of non-*C. albicans* species compared with conventional culture-based methods (116). This suggests that direct detection and identification of many *Candida* species following incubation of blood cultures is already feasible and may become the standard procedure relatively soon, although large-scale clinical evaluations in different patient groups are still required to confirm this.

NUCLEIC ACID DETECTION

Nucleic acid detection for diagnosis of fungal infection and identification of the etiological agent has been actively pursued since the early 1990s. Use of this modality directly on clinical samples offers numerous theoretical advantages, including the ability to detect organisms that are present in small numbers or that cannot be cultured, the potential for automation, removal of the risks associated with culturing Category 3/Biosafety Level 3 fungi, decreased time for identification of the agent, and the potential to detect antifungal resistance in primary clinical samples (117). Moreover, nucleic acid detection offers the potential to identify pathogens in biopsy samples that have been formalin-fixed and even wax-embedded. However, although there are many published methods (118–120), there is little consensus about the optimal methods for specimen processing, extraction of DNA, target design, and quality control. While many laboratories use "in-house" PCR methods, often with favorable results, these methods may be problematic when transferred between laboratories.

One of the diseases for which molecular detection has been very actively researched is IA. However, several issues are as yet unresolved, such as the correct specimen, the optimal sample volume and frequency of sampling, the best method for DNA extraction, the best target, and what constitutes a "positive" result (121). Sensitivity and specificity vary considerably; for aspergillosis, figures of 88 and 75%, respectively (122), and for candidiasis, 95 and 92%, respectively, have been reported (123). Use of whole blood, rRNA, or P450 multicopy targets and an *in vitro* PCR detection limit of ≤10 CFU/ml were all associated with improved assay performance for candidiasis (123).

The European *Aspergillus* PCR Initiative (EAPCRI)/ISHAM [International Society for Human and Animal Mycology]) working group has attempted to address some of these areas and to define a set of standard recommendations for diagnostic PCR for IA rather than a standard PCR assay. By performing various multicenter, multinational studies to evaluate each of the variables, they have suggested standardized conditions that should be used for PCR diagnosis of aspergillosis (124). These include using serum as the sample, using sample volumes of ≥0.5 ml, using an elution volume of <100 µl, and including various internal controls for each assay.

Some commercially available PCR assays are on the market, including Septifast by Roche (Roche Diagnostics, Indianapolis, IN), which is able to detect several species of *Candida* and *A. fumigatus*, and the MycAssay by Myconostica (Myconostica, Cambridge, UK) for diagnosis of IA. Septifast and MycAssay Aspergillus are CE-marked. Septifast was evaluated in a large number of febrile neutropenic episodes in 70 patients with hematological malignancies (125). One patient had a proven infection with *C. glabrata* but a negative PCR result, which the authors suggested may have been below the threshold of detection of *C. glabrata*, which is 100 CFU/ml. Three patients had positive PCR results for *Candida* despite negative blood cultures. There were six episodes of febrile neutropenia associated with a positive PCR result for *A. fumigatus*, of which five were associated with positive *Aspergillus* GM results. Another study found that, in two cases of probable aspergillosis, the PCR result was negative, while in five cases of possible infection, the PCR result was positive. There were four positive PCR results in patients who were not classified as having a fungal infection (126). Thus, the Septifast system has limited sensitivity and specificity and does not look promising for fungal infections.

The MycAssay Aspergillus has also been evaluated in several studies in patients in intensive care units (ICUs) (127, 128) and high-risk hematology patients (129). The assay can be used with serum as a screening test or with BAL as a diagnostic test (http://www.myconostica.co.uk/aspergillus). In the setting of hematology patients, the sensitivity ranged from 50 to 80% and the specificity from 90.5 to 100%, and the authors concluded that its performance was similar to the GM assay (129). In the ICU setting, the sensitivity was higher (86.7 to 100%), and specificities of 87.6 to 92.9% (127) were reported, with increased sensitivity when multiple samples were examined (128). The MycAssay for *Pneumocystis* has also been reported to have a sensitivity and specificity of 100% in BAL in a recent comparative study (130).

The detection of other fungal infections by nucleic acid detection is still largely research-based, although some studies have been promising. A new test, the T2Candida test, has also shown promise in detecting *Candida* in whole blood and is currently undergoing evaluation in a clinical trial (131). Although detection of invasive fungal infection remains a significant challenge, the use of nucleic acid-based detection does have potential, and further evaluation in large, clearly defined populations is required.

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Candida, *Cryptococcus*, and Other Yeasts of Medical Importance

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Yeasts are not a formal taxonomic unit but a growth form exhibited by a wide range of genetically unrelated fungi, ranging from *Exophiala*, the genus of black yeast, to *Histoplasma*, the thermally dimorphic fungus that displays a yeast phase in human tissue. The genera discussed in this chapter are yeasts that have been classified in either the Ascomycota or the Basidiomycota.

TAXONOMY OF YEASTS

Yeast taxonomy is continually evolving, and yeasts are currently being placed into taxonomic categories using DNA sequencing and phylogenetic analysis (see chapter 113). Taxonomic analysis has recently delineated "cryptic species" or "species complexes," groups of organisms that are phenotypically and morphologically indistinguishable but can be separated based on DNA sequencing analysis at particular target sequences. In some cases, each member of a species complex has been given a separate species name, such as *Candida metapsilosis*, *Candida orthopsilosis*, and *Candida parapsilosis*. In other cases, the species have not been formally separated. Kurtzman and Robnett (1) and Fell (2) have provided guidance on the phylogenetic relationships among ascomycetous and basidiomycetous yeast, respectively, but there are still no formal rules for the degree of DNA sequence concordance necessary for two isolates to be considered identical or different species.

A major recent taxonomic development that will affect the clinical community is the end of "one fungus–two names," the practice of assigning separate names to the anamorph (asexual) and teleomorph (sexual) names of an organism. The use of DNA-based methods for identifying organisms rather than the demonstration of sexual structures has brought an end to this practice. Although the International Botanical Congress, the nomenclature authority for fungi, has not yet defined formal nomenclature rules, it appears that the correct name for a given organism will be the earliest name published according to the rules of legitimacy, and organisms previously designated with two names should have the earliest name considered legitimate and valid (3). Input from the clinical community will be required to determine the name to be retained (discussed in more detail in reference 4).

The laboratorian should be familiar with both anamorph and teleomorph names for organisms. Historically, the teleomorph name has been used for an organism when appropriate sexual structures (e.g., asci, ascospores) are present. The teleomorph name is also given preference in some DNA sequence databases. The anamorph name is used when a sexual stage cannot be demonstrated or does not occur. Generally, the anamorphic state is the one recovered in the clinical laboratory. Lists of valid anamorph and teleomorph names can be found at www.mycobank.org. Recent taxonomy changes have also been reviewed (5).

Ascomycetous Yeasts

Genus *Blastoschizomyces*

Blastoschizomyces capitatus is a taxon derived from the combination of the obsolete taxa *Blastoschizomyces pseudotrichosporon* and *Trichosporon capitatum* (6). A synonymous name is *Saprochaete capitata*. The teleomorph is *Magnusiomyces capitatus* (7). Older names for this fungus are *Geotrichum capitatum*, *Trichosporon capitatum* (anamorph), and *Dipodascus capitatus* (teleomorph).

Genus *Candida*

The heterogeneous genus *Candida* currently belongs to the order Saccharomycetales within the ascomycetes. The genus contains approximately 200 species, but phylogenetic advances will certainly result in the remaking of this genus. Due to the uncertainty surrounding its taxonomy, this manual uses anamorph names for members of this genus, with teleomorph names displayed in parentheses, as appropriate.

Candida albicans

Candida albicans remains the major pathogen in this group. A number of species have been merged into *Candida albicans*, including *Candida claussenii* and *Candida langeronii* (8). *C. africana* is a germ-tube positive, chlamydospore-negative yeast closely related to *Candida albicans* (9), but its precise taxonomic placement remains uncertain. Recent multilocus sequence typing placed *C. stellatoidea* type I with *C. africana* in a group of strains highly distinct from most *C. albicans* (10), but many authors consider *C. africana* a biovar of

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C. albicans. *C. stellatoidea* type II has been redefined as a sucrose-negative variant of *C. albicans* (11, 12). *Candida dubliniensis* has many phenotypic and genotypic similarities to *Candida albicans*.

Non-*C. albicans* Species

Most notable in this group is the designation of species complexes. *Candida parapsilosis* is now considered a species complex made up of three species: *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* (13). The newly named genus *Meyerozyma* includes *Candida guillermondii* (teleomorph *Meyerozyma guilliermondii*) and *Candida fermentati* (teleomorph *Meyerozyma caribbica*) (14, 15). The *Candida glabrata* species complex includes *C. glabrata* and the closely related but phenotypically indistinguishable *Candida bracarensis* (16) and *C. nivariensis* (17). The *Candida rugosa* species complex includes the species *C. rugosa*, *C. pseudorugosa*, *C. pararugosa*, and *C. neorugosa*. *C. pseudorugosa* could be differentiated from *C. rugosa* by ulitization of xylose, glycerol, and sorbitol, and the additional utilization of ribitol differentiated *C. neorugosa* (18). Other species that are frequently reported using their teleomorph names (in parentheses) include *Candida ciferri* (*Trichomonoascus ciferrii*), *Candida famata* (*Debaryomyces hansenii*), *Candida guilliermondii* var. *membranifaciens* (*Kodamaea ohmeri*), *Candida kefyr* (*Kluyveromyces marxianus*), *Candida krusei* (*Pichia kudriavzevii*), *Candida lipolytica* (*Yarrowia lipolytica*), *Candida lusitaniae* (*Clavispora lusitaniae*), *Candida pelliculosa* (*Wickerhamomyces anomalus*), and *Candida utilis* (*Lindnera jadinii*). *Candida norvegensis* and *C. zeylanoides* have no known teleomorph. These nomenclature changes resulted from a variety of taxonomic reassignments. For example, in the genus *Pichia*, the number of species was reduced from more than 100 to about 20 (19).

Minor species known from single case reports include the species *Candida aaseri* and *C. pseudoaaseri*, which are close genetic relatives (20). *Candida subhashii* is genetically most closely related to the *C. parapsilosis* species complex, *C. tropicalis*, *C. albicans*, and *C. dubliniensis* (21). *Candida auris* was originally misidentified as *Candida haemulonii* and shares a phylogenetic relationship with this species (22).

Genus *Saccharomyces*

Although the wine-making industry has spent considerable effort investigating the taxonomy of the genus *Saccharomyces*, the single medically relevant species is *Saccharomyces cerevisiae*. The former species *S. boulardii* has been placed into synonymy with *S. cerevisiae*.

Basidiomycetous Yeasts

Genus *Cryptococcus*

A number of phylogenetic studies have resulted in the designation of *Cryptococcus neoformans* and *C. gattii* as separate species (23). Five serotypes have been recognized within these two species. Serotype A was proposed by some as *C. neoformans* var. *grubii*, and by others as the distinct species *C. grubii*. Its species status is still unresolved. Serotype D was recognized as *C. neoformans* var. *neoformans*. The A/D serotype was recognized as an intervarietal *grubii/neoformans* hybrid. Serotypes B and C were recognized as the distinct species *C. gattii*. Serotypes A and D were found to produce the teleomorphic state *Filobasidiella neoformans*, and serotypes B and C produce the teleomorph originally named *Filobasidiella bacillispora*.

Several non-*C. neoformans* species have been described as agents of human infection, including *C. adeliensis*, *C. albidus*, *C. curvatus*, *C. flavescens*, and *C. laurentii*. *C. uniguttulatus* is sometimes described using its teleomorph *Filobasidium uniguttulatum*.

Genus *Malassezia*

Fourteen *Malassezia* species have been isolated from human and animal skin. The species most commonly seen in the clinical laboratory include *M. furfur*, *M. globosa*, and *M. pachydermatis* (24). *Malassezia* species have a basidiomycetous affiliation within the sub-phylum Ustilaginomycotina (2, 24). Species may be distinguished using a variety of methods, but DNA sequencing remains the most reliable.

Genus *Pseudozyma*

At least 20 *Pseudozyma* species are recognized, most of which are environmental organisms. This genus is classified in the family Ustilaginaceae, and its members are close relatives of *Ustilago maydis* and other smut fungi. The major human pathogens are *Pseudozyma aphidis*, *P. antarctica*, *P. parantarctica*, and *P. thailandica*.

Genus *Rhodotorula*

Although more than 60 species of *Rhodotorula* are listed in the Mycobank database, the major clinically relevant species are *Rhodotorula mucilaginosa*, *R. minuta*, and *R. glutinis*.

Genus *Sporobolomyces*

Sporobolomyces are basidiomycetous yeasts that are environmental organisms. Three species have been isolated from human infection: *S. roseus*, *S. holsaticus*, and *S. salmonicolor*, the latter two species having the teleomorphs *Sporidiobolus johnsonii* (homothallic) and *Sporidiobolus salmonicolor* (heterothallic), respectively.

Genus *Trichosporon*

Although 37 valid species are listed in the Mycobank database at this writing, most systemic human infections are caused by one of a smaller number of species: *T. asahii*, *T. asteroides*, *T. cutaneum*, *T. dermatis*, *T. inkin*, *T. jirovecii*, *T. loubieri*, *T. mucoides*, *T. mycotoxinivorans*, *and T. ovoides* (25). The name *T. beigelii* is an invalid name and is no longer used.

DESCRIPTION OF THE AGENTS

Yeasts are unicellular, eukaryotic, budding cells, generally round to oval, or, less often, elongate or irregular in shape. They multiply principally by the production of blastoconidia (buds), such that a typical medically important yeast is composed of a progenitor cell with one or more attached progeny. When blastoconidia are produced one from the other in a linear fashion without separating, a structure termed a pseudohypha is formed. Under certain circumstances such as growth under reduced oxygen tension, some yeasts may produce true septate hyphae.

Cultures of yeasts are moist, creamy, or glabrous to membranous in texture. Several produce a capsule that makes the colony mucoid. With rare exceptions, aerial hyphae are not produced. Colonies may be hyaline, brightly colored, or darkly pigmented due to the presence of melanins. The latter group, referred to as phaeoid fungi and belonging to the class Eurotiomycetes, is discussed in chapter 124; also, dimorphic fungal pathogens possessing a yeast phase in tissue are discussed in separate chapters.

Yeasts are generally identified by observing the macroscopic and microscopic features mentioned above. In the routine laboratory, biochemical tests in the forms of manual kits or automated instruments are used to obtain a species identification. However, for some organisms, this phenotypic identification may be of low certainty. These isolates may represent separate species not defined by the phenotypic database used by the test instrument. Furthermore, biochemical and morphologic tests can be used successfully to place an unknown organism within a species complex but may not be able to identify the particular species within that complex to which the isolate belongs. Molecular methods (DNA- or MALDI [matrix assisted laser desorption ionization]-based) are usually required for this purpose (discussed below).

Ascomycetous Yeasts

Genus *Blastoschizomyces*

Macroscopically, colonies are glabrous with radiating edges, white to cream-colored, and shiny. Microscopically, isolates produce true hyphae, pseudohyphae, and annelloconidia resembling arthroconidia (Fig. 1f). Based on morphological features alone, *B. capitatus* can be difficult to separate from *Trichosporon* spp. and physiological tests are needed. *B. capitatus* is nonfermentative and can be separated from *Trichosporon* spp. by growth on Sabouraud glucose agar at 45°C, on cycloheximide-containing agar at room temperature, and failure to hydrolyze urea.

Magnusiomyces capitatus, the teleomorph of *Blastoschizomyces capitatus*, is heterothallic, and asci are round to ellipsoidal, containing hyaline ellipsoidal ascospores. Only glucose, galactose, glycerol, D, L-lactate, and succinate assimilation tests are positive; hyphae, arthroconidia, and clavate conidia with truncated bases may be seen. The organism grows at 37°C.

Genus *Candida* and Its Teleomorphs

Blastoconidia of *Candida* spp. vary in shape, from round to oval to elongate. Occasional initial isolates, especially from patients receiving antimicrobial agents, may be highly pleomorphic. Asexual reproduction is by multilateral budding, and true mycelium may be present. Several species of *Candida*, most notably *C. albicans*, are diploid (26).

Appearance of pseudohyphae and attachment of blastoconidia are important characteristics to observe when identifying *Candida* spp. Figure 1 illustrates these morphologic features and other characteristics of the most common clinical species. Observation of germ tubes and chlamydospores is also helpful in identifying *C. albicans*. Growth on fungal media can be detected as early as 24 hours; however, colonies usually are visible in 48 to 72 hours as white to cream colored or tan. They are creamy in texture and may become more membranous and convoluted with age. Occasionally, initial isolates of *C. albicans* on Sabouraud glucose agar are wrinkled or rugose but revert to smooth colonies on subculture. In our experience, many isolates of *C. albicans* produce "colonies with feet" (i.e., colonies with short marginal extensions) on blood agar, while most other yeasts do not, with the exception that this might be observed in 25% of isolates of *C. tropicalis* and *C. krusei* (27). These colonies should not be used to perform germ tube tests because hyphal/pseudohyphal cells will be present in the inoculum.

Colonies of *C. krusei* tend to spread radially on Sabouraud dextrose agar and blood agar, which can be a useful clue to the identification of the organism. Most *Candida* spp. grow well aerobically at 25 to 30°C, and many grow at 37°C or above.

A reddish colony variant of *C. glabrata*, which would otherwise have been confused with *Rhodotorula*, has been described (28). Bile-dependent strains of *C. glabrata* have also been isolated from urine (29). These isolates either do not grow on routine media or appear as pinpoint colonies on MacConkey agar containing bile. *Candida nivariensis* can be distinguished from the closely related *C. glabrata* by its inability to assimilate trehalose. Although *C. nivariensis* was originally considered less susceptible to azoles and flucytosine compared with *C. glabrata*, this difference has been questioned (30, 31). *Candida bracarensis* also resembles *C. glabrata*, but it produces a different color on CHROMagar and can ferment trehalose (16, 17).

Distinguishing *C. albicans* from *C. dubliniensis* can be important, especially if the patient has been exposed to fluconazole (32–34). Several single tests have been suggested to help differentiate these two species from each other. These include: growth at 45°C (positive for *C. albicans*), reduction of 2,3,5-triphenyltetrazolium chloride (positive for *C. dubliniensis*), production of a dark green color on CHROMagar (*C. dubliniensis*), production of β-glucosidase (*C. albicans*), rough colony formation on bird seed (thistle seed, niger seed) agar (*C. dubliniensis*), and abundant chlamydoconidia on Staib agar (*C. dubliniensis*) (34, 35). These tests are generally not rapid and are not definitive at separating these species (e.g., some isolates of *C. albicans* do not grow at 45°C). Other differential agar media have been suggested (34). An agglutination test has been developed that distinguishes between these two species. The Bichro-Dubli test (Fumouze Diagnostics, France) identified eight isolates of *C. dubliniensis* among 229 isolates recorded as *C. albicans* on the basis of their CHROMagar color (36). Molecular testing is required to obtain a definitive identification (34).

There is evidence that *C. africana* (*C. albicans* var. *africana*) is often misidentified as *C. albicans* on the Vitek 2 ID-YST system (37). This species is germ-tube positive but chlamydospore negative, slower to grow than *C. albicans*, and unable to assimilate *N*-acetyl glucosamine, trehalose, and lactate (9).

Candida famata (teleomorph *Debaromyces hansenii*) may be grayish-white to yellowish in culture, soft, shiny or dull, smooth or wrinkled. Budding yeasts and, rarely, pseudohyphae may be seen. Conjugation between mother cell and bud occurs, and asci containing one to four round or oval warty ascospores are formed. Fermentation reactions are variable, urease is negative, nitrate is negative, inositol is negative, and the maximum growth temperature is 35°C. However, growth at 37°C is not a reliable characteristic to distinguish the closely related *D. hansenii* from *D. fabryi*, while riboflavin production is more specific to *D. fabryi* and *D. subglobosus* (teleomorph of *Candida famata* var. *flareri*) (38). *C. famata* is often misidentified as *Candida guilliermondii* (teleomorph *Meyerozyma guilliermondii*), but these species can be distinguished by the assimilation of D,L-lactate and lack of pseudohyphal formation in *C. famata* and the reverse for *C. guilliermondii*, although the production of pseudohyphae may not occur in all *C. guilliermondii* strains (39).

Candida lipolytica (teleomorph *Yarrowia lipolytica*) is infrequently isolated, although it appears to be an emerging pathogen. It is urease positive, and isolates vary in their susceptibility to amphotericin B, fluconazole, itraconazole, and caspofungin. *C. lusitaniae* cells are oval or elongated, multilateral budding occurs on a narrow base, and pseudohyphae are produced. Colonies are white to cream, smooth, shiny or, occasionally, dull. The

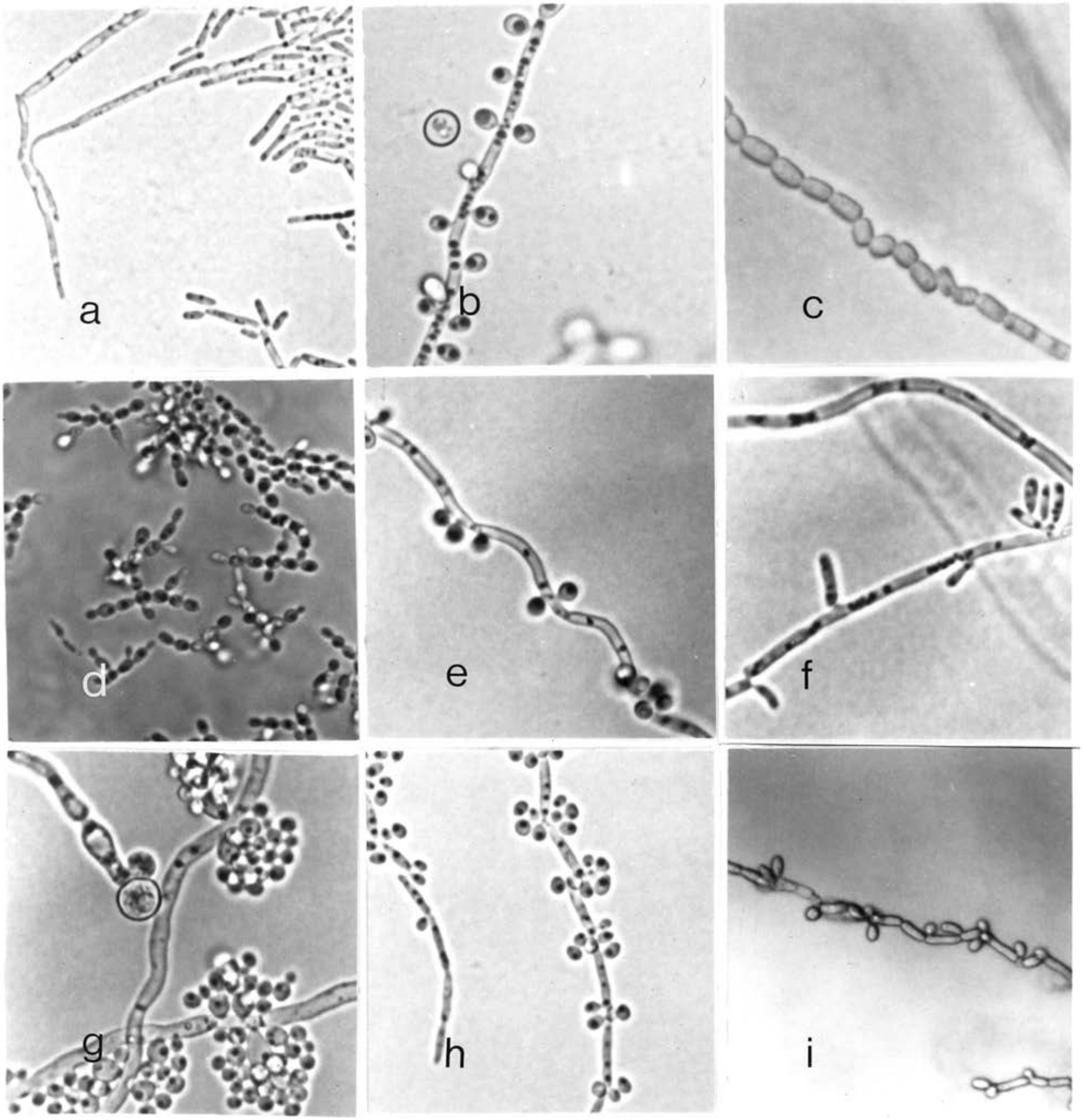

FIGURE 1 Morphological features of some yeast and yeast-like organisms on cornmeal agar at 24 to 48 hours and ambient temperature. (a) *Candida krusei*: extremely elongated, rarely branched pseudohyphae; few blastoconidia. (b) *Candida tropicalis*: blastoconidia formed at septa and between septa. (c) *Geotrichum candidum*: arthroconidia. (d) *Candida guilliermondii*: chains of blastoconidia forming sparse pseudohyphae in a young culture. (e) *Candida lusitaniae*: short, distinctly curved pseudohyphae with blastoconidia formed at, and occasionally between, septa. (f) *Blastoschizomyces capitatus*: true hyphae and annelloconidia resembling arthroconidia. (g) *Candida albicans*: blastoconidia, chlamydospores, true hyphae, and pseudohyphae. (h) *Candida parapsilosis*: elongated, delicately curved pseudohyphae with blastoconidia at septa. (i) *Trichosporon* spp.: blastoconidia formed at the corners of arthroconidia. Magnification, ×370. (Courtesy of B. A. Davis.)
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organism is heterothallic, and conjugation of opposite mating types produces asci that quickly disintegrate, releasing one or two smooth clavate ascospores. Growth occurs at 42°C. Fermentation reactions are variable, but *C. lusitaniae* grow on a variety of sugars, including sucrose, but not on mannitol or inositol. Nitrate and urease are negative.

Individual species of *Candida* with teleomorphs in *Pichia*, *Kodamaea*, *Meyerozyma,* and *Wickerhamomyces* (formerly *Pichia* species) are either homothallic or heterothallic. *Candida pelliculosa* (teleomorph *W. anomalus*) is heterothallic, but the sporogenous diploid form occurs naturally and is the one usually recovered from clinical specimens. These diploid cells convert to form asci, which disintegrate to reveal one to four hat- or Saturn-shaped ascospores (Fig. 2a). Variations in colony morphology may cause confusion with *Cryptococcus* and *Candida* spp. Texture may be smooth or wrinkled, and color may be white, cream, or tan. Microscopically, multilateral budding cells are observed; yeasts and pseudohyphae may be seen. Carbohydrate assimilation and fermentation studies or molecular analysis are needed to identify the individual species, although urease is negative, nitrate is positive, inositol is negative, and growth at 37°C can be variable.

Genus *Saccharomyces*

Saccharomyces cerevisiae is the most common species of this genus recovered in the clinical laboratory. Multilateral budding yeast cells are round to oval, and short rudimentary (occasionally well-developed) pseudohyphae may be formed. Ascospore production can be enhanced easily by growing the yeast on Fowell's acetate agar (see chapter 115) for two to five days at room temperature. Asci contain one to four round, smooth ascospores (Fig. 2c). Other physiologic properties are listed in Table 1. Assimilation of raffinose by *S. cerevisiae* is noteworthy. Very few yeasts encountered in the clinical laboratory utilize this carbon source.

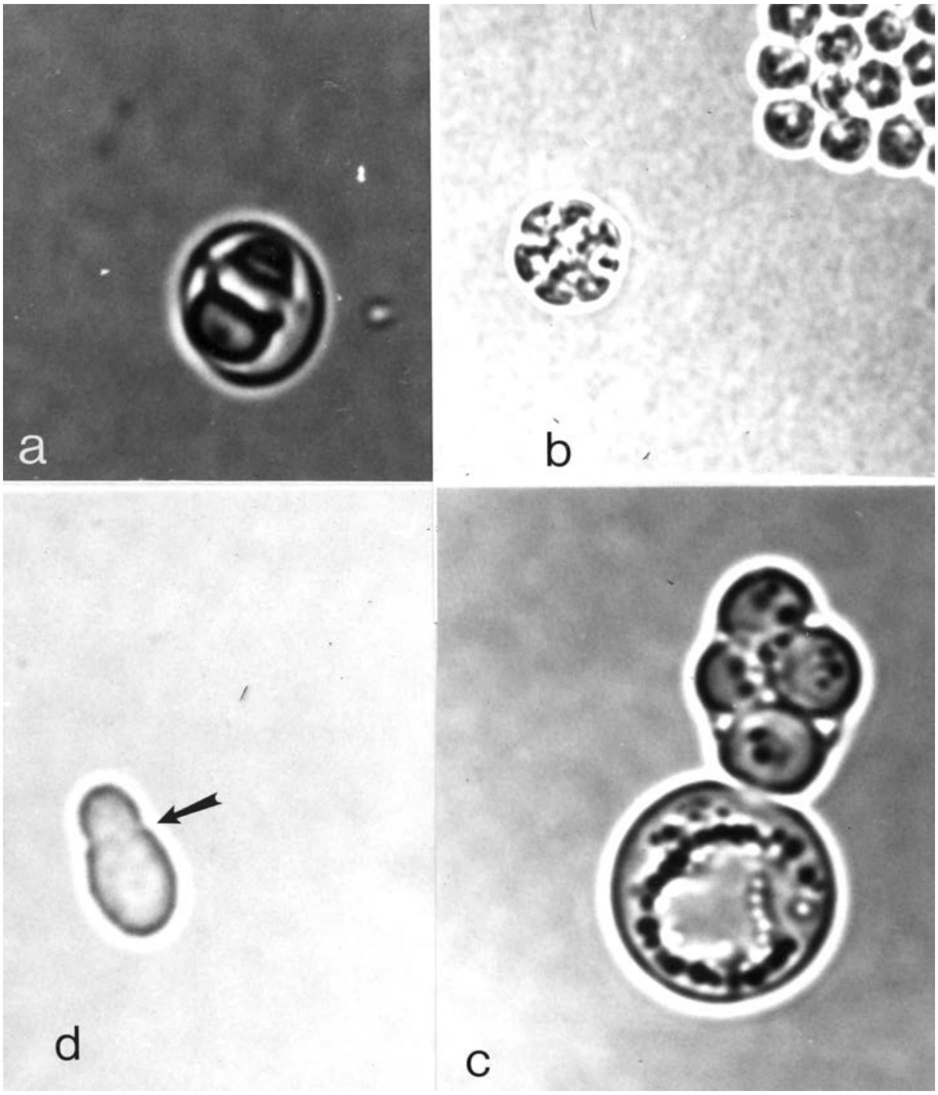

FIGURE 2 Diagnostic features of selected yeasts. (a) Ascus of *Pichia anomala* containing hat-shaped ascospores; (b) sporangium of *Prototheca wickerhamii* containing sporangiospores; (c) *Saccharomyces cerevisiae* with vegetative cell and ascus containing four globose ascospores; (d) bottle-shaped, budding yeast demonstrating annelloconidium and collarette (arrow) of *Malassezia furfur*. Magnification, ×1,000. (Courtesy of B. A. Davis.) doi:10.1128/9781555817381.ch117.f2

TABLE 1 Cultural and biochemical characteristics of yeasts frequently isolated from clinical specimens[a]

Species	Growth at 37°C	Pellicle in broth	Pseudo- or true hyphae	Chlamydospores	Germ tubes	Capsule, India ink	Assimilation of:												Fermentation of:						Urease	KNO_3 utilization	Phenol oxidase	Ascospores
							Glucose	Maltose	Sucrose	Lactose	Galactose	Melibiose	Cellobiose	Inositol	Xylose	Raffinose	Trehalose	Dulcitol	Glucose	Malose	Sucrose	Lactose	Galactose	Trehalose				
Candida albicans	+	−	+	+[b]	+	−	+	+	+*	−	+	−	−	−	+	−	+	−	F	F	−	−	F	F	−	−	−	−
C. *catenulata*	+*	−	+	−	−	−	+	+	−	−	+	−	−	−	+	−	−	−	F*	−	−	−	−	−	−	−	−	−
C. *dubliniensis*	+	−	+	+[b]	+	−	+	+	+	−	+	−	−	−	+*	−	+	−	F	F	−	−	F	F	−	−	−	−
C. *famata*	+	−	−	−	−	−	+	+	+	+*	+	+	+	−	+	+	+	+*	W	−	W	−	−	W	−	−	−	−*
C. *glabrata*	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	−	F	−	−	−	−	F	−	−	−	−
C. *guilliermondii*	+	−	+	−	−	−	+	+	+	−	+	+	+	−	+	+	+	+	F	−	F	−	F*	F	−	−	−	−*
C. *kefyr*	+	−	+	−	−	−	+	−	+	+	+	−	+*	−	+*	+	−*	−	F	−	F	F*	F	−	−	−	−	−
C. *krusei*[c]	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	F	−	−	−	−	−	+*	−	−	−*
C. *lambica*	+*	+	+	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	F	−	−	−	−	−	−	−	−	−*
C. *lipolytica*[c]	+	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−*
C. *lusitaniae*[d]	+	−	+	−	−	−	+	+	+	−	+	−	+	−	+	−	+	−	F	−	F	−	F	F	−	−	−	−*
C. *parapsilosis*[e]	+	−	+	−	−	−	+	+	+	−	+	−	−	−	+	−	+	−	F	−	−	−	−	−	−	−	−	−
C. *pintolopesii*[f]	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	F	−	−	−	−	−	−	−
C. *rugosa*	+	−	+	−	−	−	+	−	−	−	+	−	−	−	+*	−	−	−	−	−	−	−	−	−	−	−	−	−
C. *tropicalis*[d,e]	+	+	+	−[g]	−	−	+	+	+	−	+	−	+	−	+	−	+	−	F	F	F	−	F*	F*	−	−	−	−
C. *zeylanoides*	−	−*	+	−	−	−	+	−	−	−	−*	−	−*	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Cryptococcus neoformans	+	−	R	−	−	+	+	+	+	−	+	−	+	+	+	+*	+	+	−	−	−	−	−	−	+	−	+	−
C. *albidus*	−*	−	−	−	−	+	+	+	+	+*	+*	+	+	+	+	+	+	+*	−	−	−	−	−	−	+	+	−	−
C. *laurentii*	+*	−	−	−	−	+	+	+	+	+	+	+*	+	+	+	+*	+	+	−	−	−	−	−	−	+	−	−	−
C. *luteolus*	−	−	−	−	−	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	−	−
C. *terreus*	−*	−	−	−	−	+	+	+*	−	+*	+*	−		+	+	+	+	−*	−	−	−	−	−	−	+	+	−	−
C. *uniguttulatus*	−	−	−	−	−	+	+	+	+	−	−*	−	−*	+	+	+*	−*	−	−	−	−	−	−	−	+	−	−	−
Rhodotorula glutinis	+	−	−	−	−	−*	+	+	+	−	+*	−	+	−	+	+	+	−	−	−	−	−	−	−	+	+	−	−
R. *rubra*	+	−	−	−	−	−*	+	+	+	−	+	−	+*	−	+	+	+	−	−	−	−	−	−	−	+	−	−	−
Saccharomyces cerevisiae	+	−	−*	−	−	−	+	+	+	−	+	−	−	−	−	+	+*	−	F	F	F	−	F	F*	−	−	−	+
Candida pelliculosa	+*	−	−	−	−	−	+	+	+	−	+	−	+	−	+	−	+	−	F	F*	F	−	F	−	−	+	−	+
Geotrichum candidum[h]	−*	+	+	−	−	−	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Blastoschizomyces capitatus	+	+	+	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Prototheca wickerhamii[h]	+	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
Sporobolomyces salmonicolor	+*	+	+*	−	−	−	+	+	+	−	+*	−	+	−	W	−	+	−	−	−	−	−	−	−	+	+	−	−
Trichosporon asahii	+		+	−	−	−	+	+	+*	+	+	−	+	+*	+*	−	+*	−	−	−	−	−	−	−	+	+	−	−
Trichosporon mucoides	+		+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	−	−
Trichosporon ovoides	+*		+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	+	−	−	−

[a] Modified from references 268 and 269. +, growth greater than that of the negative control; −, negative reaction; *, some isolates may give the opposite reaction; R, rare; F, the sugar is fermented (i.e., gas is produced); W, weak reaction.

[b] C. *albicans* typically produces single and no more than two terminal chlamydospores, while some isolates of C. *dubliniensis* will produce terminal chlamydospore in pairs, triplets and clusters. See discussion in text for additional methods to differentiate C. *dubliniensis* and C. *albicans*.

[c] C. *lipolytica* assimilates erythritol; C. *krusei* does not. Maximum growth temperatures 43 to 45°C for C. *krusei* and 33 to 37°C for C. *lipolytica*.

[d] C. *lusitaniae* assimilates rhamnose; C. *tropicalis* usually does not.

[e] C. *parapsilosis* assimilates L-arabinose; C. *tropicalis* usually does not.

[f] C. *pintolopesii* is a thermophilic yeast capable of growth at 40 to 42°C.

[g] Rare strains of C. *tropicalis* produce teardrop-shaped chlamydospores.

[h] Not yeasts but may be confused with several yeast genera.

Basidiomycetous Yeasts

Genus *Cryptococcus*

Cryptococcus species are round to somewhat oval yeastlike fungi ranging greatly in size, from 3.5 to 8 μm or more in diameter, with a single bud and a narrow neck between the parent and daughter cell (Fig. 3). Unusually large yeast cells (up to 60 μm) have been observed, and this size appears to be associated with higher incubation temperatures (40). Occasionally, several buds are seen; rarely, pseudohyphae are observed. The cell wall is quite fragile, and it is not unusual to find collapsed or crescent-shaped cells, especially in stained tissue sections. Cells are characterized by the presence of a polysaccharide (galactoxylomannan) capsule varying from a wide halo to a nearly undetectable, lighter zone around the cells, depending on

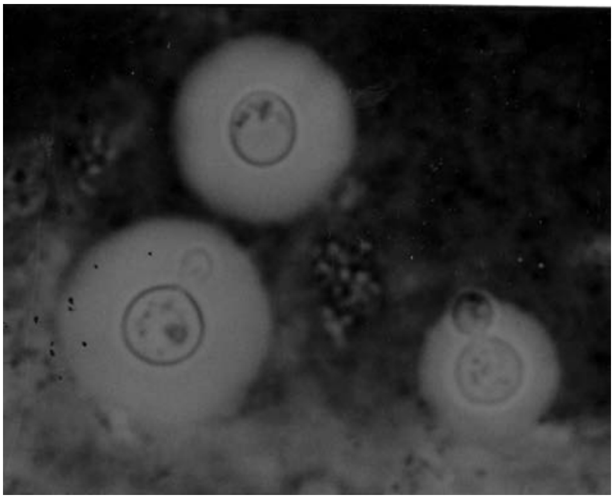

FIGURE 3 India ink preparation of *Cryptococcus neoformans*. Magnification, ×400. (Courtesy of E. S. Jacobson.) doi:10.1128/9781555817381.ch117.f3

the strain and the medium used. Colonies typically are mucoid due to the presence of capsular material, become dry and duller with age, and exhibit a wide range of color (cream, tan, pink, yellow) that may darken with age. Strains possessing only a slight capsule may appear similar to colonies of *Candida*.

Standard laboratory tests do not differentiate among the five serotypes of *C. neoformans* and *C. gattii*. However, canavanine-glycine-bromthymol blue (CGB) agar has been recommended for separating serotypes A and D from serotypes B and C (41, 42).

All members of the genus produce urease, utilize various carbohydrates, and are nonfermentative aerobes. Separation of species is based on assimilation of various carbohydrates and KNO_3 reduction (Table 1). *C. neoformans* and *C. gattii* may be distinguished from other species of *Cryptococcus* by these biochemical studies, as well as by growth at 37°C, which may also be seen with *C. albidus* and *C. laurentii*. *C. neoformans/gattii* may be differentiated from other yeasts and other species of *Cryptococcus* by their production of brown colonies on birdseed agar, although with occasional isolates (particularly serotype C), the production of phenol oxidase (part of the melanin synthesis pathway) may have to be induced. Differentiation from the genus *Rhodotorula*, which also occasionally forms a capsule, produces urease and is nonfermentative, usually is accomplished by noting utilization of inositol (Table 1) and the absence of carotenoid pigments characteristically present in *Rhodotorula*.

Genus *Malassezia*

Most species require lipid for growth; only *M. pachydermatis* can grow independently of lipid supplementation of the media. Van Abbe (43) and Leeming and Notman (44) have designed media optimal for the growth and isolation of all of the *Malassezia* spp. (see chapter 115 for recipes). These media are not commercially available, and the alternative of overlaying Sabouraud glucose agar with a few drops of sterile olive oil has been used, but some of the more fastidious species do not survive on this medium (24, 45). The inoculated media should be incubated aerobically at 32 to 35°C in a moist atmosphere for up to 2 weeks to permit development of the slower-growing species. Colonies are cream to beige in color, smooth to deeply folded, and have a brittle texture that is frequently difficult to suspend.

Malassezia is distinguished microscopically from other yeasts by the formation of a prominent monopolar bud scar or collarette resulting from the continued formation of daughter cells at that site (Fig. 2d). These structures are absent from *C. glabrata*, which could otherwise be confused with *Malassezia* species due to its small size and typical unipolar budding. Sympodial budding has been observed in cultures of *M. sympodialis* and *M. japonica*. The species vary in shape from spherical to oval or elongated, have thick cell walls, and range in length from 1.5 µm to 8 µm. Identification of the individual species is not routinely attempted but can be achieved based on morphology; utilization of certain lipids, including Tween 20, 40, and 80 and cremophor (castor oil); and reactions with esculin and catalase (24, 45) (Table 2). Organisms that are identified as *Candida lipolytica* (glucose, glycerol, and sorbitol positive) with the API 20C AUX (bioMérieux SA, Marcy l'Etoile, France) may actually be *M. pachydermatis* (24). In this case, morphology is helpful.

Genus *Pseudozyma*

Members of the genus *Pseudozyma* are basidiomycetous plant pathogens and are usually found in the environment. They

TABLE 2 *Malassezia* species phenotypic characteristics based on Cabanes et al. (270) and Ashbee (121)[a]

Species	Cell shape	Lipid requiring	Tween 20	Tween 40	Tween 60	Tween 80	Cremophor	Catalase	Growth at 37°C	Bud type
M. caprae	Round/oval	+	−	+	+	v	−	+	w	Narrow base
M. cuniculi	Round	+	−	−	−	−	−	+	+	Narrow base
M. dermatis	Oval/round	+	+	+	+	+	w	+	+	ND
M. equina	Oval	+	w	+	+	+	−	+	w	Narrow base
M. furfur	Oval/round	+	+	+	v-	+	v	+	+	Broad base
M. globosa	Round	+	−	−	−	−	−	+	w	Narrow base
M. japonica	Round/oval	+	−	v	+	−	ND	+	+	Sympodial
M. nana	Oval	+	v	+	+	v	−	+	+	Narrow base
M. obtusa	Oval/round	+	−	−	−	−	−	+	w	Broad base
M. pachydermatis	Oval	−	+	+	+	+	v	v	+	Broad base
M. restricta	Round/oval	+	−	−	−	−	−	−	w	Narrow base
M. sloofiae	Oval/round	+	v	+	+	w	−	+	+	Broad base
M. sympodialis	Oval	+	w	+	+	+	w	+	+	Sympodial
M. yamatoensis	Oval	+	+	+	+	+	ND	+	+	Narrow base

[a] +, positive; −, negative; w, weak; v, variable; ND, not determined.

form moist, beige to tan, wrinkled colonies on routine media. *P. aphidis* forms rough green colonies on CHROMagar *Candida* medium after 48 hours of incubation at 37°C. Growth is inhibited on cycloheximide-containing media. Microscopically, they demonstrate fusiform, spindle-shaped blastoconidia. The germ tube test is negative, and chlamydoconidia are not formed, although true hyphae may be seen on morphology agars. Isolates hydrolyze urea and assimilate *myo*-inositol and D-glucuronate (46). Commercial assimilation tests may give inconclusive results due to the absence of this genus from their databases.

Genus *Rhodotorula*

Rhodotorula spp. share many similar physiologic and morphologic properties with *Cryptococcus* spp. Both are round to oval-shaped, multilateral budding yeasts with capsules, produce urease, and fail to ferment carbohydrates. *Rhodotorula* spp. differ from cryptococci in their inability to assimilate inositol and their obvious carotenoid pigment. When a capsule is present, it is typically small, unlike *C. neoformans*. The most common pathogens are *R. mucilaginosa* and *R. glutinis*. *R. mucilaginosa* can be distinguished from *R. glutinis* by nitrate assimilation, as the former is negative. This is a useful additional test, as occasionally, commercial kits fail to distinguish these species (47).

Genus *Sporobolomyces*

Colonies are soft and shades of salmon pink. Budding oval to ellipsoidal yeasts, pseudohyphae, and ballistoconidia on large sterigmata may be seen. When ballistoconidia are ejected, they can be found on the inside of the lid of the Petri dish. Fermentation is absent and assimilation variable, with sucrose positive, urease positive, nitrate positive, and inositol negative; growth at 37°C is variable.

Genus *Trichosporon*

Trichosporon yeasts grow easily on standard mycological laboratory media, do not ferment carbohydrates, can assimilate various complex sugars, and are urease positive. Colonies form usually within a week on solid media and are cream colored and smooth. They may become dry, moist, shiny, folded, cerebriform, elevated, with or without marginal zones with age.

They produce blastoconidia of various shapes, well-developed hyphae, pseudohyphae, and arthroconidia (Fig. 1i). *T. loubieri* also produces one- and two-celled giant cells (48), while *T. mycotoxinivorans* produces giant cells with as many as eight cells, depending on the growth medium (Hazen, Moore, Padgett, unpublished observation). In cases in which a *Trichosporon* isolate produces only a few blastoconidia, differentiation from *Geotrichum* spp. may be difficult. Inoculation of malt extract broth at room temperature will encourage blastoconidia production in *Trichosporon* spp., usually within 48 to 72 hours. These morphological and physiological characteristics have been used to distinguish between the species in the laboratory (25, 49) (Table 3).

EPIDEMIOLOGY AND TRANSMISSION

Medically important yeasts are found on humans and warm-blooded animals and in the environments they inhabit (reviewed in reference 26). Some yeasts, such as *Candida albicans*, *Candida glabrata*, and most likely *Candida dubliniensis*, appear to be obligatory saprobes of humans and some warm-blooded animals. Analysis of the oral mycobiome suggests that just three fungi, *C. albicans*, *Cladosporium cladosporioides*, and *S. cerevisiae*, colonize more than 25% of healthy adults (50). Environmental sampling of foods, plants, potable water, and juices (pasteurized and unpasteurized) have revealed an amazing array of yeast species known as opportunistic pathogens. In all cases, the presence of these yeasts could be attributed to direct and indirect warm-blooded animal contamination. For example, Arias et al. (51) noted the presence of *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae*, *C. zeylanoides* and several other yeast species in single-strength orange juice that had been pasteurized but subsequently contaminated. These observations make clear that immunocompromised patients are repeatedly exposed to potential yeast pathogens other than those residing as part of their normal microbiota, and care should be exercised in preparing foods and in monitoring the level of contamination associated with devices or creams that may be applied to the patient (52, 53). Parenteral nutrition fluids and devices are particularly prone to contamination with yeasts, espe-

TABLE 3 Characteristics of clinically important *Trichosporon* species[a]

	T. asahii	*T. asteroides*	*T. cutaneum*	*T. inkin*	*T. loubieri*	*T. mucoides*	*T. mycotoxinivorans*	*T. ovoides*
Melibiose	−	−	+	−	+	+	+	−
Raffinose	−	−	+	−	+	+	+	V
L-Rhamnose	+	−	+	−	V	+	+	+
L-Arabinitol	+	+	+	−	V	+	W	−
Galactitol (dulcitol)	−	−	−	−	−	+	+	−
Ribitol (adonitol)	V	V	+	−	?	+	?	−
Sorbitol	−	V	+	−	V	+	+	−
Xylitol	V	+	+	−	V	+	W[a]	V
Growth at 37°C	+	V	−	+	+	+	+	+
Growth at 42°C	−	−	−	V	+	−	−	−
Urease	+	+	+	+	+	+	+	+
0.01% cycloheximide	+	V	−	V	+	+	+	+
0.1% cycloheximide	−	V	−	−	+	+	+	−
Appressoria (on slide culture)	−	−	−	+	−	−	−	+
Fusiform giant cells	−	−	−	−	+	−	+	−

[a] Modified from references 48, 49, 145, and 268.

[b] +, growth greater than negative control; −, negative; V, variable; W, weakly positive; ?, not reported.

cially C. *parapsilosis* (54). It is also noteworthy that the hospital environment can contribute to the development of colonization with *Candida* species as well as facilitate replacement of colonizing less virulent species with more virulent species (55).

Person-to-person transmission has a negligible impact on disease development, except in nosocomial outbreaks in which hands or fomites are often the source. Invasive yeast infections are associated with opportunism; thus, the yeasts causing disease must be present when the conditions are such that disease can be initiated. Several studies have shown that transmission of yeasts through sexual contact (including oral) does occur, but establishment of the transferred organisms in the recipient is affected by a variety of factors, most notably the recipient's current normal microbiota, immunological status, and antifungal status (56, 57).

Genus *Blastoschizomyces*

This environmental yeast is usually found in climates with hot, dry summers and mild, wet winters. Most infections have been reported from Southern Europe, particularly in Italy, Spain, and France below 44° northern latitude. However, recently, five cases of disseminated infection have been reported from central Switzerland: three from leukemia patients, one renal-pancreas transplant, and one continuous ambulatory dialysis patient (58). The authors propose that this could reflect changes in global temperatures and movement of people from areas of endemicity.

Genus *Candida*

Candida species are ubiquitous yeasts, being found on many plants and as normal flora of the alimentary tract of mammals and mucocutaneous membranes of humans (26). Essentially, all areas of the gastrointestinal (GI) tract of humans can harbor *Candida*. The most commonly isolated species (50 to 70% of yeast isolates) from the GI tract of humans is C. *albicans*, followed by C. *tropicalis*, C. *parapsilosis*, and C. *glabrata*.

Candidemia is the third or fourth most common cause of hospital-acquired bloodstream infection in the United States and much of the rest of the developed world and accounts for more than half of all episodes of sepsis in non-neutropenic patients in an intensive care unit (ICU) or surgical ward (59, 60). Most candidemia is caused by one of five species: *Candida albicans*, C. *tropicalis*, C. *parapsilosis*, C. *glabrata*, or C. *krusei*, although the proportions of each species vary in different countries and different patient populations around the world. In Latin America, C. *albicans* (37%), C. *parapsilosis* (26%), and C. *tropicalis* (17%) were the leading agents (61). The species distribution in Asian countries, with a high proportion of C. *tropicalis*, is closer to that of Latin America (62, 63). Very few studies from Africa exist (64). In the United States, the implementation of stringent protocols for the placement of central line catheters has contributed enormously to decreases in the prevalence of candidemia in many institutions (65). However, the proportion of candidemia caused by non-*albicans* species, particularly C. *glabrata*, is rising in the United States. The PATH (Prospective Antifungal Therapy) Alliance registry reported results from more than 3,000 patients from 2004 to 2008, showing that the proportion of candidemia caused by non-*albicans Candida* species (58%) was higher than that caused by C. *albicans* (42%). C. *glabrata* candidemia was the highest caused by a non-*albicans* species, at 26.7% (66). It is likely that the increase in non-C. *albicans* infections is linked to changes in the populations at risk.

Pfaller and Diekema (60) demonstrated that older adults were more likely to be infected with C. *glabrata* than children, and associated risk factors were patient age, severity of underlying disease, use of broad-spectrum antibiotics, central venous catheters, and length of stay in the ICU, with the link to the use of fluconazole only strong in cancer centers. C. *parapsilosis* has a greater incidence of bloodstream infections in children than in adults (60), but this organism is a known pathogen of the young (52).

C. *africana* was reported as a new cause of vaginitis in 2009 (37), with isolates identified from Africa, Spain, Germany, and Italy, The organism was also associated with an uncomplicated urinary tract infection in the United States (Hazen KC, Byrne T, Regi S, Poulter M, Mathers AJ, 2013, Isolation and description of *Candida africana* in the United States, Abstr 53rd Intersci Conf Antimicrob Agents Chemother, abstr M-1382 [American Society for Microbiology, Washington, DC]), and its presence in genital specimens from patients in the United Kingdom has also been confirmed (67).

Genus *Cryptococcus*

C. *neoformans* affects immunocompromised hosts worldwide with var. *grubii* (serotype A) being the most commonly isolated type, although var. *neoformans* (serotype D) is more commonly isolated in Europe (23). C. *gattii* predominantly affects immunocompetent hosts in endemic areas, although approximately 10% of AIDS cases in Botswana and parts of sub-Saharan Africa are infected with this species (68).

C. *neoformans* was first detected in the environment in the late 19th Century, when Sanfelice recovered the yeast from peach juice. Since then, however, C. *neoformans* var. *grubii* and C. *neoformans* var. *neoformans* have been most frequently associated with pigeon (and other bird) droppings and soils contaminated with these droppings. The yeast usually is not found in fresh droppings but is most evident in bird excreta that have accumulated over long periods of time on window ledges, vacant buildings, and other roosting sites (68).

The environmental habitat of C. *gattii* was originally identified as being the gum tree, *Eucalyptus camaldulensis*; however, a number of other trees have been indicated as sources. C. *gattii* has been reported from subtropical areas and from temperate areas of Europe, Australia, Papua New Guinea, New Zealand, British Columbia, Vancouver Island in Canada, and the Pacific Northwest and Southeast United States (68–70).

CLINICAL SIGNIFICANCE

Genus *Blastoschizomyces*

This organism is recognized as an emerging cause of invasive fungal disease in leukemic patients (58, 71, 72), continuous ambulatory dialysis, renal transplant (58), endocarditis (73), spondydiscitis, and osteomyelitis (74). It is widely distributed in nature, has been recovered as normal skin flora and from the gastrointestinal tract, and has been associated with onychomycosis (75). Disseminated disease, which is usually diagnosed by blood culture, is often associated with immunosuppressive conditions (76), in particular neutropenia. Mortality from invasive disease is high in neutropenic patients, and survival is associated with neutrophil count recovery.

Genus *Candida*

Candida spp. can be present in clinical specimens as a result of environmental contamination, colonization, or actual disease processes. An accurate diagnosis requires proper handling of clinical material, ensuring that specimens reach the laboratory in a timely fashion and have been taken and stored in an appropriate manner. *Candida* spp. that are normal flora can invade tissue and produce life-threatening pathology in patients whose immune defenses have been altered by disease or iatrogenic intervention.

C. albicans is the most common species isolated from nearly all forms of candidiasis (26). Contributing to its high association with disease is its high prevalence in the normal population, as described above. In addition, *C. albicans* appears to possess a number of virulence determinants that may promote successful parasitism, which includes proteases, adhesins, surface integrins, and phenotypic switching (26, 77, 78).

Only *C. tropicalis* appears more virulent than *C. albicans* when present in patients with leukemia or lymphoreticular malignant disease (79). Other medically important *Candida* spp. include *C. catenulata*, *C. ciferrii*, *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. haemulonii*, *C. kefyr*, *C. krusei*, *C. lipolytica*, *C. lusitaniae*, *C. norvegensis*, *C. parapsilosis*, *C. pulcherrima*, *C. rugosa*, *C. utilis*, *C. viswanathii*, and *C. zeylanoides* (5, 26, 80). This list is not exclusive, as other rare agents will certainly be added in the future. *C. lipolytica*, *C. lusitaniae*, and *C. krusei* have been isolated from cases of fungemia. Fluconazole is one of the primary drugs used to treat candidiasis, but *C. krusei* is inherently resistant, while isolates of *C. glabrata* may vary in susceptibility. Two other fluconazole resistant species, *C. inconspicua* and *C. norvegensis*, are rare agents of candidiasis. *C. aaseri* and *C. subhashii* have been reported in single case reports as agents of infection. A series of 12 *C. auris* fungemia cases in India has recently been published (81).

C. glabrata is emerging as a significant pathogen, with a relatively high proportion of strains exhibiting reduced susceptibility to fluconazole. Recently, strains resistant to echinocandin drugs have also been noted (82–84). *C. glabrata* is regarded as a symbiont of humans and can be isolated routinely from the oral cavity and genitourinary, alimentary, and respiratory tracts of most individuals. As an agent of serious infection, it has been associated with endocarditis (85), meningitis (86), and multifocal, disseminated disease (80, 87). It is recovered often from urine specimens and has been estimated to account for as much as 20% of *Candida* urinary tract infections (88). Because it may be slow growing, routine urine cultures should be incubated for at least 2 days. Two other yeasts that may resemble *C. glabrata* are *C. nivariensis*, which is an emerging cause of deep infection, and *C. bracarensis*, which has been isolated from AIDS patients.

C. dubliniensis is isolated from the oropharynx of HIV-positive patients but may also be recovered from blood, urine, vaginal, or other specimen sites—especially if the patient is immunocompromised. Acute pseudomembranous oral candidiasis is the presentation seen in patients with compromised cell-mediated immunity. It is most often caused by *C. albicans* and *C. dubliniensis*. It may be present in the prodromal stages of AIDS or accompany the late stages of AIDS (CD4 T cell count of <400 cells/μl) (80, 89). Mucocutaneous forms of candidiasis are often related to defects in cell-mediated immunity, while systemic spread is generally associated with neutropenia (90, 91). Thus, despite the presence of oral or esophageal candidiasis, systemic spread of *Candida* spp. organisms is uncommon in AIDS patients. When it occurs, systemic spread is associated with a drop in the neutrophil count (92).

Candida famata (teleomorph *Debaromyces hansenii*) has been isolated from pigeon droppings (93) and is an infrequent cause of fungemia and endophthalmitis (94, 95). *Candida lusitaniae* (teleomorph *Clavispora lusitaniae*) is a normal component of the human gastrointestinal tract but is an emerging opportunist, causing fungemia—mainly in patients with malignancy. Other occasional infections include peritonitis, urinary tract infection, meningitis (96), and a case report of cutaneous infection (97).

Candida pelliculosa (teleomorph *Wickerhamomyces anomalus*; formerly *Pichia anomala*) is found mainly in the environment but is an opportunistic pathogen that can colonize human skin and has been a cause of fungemia in neonates (98, 99). It has also been associated with catheter-related infections (100–102). *Hansenula* (*Pichia*) *angusta* has been recovered from the mediastinal lymph nodes of a child with chronic granulomatous disease (103). *Kodomaea ohmeri* has been increasingly reported as an agent of disease in immunocompromised patients (reviewed in 104). There have been a number of reports of human infection caused by *Pichia* species, several of which have been fully identified only by DNA sequencing, as routine microbiological tests were not conclusive: *Hansenula* (formerly *Pichia*) *fabianii* was recently described as a cause of endocarditis (105) and catheter-related fungemia (106) and *P. farinosa*, a cause of bloodstream infection in a lymphoma patient (107). Many of these reports describe agents that have since been moved to other genera.

Genus *Cryptococcus*

Cryptococcus neoformans var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii* are considered the only human pathogens, although *C. albidus* (108, 109) and a few others, including *C. adeliensis* and *C. uzbekistanensis*, have, in rare cases, been implicated in disease in severely debilitated individuals.

The clinical presentation in AIDS patients appears to be similar regardless of the species (*C. neoformans* vs. *C. gattii*) causing the infection. Initial cryptococcal infection begins by inhalation of the fungus into the lungs, usually followed by hematogenous spread to the brain and meninges. Involvement of the skin, bones, and joints is seen, and *C. neoformans* is often cultured from the urine of patients with disseminated infection. In nearly 45% of AIDS patients, cryptococcosis was reported as the first AIDS-defining illness. Because none of the presenting signs or symptoms of cryptococcal meningitis (e.g., headache, fever, malaise) are sufficiently characteristic to distinguish it from other infections that occur in patients with AIDS, determining cryptococcal antigen titers and culturing blood and cerebrospinal fluid (CSF) are useful in making a diagnosis (110). In patients without HIV infection, cryptococcosis may occur in association with underlying conditions such as lupus erythematosis, sarcoidosis, leukemia, lymphomas, Cushing's syndrome, organ transplant, and receipt of tumor necrosis factor (TNF) inhibitors.

Although meningitis is the most common presentation of *C. gattii* infection in Papua New Guinea and Australia, respiratory disease is the most common presentation in North America (111). Compromised immune status appears to be a significant risk factor for infections with some molecular types of *C. gattii* (111, 112). In North America, 38%

(Canada) to 59% (United States) of individuals infected with *C. gattii* were immunocompromised.

Genus *Malassezia*

These yeasts are isolated from the skin of warm-blooded animals and humans as commensals but can be agents of dermatological diseases. *M. sympodialis*, *M. slooffiae*, *M. globosa*, and *M. restricta* are the most frequent human colonizers (113–116). The main causative agents of the skin infection pityriasis versicolor are *M. globosa*, *M. sympodialis* and, occasionally, *M. slooffiae* or *M. furfur* (117–120). Other dermatological diseases, including seborrhoeic dermatitis and folliculitis, have been associated with *Malassezia* yeasts, often because there was a clinical response following antifungal therapy with a reduction in yeast numbers. *M. restricta*, *M. globosa*, *M. furfur*, *M. sympodialis*, and *M. obtusa* have all been recovered from seborrhoeic dermatitis patients, and *M. restricta* and *M. globosa* have been isolated from folliculitis patients (121). However, because *Malassezia* may be present on the skin in high numbers and there are no other characteristic features to guide laboratory diagnosis, the exact role of the organisms in these diseases remains unclear. There have been reports of onychomycosis caused by *Malassezia* species (122–124); however, whether these yeasts are true invaders of nail or secondary colonizers is undecided. In addition, *M. furfur* and *M. pachydermatis* have, in rare cases, caused systemic infections, usually of neonates in intensive care units (24, 45).

Genus *Pseudozyma*

The first description of this genus as a human pathogen came in 2003, when the species *Pseudozyma antarctica*, *P. parantarctica*, and *P. thailandica* were isolated from the blood of three Thai patients (reviewed in reference 125). Two additional cases of fungemia due to *P. aphidis* were reported from the United States and from India (125). Three new species were recently named as *P. alboarmeniaca* sp. nov., *P. crassa* sp. nov., and *P. siamensis* sp. nov., also isolated from blood of Thai patients (46). Risk factors are similar to those of other less common yeast infections and include neutropenia, cancer chemotherapy, thrombocytopenia, and the presence of indwelling catheters. Although the main manifestation of *Pseudozyma* infection is fungemia, the organism has also been isolated from brain abscess and pleural fluid associated with pulmonary infection (reviewed in 125).

Genus *Rhodotorula*

Rhodotorula spp. are normal inhabitants of moist skin and can be recovered from such environmental sources as shower curtains, bathtub grout, and toothbrushes (126). The species known to have caused human infections are *R. mucilaginosa*, *R. glutinis*, and *R. minuta* (80, 126). In rare instances, *Rhodotorula* spp. have been reported to cause septicemia (127), meningitis (128), systemic infection (129, 130), peritonitis associated with peritoneal dialysis (131), and sepsis related to complications from indwelling central venous catheters (132).

Genus *Saccharomyces*

Usually thought to be nonpathogenic, *Saccharomyces cerevisiae* has been reported to cause thrush, vulvovaginitis, empyema, and fungemia (80, 133). Person-to-person contact and exposure to commercial strains associated with health foods and baking may contribute to the ability of the organisms to colonize and infect human hosts (134).

Genus *Sporobolomyces*

Sporobolomyces are environmental organisms, some of which have been linked to human diseases, including dermatitis (135), respiratory disease (136, 137), AIDS (138, 139), endophthalmitis (140), and meningitis (141); reviewed in reference 142. Three species have been isolated from human infection: *S. roseus*, *S. holsaticus*, and *S. salmonicolor*.

Genus *Trichosporon*

Yeasts of this genus may be isolated from soil, animals, and humans, but most systemic human infections are usually caused by one of six species: *T. asahii*, *T. asteroides*, *T. cutaneum*, *T. inkin*, *T. mucoides*, and *T. ovoides*. *T. loubieri* is an apparently rare cause of disseminated trichosporonosis (48, 143), *T. japonicum* has been isolated from the sputum of a neutropenic child (144), and *T. mycotoxinivorans* (145) has also been associated with disseminated disease (K. C. Hazen et al., unpublished observation). White piedra is a superficial infection characterized by nodules of approximately 0.5 mm attached to the hair shafts on the head, axilla, or genital area. Infections in the genital area are usually due to *T. inkin*, and rarely encountered infections of hairs on the head are caused by *T. ovoides*. *T. asteroides* and *T. cutaneum* have been associated with superficial skin lesions, and *T. asahii*, and less often, *T. mucoides*, is the cause of disseminated infection mainly associated with neutropenic patients with hematological malignancy or receiving immunosuppressive therapy (146). Others at risk for trichosporonosis include patients with AIDS, extensive burns, or intravenous catheters or undergoing corticosteroid treatment or heart valve surgery (25, 147, 148). In addition, *T. asahii* and *T. mucoides* are thought to be the major causes of summer-type hypersensitivity pneumonitis in Japan, a condition that results from inhalation of arthroconidia present in the homes of the patients (149).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

No special practices for collection or transport of specimens from patients with suspected yeast infection need to be followed. Chapter 114 describes standard practices for collection, transport, and storage. However, when specimens are collected and sent to the laboratory, consideration should be given to the possibility that a particular fungus is highly infectious (e.g., *Coccidioides* species) or has unusual growth requirements (e.g., lipids for *Malassezia* species). Such information regarding the suspected etiologic agent should be provided to the laboratory in a manner that precedes manipulation of the specimen.

Transportation of specimens should be accomplished in less than 2 hours. If a delay is anticipated, specimens should be stored at 4°C. Yeasts can withstand normal refrigeration temperatures.

DIRECT EXAMINATION

The appropriate examination of a clinical specimen is essential before proper processing of the material. In addition, it often aids the laboratorian and the physician to obtain a

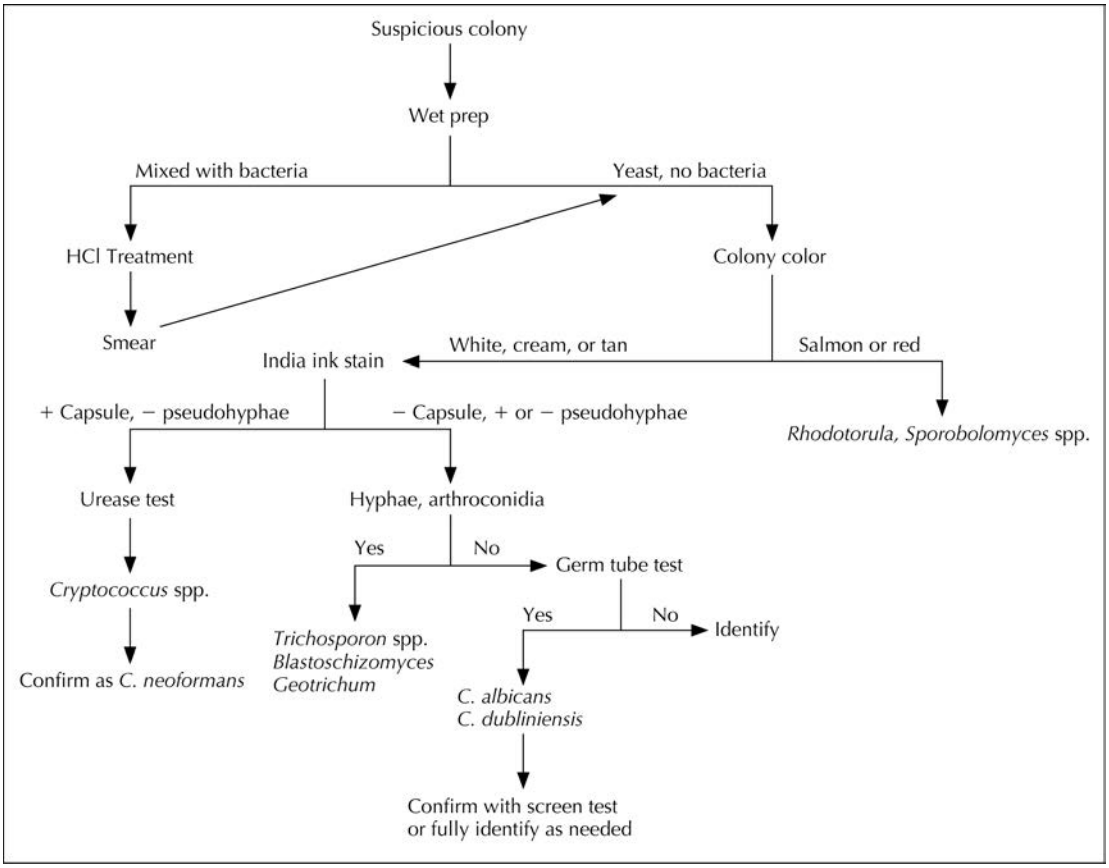
Suspicious colony
Wet prep
Mixed with bacteria
Yeast, no bacteria
HCl Treatment
Colony color
Smear
White, cream, or tan
Salmon or red
India ink stain
+ Capsule, − pseudohyphae
− Capsule, + or − pseudohyphae
Rhodotorula, Sporobolomyces spp.
Urease test
Hyphae, arthroconidia
Yes
No
Germ tube test
Cryptococcus spp.
Yes
No
Identify
Trichosporon spp.
Blastoschizomyces
Geotrichum
Confirm as *C. neoformans*
C. albicans
C. dubliniensis
Confirm with screen test
or fully identify as needed

FIGURE 4 Scheme for identification of yeasts from clinical specimens.
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preliminary identification and in either ruling in or out certain pathogenic yeasts. Some methods are universal to the preliminary observation of fungi in a specimen (e.g., Gram stain, calcofluor, 20% potassium hydroxide [KOH]). If 20% KOH is used in a preparation, neither India ink nor Gram stain may be added. Chapter 115 describes in more detail various stains and examination procedures used in the mycology laboratory.

Microscopy

In performing the microscopic examination of a specimen for yeasts, structures may be observed that may aid in identification (Fig. 4): (i) size and shape of the organism, (ii) morphology of the bud attachment site and number of buds, (iii) presence or absence of a capsule, (iv) thickness of the cell wall, (v) presence of pseudohyphae, (vi) presence of arthroconidia. A great deal of information can be gleaned from careful direct microscopic examination of an appropriately prepared and stained specimen.

KOH

Wet mounts prepared in 10 to 30% KOH solution may be used to distinguish fungi in mucoid secretions or in skin, hair, or nail. The KOH digests the proteinaceous material, leaving the fungal elements intact. Gentle warming may speed this process, but care should be taken when using strong concentrations of KOH, because the KOH rapidly crystallizes if overheated or left for prolonged periods before examination. The specimen is prepared by placing an aliquot onto a slide, adding to it a drop of KOH, and placing a coverslip on top. Ensure that enough KOH is present to completely cover the specimen. For skin and nail, the specimen may be gently squashed while removing excess KOH. In these thick materials, this makes visualization of the fungus easier. Hairs should always be viewed without squashing to obtain maximal information about the type of fungal infection. Dimethyl sulfoxide (40% vol/vol in distilled water) may be added to the KOH reagent to facilitate clearing without the need for heating or incubation, but the specimen should be examined quickly. Bubbles can be confused with yeast cells. Budding cells with internal heterogeneous material should be seen in order to enhance confidence that the object is a yeast cell. Round and oval objects lacking buds are common in tissue specimens and could be erroneously identified as yeasts.

The calcofluor stain (Blankophore, Uvitex 2B) fluoresces under filtered UV light to enhance detection of fungus as it binds to chitin in the cell wall, showing as bright green (150). A specimen may be prepared as above; the coverslip is then removed, a drop of calcofluor is added, excess fluid is removed, and the specimen is viewed. Alternatively, a drop of calcofluor can be added at the time of mounting in KOH. However, the stain may fail to penetrate thick nail samples when prepared in this way.

The diagnostic feature of pityriasis versicolor seen by direct microscopic examination of skin scrapings is the presence of short blunt-ended *Malassezia* hyphae among *Malassezia* yeasts ("spaghetti and meatballs"). Visualization of *Malassezia* species can be enhanced by the use of the stain calcofluor white with UV fluorescence.

India Ink

This stain is used to examine specimens of CSF, urine, and other body fluids for the presence of *Cryptococcus* species. Generally, it is not useful on primary specimens such as sputum or other highly cellular material that do not allow even distribution of the ink. India ink is used to visualize capsules that are transparent by bright field illumination alone, as the ink is excluded from the capsule, showing a clear halo around the yeast. Artifacts such as erythrocytes, lymphocytes, or talc from gloves appear to cause a halo effect; therefore, careful examination for the presence of yeast cells and bud formation is essential. A specimen should be prepared by placing a drop of the centrifuged sediment of a fluid specimen on a slide, adding a drop of Pelikan India ink or nigrosin solution, and placing a coverslip on top. While this method is relatively rapid and is generally satisfactory, a preferred method is to place a coverslip on the specimen and place a drop of ink on the side of the coverslip to allow the ink to diffuse underneath. This provides a gradient of ink which will contain an optimal region to detect a capsule. India ink solution should be replaced regularly as it becomes contaminated, or alternatively, a solution of 10% nigrosin in 10% formalin (using appropriate safety precautions) can be prepared.

Yeast in Tissue Sections

The appearance of fungus in histopathology sections has been reviewed (151). *C. neoformans* can be distinguished from other nondematiaceous yeasts in fixed tissue by the use of a Fontana-Masson stain, which will detect melanin precursors in the cell wall. Mucicarmine will stain the capsule, which helps to distinguish *Cryptococcus* spp. from yeasts with similar morphologies. *C. neoformans* yeast cells in tissue are typically rounder than yeasts of *Candida* species and tend to vary in size.

Yeasts can be visualized in tissue using Gomori's silver stain, although they stain blackish and some of the internal detail may be difficult to see. Periodic acid-Schiff (PAS) stain is also useful; fungal material stains reddish.

A variety of methods have been described for the detection and identification of yeast in formalin-fixed tissue blocks using DNA-based technologies (152–154). Although promising, these methods are largely in the experimental stage. The quality and quantity of extracted fungal DNA are affected by formalin fixation methods, because DNA can be crosslinked or degraded.

Antigen Detection

Signs and symptoms of invasive yeast infections generally overlap those of bacterial infections, necessitating development of rapid non-culture-based methods for diagnosis. See chapter 116 for a comprehensive review of antigen testing. Non-culture-based tests for discriminating fungal versus bacterial infection and for detection of specific genera have been developed. Of all of these tests, the cryptococcal antigen test has proven the most successful.

The unique composition of the fungal cell wall makes it particularly well suited as a focus for fungal serological tests. Many of the more common pathogenic yeasts contain β-1,3-glucan in the wall. Several commercial kits, the Fungitell (Associates of Cape Cod; available in the United States), Fungitek G (Seikagaku Corp.), B-G-Star (Maruha Nichiro Foods, Inc.), and the Wako test (Wako Pure Chemical Industries), are available for detection of β-1,3-glucan. Repeat testing of patients with negative results is recommended, as single-test positive results provide generally good sensitivities and specificities (155) and repeatedly negative tests have a high negative predictive value. Repeat testing of patients who test positive also improves the specificity of the test (155, 156). A positive test, however, does not provide information about the specific etiology, which is a weakness if tailored antifungal therapy is desired. Cryptococci do not contain β-1,3-glucan, so this test is not positive in patients with cryptococcal infection.

Genus *Candida*

With the exception of cryptococcal antigen tests for serum and CSF, efforts to develop other genus- or species-specific antigen detection systems have focused primarily on candidemia and disseminated candidiasis. No commercially available tests have been developed for body fluids and tissues other than blood and CSF. Carbohydrate antigens have provided useful targets for several commercial kits for the detection of disseminated candidiasis. Protein antigens have also been investigated. Another antigen, D-arabinitol, has been under investigation for over a decade and has been incorporated into the COBAS FARA II centrifugal autoanalyzer (Roche Diagnostic Systems) (157). While only a limited number of laboratories have evaluated D-arabinitol as a marker for candidiasis, the antigen appears promising. D-Arabinitol levels appear to correlate with therapeutic success (158).

The Platelia *Candida* Ag test (Bio-Rad) utilizes a monoclonal antibody, EB-CA1, which targets an α-1,2-oligomannoside common among multiple species of *Candida*. The test has shown reasonable sensitivity and high specificities, although they vary from study to study. Sendid et al. (159) have recommended that both the antigen and the antibody tests (Platelia *Candida* Ab) be performed to maximize early diagnosis of invasive candidiasis. The specificity of the antigen test can be further enhanced by including a test for a second antigen, β-1,2-oligomannan, which is found in only a limited number of *Candida* species: *C. albicans*, *C. glabrata*, and *C. tropicalis* (160). *C. dubliniensis* variably expresses the antigen (161). The Serion ELISA antigen *Candida* (Institut Virion/Serion GmbH; Wurzburg, Germany) is a qualitative and quantitative immunoassay for the detection of *Candida* antigen in serum or plasma. A corresponding assay, the Serion ELISA classic *Candida albicans* IgG/IgM/IgA, measures serum antibody directed against *C. albicans*. These assays were evaluated and compared with the Platelia antigen/antibody assays and β-D-glucan (162). Although the Serion antigen test discriminated between patients with candidiasis and uninfected controls, in this study its discriminatory power was lower than that of the Platelia test. These assays have been available in Europe for some time but are not approved for use in the United States.

The Pastorex *Candida* (BioRad) and Cand-Tec (Ramco Inc., Stafford, TX) are agglutination tests that detect *Candida* antigen in human specimens. Their performance has been reviewed recently (163).

Genus *Cryptococcus*

Detection of cryptococcal antigen (capsular galactoxylomannan) in serum and CSF has been available for over 2 decades and detects both *C. neoformans* and *C. gattii*. During this time, commercial tests have evolved to overcome early problems with specificity and sensitivity. The chief problem was false positives due to rheumatoid factor. Once this factor is destroyed, the sensitivity and specificity of the current generation of cryptococcal antigen tests are high. Latex agglutination-based antigen detection kits include the Cryptococcus Antigen Test (Remel, USA), Cryptococcal Antigen LA System (CALAS; Meridian Bioscience, Inc.), Latex-Cryptococcus Antigen Test (IMMY), Pastorex Crypto Plus (BioRad), the Crypto-LA test (Wampole Laboratories, NJ), and the Eiken Latex test (Eiken, Tokyo, Japan). The PREMIER Cryptococcal Antigen (Meridian) is an enzyme immunoassay (EIA)-based assay, and the newest format is a lateral flow assay (LFA) (IMMY Cryptococcal lateral flow assay, IMMY; ABACUS ALS, Australia). These tests have been recommended as the primary evaluative tool in lieu of an India ink stain for screening CSF in suspected cases of cryptococcal meningitis. This is because the sensitivity of the India ink stain is low.

False-positive tests due to causes other than rheumatoid factor have been observed. These have been associated with trichosporonosis, *Capnocytophaga canimorsus* septicemia, malignancy, *Rothia* (formerly *Stomatococcus*) bacteremia, some soaps and disinfectants, and hydroxyethyl starch (used in fluid resuscitation) (164–170). Interestingly, the cryptococcal galactoxylomannan contains an epitope that crossreacts with the galactomannan antigen of *Aspergillus* spp., which is the antigen target of the Platelia *Aspergillus* assay (171).

Monitoring a decrease in antigen titer as an indication of effective anticryptococcal therapy has been suggested. However, while the titer may decrease after initiation of therapy in non-HIV patients, it may remain greater than 200 despite microbiological clearance (172). Lu et al. (172) have suggested that a better indicator of successful therapy is the return of CSF glucose, chloride, and leukocyte count to normal limits. Mycologic sterility is also a useful indicator of successful therapy.

Specific Tests for Invasive Infections Caused by Other Yeasts

Not surprisingly, little effort has been devoted to develop serologic tests for detection of invasive infections caused by yeasts other than *Candida* and *Cryptococcus* species. With the growing awareness of bloodstream infections caused by *Trichosporon* species, specific serologic tests for their detection may be developed. Before this occurs, a possible strategy is to use the serum cryptococcal antigen test in combination with compatible signs and symptoms and underlying disease to suggest a diagnosis of trichosporonosis.

Nucleic Acid Detection

The U.S. Food and Drug Administration has approved several commercially available peptide nucleic acid-fluorescent in situ hybridization (PNA FISH) kits (AdvanDx, Woburn, MA) for identification of yeasts directly from positive blood cultures. The probes specifically detect *C. albicans*, *C. glabrata*, or *C. tropicalis* as individual species or detect a yeast species group (e.g., *C. albicans* and *C. parapsilosis* fluoresce green with the Yeast Traffic Light PNA FISH kit) in blood cultures by targeting species-specific rRNA sequences. Nucleic acid peptides that mimic 26S rRNA are used to hybridize to target rRNA. The probes are also coupled to a fluorophore that is detectable when the probe binds to its target. Several studies have shown that the *C. albicans* PNA FISH assay is 100% specific (173–175). However, the manufacturer's package insert notes (and we have observed) that the *C. albicans* probe can cross-react with *C. orthopsilosis*. In the most comprehensive study, the test was found to have excellent sensitivity (99%), specificity (100%), positive predictive value (100%), and negative predictive value (99.3%) (175). The test can rapidly (1.5 hours) indicate whether *C. albicans* is present or not and can thus indicate whether a non-*C. albicans* yeast is present (173, 174). Other PNA FISH assays are similarly rapid, although recent modifications to the probes and reagents have resulted in a second-generation assay (*Quick*FISH) that shortens the assay to 30 minutes. The test does not replace subculture because blood cultures may contain mixed species; therefore PNA FISH-positive blood cultures should be subcultured to ensure that no other yeast species is present. *C. dubliniensis* is not detected with the PNA FISH probe. With this test, laboratories can report whether a positive blood culture with yeast contains *C. albicans* within a few hours after the culture becomes positive (89).

Most assays for the direct detection of fungal DNA in human body fluids have been directed at diagnosis of invasive candidiasis. White et al. (176) and Lau (177) have reviewed methods for the rapid diagnosis of invasive candidiasis, including PCR-based methods. Avni et al. conducted a meta-analysis of 54 published studies that used whole blood for PCR diagnosis of invasive candidiasis (178) and found that the pooled sensitivity of these assays was 95% and the specificity was 92%. Zhang has recently reviewed molecular approaches for diagnosing fungal infections (179), including candidiasis. He points out that lack of standardization and validation has hindered the acceptance of these tests as diagnostic criteria for fungal infections. Furthermore, clinical outcome and cost-effectiveness data that would be helpful for laboratory decision-making are also largely lacking.

The time required to identify a pathogen could be shortened if more efficient methods of detecting fungal DNA in a specimen were developed. Real-time PCR can detect candidemia much earlier than conventional blood culture, but it does not always detect all cases of systemic infection with all species (180–182). Bennett (183) provides a balanced discussion on the use of this technique, which is likely to be used alongside blood culture protocols while it is developed further.

In Europe, the LightCycler SeptiFast Test M (Roche Diagnostics GmbH) is available to detect five species of *Candida* and *Aspergillus fumigatus* DNA in whole blood using broad-range ITS PCR and melting curves. A summary of published studies with the LightCycler SeptiFast Test M is provided in reference 163. Several other commercial tests available in Europe for experimental purposes are described in reference 163. Other assays are described in references 177 and 179. The Luminex xTAG multiplex PCR assay (Luminex Molecular Diagnostics, Ontario, Canada) detects as many as 23 fungi in a single sample. The method uses a microsphere (bead) suspension array in which the beads each contain an individual probe designed to hybridize to a different amplified fungal DNA sequence. This assay was tested for the detection of *Candida* species in positive blood culture bottles (184, 185), showing 100% sensitivity and 99% specificity. Microarrays have been tested as diagnostic tools for the detection of invasive fungal infection. Recently, the Prove-it Sepsis microarray assay (Mobidiag, Espoo, Finland) was modified to incorporate fungal targets and was found to have a sensitivity of 99% and specificity of 98% in detecting *Candida* from blood cultures (186). This assay misidentified one *C. parapsilosis* and *C. lusitaniae* isolate from 53 isolates of *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. lusitaniae*. None of these assays is approved for use in the United States.

The only U.S. FDA-approved DNA probe test is the Affirm VPIII Microbial Identification Test (BD), for use in the detection and identification of *Candida* species, *Gardnerella vaginalis*, and *Trichomonas vaginalis* nucleic acid in vaginal fluid specimens from patients with symptoms of vaginitis (187). The Affirm assay was significantly more likely to identify *Candida* than wet mount of vaginal fluid (11 versus 7%). The T2 Candida panel (T2 Biosystems, Lexington, MA) was recently approved for direct detection of five *Candida* species in blood samples. T2MR is a magnetic resonance-based system for detection of sepsis agents.

ISOLATION PROCEDURES

Processing specimens for fungal recovery is usually performed in conjunction with and as an extension of the processing procedure for recovery of bacteria. The primary difference is the selection of media used for primary plating. These media are discussed in chapter 115. Most media used for the recovery of fungi contain an antibacterial agent such as chloramphenicol or gentamicin; however, incorporation of cycloheximide (Acti-Dione) should be avoided as only *C. albicans*, some *Trichosporon* species, and *Malassezia* species are able to grow in the presence of this agent. One processing step that has unproven advantages for isolation of yeasts from sputum specimens is the use of mucolytic agents (*N*-acetyl-L-cysteine or Sputolysin).

There are, however, yeast infections for which specialized processing steps should be considered. For example, recovery of *C. neoformans* from blood or bone marrow is enhanced if lysis-centrifugation followed by inoculation onto solid media is used (188). If *C. neoformans* is suspected from a respiratory specimen, setting up primary media that include a niger seed or related medium will enhance detection.

For some yeasts, a medium supplement may be needed to either enhance or support growth during primary culture. If *Malassezia* species are suspected, the primary culture plate should be supplemented with an olive oil overlay applied with a swab before inoculation. *Malassezia pachydermatis* will grow without the overlay, but it is required to grow *M. furfur*. This may not be successful for other *Malassezia* species as they are more fastidious. The detection of bile-dependent and bile-enhanced isolates of *C. glabrata* is enhanced by the addition of 8 µg/ml of fluconazole to the primary medium. This selects for bile-dependent isolates which have defects in the ergosterol synthesis pathway and are fluconazole resistant (29, 189). This becomes a particularly powerful method when combined with a primary medium containing a chromogenic indicator used for presumptive identification of some yeasts (e.g., CHROMagar) (190).

The appropriate selection of isolation media is essential even though detection of infectious agents using molecular methods is becoming more commonplace in the diagnostic setting. Successful culture is still necessary to perform antifungal susceptibility tests, for preservation in culture collections, and for epidemiological study.

IDENTIFICATION

Use of a sound systematic approach to yeast identification is important. With the numerous yeast identification systems available today, most commonly encountered yeast species can be identified easily; however, repeat testing is sometimes necessary, and morphologic characteristics on cornmeal-Tween or similar morphology agars should be mandatory. Isolates that are problematic should be sent for DNA sequence analysis or matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) to confirm their identity.

The sections that follow outline the identification tests available, while the previous section on “Description of the Agents” contains the genus-specific detail. This topic has been reviewed (142, 163, 191, 192).

Macroscopic Characteristics of Yeasts

Most yeasts grow well (*Malassezia* species are an exception) on common mycological and bacteriological media. Growth is usually detected in 48 to 72 hours, and subcultures or lab-adapted strains may grow more rapidly. Colonies have a smooth to wrinkled, creamy appearance; some pigment may be observed initially or intensify with age. Heavily encapsulated yeasts give a very moist, mucoid appearance.

The ability of yeasts to grow at 37°C is a very important characteristic. Most pathogenic species grow readily at 25 and 37°C, while saprobes usually fail to grow at the higher temperature. Spiking around the edge of a colony grown in CO_2 is indicative of *Candida albicans* and has been reported to be more sensitive than germ tube production (193). This feature is the same as the “colonies with feet” described in the *Candida* section of “Description of the Agents.”

Pellicle growth on the surface of liquid media such as Sabouraud glucose broth or malt extract broth has been used in the past to assist with yeast identification. More recent evidence suggests that this characteristic can be variable; however, as an ancillary test, it may be helpful in identifying *Candida tropicalis* and *Candida krusei* (194).

Microscopic Characteristics of Yeasts

On isolation of a suspected yeast from a clinical specimen, the first examination should be a wet preparation of a colony. This provides an initial clue to the organism’s identity. There are several fungi that produce yeast-like colonies that are not yeasts. Observations should include size and shape

TABLE 4 Microscopic appearance of several yeasts and yeast-like fungi on morphology agar

Organism	Pseudohyphae	True hyphae	Blastoconidia	Arthroconidia	Annelloconidia	Chlamydospores	Ascospores
B. capitatus	X	X	X		X		
C. albicans/*C. dubliniensis*	X	X	X			X	
Other *Candida* species	X[a]	X[a]	X				X[a]
Cryptococcus spp.			X				
Geotrichum spp.		X		X			
Pichia spp.	X[a]		X				X
Rhodotorula spp.			X				
Saccharomyces spp.	X[a]		X				X
Trichosporon spp.	X	X	X	X			

[a] Strain variation.

of the yeast, method of bud attachment, and presence or absence of pseudohyphae, true hyphae, or arthroconidia (Table 4). Any round or slightly oval budding yeast with rare or no pseudohyphae seen in this preparation should be examined further for the presence of a capsule.

The same wet preparation used for the initial microscopic examination can be used for an India ink examination for the presence of encapsulated yeasts (Fig. 2) (see previous section). Capsular size cannot be used for identification purposes because this characteristic may be influenced by culture age, media composition, and strain variation. The presence of a capsule does not automatically ensure that the yeast is *Cryptococcus neoformans/gattii*, as other cryptococci, *Rhodotorula* spp., and rare *Candida* spp. will produce capsulelike structures. In practice, any nonpigmented, round, encapsulated yeast recovered from CSF should be considered *C. neoformans* or *C. gattii* until proven otherwise.

Purity of Cultures

Before any additional physiologic tests are performed, it is essential to ensure that the culture is pure. A Gram stain of the culture can verify purity, but often, bacterial contamination can be detected during the wet preparation examination. If the culture is mixed with bacteria, the isolate should be inoculated to a blood agar plate or Sabouraud dextrose agar containing antibiotics and individual colonies should be subcultured for purity, or alternatively, treated with hydrochloric acid (Fig. 4). The HCl procedure is performed by inoculating a colony into three tubes of Sabouraud glucose broth (each containing 5 ml). A capillary pipette is used to add 4 drops of 1 N HCl to the first tube, 2 drops to the second tube, and 1 drop to the third tube. After incubation at 25°C for 24 to 48 hours, 0.1 ml of each broth is subcultured onto fresh Sabouraud dextrose agar plates.

More than one yeast species may be recovered from a clinical specimen, especially if the specimen is from a normally nonsterile site (195). In addition, multiple yeast species may be responsible for fungemia episodes. Careful attention to colonial morphology and microscopic characteristics can offer clues to a mixed population. Subculturing individual isolates to additional media can be helpful (195), and the use of a chromogenic medium may delineate the presence of more than one yeast species.

Chromogenic Agars

Presumptive identification of one or two yeast species based on colony characteristics can be obtained with chromogenic agar media (reviewed in references 163 and 192). CHROMagar *Candida* (CHROMagar France, Paris, France, distributed in the United States by Becton Dickinson/BBL, Sparks, MD) allows differentiation of more than 10 species and is marketed for the presumptive identification of *C. albicans*, *C. tropicalis*, and *C. krusei*. Colony identification is based on the differential release of chromogenic breakdown products from various substrates following exoenzyme activity. It is important to recognize that identifications are presumptive. Evidence suggests that variation in colony appearance occurs among the species (196, 197). The directions of the manufacturer must be strictly followed, as is the case in any rapid test based on exoenzyme activity. Incubation time and temperature significantly affect the colony appearance. The medium is useful for the detection of mixed yeast infections, especially in blood, and for resolving identification problems. Other chromogenic media are now commercially available (see chapter 115 and Table 6). ChromID Candida (bioMérieux) and CandiSelect 4 (Bio-Rad, Marnes La Coquette, France) perform similarly to CHROMagar. No one medium detects all instances of mixed yeast blood cultures (198–200). Approximately 2 to 10% of *C. albicans* isolates produced white colonies rather than colored colonies on these chromogenic media, strongly supporting the concept that these media provide only a presumptive identification. Brilliance Candida Agar (formerly Oxoid chromogenic Candida agar; Thermo Fisher Scientific, Basingstoke, UK) and HiChrome Candida Differential Agar (HiMedia Laboratories, Mumbai, India) have also been introduced recently.

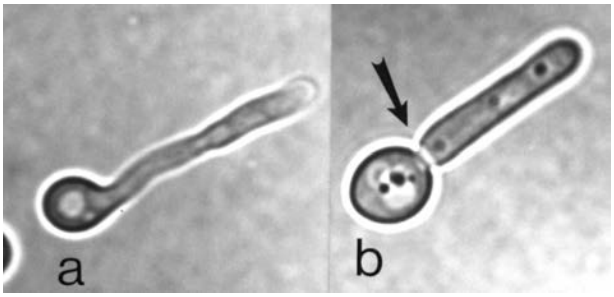

FIGURE 5 Germ tube test. (a) Germ tube formation of *Candida albicans;* (b) blastoconidial germination with constriction (arrow) of *Candida tropicalis* not seen with true germ tubes of *C. albicans*. Magnification, ×400. (Courtesy of B. A. Davis.) doi:10.1128/9781555817381.ch117.f5

Morphology Studies

While examination of a wet preparation gives a primary indication of the yeast involved, more extensive study of morphology on cornmeal-Tween agar, rice agar, PCB (potato carrot bile) agar (Bio-Rad, France) or other similar agars offer the opportunity to correlate morphologic characteristics with results of biochemical testing, as these characteristics form part of the identification code for many commercially available kits. Microscopic examination should reveal the thick-walled chlamydospores of *Candida albicans/Candida dubliniensis*; attention should be given to factors such as the size and shape of the pseudohyphae, the arrangement of blastoconidia along the pseudohyphae, and the presence of capsules(Fig. 1 and Table 4).

Germ Tube Test

One of the most valuable and simple tests for the rapid presumptive identification of *C. albicans* is the germ tube test (Fig. 5a). The test is considered presumptive because not all isolates of *C. albicans* will be germ tube positive, and false positives may be obtained, especially with *C. tropicalis*, despite well-trained staff (201). Also, *C. dubliniensis* is germ tube positive, although most isolates do not form germ tubes in a commercial synthetic germination medium (202). Microscopic observation of the preparation reveals that the short hyphal initials produced by *C. albicans* are not constricted at the junction of the blastoconidium and germ tube. *Candida tropicalis* can produce hyphal initials, but the blastoconidia are larger than those of *C. albicans*, and there is a definite constricture where the hyphal initial joins the blastoconidium (Fig. 5b). In addition to using a known culture of *C. albicans* as a positive test control, negative controls using *C. tropicalis* and *C. glabrata* also should be included. Optimum conditions are obtained by using colonies grown on Sabouraud glucose agar or blood agar at 30°C for 24 to 48 hours (unpublished observations). The test is performed by first lightly touching a colony with a bacteriological loop so that a thin film of organism is obtained, then transferring the inoculum into the test substrate (e.g., fetal bovine serum) followed by incubation at 37°C for up to 3 hours. Germ tube tests should be read between 2 to 3 hours to prevent clumping of hyphal initials and to reduce the number of false positives. In addition, a heavy inoculum of yeasts and the presence of bacteria can each lead to false-negative results.

Ascospore Formation

Homothallic yeasts recovered from clinical specimens may be present in their teleomorphic (sexual) state. In order to enhance production of ascospores by ascomycetous yeasts, cultures should be inoculated onto media such as Fowell's acetate agar (203), V-8 juice agar, or yeast extract-malt extract (YM) agar, incubated at room temperature for 2 to 5 days, and examined by wet preparation for the presence of ascospores within asci. Some mycologists prefer to perform special stains to detect ascospores. The Ziehl-Nielsen stain, routinely employed in mycobacteriology, can be used if needed; however, most ascospores can be detected easily in a drop of sterile distilled water. Ascospore production in *Saccharomyces cerevisiae* is evidenced by the presence of one to four globose spores (Fig. 2c); *Wickerhamomyces anomalus* produces one to four hat-shaped spores (Fig. 2a). Ascospore formation can aid in identification when equivocal results are obtained by other identification methods (Table 5).

Phenol Oxidase Test

This screening procedure detects the ability of *Cryptococcus neoformans* and *C. gattii* to produce phenol oxidase on substrates containing caffeic acid. The most frequently used medium is "birdseed" agar (containing niger or thistle seeds), and a number of formulations showing varying degrees of success have been described. Test performance is related to the glucose content of the medium; the more glucose in the medium, the less likely a valid test will be obtained. *C. neoformans* and *C. gattii* colonies turn a dark brown in 2 to 5 days. Because of the wide variation in media formulations, the test must be subjected constantly to quality-control measures. Some laboratories advocate the use of birdseed agar as part of the primary plating for respiratory cultures for early detection of *C. neoformans* and *C. gattii* isolates. A rapid phenol oxidase test (Remel and other vendors) utilizes a caffeic acid disk that is rehydrated, then inoculated with several yeast colonies and incubated at 35 to 37°C for up to 4 hours. The disk should be examined every 30 minutes for the production of a brown pigment. If pigment is produced, it is presumptive for *C. neoformans* or *C. gattii*; if no pigment is detected but *C. neoformans*/*C. gattii* are suspected, further identification should be pursued.

Urease

This is a test to detect a yeast's ability to produce the enzyme urease. In the presence of suitable substrates, urease splits urea, producing ammonia, which raises the pH and causes a color shift in the phenol red indicator from amber to pinkish-red. The urease test aids in the identification of the urease-positive basidiomycetous yeasts (*Cryptococcus*,

TABLE 5 Anamorph/teleomorph names and appearance of asci/ascospores of ascomycetous yeasts[a]

Anamorphic species	Previous synonym or obsolete name	Teleomorph	Hetero- or homothallic	Ascospores
Blastoschizomyces capitatus (*Saprochaete capitata*)	*Geotrichum capitatum*, *Trichosporon capitatum*, *Dipodascus capitatus*	*Magnusiomyces capitatus*	Hetero	4/ascus, hyaline, ellipsoidal, with slimy sheath when released
Candida ciferrii		*Trichomonascus ciferrii*	Hetero	1–4/ascus, helmet or hat shaped
Candida famata	*Torulopsis candida*	*Debaryomyces hansenii*	Homo	1–2/ascus, spherical with warts[b]
Candida guilliermondii	*Pichia guilliermondii*	*Meyerozyma guilliermondii*	Hetero	Variable
Candida kefyr	*Candida pseudotropicalis*, *Candida macedoniensis*	*Kluyveromyces marxianus*	Homo	1–4/ascus, crescent to reniform, agglutinates on MEA[c]
Candida krusei	*Issatchenkia orientalis*	*Pichia kudriavzevii*	Homo	1–2/ascus, spherical[d]
Candida lipolytica		*Yarrowia lipolytica*	Hetero	1–4/ascus, spherical to hat shaped protuberance on 1 or 2 ends
Candida lusitaniae	*Candida obtusa*, *Candida parapsilosis* var. *obtusa*	*Clavispora lusitaniae*	Hetero	1–4/ascus, clavate on MEA[b]
Candida norvegensis		*Pichia norvegensis*	Homo	1–4/ascus, hat shaped on acetate agar
Candida pintolopesii	*Candida slooffii*	*Kazachstania pintolopesii*	Homo	1–2/ascus, spherical to ovoid, rough to spiny
Candida pelliculosa		*Wickerhamomyces anomalus*	Hetero	1–4/ascus, hat shaped
Candida pulcherrima		*Metschnikowia pulcherrima*	Hetero	1–2/ascus, from chlamydospore, spherical with peduncle
Candida utilis		*Lindnera jadinii*	Homo	1–4/ascus, hat shaped on MEA[b]
		Saccharomyces cerevisiae	Homo	1–4/ascus, spherical or short ellipsoidal (on acetate agar)

[a] Modified from references 5 and 271.

[b] Difficult to see.

[c] MEA, malt extract agar.

[d] Difficult to induce.

Malassezia, *Pseudozyma*, *Rhodotorula*, *Trichosporon*) from the urease-negative ascomycetous yeasts (e.g., *Candida*, *Saccharomyces*, *Geotrichum*, *Blastoschizomyces*). Nearly all *Candida* spp. encountered in clinical specimens are urease negative, exceptions being *Yarrowia* (*Candida*) *lipolytica* and some strains of *C. krusei*, neither of which assimilates inositol. A rapid disk test can take 4 to 72 hours for a reaction to develop, while the more definitive urea agar slants can take from 24 to 72 hours.

Rapid Trehalose

The rapid trehalose test can provide a presumptive identification of *C. glabrata* within 3 hours. The Clinical and Laboratory Standards Institute (CLSI) has approved a rapid test procedure (document M35-A) for identification of *C. glabrata* based primarily on rapid trehalose assimilation (204). False positives occur with trehalose tests (205), but these can be reduced if the results are correlated with small cell size and failure to produce germ tubes. Several modifications of the rapid trehalose assay have been introduced (reviewed in references 163 and 192). The GLABRATA RTT (Fumouze Diagnostics Levallois Perret, France) combines hydrolysis of trehalose and maltose and includes a control (a sugar-free basal medium). *C. glabrata* hydrolyzes the trehalose rapidly, but not the maltose (which other yeasts may hydrolyze). The test provides acceptable sensitivity and specificity, but the choice of growth medium for the test inoculum influences the likelihood of obtaining false positives. Blood agar should be avoided because both *C. albicans* and *C. tropicalis* cause high false-positive results (200, 206). The most suitable media appear to be CHROMagar and Sabouraud glucose (4%) agar. Combining colony color on chromogenic agars with the GLABRATA RTT or the Rapid Trehalose test could help improve specificity (207, 208). Rapid trehalose and maltose hydrolysis have also been modified into a 30-second dipstick assay. This test provides adequate specificity and sensitivity, but these parameters are affected by the medium used to grow the test inoculum (207).

Phenotypic Systems

A variety of manual and semiautomatic single to multiphenotypic assay systems have been commercialized (Table 6 and reference 192). Most are designed to help identify the most common yeast etiologic agents. The systems vary in ease of use and accuracy. Generally, the systems designed to identify multiple genera and their species are less reliable with unusual or rare pathogens (e.g., *C. inconspicua*), although sometimes, rare isolates of common species such as *C. parapsilosis*, *C. guilliermondii,* and *C. krusei* can result in misidentification or poor species discrimination (209, 210).

Carbohydrate Assimilation Tests

The mainstay of yeast identification to the species level is the carbohydrate assimilation test (Table 1), which measures the ability of a yeast to use a specific carbohydrate as the sole source of carbon in the presence of oxygen. There are several reliable commercially available kits such as API 20C AUX (bioMérieux SA, Marcy l'Etoile, France), API ID 32C (not available in the United States), Auxacolor 2 (BioRad, Hercules, CA), and automated and semiautomated systems (e.g., ID 32C and Vitek 2 YST, produced by bioMérieux) on the market today (Table 6) that make the classic Wickerham-Burton method (211) unnecessary for routine clinical isolates. None of these tests is 100% concordant with the Wickerham-Burton method, and they do not uniformly agree with each other. Assimilation reactions provided in reference books, such as Barnett et al. (212) and Kurtzman et al. (213), are based on the Wickerham-Burton method. Because commercial assimilation tests are only similar to the Wickerham-Burton tests, their assimilation reaction profiles may differ from those provided in reference books. A recent comparison of the API ID 32C, Auxacolor, and Vitek for 251 isolates encompassing 35 species concluded they were equally good at identifying the common species, but that the Vitek system was better at identifying the rare species (214). The most recent codebook or identification software should be used with any particular assimilation system.

Carbon assimilation and, occasionally, fermentation studies are needed to differentiate species (Table 1), but rapid confirmation tests for particular species are becoming available (see "Rapid Identification of Yeasts," below, and Table 6). It is important to note that assimilation and fermentation tests may not discriminate among individual members of a species complex. Of the *Candida* spp. usually recovered from clinical specimens, *C. guilliermondii* is the only one to assimilate dulcitol (galactitol), and *C. kefyr* assimilates lactose. The assimilation of rhamnose can be helpful in separating *C. lusitaniae* from the biochemically similar but rhamnose-negative variants of *C. tropicalis* (215). For atypical isolates of *C. lusitaniae*, which can be confused with *C. tropicalis*, good growth on Trichophyton agar #1 with biotin but weak growth on vitamin-free medium indicates *C. tropicalis* (216). Certain rare strains of *C. tropicalis* may assimilate cellobiose weakly and exhibit an assimilation pattern similar to that of *C. parapsilosis*. The inclusion of arabinose is helpful, since *C. parapsilosis* readily assimilates this carbohydrate, while most strains of *C. tropicalis* do not. Urease generally is not produced, nor is KNO_3 utilized by the *Candida* species listed in Table 1. However, occasional isolates of *C. krusei* may be urease positive.

Nitrate Tests

The nitrate assimilation procedure is similar to that of the carbon assimilation test. It tests the ability of a yeast to utilize nitrate as a sole nitrogen source. The test is most beneficial when trying to identify *Cryptococcus*, *Rhodotorula*, and *Pichia* spp. In order for yeasts to utilize nitrate, they must reduce it. Nitrate reduction assays, however, have limited value for identification of yeasts and are used primarily for discrimination of *Cryptococcus* species.

Carbohydrate Fermentation Tests

Fermentative yeasts recovered from clinical specimens produce carbon dioxide and alcohol; therefore, production of gas indicates fermentation. The pH of the medium may not change. Fermentation studies are rarely needed to identify most of the commonly isolated yeasts if the mycologist is familiar with typical morphology on cornmeal agar. The test is most helpful in differentiating the various species of *Candida*; *Cryptococcus* and *Rhodotorula* spp. are nonfermentative.

Rapid Identification of Yeasts

Rapid identification of yeasts has an impact on patient management. This is particularly true given the recognition that various yeast species (e.g., *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. lusitaniae*, *C. tropicalis*, *C. neoformans*) are inherently or potentially resistant to amphotericin B, triazole agents, or echinocandins (59, 80, 83, 142) and that species-specific MIC breakpoints are now being used for the genus *Candida* (217, 218).

TABLE 6 Rapid tests (<24 h) and semirapid tests (<48 h) available for presumptive or definitive identification of yeasts following colony formation[a]

Group 1 (for single species)		Group 2 (for several species)	Group 3 (for multiple species)
Test name	Organism identified		
BactiCard Candida	C. *albicans*	Chromogenic agars (*Candida* species): CHROMagar, CandiSelect 4, Brillance Candida, chromID Candida, HiChrom Candida Differential Agar	API-Candida
Bichrolate albicans*	C. *albicans*	PNA-FISH (*Candida* species)	API 20C AUX
Fluoroplate Candida*	C. *albicans*		Auxacolor*
Germ tube	C. *albicans*		Candifast*
C *albicans* Test Kit (Murex CA 50)	C. *albicans*		Fungichrom*
MUAG Candi Kit	C. *albicans*		Fungifast*
O.B.I.S. albicans	C. *albicans*		ID 32C*
AlbiQuick test kit	C. *albicans*		RapID Yeast Plus
Glabrata RTT	C. *glabrata*		Uni-Yeast Tek
Rapid Trehalose assimilation	C. *glabrata*		Biolog YT Microplate
Bichrolate krusei*	C. *krusei*		MicroScan Rapid YS
Caffeic acid disk	C. *neoformans*		Sherlock MIS
			Vitek YBC
			Vitek 2 YST

[a]Tests not available in the United States are indicated by *.

Rapid tests are divided into those that provide definitive or presumptive identification within the same day (<24 hours) of colony formation and either are specific to a single species, are limited to a few species, or apply to multiple genera (Table 6). In general, tests listed within groups 1 and 2 of Table 6 are presumptive, and confirmation of the identification is needed.

Tests based on preformed enzymes may be acutely affected by incubation temperature (219) and are effectively limited to the more common yeast species isolated in the clinical laboratory. However, detection of specific exoenzymes can be useful for resolution of the confusion between two or three possible species. In particular, several rapid tests are available for this purpose, particularly for detection of *Candida albicans* (e.g., the Thermo Remel *Candida albicans* test kit [Thermo Fisher Scientific, Houston TX]). These tests are excellent for confirmation of a germ tube-positive organism such as *C. albicans* and could be used in place of more long-term identification tests for this purpose. However, similar results will be obtained for *C. dubliniensis*. Using these tests for confirmation purposes can provide financial savings to the clinical laboratory. Both tests depend on the production of two enzymes (proline aminopeptidase and β-galactosaminidase) instead of a single enzyme (β-galactosaminidase) which could otherwise lead to spurious results (220).

A rapid test to help distinguish some *Cryptococcus* species uses the ability to reduce nitrate on a rapid nitrate swab test (Thermo Remel). This can supplement the results of more comprehensive tests and help to distinguish *C. neoformans* and *C. gattii* (nitrate negative) from *C. albidus* and *C. terreus* (nitrate positive). Molecular-based tests and serologic tests appear to be promising rapid alternatives with higher sensitivity and specificity.

Molecular Methods

The obvious advantage of using molecular methods is speed, as results are often available within 24 hours of an isolated colony, whereas traditional methods usually take at least 48 hours. However, development of molecular databases and standardization remain issues before any of these tests become commonplace (discussed in references 179 and 221), and most of these tests will require validation and approval by regulatory agencies before they can be widely implemented.

Direct DNA Sequencing

DNA sequencing is increasingly being used for definitive identification of unknown yeast isolates. Characteristic assimilation profiles of unusual isolates may not be present in the commercially available phenotype databases, resulting in misidentifications. Also, individual members of species complexes are often not distinguished by kits based on phenotype. As a result, increasing use is being made of ribosomal DNA (rDNA) sequencing and comparison to genetic databases for conclusive identification. Two widely used databases are GenBank (http://www.ncbi.nlm.nih.gov/blast) and the Centraalbureau voor Schimmelcultures (CBS) Yeast database (http://www.cbs.knaw.nl/yeast/biolomics.aspx), with the latter containing databases for strains held in the culture collection. Lau et al. (177) highlight the need to be aware of how the genetic database being used is maintained. GenBank contains unreferreed sequences, and errors are known to occur, whereas the CBS has a curated intervening transcribed spacer (ITS) sequence database. Reference texts such as the *Atlas of Clinical Fungi* (222) have included restriction maps of ribosomal operons for described fungi. Molecular approaches and guidelines to fungal identification are described in CLSI documents MM03-02 and CLSI MM18-A (223, 224).

In order to develop a universal genetic identification system for yeasts, the most reproducible, specific, and sensitive sequence for comparison must be identified, and most research has focused on the rDNA regions. The large subunit, specifically the D1/D2 region, was shown to be highly discriminatory for the ascomycetous yeasts (1) and, similarly, for the basidiomycetous yeasts (2, 225), although differentiation of some closely related species in the latter study (225) was achieved using the ITS and intergenic sequence (IGS) regions. Other studies have also used the ITS1 and ITS2 regions (226, 227). Chen et al. demonstrated that ITS-2 sequence alone can be sufficient for discriminating *Candida* species (228). Linton et al. (229) conducted a study assessing the value of direct 26S rDNA sequencing using over 3,000 isolates submitted to the UK Mycology Reference Laboratory. All 153 isolates that could not be identified using the Auxacolor 2 kit were unambiguously identified using 26S DNA sequencing. Ciardo et al. (230) compared ITS sequencing with the ID 32C system for yeast strains. ITS sequencing identified 98% of 113 yeast isolates, while the phenotype-based system identified 87% correctly.

Two systems for DNA sequencing and analysis are commercially available. Hall et al. (231) used the MicroSeq D2 large-subunit rDNA sequencing system (ABI) to identify 131 clinical yeast isolates and compared the results with those obtained by conventional methods, including the API 20C AUX system. When 100 isolates representing 19 species of *Candida* were compared using the MicroSeq kit, 98% matched with identifications using the API 20C AUX. For the remaining isolates, representing nine species, there was 81.3% concordance between the systems. Most of the discrepancies were attributed to a lack of available data in the relevant databases. The SmartGene Fungi system (SmartGene, Lausanne, Switzerland) is another commercial database used for identifying unknown isolates. In a recent evaluation with 2,938 specimens, 79% of 169 isolates that could not be identified using phenotypic tests were identified to species level using ITS sequencing and the SmartGene IDNS database (232). About half of the sequenced isolates were common pathogens with atypical biochemical profiles, and the remainder were rare yeast species.

Pyrosequencing, a novel method for rapid determination of a short stretch of DNA sequence, has been examined as a potentially fast identification method. Montero et al. (233) identified only 69.1% of 133 isolates representing 43 yeast species by comparing the sequences of the hypervariable ITS region to traditional methods, as it was not possible to speciate the *Trichosporon* or *Cryptococcus* isolates. In contrast, Boyanton et al. (234) obtained 100% agreement between pyrosequencing and traditional identification methods for 60 isolates of the species *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, and *C. tropicalis*. Also, Borman et al. demonstrated that pyrosequencing of 25 bp of the ITS2 region correctly identified most clinically important *Candida* species (235).

For organisms that are difficult to grow or require noncommercially available materials, these methods hold great potential. For example, identification of *Malassezia* species otherwise requires both specialized growth media and phenotypic identification tests. However, more research is needed before a universal genetic database can be devised, because the D1/D2 and ITS regions do not appear to identify all yeasts. Using the ribosomal 5.8S-ITS sequence, only 6 of 15 *Debaryomyces* species were correctly identified; the remaining species were differentiated using the sequences of the ACT1 (actin) gene (236). The ribosomal IGS region is required for species identification of *Trichosporon* isolates (25).

DNA-Based Methods that Do Not Involve Direct Sequencing

Restriction enzyme analysis (REA), karyotyping using pulsed-field gel electrophoresis, and the use of species-specific probes have all been used to identify yeast species and have been reviewed (177, 192, 237). However, it must be remembered that these techniques were developed before many of the species complexes were discovered and may not be able to distinguish among them.

Microarrays for fungal identification have been produced using the ITS regions. Leinberger et al. (238) identified *C. albicans*, *C. dubliniensis*, *C. krusei*, *C. tropicalis*, *C. guilliermondii*, and *C. lusitaniae*. A further modification on this principle was able to distinguish among *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* from among 24 other fungal pathogens, including 10 other *Candida* species (239). Other rapid identification systems developed include the DiversiLab system of automated repetitive sequence-based PCR (bioMérieux). This method uses primers against noncoding repetitive sequences; amplified products are separated on a microfluidic chip to generate a fingerprint of intensity of fluorescence versus migration time. This technique was used to screen 115 clinical isolates of *Candida* species by sequence analysis of the contiguous ITS region and obtained 99% concordant results with traditional identification methods (240). Although results can be obtained within 24 hours of a pure culture being obtained, identification is dependent on a comprehensive library of species fingerprints for comparison. The Luminex multianalyte profiling platform (described earlier) has been used on a number of types of clinical specimens and enables screening of a range of fungal pathogens simultaneously. Landlinger (241) used the ITS2 region to design 75 genus and species-specific hybridization probes to detect fungal infection in blood, pulmonary biopsy specimens, and bronchotracheal secretions. Deak used this method for rapid identification of 1,182 *Candida* isolates (242).

MALDI-Based Methods

An alternative method to DNA based technologies is MALDI-TOF MS. This method requires a pure colony of yeast and produces a mass-spectrum fingerprint characteristic of the organism. Identification of fungal isolates using MALDI has been recently reviewed (243, 244). Recently, the technique was used to assess the identification of 346 yeasts directly from blood culture bottles and found 96% sensitivity for the detection and identification of *C. albicans*, 94% for *C. parapsilosis*, 87% for *C. tropicalis*, 84% for *C. glabrata*, and only 75% for *C. krusei* (245). The great advantage was that the results were available within 24 hours of the blood culture setup. However, Cassagne et al. (246) found that a direct smear of yeast culture or fast formic acid extraction yielded lower identification rates than a complete formic acid/acetonitrile extraction. As with other fingerprinting techniques, this method requires an adequate database of reference strains for comparison to the test isolate to facilitate rapid routine identification of clinical yeast isolates (247).

Troubleshooting Difficult Identifications

Laboratories encounter yeast isolates that do not fit easily into a specific species, particularly when commercial identification systems are used. Fortunately, there are key features that help separate troublesome organisms from others. For example, urease production separates basidiomycetous yeasts from ascomycetous yeasts. Maximum growth temperature, cycloheximide resistance, and the ability to assimilate cellobiose, inositol, or trehalose are helpful. For more difficult identifications, use of traditional auxanographic methods with carbon source-impregnated disks on yeast carbon base agar plates (rather than spotting the sugar on the edge of the plate, as done by Beijerink) may provide the most accurate assimilation information. Differential media such as chromogenic agars or eosin methylene blue agar (*C. glabrata* and *C. kefyr* produce distinct colonies) may sometimes help (248). Fermentation tests should also be considered, as should a second commercial system but one that uses a different endpoint (e.g., growth versus exoenzyme production). Clinically relevant isolates that consistently fail to be identified by conventional tests should be sent to a reference laboratory for DNA sequencing or MALDI analysis.

ORGANISMS RESEMBLING YEASTS

Occasionally, organisms such as moulds and algae may grow on mycological media and produce colonies that resemble those produced by yeasts. However, careful attention to morphologic characteristics differentiates them. Examples of such organisms recovered from clinical specimens that can be superficially confused with yeasts include *Geotrichum*, black yeasts, and *Prototheca* species. *Geotrichum candidum* (dry, white colonies) can be separated from *Trichosporon* spp. by carbohydrate assimilations and the lack of urease and blastoconidia production. Black yeasts (initially white to tan-colored colonies) are treated elsewhere in this *Manual*. *Prototheca* species (white to cream colored, dull or moist to mucoid colonies) are ubiquitous, achlorophyllous algae living on decaying organic material and rarely produce disease in humans and animals (249). *P. wickerhamii* is recovered most often from human infection.

TYPING SYSTEMS

In order to understand the epidemiology of infection, to distinguish between endogenous and exogenous infection (and in the latter, to locate the source), to examine transmission, or to monitor the spread of drug resistance, it is necessary to be able to distinguish between isolates within a species. Successful strain typing depends on the choice of technique(s) and experimental conditions for maximum discrimination, which has to be established for each species tested.

DNA-based typing methods began in the 1980s with REA, which required careful selection of the enzyme used to maximize discrimination. Other methods quickly followed: Southern blotting using species-specific or generic probes, karyotyping, pulsed-field gel electrophoresis-REA, and random amplified polymorphic DNA analysis. These methods are all subject to discrepancies due to mutation or laboratory reproducibility and have been reviewed (237, 250, 251). Most of these techniques have been superseded by more accurate and reproducible methods such as microsatellite typing and multilocus sequence typing (MLST). Gil-Lamaignere et al. (237) and Taylor et al. (251) have critically reviewed the limitations and applications of these techniques.

Microsatellite typing is the amplification of short tandem repeat sequences that are polymorphic, resulting in bands of varying lengths related to the number of repeats contained therein. Variation in the number of repeats occurs during DNA replication, and this provides the characteristic fingerprint. This is a fast typing method but cannot be used to estimate isolate relatedness unless the bands are sequenced and the content compared (for example, see reference 252). MLST is an accurate and reproducible method of fingerprinting that uses the sequences of six to eight selected housekeeping genes and identifies polymorphic nucleotide sites. The method can be used to establish population structures by assessing variation in sequence for a set number of genes, and this has enabled databases to be developed for interlaboratory comparisons. The method has been used to type a number of yeast species (253–255) and recently demonstrated the spread of *Cryptococcus gattii* from Vancouver Island in Canada to the northwest United States (256). MLST has been used to develop a consensus typing scheme for *C. gattii* and *C. neoformans* (257).

MLST has been used to demonstrate that most patients with *C. albicans* carried the same strain at multiple sites or over time and to confirm that microvariation could be detected over time when some isolates are stored (258). However, while MLST provided detail on the population structure of clinical isolates of *C. glabrata* from Taiwan, greater typing discrimination was achieved using PFGE-REA (259).

SEROLOGIC TESTS

Genus Candida

Invasive *Candida* infections are life-threatening, but detection by relatively noninvasive methods such as blood culture is problematic. A test for detection of antibody to *Candida* mannan is available (e.g., using the Platelia *Candida* Ab EIA kit [Bio-Rad Laboratories]), but such tests have poor sensitivity and specificity (177). These poor results are due to the presence of *Candida* species as part of the commensal flora and antibodies against cell wall components that may be found in colonized individuals. The utility of these tests is maximized when combined with detection of antigen levels because the antibody and antigen levels may show an inverse correlation during disease progression. More detail on antigen testing is in an earlier section.

ANTIMICROBIAL SUSCEPTIBILITIES

Details of susceptibility testing methods, antifungal resistance profiles, and mechanisms of yeasts and moulds are described in other chapters of this manual. Table 7 contains the MICs from a number of sources for 36 yeast species. The references in Table 7 have all used CLSI methods, with the following exceptions: Lass-Flörl et al. (260), who followed EUCAST methodology; the *Atlas of Clinical Fungi* (222); and Ashbee (121), which contains data from published work. The data on *Malassezia* from Ashbee's review have been included because there is little information on the susceptibility of these organisms. Species-specific susceptibility breakpoints have been determined for several species of *Candida* and the major classes of antifungal drugs (217, 218). Most species of *Candida* are susceptible to the echinocandins, although there is some variation within species in susceptibility to caspofungin (261, 262), and echinocandin resistance has begun to emerge in *C. glabrata* (82–84). *Candida krusei* is intrinsically resistant to fluconazole but is susceptible to posaconazole and voriconazole, while some strains of *C. glabrata* may have elevated MICs to fluconazole. *C. norvegensis* and *C. inconspicua* are also fluconazole resistant. Few *Candida* species are resistant *in vitro* to amphotericin B, although *C. lusitaniae* may exhibit secondary resistance to amphotericin B. Therefore, clinical response to this drug may be poor despite *in vitro* susceptibility tests indicating that strains are susceptible (96, 263, 264). However, clinically relevant breakpoints have not been established for susceptibility testing of yeasts against amphotericin B (265).

Reference 142 reviews current information about diagnosis, antifungal susceptibility testing, and treatment of inva-

TABLE 7 Antifungal susceptibilities[a]

Organism	MIC (μg/ml) of drug								Reference(s)
	AmB	5FC	Flucon	Itra	Vori	Pos	Casp	Terb	
Blastoschizomyces capitatus	0.5–2.0	0.25–0.5	16–32	0.12–0.50	0.25–0.5	0.03–0.25[b]	ND	ND	272, 273[c]
Candida albicans	0.06–1.0	1.0[b]	0.25–64	0.06–4	0.015–1	0.03–0.12[c]	0.015–0.125	0.03–128[b]	273,[c] 274
C. dubliniensis	0.05–0.38	0.12	0.12–64	0.015–0.5	0.008–0.5	0.03[c]	ND	ND	222, 273[c]
C. famata	0.5[b]	1.0[b]	4–8[b]	0.25[b]	0.06[b]	0.03–0.25[c]	ND	ND	273[c]
C. glabrata	0.125–4	ND	1–>64	0.125–4	0.03–1	0.03–4.0[c]	0.015–0.5	>128[b]	273,[c] 274
C. guilliermondii	0.06–32	0.06–4	0.5–>128	0.06–>8	0.06–>8	0.06–8	0.03–>8	0.625–100[b]	275, 276
C. haemulonii	0.5–32	ND	2–128	0.125–4	0.03–2	ND	0.125–0.25	ND	277
C. kefyr	0.5[b]	0.03–16[b]	0.5–1[b]	0.25–0.5[b]	0.03[b]	0.03[c]	ND	0.5–50[b]	273[c]
C. krusei	0.03–16	0.06–64	32–64[b]	0.5[b]	0.5–1.0[b]	0.03–0.25[c]	0.03–2.0	8–32[b]	273,[c] 278
C. lusitaniae	1.5–2.0	0.004–>32	0.064–6.0	0.004–0.5	0.003–0.094	0.03[c]	4–8	ND	260, 273,[c] 279
C. parapsilosis	0.125–1	1.0[b]	0.25–8	0.015–2	0.015–1	0.03–0.12[c]	0.06–2	0.125–2[b]	273,[c] 274
C. rugosa	0.5–1.0	0.12–1.0	1–8	0.03–0.12	0.03–0.06	0.03[c]	ND	ND	222, 273[c]
C. tropicalis	0.06–1	1.0[b]	0.05–32	0.03–4	0.03–1	0.03–>16[c]	0.015–0.125	1.0[b]	273[c], 274
Cryptococcus neoformans	0.5–1[b]	2–8[b]	2–8[b]	0.5[b]	0.25[b]	0.125–0.5	>8	2–8[b]	260
Kodamaea ohmeri	0.25–0.5	ND	2–32	0.125–0.5	0.3–0.5	ND	0.125–0.25	ND	280
Malassezia dermatis	0.03–0.12	ND	2	0.016–0.03	0.12	0.03–0.5[c]	ND	0.03–4	121, 273[c]
M. furfur	0.12–16	>64	2–32	0.03–25	0.03–16	0.03–32[c]	ND	0.03–50	121, 273[c]
M. globosa	0.10–4	ND	12.5–50	0.016–6.3	0.03–0.12	0.03–0.06[c]	ND	0.06–16	121, 273[c]
M. japonica	ND	ND	ND	0.016	ND	ND	ND	ND	121
M. nana	ND	ND	ND	0.016	ND	ND	ND	ND	121
M. obtusa	0.03–0.06	ND	2	0.016–1.6	0.03–0.06	0.03[c]	ND	0.03–64	121, 273[c]
M. pachydermatis	0.12	>64	4–16	0.016–6.3	0.03–0.25	0.12[c]	ND	0.03–50	121, 273[c]
M. restricta	4–8	ND	0.5–1	0.0156–6.3	0.03	0.03[c]	ND	0.06–4	121, 273[c]
M. slooffiae	0.5–8	>64	1–4	0.016–0.8	0.03–0.25	0.03[c]	ND	0.03–25	121, 273[c]
M. sympodialis	0.06–0.5	>64	0.25–16	0.016–0.2	0.03–0.125	0.03–0.06[c]	ND	0.03–6.3	121, 273[c]
M. yamatoensis	ND	ND	ND	0.016	ND	ND	ND	ND	121
Pichia anomala (current name, *Wickerhamomyces anomalus*)	0.12–1	ND	2–16	0.06–1	0.03–0.5	ND	0.03–0.25	ND	281
Rhodotorula glutinis	0.25–0.5	ND	64	2–16	2–16	0.06[c]	ND	ND	273,[c] 282
R. mucilaginosa	0.5–1	ND	0.5–>64	0.25–4	0.25–4	ND	ND	ND	222
Saccharomyces cerevisiae	0.5[b]	0.125[b]	0.25–>64	0.015–>4	0.03[b]	0.015–>4	4–8	0.125[b]	260, 283
Sporobolomyces salmonicolor	0.25–16	ND	8–64	0.25–0.5	0.12–0.25	ND	ND	ND	282
Trichosporon asahii	025–1.0	22.2[d]	1–16	0.12–1	0.12–1	0.03–0.25[c]	>8	ND	222,[d] 260, 273,[c] 282
T. asteroides	0.25–4.0	0.5–64	0.125–1	0.03–0.125	0.03–0.06	ND	2–>16	ND	284
T. cutaneum	0.012	6.25	2.2	0.01	ND	ND	ND	ND	222
T. mucoides	1	50[d]	2–64	0.25–2	0.12–0.16	ND	ND	ND	222,[d] 282
T. ovoides	0.03	35.3	4.4	0.02	ND	ND	ND	ND	222

[a] Abbreviations: AmB, amphotericin B; 5FC, 5-fluorocytosine; Flucon, fluoconazole; Itra, itraconazole; Vori, voriconazole; Pos, posaconazole; Casp, caspofungin; Terb, terbinafine; ND, not determined.

[b] MICs taken from http://www.mycology.adelaide.edu.au/Laboratory_Methods/Antifungal_Susceptibility_Testing/astprofiles.html.

[c] MICs taken from reference 273 for posaconazole.

[d] MICs taken from reference 222 for 5-fluorocytosine.

sive fungal infections due to rare yeasts other than *Candida* species. MICs of the six common human *Trichosporon* species indicate that all species have resistance to 5-fluorocytosine (6.25 to 50 μg/ml), have higher MICs to fluconazole than most *Candida* species (0.15 to 25 μg/ml), and are variably susceptible to amphotericin B (0.01 to >4 μg/ml), ketoconazole, and itraconazole (0.1 to 0.6 μg/ml) (49). Echinocandins are considered ineffective against *Trichosporon* and *Cryptococcus* species.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

As taxonomic relationships and rules are revised, the names of organisms change. To prevent confusion, it is recommended that clinical reports include the more familiar species names alongside any new classification being used. At this time, rather than change the name of *Candida* genus organisms from names that are deeply entrenched in the literature and in the minds of physicians and laboratorians, this *Manual* has retained their *Candida* genus taxon as well as the teleomorph name. This will likely change in the future, and laboratorians should familiarize themselves with newer names as they are introduced.

As in clinical bacteriology, the significance of isolating a species known to be a member of the normal microbiota or a common contaminant depends on numerous factors, including but not limited to the patient's underlying disease, geographic and hospital environment, specimen site, preceding antimicrobial and device management, specimen collection and quality, and specimen processing. In general, the isolation of yeasts from sterile body sites is suggestive of infection, but the influence of the method of collection (e.g., *C. albicans* in CSF collected by lumbar puncture) must be considered. The more challenging decision about significance is when the yeast is present in specimens obtained from nonsterile body sites.

Review of the literature reveals a surprising lack of evidence-based studies regarding development of criteria for establishing significance in nonsterile body sites. Clinical microbiology laboratories typically apply bacterial criteria to assess specimens for possible fungal infection. An example of this is to not report yeasts unless they are predominating (over bacteria). However, there are several problems with this approach. First, yeasts are not as abundant as bacteria in the normal microbiota, so an increase in relative abundance may reflect yeast infection. Roughly 10 times more bacteria than yeast can occupy the same volume of tissue. Secondly, a specimen from a patient who is receiving antibacterial agents may contain more abundant yeasts, the reporting of which may mislead the patient's physician about the yeasts' significance. Third, when the abundance of a yeast is considered insufficient to warrant further investigation, the species may not be reported. This could lead to unfortunate consequences because certain yeasts, such as *C. tropicalis*, are considered particularly aggressive in immunocompromised patients.

Two specimens that are particularly problematic are sputum and urine (clean catch, indwelling catheter, and "in-and-out" catheter). The significance of *Candida* species in sputum, regardless of quantity, has been questioned (266, 267). The presence of these yeasts in sputum has little significance as the respiratory tract is frequently colonized by *Candida* species in patients receiving ventilatory support. The latest Infectious Diseases Society of America guidelines recommend that antifungal therapy not be initiated on the basis of a positive respiratory tract culture, and in cases where *Candida* pneumonia is suspected, histopathological evidence should be sought (59). Similarly, the presence of a few *C. neoformans* colony-forming units in sputum does not necessarily imply etiologic significance unless patient information strongly suggests cryptococcosis. To prevent unnecessary testing but still provide useful information to physicians, Barenfanger et al. (266) recommend reporting "yeast, not *Cryptococcus*" for all respiratory secretion specimens in which a rapidly growing yeast is obtained and confining full identification for patients in whom candidal pneumonia was indicated by histopathology. Patients for whom the limited form of identification was used were found to experience a shorter hospital stay, received fewer antifungals, had a lower mortality rate, and incurred fewer expenses. *Cryptococcus* species are ruled out using a rapid urease test. This approach allows physicians the opportunity to request further identification if desired. A similar approach to rapidly growing yeasts isolated from routine urine specimens (urinary tract infection is the only presentation) could also be used. However, in this situation, differentiation of *C. glabrata* from other common yeast uropathogens could be achieved by using one of the simple rapid tests.

The significance of yeasts in urine must be examined in the context of the clinical setting and whether therapy would be desirable (59). If the patient is asymptomatic and there is no predisposing condition, the situation should be monitored. If a predisposing condition is identified, treatment may be justified, but for patients with higher risk factors such as neonates or immunocompromised patients with fever and risk of candidemia, treatment should be commenced. Fluconazole is the front-line therapy unless *C. krusei* or *C. glabrata* infection is suspected; in these cases, irrigation of the bladder with amphotericin B may be indicated (59).

Although yeast species express different antifungal profiles, especially to the expanding spectrum of new antifungal agents (see reference 142 and Table 7), the most commonly isolated *Candida* species are generally susceptible to the triazoles, the polyenes, and the echinocandins. The two exceptions for the triazoles are *C. glabrata* and *C. krusei* (*C. parapsilosis* isolates are occasionally resistant to the echinocandins). *C. krusei* is relatively rarely isolated from infections. Thus the need to identify rapidly growing yeasts present in clinically significant quantities from nonsterile sites can be limited to performing a rapid screen for *C. glabrata*. It is more important to obtain a yeast's antifungal susceptibility profile if there is failure to respond to antifungal therapy or if an azole-resistant isolate is suspected (59).

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Pneumocystis

MELANIE T. CUSHION

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The yeast-like fungi in the genus *Pneumocystis* are extracellular, host-obligate, host-specific, and typically restricted to the lung tissues of mammals, although extrapulmonary manifestations have been reported (1, 2). Once known collectively by the single genus and species "*Pneumocystis carinii*," it is now understood that distinct species of *Pneumocystis* infect different mammalian hosts. Current evidence suggests *Pneumocystis* can exist with little consequence in hosts with intact immune systems (3), but debilitation of the immune system, induced by various means including infectious or immunosuppressive agents, congenital defects, or malnutrition, can lead to organism proliferation within the lung alveoli, colonization, and potentially a lethal pneumonia if untreated. No species of *Pneumocystis* can be cultivated continuously outside the mammalian lung, impeding diagnostic capabilities as well as basic scientific research. Limited therapy is available with which to treat the pneumonia, since these fungi are not susceptible to such antifungal drugs as amphotericin B, the azoles, or the echinocandins.

TAXONOMY

Taxonomic problems have plagued the organisms known as "*Pneumocystis carinii*" since their original description in 1909 by Carlos Chagas, who mistakenly identified the cyst forms as life cycle stages of the protozoan parasite *Trypanosoma cruzi*. In 1914, these organisms were provided an identity of their own and given the binomial epithet that reflected their predilection for the lung, *pneumo-*; the characteristic morphological form, *-cystis*; and, to honor the Italian investigator Antonio Carini, who provided the slides for study, *carinii*. The *P. carinii* organisms were presumed to be a protozoan parasite at the time of identification; the question of their potential fungal nature was first raised in the 1950s, and the controversy of their protozoan or fungal nature continued to the late 20th century. A more detailed early history of *Pneumocystis* identification and nomenclature can be found in reference 4.

The most recent phylogenetic classification based on gene comparisons with other fungi places *Pneumocystis carinii* in the phylum Ascomycota; subphylum Taphrinomycotina; class Pneumocystidomycetes; order Pneumocystidales; genus *Pneumocystis* (5). Within the Taphrinomycotina are the genera *Taphrina* (plant pathogens), *Neolecta* (associated with trees; may be parasitic), *Pneumocystis*, and *Schizosaccharomyces* (fission yeasts).

Pneumocystis species all appear to contain similar life cycle stages, although the clusters of organisms removed from the lungs of the different species can vary in presentation and size. For example, clusters of *P. jirovecii* can be much larger than those obtained from rodent lungs, stain more intensely with Wright-Giemsa-like stains, and form multilayered "mats" composed of several layers of organisms that hinder identification of individual life cycle stages (see Fig. 3B, D, and F for example). The term "*Pneumocystis carinii*" was thought to represent a single zoonotic species until 1976, at which time Frenkel described serological differences between human- and rat-derived organisms that he suggested were representative of distinct species (6). It is now clear that the organism first identified as *Pneumocystis carinii* is actually a collection of many species within the genus *Pneumocystis* that likely number in the hundreds to thousands. Almost every mammal examined to date appears to harbor at least one species of *Pneumocystis* that is not found in any other mammal. Five *Pneumocystis* species have been formally described according to the International Code of Botanical Nomenclature: *Pneumocystis carinii* and *P. wakefieldiae* are found in rats; *P. murina* is found in the lungs of mice; *P. jirovecii* is found in humans (4); and *P. oryctolagi* resides in rabbits (7). The name *P. jirovecii*, though first resisted by some groups, has gained widespread acceptance within the scientific and clinical communities (8).

DESCRIPTION OF THE AGENTS

The terminology used to describe the various life cycle stages of *Pneumocystis* bears remnants of its earlier classification as a protozoan parasite. This discussion uses terms more suitable for its fungal identity, but identifies those commonly found in the literature for continuity. Three developmental forms are generally recognized: the trophic form (trophozoite), 1 to 4 μm (Fig. 1A); the sporocyte (precyst), 5 to 6 μm (Fig. 1B); and the ascus (cyst), 5 to 8 μm (Fig. 1C–E). *Pneumocystis* spp. reproduce extracellularly within the mammalian lung alveoli. The trophic forms appear ameboid in structure in electron micrographs, but in freshly prepared specimens they are ellipsoidal and often occur in clusters with other trophic forms and developmental stages. The nucleus and often the mitochondrion are visible in rapid Wright-Giemsa-stained specimens by light microscopy. The trophic forms do not stain with fungal stains designed to complex with the cell wall, such as methenamine silver. The sporocyte is smaller than the mature cyst and frequently

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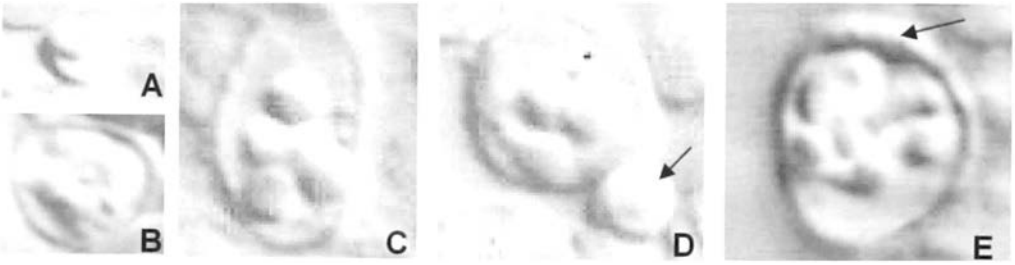

FIGURE 1 Major developmental forms of *Pneumocystis*. (A) Single trophic form; (B) sporocyte (precyst); (C) cyst with four visible spores (intracystic bodies); (D) cyst with three intracystic spores and a spore that has apparently excysted (arrow); (E) cyst with localized thickening of the cell wall (arrow) and eight visible spores. Nomarski Interference Contrast microscopy; magnification ×1,000. doi:10.1128/9781555817381.ch118.f1

oval in shape. This stage contains a rigid cell wall that is lacking in the trophic forms and is stained with methenamine and other fungal wall stains. At the sporocyte stage, the nuclei are at varying levels of nuclear division (from two to eight nuclei) but have not yet been compartmentalized into separate spore structures. Aggregates of mitochondrion can also be seen in this stage. The mature cyst is spherical in shape; it contains eight spores (although these may not all be visible) and a thick cell wall that excludes certain stains such as the rapid Wright-Giemsa, and it can be visualized with cell wall-complexing stains such as methenamine silver. The cyst/ascus is considered the diagnostic morphological form. All developmental forms are often found in large, multilayered, tightly adherent aggregates or clusters in clinical specimens, making identification of each stage difficult (see Fig. 3B, D, and F).

EPIDEMIOLOGY AND TRANSMISSION

Early animal studies showed that *Pneumocystis* infection was likely transmitted by an airborne route. Immunosuppressed, *Pneumocystis*-free rats could acquire the infection from infected rats housed in the same room or from infected cage mates (9). Recent air sampling studies using quantitative PCR, conducted around patients admitted with *Pneumocystis* pneumonia (PCP), showed the highest levels of detection within 1 m of the patients' heads (10). Although levels decreased with increasing distance from the patient source, samples were still positive in corridors outside the patients' rooms. Importantly, the genotypes of the *P. jirovecii* infecting the patients were the same as the genotypes outside the immediate patient area, confirming that the potential sources of transmission were these patients. These data provide evidence that supports historical and more recent descriptions of outbreaks in renal transplant units (11) and other clinical settings where patient-to-patient transmission can occur. The agent of transmission appears to be the cyst (ascus) form, as experiments using mice with infections composed almost exclusively of trophic forms could not transmit the infection (12).

Putative Life Cycle

Histochemical and ultrastructural studies form the basis of the current understanding of the life cycle of *Pneumocystis*, due to a historic lack of a long-term cultivation method outside the lung. Thus, any life cycle should be considered presumptive until it is possible to perform definitive kinetic analyses. There is no evidence for an intracellular phase, although the organisms can be frequently observed within macrophages as a result of the host response to the infection. Despite numerous attempts to find an environmental cycle or external reservoir for *Pneumocystis*, none has been identified. A growing body of evidence suggests that the reservoir for *Pneumocystis* is its mammalian host, a situation similar to other host-dependent pathogens like *Entamoeba histolytica* or *Mycobacterium tuberculosis*. Studies in humans and animal models support a role for neonates and immune-competent hosts as potential reservoirs that are colonized transiently or longer term (3).

A schematic of a proposed life cycle, based on a number of histological and ultrastructural studies, can be found in Fig. 2, with a detailed description provided in the legend. The various life cycle stages of *Pneumocystis* are most often found together in very large adherent clusters that resemble biofilms *in vivo* (Fig. 2B). The *in vitro* formation of biofilms by *P. murina* and *P. carinii* provides support for the formation of these structures *in vivo*, which could be responsible for intransigent infections that are unresponsive to current therapies (13, 14). Trophic forms are presumed to be the vegetative stages of the *Pneumocystis* life cycle and reproduce asexually by binary fission, not budding as most yeast do. It is likely that they also participate in the sexual mode of reproduction using a process similar to yeast mating type systems, although these processes are not fully defined. Several fungal meiosis-specific and mating type gene homologs have been identified in cDNA and genomic *P. carinii* databases, lending credence to the existence of these processes (15, 16). Subsequent to mating process and karyogamy (nuclear fusion), the zygotic nucleus undergoes meiosis and sporogenesis is initiated, resulting in formation of the precyst, or sporocyte. Following meiosis, an additional mitotic replication occurs, with subsequent compartmentalization of the nuclei and organelles into eight ascospores. The end product of sporogenesis is the spherical cyst, or ascus. The process of spore release has not been described, but may involve a localized thickening at one pole of the ascus. It should be noted that, unlike other fungi, all the developmental stages of *Pneumocystis* contain a double membrane.

Transmission

Evidence from several different studies suggests that: (i) *Pneumocystis* is transmitted by an airborne route; (ii) it is likely to be acquired early in life; (iii) it is transmitted among immunologically intact individuals; (iv) immunosuppressed and infected hosts are able to transmit the infection to immunologically intact hosts; and (v) transmission requires a short period of exposure and low numbers of organisms.

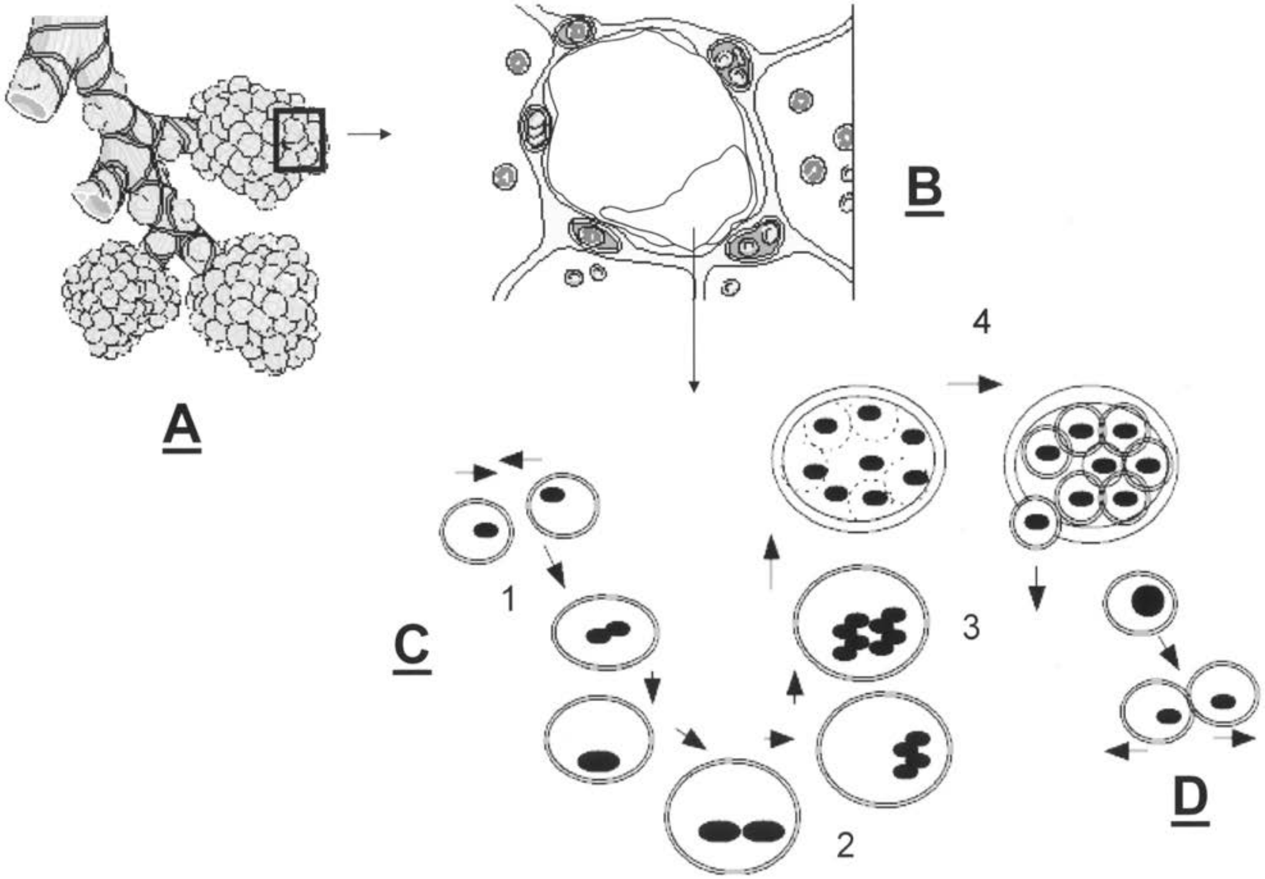

FIGURE 2 Proposed life cycle of *Pneumocystis*. (A) The primary site of *Pneumocystis* infection is the lung alveoli. Three clusters of alveoli are illustrated. An expanded schematic of an alveolus (box) is shown in panel B. (B) Single alveolus with *Pneumocystis* organisms depicted as a hatched shape attached to the cells lining the alveoli in some areas (type I pneumocytes) and unattached to other alveolar cells (type II pneumocytes). (C) Putative sexual cycle of *Pneumocystis* in the lung alveoli: (1) opposite mating types fuse and undergo karyogamy resulting in a diploid zygote; (2) the zygote then undergoes meiosis resulting in four nuclei; (3) additional postmeiotic mitosis increases the number of nuclei to eight; (4) the nuclei and mitochondria (not shown) are compartmentalized by invagination of the inner plasma membrane, resulting in eight spores. Spores are released from the ascus (cyst) and presumably enter into the vegetative phase of the cycle. (D) Asexual replication cycle of *Pneumocystis*. Trophic forms undergo binary fission after mitotic replication of the nucleus. (Drawn with SmartDraw Suite, ed. 7.3.)
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Experimental evidence indicates that very few organisms are required to initiate *Pneumocystis* infection and that the fungi are very efficient in their method of transmission. Studies have shown that fewer than 10 *P. carinii* organisms were sufficient to establish a fulminant infection in immunosuppressed rats (17) and a 1-day period of exposure was all that was needed to transmit the infection from an infected *scid/scid* mouse to an uninfected *scid/scid* mouse (18). The widespread prevalence of *Pneumocystis* in commercial colonies housing healthy rats not only supports the very efficient dissemination of the infection throughout the members of a colony, but also shows that the organism thrives in the immune-competent host (19). Transmission from infected patients to immune-competent health care workers has been reported (20). Evidence that healthy human populations may also serve as reservoirs or sources of infection is accumulating. A recent study conducted in humans without underlying lung disease or immunosuppression found that 20% of the oropharyngeal wash samples from 50 individuals were positive for *P. jirovecii* by nested PCR targeting the mitochondrial large subunit ribosomal gene (21). In a study of 851 non-HIV–infected patients with pneumonia in China, *P. jirovecii* was detected in 14.5% by methenamine silver staining and in 24% by PCR (22).

Epidemiology

Beard et al. reported that the expansion of *P. jirovecii* carrying a double mutation in the DHPS gene in selected HIV-infected human populations provided strong support for transmission of *P. jirovecii* via a person-to-person route, and that this was illustrative of a positive selective mechanism as well (23). Previous and recent epidemiological surveys showing the clustering of specific *P. jirovecii* genotypes with patients' place of residence or in clinical settings (many in renal transplant units) are consistent with the hypothesis of person-to-person transmission of *P. jirovecii* via the airborne route (11, 24). A report from Japan documented a PCP outbreak in a renal transplantation unit with 27 cases in a single year that could be traced by molecular methods back to the outpatient clinic (25). The median incubation time was estimated to be 53 days, with a range of 7 to 188 days. Several such outbreaks have been reported over the past 2 decades, providing a cautionary note for immunosuppressed patients gathering in a community setting, as well as for appropriate prophylaxis therapy for susceptible populations. In some cases, the mortality rate was as high as 50% (26). Surprisingly, investigators recently showed that most cases of *Pneumocystis* colonization in renal transplant patients were detected beyond 2 years after transplant rather than within the initial 2-year time period (26). However, a recent report on the epidemiology of *P. jirovecii* colonization in families showed only a 3.3% detection in children of HIV-infected adults, in which their colonization rate was 11.4%, suggesting that merely close contact with potential reservoirs is not sufficient to induce this state (27).

Serological studies performed in the 1970s through the 1980s showed *P. jirovecii* was acquired early in life (28, 29). Most human beings become seropositive to *P. jirovecii* organisms or antigens by the age of 2 to 4 years. Vargas et al. detected *P. jirovecii* DNA in nasopharyngeal aspirates in 24 of 72 infants (32%) suffering from mild respiratory infections (30). Seroconversion developed in 67 of 79 (85%) of the same cohort of infants by 20 months of age. Thus, serological testing has not been useful for the diagnosis of PCP.

Colonization

Emerging reports of the detection of *P. jirovecii* in populations without underlying immunosuppression, as well as populations who have chronic underlying diseases that have not been historically associated with its presence, may suggest colonization or expansion of host range (3, 31–33). Colonization, carriage, asymptomatic infection, and subclinical infection have all been used to describe the presence of *Pneumocystis* organisms or DNA in the absence of PCP. The effects of the presence of low numbers of *P. jirovecii* on the host have yet to be determined, and the role of carriage in mild respiratory infections, chronic lung disease, and progression to PCP are now being actively investigated. The most common underlying conditions associated with the presence of *P. jirovecii* in non-HIV-positive individuals include asthma and chronic lung diseases, chronic obstructive pulmonary disease, cystic fibrosis, Epstein-Barr virus infection, lupus erythematosis, high-dose corticosteroid therapy, anti-tumor necrosis factor-α therapies for rheumatoid arthritis, thyroiditis, ulcerative colitis, and pregnancy. Children appear to have a higher rate of colonization than adults do. Serological studies showed early exposure occurred during the first few years of life; 32% of colonized infants manifested mild respiratory symptoms in a large study in Chile (30). Studies from the same group suggested an association with sudden infant death syndrome, but this was not supported by subsequent investigations. Should the presence of *P. jirovecii* be confirmed as a causal agent in the underlying pathogenesis of any of these diseases, treatment of this cofactor could improve patient outcome.

CLINICAL SIGNIFICANCE

PCP remains the leading opportunistic infection associated with AIDS patients, even in the era of highly active antiretroviral therapy (HAART) (34). The mortality rate associated with PCP prior to and after the era of HAART (1996 forward) has not changed significantly in the United States, with an average of 10 to 13.5% (35, 36). In developing countries and within urban American cities, the mortality is much higher despite the availability of HAART (37, 38). The mortality rate for a medically underserved population in Atlanta, Georgia, from 1996 to 2006 was 37%, while patients who required aggressive intervention such as mechanical ventilation experienced an 80% mortality rate from PCP (38). In patients with cancer and other non-HIV diseases, there has been little improvement in mortality rates, and often these patients fared more poorly than patients with HIV. In one such study, the mortality of non-HIV-infected patients with PCP was 48% while HIV-infected patients experienced a 17% mortality (39). The mortality rate of *P. jirovecii* pneumonia in rheumatoid arthritis patients receiving eight immunosuppressive therapies in Japan averaged about 15.5%, with the highest mortality in patients who received tacrolimus; only one patient treated with golimumab developed PCP and did not die (40). Although the incidence of PCP in pediatric populations has declined, PCP and its association with immune reconstitution inflammatory syndrome (IRIS) (41) cause significant clinical problems in these populations and vulnerable adult populations worldwide.

Within the mammalian lung, the trophic forms of *Pneumocystis* adhere to the type I pneumocytes, presumably through macromolecular bridges (42–44). Type I cells are responsible for the gas exchange between the alveolar capillaries and the alveolar lumen. Besides attachment to the type I cells, the various developmental stages of the parasite adhere to one another, producing large clusters that extend outwardly into the alveolar lumen. In severe, untreated infections, most of the alveoli are filled with organisms. Direct attachment to the cells responsible for gas exchange combined with the accumulation of organisms within the alveoli results in impaired gas exchange and altered lung compliance, as well as other physiologic changes associated with the pneumonia, such as hypoxia (45).

Histopathologic findings can be characterized by two traits: (i) alveolar interstitial thickening and (ii) a frothy eosinophilic honeycombed exudate in the lumina of the lung. The interstitial thickening is a result of hyperplasia and hypertrophy of the type II pneumocyte, interstitial edema, mononuclear cell infiltration, and in some cases mild fibrosis. The exudate is apparent upon staining with hematoxylin and eosin, which does not stain the organisms but clearly permits visualization of the eosinic exudate. Methenamine silver or another yeast cell wall stain must be used to visualize the cyst form of the organism, considered the diagnostic stage.

Immune Reconstitution Inflammatory Syndrome

Treatment with antiretroviral therapy improves immune function, with a concomitant increase of CD4 cells within a few months after start of therapy. This improvement in immune responses can be accompanied by a paradoxical, exaggerated inflammatory response manifested against infectious or noninfectious agents that often results in clinical worsening, referred to as IRIS (41). IRIS occurs more frequently in adults, but has been reported in children. In adults, IRIS has been associated with patients harboring mycobacterial infections; PCP; cryptococcal infections; cytomegalovirus, varicella virus, or herpes simplex virus infections; or progressive multifocal leukoencephalopathy.

Presentation in Children

PCP is a common manifestation among HIV-infected children. The highest incidence occurs during the first year of life, peaking at 3 to 6 months of age (41). Although a significant and dramatic decline in PCP infection rates in U.S. infants has been reported by the Centers for Disease Control and Prevention (CDC) and Perinatal AIDS Collaborative Transmission Study *Pneumocystis*, it remains a deadly neonatal disease in Africa, where postmortem analysis showed that 44% of children who died between 2000 and 2001 had PCP (46). Recent data indicate that *P. jirovecii* is a significant cause of pneumonia in HIV-infected children without appropriate antiretroviral therapy or prophylactic treatment, with high mortality rates ranging from 28 to 63% (47).

PCP was first described in children and was considered a pediatric infection in its early history (48). Prominent epidemics of "interstitial plasma cell pneumonia" in undernourished children housed in suboptimal conditions after World War II were manifestations of the disease that was later identified as *P. jirovecii* pneumonia. The infection in these children was characterized by a plasma cell infiltrate, in contrast to the type II cell hypertrophy and scanty mononuclear infiltrate described in adults with the pneumonia.

Clinical features of PCP in children include fever, tachypnea, dyspnea, and cough. Onset can be acute or subtle, associated with nonspecific conditions such as mild cough, loss of appetite, diarrhea, and weight loss. Fever may or may not be present, but most children will exhibit rapid breathing with short shallow breaths at the time the pneumonia is visible by radiographic methods. Bilateral basilar rales, respiratory distress, and hypoxia are often evident on physical exam. In HIV-infected children, four clinical variables are independently associated with PCP: age younger than 6 months; a respiratory rate of greater than 59 breaths per minute; arterial percentage hemoglobin saturation less than or equal to 92%; and the absence of vomiting (41, 49).

Most children manifest frank hypoxia, with low arterial oxygen pressure of less than 30 mm Hg. The $CD4^+$ count is often less than 200 cells/mm^3, but can be higher. Children older than 5 years have a percent $CD4^+$ of less than 15%. Like adults, children will often have bilateral diffuse parenchymal infiltrates with a ground-glass appearance, but such manifestations may be altogether lacking or mild.

Presentation in Adults

Adults with *Pneumocystis* pneumonia frequently present with dyspnea, nonproductive cough, inability to breathe deeply, chest tightness, and night sweats (45). A low-grade fever (e.g., 38.5°C) and tachypnea are often present, while hemoptysis or sputum production is rare. On physical examination, few pronounced abnormalities are detected, but various degrees of respiratory distress, small respiratory volumes, and fine basilar rales can be observed. Patients with AIDS often have a more insidious progression to clinical disease than patients who are not infected with HIV but are immunosuppressed.

The chest radiograph may appear normal or reflect a disease state. Diffuse, symmetrical, interstitial infiltrates are most commonly present, while focal infiltrates, lobar consolidations, cavities, and nodules are less common. Infiltrates in early infection may be widely distributed, but consolidation increases as the disease progresses. Administration of aerosolized pentamidine as a prophylactic measure has been associated with increased frequency of apical infiltrates and pneumothoraces. The severity of abnormalities on the chest radiograph is considered prognostic and can be correlated with higher mortality.

If the radiograph is normal or unchanged from a prior radiograph, a test for the diffusing capacity of the lung for carbon monoxide (DL_{CO}) is recommended if the patient's symptoms consist of a nonproductive cough or shortness of breath, with or without fever. If the DL_{CO} (corrected for hemoglobin) is ≤75% of the predicted value or decreased ≥20% from baseline, the patient should undergo diagnostic evaluation or bronchoscopy or both (50).

The oxygenation impairment induced by pneumocystosis can be detected in most patients by a widening of the alveolar-arterial oxygen gradient [$(A\text{-}a)DO_2$] correlated with severity of disease and respiratory alkalosis (50). However, it should be noted that a significant number of patients can have a normal $(A\text{-}a)DO_2$ gradient at rest. Impaired diffusing capacity, alterations in lung compliance, total lung capacity, vital capacity, and hypoxemia are other physiologic changes that may be associated with *P. jirovecii* infection (45). Abnormalities in surfactant proteins also occur in infected individuals with increases in surfactant protein A and D most notable, since these proteins are considered components of innate immunity (51, 52).

High-Resolution Computerized Tomography

Chest radiography (X-ray) has been used to diagnose PCP, but the findings of "ground-glass" infiltrates and other characteristics are not definitive for the pneumonia, and about one third of patients with PCP have normal radiographic findings. High-resolution computerized tomography (HRCT) of PCP patients reveals extensive ground-glass opacity with a central distribution in a background of interlobular septal thickening, a mosaic pattern, or diffuse distribution in some patients (53). In some rare cases, unusual multiple nodular changes are observed (54). Pulmonary cysts of varying shapes and sizes are increasingly detected in up to one-third of patients on chemoprophylaxis. In some cases, a reversed halo sign may be indicative of PCP. Such a finding is observed as a round area of ground-glass attenuation surrounded by a crescent or ring of consolidation. First described as being specific for cryptogenic organizing pneumonia, it can be observed in several other infectious and noninfectious diseases.

Extrapulmonary Pneumocystosis

The incidence of *Pneumocystis* organisms in sites other than the lung has been reported in 0.6 to 3% of postmortem examinations of patients with pulmonary *P. jirovecii* infections (1, 2, 55). This is likely an underestimate due to the limited number of autopsies currently performed and the lack of suspicion of extrapulmonary pneumocystosis. Methenamine silver staining or immunofluorescent kits served to identify the cyst stage of *Pneumocystis* in tissue samples and fine-needle aspirates in antemortem cases. The lymph nodes were the most frequent site of extrapulmonary involvement in a series of 52 patients (44%), followed by the spleen, bone marrow, and liver (33%). *P. jirovecii* has been detected in the adrenal glands, gastrointestinal tract, genitourinary tract, thyroid, ear, liver, pancreas, eyes, skin, and other sites. Infection of multiple extrapulmonary sites was associated with a rapidly fatal outcome. Pathological findings correlated with the organ where the infection was present. For example, retinal cotton wool spots were reported in infected eyes, and pancytopenia was observed in patients with bone marrow lesions.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

P. jirovecii pneumonia should be considered in any immunocompromised patient who presents with fever, respiratory symptoms, or infiltrates on chest X-ray. While mostly restricted to the lung, *P. jirovecii* have been found as extrapulmonary masses (e.g., pleura, intra-abdominal) in HIV-positive patients and should be included in a differential diagnosis in these patients. As no symptoms are specific for *P. jirovecii* pneumonia, a definitive diagnosis is usually made by morphologic identification of the organism, though molecular methods are gaining in usage. Algorithms for clinical evaluation and treatment of PCP and differential diagnosis for HIV-associated pneumonias have been published and the reader is referred to these reviews for further details (45, 56, 57).

Because *P. jirovecii* cannot be cultured, the diagnosis of PCP relies on efficient sampling as most diagnostic methods still rely on microscopic techniques. The fungus can be detected in a variety of respiratory specimens, including induced sputum, bronchoalveolar lavage fluid (BALF), tracheal aspirate fluid, tissue obtained by transbronchial biopsy, cellular material obtained by bronchial brush, pleural fluid, and tissue obtained by open thorax lung biopsy. The diagnostic yield is dependent on the underlying disease state of the patient and the expertise of the staff obtaining the sample. In some hospitals where the staff are trained to obtain sputum samples and there is a large population with AIDS, 80% of diagnoses of PCP were made from induced sputum (58). In contrast, the diagnostic yield from non-AIDS patients can be quite low and bronchoalveolar lavage or other methods may be needed to ensure an appropriate diagnosis (59). Multiple slides from non-AIDS patients should be examined, especially when there is a high degree of suspicion. Hospitals serving a diverse population often rely on methods other than induced sputum. A notable drawback of using induced sputum as the primary procedure for the diagnosis of PCP is the lack of information concerning other infections or disease processes that may be present in the lung. The diagnosis of extrapulmonary pneumocystosis relies on accurate sampling of the infected organ and subsequent staining of the histological sections. For infants and young children who are unable to produce sputum or in whom this method is not warranted, tracheal aspirate fluid, open thorax lung biopsy, or respiratory specimens must be obtained by a special pediatric bronchoscopy service (30, 60).

Bronchoalveolar Lavage

Fluid obtained by bronchoalveolar lavage is sufficiently liquid and does not require treatment with mucolytic agents. Twenty to 30 ml should be concentrated by centrifugation at 3,000 × *g* for 15 min. The resultant pellet is reconstituted in 0.5 to 1.0 ml of buffer or saline, and a sterile wooden applicator is used to smear the sediment on glass slides, which are then fixed in absolute methanol, acetone, or a commercial fixative in rapid staining kits. Samples in limited quantities can be concentrated by use of a Cytospin Centrifuge (ThermoScientific, Waltham, MA) or equivalent. Addition of 1 drop of 22% bovine serum albumin to 500 μl of sample will aid in adherence to the slide. Slides are air dried and then processed for staining. Morphological criteria for recognition of *P. jirovecii* stained by various procedures are discussed below and summarized in Table 1.

Induced Sputum Collection

Sputum collection is best done in a centralized facility by pulmonary function laboratory technicians, respiratory therapists, or specially trained assistants. Deep inhalation of nebulized 3% sodium chloride solution by the patient will result in osmotic accumulation of fluid in and irritation of the respiratory passages with subsequent coughing and expectoration of bronchoalveolar contents. The patient should vigorously brush the teeth, tongue, and gums with a toothbrush and normal saline for 5 to 10 minutes prior to sputum induction, followed by thorough rinsing, to remove as much cellular debris of oral origin as possible. Toothpaste should not be used, as it can interfere with subsequent processing and staining of the specimen. The induced sputum specimen is usually mucoid and translucent in appearance; only rarely is it purulent. When *P. jirovecii* clusters are present in the unstained sputum, they are typically 0.1 to 0.2 mm in diameter and cream to light tan in color. Sputum smeared directly on slides and subsequently stained was shown to be a less sensitive method than treatment with a mucolytic agent followed by concentration of the specimen (61).

Induced sputum is mucolyzed by the addition of an equal volume (at least 2 ml) of freshly prepared 0.0065 M dithiothreitol (Stat-Pak Sputolysin; Caldon-Biotech, Carlsbad, CA) or 0.5% *N*-acetyl-L-cysteine and incubation on a rotary shaker at 35°C with intermittent vigorous vortexing until the specimen is almost completely liquefied (complete liquefaction will lead to dispersal of the *P. jirovecii* clusters, making microscopic detection more difficult). Some protocols require the addition of a clearing reagent for optimal results (e.g., the Light Diagnostics *Pneumocystis carinii* DFA Kit [Millipore, Billerica, MA]). The specimen is then concentrated by centrifugation at 3,000 × *g* for 5 min and the sediment is smeared on glass slides, which are air dried and heat fixed by exposure to a heating block (50 to 60°C). Prolonged heat fixation is important in fixing the material to the slide (~30 min), since most of the natural cellular adhesions are removed during mucolysis. Slides containing the fixed material are then stained and examined microscopically (see below). Samples can be concentrated using a Cytospin centrifuge as described for BALF (above).

Open Thorax Lung Biopsy

Open thorax lung biopsy is the most invasive of the sampling procedures and is not routinely performed. This technique also suffers from a lack of sensitivity (57). However, should the laboratory receive such a specimen, the tissue should be blotted onto sterile gauze to reduce excess fluid (which would interfere with a diagnostic imprint) and used to make touch imprints by pressing several cut surfaces onto sterile glass slides. The remainder of the tissue can be used for histological sections, for microbiological cultures, or for nucleic acid extractions and subsequent PCR techniques. Glass slides with touch imprints should be air dried and then treated with absolute methanol or fixatives included in commercial kits of rapid Wright-Giemsa-like stains, such as Protocol Hema 3 (Fisher Scientific Inc., Cincinnati, OH). Infected tissue stained in this manner will reveal the presence of clusters of trophic forms and cysts with reddish purple nuclei and blue cytoplasm; cysts are surrounded by a halo of dye exclusion (see "Microscopic Identification" for a more detailed description). Slides should be fixed in acetone or a vendor-recommended fixative for immunofluorescent staining. Alternatively, a more concentrated sample can be achieved by a tissue homogenization using tabletop instruments like the gentleMACS Dissociator (Miltenyi Biotec) or the Stomacher Lab Blender (Tekmar Inc., Cincinnati, OH) following vendor recommendations for tissue type and weights. Slides are prepared with 10-μl drops of the homogenate, which are then air dried, fixed, and stained as desired.

TABLE 1 Comparison of stains used to detect *P. jirovecii*

Stain	Time to perform stain	Cyst wall	Trophic and other forms	Advantage(s)	Disadvantage(s)
Giemsa	30 to 60 min	Unstained; cyst wall appears as a clear ring around spores/intracystic bodies	Nuclei stain red-purple; cytoplasm stains light to dark blue, depending on thickness and depth of cluster	Inexpensive; stain simple to perform; stains all life cycle stages of *Pneumocystis*; stains most other pathogens (e.g., bacteria, parasites, fungi) and host cells	Experienced reader required to distinguish *Pneumocystis* clumps from stained host cells
Rapid Giemsa-like stains (e.g., Diff-Quik, Hema 3)	<5 minutes				
Fluorescein-conjugated monoclonal antibody kits (direct and indirect immunofluorescence)	15 to 30 min	Stains and fluoresces apple green; cyst contents usually unstained (appear black) or dull; fold in cyst wall sometimes apparent, giving a crinkled, raisin-like appearance	Stained; appear as small polygons or spheres outlined in apple green; nuclei may stain; clusters can stain with a diffuse green glow	Recommended for less experienced personnel; immunofluorescent staining is sensitive and specific for *Pneumocystis*	Requires fluorescence microscope; reagents are expensive
Methenamine silver (Gomori/Grocott)	30 min (microwave); 1 to 2 h (rapid); 6 to 24 h (conventional)	Stains brown to black; cyst wall thickenings (double comma) and fold in the cyst wall stain dark brown to black; does not differentiate empty cysts from cysts with spores	Unstained	Easy to detect cysts; host cells not stained	Prolonged staining time for conventional method; moderate costs; strong acids used; only the cyst form is stained; stains other fungi
Toluidine blue O/cresyl echt violet	1 to 6 h	Stains violet to purple; cyst wall thickenings and folds stain darker violet to purple; does not differentiate empty cysts from cysts with spores	Unstained	Easy to detect cysts; host cells not stained	Prolonged staining time; moderate costs; strong acids used; only the cyst is stained; stains other fungi
Calcofluor white	<5 min	Stains blue-white or green, depending on filter; cyst wall and thickenings intensely fluorescent	Unstained	Cyst fluoresces brilliantly; simple to perform; inexpensive	Requires fluorescence microscope; strong alkali used; only cyst is stained; stains other fungi; some expertise is required to distinguish *Pneumocystis* cysts from other fungi
Gram-Weigert	<5 min	Unstained wall; intracystic bodies stain purple	Trophic forms faintly visible	Commonly available in cytopathology laboratories	Faint staining; can be overcome by experienced observer, but better stains are available
Papanicolaou	1 to 6 h	Unstained wall; intracystic bodies stain purple	Trophic forms faintly visible	Commonly available in cytopathology laboratories	Faint staining; can be overcome by experienced observer, but better stains are available

Transbronchial Biopsy

Fiber optic bronchoscopy with transbronchial biopsy is the most common invasive technique for collection of tissue; however, like open thorax lung biopsy, this procedure is rarely performed today. Tissue should be handled as described for open thorax lung biopsy above.

Nasopharyngeal Aspirates and Oropharyngeal Washes

A number of studies in humans reported the ability to detect *P. jirovecii*-specific DNA in nasopharyngeal aspirates (NPA) (30, 62) and oropharyngeal washes (OW) (63) after amplification by PCR with *P. jirovecii*-specific primers. These minimally invasive techniques have been shown to have high sensitivity, specificity, positive predictive value, and negative predictive values for diagnosis of *P. jirovecii* pneumonia versus microscopic methods when used in conjunction with quantitative PCR techniques. Handling of OW and NPA is presented here since the use of PCR for the diagnosis of infectious diseases is becoming widespread. These methods will be extremely useful for sampling of pediatric populations in which more invasive techniques of sampling are problematic or where the yield is low.

OW samples are obtained by gargling with 10 ml of sterile physiologic saline (0.9% NaCl) for a period of 1 min (21). Samples are then centrifuged at 2,900 × *g* for 5 min and kept frozen at −20°C until DNA is extracted. After digestion with proteinase K at 56°C for 2 h, DNA or RNA can be extracted using any number of commercial kits available.

NPA are collected with a suction catheter and sterile saline (30). If the amount of collected specimen is small or it is highly viscous, sterile saline should be washed through the catheter to dilute the specimen or added to the final collection tube. The sample can then be treated with a mucolytic agent and subsequently stained (as described above) or prepared for the PCR by DNA or RNA extraction (described below).

DIRECT EXAMINATION AND IDENTIFICATION

Microscopy

A variety of stains have been used for the identification of *P. jirovecii* organisms. One of the most common stains used by pathologists for tissue sections, hematoxylin and eosin, does not stain the organism but rather the foamy exudates within the lung alveoli, often described as honeycombed in appearance. Stains that illustrate the morphology of the organism by microscopic examination are those used for diagnosis in the clinical laboratory.

A common staining procedure used for the diagnosis of PCP is methenamine silver, which also stains other fungi (Table 1; Fig. 3C). Cell wall and membrane polysaccharides of fungi are oxidized to aldehydes by treatment with periodic acid, which in turn reduce the silver ion to metallic silver at alkaline pH. Addition of gold salts stabilizes the complex and excess silver is removed by a sodium thiosulfate rinse. Variations of the methenamine silver stain (e.g., Grocott, Gomori) are used for both tissue sections and bodily fluids such as BALF or induced sputum (64–66). Disadvantages to the use of the silver staining process have been the instability of solutions, the capricious nature of the metal impregnation, and the length of time required for staining (1 to 2 h or 6 to 24 h; see Table 1). These disadvantages have been largely overcome with kits using standardized laboratory microwave ovens for controlled processing and supplied reagents (e.g. ACCUSTAIN Modified GMS Microwave staining kit; Sigma-Aldrich, St. Louis, MO). Unlike other pathogenic fungi, *P. jirovecii* does not bud, and this feature can be used to discriminate between these organisms and other fungi found in the lung that do bud, e.g. *Histoplasma capsulatum*. Silver-stained cysts have a distinctive, black, cup-shaped morphology against green-colored host cell architecture. In some staining reactions, cyst wall thickenings appear as a double-comma morphology. More often, the folds in the wall stain a dark brown to black to produce a crinkled, raisin-like appearance (Fig. 3C). Intracystic daughter forms cannot be seen with this stain, and cysts that are empty (nonviable) appear the same as those with the full contingent of eight spores.

Other stains that complex with components of the cyst wall include periodic acid-Schiff, toluidine blue, cresyl echt violet, and calcofluor. The reactions to some of the more commonly used stains are described in Table 1. Cresyl echt violet stain produces results similar to toluidine blue O (Fig. 3E) and has the same drawbacks, since a mixture of sulfuric acid and glacial acetic acid is necessary for the step prior to staining with the dye. Cysts stained by toluidine blue are similar in appearance to those stained with methenamine silver, except for the light purple color. Staining with calcofluor, whether in commercial kits (e.g., Fungifluor; Polysciences, Inc., Warrington, PA) or prepared in-house, can produce variable effects. Designed to detect only the cysts, excitation at 420 to 490 nm with a suppression filter of 515 nm produces a yellow-green or apple-green fluorescence, often with a characteristic double-parentheses staining body within the cyst (Fig. 3A). Excitation in the UV range (340 to 380 nm) with a suppression filter of 430 nm produces a fluorescent blue color that is not as intense and is sometimes difficult to visualize. Refer to the vendor instructions for optimal filter requirements.

In contrast to the cyst wall stains, Giemsa and rapid Giemsa-like stains do not stain the cyst wall, but instead stain the nuclei of all the various life cycle stages a reddish purple and the cytoplasm a light blue (Fig. 3B). The cyst wall excludes the dyes and appears with a circumscribed clear zone surrounding the reddish purple nuclei of the daughter forms within. Note the thick mat type of appearance, characteristic of the human infection. Lung cells are often present; their nuclei are much larger than those of *Pneumocystis* and stain a deep reddish purple (Fig. 3B). The rapid variants of the Giemsa stain are recommended for the diagnosis of PCP using BALF, induced sputum, or impression imprints because of the low cost, ease, and rapidity of the staining procedure. Commercial kits such as Protocol Hema 3 (Fisher Scientific Co., Cincinnati, OH) produce similar results. The staining procedure requires less than a minute to perform and all forms of the organism are detected. Since there are approximately 10-fold more trophic forms than cysts, the sensitivity of detection is likely to be increased. This stain also permits assessment of specimen quality of BALFs by demonstration of host alveolar macrophages, which should be present in a productive sample. In addition, the distinctive Giemsa-stained morphological appearance of other organisms likely to be encountered in the lung environment, such as *H. capsulatum*, permits rapid diagnosis of pulmonary infections caused by these pathogens which may not be detected with other stains. Because background host cells will also stain, training and expertise in interpret-

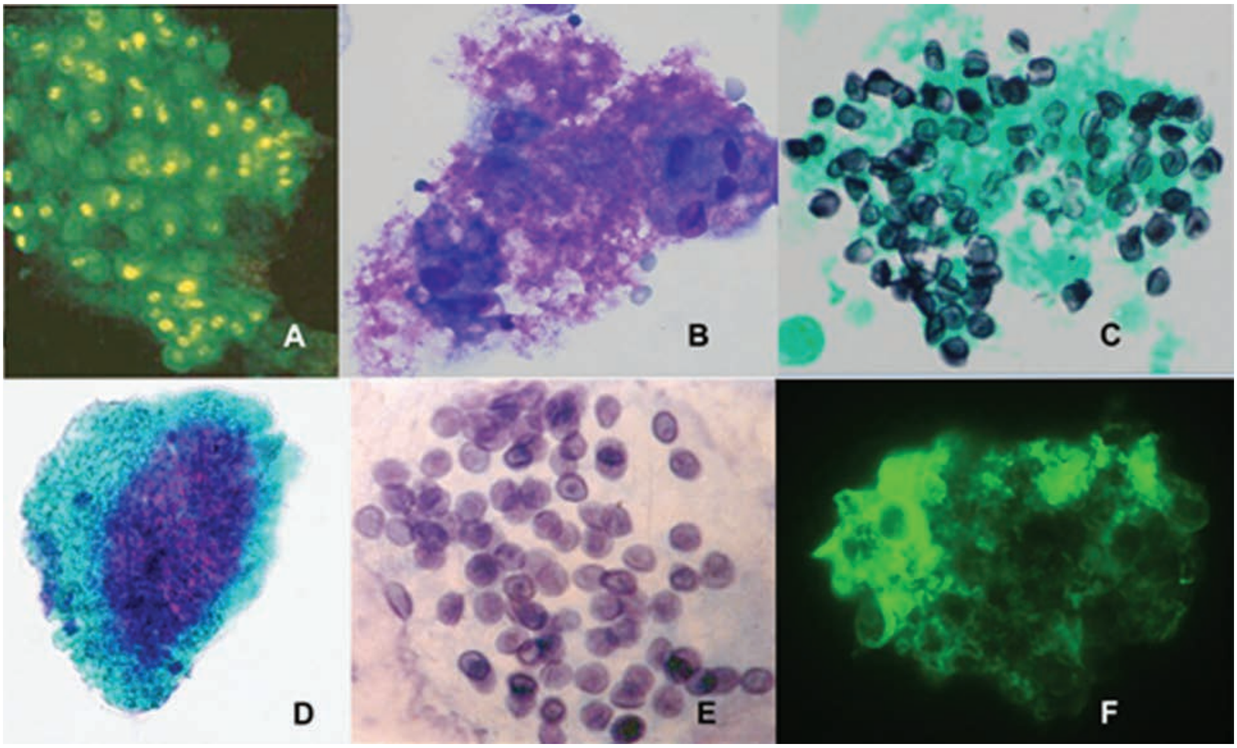

FIGURE 3 Morphology and tinctorial characteristics of *P. jirovecii* in clinical samples stained with various stains (magnification, × 960, unless stated otherwise). (A) Calcofluor stain of BAL fluid. Cyst walls with internal thickenings (double comma) are highly fluorescent (color varies with barrier filter used). (B) Rapid Giemsa-like (Diff-Quick) stain of BALF. Thick cluster of mostly trophic forms (2 to 3 μm) with small reddish-purple nuclei and light blue to red-violet cytoplasm. Boundaries of trophic forms are rarely discernible with this stain. Trophic forms overlay each other to produce darker staining blue cytoplasm. Large dark purple host nuclei are admixed in the cluster. (C) Gomori methenamine silver stain (Grocott) of organisms from BALF. Cyst walls and thickenings (double comma) can be observed as well as collapsed, cup shapes and crinkled raisin-like appearance. Note the lack of budding. Trophic forms are not stained with silver-based stains. (D) Papanicolaou's stain of BALF. Note the distinctive alveolar cast morphology; magnification, ×400. (E) Toluidine blue O stain of *P. jirovecii* in BALF. Cyst walls are stained light purple. The crinkled appearance of the cysts is illustrated with this stain, as well as darker central staining body. Note the lack of budding with this and other cyst wall stains. Trophic forms are not stained. (F) Direct fluorescent antibody stain of organism cluster from BAL. Note apple-green fluorescence distributed unevenly over the cluster, with accumulation on a cyst wall (lower left of cluster). Structures within the cysts are unstained and appear black. doi:10.1128/9781555817381.ch118.f3

ing cellular elements in Giemsa-stained preparations is necessary. Laboratories with a lower volume of *P. jirovecii* specimens may prefer to use immunofluorescent staining or one of the other stains described in Table 1.

Direct and indirect fluorescein-conjugated monoclonal anti-*P. jirovecii* antibodies used for immunofluorescent assay (IFA) are targeted to a family of surface glycoproteins that contain both common and distinct epitopes, within and among *Pneumocystis* species (67). Depending on the monoclonal antibody supplied with the kit, staining may target only the cyst form or all forms of the organism. Since trophic forms are more numerous than cysts, kits using those antibodies directed to all forms of the organism, such as Bio-Rad's MONOFLUO *Pneumocystis jirovecii* IFA Test Kit, a direct immunofluorescence assay for the detection of all *P. jirovecii* life cycle forms, are more sensitive. The typical fluorophore conjugated to the antibody or used in an indirect assay is fluorescein isothiocyanate, which produces a brilliant apple green color. The staining reaction shows a diffuse surface pattern distributed over the entire cluster of organisms (Fig. 3F) and often stains the matrix in which the organisms are embedded. Single cysts will usually appear with a distinctive rim of fluorescence and duller interior fluorescence. It should be noted that kits using a direct staining procedure may not react with *P. jirovecii* on slides fixed in ethanol, and fixation in acetone or vendor recommendations for fixation should be followed (68).

Papanicolaou's stain, frequently used for cytopathological specimens, stains the clusters of extracellular organisms a greenish color, although thick clusters of organisms can collect the stain and appear bicolored with pink to purple and green/turquoise staining as in Fig. 3D. A diagnostic criterion is the presence of distinctive alveolar casts, as shown in panel D. Organism architecture is better observed with the Giemsa-like stains. Gram's stain produces a negative (pink) reaction with poorly defined organism morphology.

Serum (1→3)-β-D-glucan

An assay that measures the serum levels of (1→3)-β-D-glucan in patients suspected to have *P. jirovecii* is proving to be a successful diagnostic modality, especially for patients who cannot produce appropriate sputum samples or where bronchoscopy may not be safe (69). (1→3)-β-D-Glucan is a component of the ascus (cyst) cell wall and is secreted in significant amounts during infection with *Pneumocystis* spp. Except for *Cryptococcus* and Mucormycetes, most other fungi also secrete (1→3)-β-D-glucan during infection, but the high amounts observed during infection with *P. jirovecii* have proven to be a useful diagnostic modality. A licensed detection kit uses a modification of the *Limulus* amebocyte lysate (LAL) pathway. The Fungitell Assay reagent (Associates of Cape Cod, Falmouth, MA) is processed to eliminate factor C and is therefore specific for (1→3)-β-D-glucan (http://www.acciusa.com/clinical/fungitell/index.html). The reagent does not react with other polysaccharides, including beta-glucans with different glycosidic linkages. Normal human serum contains low levels of (1→3)-β-D-glucan, typically 10 to 40 pg/ml, presumably from commensal yeasts present in the alimentary canal and gastrointestinal tract. At present, the Fungitell assay is indicated for presumptive diagnosis of fungal infection and should be used in conjunction with other diagnostic procedures. Other kits that measure (1→3)-β-D-glucan are available from Seikagaku Corp., Tokyo, Japan, and from Wako Pure Chemical Industries Ltd., Osaka, Japan, but are not yet approved by the U.S. Food and Drug Administration.

It is very important to follow the vendor's instructions for handling of patient serum as (1→3)-β-D-glucan can be an environmental contaminant. Serum is the only sample type that is currently approved for use with the Fungitell assay. Serum that is hemolyzed, lipemic, or visually icteric or turbid is not suitable for use with the Fungitell assay. Because a fungal infection is a dynamic process, repeat testing, typically 2 to 3 times per week, improves sensitivity.

Values below 60 pg/ml are considered negative for fungal infection; those between 60 and 79 pg/ml are "indeterminate," while values of >80 pg/ml are positive for a fungal infection.

Cutoff levels specifically for the diagnosis of *P. jirovecii* pneumonia vary slightly. Recent studies have reported that elevated plasma (1→3)-β-D-glucan levels of >80 pg/ml have a high predictive value for diagnosis of *P. jirovecii* pneumonia in AIDS patients with respiratory symptoms (70), while another study suggested a threshold of 100 pg/ml (71). In a recent meta-analysis that evaluated the use of (1→3)-β-D-glucan for diagnosis of *P. jirovecii* pneumonia in a variety of patient settings, the sensitivity was found to be 94.7% using a range of cutoffs (72). The recommendation from this study was to use a slightly higher cutoff level than the >80 pg/ml that is recommended in general for fungal infections, e.g., 100 pg/ml.

It should be cautioned that measurement of (1→3)-β-D-glucan levels has not been shown to track with therapeutic efficacy and should not be employed for this purpose. Also, although a high (1→3)-β-D-glucan level is likely to be diagnostic for *P. jirovecii* pneumonia, another confirmatory test is recommended at this time.

Nucleic Acid Detection

Amplification of *P. jirovecii* DNA for the diagnosis of PCP in the clinical laboratory setting is increasingly becoming a routine diagnostic assay. With the advent of real-time PCR, detection of *P. jirovecii* is a more sensitive and specific method than microscopic detection methods and the standard PCR or nested PCR techniques (73, 74). However, with this high degree of sensitivity, diagnosis should be in conjunction with clinical manifestations of PCP, as colonization with these organisms can be detected by these highly sensitive assays (3). PCR methods can also be used for identification of potentially resistant strains or species of the organism by targeting of specific genes such as dihydropteroate synthase, for mutations associated with resistance (23). Although the significance of infections with multiple genotypes of *P. jirovecii* is currently unclear, PCR-based detection systems would also be able to detect PCP caused by single or multiple genotypes. Quantitative PCR tests for PCP are being conducted by commercial firms such as ViraCor IBT laboratories (www.viracor.com), but such tests have not been cleared or approved for diagnostic use in the United States by the Food and Drug Administration. Samples must be sent to the commercial vendor at this time. Detection limits are from 84 to 1 × 10^8 copies/ml for BAL, bronchial wash, nasopharyngeal wash, or nasopharyngeal aspirate and 213 to 1 × 10^8 copies/ml for whole blood.

In-house detection of *P. jirovecii* by PCR typically targets the large ribosomal subunit of the mitochondrial rRNA (mtLSUrRNA), intertranscribed spacer regions (ITS) or the major surface glycoprotein (MSG) family of genes (71, 75). The mtLSUrRNA and MSG genes are present in multiple copies per organism and provide the most sensitive target for detection. DNA can be isolated from clinical samples using commercial kits such as the PhaseLock System (Eppendorf Scientific, Westbury, NY) or a series of products designed for the clinical laboratory by vendors such as Qiagen (Valencia, CA). The primers targeting the mtLSUrRNA and PCR conditions are listed in Table 2 for standard PCR.

A commercial real-time PCR assay, the MycAssay Pneumocystis kit (Myconostica Ltd., Cambridge, United Kingdom), was evaluated against direct immunofluoresence microscopy, real-time PCR (*cdc2* gene of *P. jirovecii*), and conventional PCR directed to the mtLSUrRNA gene (74). BAL samples were liquefied with BD BBL MycoPrep (BD Diagnostics, MD) prior to DNA extraction using a MycXtra fungal DNA extraction kit (Myconostica). The kit contains proprietary reagents including primers and a molecular beacon targeting the mtLSUrRNA. The test is considered positive if the cycle threshold (CT) value is <39.0 and negative if the CT is 39.0 or undetermined (failure to cross the threshold) without exponential amplification, while exponential amplification with CT values of <39.0 is considered positive. The MycAssay was superior to all other methods, including a sensitivity and specificity of 100% and 86% for the *cdc2*-real time PCR assay, 100% and 98.5% for the mtLSUrRNA conventional PCR and sequencing assay, and 100% positive and negative predictive values for the MycAssay. At present, the MycAssay is not for sale for diagnostic use in the United States.

Another highly sensitive and almost fully automated real-time PCR assay targets the multicopy MSG genes on a BD MAX platform (BD Diagnostics Sparks, MD) (75). After treatment with dithiothreitol for liquefaction of respiratory specimens, the samples are loaded into extraction tubes and DNA or RNA is automatically extracted and added to the PCR master mix, where the MSG target gene, internal control (Texas Red), and a sample process control (Cy5) are amplified. The limit of detection is 10 copies/ml of a plasmid per PCR that equated to 500 copies in BALF. Accurate quantitation was observed over a 7- to 8-log range. This fully automated method showed a good quantitative correlation with the reference PCR (R^2 = 0.82). This method permits pre-aliquotting of PCR reagents; allows the use of the entire system outside a specified PCR area, reducing cross-contamination; and requires little technician input, reducing potential human error. Like other nucleic acid-based detection methods, the BD Max platform has not yet received FDA approval. In addition, use of such kits demands evaluation in the clinical setting and should be approached with caution. Thresholds of each assay to distinguish colonization versus infection remain a concern. In a recent study using real-time PCR with mtLSUrRNA as the target gene, cutoff values of 1.6 × 10^3 copies/ml and an upper cutoff level of 2 ×10^4 copies/ml achieved 100% sensitivity and 100% specificity for determining colonization versus infection. Combined with a 100-pg/ml threshold for (1→3)-β-D-glucan, these suggested copy numbers could discriminate PCP from colonization (71).

ISOLATION PROCEDURES

Detection of *Pneumocystis* by growth in artificial media or tissue culture is not a diagnostic option, since no species of this genus can be continuously cultivated outside the mammalian lung.

TYPING SYSTEMS

There is no consensus for a typing system for *P. jirovecii*.

SEROLOGIC TESTS

Serological assays to detect anti-*P. jirovecii* antibodies are useful for epidemiological studies, but not for diagnosis of PCP. Most human beings become seropositive for *P. jirovecii* antibodies early in their childhood, between 2 and 4 years of age, and likely come in contact with the organism many times over their lifetime. In some cases, a rise in antibody titer can be detected in some PCP patients over time; in others, antibody titers can just as frequently drop or remain the same.

A number of other laboratory tests have been used for the diagnosis of PCP, but do not provide a definitive diagnosis. These include an increased arterial-alveolar gradient, an elevation of serum lactic dehydrogenase levels, and gallium and diethylenetriamine pentaacetic scans. The latter two tests are not routinely used due to higher costs. Reduced S-adenosylmethionine (AdoMet) levels have been reported to reflect infection with *P. jirovecii* (76, 77); however, another study conducted to evaluate the diagnostic utility of AdoMet versus (1→3)-β-D-glucan levels found that AdoMet levels did not discriminate between infected and noninfected patients, while (1→3)-β-D-glucan levels correlated with a high level of sensitivity and specificity using a cutoff of 60 pg/ml (78).

ANTIMICROBIAL SUSCEPTIBILITIES

Two drugs comprise the mainstay of therapy for acute PCP, trimethoprim-sulfamethoxazole (TMP-SMX) and pentamidine isethionate (79). Secondary treatments, such as atovaquone and clindamycin-primaquine, have been used for milder forms of the disease, and treatment with corticosteroids has been used to improve the clinical outcome in some patients. However, there have been significant rates of relapse and recurrence with such second-line therapies

TABLE 2 PCR detection of *Pneumocystis*: suggested primers and conditions

Gene target	Primers	Sequence (5′ to 3′)	Expected product size (bp)	Conditions
mtLSUrRNA				
Primary[a]	pAZ102-E	GATGGCTGTTTCCAAGCCCA	346	94°C × 1 min; 55°C × 1 min; 72°C × 2 min; 40 cycles; termination: 72°C × 5 min
	pAZ102-H	GTGTACGTTGCAAAGTACTC		
Nested[b]	pAZ102-X	GTGAAATACAAATCGGACTAGG	267	94°C × 1 min; 55°C × 1 min; 72°C × 2 min; 35 cycles; termination: 72°C × 5 min
	pAZ102-Y	TCACTTAATATTAATTGGGGAGC		
DHPS[c]				
Round 1	F1	CCTGGTATTAAACCAGTTTTGCC		94°C × 5 min; 92°C × 30 s; 52°C × 30 s; 72°C × 1 min; 35 cycles; termination: 72°C × 5 min
	B_{45}	CAATTTAATAAATTTCTTTCCAAATAGCATC		
Round 2	A_{HUM}	GCGCCTACACATATTATGGCCATTTTAAATC	300	94°C × 5 min; 92°C × 30 s; 55°C × 30s; 72°C × 1 min; 35 cycles; termination: 72°C × 5 min
	BN	GGAACTTTCAACTTGGCAACCAC		

[a] Mitochondrial large subunit rRNA; from reference 89.
[b] From reference 90.
[c] Dihydropteroate synthase gene; from references 23 and 91.

(80). TMP-SMX is a drug combination that targets the enzymes dihydrofolate reductase (trimethoprim) and dihydropteroate synthase (sulfamethoxazole), both integral steps in the folic acid pathway. The standard therapeutic dose of TMP-SMX is 15 to 20 mg/kg intravenously or orally daily in three or four divided doses. Steroids are an important adjunctive therapy for patients with moderate to severe infections associated with HIV infection. It is thought that deleterious inflammatory responses are reduced with their administration. Steroids are usually given in high doses for 5 to 7 days, with a reduction in dose and continued treatment for an additional 2 weeks. Steroid treatment may be started at the same time as therapy and withdrawn before antimicrobial treatment is complete. The drug of first choice for PCP prophylaxis is also TMP-SMX, with a daily or thrice-weekly administration of 960 mg (81). If patients are intolerant to TMP-SMX, oral dapsone (100 mg/day), aerosolized pentamidine (300 mg every 4 weeks), or oral atovaquone (at least 1,500 mg/day) is recommended.

Pentamidine is a cationic diamidine that was first used to treat African trypanosomiasis, or sleeping sickness, and later found to be efficacious as PCP therapy. The mode of action of this drug is not known but may involve suppression of mitochondrial activity, inhibition of topoisomerases, or binding of the minor groove of DNA (82). Pentamidine and TMP-SMX have significant side effects including nephrotoxicity and, in the case of TMP-SMX, severe rash, fever, and neutropenia that often necessitate a change to alternative treatment. In a recent study of HIV-1-infected patients with first episode PCP, only 64% completed TMP-SMX treatment (83). Neither drug is considered pneumocysticidal. Administration of pentamidine via an aerosolized route delivers the drug efficiently to the areas of infection, but it has been shown to be less effective than other drugs in the treatment of PCP as well as being associated with dispersal of *P. jirovecii* and other respiratory pathogens (12).

PCP remains refractory to most common antifungal drugs such as the azoles or amphotericin B. Echinocandins, (1→3)-β-D-glucan inhibitors, are a relatively new family of antifungal drugs that are fungicidal against candidal infections and fungistatic against *Aspergillus* infections (84). Reports of the efficacy of echinocandins for PCP have been contradictory, due in large part to the anecdotal nature of the reports (85, 86). Systematic studies of three clinically available echinocandins—caspofungin, anidulafungin, and micafungin—in rodent models of PCP revealed dramatic reductions in cysts, but much less of an effect on trophic forms, suggesting that use as monotherapies would not be efficacious, but a combination with TMP-SMX may provide beneficial to some patients (12).

Mutations in the DHPS gene of *P. jirovecii* associated with sulfa resistance in other pathogens have been identified in about 50% of the PCP isolates in certain geographic areas (23). The presence of the mutations in the DHPS gene of *P. jirovecii* was associated with previous TMP-SMX therapy, but the impact of the mutations in terms of outcome and response to therapy is not yet clear.

It may become desirable in the future to track the emergence of *P. jirovecii* strains that are resistant to TMP-SMX, or to evaluate the potential for therapeutic response. In anticipation of this goal, the primers targeting the regions of the dihydropteroate gene (DHPS) associated with sulfa resistance in other pathogens are shown in Table 2. The nucleotide (nt) positions in which the mutations occur are at nt 165 and nt 171. Changes at these nucleotide positions result in changes in amino acids. The following are the four genotypes for this target gene: genotype 1, nt 165 (A)/nt 171 (C) = Thr/Pro; genotype 2, nt 165 (G)/nt 171 (C) = Ala/Pro; genotype 3, nt 165 (A)/nt 171 (T) = Thr/Ser; and genotype 4, nt 165 (G)/nt 171 (T) = Ala/Ser. The last genotype, GT, represents a double mutation in the DHPS gene that has been associated with drug resistance and is emerging as the dominant genotype for *P. jirovecii* isolates in some areas (17). Although atovaquone resistance in PCP has been associated with mutations in the mitochondrial cytochrome b_1 gene (87), resistance to TMP-SMX is much more problematic, and identification of mutations in the DHPS gene will likely be the more critical genetic region to evaluate in the clinical setting.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

The microscopic demonstration of *P. jirovecii* in tissue and fluids by staining with Gomori methenamine silver or a rapid variant of the Wright-Giemsa stain, by IFA, or by other stains such as Papanicolaou stain should be considered sufficient for diagnosis. In many cases, the fungi are present as large clusters of organisms, in which it may be difficult to differentiate the life cycle stages within the dense assemblage by using the Wright-Giemsa or Papanicolaou stains. Because of this, it is recommended that a stain which only visualizes the cyst form of *Pneumocystis* (e.g., Gomori methenamine silver) be used for diagnosis because it is easier to interpret. The rapid stains can be used as a preliminary diagnostic technique, followed by the definitive cyst stain. IFA can be helpful in laboratories that are less familiar with *P. jirovecii* morphology, but this requires a fluorescent microscope, which may not always be available. The outcome of IFA staining depends on the monoclonal antibody target. Some kits use monoclonal antibodies targeting the surface glycoprotein present on all of the life cycle stages, but these also stain the dense matrix in which *P. jirovecii* is embedded, resulting in a highly fluorescent mass with little detail. Since there are no other species of the genus that are known to cause pneumonia in humans, the presence of these fungi can be reported as *P. jirovecii* or *Pneumocystis* spp. The name "*Pneumocystis carinii*" should not be used, as this species infects rats. Treatment should be initiated upon demonstration of *P. jirovecii* by microscopic methods and based on clinical evaluation of the patient. Moderate to severe PCP is treated with a combination of corticosteroids and intravenous TMP-SMX, clindamycin/primaquine, or pentamidine. Mild to moderate PCP is treated with TMP/SMX, TMP-dapsone, pentamidine, atovaquone, or clindamycin/primaquine(50).

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Aspergillus and *Penicillium*[*]

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ASPERGILLUS SPECIES

Taxonomy

The genus *Aspergillus* is classified in the family Trichomaceae of the Ascomycota. Taxonomic assignment of members of this genus has evolved substantially with over 250 species now described (1, 2; http://www.aspergilluspenicillium.org). The majority (64%) of species show no sexual reproduction (3), with the anamorphic (asexual) genus *Aspergillus sensu stricto* phylogenetically related to the anamorphic genera *Penicillium sensu stricto* and *Paecilomyces* (4).

Raper and Fennell in 1965 first classified 132 *Aspergillus* species into 18 subgroups (*Aspergillus candidus*, *A. clavatus*, *A. cervinus*, *A. cremeus*, *A. flavipes*, *A. flavus*, *A. fumigatus*, *A. glaucus*, *A. nidulans*, *A. niger*, *A. ochraceus*, *A. ornatus*, *A. restrictus*, *A. sparsus*, *A. terreus*, *A. ustus*, *A. versicolor*, and *A. wentii*) and 18 varieties based on their morphological characters (5). With advancements in molecular phylogeny, however, taxonomic changes in species classification have been made, resulting in new subdivisions, subgenera, and sections (6–8).

Most recently, using multigene phylogeny based on four genetic loci—(i) β-tubulin, (ii) calmodulin, (iii) internal transcribed spacer (ITS) and large subunit (LSU) of the rDNA gene cluster, and (iv) RNA polymerase II 2 (RPB2)—a need for further taxonomic revision was demonstrated. Using this approach, Peterson established five subgenera, *Aspergillus*, *Circumdati*, *Fumigati*, *Nidulantes*, and *Ornati*, which he subdivided into 16 sections (section *Aspergillus*, *Candidi*, *Cervini*, *Clavati*, *Circumdati*, *Cremei*, *Falvi*, *Flavipedes*, *Fumigati*, *Nidulantes*, *Nigri*, *Restricti*, *Sparsi*, *Terrei*, *Usti*, and *Versicolores*), either retaining formerly recognized sections or reclassifying them (9). This restructuring resulted in a number of important changes within the genus, including the fact that specific groups of aspergilli now contain uniseriate and biseriate species as well as species characterized by different colony colors. This was not the case previously, when phenotypic characteristics such as colony color were used as key features to separate different groups of aspergilli. The application of this polyphasic phylogenetic species concept has led to the discovery and description of new cryptic or sibling species within the most frequent pathogens, e.g., *A. lentulus* (10) and *A. alabamensis* (11). In the latest taxonomic revision, Samson and Varga (2) extended the work of Peterson (9) and grouped more than 250 species into eight subgenera (*Aspergillus*, *Fumigati*, *Circumdati*, *Candidi*, *Terrei*, *Nidulantes*, *Warcupi*, and *Ornati*) which are subdivided into sections or species complexes.

It is now obvious that it is necessary to establish sequence-based associations between the anamorph and teleomorph (sexual) stages for aspergilli (where relevant) independent of whether mating occurs. At the time of writing, the anamorphic species of the genus *Aspergillus* are associated with nine teleomorphic genera (*Chaetosartoria*, *Dichotomomyces*, *Emericella*, *Eurotium*, *Fennellia*, *Neocarpenteles*, *Neopetromyces*, *Neosartorya*, and *Petromyces*) within the taxon Trichocomaceae (2, 12). Peterson had suggested to split the genus *Aspergillus* based on the teleomorph states associated with particular monophyletic lineages (9); however, this would have deemphasized the most common morphological features associated with the genus *Aspergillus*. With the establishment of the "one fungus = one name" principle in 2011 at the International Botanic Congress in Melbourne, Australia, as a new "International Code of Nomenclature for Algae, Fungi, and Plants" (13), and the abolishment of the priority of the teleomorph designation for fungi (14), the naming of *Aspergillus* species has been debated. The *Aspergillus* working group in 2012 agreed to retain the name *Aspergillus* and not adopt the teleomorph nomenclature (*sensu* Raper and Fennell [5]). This decision has significant consequences, as it results in the loss of the well-known teleomorph genera *Emericella*, *Eurotium*, and *Neosartorya*. The argument for maintaining *Aspergillus* for the whole genus is ensuring stability for nomenclature of most medically important *Aspergillus* species, and their clinical interpretation. For the present, discussions are ongoing whether to use the anamorph name for the whole genus or to split the genus according to teleomorph form; this chapter follows the current official view on nomenclature by using the anamorphic name followed by its teleomorph stage in parentheses (for more information, see http://www.aspergilluspenicillium.org).

Of known *Aspergillus* species, the majority of human infections are caused by *A. fumigatus* (teleomorph synonym: *Neosartorya fumigata*), followed by *A. flavus* (teleomorph synonym: *Petromyces flavus*), *A. terreus*, and *A. niger*. In addition, at least another 48 *Aspergillus* species have been implicated in disease (15). These uncommon but important pathogens are shown in Table 1. The phylogenetic relationships, based on ITS1 and ITS2 rDNA sequence analysis, of most of these species are shown in Fig. 1.

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[*] This chapter incorporates material from chapter117 by S. Arunmozhi Balajee and Mary E. Brandt in the 10th edition of this *Manual*.

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TABLE 1 Known uncommon pathogenic *Aspergillus* species

Anamorph	Teleomorph (synonym)
Aspergillus acidus	
A. aculeatu	
A. alabamensis	
A. alliaceus	*Petromyces alliaceus*
A. avenaceus	
A. brasiliensis	
A. caesiellus	
A. calidoustus	
A. candidus	
A. carneus	
A. chevalieri	*Erotium chevalieri*
A. clavato-nanicus	
A. clavatus	
A. conicus	
A. deflectus	
A. fischerianus	*Neosartorya fischeri*
A. flavipes	*Fennellia flavipes*
A. fumigatiaffinis	
A. fumisynnematus	
A. glaucus	*Eurotium herbariorum*
A. granulosus	
A. hollandicus	*Eurotium amstelodami*
A. janus	
A. japonicas	
A. lentulus	
A. nidulans	*Emericella nidulans*
A. niveus	*Fennellia nivea*
A. nomius	*Petromyces nomius*
A. ochraceopetaliiformis	
A. ochraceus	
A. oryzae	
A. parasiticus	
A. penicilloides	
A. persii	
A. reptans	*Eurotium repens*
A. restrictus	
A. rubrobrunneus	
A. rugulovalvus	*Emericella rugulosa*
A. sclerotiorum	
A. sydowii	
A. tamari	
A. tetrazonus	*Emericella quadrilineata*
A. spinosus	*Neosartorya spinosa*
A. thermomutatus	*Neosartorya pseudofischeri*
A. tritici	
A. tubingensis	
A. udagawae	*Neosartorya udagawae*
A. unguis	*Emericella unguis*
A. ustus	
A. versicolor	
A. viridinutans	
A. wentii	

Description of the Agent

Aspergillus species are ubiquitous. They grow rapidly and form powdery colonies on mycological media. Colony color varies with species and is influenced by growth conditions and factors such as whether vegetative hyphae, conidial heads, and/or sexual structures are present. The color of the aerial part of the colony may also be different from the pigment exhibited by the portion in the growth medium. The growth rate of *Aspergillus* species and their colony diameter in media and at a certain age are important features to aid species identification, as well as the appearance of the colony at its margins. The margins can be sharply delineated or thin and diffuse, smooth over its entirety or irregularly lobed, and submerged or aerial. Colony texture of the surface which ranges from velvety, floccose to granular, and which may show a zonation, is also used to assist with species designation (15) (see "Identification").

Aspergillus spp. form nonseptate stipes or conidiophores (walls), which commonly terminate in a vesicle (distinct inflated part) from which phialides are produced synchronously. They reproduce via conidia, which are formed in dry chains on the end of the phialides. The phialides can be formed directly from the vesicle (uniseriate) or alternatively they can form metulae (intermediate series of cells) (biseriate) (15). Figure 2 provides a schematic of the more important structural features of aspergilli. Morphological characteristics of clinically relevant sections/species are listed in Table 2.

Epidemiology and Transmission

Aspergillus species are environmental saprophytes that thrive in decaying vegetation and are readily cultured from soil, water, certain foods, and air. Although conidia are continually inhaled from the environment, they are eliminated from healthy hosts via mucociliary clearance or following phagocytosis by alveolar macrophages (16). In hosts with altered lung function, they may colonize the respiratory tract or cause allergic or invasive pulmonary disease. Severely immunocompromised individuals are at particular risk of invasive pulmonary aspergillosis, which may also spread to contiguous sites or disseminate to other body sites via the blood stream (17, 18).

In health care facilities, *Aspergillus* has been cultured from unfiltered air, ventilation systems, dust, food, hospital water supplies, and related wet environments, including shower outlets (19–21). Numerous outbreaks of hospital-acquired invasive infection have been described, mostly following inhalation of airborne spores originating from construction or demolition activities (22, 23). Aerosolization of water from contaminated sources or surfaces may also occur (20, 21, 24). Warris and Verweij have proposed control measures for the prevention of waterborne infection in hospitals, though the importance of water as a source of hospital outbreaks remains uncertain (24). Most outbreaks are associated with invasive pulmonary aspergillosis, but clusters of surgical wound and skin infections have also been described (25). In some studies, identification of a common source was supported by molecular genotyping (19, 22), though in addition to clonally related genotypes and microvariants, multiple genotypes have typically been found in clinical and environmental samples (26). Not all researchers have detected genetic relatedness between environmental and clinical isolates (24). Concentrations of airborne fungi in patient-care areas during outbreak investigations have ranged from 0 to >100 spores/m^3. Though higher concentrations of airborne spores have been associated with outbreaks (26), a threshold below which patients are considered safe has not been determined. Determining this threshold may not be possible due to use of variable sampling methods, rapid temporal variation in spore counts, delayed sampling as a result of delays in clinical presentation after exposure, differences in host susceptibility to infection, and other factors (19). The Centers for Disease Control and Prevention and the Healthcare Infection Control Practices

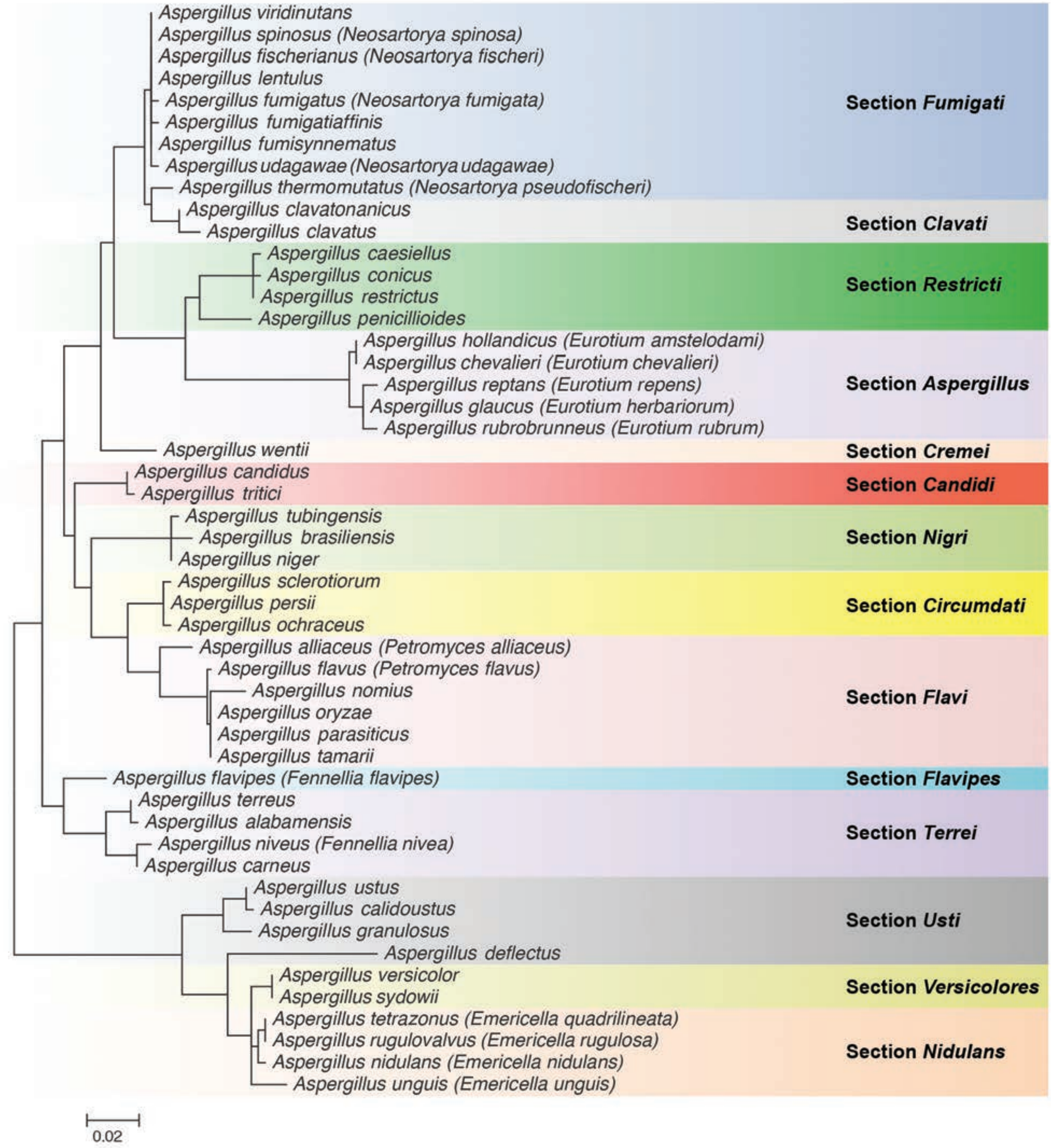
Aspergillus viridinutans
Aspergillus spinosus (Neosartorya spinosa)
Aspergillus fischerianus (Neosartorya fischeri)
Aspergillus lentulus
Aspergillus fumigatus (Neosartorya fumigata)
Aspergillus fumigatiaffinis
Aspergillus fumisynnematus
Aspergillus udagawae (Neosartorya udagawae)
Aspergillus thermomutatus (Neosartorya pseudofischeri)
Aspergillus clavatonanicus
Aspergillus clavatus
Aspergillus caesiellus
Aspergillus conicus
Aspergillus restrictus
Aspergillus penicillioides
Aspergillus hollandicus (Eurotium amstelodami)
Aspergillus chevalieri (Eurotium chevalieri)
Aspergillus reptans (Eurotium repens)
Aspergillus glaucus (Eurotium herbariorum)
Aspergillus rubrobrunneus (Eurotium rubrum)
Aspergillus wentii
Aspergillus candidus
Aspergillus tritici
Aspergillus tubingensis
Aspergillus brasiliensis
Aspergillus niger
Aspergillus sclerotiorum
Aspergillus persii
Aspergillus ochraceus
Aspergillus alliaceus (Petromyces alliaceus)
Aspergillus flavus (Petromyces flavus)
Aspergillus nomius
Aspergillus oryzae
Aspergillus parasiticus
Aspergillus tamarii
Aspergillus flavipes (Fennellia flavipes)
Aspergillus terreus
Aspergillus alabamensis
Aspergillus niveus (Fennellia nivea)
Aspergillus carneus
Aspergillus ustus
Aspergillus calidoustus
Aspergillus granulosus
Aspergillus deflectus
Aspergillus versicolor
Aspergillus sydowii
Aspergillus tetrazonus (Emericella quadrilineata)
Aspergillus rugulovalvus (Emericella rugulosa)
Aspergillus nidulans (Emericella nidulans)
Aspergillus unguis (Emericella unguis)
Section Fumigati
Section Clavati
Section Restricti
Section Aspergillus
Section Cremei
Section Candidi
Section Nigri
Section Circumdati
Section Flavi
Section Flavipes
Section Terrei
Section Usti
Section Versicolores
Section Nidulans
0.02

FIGURE 1 Maximum likelihood tree of pathogenic *Aspergillus* species based on internal transcribed spacer (ITS) 1 and ITS2 sequences of the rDNA gene obtained from GenBank using the program MEGA ver. 5.2 (231). Teleomorph names are given in parentheses.
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Advisory Committee have published guidelines on prevention of aspergillosis in health care facilities (23).

A. fumigatus is the commonest cause of invasive aspergillosis in developed and developing countries, accounting for at least 65% of cases, especially in the highest risk group, patients with hematological malignancies (27–30). Other pathogenic species include *A. flavus*, *A. terreus*, *A. niger*, and, infrequently, *A. nidulans* and *A. ustus* (31). *A. flavus* infections are more common in developing countries and in arid climates where they comprise the majority of cases of allergic aspergillosis, trauma-associated endophthalmitis, and sino-orbital-cerebral disease (28, 32, 33). Nosocomial outbreaks of aspergillosis are most common in patients with hematological cancers and are almost always caused by *A. fumigatus* or *A. flavus* (19). In the U.S.-based Transplant-Associated Infection Surveillance (TRANSNET) study conducted from 2001

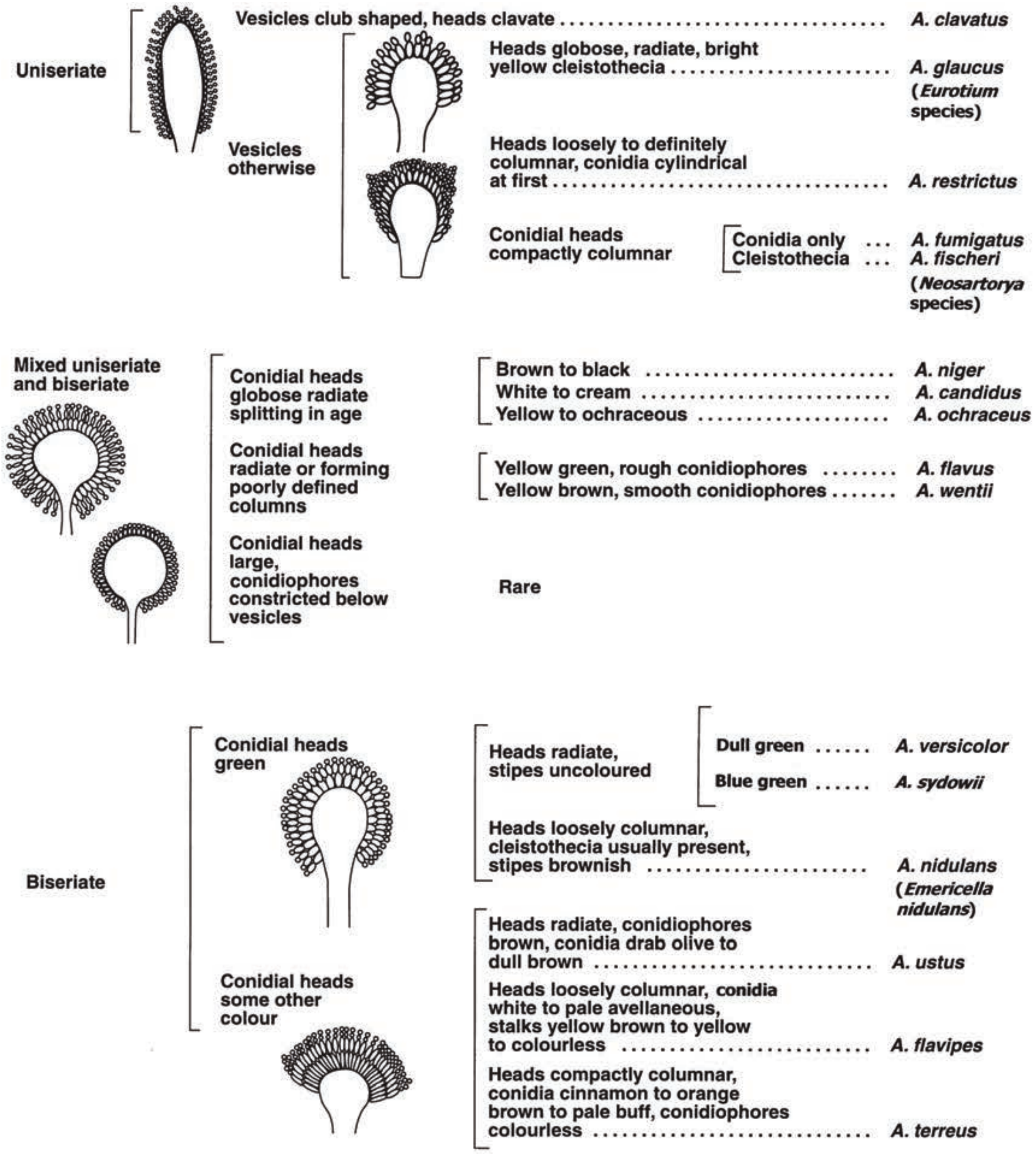
Uniseriate
Vesicles club shaped, heads clavate .... A. clavatus
Vesicles otherwise
Heads globose, radiate, bright yellow cleistothecia .... A. glaucus (Eurotium species)
Heads loosely to definitely columnar, conidia cylindrical at first .... A. restrictus
Conidial heads compactly columnar
Conidia only ... A. fumigatus
Cleistothecia ... A. fischeri (Neosartorya species)
Mixed uniseriate and biseriate
Conidial heads globose radiate splitting in age
Brown to black .... A. niger
White to cream .... A. candidus
Yellow to ochraceous .... A. ochraceus
Conidial heads radiate or forming poorly defined columns
Yellow green, rough conidiophores .... A. flavus
Yellow brown, smooth conidiophores .... A. wentii
Conidial heads large, conidiophores constricted below vesicles
Rare
Biseriate
Conidial heads green
Heads radiate, stipes uncoloured
Dull green ...... A. versicolor
Blue green ...... A. sydowii
Heads loosely columnar, cleistothecia usually present, stipes brownish .... A. nidulans (Emericella nidulans)
Conidial heads some other colour
Heads radiate, conidiophores brown, conidia drab olive to dull brown .... A. ustus
Heads loosely columnar, conidia white to pale avellaneous, stalks yellow brown to yellow to colourless .... A. flavipes
Heads compactly columnar, conidia cinnamon to orange brown to pale buff, conidiophores colourless .... A. terreus

FIGURE 2 Key to representative species of *Aspergillus*. doi:10.1128/9781555817381.ch119.f2

to 2006, 67% of isolates were identified as *A. fumigatus*; cryptic species of *A. fumigatus* comprised 6% of this complex (30).

Clinical Significance

Aspergilli can cause disease as a result of ingestion of mycotoxins or other metabolites; direct inoculation into traumatized skin, eyes, or other sites; and inhalation of spores causing hypersensitivity or invasive disease. Aspergillosis most commonly involves the lungs but almost every organ can be involved as a result of primary infection, contiguous spread, or dissemination (34, 35). Lung infection presents as a spectrum of disease, depending on the extent of immunosuppression, genetic factors, and structural abnormalities within the respiratory system. Certain species are associated with specific forms of disease (Table 3).

Invasive aspergillosis is the second most common hospital-acquired fungal infection, after invasive candidiasis (36), and is associated with high morbidity and mortality especially in patients with hematological malignancies or allogeneic stem cell or lung transplants (37, 38). Other groups at increased risk and with poor outcomes include patients who have had heart, liver, and, less commonly, renal transplants; patients receiving immunomodulators such as infliximab or tumor necrosis factor alpha inhibitors; and patients with advanced AIDS, malnutrition, or other underlying immune deficiencies, including patients with a primary neutrophil defect. Critically ill patients and patients with chronic respiratory tract disease are also at risk of invasive pulmonary aspergillosis (18, 27, 28–30, 39).

In diagnosing aspergillosis, positive cultures (or antigen- or nucleic acid-based tests) for *Aspergillus* from clinical speci-

TABLE 2 Characteristics of some medically important *Aspergillus* species grown on identification media[a]

Section[b] and species	Seriation		Colony color	Microscopic features[c]	Comments[d]
	Uniseriate	Biseriate			
Fumigati					
Aspergillus fumigatus sensu stricto (teleomorph: *Neosartorya fumigata*)	+		Dark blue-green to gray-turquoise; slate gray with age; reverse variable (Fig. 3B)	Conidiophore: up to 300 µm long and 5–8 µm wide, smooth, noncolored or greenish Vesicle: dome-shaped, 20–30 µm diameter, phialides on upper half only Head: strongly columnar Conidia: subglobose to globose, smooth to echinulate, 2–3.5 µm diameter (Fig. 3C)	Characteristic blue-green-colonies, growth at 50°C (growth temperature range: 15–50°C) Columnar heads with single layer of phialides Sterile white fast-growing or glabrous cerebriform slow-growing variants confirmed by thermotolerance and DNA sequencing
A. lentulus	+		Suede-like to floccose, usually white, interspersed with gray-green colonies; reverse yellow, no diffusible pigment (Fig. 3D)	Stipes: 250–300 µm long, 2–7 µm wide, smooth, sometimes sinuous and often constricted at the neck, colorless Vesicle: diminutive, 8–10 µm wide, hyaline, subclavate or subglobose, fertile over only half of the area, few short flask-shaped phialides Head: short columnar Conidia: fewer than 6 or 7 per chain, globose to ellipsoidal, rough with ornamentation, 2.5–3 µm diameter, bluish to olive green (Fig. 3E)	Cryptic relatively new species within *A. fumigatus* species complex. Slow-sporulating, white Does not grow at 50°C (growth temperature range 10–45°C)
A. thermomutatus (teleomorph: *N. pseudofischeri*)	+		Suede-like to floccose, usually white to pale yellow with slow to poor conidiation	Morphologically similar to *A. fumigatus sensu stricto*	Cryptic relatively new species within *A. fumigatus* species complex. Slow-sporulating, white
A. udagawae (teleomorph: *N. udagawae*)	+		Suede-like to floccose, usually white to pale yellow with slow to poor conidiation	Morphologically similar to *A. fumigatus sensu stricto*	Cryptic relatively new species within *A. fumigatus* species complex. Slow-sporulating, white
Flavi					
A. flavus (teleomorph: *Petromyces flavus*)	+	+	Yellow to dark yellowish green (Fig. 3F)	Conidiophore: 400–850 µm long, 20 µm wide, roughened, uncolored Vesicle: subglobose/globose, 25–45 µm diameter Head: loosely radiate or splitting into columns with age (Fig. 3G) Conidia: globose or ellipsoidal, roughened, 3–6 µm diameter	Heads vary in size and seriation, growth usually enhanced at 37°C, brown to black sclerotia may be present, colony color influenced by culture medium additions such as yeast extract Toxigenic
Flavipedes					
A. flavipes (teleomorph: *Fennellia flavipes*)		+	White with patches of yellow or pale grayish buff; reverse yellow to golden brown	Coniodiophore: 150–400 µm long, 4–8 µm wide, smooth to rough, uncolored to pale brown Vesicle: subglobose, 10–290 µm diameter Head: radiate to loose columnar Conidia: globose, smooth, 2–3 µm diameter Cleistothecia and Hülle cells rare	Distinguished from *A. terreus* by slower-growing colonies, metulae usually formed over entire vesicle with radiate to loosely columnar heads

(Continued on next page)

TABLE 2 (*Continued*)

Section[b] and species	Seriation		Colony color	Microscopic features[c]	Comments[d]
	Uniseriate	Biseriate			
Nidulantes					
A. *nidulans* (teleomorph: *Emericella nidulans*)		+	Dark green if mainly conidial; buff to purple brown if cleistothecial; reverse: deep red to purple	Conidiophore: 70–150 μm long, 3–6 μm wide, smooth, brown Vesicle: hemispherical, 8–12 μm diameter Head: phialides on upper part, columnar Conidia: globose, rough, 3–4 μm diameter Cleistothecia reddish brown, globose, 100–250 μm (Fig. 3H) Hülle cells globose Ascospores lenticular with 2 longitudinal crests ca. 5 μm long, red purple	Distinguished by red-brown cleistothecia, abundant Hülle cells, red-purple ascospores with 2 crests, short conidia, and short metulae Toxigenic
A. *tetrazonus* (teleomorph: *Emericella quadrilineata*)		+	Olive green to grayish purple; reverse purple	Conidial heads, ascocarps and Hülle cells similar to those of *E. nidulans*. Ascospores also similar but have 2 major and 2 minor equatorial crests	Distinguished from *E. nidulans* by 4 crests on ascospores
Nigri					
A. *niger*		+	Black with white margin, yellow surface mycelium; reverse uncolored or pale yellow	Conidiophore: 400–3,000 μm long, 15–20 μm wide, smooth, uncolored to brownish near tip Vesicle: globose, 30–75 μm diameter Head: radiate then splitting into columns with age Conidia: globose with thick walls, brownish black, rough, 4–5 μm diameter	Frequent cause of otomycosis, sometimes associated with intracavitary colonization, especially in diabetes mellitus
Terrei					
A. *terreus*		+	Tan to cinnamon brown	Conidiophore: 100–150 μm long, 4.5–6 μm wide, smooth, uncolored Vesicle: dome shaped, 10–16 μm diameter, phialides on upper half Head: columnar Conidia: globose/subglobose, smooth, 2 μm diameter, solitary single-celled conidia commonly formed sessile on submerged hyphae	Distinctive features: cinnamon-colored colonies, columnar heads and solitary accessory conidia Often resistant to amphotericin B
Usti					
A. *ustus*		+	Brown-gray or olive gray; reverse yellow, dull red or purplish	Conidiophore: 75–400 μm long, 4–7 μm wide, smooth, becoming brown Vesicle: globose/subglobose, 7–16 μm diameter, fertile over upper two-thirds Head: radiate to loosely columnar Conidia: globose, rough, 3–4.5 μm diameter Irregular Hülle cells often present (Fig. 3I)	Hülle cells are distinguishing feature when present. Brown conidiophores
A. *deflectus*		+	Slow growing. Mouse gray with pinkish margins or patches of yellow	Conidiophore 40–125 μm long, 2.5–3.5 μm wide, smooth, red-brown Vesicle: hemispherical, 5–7 μm in diameter, bent at right angle to stipe Head: phialides on upper surface, columnar Conidia; globose, 3–3.5 μm diameter, smooth to rough Hülle cells sometimes present	Vesicle bent almost at right angle to stipe is distinguishing feature. Rare human pathogen

(*Continued on next page*)

TABLE 2 Characteristics of some medically important *Aspergillus* species grown on identification media[a] (*Continued*)

Section[b] and species	Seriation		Colony color	Microscopic features[c]	Comments[d]
	Uniseriate	Biseriate			
Versicolores					
A. *versicolor*		+	Green/gray-green or tan with patches of pink or yellow; reverse variable, often deep red	Conidiophore: 200–400 µm long, 5 µm wide, smooth, uncolored, yellow or pale brown Vesicle: ovate to elliptical, 9–16 µm diameter Head: radiate to loosely columnar Conidia: globose, echinulate, 2.5–3 µm diameter Hülle cells globose	Distinguished by slow growing, green-tan or variably colored colonies and small biseriate vesicles. Toxigenic
Less common pathogens: *Aspergillus*					
A. *glaucus* (teleomorph: *Eurotium herbariorum* and other spp.)	+		Deep green mixed with bright yellow; reverse uncolored or pale yellow	Conidiophore: 200–300 µm long, 7–12 µm wide, smooth, uncolored to pale brown Vesicle: globose, 15–30 µm diameter Head: large radiate Conidia; subglobose, echinulate, 5 µm diameter Cleistothecia: thin walled, yellow, globose, 75–150 µm ascospores smooth or rough, with furrow and rounded or frilled crests	Formerly called glaucus group Osmophilic Reproduction enhanced on high-sugar media Growth poor at 37°C Readily identified by ascospore morphology rather than by conidial head features Members include E. *umbrosus*, E. *ruber*, E *repens* E. *umbrosus* is reported as cause of farmer's lung (anamorph A. *glaucus*)
Restricti					
A. *restrictus*	+		Dull olive green to brownish green, very slow growing	Conidiophore: 80–200 µm long, 4–8 µm wide, smooth or rough, uncolored Vesicle: hemispherical, 8–20 µm diameter Head: phialides on upper third, columnar Conidia: cylindrical to ellipsoidal, rough, 4–7 µm long, 3–4 µm wide	Very slow growth on standard media distinguishes this from A. *fumigatus*, slightly enhanced growth on high-sugar media, no growth at 37°C, cylindrical conidia developing to long, adherent columns
Candidi					
A. *candidus*	+	+	White to cream	Conidiophore: mostly 200–500 µm long, 7–10 µm wide, smooth to rough, uncolored Vesicle: globose/subglobose, 17–35 µm diameter Head: fertile over entire surface, radiate Conidia: globose, smooth, 3–4 µm diameter Sclerotia: sometimes, present, reddish purple	Distinguished from all colored aspergilla by white, slow-growing colonies; however, be cautious about white, slow-growing forms of poor sporulators of A. *fumigatus* species complex.

[a] Modified from previous versions of this chapter.

[b] Modern concepts have replaced group names with subgenera and sections.

[c] Refer to "Taxonomy" above for description of terms.

[d] Only species producing potent toxins are noted as toxigenic, but other species may produce toxins of lesser significance.

mens may not in themselves indicate a pathological process. The distinction between colonization of the respiratory tract and invasive disease is particularly difficult in highly immunosuppressed patients and those with chronic airway disease (40, 41), but the probability of requiring treatment increases with increasing immunosuppression. To enable standardization in assisting diagnosis, and for the purpose of clinical trials in patients with hematological malignancies, consensus definitions of proven and probable aspergillosis are published by the European Organization for Research and Treatment of Cancer and the Mycoses Study Group (EORTC/MSG). These incorporate host immune status, lung imaging (computerized tomography [CT]) findings, antigen-based biomarker tests, histology and culture findings, but not as yet nucleic acid detection tests (42). Criteria have also been developed for diagnosis of invasive aspergillosis in patients with chronic obstructive lung disease and for the entity of chronic pulmonary aspergillosis (43, 44). Demonstration of

TABLE 3 Clinical categorization of *Aspergillus* infection and major causative species[a]

Clinical presentation	Syndromes	Associations
Allergic (hypersensitivity)	Allergic bronchopulmonary aspergillosis (ABPA); SAFS[b]	*Aspergillus fumigatus* (*A. terreus*); *Aspergillus* spp.; asthma
Saprophytic	Aspergilloma	*A. fumigatus*; preexisting cavities, tuberculosis
	Otomycosis	*A. niger* is predominant cause
	Onychomycosis	*A. versicolor* complex/*A. sydowii* are common causes
Semi-invasive[c] (hyphal invasion of tissue)	Chronic necrotizing pulmonary aspergillosis	*A. fumigatus*; structural lung disease
	Keratitis	*A. fumigatus*, *A. flavus*, and other species
Invasive[d]	Rhinosinusitis (rhino-orbital/cerebral disease) (acute invasive, chronic invasive, chronic granulomatous forms; aspergilloma)	*A. fumigatus*, *A. flavus* *A. flavus* is the commonest cause in developing countries. Granulomatous sinusitis (Sudan, India, Pakistan, Saudi Arabia) *A. flavus* (90%), *A. fumigatus*, *A. niger* *A. fumigatus*, *A. flavus*, *A. nidulans*, *A. terreus*
	Invasive bronchial aspergillosis (superficial, pseudomembranous or ulcerative tracheobronchitis)	Lung transplantation, AIDS
	Invasive pulmonary aspergillosis	*A. fumigatus*, *A. terreus*, *A. flavus*
	Hyphal invasion (pyogranulomatous inflammation/necrosis)	Immunosuppression, no neutropenia: GVHD, SOT, CGD, HIV/AIDS, corticosteroids
	Angioinvasion (coagulative necrosis, hemorrhagic infarction)	Prolonged, severe neutropenia

[a] References 34 and 35. Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; CGD, chronic granulomatous disease; IPA, invasive pulmonary aspergillosis; GVHD, graft-versus-host disease; SAFS, severe asthma with fungal sensitization; SOT, solid organ transplant.

[b] SAFS can be caused by fungi other than *Aspergillus*; does not meet serological criteria for ABPA (35).

[c] As defined by Chakrabarti et al. (28) and Thompson and Patterson (17).

[d] Classified by Hope and Denning (34) on the basis of tissue invasion by hyphae (with or without angioinvasion).

hyphae in tissue or other clinical samples, and culture of a heavy growth of *Aspergillus* from a single specimen or the same species from multiple specimens, point to invasive disease in the appropriate clinical context. Culture of at least three good-quality sputum specimens is recommended whenever fungal infection is suspected. Antigen- and nucleic acid-based diagnostics are discussed below.

Collection, Transport, and Storage of Specimens

Methods to collect appropriate clinical specimens (see chapter 114) are especially important for diagnosis of invasive pulmonary aspergillosis due to uncertainty in differentiation of *Aspergillus* colonization (or even contamination) from *Aspergillus* disease. Diagnosis can be difficult (36, 45) and appears to be most problematic in nonneutropenic hosts and in patients with compromised airways (e.g., lung transplant recipients). The most convincing evidence of aspergillosis is provided by recovery of the fungus from lung biopsy tissue and other normally sterile sites and/or by histopathologic demonstration of hyphae in tissue. Yet nonsterile specimens such as sputum and bronchoalveolar lavage (BAL) fluid represent the more frequent specimens submitted for culture where recovery of aspergilli may reflect colonization (46). Culture results should be interpreted in combination of an assessment of clinical, radiographic, and other diagnostic tests.

Other than the respiratory tract, *Aspergillus* can be recovered from culture of any body site including sinus, cerebrospinal fluid (CSF), skin, other tissue, the eye, and heart valves. In *Aspergillus* endocarditis, as with other invasive infections, blood cultures are typically negative. Conversely, positive blood cultures for *Aspergillus* are often indicative of contamination even in populations at high risk for invasive aspergillosis (47), although they uncommonly are helpful in disseminated infection (48). All specimens should be transported to the laboratory as soon as practicable for culture and other diagnostic testing. For detection of *Aspergillus* DNA in archived samples, such as paraffin-embedded (PE) tissue, tissue should be sent in thin (approximately 10 × 10 μm) sections in a sterile receptacle (49). It is advisable to discard the first section due to possible contamination with fungal spores.

Direct Examination

Microscopy

Microscopic examination of fresh and/or stained specimens of tissue, BAL fluid, sputum, tissue aspirates, and other specimens may reveal fungal structures, but this method is insensitive and specimens are often positive only in advanced disease. Hyphal elements can be observed using routine potassium hydroxide preparations, or with a fluorescent stain such as calcofluor white or Blankophor P. In tissue biopsy sections, hyphae may be seen when using specific fungal stains including Gomori methenamine silver (GMS) or Grocott's stain and periodic acid-Schiff (PAS). In hematoxylin and eosin (H&E)-stained tissue, hyphae can also be visualized, although care must be taken not to traumatize tissue. Viable hyphae are typically basophilic to amphophilic while damaged or necrotic hyphae are more often eosinophilic (50).

Although species identification of *Aspergillus* is not possible, microscopy provides relevant preliminary diagnostic information; some assessment of fungal morphology and demonstration of fungal hyphae in tissue is a requirement of the EORTC/MSG classification for a "proven" or "definite" fungal infection (42). *Aspergillus* hyphae are typically hyaline, septate, and 6 µm in diameter and branch dichotomously at acute (≈45°C) angles, although not all the above features may be evident (see Fig. 3A for an H&E-stained section of lung). Hyphae have smooth parallel walls with no or slight constrictions at the septa (15, 50). Where invasion has occurred, hyphae may extend throughout the tissue and form parallel or radial arrays. Aspergilli in chronic lung cavities grow as tangled masses of hyphae and may exhibit atypical hyphal features such as swellings measuring up to 12 µm in diameter and/or absence of conspicuous septa. Unusual features such as swelling of the terminal portion of the hyphae may also be seen in patients who have received antifungal therapy, especially with the echinocandin class.

Adventitial forms (conidia, ascomata, and other asexual structures [Fig. 2]) of *Aspergillus* may also be recognized in histopathologic sections and may allow presumptive identification of the fungus. For example, in tissue *A. terreus* displays distinctive aleurioconidia along the lateral hyphal walls (51). Calcium oxalate crystals have been associated with *A. niger* infection (52). However, culture, immunohistochemical staining, or nucleic acid amplification is required to identify the pathogen. Specificity of microscopic methods is low for distinguishing non-*Aspergillus* hyaline moulds from *Aspergillus* (53). Techniques, such as *in situ* hybridization with a specific fluorescent antibody that binds to fungal elements in tissue, may aid diagnosis; in one study, *Fusarium* was distinguished from *Aspergillus* elements in tissue sections with a 100% positive predictive value (PPV) (54), although the use of immunohistochemistry has not been validated for this purpose.

Antigen Detection

Galactomannan (GM) is a major polysaccharide constituent of the cell walls of *Aspergillus*, most *Penicillium* (and/or *Talaromyces*) species, and certain other moulds, which is released *in vivo* from growing hyphae. Commercial assays for GM are well validated. The Platelia *Aspergillus* enzyme linked immunosorbent assay (ELISA) (BioRad Laboratories, Hercules, CA) is a double sandwich EIA that utilizes a rat monoclonal antibody (mAb) EBA-2, directed against the galactofuranoside side chain of the GM antigen (reviewed in reference 55), and it is data on this test that resulted in inclusion of GM in the revised EORTC/MSG definitions of invasive aspergillosis in immunosuppressed patients (42). These definitions were developed for use in clinical and epidemiological research; in clinical practice, they must be taken in context with clinic-pathologic descriptions and classifications (34) (see "Clinical Significance").

Although the EORTC/MSG definitions include GM detection in plasma, serum, BAL fluid, or CSF, extensive clinical validations have only been performed for serum and, following release of a newer format of the above assay from Bio-Rad, BAL fluid, especially in patients with hematological malignancies (for reviews and meta- and systematic analyses, see references 55–60). Based on a meta-analysis, the sensitivity and specificity of serum GM, using a cutoff optical density index (ODI) of 0.5 for the diagnosis of invasive aspergillosis, are 71 and 89%, respectively, with a low PPV of 26 to 53% but high negative predictive value (NPV) of 95 to 98% (56), suggesting that GM is more suitable as a screening to exclude aspergillosis than as a diagnostic test. The sensitivity and specificity of serum GM testing vary substantially in different studies, mainly due to differences in study design and study populations. It is much more sensitive when used in neutropenic and hematopoietic stem cell transplant recipients than in patients with solid organ transplants (61, 62) or critically ill patients with chronic obstructive pulmonary disease, presumably because of the higher incidence of angioinvasion in neutropenic patients (63, 64). As a diagnostic test in patients with hematological malignancies, the specificity and PPV were increased to 98.6% when two consecutive samples were positive at an ODI of at least 0.5 (65). It has been recommended that a single positive GM index of ≥0.7 or two consecutive samples of ≥0.5 should prompt a diagnostic workup (65). Use of mould-active antifungal agents reduces the sensitivity of the serum GM test (66), and false positives have occurred in patients receiving piperacillin-tazobactam or amoxicillin-clavulanate in association with *Bifidobacteria* in the gut of neonates and with ingestion of foods containing GM (63). Some non-*Aspergillus* moulds may yield positive GM results (*Talaromyces marneffii*, *Histoplasma capsulatum*, *Fusarium oxysporum*, *Paecilomyces*, and *Alternaria* species) (59).

Recently three systematic reviews/meta-analyses of the utility of GM in BAL fluid in patients with hematological malignancies or mixed comorbidities were published (58–60). All acknowledged limitations of the studies analyzed but concluded that the BAL GM assay was more sensitive than serum GM, though different cutoff ODIs for BAL positive samples were recommended: 1.0 by Zou et al. (58), and 1.5 by Heng et al. (59). Data regarding the effect of mould-active agents on level of BAL-GM are conflicting, and standardization across studies is needed to resolve these differences (59). There is emerging evidence that BAL GM is more sensitive and specific than serum GM in critically ill patients with chronic obstructive pulmonary disease, though a proposed cutoff of 0.8 for the ODI requires confirmation (67).

Aspergillus Antigen Detection Using Lateral Flow Technology

Recently, a simple test that is more specific than the GM assay and that does not require specific equipment has been developed as a rapid diagnostic test, although the current need for a pretreatment step in testing serum (but not BAL fluid) samples reduces its utility as a point-of-care test. The test relies on binding of a mouse monoclonal antibody (Mab JF5) to an extracellular glycoprotein of *Aspergillus*, which is present in cell walls of growing germ tubes and secreted at growing hyphal tips but absent from ungerminated conidia (68, 69). The Mab JF5 does not cross-react with fungal antigens that cross-react with Mab EB-A2 used in the GM assay, namely, those of *T. marneffei*, *H. capsulatum*, *F. oxysporum*, and *Alternaria* species. However, both antigen tests demonstrate cross-reactivity against *Paecilomyces* and non-*Talaromyces Penicillium* species (68) (see "*Penicillium*"). Testing of samples from patients with hematological malignancies suggests that the *Aspergillus* lateral flow assay is as specific as, but may be more sensitive than, GM; that it can be used on BAL samples; and that both sensitivity and specificity may be improved by using it in combination with PCR-based assays (70–72). It should be noted that, at the time of writing, the *Aspergillus* lateral flow test is not widely available.

(1,3)-β-D-Glucan

Other than tests to detect *Aspergillus* GM and, now, novel *Aspergillus*-specific antigens (68), colorimetric detection of

(1,3)-β-D-glucan in the serum of patients represents another strategy for diagnosis of invasive aspergillosis. As with *Aspergillus* GM, detection of (1,3)-β-D-glucan has been added as a diagnostic criterion in the EORTC/MSG assessment for probability of invasive aspergillosis (42). This antigen is a major component of the fungal cell wall, and circulating (1,3)-β-D-glucan can be quantified in the serum of patients with invasive aspergillosis (73–76). Concentrations approaching 1 pg of antigen/ml can be detected, with a level of ≥80 ng/ml (Fungitell Assay; Associates of Cape Cod, Inc., Falmouth, MA) considered indicative of fungal infection.

However, the inability of the test to discriminate between *Aspergillus*-related infections and those caused by other fungi—i.e., its low PPV (approximately 50% at best)—is a key limitation of the assay for diagnosing aspergillosis. The sensitivity, specificity, and NPV of the assay for any systemic fungal infection are described to be approximately 93, 77, and 98%, respectively (73, 74). In a meta-analysis of 16 studies encompassing a variety of fungal infections in hematology and other patient populations, the pooled sensitivity was 76.8%, specificity was 85.3%, positive likelihood ratio was 5.2, and negative likelihood ratio was 0.29 (77). Data indicate that the sensitivity for invasive aspergillosis ranges widely, from 60 to 100%. Despite these limitations, the serum (1,3)-β-D-glucan test may be useful as a negative predictor of aspergillosis if combined with more specific tests; exposure to mould-active antifungal agents reduces the sensitivity of the test (75–77). Conversely, false-positive results are common under conditions of hemodialysis (cellulose membranes), treatment with immunoglobulin products, and exposure to glucan-containing material (55, 77). Cross-reactivity with amoxicillin-clavulanic acid and cell wall components of Gram-positive organisms has occurred. Test results should be interpreted within the clinical context, and further validation is required to determine its usefulness for monitoring therapeutic response.

Nucleic Acid Detection

Aspergillus DNA can be detected in clinical specimens either by nucleic acid amplification methods specifically targeting *Aspergillus* or by panfungal PCR assays followed by DNA sequencing for species identification. The former has the better sensitivity and faster turnaround time (no need for DNA sequencing), but panfungal assays have broader diagnostic potential. Both strategies may identify *Aspergillus* spp. directly in tissue, blood, CSF, and respiratory samples (49, 78–80) with good overall sensitivity and specificity, although with wide interstudy variability in performance (40 to 100% for sensitivity; 60 to 100% for specificity) (reviewed in reference 55). Badiee et al., studying 194 hematology patients with proven/probable aspergillosis, verified values for molecular detection of *Aspergillus* spp. of 66, 96, 63, and 97%, respectively, for sensitivity, specificity, PPV, and NPV (80). Technical advances in amplicon detection methods have enabled real-time detection and quantification of *Aspergillus* DNA using TaqMan, LightCycler, high resolution melt curve analysis, or nucleic acid sequence-based amplification; these methods are detailed elsewhere (55, 81–83). Yet the clinical utility of *Aspergillus* PCR tests remains incompletely defined.

Prior to initiatives to formally address the limited clinical application of *Aspergillus* PCR assays (see below), Mengoli et al. undertook a systematic review to assess assays for their (i) diagnostic value (specifically diagnostic odds ratios [DORs]) or (ii) role in screening for invasive aspergillosis (84). Sixteen studies that employed PCR on blood, serum, or plasma samples (>10,000 samples from 1,618 patients at high risk for invasive aspergillosis) were included. The meta-analysis showed that a single PCR-negative result was sufficient to exclude a diagnosis of proven/probable aspergillosis if PCR was used as a screening tool from the start of the at-risk period. Two positive PCR results are required to maximize specificity. Calculated pooled sensitivity was 75% (95% confidence interval [CI], 54 to 88), specificity was 87% (95% CI, 78–93), PPV was 15.2 to 81%, and NPV was >97%. The at-risk populations studied varied, and there was great heterogeneity in PCR methodology, nature of samples (e.g., whole blood versus serum), and volume of sample tested (84). Indeed, 1-ml serum volumes when compared with standard 100 μl volumes yielded superior sensitivity (100% versus 76.5%) (85).

PCR performed on BAL samples has good potential to assist diagnosis of invasive pulmonary aspergillosis (86–88). A recent evaluation of a real-time pan-*Aspergillus* PCR in lung transplant patients showed a sensitivity and specificity of 100 and 88%, respectively, with similar performance characteristics noted for an *A. fumigatus*-specific PCR (86). Despite assay differences, studies have demonstrated a high NPV and good sensitivity, with variable PPVs reflecting differences in diagnostic certainty.

Because of these limitations, the European *Aspergillus* PCR Initiative (EAPCRI) working group of the International Society for Human and Animal Mycology (ISHAM) has devised standards for *Aspergillus* PCR which would allow clinical validation and subsequent wide use of a PCR assay. Notably, the following factors are critical in influencing the quality and success of *Aspergillus* PCR assays: the type of specimen, volume of specimen, the DNA fungal extraction method, and potential for automation of the PCR process itself (to increase sensitivity). This international collaboration thus far has established a recommended methodological process to detect *Aspergillus* in EDTA-blood using standardized DNA extraction (89, 90). Guidelines for DNA extraction from serum specimens and for *Aspergillus* DNA detection have also been published (90). There was no significant difference in sensitivity between the use of whole blood or serum for PCR-based diagnosis of aspergillosis, although in one study there was a trend for whole blood to be more sensitive (85% versus 79%) and to show earlier positive results (36 days versus 15 days) compared with serum (91). Against this finding is the reduced false-positivity rate using serum, which is also easier to process. The optimum sample type should be determined by the local requirement.

A further advance is the commercial MycAssay *Aspergillus* assay (Myconostica, Manchester, UK), a real-time platform that detects *Aspergillus* spp. and allows standardized detection using quality-controlled reagents. However, the kit does not distinguish between individual species and is not cleared by the U.S. Food and Drug Administration (FDA) for use. When tested on lung and other tissue samples containing *Aspergillus*, the sensitivity was 82% and specificity was 79% relative to microscopy, and 90% and 64%, respectively, compared to culture (92). In high-risk hematology patients, the kit had a sensitivity of 65% and specificity of 95% when performed on serum and BAL fluid (93, 94). In nonhematology patients with invasive lung aspergillosis, the sensitivity, specificity, PPV, and NPV on lower respiratory tract samples were 86.7, 87.6, 34.1, and 92.2%, respectively; sensitivity increased when multiple samples were analyzed (95). The median time to detection of *Aspergillus* was 4 hours. At present, the kit should be used to complement culture methods to ensure accurate species identification.

Clinical Utility of Combined Antigen Detection and Nucleic Acid Amplification Test

The above descriptions of GM/(1,3)-β-D-glucan antigen and *Aspergillus* PCR testing for the diagnosis of invasive aspergillosis have highlighted a number of limitations of both strategies. Test performance can vary considerably, and the (1,3)-β-D-glucan test is not specific for *Aspergillus* spp. Much progress has been made in standardizing *Aspergillus* PCR procedures, although more work is required prior to enabling its wider deployment.

Nonetheless, it is clear from numerous publications that it is the use of these tests in combination that assists in the earlier diagnosis of invasive aspergillosis. In a recent randomized trial of the *Aspergillus* GM and *Aspergillus* PCR versus culture and histology methods in high-risk hematology patients, the use of GM and PCR to direct therapy reduced the use of empiric antifungals, enabled more diagnosis of probable aspergillosis a median of 4 days earlier, and was most effective in patients receiving fluconazole, itraconazole, or no antifungal prophylaxis (96). Rogers et al. demonstrated that addition of PCR to GM monitoring in the setting of hematological malignancy provided greater diagnostic accuracy for invasive aspergillosis (97). Others have reported that GM or PCR can diagnose invasive aspergillosis a median of 2 to 9 days earlier than culture and high-resolution CT scans in up to 88.8% of patients (98, 99). An early retrospective clinical evaluation of the *Aspergillus* lateral flow test gave good clinical performance, especially if combined with PCR (100% sensitivity and 100% specificity) for proven/probable aspergillosis (72).

Isolation Procedures

Aspergillus species are not fastidious in their growth requirements. They are typically easily recovered on standard mycological media and will also grow on bacteriological media such as blood agar. On occasions where respiratory tract specimens may be inoculated on media selective for certain non-*Aspergillus* moulds (e.g., dichloran rose bengal agar for selective isolation of *Scedosporium* species [100]), and where the presence of potential *Aspergillus* organisms may be clinically relevant, it should be noted that growth of aspergilli is inhibited on these media.

Most identification schemes utilize specific media and incubation conditions for the description of characteristics, such as colony color and size (diameter) (Fig. 3). *Aspergillus* species usually grow without difficulty at 25°C but also at higher temperatures. Although isolation procedures do not require specific biohazard precautions, it is good laboratory practice to handle all culture material in a class II biosafety cabinet to avoid dispersal of spores through the environment and cross-contamination. The culture process usually requires several days.

Identification

Culture-based identification of aspergilli remains important. Diagnostic features of the more common pathogenic species/species complex are summarized in Table 2. For keys to taxa and additional information, see also de Hoog et al. (15). Figure 2 presents a diagrammatic key to several species. Some species commonly produce sexual structures (i.e., cleistothecia and ascospores), which may aid identification. The respective teleomorph names of common *Aspergillus* species are given in parentheses (e.g., *Emericella*, *Neosartorya* [Table 2]).

Phenotypic identification of *Aspergillus* requires the assessment of both macroscopic and microscopic morphological characteristics. Isolates are usually inoculated at three points on potato dextrose agar, Czapek Dox agar (with or without 20 to 30% added glucose) or 2% malt extract agar and incubated at 25°C (6, 15). These media are necessary since aspergilli tend to reproduce in the asexual form when grown on Sabouraud dextrose agar (SDA). Most species sporulate within 7 days, but the teleomorph form takes longer to develop. Descriptions are primarily based on colony pigmentation and morphology of the conidial head with reference to those in expert monographs (4, 8).

Macroscopic Features

Colony diameters on standard media at 25 and 37°C, obverse and reverse colors, texture, topography, and presence of exudate droplets and diffusible pigments may be recorded after 7 days (see Fig. 3B–3I) (15, 101). Isolates not identifiable should be retained for longer for possible development of ascomata or other structures that may aid identification.

Microscopic Features

Microscopic mounts are best made using sticky tape flag or slide culture preparations mounted in lactophenol cotton blue (101). A drop of alcohol is usually required to remove bubbles and excess conidia. Key features are hyaline hyphomycetes showing distinctive conidial heads with flask-shaped phialides arranged in whorls on a vesicle (Fig. 2). Specifically, important features are differences in size, shape, color, and wall ornamentation of various structures including the stipe, shape and size of vesicles, and arrangement of phialides. A stipe or conidiophore arises either directly from the vegetative hyphae or from a specialized hyphal cell called a foot cell; it is typically nonseptate and varies in color, length, and wall ornamentation according to spe-

FIGURE 3 (A) H&E-stained section of lung showing *Aspergillus* hyphae with smooth hyaline which are septate and branch dichotomously at acute angles. (B) Colonies of *Aspergillus fumigatus* on potato dextrose agar showing typical blue-green surface pigmentation with a suede-like surface consisting of a dense felt of conidiophores. (C) Conidiophores of *Aspergillus fumigatus* are columnar (up to 400 × 50 μm long) but may be shorter and are uniseriate. The vesicle is conical shaped and sports a singe row of phialides on the upper two thirds. Conidia are produced in basipetal succession with long chains and are globose to subglobose. (D) Culture of *Aspergillus lentulus*. Colonies are suede-like to floccose, white, and interspersed with gray-green patches of conidia (conidiation is slow to poor). (E) Conidiophores of *Aspergillus lentulus* are short, columnar, and uniseriate. Stipes are of narrower width than those of *Aspergillus fumigatus*. Vesicles are subglobose and are smaller (6 to 25 μm diameter) than those of A. *fumigatus* (10 to 26 μm). (F) Culture of *Aspergillus flavus*. Colonies are granular, flat, often with radial grooves, yellow at first but quickly becoming bright to dark yellow-green with age. (G) Biseriate head of *Aspergillus flavus*. (H) *Aspergillus nidulans* (*Emericella nidulans*) showing cleistothecia. (I) *Aspergillus ustus* showing biseriate head, brown stipe, and Hülle cells.
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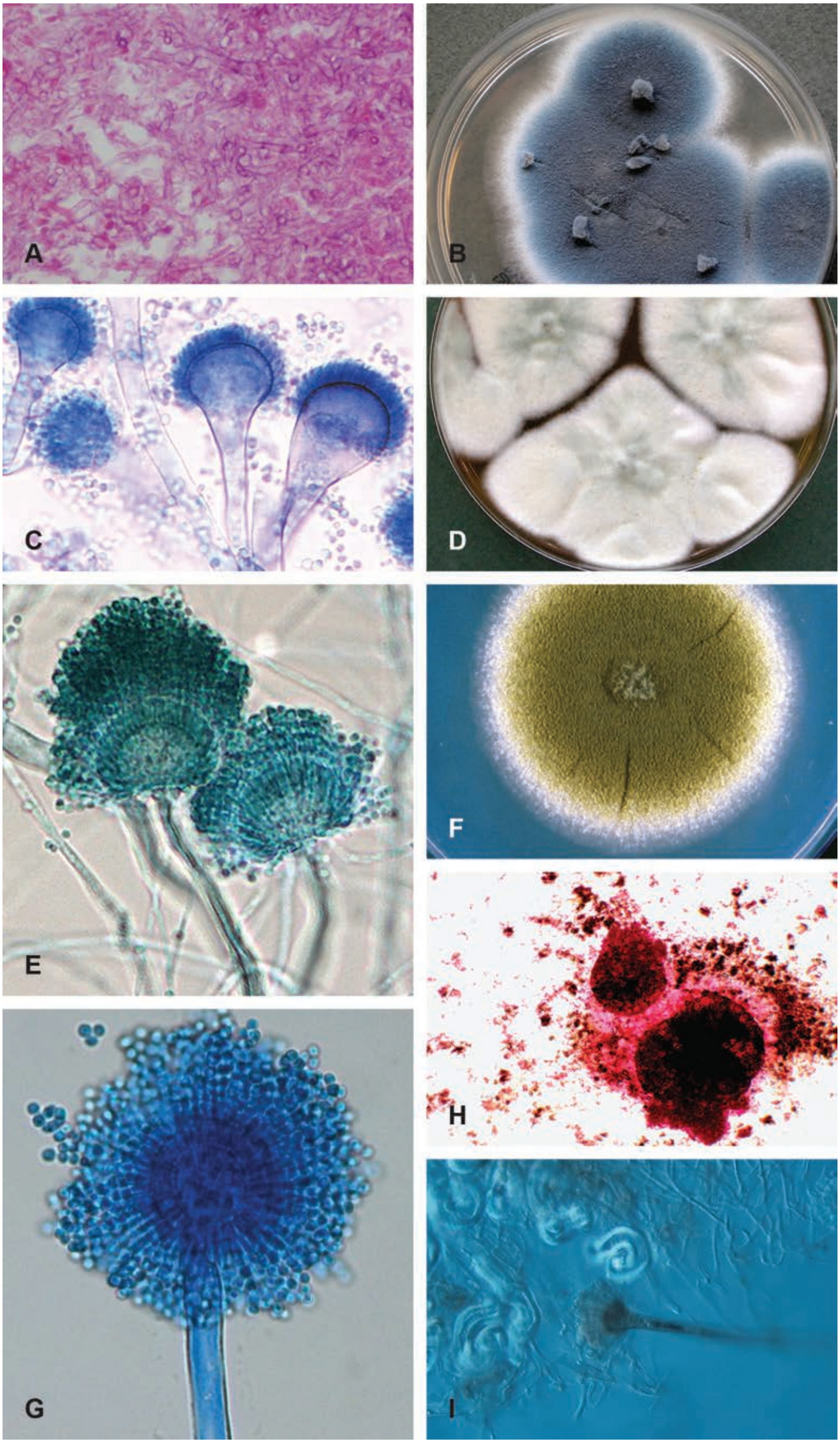
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cies. The stipe ends in a variably shaped (globose, subglobose, hemispherical, pear-shaped or clavate [club-shaped]), swollen vesicle (Fig. 2); either the entire vesicle or its upper portion is covered with phialides which give rise to the conidia. Conidial head morphology in *Aspergillus* is either *uniseriate* in arrangement, where the phialides arise directly from the vesicle, or *biseriate*, where they arise from an intermediate series of cells, the metulae, or a combination of both, as occurs in *A. flavus* (Fig. 2, Table 2).

The conidia themselves may be borne in a single column (columnar), or columns may be split with some arising at right angles to the stipe (radiate). A dissecting microscope is required to make this distinction. Conidia are usually ellipsoidal or globose, may be echinulate, and vary in size, color, and wall markings depending on the species. At times, contrast microscopy may be required to delineate morphology (102).

Sclerotia are firm, fruiting-body-like structures comprising swollen hyphal cells but without internal spores. Hülle cells are variably globose with thick refractile walls (Fig. 3I). They commonly occur within the growing mycelium near the colony center, where their presence is indicated by droplets of exudate. They are often associated with cleistothecial ascomata (Table 2). The interiors of cleisthothecia are filled with asci and ascospores. Shape, color, size, and wall features of these structures are important in distinguishing the different teleomorph forms.

Poorly Sporulating Species or Variants

Where sporulation is poor, identification may be problematic, although incubation at 25°C in light and allowing the cultures to experience the normal diurnal cycles of light and dark may help to induce sporulation (102, 103). Poorly sporulating "*A. fumigatus*" isolates are not uncommonly recovered from clinical specimens (8, 102). When this occurs, *A. fumigatus* species complex could be considered if an atypical, sometimes waxy or cerebriform mould was grown at 45°C (all members of section *Fumigati* grow at this elevated temperature [Table 2]); it is now well established that various cryptic species such as *A. lentulus* and other closely related sibling species of *A. fumigatus* demonstrate a poorly sporulating phenotype (10, 102). *A. lentulus* does exhibit smaller vesicles (Table 2, Fig. 3E) and, unlike *A. fumigatus*, cannot grow at 48 or 50°C, but in practice it cannot be distinguished by morphological methods alone from *A. fumigatus* without scanning electron microscopy (SEM) or other sophisticated microscopy. As *A. lentulus* has low *in vitro* susceptibilities to the azoles, amphotericin B, and caspofungin (10), it is important to accurately identify all clinically significant isolates. Since the discovery of *A. lentulus* as a new species, subsequent screening studies of "*A. fumigatus*" isolates have uncovered pathogens misidentified as *A. fumigatus*, including *N. pseudofischeri* and *N. udagawe* (30, 102); these isolates also demonstrate variable susceptibility to antifungal drugs *in vitro*. As for *A. lentulus*, phenotypic identification methods will not differentiate them from *A. fumigatus*. For these, for other cryptic species of the *A. fumigatus* complex, and also for members of other *Aspergillus* species complexes, DNA sequencing (see below) of one or more sections of the fungal genome is required for species identification.

Molecular Identification

Where conventional phenotypic methods cannot identify *Aspergillus* isolates or identification is uncertain (e.g., with nonsporulating isolates), DNA sequencing can be helpful. In particular, the ITS, including the 5.8S rRNA gene regions, has emerged as the most common target(s) for molecular identification (104, 105); the ITS regions are generally more discriminatory than the D1-D2 domain of the 28S subunit for the identification of aspergilli. Other gene targets useful in species identification include the β-tubulin, rodlet A, and mitochondrial cytochrome *b* genes (10, 102). ITS sequencing is of clinical utility in identification of *Aspergillus* to species complex level but often cannot discriminate between species within the complex. The ISHAM *Aspergillus* Working Group recommends the use of a comparative sequencing-based identification method that uses the ITS regions for species complex-level identification and of a protein coding locus (β-tubulin region) for identification of species within each *Aspergillus* species complex (106). This approach successfully identified 218 *Aspergillus* isolates collected for TRANSNET, USA (30).

Non-sequence-based identification methods include real-time PCR and *Aspergillus* species-specific microsphere-based assays using Luminex xMAP technology (Luminex Corp., Austin, TX). Alonso et al. developed a real-time PCR followed by combined probe hybridization and high-resolution melt analysis to identify section *Fumigati* and non-*Fumigati* *Aspergillus* isolates; the detection limit was 10^2 conidia/ml (82). Microsphere-based Luminex (Luminex Corp.) assays have also enabled species complex level identification as well as differentiation of species within the *A. fumigatus* complex (107).

The preciseness of reporting of an identification of *Aspergillus* depends on (i) clinical need to accurately identify the fungus species (e.g., from a tissue biopsy compared with recovery from sputum) and (ii) access to molecular identification methods. In smaller laboratories, the use of the term "species complex" aids correct identification since isolates are relatively reliably identified to the section level by morphological methods. For example, a laboratory can identify a fungus as "*A. fumigatus* species complex" by phenotypic methods and indicate that molecular identification is required to identify the isolate as a species within the complex.

Mass Spectrometry Identification Methods

Identification of mould cultures using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is increasingly undergoing evaluation to reduce the turnaround time for identification of *Aspergillus*. Despite initial technical difficulties posed by the relatively complicated morphology and *Aspergillus* cell wall, the technology has utility in identifying isolates not only to the species complex level, but also to identify species, and in some cases, to delineate strain variation (108–115).

Bille et al. reported an overall identification rate for *Aspergillus* spp. of 98.4% (63/64 isolates) using the Andromas system (Andromas SA, Paris, France) and Alano et al. reported an identification rate of 98.6% (138/140 isolates) (111, 112). In another study, 82% of 44 *Aspergillus* isolates were identified correctly (resolved by gene sequencing) using the Vitek-MS platform (bioMerieux, France); correct identification was contingent on appropriate spectra being contained in the database (110). Hettick et al. used MALDI-TOF MS to derive spectra for 12 *Aspergillus* species and five different strains of *A. flavus;* classification of each species and strain of *Aspergillus* tested was achieved with 100% accuracy (113). When analyzing invasive *Aspergillus* isolates, Pan et al. reported that more of the differential spectral peaks were present at the second stage of sporulation, which contains differentiated structures, than at the first stage, which is comprised primarily of vegetative hyphae (114).

All studies have stressed the need for adequate sample preparation for MALDI-TOF MS and the construction of libraries of robust spectra from known strains, independent of the MALDI-TOF MS system (reviewed in reference 108). Use of MALDI-TOF MS has not been approved for the identification of *Aspergillus* or other moulds by the FDA.

Typing Systems

Genotyping of *Aspergillus* is central to the understanding of epidemiological relationships between clinical and environmental isolates, and to identify the origin and track the spread of nosocomial infections (see "Epidemiology and Transmission" above), especially in responding to public health situations (116). Genotyping also assists in determining if a patient is colonized with the same strain over long periods or becomes recolonized by different strains. *Aspergillus* may be genotyped by a range of molecular typing techniques; these include random amplified polymorphic DNA (RAPD) analysis, restriction fragment length polymorphism (RFLP) analysis, DNA fingerprinting, PCR fingerprinting, amplified fragment length polymorphism (AFLP) analysis, multilocus microsatellite typing (MLMT), multilocus sequence typing (MLST), and cell surface protein (CSP) gene typing.

RAPD analysis was first used to differentiate isolates of *A. fumigatus* (117) but has also been applied to genotype other species including *A. flavus* (118) and *A. tereus* (119). RFLP analysis was likewise first applied to genotype *A. fumigatus* using XhoI and SalI enzymes and then for species identification among a heterogeneous *Aspergillus* group, where it correctly identified *A. flavus* isolates (120). RFLP analysis does not enable strain differentiation within a species.

DNA fingerprinting has utility in genotyping using probes such those containing retrotransposon-like elements, namely, Afut1, Afut2, and Afut4 (121–123), while clinical and environmental *A. flavus* isolates from a neonatal intensive care unit have been successfully typed with the species-specific probe pAF28 (124). AFLP analysis is another approach that has determined genetic diversity within *A. fumigatus* (125) and within the sections *Flavi* (126) and *Nigri* (127). More recently, MLMT analysis using four, then later nine, different microsatellite markers was developed to form the STRAf scheme for genotyping *A. fumigatus* (128); this approach has been extended to include other species including *A. flavus* (seven markers [129]), *A. niger* (six markers) and *A. nidulans* (seven markers) (130, 131). MLST analysis with a panel of seven genetic loci has also been applied for strain typing of *A. fumigatus* (132). Finally, a single-locus sequence typing scheme, detecting tandem repeats in a gene encoding a putative cell surface protein, CSP, has been developed (133). Strain variation is a result of both repeat number variation and nucleotide sequence variation. In one study, CSP typing was compared with PCR fingerprinting using minisatellite (core sequence of the bacteriophage M13) and microsatellite [$(GTG)_5$ and $(GACA)_4$] specific single primers and MLMT analysis to differentiate between *A. fumigatus* strains; only MLMT separated all clinical and environmental isolates implicated in a case cluster (22).

RAPD and AFLP studies in particular have been hampered by low interlaboratory reproducibility due to their reliance on pattern-based typing techniques. This limitation has been overcome by using the highly reproducible and discriminatory MLMT, MLST, and CSP typing techniques, which yield unambiguous and portable data. Microsatellite based approaches and MLST analysis are species specific.

Serologic Tests

The detection of *Aspergillus* GM and *Aspergillus*-specific recombinant proteins in serum and other body fluids is discussed in "Antigen Detection," above. Methods to detect other *Aspergillus* antigens or anti-*Aspergillus* antibodies against these antigens in patients with aspergillosis, including reactivity in skin tests, are also developed, but their clinical utility is less well defined. The main reasons for this have traditionally been related to the nature and purity of the antigen. Although antigen preparations may be available commercially, their quality is variable and they demonstrate cross-reactivity with non-*Aspergillus* antigens. However, hybridoma technology and monoclonal antibody-based detection systems may be changing the state of immunodiagnosis of aspergillosis.

A number of reviews detail *Aspergillus* antigens that have been evaluated as candidates for incorporation into immunoassays (68, 133, 135). Antigens tested include somatic preparations, either crude or purified whole cell mycelial extracts, *Aspergillus* metabolites, mannoproteins, and more recently, expressed and purified recombinant antigens. Of purified somatic antigens, one, a 19-kDa basic protein, was shown to be a major circulating antigen in the urine of patients with invasive aspergillosis (68). This protein was also present in the serum of patients with aspergillomas. Molecular characterization has subsequently shown it to be Aspf1, an IgE-binding protein (and potent allergen) related to the mitogillin family of cytotoxins. Other potential serodiagnostic antigens include an 88-kDa dipeptidyl peptidase, a 33-kDa alkaline protease, the recombinant mannoproteins antigens Afmp1 and Afmp2, and a recombinant 19-kDa Cu-Zn superoxide dismutase (68, 136, 137). Various test formats have been exploited to detect antibodies to these antigens. Double immunodiffusion and counterimmunoelectrophoresis were initially used and still are, as they are simple to perform (134). In many laboratories, these are now replaced by enzyme immunoassays (ELISA) and immunofluorescence assays, including commercial systems.

However, rather than detecting antigen, most serological evaluations have focused on detecting the humoral response to these antigens. Although many assays proved sensitive, their routine use has been limited by substantial interlaboratory variability, cross-reactivity between different fungi, and the type of antigen used in the assay, but probably most importantly, the inability to mount diagnostic antibody responses in immunocompromised hosts. Nonetheless, in one study, IgG anti-*A. fumigatus*-specific mitogillin was detected by ELISA in the serum of all (32 of 32) patients with aspergilloma and in 64% (31 of 42) of patients with invasive lung aspergillosis (138); serum IgG and IgM antibodies were found in only 1.3% of healthy volunteers. However, the optimal cutoff value for the ELISA is not clearly established. Humoral antibody responses to Afmp1 detected by ELISA were demonstrated with high sensitivity (100%) for patients with aspergilloma, whereas in invasive aspergillosis the sensitivity was only 33.3% (137). Combined immune responses to Afmp1 and Afmp2 may increase sensitivity (139). The measurement of these antibody responses is largely restricted to allergic forms of aspergillosis and aspergilloma. However, recent work showed that total antibody specific to *Aspergillus*-specific thioredoxin reductase (GliT) was detected in nonneutropenic patients with invasive aspergillosis with a sensitivity of 81% and specificity of 96% (140). Further studies are required to determine the diagnostic potential of these assays.

Detection of circulating antibodies may also be useful in aiding diagnosis of allergic aspergillosis in patients with cystic fibrosis and in those with severe asthma with fungal sensitization (SAFS) (Table 3). Anti-*A. fumigatus* IgE and IgG and *A. fumigatus* precipitins are established markers of ABPA and are criteria included in its diagnosis (141). Further, immunoglobulin subclass measurements may add valuable information. Recently, detection of specific IgE to recombinant *A. fumigatus* allergens, such as those of the rAspf family, and detection of thymus activation-regulated chemokine TARC/CCL17 have been identified as new biomarkers of ABPA (142). Consensus opinion indicates that measurements of serum IgG subclasses, especially IgG4, against *A. fumigatus* are useful in screening for and classifying ABPA, at least in cystic fibrosis patients, whereas IgA measurement could be useful to determine prognosis of ABPA (142, 143). Quantitation of serum IgG4 may improve specificity of diagnosis. Further, the biomarker TARC has shown greater test accuracy for ABPA diagnosis compared to antibody responses. Anti-*A. fumigatus* IgE and IgG may be measured by commercial assays such as the ImmunoCap assay (Phadia, Uppsala, Sweden) and Platelia *Aspergillus* IgG assay (Bio-Rad, Marnes-la-Coquette, France); in a recent study, both these two ELISA assays were more sensitive than counterimmunoelectrophoresis (CIE; >93% versus 63%) in diagnosing ABPA (144).

Antimicrobial Susceptibilities

Drugs Used to Treat Invasive Aspergillosis

Amphotericin B, the triazoles (voriconazole and posaconazole), and echinocandins form the backbone for treatment of invasive aspergillosis, although itraconazole may be useful in the more chronic forms of disease. Consensus opinion informed by a landmark randomized, controlled trial recommends voriconazole as first-line therapy (145) for invasive lung aspergillosis as well as extrapulmonary and disseminated infection (146). Oral voriconazole is preferred for "step-down" or maintenance therapy. Other antifungals approved for treating aspergillosis are lipid amphotericin B formulations (e.g., the liposomal compound, for primary therapy or salvage treatment) (146) and the echinocandins, although only caspofungin is licensed for this indication in many countries (147) (also for primary/salvage therapy). Posaconazole, amphotericin B, and caspofungin have been used in salvage therapy, all with response rates between 40 and 50% (146). Though there are insufficient data to recommend antifungal combinations for primary/salvage treatment, a clinical trial of voriconazole versus voriconazole-anidulafungin for initial treatment of proven/probable aspergillosis showed a trend towards improved survival in hematology patients (148). For detailed treatment practices for aspergillosis, the reader is referred to the 2010 Infectious Diseases Society of America guidelines document (146).

In Vitro Antifungal Susceptibility Testing

In vitro susceptibility testing of *Aspergillus* isolates may help guide therapy. Resistance to azoles, the only orally active group of antifungals for aspergillosis, used to be uncommon and was limited to patients receiving long-term azole therapy or prophylaxis (149); in one study, there was a positive correlation between prolonged itraconazole use and high MICs of posaconazole (150). Given the increasing azole resistance among both clinical and environmental *A. fumigatus* isolates in some countries (151), routine testing of aspergilli for antifungal susceptibility in these regions is important for clinical decisions and to monitor trends. Rates of azole resistance were 5.3% in the Netherlands (152), 5.8% in the ARTEMIS global surveillance study (153), and up to 20% in the United Kingdom, although the U.K. study contained many isolates from patients with chronic aspergillosis who had received long-term azole therapy (154). Knowledge of susceptibility patterns for new and cryptic species is likewise important.

The majority of azole-resistant *A. fumgatus* (and other *Aspergillus* species) isolates were found to contain an alteration in the target protein sterol 14a-demethylase (or Cyp51), inhibiting drug binding. These changes are as a result of single nucleotide polymorphisms in the gene *CYP51A* encoding the protein leading to amino acid substitutions. Mutational hot spots confirmed to cause resistance have been located at amino acid positions G54 and L98 among others, of which the L98 alterations require a tandem repeat (TR) in the promoter region of *CYP51A* to cause resistance, the TR/L98 mutation (151). Mechanisms of azole, as well as echinocandin, resistance are detailed in chapter 131.

Methodologies and MIC Determinations

In the past few years, standardization of two reference methods for susceptibility testing of conidium-forming moulds has provided guidelines for antifungal susceptibility testing of *Aspergillus*. These methods are (i) the Clinical Laboratory Standards Institute (CLSI) M38-A2 methodology and (ii) the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard (155, 156) (see chapter 131). With either approach, MIC determinations (and minimum fungicidal determinations) are relatively straightforward for azoles and amphotericin B due to the growth-versus-no-growth pattern of inhibition, and MICs obtained by these reference methods are in close agreement (151).

Interpretative CLSI breakpoints to recognize susceptibility or resistance have not yet been established for any drugs for *Aspergillus*. Nonetheless, amphotericin B, itraconazole, voriconazole, posaconazole, and the echinocandins are active *in vitro* and demonstrate the lowest MIC values (157, 158). Using EUCAST methodology, clinical breakpoints (CBPs) for amphotericin B, voriconazole, posaconazole, and itraconazole have been published, primarily for *A. fumigatus* (159, 160); epidemiological cutoff MIC values (ECOFF) of wild-type *Aspergillus* strains have also been published (161, 162). The derivation of CBPs and ECOFF values is detailed in chapter 131. Importantly, MICs may vary with species and with geographic region. However, *A. terreus* is considered resistant to amphotericin B, as this species is a poor target for this drug; MICs are as high as 16 μg/ml and susceptibility testing is not required for this species (160). Infections due to *A. terreus* are expected to be associated with poorer responses to amphotericin B compared with those caused by, e.g., *A. fumigatus*. Overall, there is no clear correlation of amphotericin B MICs with clinical response since there is a narrow distribution of MICs with no clear resistance phenotype (163).

With regard to the correlation of azole MICs and clinical outcomes, earlier work indicated that there was good correlation between high MICs and treatment failure in animal models of invasive aspergillosis (164). Recently, voriconazole MICs were predictive of treatment results in *A. terreus* murine infections (165). However, in another study there was no relationship between increasing MICs and response to voriconazole in mice with *A. fumigatus* infection (166). Baddley et al. observed that MICs of voriconazole exceeding the ECOFF value (i.e., 1 μg/ml) were not associated with increased mortality in patients with *A. fumigatus* infection receiving voriconazole (167). For other *Aspergillus* species, correlation of azole MICs and outcome is also uncertain (168).

Susceptibility endpoints for the echinocandins against *A. fumigatus* by CLSI methodology may be uncertain due to significant trailing growth. Thus, a minimum effective concentration (MEC) is employed, where the MEC is the lowest concentration leading to macroscopically aberrant growth as microcolonies or granular growth when compared to the growth control wells. MEC determination is time-consuming and requires a microscope to determine morphological changes, and interlaboratory reproducibility is suboptimal. Correlation between MECs and *in vivo* outcomes is uncertain. One report found clinical failure and *in vivo* resistance in an animal model for an isolate with an MEC in the wild-type range (169). There are no CBPs for the echinocandins with EUCAST.

In practice, both reference methods may be performed by specialist mycology laboratories, but they are not suitable for use in most clinical laboratories as they require extensive training. Commercial kits for susceptibility testing of *Aspergillus*, such as the Etest (AB Biodisk, Solna, Sweden), are often useful. The Etest has excellent correlation with the CLSI reference method for voriconazole (170) and between 80 and 100% agreement for itraconazole and posaconazole (171, 172). The Sensititre Yeast YO10 system (incorporating prefilled trays of nine antifungal agents [Trek Thermofisher Diagnostics, Cleveland, OH]) is based on the CLSI method. The visual endpoint allows easier reading, and it shows good correlation with the azoles and amphotericin and the echinocandins (172). Another approach is to use azole-containing agars, for example, itraconazole at 4 mg/liter, to screen for azole resistance; this technique has been evaluated for *A. fumigatus* (151). Cross-resistance between azoles occurs in up to 74% of cases (151, 154). Research methods include isothermal microcalorimetry for real-time susceptibility testing (173).

Evaluation, Interpretation, and Reporting of Results

The diagnostic mycology laboratory's aims are to provide timely, clinically meaningful information using accurate and and up-to-date methods. Criteria have been proposed by the EORTC/MSG for the analysis of patient data in clinical trials to help to standardize interpretation of results. Identification of *Aspergillus* fungi to species level should be undertaken when they are recovered from sterile specimens and from patients at high risk of invasive aspergillosis. Certain species may be more resistant to antifungal agents. *A. terreus* complex is resistant to amphotericin B, while the *A. nidulans* complex and *A. ustus* are less susceptible to many agents compared to *A. fumigatus* (51, 174). *Aspergillus* recovered from nonsterile specimens including BAL fluid in immunecompetent patients may not be responsible for disease and may not require species identification. Workup of isolates should involve dialogue between the requesting clinician and the microbiologist.

Diagnosis of invasive aspergillosis is difficult, as clinical and radiological features are nonspecific. Although diagnosis is straightforward when *Aspergillus* is visualized in and cultured from clinical specimens, more often it relies collectively on non-culture-based tests in the setting of appropriate host risk factors, clinical presentation, and radiological findings. The conventional approach for patients at risk for aspergillosis is to administer antifungal agents empirically when a diagnosis has not yet been made. The recent evaluation of the utility of biomarkers of infection, in particular *Aspergillus* GM and PCR, either alone or in combination, has led to the increasing adoption of a preemptive antifungal therapy approach. Using these biomarkers, prospective screening for infection in feasibility studies and one randomized, controlled clinical trial demonstrates the utility of further diagnostic workup (96, 98) and has led to earlier diagnosis of aspergillosis without decreasing mortality.

Despite progress made by the EUCAST movement in defining CBPs and ECOFF values for antifungal agents, correlation between MICs and clinical outcomes is not yet established. Interpretation of MIC testing of *Aspergillus* remains problematic. Nonetheless, given the rise in azole resistance among *A. fumigatus* in some countries, susceptibility testing to azoles against this species is warranted in these regions and if there is therapeutic failure. The widespread use of EUCAST methodology is limited by the expertise and training needed to perform these tests. Useful information regarding expected antifungal susceptibility may be obtained by identifying the fungus to species level (e.g., *A. lentulus*).

PENICILLIUM SPECIES

Taxonomy and Position of *Talaromyces marneffei* (formerly *Penicillium marneffei*)

Early work on the taxonomy of the genus *Penicillium* (otherwise referred to as *Penicillium sensu lato*) is based on the studies of Thom (175) and Raper and Thom (176). The genus contains more than 300 species (http://www.aspergillluspenicillium.org). These species have traditionally been separated based on their morphology, specifically conidiophore branching, into four anamorphic subgenera—*Aspergilloides*, *Furcatum*, *Penicillium*, and *Biverticillium*—within the family Trichocomaceae (4, 177). However, more recent multi-gene phylogenetic analyses have shown that three of these subgenera, *Aspergilloides*, *Furcatum*, and *Penicillium* (i.e., *Penicillium sensu stricto*), are polyphyletic, whereas the fourth, *Biverticillium*, forms a monophyletic group (178), which is characterized by the presence of symmetrical biverticillate conidiophores (179). The distinctive morphological and physiological features of *Biverticillium* have resulted in its evolving taxonomic status and suggestions to separate the subgenus *Biverticillium* from *Penicillium sensu stricto* (4, 177–179).

Rationale for Separation of Members of *Biverticillium* from *Penicillium sensu lato*

Penicillium sensu lato is associated with two teleomorphic genera: *Eupenicillium* (now synonymous with *Penicillium senso stricto*) and *Talaromyces* (now the teleomorph stage of *Biverticillium*). These two teleomorph genera are separated by distinctive ascomata. The sclerotium-like ascomata of *Penicillium sensu stricto* are thick-walled isodiametric cells maturing over several months and forming in most cases no ascospores, whereas *Talaromyces* cells are characterized by soft ascomatal walls comprising multiple layers of interwoven hyphae leading to mature ascomata within weeks. In comparison with *Penicillium senso stricto*, the members of *Talaromyces* often have darker green conidia, produce pigmented and encrusted aerial hyphae, and show yellow, orange, or red to purple colony color on reverse. Additionally, phylogenetic studies have increasingly provided evidence that *Penicillium sensu stricto* and *Talaromyces* should be considered subfamilies as demonstrated by genetic relatedness employing the fungal rRNA and calmodulin genes (180–183). The subdivision of these genera was finally confirmed using a multigene approach (178).

As true for "*Aspergillus*," the ability to correlate anamorphic and teleomorphic genera with modern taxonomic tools has led to the one fungus = one name concept (13). By removing the primacy of the teleomorph name over the anamorph-typified name (14), this has likewise had a major impact on what was known as the genus *Penicillium*. The name *Penicillium* is now used for *Penicillium sensu stricto* (i.e., comprising the subgenera *Aspergilloides*, *Furcatum*, and *Penicillium*) while the term *Talaromyces* is now defined as a pleomorphic genus, representative of the anamorphic species formerly within the *Penicillium* subgenus *Biverticillium* (184).

It is notable that the only species known to be a human and animal pathogen is *Talaromyces* (formerly *Penicillium*) *marneffei*, a member of the subgenus *Biverticillium*. This species is dimorphic, growing as a yeast at 37°C in the host and as a filamentous fungus at 25°C in the environment. As a result of the revised nomenclature, *P. marneffei* is now correctly classified as *T. marneffei* (Segretain, Capponi & Sureau) Samson, Yilmaz, Frisvad & Seifert, comb. nov. MycoBank MB560656 (184, 185), rendering *P. marneffei* [Segretain, Capponi & Sureau *apud* Segretain (1959)] the basonym of the new taxonomic assignment.

Epidemiology and Transmission

T. marneffei is an emerging pathogen and is a particular health problem in areas of endemicity in tropical Asia, especially Thailand, northeastern India, China, Hong Kong, Vietnam, and Taiwan (186–189). The arrival of HIV/AIDS changed the rarity of human penicillinosis marneffei; mortality is high where the presence of infection is an AIDS-defining illness.

The ecological reservoir of *T. marneffei* remains an enigma. A key question is whether human disease occurs as a result of zoonotic (animal) or sapronotic (environmental) transmission. Although *T. marneffei* was initially isolated from a bamboo rat, *Rhizomys sinensis*, in Vietnam in 1956 (190), exposure to soil and decaying material especially under humid and rainy conditions is likely the critical risk factor (186, 191); agricultural occupations have been independently associated with increased risk (192). Yet, despite extensive efforts, attempts to recover the organism from soil have met with only limited success (186, 193) and proof of an environmental reservoir is still lacking. Conversely, it has been recovered from environments associated with bamboo rats of the genera *Rhizomys* and *Cannomys*, suggesting these rodents are a key facet of the *T. marneffei* life cycle. When MLMT analysis was used to genotype this fungus, isolates from bamboo rats and humans shared identical genotypes (186, 194). This suggests that either rodents are vectors for human infections or both humans and rodents are infected from an as-yet-unidentified environmental source.

Patients are infected by inhalation of *T. marneffei* conidia, thus pulmonary alveolar macrophages are the primary host defense. The fungus proliferates within phagocytic cells, resulting in granulomas in immune-competent hosts. In HIV/AIDS, infection typically develops in advanced HIV infection where the CD4+ cell count is <50 cells/ml.

Clinical Significance

T. marneffei now ranks as the third most common opportunistic infection in HIV/AIDS in regions of endemicity (187). Its prevalence has increased in areas where HIV infection is on the rise (195). Although uncommon, infections have been reported in other immunocompromised patients, including those with kidney transplants and systemic lupus erythematosus (196–198). Immunosuppressed patients traveling to areas of endemicity should be vigilant with regard to risks of penicilliosis (198). Disseminated infection in HIV/AIDS is typical with involvement of the skin (60 to 85% of cases), lungs (pneumonia, including cavitation), liver, and joints. Most cases present with fever, weight loss, lymphadenopathy, and skin lesions—these are most often on the face, neck, and oral cavity, but they can affect any region including the genitalia (186). The rash is typically an umbilicated papular rash with central necrosis.

The fungus can be isolated from skin lesions or from blood, bone, or bone marrow. Presumptive diagnosis is made by visualization of yeast-like *T. marneffei* cells in Wright-stained smears of skin lesions or in biopsy specimens from these sites (199, 200).

Collection, Transport, and Storage of Specimens

Methods of collection, transport, and storage of specimens are similar to those for other fungal pathogens (see chapter 114).

Direct Examination

Microscopy

Direct visualization of the organism in tissue or fine needle aspiration (FNA) samples of affected sites establishes presumptive diagnosis. Rapid diagnosis is enabled through cytological examination of lung biopsy imprint smears, lung aspirates, lymph node aspirates, skin smears, and even sputum. The yeast form is typically stained by methenamine silver or periodic acid-Schiff stain but can also be visualized by Giemsa and H&E stains (189). Wright-stained bone marrow aspirates, often performed in the workup of HIV-positive patients with unexplained fever, are useful in these patients. Histiocytes are seen, with or without granuloma formation, and may contain few to many intracellular yeast-form cells (201); these are oval to elliptical (3 to 8 μm in diameter) with a distinctive clear central septum (202). This and the absence of budding help distinguish the condition from histoplasmosis and toxoplasmosis (15, 200). A specific indirect fluorescent-antibody reagent for rapid identification of *T. marneffei* in histologic sections using rabbit antiglobulin against yeast antigens of *T. marneffei* has also been developed (203).

Nucleic Acid Detection

A number of molecular techniques have been developed to directly detect and identify *T. marneffei* in clinical specimens. Many are in-house assays. Methods include single-step PCR and nested and seminested PCR assays to detect *T. marneffei* in PE tissue, skin, blood, and other specimens. Most assays targeted the *T. marneffei* 18S rRNA and ITS gene region (204–207). The ability of one nested 18S rRNA PCR assay to directly identify the fungus in PE tissue was evaluated using inner primers (Pm1 and Pm2) specific to *T. marneffei*. Amplification of a species-specific fragment of approximately 400 bp was successful for tissue from all 14 patients studied as well as for tissue from 10 bamboo rats with *T. marneffei* infection, with an analytical sensitivity of 14 fg/μl (206). In other studies, a multiplex ligation-dependent probe amplification (MLPA) assay detected *T. marneffei* DNA in PE tissue with high specificity (207), as has a loop-mediated isothermal amplification assay (208).

Isolation Procedures

Culture remains the gold standard method of diagnosis, even though it is slow and requires collection of clinical specimens with invasive procedures. *T. marneffei* is easily cultured from clinical specimens and grows well on SDA without cycloheximide; culture of bone marrow is the most sensitive (100%) followed by skin (90%) and blood culture (76%) (189). Mould-to-yeast conversion is achieved by subculturing onto brain-heart infusion agar and incubating at 37°C (202). Identification of *T. marneffei* is based on colony morphology (see below). In Europe, Australia, and New Zealand, *T. marneffei* is classed as a risk group 3 (RG-3) pathogen and represents a biohazard to laboratory personnel. Therefore, specimens and cultures should be handled in a containment level 3 (or above) facility to avoid inhalation of conidia or accidental inoculation into skin.

Identification

Members of the genus *Talaromyces* are ubiquitous laboratory moulds and are grown easily from most nonsterile specimens, especially respiratory specimens. Differentiation of *T. marneffei* from other penicillia is not usually problematic since *T. marneffei* is thermally dimorphic. Diagnosis is made by observing the conversion of the mould to yeast form at 37°C or the reverse at 25°C.

Colonies on SDA at 25°C are fast growing, downy, and white with yellowish-green conidial heads. Colonies become grayish pink to brown with age and produce a diffusible brown-red pigment particularly evident on the reverse (Fig. 4), which is an early indication that an isolate may be *T. marneffei*. However, red pigment may be produced by nonpathogenic *Penicillium* species including *P. citrinum*, *P. janthellum*, *P. purpurogenum*, and *P. rubrum*. Colonies on brain heart infusion agar (37°C) are rough, glabrous, and tan-colored. Microscopic yeast-like cells are spherical to elliptical and are 2 to 6 µm in diameter.

Morphology of *T. marneffei* is distinctive, with hyaline smooth-walled conidiophores with terminal verticils comprising three to five metulae (secondary branches) with conidia, each bearing three to seven phialides. Conidia are globose to subglobose, 2 to 3 µm in diameter, smooth walled, and produced in basipetal succession from the phialides, producing a typical "penicillus" or brush-like fruiting structure (15, 202).

Together with direct detection of the fungus in clinical specimens, molecular tools enable *T. marneffei* cultures to be identified by PCR in combination with DNA sequencing or probe hybridization and distinguished from other *Penicillium* species that may be incidentally present. Since species identification can be problematic within the genera *Penicillium* and *Talaromyces*, a polyphasic approach is recommended. Morphological approaches should be combined with molecular strategies. Assays typically amplify one or more of the 18S rRNA, ITS region, β-tubulin, calmodulin, actin, or RNA polymerase genes (186). Introduction of molecular methods of identification would depend on clinical need.

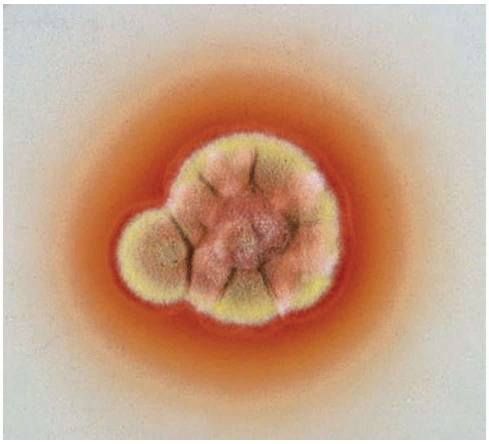

FIGURE 4 White colonies of *Talaromyces marneffei* on Sabouraud dextrose agar (25°C) with yellow-green conidial heads. With age, the diffusible brownish-red to wine-red pigment is evident. doi:10.1128/9781555817381.ch119.f4

Typing Systems

A number of typing systems have been developed to (i) study the population structure and genetic diversity of *T. marneffei* and (ii) investigate clusters of infection should they arise. A MLMT system was developed using 23 genetic loci (209) and applied to clinical isolates from Southeast Asia; 21 of 23 tested loci were polymorphic, revealing a high degree of genetic diversity. A clear separation between the population of strains from the eastern region (China, Hong Kong, Indonesia) and from the western region (Thailand, India) was observed, with the eastern clade being more polymorphic than the western clade (210). An independent study confirmed the clustering of isolates from these regions into two clades (211). The separation of these clades may be due to potential isolation of these populations or for reasons not yet known, and/or resulting from a clonal reproductive mode.

In addition to MLMT, MLST analysis incorporating eight genetic loci (transcription factor [*AbaA*], catalase [*CpeA*], homeodomain transcription factor [*StlA*], isocitrate lyase [*Icl1*], polyaromatic amino acid biosynthesis [*PAA*], NADH-dependent glutamate synthase [*NGS*], lovastatin nonaketide synthase [*LNS*], a cell wall mannoprotein [MP1], and a gene fragment of the cytochrome oxidase subunit 1 gene [*COX1*] of the *T. marneffei* mitochondrial genome) has also been developed as a typing tool. In one study, no nucleotide polymorphisms were observed within the *COX1*, *AbaA*, and *NGS* loci. However, the remaining five loci showed a high degree of genetic diversity and again indicated geographic separation between isolates from China or Thailand, but also mixed clades. Identical MLST types between different patients have been shown, supporting an asexual mode of reproduction (212). Other investigations have found identical MLMT types among strains from humans and bamboo rats; however, the role of contact with bamboo rats or bamboo rat burrows in the transmission of human disease remains unclear (186, 213).

Serologic Tests

Serologic tests may help in diagnosis of infection, although no standardized commercial tests are available. Assays have been developed to detect antibodies to *T. marneffei*, *T. marneffei* antigen, or both in serum and other body fluids in various test formats including immunodiffusion, indirect immunofluorescence, and ELISA (186, 199).

Immunodiffusion tests to detect precipitin antibodies using *T. marneffei* mycelial exoantigens had low sensitivity when tested on serum in HIV-positive patients (214). Using an indirect fluorescent approach for detecting IgG antibodies against germinating conidia and yeast forms, patients with *T. marneffei* infection were shown to have higher titers (>160) than those without (≤40) (215). An ELISA-based antibody test using recombinant *T. marneffei* mannoprotein (Mp1p) diagnosed infection with 80% sensitivity and 100% specificity (216). In another study, an ELISA-based test detected and measured Mp1p in sera of patients with a sensitivity of 65%; however, combining Mp1p-based antigen and antibody testing yielded a sensitivity of 88%, PPV of 100%, and NPV of 96% (217), as also found by an additional study, which reported a sensitivity of 100% (218).

Other methods of serum antigen detection include a *T. marneffei*-specific latex agglutination test (sensitivity 76%). When used with the immunodiffusion test, the sensitivity increased to 82%. Specificity of both test formats is 100% (214). False-positive results have been rarely observed using the Platelia *Aspergillus* ELISA for GM, which uses a monoclonal antibody to *A. fumigatus* (219). ELISAs have also been used to detect and quantitate *T. marneffei* antigen in urine (sensitivity 97%, specificity 98% at a cutoff titer of 1:40) (220). A capture ELISA was evaluated with 53 patients with culture-confirmed penicilliosis and 240 controls. The diagnostic sensitivity, specificity, and accuracy of the ELISA when tested on serum were 92.45, 97.5, and 96.59%, respectively (221).

Antimicrobial Susceptibilities

Early treatment of penicillosis marneffei is critical to reduce mortality of this infection. *In vitro* susceptibilities help guide therapy despite the absence of formal clinical breakpoints for antifungal drugs. *T. marneffei* appears to be susceptible *in vitro* to itraconazole, ketoconazole, flucytosine, and voriconazole. Amphotericin B is less active, and fluconazole is the least active. Some strains have high MICs to fluconazole (up to 16 mg/liter) and are considered resistant (222, 223). Testing of 39 *T. marneffei* strains from China revealed that voriconazole MICs were low (MIC range, 0.004 to 0.25 mg/liter; geometric mean [GM], 0.04 mg/liter). The GM MICs of itraconazole, terbinafine, amphotericin B, and fluconazole were 0.11, 0.15, <0.65, and 4.1 mg/liter, respectively (224). Clinical and microbiological responses appear to correlate with susceptibility to azoles, but the results for amphotericin B are difficult to assess. Amphotericin B, voriconazole, and itraconazole are often used to treat penicillosis. The echinocandins are also active against *T. marneffei*; *in vitro* the drugs are more active against the mycelial, rather than yeast, form (223, 225). Micafungin has demonstrated synergy with itraconazole (65% of strains) and amphotericin B (50%) but not with fluconazole (226). Posaconazole and terbinafine may be clinically useful, but more data are required.

Other *Penicillium* (and *Talaromyces*) Species

As many *Penicillium* species are completely or strongly inhibited at 37°C, they have rarely caused human infection. Even repeated isolation of *Penicillium* from patient specimens does not necessarily indicate an etiological role. This includes the isolation of the fungus in patients with chronic lung disorders, including bronchiectasis and cystic fibrosis, where *Penicillium* can colonize airways for prolonged periods. The role of penicillia in allergy and hypersensitivity pneumonitis is well established (227). Nonetheless, infections due to a range of *Penicillium* and *Talaromyces* species, resulting in keratitis, otomycosis, peritonitis, pneumonia, and endocarditis, are reported, typically after the fungus is repeatedly isolated. Species implicated in infection include *P. citrinum*, *P. commune*, *P. chrysogenum*, *P. aurantiogriseum*, *P. brevicompactum*, *T. verrucosum*, *T. piceus*, *T. pururogenus*, and *T. rugulosus* (15, 228, 229).

Identification of *Penicillium* species requires expertise with growth of an isolate on several media and careful microscopic examination. No thermal dimorphism is displayed. Isolates are inoculated at three points on Czapek Dox agar, 2% malt extract agar, and/or 25% glycerol nitrate agar and incubated at 25°C. Colonies are fast-growing, in shades of green (sometimes white), consisting of a dense felt of conidiophores. Most species sporulate within 7 days. Microscopic mounts are best made using a sticky tape flag or slide culture mounted in lactophenol cotton blue. A drop of alcohol is often needed to remove bubbles and excess conidia. Molecular techniques may be required for species identification. The phylogenetic relationships of penicillia based on the ITS1/2 region are shown in Fig. 5.

Evaluation, Interpretation, and Reporting of Results

T. marneffei is the only member of the genus *Talaromyces* that has been linked unequivocally to human infection. Diagnosis of penicillosis marneffei should include positive culture, direct smear, and molecular detection/identification in a patient with an appropriate history of exposure to the area of endemicity. Most other *Penicillium/Talaromyces* species have low potential for causing human infection.

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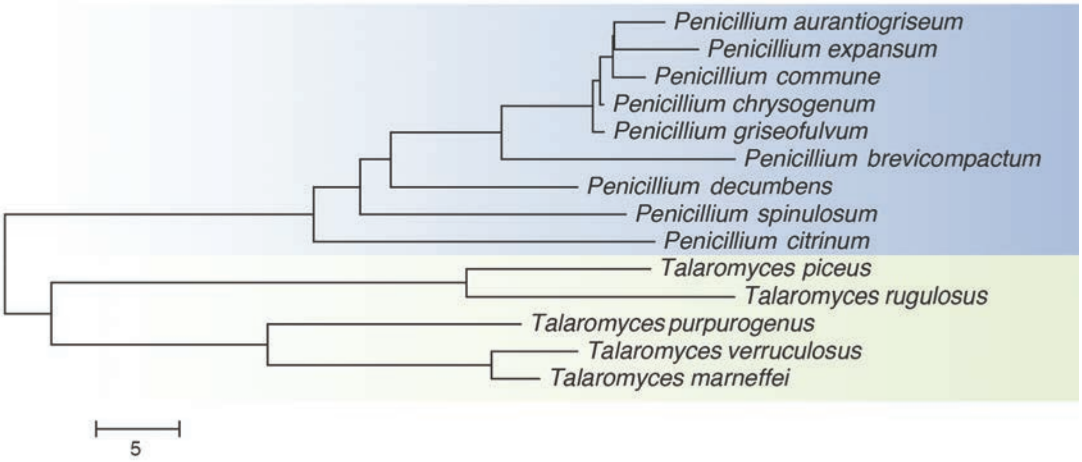

FIGURE 5 Neighbor joining tree of pathogenic *Penicillium* and *Talaromyces* species based on ITS1/2 sequences of the rDNA gene cluster obtained from GenBank using MEGA ver. 5.2.
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Fusarium and Other Opportunistic Hyaline Fungi*

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The opportunistic hyaline or lightly colored moulds (also referred to as moniliaceous) constitute a phylogenetically diverse group of common to rare anamorphic (asexual) and teleomorphic (sexual) fungi that typically occur as saprobes in soil, in air, on plant litter, or as facultative plant pathogens. Some may be recovered from patients without having any clinical significance. Others are isolated infrequently enough to challenge the proficiency of a diagnostic laboratory, and critical assessment is required to evaluate the significance of their recovery. While several of the genera treated in this chapter include species that have either lightly colored or dark (melanized) conidia, the emphasis is on those fungi that grow in tissue in the form of hyaline or lightly colored, septate hyphae.

The term "fusariosis" is used to define infections caused by species of *Fusarium* (1) (Table 1), but the practice of coining disease names based on the fungal genus is disadvantageous for infections caused by uncommon or rare pathogens. The wide variety of fungi involved makes it difficult to place the organisms into accessible groups, and problems arise when fungal names are changed. To avoid unnecessary name changes for disease names based on a genus, two major disease groups have been proposed: hyalohyphomycosis and phaeohyphomycosis (2). Although the groups were defined as encompassing similar clinical spectra, they were distinguished by the presence of septate hyphae in tissue without (hyalohyphomycosis) or with (phaeohyphomycosis) pigmentation or melanin in the fungal cell wall. However, some fungi, such as *Scedosporium* species and *Neoscytalidium dimidiatum* (formerly *Scytalidium dimidiatum* [*Nattrassia mangiferae*]) (3), which form darkly pigmented colonies and conidia *in vitro*, produce hyaline or lightly pigmented hyphae in tissue. Similarly, rare fusariosis cases have been reported to produce darkly pigmented colonies *in vitro* and *in vivo* (4). The Masson-Fontana silver stain helps to detect melanin pigmentation of fungal elements in tissue, but the results are not always conclusive (see also the discussion in chapter 115). Some fungi with variable pigmentation may stain faintly or inconsistently. In practice, the terms for the disease categories have been used to designate infections caused by fungi that are either hyaline or pigmented (melanized, phaeoid, dematiaceous) *in vitro*. Although it may be useful to have terms for broad categories of mycotic diseases, there are problems in categorizing fungi by color. A subcommittee of the International Society for Human and Animal Mycology (ISHAM) has suggested that fungal diseases be named by providing a specific description of the pathology and naming the causative agent, e.g., subcutaneous cyst caused by fungus *x* (5, 6).

TAXONOMY AND IDENTIFICATION

The opportunistic hyaline moulds include an ever-increasing number of genera. Most do not produce a teleomorph (meiotic or sexual stage) in culture and comprise taxa that are either anamorphs of the Ascomycota and Basidiomycota or genera for which no sexual state has been described (see chapter 113). Today the relationships between many anamorphs and their sexual relatives are known through discovery of teleomorphs or are inferred by phylogenetic analysis of DNA sequence data. This knowledge is extremely important in understanding fungal relationships and has allowed placement of asexual fungi next to their sexual relatives in fungal phylogenies. Rapid developments in this area have led to significant changes in genus and species concepts, particularly among the fusaria (7–17). Teleomorphs may develop in culture from homothallic, self-fertile species (see chapter 113); however, they may be difficult to obtain without the use of specialized media and extended incubation. Recent modifications relative to the naming of pleomorphic fungi, however, have prohibited the use of this dual nomenclature after 1 January 2013 (18, 19), and many changes regarding accepted taxa are expected. One such significant change is the proposed unitary use of the name *Fusarium* over various teleomorph names (20), in part because fewer than 20% of fusaria are known to reproduce sexually. In this chapter, the teleomorph name will continue to be used for species that are identified mainly by their sexual structures.

Most of the pathogenic moulds considered in this chapter are classified in the form class Hyphomycetes (genera which bear their conidia freely) (21–23). Phenotypic identification of Hyphomycetes is based on morphology of the conidia and the mechanisms by which conidia are formed (conidiogenesis; see chapter 113). Three basic tools are necessary for practical observation of these features. (i) An ocular micrometer is essential for determining sizes of conidia or sexual spores when present. Identification of moulds often requires comparison with published taxonomic descriptions in which size is often a key criterion for species distinction. (ii) A dissecting microscope with magnifications of up to

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TABLE 1 Classification of *Fusarium* infections[a]

I. Normal host
  A. Keratitis
    a. Trauma and penetration of cornea
    b. Contamination of soft contact lenses, solutions, cases
    c. Local immunosuppression by corticosteroid drops
  B. Onychomycosis[b]
    a. Distal subungual lesion in toenails in females
    b. Lateral subungual onychomycosis
    c. Proximal subungual onychomycosis
    d. Paronychia-like reaction in proximal nail fold
  C. Intertrigo[b]
  D. Tinea pedis[b]
    a. Interdigital infection
  E. Hyperkeratotic plantar lesions[b]
  F. Skin infections[b]
  G. Surgical wound infections
  H. Burns
  I. Ulcers
  J. Otitis media
  K. Peritonitis (continuous ambulatory peritoneal dialysis)
  L. Catheter-associated fungemia
  M. Fungemia with or without organ involvement
  N. Pneumonia
  O. Sinusitis
  P. Septic arthritis
  Q. Thrombophlebitis
  R. Endophthalmitis
  S. Osteomyelitis
II. Immunocompromised host
  A. Endophthalmitis
  B. Sinusitis
  C. Pneumonia
  D. Skin involvement
  E. Fungemia
  F. Disseminated infection
  G. Brain abscess
  H. Peritonitis

[a] Adapted from references 56, 65, 72, and 99. Patients infected with other fungi discussed in this chapter may present with similar clinical syndromes, and fungal structures in tissue may resemble those of *Aspergillus* species.

[b] Patients from which a dermatophyte has not been isolated.

×60 and basal illumination is useful for the examination of colonies in plates or tubes for the presence of conidia in chains or slimy heads, specialized structures, such as Hülle cells, sclerotia, and conidiomata, or sexual fruiting bodies forming under the aerial mycelium or embedded in the agar. (iii) Microscopic mounts that allow observation of how a fungus forms its conidia also are necessary. Slide culture preparations are excellent for many fungi and generally necessary for those with small, delicate conidia (24, 25); however, rapidly growing species, such as those in the genus *Trichoderma*, should be examined early by tease mounting. Morphologic features important for identification of conidial fungi include (i) conidium size, shape, and pattern of septation; (ii) the color of the conidia and conidiophore, whether light (hyaline) or dark (melanized or phaeoid); (iii) developmental aspects of conidiogenesis, including the nature of the conidiogenous cell; (iv) the mechanism of conidium liberation or dehiscence; and (v) the structure of the conidioma (if present). Differences in conidial shape and septation are useful characters for preliminary distinction and have been used traditionally for grouping conidial fungi. Conidia may be nonseptate or may have one or more septa. Some fungi produce septate and nonseptate conidia. In genera such as *Fusarium*, large multiseptate and much smaller septate or nonseptate conidia are referred to as macro- and microconidia, respectively. Conidia also vary in shape. Some macroconidia are long and narrow, as with *Fusarium*. Development of a conidium may occur by conversion of an existing cell or several cells (thallicarthric or arthrosporic) or may involve new wall building or blowing out of a portion of the wall (blastic). Conidiogenesis usually occurs at a particular location on a conidiogenous cell. If development occurs at a site that remains fixed and gives rise to more than one conidium, then the site is referred to as stable or determinate. If conidia develop at new points on the conidiogenous cell (or axis), then the site is described as unstable or indeterminate. New sites may occur on an axis which lengthens (progressive) or shortens (retrogressive). Depending on the species, conidiogenous cells may produce a single conidium or multiple conidia. Sympodial development involves the development of a single conidium at successive sites on a lengthening axis. Phialides and annellides are conidiogenous cells that are specialized to produce multiple conidia. Conidia are produced successively (serially) and develop in slimy masses or in chains, with the youngest at the base of the chain (basipetal). Although it is sometimes difficult to differentiate between the two types of cells, the annellide elongates and sometimes narrows during the formation of each new conidium, leaving an often imperceptible series of rings or scars on the conidiogenous cell. Scrutiny of the conidiogenous locus by use of an oil immersion objective may be necessary to make this distinction. Holoblastic conidiogenesis, in which the inner and outer cell walls are involved, usually results in the formation of acropetal chains, with the youngest conidium at the tip of the chain. The distinction between acropetal and basipetal chains may be revealed by comparison of the sizes and wall morphologies of the distal and proximal conidia in the chain. The youngest conidium is recognizable by its smaller size, lighter color if the conidia are pigmented, and differences in wall ornamentation if the conidia are roughened. Some conidiogenous cells form multiple conidia simultaneously over the surface of the swollen cell. When mature, conidia detach by fission of a double septum (schizolytic dehiscence) or by sacrifice of a supporting cell (rhexolytic dehiscence), either by fracture of a thin-walled region or by lysis of the supporting cell. Lytic dehiscence typically occurs in the dermatophytes and related fungi.

The presence of different spore states can make identification difficult. Careful examination and sometimes subculture via a single spore or hyphal tip are required to assess whether the spore types represent different states of the same fungus or whether the isolate is contaminated with more than one species. Moulds having more than one independent stage are called pleomorphic fungi. Different conidial forms of the same fungus are called synanamorphs. These may be represented by simple yeast or yeastlike stages or by the formation of complex conidiomatal structures, such as sporodochia (conidiophores crowded on a compact mass of hyphae or a hyphal stroma), synnemata (conidiophores aggregated into a compound stalk), or pycnidia (conidiogenous cells formed inside a round or oval fruiting body). Expression of a synanamorph may be influenced by the agar medium used and may be lost upon repeated subculture.

The number of hyaline fungal species that have been reported to cause opportunistic infections in humans and other animals is increasing, and it is beyond the scope of this chapter to describe them all. Some reports identify the fungus only to the genus level, while others provide the fungus name but without salient details of its colony and microscopic features. A continuing and vexing problem is that authenticity of reports cannot be verified if the fungus is inadequately described and illustrated and if isolates are not deposited in publicly accessible culture collections. This chapter describes the salient colony and microscopic features of medically important species in the genus *Fusarium* (Table 2) and other selected hyaline opportunists (Table 3). Detailed descriptions of species listed are found in several reference manuals (15, 24–34) as well as the current literature. Due to the rapid discovery of novel species via multilocus sequence typing (MLST) over the past decade, most clinically important species within *Fusarium* appear to be undescribed (35). Two Internet-accessible websites have been developed for identifying *Fusarium*, Fusarium-ID (http://isolate.fusariumdb.org/index.php) (36) and the Fusarium MLST database (http://www.cbs.knaw.nl/fusarium/). Identifications are conducted via BLASTn searches of either database using a partial translation elongation factor (1α) sequence of the unknown as the query or sequences from several other loci.

CLINICAL SIGNIFICANCE

The spectrum of disease caused by hyaline moulds is diverse. The disease is determined largely by the local and general immunologic and physiologic state of the host and may be symptomatic or asymptomatic. In most instances, the portal of entry for fungal propagules is likely by way of the lungs or through a break in the epidermis due to trauma. Exceptions to this include introduction by means of contaminated surgical instruments, intraocular lenses, prosthetic devices, or other contaminated materials or solutions associated with surgery or routine health care. Individuals whose resistance is lowered as a result of a severe debilitating disease or immunosuppressive therapy typically suffer from invasive pulmonary or paranasal sinus infection, but in some instances, the infecting fungus may spread to surrounding tissues or disseminate to virtually any organ. Fungemia is uncommon except in disseminated *Fusarium* infections. Noninvasive forms of infection also have been noted in debilitated individuals as well as in individuals with apparently normal defense mechanisms. In such cases, the fungus colonizes a preexisting cavity in the lungs, such as an ectatic bronchus, a tuberculous cavity, or a lung cyst. Other clinical syndromes usually occurring in immunocompetent individuals include chronic sinusitis, onychomycosis, subcutaneous abscess, keratitis, otomycosis, and allergic manifestations, including bronchopulmonary mycosis and sinusitis in atopic patients.

Although the majority of saprobic and plant-pathogenic moulds are not considered pathogenic for humans and other animals and appear unlikely to be able to adapt to or take advantage of risk factors predisposing individuals to opportunistic infection, those capable of growing at or near body temperature must be considered to have latent pathogenic capability. The diversity of fungi that have invaded human tissue has increased dramatically in recent years, as reflected by new reports of proven infection. Moreover, certain fungi are isolated often enough to be suspicious for pathogenic potential. Still, there is a need for definitive evidence of infection due to a normally saprobic mould. The laboratory procedure for confirming fungal etiology includes (i) detection in the specimen of hyphae that are compatible with the morphology of the isolated mould, (ii) isolation of several colonies of the fungus or isolation of the same fungus from two or more specimens over time, (iii) accurate identification of the isolated mould, and (iv) confirmation of the mould's ability to grow at or near body temperature. Species of *Fusarium* or other hyaline moulds isolated from all deep tissue or body fluids must be considered potential invasive opportunistic pathogens. No fungal isolate should be discarded as a contaminant without thorough examination of the clinical specimen. Quality control measures to ensure that isolation media are not contaminated and inspection of the slant or plate to evaluate where the fungus is growing relative to where the specimen was placed may also be important in evaluating whether an isolate is involved in disease. Close communication between microbiologists and physicians also is essential, especially for rare or unusual opportunists seen in individuals maintained on long-term immunosuppressive therapy.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Clinical specimens from patients suspected of having mycosis should be collected with prudence and transported to the laboratory and processed as soon as possible by using the standard procedures described in chapter 114 of this *Manual*. Because of the diverse clinical manifestations, various sites may need to be examined for fungal elements. Biopsy material, transtracheal aspirates, and sputum samples collected in the early morning all may be useful specimens for the isolation and detection of hyaline moulds, as are infected nails. Swabs taken from mucous membranes and skin lesions are not recommended. Although the reliability of determining whether an isolate is a possible pathogen is increased if cultures of two different specimens yield the same organism, a tissue biopsy specimen is more informative since the significance of culture can be verified by histological examination of the tissue. While blood cultures are generally of limited use for the detection of invasive hyaline moulds that produce dry conidia (microconidia in chains), such as *Aspergillus* (37), *Fusarium* and other species with slimy conidia (microconidia in clusters or balls) may be reliably detected (38). For optimum recovery, the specimen should be inoculated onto several types of media and incubated at 28 to 30°C. Opportunistic moulds are variably sensitive to cycloheximide, so media containing this selective agent should be used cautiously. Suspicious isolates, especially of uncommon species, should be tested for their ability to grow at 35 to 37°C. Potentially neurotropic species may also grow at 40°C and beyond (39, 40).

FUSARIUM SPECIES

Taxonomy

Fusarium species are cosmopolitan soil saprobes and facultative plant pathogens that can cause infection or toxicosis in humans and other animals (15, 26, 41, 42). They belong to the order Hypocreales, family Nectriaceae. Clinically significant species are mostly heterothallic, with only the anamorphic (asexual) state seen in culture. Teleomorphs occur in *Gibberella*, *Neocosmospora*, and other genera; however, these taxa should be reported under the *Fusarium* anamorph name (20). Several molecular phylogenetic studies have shown that organisms once considered individual species by morphologic features are now known to represent species complexes (SCs) (7, 8, 14–17, 36, 43, 44). Multilocus phylogenetic studies have revealed that the most frequently recovered clinically important fusaria are nested within the *Fusarium solani* species complex (FSSC) (Fig. 1A). This SC comprises over 60 phylogenetically distinct

TABLE 2 Key phenotypic features of clinically significant *Fusarium* species[a]

Complex	Colonial form	Characteristic(s) of:				
		Sporodochia[b]	Conidia	Macroconidia[c]	Microconidia	Chlamydospores
FSSC						
F. petroliphilum (FSSC 1), *F. keratoplasticum* (FSSC 2), and several other unnamed species (Fig. 1A)[d]	Mostly creamy, occasionally slightly blue-green, reddish, or lavender; floccose; rapid growth	Creamy in confluent pionnotes	Long monophialides	Multiseptate; abundant; stout; thick walled; dorsal and ventral surfaces only are almost parallel	Abundant; mostly 0–1 septum; oval to kidney shaped in false heads	Present, single, and in pairs
F. falciforme (FSSC 3+4, formerly *Acremonium falciforme*)	Creamy to pale brown, glabrous to velvety; slow growth; lavender reverse on SDA	Seldom seen on PDA	Long monophialides	Poor conidial production; most lack foot cells	1 to 3 celled	Present, often pale brown
F. lichenicola (formerly *Cylindrocarpon lichenicola*)	Initially white and then pale yellow to light brown; floccose; rapid growth	Seldom seen	Long monophialides	Straight; multiseptate; rounded at apices; truncate basal cells	Absent	Short chains and clusters, brown, rough
F. neocosmosporiellum (formerly *Neocosmospora vasinfecta*)[e]	Flat, thin, almost transparent; becoming punctate with production of orange to pale-brown perithecia	Absent	Long monophialides	Absent on PDA	Similar to those seen in *F. petroliphilum*	As in *F. petroliphilum*
FOSC						
F. oxysporum (Fig. 1B)[f]	White to lavender, salmon tinge, lavender reverse; floccose; rapid growth	Orange; erumpent	Short monophialides	Multiseptate; slightly sickle shaped; thin walled; delicate	Mostly 0 septa; oval to kidney shaped, in false heads only	Present, abundant, single and in pairs
FFSC						
F. verticillioides (formerly *F. moniliforme*) (Fig. 1C)	White to lavender with lavender reverse; floccose; rapid growth	Usually absent on PDA; tan to orange on CLA	Medium-length monophialides	Multiseptate; almost straight	0–1 septum; oval to clavate; truncate; occur in false heads and chains	Absent
F. thapsinum	Morphologically indistinguishable from *F. verticillioides* except for diffusing yellow pigment on PDA (not produced by all strains)	Same as for *F. verticillioides*	Same as for *F. verticillioides*	Same as for *F. verticillioides*	Same as for *F. verticillioides*	Same as for *F. verticillioides*
F. napiforme	White to lavender, lavender reverse	Usually absent on PDA; tan on CLA	Medium-length monophialides	Multiseptate; falcate to almost straight	0–1 septum; ovoid to pyriform (pear shaped) to napiform (beet shaped) in false heads and short chains	Sparse, short chains or clusters
F. proliferatum (Fig. 1D)	White to lavender, lavender reverse; floccose; rapid growth	May be absent on PDA; tan on CLA	Monophialides and polyphialides	Multiseptate; falcate to almost straight	Oval to pyriform; truncate; occur in false heads	Absent
F. nygamai	White to lavender, lavender reverse; floccose; rapid growth; an orange to violet spore mass is common centrally	Orange on CLA	Monophialides and polyphialides; however, polyphialide production is variable	Multiseptate; falcate to almost straight; thin walled	Oval to clavate; mostly 0 septa in false heads and short chains (up to 20 conidia in length)	Few to abundant, single, chains, clusters; smooth or rough, hyaline to yellow

(Continued on next page)

TABLE 2 *(Continued)*

Complex	Colonial form	Characteristic(s) of:				
		Sporodochia[b]	Conidia	Macroconidia[c]	Microconidia	Chlamydospores
FCSC						
F. chlamydosporum	White to pink to carmine, brown centrally with production of chlamydospores; floccose; rapid growth	Uncommon on PDA; tan to orange on CLA	Short monophialides and short polyphialides, often with 3 openings	Rare except on sporodochia	0–2 septa; fusiform, apiculate; may be slow to form in some strains on PDA	Abundant, brown, rough, in chains and clusters
FDSC						
F. dimerum (Fig. 1E)	Slimy, yeastlike due to conidial masses; aerial mycelium is sparse to absent; salmon colored to light orange, reverse is the same or pale yellow; slow growth	Orange; well developed on CLA	Monophialides; conidiation occurs on an agar surface from lateral phialidic pegs	Abundant; 0–1 septum, with septum in middle; curved	Ellipsoidal to ovoidal to curved; mostly 1 celled	Derived from macroconidia; hyphal chlamydospores are rare or absent
F. delphinoides (Fig. 1F)	Similar to *F. dimerum* except for reverse, which may be speckled with red-brown clumps of pigment	Same as for *F. dimerum*	Same as for *F. dimerum*	Can have 2 septa; when there is 1 septum, the septum is off-center	Same as for *F. dimerum*	Same as for *F. dimerum*
FIESC						
Fusarium sp.[g]	Buff to light brown, reverse is salmon colored; floccose; rapid growth	Orange; produced by some strains on CLA	Monophialides and polyphialides	Those produced in aerial mycelium are almost straight; those produced in pionnotes[h] or sporodochia are curved	0 septa; sparse or absent	Sparse, intercalary, single cells or chains

[a] On potato dextrose agar (PDA) after 4 days of incubation at 25°C unless otherwise noted. See references cited in the text for a more detailed description of the features noted in this table. The list is not all-inclusive. CLA, carnation leaf agar; SDA, Sabouraud dextrose agar.

[b] Cushion-shaped mats of hyphae, conidiophores, and macroconidia.

[c] The most characteristic macroconidia for all species are those formed in sporodochia on CLA; macroconidia, also formed in aerial mycelium on PDA, are often smaller.

[d] At least 20 species within the FSSC have been implicated in mycoses of humans and other animals. *Fusarium petroliphilum* (FSSC 1), *F. keratoplasticum* (FSSC 2), and *F. falciforme* (FSSC 3+4) account for most infections caused by members of the FSSC (12, 46).

[e] This microconidial, self-fertile, or homothallic *Fusarium* species frequently produces perithecial fruiting bodies in culture. Perithecial walls are of the textura angularis type, and asci are cylindrical and eight spored (30 to 100 by 11 to 15 μm); ascospores are one celled, thick walled, rough, yellow to brown, and globose to ellipsoidal (10 to 15 by 7 to 12 μm).

[f] The FOSC is phylogenetically diverse and comprises at least 26 two-locus sequence types associated with mycoses (49), including the widespread clonal lineage ST 33, recovered from hospital and other plumbing systems (14, 44).

[g] Of the 20 species within the FIESC that have been implicated in mycotic infections of humans and other animals (13), Latin binomials can be applied with confidence only to *F. equiseti* (FIESC 14) and *F. lacertarum* (FIESC 4). Although the names *F. incarnatum*, *F. pallidoroseum*, and *F. semitectum* are commonly applied to members of the FIESC, these names should not be used until it is determined what species they represent.

[h] A flat mass of macroconidia having a greasy or fatty appearance.

species within three major clades, with the human isolates restricted to clade 3 (12, 45). Clade 3 was also shown to be associated with four major species-level lineages, designated groups 1 through 4, with many strains clustered in groups 1 and 2 (8, 17, 46). Subsequently, more-robust typing studies identified 34 species within clade 3 of the FSSC (12). The FSSC currently also includes *Fusarium petroliphilum* (FSSC 1), *Fusarium keratoplasticum* (FSSC 2) (47), *Fusarium falciforme* (FSSC 3+4), and *Fusarium neocosmosporiellum* (formerly *Neocosmospora vasinfecta*) (48). Although the *Fusarium oxysporum* SC (FOSC) is best known as an economically devastating group of vascular wilt pathogens on a wide range of agriculturally important plant hosts (49), it is also the second-most-common *Fusarium* SC responsible for human disease (Fig. 1B). The FOSC is also phylogenetically diverse (50). Multilocus sequence typing and amplified fragment length polymorphism (AFLP) analyses of over 100 isolates from the environment, hospital bronchoscopy and water sources, and clinical isolates revealed a geographically widespread clonal lineage comprising >70% of isolates genotyped (14). These studies have further supported hospital water distribution systems as a potential source of nosocomial infection (51, 52). Several medically important species are included within the species-rich *Fusarium fujikuroi* SC (FFSC), as defined by multilocus genotyping (7, 9, 10). These include *Fusarium verticillioides* (Fig. 1C), *Fusarium proliferatum* (Fig. 1D), *Fusarium napiforme*, and *Fusarium nygamai*. Another morphologically and phylogenetically diverse group of fusaria includes those previously referred to as *Fusarium semitectum* or *Fusarium incarnatum* and now known to reside in the *Fusarium incarnatum-Fusarium equiseti* species complex (FIESC) (11). Multilocus phylogenetic studies have demonstrated that this complex encompasses at least 28 species (13). The *Fusarium chlamydosporum* SC

TABLE 3 Key phenotypic features of selected hyaline moulds[a]

Genus	Key features	Etiologic agents	Comments
Homothallic ascomycetes[b]			
Achaetomium	Colonies are fast growing, white to yellowish with pink diffusible pigment (Fig. 1G). Conidia are formed from minute phialides. Ascomata perithecia bear thin-walled setae. Ascospores are brown, smooth, and fusoidal.	*A. strumarium*	Growth at 42°C, neurotropic; compare with *Chaetomium*. Also see chapter 124.
Aphanoascus	Colonies are moderately fast growing, yellowish white, granular, and cycloheximide tolerant. Cleistothecia are globose, contain roughened ascospores, and are associated with a *Chrysosporium* anamorph consisting of terminal 1-celled sessile conidia and alternate arthroconidia.	*A. fulvescens*	Compare with *Chrysosporium* and dermatophytes.
Cephalotheca	Colonies are moderately fast growing, velvety to lanose, and orange-gray with a light-brown reverse. This ascomycete is homothallic and forms black, superficial, ciliated cleistothecia and small, brown, kidney-shaped, foveolate (delicately pitted) ascospores (Fig. 2D); it is associated with a *Phialemonium*-like anamorph (Fig. 2E).	*C. foveolata*	UV light and extended incubation enhance ascoma production.
Chaetomium	Colonies are fast growing and yellowish green to gray. Anamorphs are absent, or conidia are formed from phialides. Ascomata perithecia bear coiled, straight, branched brown, or indistinct setae. Ascospores are lemon shaped, brown, and smooth.	*C. atrobrunneum*, *C. globosum*, *C. perlucidum*	*C. atrobrunneum* and *C. perlucidum* grow at 42°C and are neurotropic. Also see chapter 124.
Gymnascella	Colonies are yellowish white, becoming bright yellow or yellowish green or orange (Fig. 1H and 2A). Ascospores are borne in naked clusters, smooth, and pale yellow. Anamorphs are absent. Ascospores of *G. hyalinospora* are oblate and yellowish (Fig. 2B); those of *G. dankaliensis* are reddish orange and ornamented with a thickened polar band and minute thickenings on the sides.	*G. hyalinospora*, *G. dankaliensis*	*G. hyalinospora* is also known as *Narasimhella hyalinospora*; it may give positive results for *B. dermatitidis* with the Gen-Probe DNA probe.
Microascus	Colonies are moderately fast growing, hyaline, or gray-brown to black. Ascomata are perithecia with necks (Fig. 2C) and contain yellowish to reddish-orange (straw-colored) ascospores extruded in cirri; they are associated with hyaline or phaeoid *Scopulariopsis* anamorphs.	*M. cirrosus*, *M. cinereus*, *M. trigonosporus*, *M. manginii*	Ascospore shapes for the first 3 species listed, which are dark, heart shaped, orange section shaped, and triangular, respectively.
Thermoascus	Colonies are yellow-orange to reddish-orange and woolly to granular. They are thermophilic, with growth to 50°C. Ascomata are nonostiolate; ascospores are pale yellow, elliptical, thick walled, and rough. *Paecilomyces* anamorphs, *P. crustaceous*, and *P. taitungiacus* have conidia that are initially rectangular but become elliptical to subglobose.	*T. crustaceus*, *T. taitungiacus*	*T. taitungiacus* fails to grow at 20°C and has irregularly verrucose ascospores. *T. crustaceus* has finely echinulate ascospores.
Filamentous basidiomycetes			
Hormographiella	Colonies are fast growing, white, amber to tan, and woolly; they are cycloheximide sensitive and benomyl resistant. Conidiophores are short and bear short fertile branches. Arthroconidia are schizolytic, thin-walled, single celled, and often adherent around the conidiophores. Sclerotia are sometimes present. This basidiomycete is an anamorph.	*H. aspergillata* (anamorph of *Coprinus cinereus*), *H. verticillata*	Differs from *A. kalrae* by cycloheximide sensitivity and a high growth rate.
Inonotus	Colonies are fast growing, woolly, and yellowish-orange; they are cycloheximide and benomyl resistant. Setal hyphae are thick walled, and hyphal swellings are present; conidia are absent (Fig. 3A). Identification should be confirmed with sequencing.	*I. tropicalis*	Synonym *Phellinus tropicalis*; wood-destroying poroid basidiomycete.
Oxyporus	Colonies are moderately fast growing at 35°C, but growth is poor at 25°C; they are white and woolly. Conidia are absent. Identification should be confirmed with sequencing.	*O. corticola*	White-rot decay fungus of woody angiosperms and gymnosperms.
Quambalaria	Colonies are cycloheximide sensitive, moderately fast growing, and white to lavender; a red diffusible pigment is often present. Conidiophores are solitary and form conidia sympodially on small denticles on the sides or at the tips. Primary conidia bear 1–3 secondary conidia.	*Q. cyanescens*	Synonyms include *Fugomyces cyanescens*, *Sporothrix cyanescens*, and *Cerinosterus cyanescens*.

(Continued on next page)

TABLE 3 *(Continued)*

Genus	Key features	Etiologic agents	Comments
Schizophyllum	Colonies are fast growing, white, woolly or cottony, and cycloheximide sensitive, and growth is enhanced at 37°C. They are usually sterile. Hyphae bearing clamp connections resemble aspergilli and short, thin pegs or spicules, but both may be absent (Fig. 2G). Clamped isolates usually develop fan-shaped, gilled basidiocarps (mushrooms) on sporulation media after 3–6 weeks (Fig. 2H).	*S. commune*	Clinical picture resembles aspergillosis and clampless isolates by histopathology (Fig. 2F).
Sporotrichum	Colonies are fast growing, white to buff to beige, and woolly. Conidiophores form with profuse branching, with each branch terminating with a conidium. Conidia are ellipsoidal, truncate, thick walled, and smooth. Large (20–60 µm) globose chlamydospores and arthroconidia are also present. This basidiomycete is an anamorph.	*S. pruinosum* (anamorph of *Phanerochaete chrysosporium*)	Large globose chlamydospores similar to those produced by *Emmonsia parva*; can be seen in sputum.
Hyphomycetes			
Acremonium	Colonies are slow growing (usually <3 cm in 10 days), often white, cottony, fasciculate (spiky), glabrous or moist, and pink or salmon colored. Conidiogenous cells are solitary, slender (ca. 2 µm wide), mostly unbranched, awl (needle)-shaped phialides. Conidia are 1 celled and straight or curved and grow in slimy masses.	*A. sclerotigenum*, *A. egyptiacum*, *A. implicatum*, *A. recifei*, *A. persicinum*, *A. fusidioides*, *Acremonium* spp.	Differs from *Fusarium* by having a low growth rate, narrower hyphae (mostly <2 µm in width), and more-slender and needle-like phialides. Compare with *Coniochaeta* and *Phialemonium*.
Acrophialophora	Colonies are fast growing, white, and dark to grayish brown centrally. Conidiophores are brown, long, seta-like, and echinulate. Conidiogenous cells are flask-shaped phialides borne near the tip of conidiophores or on vegetative hyphae. Conidia occur in long chains and are 1 celled, lemon shaped, smooth, or rough, and distinct spiral bands may be visible.	*A. fusispora*	Growth at 40°C. Potentially neurotropic species. Compare with *Paecilomyces* spp., *Scedosporium prolificans*, and *Phialosimplex*.
Arthrographis	Colonies are slow growing and cycloheximide tolerant. They are initially white and yeastlike but become hyphal and buff with a yellow reverse. Growth is enhanced at 37°C. Conidiophores are dendritic (treelike), bearing lateral branches. Arthroconidia are formed by fragmentation of branches or from undifferentiated hyphae.	*A. kalrae*	Compare with *Onychocola* and *Hormographiella*.
Beauveria	Colonies are slow to moderately fast growing and yellowish white. Conidiogenous cells are solitary (in whorls or in sporodochia) and basally swollen, proliferating sympodially at the tip in a zigzag (geniculate) fashion. Conidia are 1 celled and subglobose.	*B. bassiana*	Compare with *Engyodontium album* and *Sporothrix*. A biological control agent for insects.
Chrysosporium	Colonies are slow to moderately fast growing and yellowish white (Fig. 3B). Conidia are single celled and smooth to rough; aleurioconidia are sessile or at the ends or on the sides of unswollen stalks (Fig. 3C). Arthroconidia are sometimes present. (See *Aphanoascus* and *Emmonsia*.)	*C. zonatum*, *C. ophiodiicola* (anamorph of *Nannizziopsis vriesii*)	Reports of infection by unnamed species are difficult to evaluate because isolates are not adequately described to confirm etiology.
Coniochaeta	Colonies are white to salmon, moist or fasciculate (spiky), or tan, darkening to black in patches. Conidiogenous cells are adelophialides (short, stumpy phialides without a basal septum), as well as awl-shaped phialides. *C. mutabilis* forms brown chlamydospores, while *C. hoffmannii* does not.	*C. hoffmannii*, *C. mutabilis*	Differs from *Acremonium* by predominance of adelophialides. Hyphal elements usually are reported as hyaline. Also see chapter 124.
Cylindrocarpon	Colonies are fast growing, felty or cottony, yellowish white, tan, orange, or purple and sometimes diffuse pigments. Conidiogenous cells are awl-shaped phialides with a single opening; they are solitary in branched structures or in sporodochia (conidiophores are crowded in a compact mass of hyphae). Macroconidia are straight or slightly curved with rounded ends and multicelled; microconidia are not clearly distinguished from macroconidia. Chlamydospores are sometimes present.	*C. destructans*, *C. cyanescens*	Distinguished from *Fusarium* by rounded apical cells and the absence of foot cells.

(Continued on next page)

TABLE 3 Key phenotypic features of selected hyaline moulds[a]

Genus	Key features	Etiologic agents	Comments
Engyodontium	Colonies are slow to moderately fast growing and yellowish white. Conidiogenous cells are solitary or borne in whorls and basally swollen; they taper at the tip and proliferate sympodially in a zigzag (geniculate) fashion. Conidia are 1 celled and subglobose.	*E. album*	Compare with *Beauveria*.
Metarhizium	Colonies are moderately fast growing, becoming olivaceous green or buff. Conidiogenous cells are cylindrical phialides borne on verticillately or irregularly branched conidiophores formed on sporodochia. Conidia are cylindrical, smooth, and yellowish-green, and they form in adherent columns. Irregularly shaped appressoria may be present.	*M. anisopliae*	Biological control agent for insects.
Myceliophthora	Colonies are fast growing, thermophilic (up to 50°C), and cinnamon brown; 1 to 3 conidia are formed on small denticles borne on the sides or at the ends of short, swollen stalks (Fig.3E). Conidia are initially smooth and hyaline but brown and rough at maturity.	*M. thermophila*	Differs from *Chrysosporium* by swollen stalks.
Myriodontium	Colonies are moderately fast growing, yellowish white, often zonate, and powdery. Conidia are formed at the ends of narrow stalks borne at right angles to the fertile hyphae.	*M. keratinophilum*	Differs from *Chrysosporium* by stalks borne at right angles.
Onychocola	Colonies are restricted, raised, yellowish white to grayish white, and cycloheximide tolerant (Fig. 3F). Conidia are 1- or 2-celled cylindrical or swollen arthroconidia forming in adherent chains; they detach by schizolysis or lysis of thin-walled cells. Brown knobby setae are often present (Fig. 3G).	*O. canadensis*	Differs from *Neoscytalidium dimidiatum* (see chapter 124) and *Hormographiella* by having adherent chains, slow growth, and cycloheximide tolerance.
Paecilomyces	The section on *Paecilomyces* includes the thermotolerant *P. variotii* and anamorphs of *Byssochlamys* and *Thermoascus*, with fast-growing yellowish brown, buff, or orange colonies (Fig. 3H). Conidiogenous cells are phialides formed on verticillately branched conidiophores. Conidia are single celled and occur in chains.	*P. variotii*, *P. formosus*, *P. javanicus*, *P. fumosoroseus*	See *Thermoascus* for additional anamorphic species.
Phialemonium	Colonies are slow growing and white to grayish. Conidiogenous cells are adelophialides (short, stumpy phialides without a basal septum), as well as awl-shaped phialides. *P. ovobatum* produces a green diffusible pigment.	*P. obovatum*, *P. atrogriseum*, *P. inflatum*, *P. globosum*	Compare with *Acremonium* and *Coniochaeta*. Also see chapter 124.
Phialemoniopsis	Colonies are slow growing and yellowish white to grayish (Fig. 4C). Conidiogenous cells are adelophialides and awl-shaped phialides.	*P. curvata*, *P. pluriloculosa*, *P. cornearis*, *P. ocularis*	Compare with *Phialemonium* and *Coniochaeta*.
Phialosimplex	Colonies are moderately fast growing, pale, white, and creamy to yellowish white; they sometimes fasciculate centrally with occasional sectoring. Some species produce diffusible yellow pigment (Fig. 4D). They are inhibited by cycloheximide. Conidiogenous cells are mostly monophialides, narrow, and swollen at the base (Fig. 4E). Conidia are borne in long chains or heads, hyaline, subglobose, pyriform, obovoid or ovoid, and truncate. Chlamydospores and sclerotia are present or absent.	*P. caninus*, *P. chlamydosporus* (synonym, *Sagenomella chlamydospora*), *P. sclerotialis*	Compare with *Acrophialophora fusispora*.
Purpureocillium	*Purpureocillium* exhibits slower growth than *Paecilomyces* spp., with maximum growth at 25–33°C; conidia are lilac colored (Fig. 4A), and chlamydospores are absent (Fig. 4B).	*P. lilacinum*	Compare with *Paecilomyces* and *Rasamsonia*.
Rasamsonia	Colonies are moderately fast growing, with enhanced growth at 35°C. They are creamy to buff colored. Stipes and metulae are rough. Cuneiform (wedge-shaped) to ellipsoidal conidia are borne from rough-walled phialides lacking narrow necks, as with *Penicillium* or *Paecilomyces* (Fig. 3D).	*R. argillacea*	Synonym *Penicillium argillaceum*/*Geosmithia argillacea*. Compare conidia with those of *P. crustaceus*.
Sarocladium	*Sarocladium* is phylogenetically distinct from *Acremonium*. Several species yield ochre-brown colors on SDA. Conidiogenous cells are adelophialides or long slender phialides.	*S. kiliense*, *S. strictum*, *S. bacillisporum*	Compare with *Acremonium*.

(Continued on next page)

TABLE 3 *(Continued)*

Genus	Key features	Etiologic agents	Comments
Scopulariopsis	Colonies are white, buff, and gray-brown to black. Conidiogenous cells are annellides formed on branched conidiophores with 1 or 2 levels of branching. Conidia are 1 celled, globose, or ellipsoidal and occur in chains. *S. brevicaulis* has buff or tan granular colonies, with thick-walled smooth to coarsely roughened conidia that are truncate at the base and rounded or pointed at the tip (Fig. 4F). *S. candida* has white colonies and smooth conidia.	*S. brevicaulis*, *S. candida*, *S. acremonium*	See also *Microascus* and chapter 124 for phaeoid species.
Trichoderma	Colonies are fast growing, cottony or woolly, and white, becoming yellowish green to dark green, sometimes with a diffusing yellow pigment (Fig. 4G). Conidiogenous cells are flask-shaped or cylindrical phialides that occur singly or in whorls; conidia are green, smooth, and ellipsoidal to subglobose (Fig. 4H).	*T. longibrachiatum*, *T. citrinoviride*, *T. harzianum*	The report concerning *T. viride*, *T. koningii*, and *T. pseudokoningii* is not substantiated.
Coelomycetes			
Colletotrichum	Colonies are fast growing, woolly, tan to gray-brown, occasionally greenish, and sometimes lavender, with honey-colored masses of conidia. Brown appressoria are usually present; conidiomata (fruit bodies) are acervular, conidiogenous, phialidic cells. Conidia are hyaline, aseptate, and straight to curved; they also form in aerial mycelium. Setae and sclerotia may be present.	*C. gloeosporioides*, *C. coccodes*, *C. dematium*, *C. crassipes*	Colonies may be dark, with better sporulation on potato carrot agar. Compare *C. dematium* with *Fusarium*.
Phoma	Colonies are pale tan to gray-brown and woolly. Conidiomata are pycnidial, usually dark, separate, or aggregated. Ostioles occur singly or in groups, are immersed or semi-immersed, and are mostly thin-walled. Conidiogenous cells are phialidic, and conidia are one celled, small, hyaline, and often guttulate.	Several species are reported in the literature, but most are not well documented.	Morphologically similar to *Pleurophoma*. Species-level identification is difficult. See chapter 124 for additional coelomycetous genera.

[a] Species listed produce their teleomorph (sexual structures) in culture.

[b] On potato dextrose agar (PDA) after 4 days of incubation at 25°C unless otherwise noted. See references cited in the text for a more detailed description of the features noted in this table. The list is not all inclusive.

(FCSC) is also phylogenetically diverse and represents four species based on multilocus genotyping (13). Similarly, the *Fusarium dimerum* species complex (FDSC) (Fig. 1E and F) currently contains seven named and five unnamed species (53). Members of the FDSC are unlike those in the aforementioned SCs in that they frequently display slower growth, and aerial mycelium is either absent or only sparsely developed, so colonies appear mucoid to slimy (usually some shade of orange).

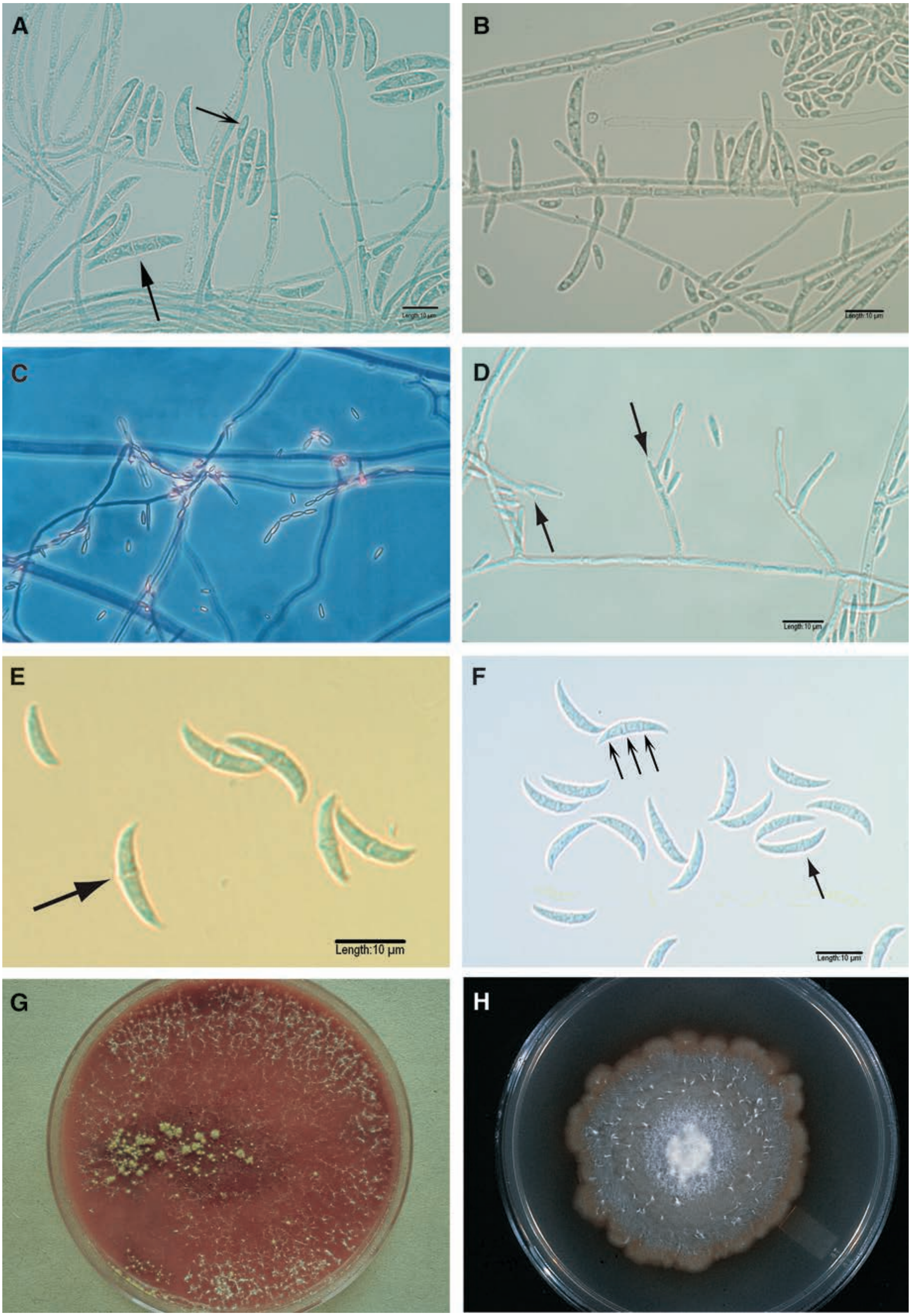
Description of Agents

As can be seen from the taxonomic discussion, the *Fusarium* species cited as etiologic agents of human and animal diseases mostly fall within six SCs. Based upon our current understanding of the genus, and recognizing the likelihood of future taxonomic changes, the more common species within these complexes are as follows: FSSC, *Fusarium falciforme*, *Fusarium petroliphilum*, *Fusarium keratoplasticum*; FOSC, *Fusarium oxysporum* (phylogenetically diverse but the species limits are not resolved currently); FFSC, *Fusarium verticillioides*, *Fusarium thapsinum*, and *Fusarium proliferatum*; FCSC, *Fusarium chlamydosporum*; FDSC, *Fusarium dimerum* and *Fusarium delphinoides*; and FIESC, unnamed species frequently reported incorrectly as *Fusarium incarnatum*/*Fusarium pallidoroseum*/*Fusarium semitectum*. See also Table 2.

Epidemiology and Transmission

There are numerous toxic secondary metabolites produced by *Fusarium* species that have been implicated in human disease, especially associated with the consumption of contaminated grain (see chapter 126). Alimentary toxic aleukia was described in individuals who ate grain contaminated with trichothecene mycotoxins produced by *Fusarium sporotrichioides* or *Fusarium poae* (54). Systemic effects of exposure to inhaled mycotoxins, in addition to orally ingested mycotoxins, have also been attributed to *Fusarium*, although these effects have been much less characterized (55). The more common clinical presentation in human disease is associated with fusarial conidia gaining access to the host and germinating and subsequent tissue invasion by hyphae. The portal of entry, however, is unknown in most cases of invasive infection. Ingestion or access through mucosal membranes may occur in some (56, 57). Disseminated infection may also follow onychomycosis, often associated with cellulitis (58). Hospital water supply and distribution systems have also been implicated as a source of infection in immunocompromised patients by Anaissie et al. (51) and O'Donnell et al. (14). The most common mode of transmission, however, appears to be inhalation of airborne conidia (59).

Although invasive infection by *Fusarium* is uncommon, an increased incidence of disseminated infection has been seen in neutropenic patients with hematologic cancer, in recipients of solid-organ transplants, and in recipients of allogeneic hematopoietic stem cell transplants (HSCTs) (1, 38, 42, 57, 60–69). The incidence of fusariosis among allogeneic HSCT recipients varied between 4.21 and 5.0 per 1,000 cases in related, human leukocyte antigen (HLA)-matched transplant recipients and 20.19 per 1,000 cases in HLA-mismatched transplant recipients (68). Among allogeneic HSCT recipients, a trimodal distribution was observed: a first peak before engraftment, a second peak at a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median of 62 days after transplantation, and a third peak >1 year after transplantation. The actuarial survival was 13% (median, 13 days), with persistent neutropenia (hazard ratio of 5.43) and use of corticosteroids (hazard ratio of 2.18) being the most important factors associated with poor outcome (65, 68).

Clinical Significance

Fusarium species can cause a spectrum of diseases in both healthy and immunocompromised hosts. A frequent infection in the normal host is keratitis resulting from trauma and penetration of the cornea, contamination of soft contact lenses and/or solutions (11, 46, 66, 70–72), or local immunosuppression by corticosteroid drops (66). The keratitis outbreaks recognized in Hong Kong (72), Singapore (71), and the United States (46) in 2005 and 2006 in contact lens wearers using ReNu with MoistureLoc solution (Bausch & Lomb, Rochester, NY) resulted in the largest investigation and molecular characterization of fusarial keratitis isolates to date and clearly demonstrated that these strains occurred in nearly the full spectrum of clinically significant fusarial SCs. Other presentations in the immunocompetent host include those listed in Table 1. Cutaneous lesions in various stages of evolution (68, 73), fungemia (74), rhinocerebral involvement, pneumonia, endogenous endophthalmitis (75, 76), or combinations of these are common clinical findings in disseminated fusarial disease (38, 42, 57, 65–69, 77, 78). Unlike in invasive cases of aspergillosis, recovery of *Fusarium* species from the blood in disseminated disease approaches 60% (38, 67). Members of the FSSC (typically reported incorrectly as *Fusarium solani*) are the most frequently cited agents of disease, and most cases involve keratitis (26, 79). Mayayo et al. (80) consider members of this SC to be the most virulent, and it has been suggested that production of cyclosporine may contribute to their pathogenicity (81). Members of the FOSC (14) and *Fusarium verticillioides* (82) in the FFSC appear to be the next-most-common organisms recovered, with a similar spectrum of infection. *Fusarium verticillioides* was the most frequently isolated species in deep-seated infections in Italy (83). Given our current understanding of the genus, it is now difficult to attribute older clinical case reports to the newly defined species. See references 26, 35, 53, 83, and 84 for a more complete list of *Fusarium* species known as etiologic agents in human and animal disease.

Collection, Transport, and Storage of Specimens

Methods of collection, transport, and storage of specimens are detailed in chapter 114. Like invasive aspergillosis, invasive *Fusarium* infections are difficult to diagnose and usually require a combination of clinical, cultural, and radiographic findings. However, unlike *Aspergillus*, *Fusarium* is frequently recovered from blood, nails, and skin lesions of immunocompromised or immunosuppressed patients (56, 85). The recovery from a normally sterile site and microscopic evidence of invasive growth in tissue provide the most convincing evidence of invasive fusariosis.

Direct Examination

Microscopy

In direct examination of tissues, hyphae of *Fusarium* species resemble those of *Aspergillus*, *Paecilomyces*, or *Scedosporium* species in size (3 to 6 mm in width), septation, branching pattern, and predilection for vascular invasion. The hyphae are irregular in width and may show areas of collapse. Hyphae exhibit both dichotomous branching (i.e., branching at 45°), as is commonly seen in invasive aspergillosis, and branching at right angles (86, 87). Microconidia and, rarely, macroconidia and budding cells, as well as phialides, may be found in blood vessel lumens or in aerated tissues (87). Immunohistological methods may help to distinguish between infections caused by some hyaline moulds, but cross-reactivity is problematic and serological tests are not in common use (88). Definitive diagnosis requires isolation and identification of the fungus, along with demonstration of fungal elements in tissue.

Antigen Detection

No commercial system is available for detection of genus-specific or species-specific antigens released by *Fusarium* species in human infection. The diagnostic utility of polyclonal fluorescent-antibody reagents to members of the FSSC in tissue sections from patients with invasive fusariosis was evaluated, but extensive cross-staining was observed with sections containing aspergilli, *Purpureocillium lilacinum*, and *Pseudallescheria boydii* (88).

There is one report that describes 9 out of 11 hematological patients with disseminated or deep-seated *Fusarium* infection who had repeated serum-positive reactions with the Platelia *Aspergillus* galactomannan (GM) enzyme-linked immunosorbent (EIA) assay (Bio-Rad, Marne La Coquette, France) in the absence of *Aspergillus* spp. (89). Exoantigen extracted from *Fusarium* species, including *F. oxysporum*, *F. proliferatum*, *F. falciforme*, and *F. verticillioides*, produced positive reactions when tested undiluted by the GM EIA assay *in vitro* (89), suggesting that *Fusarium* species may produce an exoantigen that is cross-reactive with the Platelia *Aspergillus* assay. Antigens prepared from *F. verticillioides* were also found to cause borderline cross-reactivity with the Platelia *Candida* antigen assay (Bio-Rad) (90). This reactivity appeared to be specific for *F. verticillioides*, since extracts prepared from members of the FSSC and FOSC showed no cross-reactivity *in vitro*.

Although (1→3)-β-D-glucan is not immunogenic, it is an important cell wall polysaccharide in most fungi that can be detected in serum via activation of a β-D-glucan-

FIGURE 1 (A) Member of the FSSC. Microconidia are borne on long monophialides (thin arrow). Macroconidia borne in the aerial mycelium are also present (thick arrow). (B) Member of the FOSC bearing microconidia on short monophialides. A few macroconidia are also present. (C) Chains of microconidia produced by *Fusarium verticillioides* (formerly *Fusarium moniliforme*) in the FFSC. (D) *Fusarium proliferatum* in the FFSC. Note polyphialides (arrows) with more than one opening not delimited by a septum. Truncate conidia that have been borne in chains as well as false heads are also present. (E) Macroconidia of *Fusarium dimerum* in the FDSC. Note that conidia are two celled and that the septum is in the middle. (F) Macroconidia of *Fusarium delphinoides* in the FDSC. Note that the septum in two-celled conidia is off-center (thick arrow) and that a macroconidium with three septa (thin arrows) and four cells is also present. (G) Colony of *Achaetomium strumarium* showing yellowish surface mycelium and pink pigment on cornmeal agar after 5 weeks. (H) An isolate of *Gymnascella hyalinospora* described in reference 137 was glabrous and sterile when grown on potato dextrose agar for 15 days at 30°C. doi:10.1128/9781555817381.ch120.f1 sensitive proteolytic coagulation cascade. Circulating (1→3)-β-D-glucan can be detected in the blood of patients with *Fusarium* fungemia and pneumonia (91, 92), although the presence of this marker is not specific for *Fusarium* (93). The sensitivity for detection of *Fusarium* infection was found to be 100% at a cutoff value of 60 pg/ml among three patients with invasive fusariosis (92). The clinical experience of (1→3)-β-D-glucan detection in patients with invasive fusariosis is limited, but the test is of value for patients suspected of having invasive fungal infection as part of their diagnostic workup. In a mouse model, (1→3)-β-D-glucan remains high throughout the infection, suggesting that this marker may provide a specific and sensitive diagnostic tool for the detection of invasive fusariosis (94). False-positive reactions of (1→3)-β-D-glucan are known to occur in some patients. These include subjects who had experienced renal failure and who were undergoing hemodialysis with cellulose membranes (95), subjects treated with certain immunoglobulin products (96), and specimens (or subjects) exposed to glucan-containing gauze or related materials (97). This underscores the need to combine (1→3)-β-D-glucan monitoring with clinical examination of the patient and other diagnostic procedures, such as high-resolution computed tomography scans, when making a diagnosis.

Nucleic Acid Detection Techniques

While most fusaria can be identified as members of this genus by means of microscopic and macroscopic features, accurate identification of most isolates to the species level requires molecular data. However, with the exception of two Web-accessible databases dedicated to the identification of fusaria, only a very limited number of molecular tools have been developed. In one study using PCR, *Fusarium* DNA was detected in tissues in a mouse model of disseminated fusariosis (98). In another study, a panfungal PCR was designed based on the 18S rRNA gene sequence and applied to ocular specimens from three patients suspected of having bacterial or fungal endophthalmitis. Although *Fusarium* was detected in spiked specimens, no clinical samples from patients with *Fusarium* endophthalmitis were analyzed (99). PCR-based identification was also applied to *Fusarium* isolates obtained from patients with onychomycosis (100). The *Fusarium* species identified were the same as those known to cause disseminated fusariosis in immunocompromised patients.

Panfungal PCR systems that detect a wide range of fungi, including *Fusarium*, have been developed. In one study, the PCR was positive with blood samples from a patient with an invasive fusariosis and blood cultures positive for a member of the FSSC (101). Luminex microbead hybridization technology using 75 genus-specific hybridization probes has recently been reported to detect a variety of fungal pathogens from blood and pulmonary samples, including *Fusarium* (102). As with other opportunistic fungi, molecular methods, such as PCR, appear to have promise for the detection and identification of fusaria causing infections; however, the value of these tools in the management of patients remains to be investigated.

Isolation Procedures

In general, *Fusarium* species can be recovered easily on routine mycological media without specific growth requirements. Given the aggressive nature of fusaria in neutropenic, immunocompromised individuals, however, the use of a medium for direct plating of specimens that can aid an early diagnosis by displaying consistent morphological features, such as potato dextrose agar (PDA), is highly recommended. Most *Fusarium* species grow rapidly on PDA in the absence of cycloheximide, which can be inhibitory. See chapter 114 for detailed information on specimen collection and processing.

Identification

For the limited number of *Fusarium* species that can be identified using phenotypic data, microscopic features of phialide shape, number of openings on the phialides (i.e., monophialides or polyphialides), formation of conidia in heads or chains, micro- and macroconidial shape and septation, the presence and arrangement of chlamydospores, colony features (including growth rates and color of colony obverse and reverse) (103), and color of conidial masses are important characters. However, considerable proficiency is required to identify *Fusarium* species with certainty, and a reference laboratory should be consulted. Although fusaria grow well on most mycological media, the medium can profoundly influence the colony morphology, color, and conidium development. Synthetic nutrient agar, PDA, and tap water agar supplemented with either sterilized carnation leaves or potassium chloride are widely employed (42, 104). Use of a rich medium to maintain isolates can result in cultural degeneration. Alternatively, molecular tools to help identify clinical *Fusarium* isolates are being developed (35, 100, 105). With the wide application of matrix-assisted laser desorption–ionization time of flight mass spectrometry (MALDI-TOF MS) in clinical microbiology laboratories, this technique may allow for the rapid and accurate genus- and species-level identification of *Fusarium* isolates (106, 107). A recently recommended biosafety level (BSL) classification for fusaria is either BSL 1 or BSL 2, depending upon the species (26). Clinical isolates should be handled in a biological safety cabinet.

Typing Systems

As with *Aspergillus* and *Candida*, multilocus DNA sequence typing (MLST) systems have been developed to differentiate clinically and veterinarily important isolates of *Fusarium* (43, 44, 108, 109). Recently, the Clinical and Laboratory Standards Institute published a document for identification of bacteria and fungi by DNA target sequencing (110), and a consortium of international experts assembled as an ISHAM working group on fungal identification has provided clinical laboratories with initial recommendations for molecular typing of *Aspergillus*, *Fusarium*, and the Mucorales (111). These single-locus (internal transcribed spacer [ITS]) guidelines for placing fusaria within an SC are based upon knowledge gained from multilocus sequencing (*ITS-LSU*, *EF-1*α, β-*tubulin* [β-*TUB*], *calmodulin* [CAM], and *RPB2*) analyzed by phylogenetic methods (8–14). These guidelines propose that if the ITS sequence yields a >99% identity with a type or reference strain by comparative sequence analysis with GenBank/EMBL/DDBJ sequences, then the isolate can be placed within one of the six SCs. DNA sequence data from some of the loci mentioned above are essential for identification of most clinically important fusaria to the species level (111). In addition to the NCBI's GenBank, two dedicated websites are available for identifying fusaria via the Internet (35, 36).

Serologic Tests

Conventional serologic tests have been developed and used to measure exposure of specific patient populations to *Fusarium*, most commonly in the setting of occupational exposure or indoor dampness problems (112–114). In these populations, immunoglobulin G (IgG) directed against *Fusarium* can be detected. Elevated levels of IgE and IgG antibody directed against *F. oxysporum* were found in a patient with allergic bronchopulmonary mycosis due to this pathogen (115). Anti-*Fusarium* antibodies have not been evaluated as a diagnostic tool for patients with invasive fusariosis.

Antimicrobial Susceptibilities

The typical antifungal susceptibility profiles of *Fusarium* species indicate relative resistance to most antifungal agents (116). The MICs of amphotericin B and triazoles are elevated relative to those seen for *Aspergillus* species. *In vitro* studies show relatively high MICs of amphotericin B and itraconazole for members of the FSSC (MIC_{90}s, 4 and >8 μg/ml, respectively) and FOSC (MIC_{90}s, 1.0 and >8 μg/ml, respectively) (117). Universal *in vitro* resistance was found in four clades in the FSSC (118), and testing of this SC by the European Committee on Antimicrobial Susceptibility Testing method indicated a lack of activity by any agent tested (119). Voriconazole, posaconazole, itraconazole, and ravuconazole show varied to no *in vitro* activity against clinical isolates of *Fusarium* (120, 121). In one series, voriconazole was active against an unidentified member of the FSSC (reported as *F. solani*), *F. oxysporum*, *F. proliferatum*, and *F. verticillioides* (reported as *Fusarium moniliforme*) (MIC_{90}, 2 μg/ml) but was not fungicidal against most isolates (122, 123). Although voriconazole and posaconazole exhibit only modest activity *in vitro* against isolates of *Fusarium*, both of these triazoles have been used successfully in some patients with amphotericin B-refractory fusariosis (124–126). The echinocandins show no meaningful activity against *Fusarium in vitro* (127, 128); the *fks1* gene appears to confer intrinsic resistance to echinocandins in an unidentified member of the FSSC (129). *In vitro* testing of less frequent *Fusarium* species in the FFSC, FCSC, and FIESC showed that terbinafine was the most active agent against all species except for a member of the FIESC (reported as *F. incarnatum*), against which amphotericin B was the most active agent (84, 130). In contrast, terbinafine showed high MIC_{90}s against members of the FSSC and FOSC (131). Synergy studies of a murine model with *F. oxysporum* showed prolonged survival and reduced fungal burden with a combination of amphotericin B and posaconazole (132); silver nitrate also exhibited *in vitro* activity against several fusaria from ocular infections (133).

Evaluation, Interpretation, and Reporting of Results

Early diagnosis of fusariosis is often key to appropriate management strategies, and the recovery of a *Fusarium* species should be reported to the clinician long before a final identification is made. With few exceptions, fusaria identified by phenotypic features and/or ITS ribosomal DNA (rDNA) sequence data should be reported as members of one of the SCs.

OTHER OPPORTUNISTIC HYALINE MOULDS

Taxonomy

Other opportunistic hyaline moulds known to cause human or animal disease are distributed among various taxonomic groupings, as evidenced in Table 3. A few are homothallic ascomycetes and filamentous basidiomycetes; however, the majority are anamorphic members of the Ascomycota. Most are hyaline or only lightly pigmented; however, some, like *Acrophialophora*, become dark centrally. Others, such as *Phialemonium*, *Phialemoniopsis* (134), and *Coniochaeta* (formerly *Lecythophora*) (135), have been treated as agents of phaeohyphomycosis but are included here as they are frequently pale in culture. A few coelomycetous genera, such as *Colletotrichum* and *Phoma*, are also included. Unlike the hyphomycetes, which bear conidia freely, coelomycetes produce conidia within semienclosed or enclosed structures known as acervuli or pycnidia, respectively. Coelomycetes are frequently acquired as a result of some type of implantation of the fungus following trauma rather than inhalation (136, 137).

Description of Agents

Ascomycetes

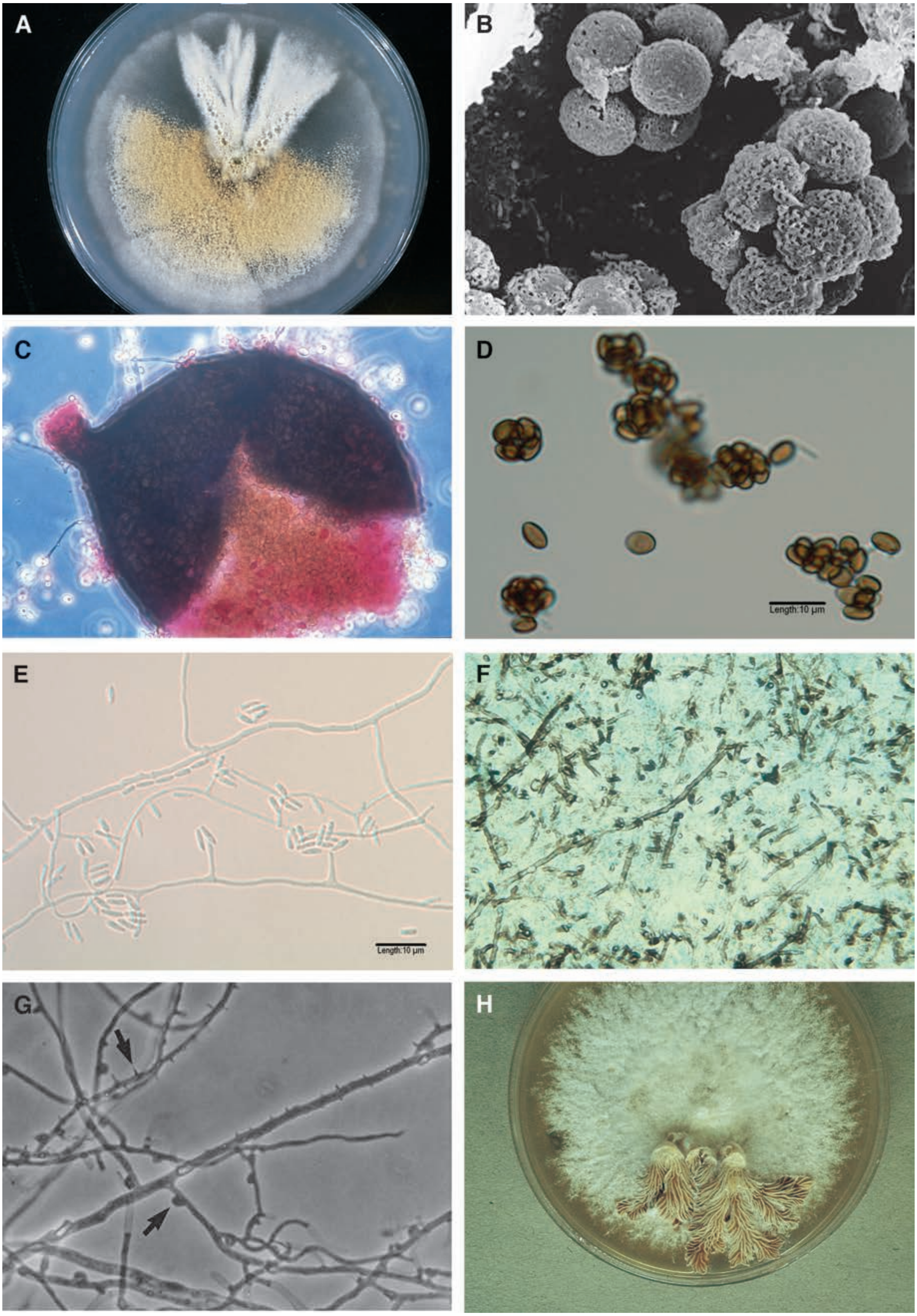
Homothallic Ascomycetes

Homothallic ascomycetous genera that may be seen in culture include those producing cleistothecia (ascomata without openings or ostioles), such as *Thermoascus*, *Aphanoascus*, and *Cephalotheca*, those that produce perithecia (ascomata with ostioles), such as *Achaetomium* (Fig. 1G), *Chaetomium*, and *Microascus* (Fig. 2C), and those whose ascospores are borne in naked clusters, as in *Gymnascella*. *Thermoascus* species are nonostiolate, thermophilic ascomycetes that grow to 50°C. They produce pale-yellow, elliptical, thick-walled ascospores and have anamorphs in the genus *Paecilomyces*. *Aphanoascus* is a keratinolytic ascomycete characterized by yellowish, lens-shaped reticulate ascospores and a *Chrysosporium* anamorph. *Cephalotheca foveolata* is characterized by cleistothecial ascomata covered with yellow to brown hairs, foveolate ascospores that are delicately pitted or dimpled, as seen by scanning electron microscopy (Fig. 2D), and a *Phialemonium*-like anamorph (Fig. 2E) (138). *Achaetomium* and *Chaetomium* produce brown lemon-shaped to fusiform ascospores within ascomata that are ornamented with hairs (or setae), especially around the upper part near the opening. Anamorphs are uncommon. *Microascus* species produce yellowish to reddish-orange, variously shaped ascospores extruded in long cirri (like toothpaste squeezed from a tube) and have anamorphs in the genus *Scopulariopsis*. Species identification in the genus *Microascus* is based primarily on features of the perithecia, such as size and length of necks, and ascospore shape. *Gymnascella* species produce clusters of ascospores surrounded by yellow or orange filaments, but differentiated ascomata are not formed (Fig. 1H and 2A and B). Both a case isolate and the reference isolate of this species displayed false-positive results in the Gen-Probe test for *Blastomyces dermatitidis* (139).

Basidiomycetes

Schizophyllum and Other Genera

Filamentous basidiomycetes are uncommon causes of human disease and are still poorly characterized. However, those that have been well documented are included in genera that may remain sterile in culture. A clinical isolate may be confirmed as a basidiomycete by the presence of clamp connections on the hyphae, but these diagnostic structures may be lacking. A nonsporulating hyaline mould may be suspected as a basidiomycete when the isolate is fast growing, displays growth on benomyl agar, grows at 37°C, and fails to grow on medium with cycloheximide (140). A positive urease test result may also suggest basidio-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mycetous affinities; however, bacterial contamination should always be considered when evaluating positive results.

Schizophyllum commune is recognized as a significant cause of allergic sinusitis, allergic bronchopulmonary mycosis, and related allergic disease and as an occasional cause of invasive infection in both immunocompetent and immunosuppressed patients (141). In culture, isolates of *Schizophyllum commune* may be dikaryotic, producing diagnostic spicules and clamp connections on the hyphae (Fig. 2G), as well as basidiocarps (mushrooms) when incubated under light (Fig. 2H). Other isolates are monokaryotic, remain sterile, and lack clamps and sometimes spicules. When spicules are absent, hyphae of *S. commune* then resemble those of *Aspergillus* species or other moulds both in culture and in tissue (140, 142, 143) (Fig. 2F). Monokaryotic isolates can be difficult to identify, and techniques such as vegetative compatibility tests or sequencing of the ITS rRNA may be required to confirm their identification as *S. commune* (143, 144).

Other uncommon basidiomycetes causing disease in humans and other animals include *Inonotus tropicalis* (Fig. 3A) (145, 146), *Oxyporus corticola* (147), *Ceriporia lacerata* (148), *Irpex lacteus* (149), a *Perenniporia* sp. (150), *Volvariella volvacea* (151), *Coprinus* species with arthroconidium-forming *Hormographiella* anamorphs (152, 153), and the *Sporothrix*-like organism previously reported as *Sporothrix cyanescens* (144), *Cerinosterus cyanescens* (154), and *Fugomyces cyanescens* (31). Phylogenetic studies have placed the last species in the family Quambalariaceae as *Quambalaria cyanescens* (155). Another basidiomycete whose pathogenic potential remains to be determined is *Sporotrichum pruinosum*, the anamorph of *Phanerochaete chrysosporium* (156).

Hyphomycetes

Scopulariopsis

The annellidic genus *Scopulariopsis* (Fig. 4F) contains both lightly colored and darkly pigmented species, some of which have teleomorphs in the genus *Microascus* of the Microascaceae (27, 157, 158) or the genus *Kernia*. Members of the genus *Scopulariopsis* are common soil fungi that are noted for their deterioration of cellulosic substrates. Of the 30 species known, only a few are reliably reported from human infections; however, several new species in both genera are currently being described based on molecular phylogenetic data.

Acremonium, Sarocladium, Coniochaeta, Phialemonium, and *Phialemoniopsis*

Fungi belonging to the genera *Acremonium*, *Sarocladium*, *Coniochaeta* (formerly *Lecythophora*), and *Phialemonium* form single-celled conidia in slimy masses or chains from long slender phialides or short, intercalary conidiogenous cells referred to as adelophialides; conidia are also formed from sporodochium-like masses in *Phialemoniopsis*. The polyphyletic genus *Acremonium*, formerly called *Cephalosporium*, includes approximately 100 species associated with soil, insects, sewage, rhizospheres of plants, and other environmental substrates. Teleomorphs, where known, are in the genera *Nectria*, *Emericellopsis*, and *Thielavia*, which are placed in different ascomycete orders. Teleomorph connections and molecular data provide evidence for some restructuring of the genus along phylogenetic lines (26, 159, 160). The most frequently seen clinical species in the United States include *Acremonium kiliense* and *Acremonium sclerotigenum*-*Acremonium egyptiacum* (161). A more recent phylogenetic overview of *Acremonium* and related taxa has placed several clinical species in the genus *Sarocladium*, e.g., *S. kiliense*, *S. strictum*, and *S. bacillisporum* (162). The genus *Lecythophora*, which contained two significant species, *L. hoffmannii* and *L. mutabilis*, has been transferred to the genus *Coniochaeta* based on the name's priority (and in keeping with elimination of a dual nomenclature after 1 January 2013) and now contains nine species, including *C. hoffmannii* and *C. mutabilis* (135). Human etiologic agents in the genus *Phialemonium* previously included *P. obovatum* and *P. curvatum* (26). *P. obovatum* is retained in the genus, and new species added include *Phialemonium atrogriseum* (*Acremonium atrogriseum*), *Phialemonium inflatum* (*Paecilomyces inflatus*), and *Phialemonium globosum*. However, species that produced sporodochium-like structures (species previously considered *Phialemonium curvatum*) have been transferred to the genus *Phialemoniopsis*, which now includes *P. curvata*, *P. pluriloculosa*, *P. cornearis*, and *P. ocularis* (134). Three species of *Phialemonium* were formerly distinguished by conidial shape and colony color, including a green diffusing pigment in *P. obovatum* (163). However, based on PCR-restriction fragment length polymorphism banding patterns that indicated a close relationship between *P. curvatum* and *P. dimorphosporum*, these two species were synonymized (164). Subsequently, these species were transferred to *Phialemoniopsis* and are distinguished from other genera by the presence of sporodochium-like conidiomata. As many of the aforementioned genera are very similar morphologically, identification to the species level is very difficult without molecular characterization. Thus, many reports of infection are based on unidentified species (15, 160). The genus *Phaeoacremonium*, including *P. parasiticum* and some other species associated with human infection, is distinguished from *Acremonium* by its brownish-pigmented hyphae and conidiophores (see chapter 124). The genera *Coniochaeta* and *Phialemonium* differ from *Acremonium* by their formation of short, stumpy phialides without basal septa (called adelophialides) in addition to the more spindle-shaped phialides (163), but these distinctions are not always readily observed. *Coniochaeta mutabilis* differs from *C. hoffmannii* in forming brown accessory chlamydospores on sporulation media.

FIGURE 2 (A) Same isolate of *Gymnascella hyalinospora* as in Fig. 1H turned yellow on oatmeal agar, with clusters of ascospores after 15 days at 30°C. (B) Ascospores of *Gymnascella hyalinospora* observed by scanning electron microscopy. Magnification, ×6,000. (C) Perithecium of a species of *Microascus*. (D) Brown ascospores of *Cephalotheca foveolata* that formed after 8 weeks on carnation leaf agar at 25°C. (E) *Phialemonium*-like anamorph of *Cephalotheca foveolata* showing adelophialides (reduced phialides without a septum) and ellipsoidal conidia. (F) Tissue section stained with Gomori methenamine silver stain showing monokaryotic (clampless) hyphae of *Schizophyllum commune* in a pulmonary fungus ball. (G) *Schizophyllum commune* in slide culture preparation showing clamp connections and narrow pegs or spicules (arrows). Magnification, ×580. (H) Dikaryotic culture of *Schizophyllum commune* showing development of gilled fruiting bodies on potato dextrose agar after 7 weeks in the light.
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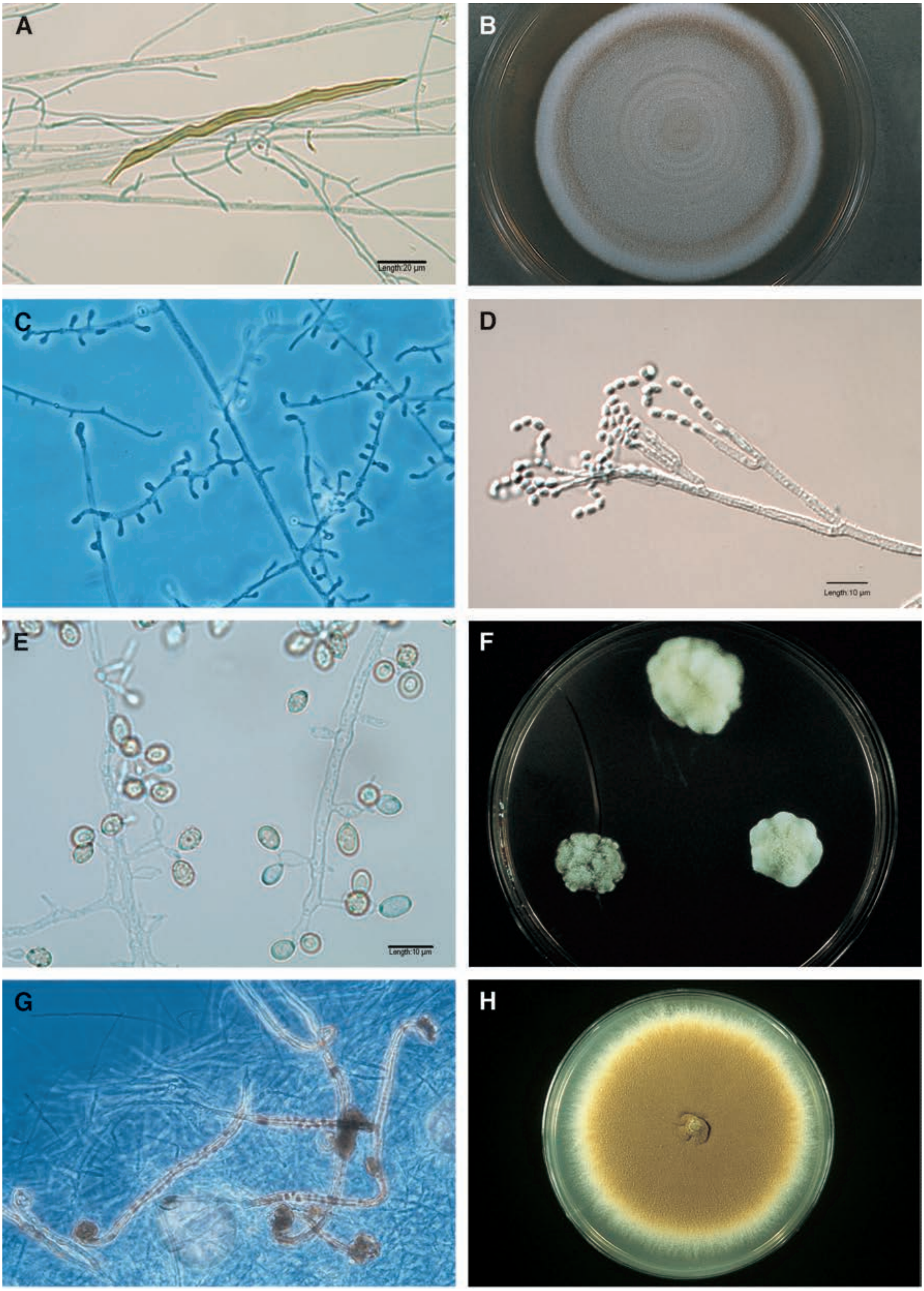
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Arthrographis, Onychocola, and *Scytalidium*

The genera *Arthrographis*, *Onychocola*, and *Scytalidium* are hyaline, arthroconidium-forming fungi. The thermotolerant *Arthrographis kalrae* is a rare opportunist recovered from skin, lung, corneal ulcer, and sinus (31, 165, 166). Because initial growth is often yeastlike, an isolate may not be recognized as a pleomorphic mould and thus incorrectly subjected to tests commonly used for yeast identification. *Onychocola canadensis* (Fig. 3F and G) is a cycloheximide-tolerant hyphomycete. *Scytalidium cuboideum* (*Arthrographis cuboidea*), recently reported from respiratory specimens, including a lung mass and a nasal sinus, is differentiated from other species in the genus by orange colonies and its yellow cuboid arthroconidia. Although it was not previously documented to cause disease, growth at 37°C suggests potential pathogenicity (167).

Beauveria and *Engyodontium*

Beauveria and *Engyodontium* species produce solitary conidia borne sympodially. The *Beauveria bassiana* species complex comprises insect pathogens with limited virulence for humans (168). *Engyodontium album* is closely related (26) and was segregated from the genus *Beauveria*.

Chrysosporium, Myceliophthora, Myriodontium, and *Nannizziopsis*

Members of the genera *Chrysosporium* (Fig. 3B and C), *Myceliophthora*, *Myriodontium*, and *Nannizziopsis* (Fig. 3E) produce solitary, usually single-celled conidia that are called aleurioconidia because of their lytic method of conidium dehiscence (24). Members of these genera are related to the dermatophytes and the dimorphic pathogens, sharing with them a tolerance to cycloheximide and producing teleomorphs that are classified in the ascomycete order Onygenales (24, 30). The recently described family within the Onygenales, Nannizziopsiaceae, contains several species of *Nannizziopsis* that are pathogens primarily of various reptiles (169).

Metarhizium and *Trichoderma*

A multilocus phylogenetic analysis of the *Metarhizium anisopliae* complex, an insect pathogen of wide distribution, revealed that it comprises multiple species-level lineages (170). A taxonomic revision of the genus was made by Driver et al. (171). Conidia are produced in chains that adhere together, unlike with *Trichoderma* species, which form conidia in slimy heads. Morphological criteria for recognition of the *Trichoderma longibrachiatum* SC include (i) fast-growing yellowish-green colonies with a radius greater than 35 mm at 40°C after 3 days of growth on potato dextrose agar; (ii) a strong yellow diffusing pigment present at 30°C but absent at 40°C; (iii) hyphae sparingly branched and forming phialides that are mostly solitary, long, and gradually tapered (cylindrical); and (iv) smooth oblong to ellipsoidal conidia (Fig. 4G and H). Intercalary phialides and chlamydospores are common. Some reports concerning *Trichoderma pseudokoningii* may actually refer to *Trichoderma citrinoviride*, a thermotolerant species closely related to *T. longibrachiatum* (26, 172). An oligonucleotide barcode program, *Trich*OKEY, was published in 2006 for sequence-based identification (173).

Paecilomyces, Purpureocillium, Acrophialophora, Phialosimplex, and *Rasamsonia*

The polyphyletic species of *Paecilomyces* occur worldwide as soil saprophytes, insect parasites, and agents of biodeterioration. Recent studies show that the medically significant *Paecilomyces variotii* is actually a complex comprising five species, with *P. variotii* and *P. formosus* being the most important clinically (174). Their affinities are within the ascomycete family Trichocomaceae, which also includes *Penicillium* and *Aspergillus*; some other *Paecilomyces* species have teleomorphs in the genus *Thermoascus* (Thermoascaceae). The colony (Fig. 3H) and morphological features of the *P. variotii* complex are described in Table 3. A similar species not confirmed as an agent of infection, *P. marquandii*, displayed a yellow diffusing pigment and failed to grow at 37°C (141).

Purpureocillium is a new genus for the medically important *Paecilomyces lilacinus* now placed within the family Ophiocordycipitaceae (175). Colonies are typically lilac in color, the growth rate is lower than for *Paecilomyces* spp., the maximum growth temperature is 25 to 33°C, and chlamydospores are absent.

Acrophialophora is a thermotolerant and potentially neurotropic genus widespread in temperate to tropical regions. Colonies are initially pale but darken centrally at maturity. Also described under the name *Paecilomyces fusisporus*, it differs by producing unbranched, erect, brown, echinulate conidiophores that are fertile at the apex and anchored by a foot cell and basally swollen monophialidic but occasionally polyphialidic conidiogenous cells. Conidia are borne in chains, and distinct spiral bands may be present (176). The organism may superficially resemble *Scedosporium prolificans* (177, 178).

Phialosimplex caninus is a newly described species having phylogenetic affinity to the Trichocomaceae, seen primarily in dogs (179). Colonies are moderately fast growing, white to gray to yellowish, and distinguished from other species in this genus by production of a yellow diffusible pigment (Fig. 4D). Hyaline truncate conidia that are subglobose to pyriform to ovoid are borne in long chains or heads from single, narrow, mostly monophialidic conidiogenous cells (Fig. 4E). *Sagenomella chlamydospora* (180) and *Sagenomella sclerotialis* (181) were transferred to *Phialosimplex* as *Phialosimplex chlamydosporus* comb. nov. and *Phialosimplex sclerotialis* comb. nov., respectively.

Rasamsonia is a new genus in the family Trichocomaceae that accommodates thermotolerant or thermophilic species segregated from *Geosmithia* (182). It is distinguished from *Penicillium* species by roughened stipes, metulae, and phialides and by cuneiform to ellipsoidal conidia (Fig. 3D). *Rasamsonia argillacea* under the name *Geosmithia argillacea* caused a disseminated disease in a German shepherd dog

FIGURE 3 (A) Setal hyphae of *Inonotus tropicalis* in a slide culture preparation on potato flakes agar at 10 days and 25°C. Bar, 20 µm. (B) Colony of *Chrysosporium zonatum* on potato dextrose agar after 14 days at 37°C. (C) Conidia of *Chrysosporium zonatum* formed on short curved stalks. (D) Rough-walled stipe, metulae, and phialides of *Rasamsonia argillacea*. Note also that conidia are initially cuneiform (wedge shaped). Bar, 10 µm. (E) Conidia of *Myceliophthora thermophila* in various stages of maturity. Mature conidia are dark and rough. Bar, 10 µm. (F) Culture of three different isolates of *Onychocola canadensis* after 5 weeks on Mycosel agar. (G) Setae (appendages) of *Onychocola canadensis*. (H) Colony of *Paecilomyces variotii* on potato dextrose agar after 7 days.
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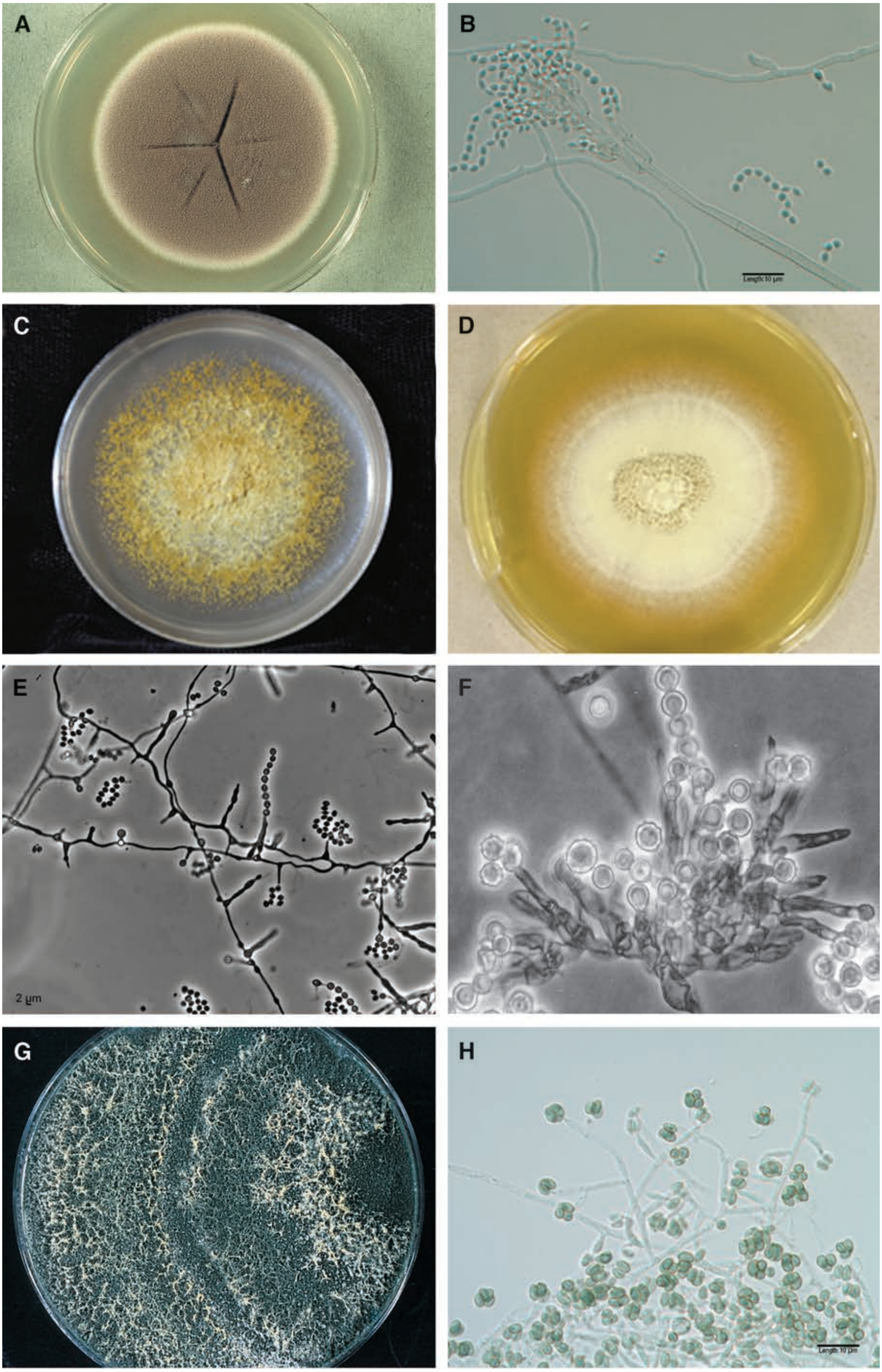
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(183) and is an emerging agent in cystic fibrosis patients (184) and those with chronic granulomatous disease (185). A recent phylogenetic study of the genus has differentiated this complex into 6 clinically important species.

Coelomycetes

Colletotrichum and *Phoma*

Coelomycetous fungi are being documented as agents of disease more frequently, particularly in patients maintained on long-term immunosuppressive therapy. Infections are typically acquired through some type of direct implantation of the fungus, unlike with the hyphomycetous genera, which are acquired by inhalation. Two of the more common genera addressed here are *Colletotrichum* and *Phoma*. *Colletotrichum* species are acervular coelomycetes occasionally recovered as agents of disease. They produce fast-growing colonies of various shades that are most easily recognized in the laboratory by the production of brown, variably shaped appressoria. Honey-colored masses of conidia as well as setae and sclerotia in some species may be present in culture. Cano et al. described the salient features seen in clinical strains (186). Species in the genus *Phoma* are pycnidial coelomycetes that may be pale or darker in culture. They are usually recognized by small dark pycnidia that form on the surface or are immersed in the agar. Boerema et al. published a *Phoma* identification manual (187); however, species differentiation is best handled in a reference laboratory. A recent review of coelomycetous fungi seen in clinical laboratories cites several species documented to cause disease, gives salient features for their phenotypic identification, and provides *in vitro* antifungal susceptibility data for selected genera/species (188). Also see chapter 124 for additional coelomycetous genera.

Epidemiology and Transmission

The methods of transmission and sources of infection for the other opportunistic hyaline moulds are similar to those seen in aspergillosis and fusariosis, and acquisition is typically through inhalation or traumatic implantation. Coelomycetous fungi, although ubiquitous, are mostly reported from cases of keratitis and subcutaneous mycoses in compromised individuals (189) and appear to be acquired primarily through external inoculation (136).

Clinical Significance

Ascomycetes

Homothallic Ascomycetes

Species of *Chaetomium* and *Achaetomium* (Fig. 1G) are neurotropic agents causing cerebral infection (39, 40, 190), while *Chaetomium globosum* occurs most commonly as a contaminant or as a rare agent of onychomycosis (6, 31). *Microascus* spp. are also agents of deep infections, including endocarditis (191) and a brain abscess (192) by *Microascus cinereus*, disseminated infections by *Microascus cirrosus*, and a fatal pneumonia by *Microascus trigonosporus* (193). The genus *Microascus* is currently being revised with the expectation that novel clinically relevant species will be described. Additional rarely implicated genera include *Gymnascella* (Fig. 1H and 2A and B) and *Cephalotheca* (Fig. 2D and E). Please see references 26, 139, and 191–195.

Basidiomycetes

Schizophyllum and Other Genera

Filamentous basidiomycetes, as well as species of smuts that may appear yeastlike (Ustilaginaceae and Tilletiaceae), are commonly isolated from respiratory specimens and sometimes from blood; however, their significance can be difficult to evaluate (140, 196).

Schizophyllum commune is recognized as a significant cause of allergy-related sinusitis and pulmonary disease, including allergic bronchopulmonary mycosis and bronchial mucoid impaction (140, 142, 143, 197–200) as well as infections of the brain, lungs, and buccal mucosa in both immunocompetent and immunosuppressed patients (31, 140, 199, 201, 202). *Coprinus cinereus* or its anamorph *Hormographiella aspergillata* has been reported from prosthetic-valve endocarditis, fatal lung infections in leukemic patients, a lung abscess in a patient with non-Hodgkin's lymphoma, keratomycosis in a dog, and cutaneous lesions (31, 152, 153, 203, 204). *Inonotus tropicalis* was reported as an agent of osteomyelitis in a patient with X-linked chronic granulomatous disease (145, 146) and has also been seen in an additional patient with this disease (D. Sutton, unpublished data). *Ceriporia lacerata* was reported in four cases from pulmonary sites (148), *Irpex lacteus* was recovered from a pulmonary abscess in an immunosuppressed child (149), a *Perenniporia* sp. was the etiologic agent in a pulmonary fungal ball (150), and *Volvariella volvacea* incited invasive disease and death in a Hodgkin's lymphoma patient (151).

Hyphomycetes

Scopulariopsis

Scopulariopsis brevicaulis (Fig. 4F) and other species are occasional agents of onychomycosis (24, 30, 157). They are also rarely invasive, causing otomycosis, keratitis, prosthetic-valve endocarditis, sinusitis, pneumonia, brain abscess, and subcutaneous and bone invasion in immunocompetent and immunosuppressed individuals (26, 194, 205–209). *Scopulariopsis candida* and *S. acremonium* have been reported from invasive sinusitis, but few details concerning the latter species were provided in the report (210, 211). Brain abscess caused by *Scopulariopsis brumptii* and invasive cutaneous infection caused by *Microascus cirrosus* have been reported to occur in liver transplant and bone marrow recipients, respectively (194, 212). A case of fatal *S. acremonium* was reported to occur in a lung transplant recipient (213). As noted above for *Microascus* spp., the genus is currently being revised.

FIGURE 4 (A) Colony of *Purpureocillium lilacinum* on potato dextrose agar after 14 days. (B) Verticillate conidiophores of *Purpureocillium lilacinum* bearing whorls of phialides. Bar, 10 μm. (C) Yellowish colony of *Phialemoniopsis curvata* on potato flakes agar after 14 days at 25°C. (D) Colony of *Phialosimplex caninus* with yellow diffusible pigment on potato dextrose agar after 21 days at 30°C. (E) Conidia of *Phialosimplex caninus* borne in long chains or heads from simple basally inflated phialides. Bar, 2 μm. (F) Rough-walled conidia in chains formed on annellides in *Scopulariopsis brevicaulis*. Note the branched conidiogenous apparatus. Magnification, ×580. (G) Colony of *Trichoderma longibrachiatum* on potato dextrose agar after 4 days at 37°C. Note that the plate was inoculated on one side. (H) Green, oval conidia of *Trichoderma longibrachiatum*. Bar, 10 μm.
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Acremonium, *Sarocladium*, *Coniochaeta*, *Phialemonium*, and *Phialemoniopsis*

Many reports concerning *Acremonium* involve infections of the nail, skin, eye, or mycetoma (see also chapter 125) (24, 26); however, many of these species are now classified in other genera. Localized and disseminated infections occur in patients following valve replacement, dialysis, or transplantation or in patients with hematologic or solid organ malignancies (6, 15, 26, 160, 214, 215). Fungemia is common. Several cases of invasive *Sarocladium* (*Acremonium*) *strictum* have been reported (216–218). *C. mutabilis* was reported to cause prosthetic-valve endocarditis in a diabetic patient (219), and *C. hoffmannii* was reported to cause chronic sinusitis in a HIV-infected patient (220). *Phialemonium obovatum* was an agent of endocarditis (221), while arthritis, fungemia, endovascular infections, and ophthalmitis have been reported for *Phialemoniopsis curvata* (*Phialemonium curvatum*) (164, 222–224).

Arthrographis, *Onychocola*, and *Scytalidium*

The thermotolerant *Arthrographis kalrae* is a rare opportunist recovered from skin, lung, corneal ulcer, and sinus (31, 165, 166). *Onychocola canadensis* (Fig. 3F and G) causes distal subungual onychomycosis or, less commonly, white superficial onychomycosis and infection of the glabrous skin (24, 30, 225, 226). Although *O. canadensis* is a relatively uncommon agent of onychomycosis, more than 60 isolates from nails have been recorded from New Zealand, Australia, Europe, and the United States, with two additional cases from Spain (227). The growth of *S. cuboideum* at 37°C suggests its pathogenic potential (167).

Beauveria and *Engyodontium*

Beauveria bassiana has caused several cases of fungal keratitis (228, 229). A recent molecular study comparing clinical keratitis isolates with Environmental Protection Agency-registered strains showed that these strains were unrelated (230, 231). An isolate that failed to grow at 35°C also caused disseminated disease in a patient with acute lymphoblastic leukemia (232). *Engyodontium album* was the cause of endocarditis (233).

Chrysosporium, *Myceliophthora*, and *Nannizziopsis*

The thermotolerant *Chrysosporium zonatum* is an etiologic agent of human pneumonia and osteomyelitis (234) (Fig. 3B and C). In a review of human infections caused by *Emmonsia* and *Chrysosporium*, those caused by *Chrysosporium* typically occur in immunocompromised individuals (235). The thermophilic species *Myceliophthora thermophila* (Fig. 3E) has been reported to cause fatal aortic vasculitis in two patients, was isolated from the brain of a patient who developed a bacterial cerebral abscess after trauma, and is an agent of severe osteomyelitis following a pitchfork injury (236–239). While some *Chrysosporium* species pathogenic for reptiles have been retained in this genus, i.e., *Chrysosporium ophiodiicola* (240), others, such as the *Chrysosporium* anamorph of *Nannizziopsis vriesii* and *Chrysosporium guarroi* (241), have been transferred to the genus *Nannizziopsis*.

Metarhizium and *Trichoderma* Species

The *Metarhizium anisopliae* complex contains multiple phylogenetically distinct species that are widely distributed insect pathogens. They are also documented to cause keratitis, sinusitis, invasive infections, and disseminated skin lesions (242–245). Species of *Trichoderma* in the section *Longibrachiatum*, which includes *T. longibrachiatum* (Fig. 4G and H) and *T. citrinoviride*, appear to be the most important pathogenic species (246–249).

Paecilomyces, *Purpureocillium*, *Acrophialophora*, *Phialosimplex*, and *Rasamsonia* Species

Clinical manifestations of both *Paecilomyces variotii* and *Purpureocillium lilacinum* include cutaneous and subcutaneous infections, pulmonary infection, pyelonephritis, sinusitis, cellulitis, endocarditis, and fungemia in both immunocompetent and immunocompromised patients (6, 15, 26, 250–252). The clinical manifestations, treatment options, and outcomes for *P. lilacinum* infections have been reviewed (253, 254). The species has also been noted to reside in water distribution systems, including those of a bone marrow transplant unit (175). *Acrophialophora fusispora* was the etiologic agent in a brain abscess in a child with leukemia (176) and has been recovered in pulmonary infections (255, 256) and cases of keratitis (255). It also appears to be a frequent colonizer in patients with cystic fibrosis (257, 258). *Phialosimplex caninus* sp. nov., seen primarily in dogs, has also been recovered from pleural fluid and tissue from a human (179). *Rasamsonia argillacea* (formerly *Geosmithia argillacea*) has been described as an emerging cause of invasive fungal infection in human chronic granulomatous disease (259) and has also been reported to cause a pulmonary and aortic-graft infection in an immunocompetent patient (260).

Coelomycetes

Colletotrichum and *Phoma*

Colletotrichum species are primarily phytopathogens but occasionally are recovered as agents of keratitis (26). There are also rare reports of subcutaneous infection following trauma (189). A case of phaeohyphomycotic cysts caused by *Phoma* species was reported in a patient (261).

Collection, Transport, and Storage of Specimens

Methods of collection, transport, and storage of specimens are detailed in chapter 114. As with other invasive mycoses, infections are difficult to diagnose and usually require a combination of clinical, culture, and radiographic findings. The recovery from a normally sterile site and microscopic evidence of invasive growth in tissue provide the most convincing evidence of disease.

Direct Examination

Microscopy

Histopathological findings for most opportunistic hyaline moulds are typically indistinguishable from species of *Aspergillus*, *Fusarium*, and *Pseudallescheria* (208). Ascospores may occasionally be observed, as was demonstrated with *Gymnascella* (139), and clamp connections may also be present in tissue sections with *Schizophyllum commune* (201). Budding forms may also rarely be seen (87, 214, 215).

Antigen Detection

Detection of (1→3)-β-D-glucan (91, 93) in patients with invasive hyalohyphomycosis may assist with an early diagnosis, but monitoring of this marker needs to be combined with clinical examination of the patient and other diagnostic procedures, such as high-resolution computed tomography scanning.

Nucleic Acid Detection

Luminex microbead hybridization technology has recently been reported to detect a variety of fungal pathogens from clinical blood and pulmonary samples (102). This method appears to have promise for the early detection and identification of various invasive fungal pathogens.

Isolation Procedures

Opportunistic hyaline moulds are usually cultured easily on routine mycological media, and there are no specific growth requirements; however, media with and without cycloheximide should be employed. The fungicide benomyl at a final concentration of 10 mg/ml in the culture medium can be useful to distinguish filamentous basidiomycetes, which are tolerant to benomyl, from ascomycetes, which may be sensitive to benomyl (262). Coelomycetes grow well on most fungal media; however, they are notorious for remaining sterile without extended incubation (up to several weeks for some genera) (136). See chapters 114 and 115 for detailed information on appropriate media for initial plating and isolation.

Identification

More-detailed descriptions of these hyaline fungi are found in several identification manuals and in the references cited therein (24–33). Many can be identified to the genus level with little difficulty; however, identification of most clinically relevant moulds to the species level requires DNA sequence data. Furthermore, MALDI-TOF MS has the potential capability of speeding up genus- and species-level identification (106). See Table 3 for salient phenotypic features of the organisms reviewed.

Typing Systems

Although comparative sequence analysis of clinical isolates is becoming more common in large tertiary-care and research centers, it is far from standardized. Various methods of DNA extraction are available (263), and several different genes or portions thereof may be sequenced (8–14, 17, 53, 110, 111, 264, 265). The use of DNA sequence data to identify isolates that remain sterile in culture is largely dependent on the accuracy of and interpretation of data in publicly accessible databases, such as the NCBI's GenBank. The validity of this approach was demonstrated by DNA sequence-based identification of several nonsporulating moulds (266); this approach will continue to be used more widely. It should be highlighted, however, that numerous database entries in public databases are incorrect, making comparative sequence analysis without phenotypic correlation a challenge that requires that the top sequence matches be examined critically (111). On the other hand, molecular characterization has become the "gold standard" for classification of species for taxonomic categorization and will continue to provide a better understanding of the evolutionary relationships of clinically significant fungi (8–14, 53, 267).

Serologic Tests

Serologic procedures currently have little clinical utility in the diagnosis of uncommon hyaline opportunistic fungi.

Antimicrobial Susceptibilities

Uncommon hyaline moulds display various antifungal susceptibility patterns. Susceptibility testing of uncommon moulds is useful for empirical antifungal therapy; however, patient isolates should be assessed individually for appropriate patient management. For published *in vitro* data, see references relating to the genera of interest. *Chaetomium* infections that failed to respond to treatment with amphotericin B alone or in combination with itraconazole have been reported (268). However, voriconazole and the experimental triazoles ravuconazole and albaconazole showed potent activity *in vitro* against *Chaetomium* spp., with MICs less than 0.5 μg/ml (268); however, the echinocandin micafungin was not active *in vitro*. Evaluation of antifungal activity against 44 clinical isolates of filamentous basidiomycetous fungi, including *Schizophyllum commune* ($n = 5$), *Coprinus* spp. ($n = 8$), *Bjerkandera adusta* ($n = 14$), and sterile, uncharacterized basidiomycetes ($n = 17$), demonstrated low MICs of amphotericin B, itraconazole, voriconazole, and posaconazole, in contrast to those of fluconazole and flucytosine (269). No statistically significant differences among the genera were noted.

Antifungals, including amphotericin B and itraconazole, have limited *in vitro* activity against *Scopulariopsis* spp., and conflicting results have been reported for voriconazole and terbinafine (131, 270). A promising interaction was observed between terbinafine and fluconazole, itraconazole, and voriconazole *in vitro* against isolates of *S. brevicaulis*, although clinical experience with combination therapy is very limited (271). When *Paecilomyces* species were evaluated by the European Committee on Antimicrobial Susceptibility Testing methodology (119, 272), amphotericin B, itraconazole, and the echinocandins showed poor activities against 27 strains of *P. lilacinum*; however, the newer triazoles voriconazole, ravuconazole, and posaconazole and the allylamine terbinafine showed low MIC_{90}s. In contrast, for 31 strains of *P. variotii*, the MICs of all of the above agents except voriconazole and ravuconazole were low (273).

Evaluation, Interpretation, and Reporting of Results

The early diagnosis of invasive hyalohyphomycosis caused by fusaria and other hyaline moulds is often key to appropriate management strategies. Identification of uncommon hyaline moulds should always be evaluated in light of the patient's immune status as well as the anatomic site of recovery and frequency of isolation.

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The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned. The USDA is an equal opportunity provider and employer.

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Agents of Systemic and Subcutaneous Mucormycosis and Entomophthoromycosis

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Mucormycosis and entomophthoromycosis are invasive fungal infections caused by environmental nonseptate filamentous fungi. Mucormycosis is caused by the ubiquitous *Mucorales* and mostly occurs in immunocompromised patients or those with diabetes mellitus. These fungi are responsible for rhinocerebral, pulmonary, cutaneous, or disseminated infections characterized by angio-invasion and necrosis and severe prognosis despite current antifungal and surgical therapies. Entomophthoromycosis, which occurs mostly in immunocompetent hosts, is caused by members of *Entomophthorales* (responsible for conidiobolomycosis) and *Basidiobolales* (responsible for basidiobolomycosis), both mostly found in warm climates. Entomophthoromycosis presents as subcutaneous infections with favorable outcome after prolonged azole therapy, although it is potentially associated with disfiguring lesions.

TAXONOMY

The classification of the kingdom of *Fungi* has been constantly modified over the last decades. Recently, a comprehensive phylogenetic study based on multigene sequence analyses (1) proposed a classification that accepts one kingdom, one subkingdom, seven phyla, 10 subphyla, 35 classes, 12 subclasses, and 129 orders. The most important modifications were the creation of subphylum *Dikarya*, which includes members of *Ascomycota* and *Basidiomycota* and the redistribution of taxa located in phyla *Zygomycota* and *Chytridiomycota*. The etiological agents responsible for mucormycosis (order *Mucorales*) and those responsible for entomophthoromycosis (orders *Entomophthorales* and *Basiodiobolales*) were traditionally assigned to the lower fungus phylum *Zygomycota*. In the study by Hibbett et al., this phylum is no longer accepted due to its polyphyletic nature (1). Taxa are redistributed between *Glomeromycota* and four new subphyla of uncertain position (*incertae sedis*): *Mucoromycotina* (which includes three orders; the core group of *Mucorales*, *Endogonales*, and *Mortierellales*), the *Entomophthoromycotina* (with the order *Entomophthorales*), the *Zoopagomycotina* (with the order *Zoopagales*), and finally, the subphylum *Kickxellomycotina* (which includes the orders *Kickxellales*, *Dimargaritales*, *Harpellales*, and *Asellariales*).

Over the last years, phylogenetic interactions among members of the order *Mucorales* have been studied (2–4). These studies have mainly shown the polyphyletic nature of families (*Thamnidiaceae*, *Mucoraceae*, and *Chaetocladiaceae*) and genera such as *Absidia* and *Mucor*. The family structure of *Mucorales* was recently studied based on the phylogenetic analysis of four markers (5). *Rhizopodaceae* is one of the newly erected families, which include the genus *Rhizopus* and other pathogenic genera.

Revisions of other genera have been published in recent years and will be detailed below (3, 6–10). Of note, the first analysis of a genome sequence from a member of the *Mucorales* (*Rhizopus arrhizus*) was published in 2009 (11). The genome annotation for the species *Mucor circinelloides* var. *circinelloides* is now available (http://www.broadinstitute.org).

Table 1 displays the *Mucorales* species that have been described as human pathogens or potential human pathogens considering the new family structure published by Hoffmann et al. (5).

Recently, the subphylum *Entomophthoromycotina* was elevated to the phylum *Entomophthoromycota*. The monophyly of these organisms was then confirmed by multigene phylogeny analysis (12, 13). This newly erected phylum comprises more than 250 species distributed among three classes (*Basidiobolomycetes*, *Neozygitomycetes*, and *Entomophthoromycetes*) and six families (*Basidiobolaceae*, *Neozygitaceae*, *Ancylistaceae*, *Completoriaceae*, *Entomophthoraceae*, and *Meristracraceae*). There are more than 25 species in the genus *Conidiobolus* but only 3 (*C. coronatus*, *C. lamprauges*, and *C. incongruus*) have been recovered from clinical specimens, while in the genus *Basidiobolus*, *Basidiobolus ranarum* is the only species of the genus to provoke human disease (14, 15).

MUCORMYCOSIS

Epidemiology and Transmission

Mucorales are ubiquitous fungi widely distributed in the environment (soil, plants, and decaying organic material) (16). They are frequent pathogens of plants and contaminants of grains and food such as fruit or bread. Airborne spores are considered the infectious particles responsible

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TABLE 1 Species of *Mucorales* (subphylum *Mucoromycotina*) involved in human mucormycosis

Family	Genus	Species
Lichtheimiaceae	*Lichtheimia*	*L. corymbifera* (syn. *Mycocladus corymbifer, Absidia corymbifera*)
		L. ramosa (syn. *Absidia ramosa*)
		L. ornata (syn. *Absidia ornata*)
	Rhizomucor	*R. pusillus*
Mucoraceae	*Mucor*	*M. circinelloides*
		M. indicus
		M. velutinosus
		M. irregularis (formerly *Rhizomucor variabilis* var. *variabilis*)
		M. ramosissimus
	Actinomucor	*A. elegans*
	Cokeromyces	*C. recurvatus*
Saksenaeaceae	*Saksenaea*	*S. vasiformis*
		S. erythrospora
		S. oblongispora
	Apophysomyces	*A. elegans*
		A. trapeziformis
		A. ossiformis
		A. variabilis
Rhizopodaceae	*Rhizopus*	*R. microsporus*
		R. arrhizus (syn. *R. oryzae*)
		R. schipperae
Syncephalastraceae	*Syncephalastrum*	*S. racemosum*
Cunninghamellaceae	*Cunninghamella*	*C. bertholletiae*
		C. echinulata

for disease, particularly in immunocompromised individuals, explaining the most frequent body localizations (skin, sinuses, and lungs). Of note, immunocompetent patients may develop posttrauma skin infections, representing up to 18% of all mucormycosis cases in a comprehensive study of cases diagnosed in France (17). In addition, necrotizing cutaneous cases have been recently reported after a tornado in Joplin, MO (18), or as a cause of infections following combat-related injuries in Afghanistan (19). These species are easily found as laboratory contaminants and can be a source of nosocomial infections, including outbreaks (20–22). Specific geographical distribution and environmental niches will be described for relevant species in the corresponding section.

Some reports have suggested an increasing incidence of mucormycosis based on single-center studies (23–27). In a retrospective analysis of hospital records in France, our group recently provided a population-based estimate of mucormycosis incidence and trends for the country over a 10-year period (28). We then better estimated the real burden of mucormycosis infections through a capture-recapture method using two available sources of information (29) and found different incidences according to the region, suggesting local ecological specificities at a country level (30). The incidence significantly increased over time with an average $0.9/10^6$ annual incidence rate different from the incidence reported in a population-based study in California in 1992 to 1993 ($1.7/10^6$) (31) and in Spain in 2005 ($0.43/10^6$) (32). The sex ratio (1.8 male versus 1.1 female) and mean age (38.8 versus 57.1 years) differ in the review of 929 cases published between 1940 and 1999 and in France (28, 33).

Diabetes is a major risk factor (23% in France and up to 36% of the cases elsewhere, 20% with type 1 diabetes) (17, 33, 34), with a significant increase in the incidence of diabetes-associated mucormycosis recently documented in France (28) and in a tertiary care center in North India (35). In the latter study, 131/178 cases were observed in uncontrolled diabetic patients (73.6%), and more importantly, mucormycosis led to the diagnosis of diabetes in 56 cases (42.7% of diabetes cases). Diabetes mellitus was also found in 15% (type 1 = 13%) of 157 pediatric cases (36). Diabetes was an independent risk factor for mucormycosis in patients with leukemia and/or bone marrow transplantation (24) and significantly influenced the occurrence of mucormycosis during solid organ transplantation (SOT) (17, 37).

Among patients with hematological malignancies, which represented 50% of the cases in France (17), those with acute leukemia (profound neutropenia or relapse) or allogeneic stem cell transplantation are more prone to develop mucormycosis. Mucormycosis most often occurs more than 3 months after transplant in the setting of graft versus host disease (24, 25, 27, 38–42). Mucormycosis now represents 7 to 8% of invasive fungal infections in bone marrow transplant patients (43, 44). An increased incidence of mucormycosis was observed in patients with hematological malignancies or stem cell transplant in France (28), as already noted in the 1990s in one U.S. center (27). In that population, the potential role of prior exposure to antifungals lacking activity against *Mucorales*, such as voriconazole and caspofungin, has been reported (24, 25, 37, 45–48).

Mucormycosis represents 2% of invasive fungal infections during SOT, mostly in kidney transplantation (49). In a recent international study of SOT recipients, renal failure, diabetes mellitus, and prior voriconazole and/or caspofungin use were associated with a higher risk whereas tacrolimus was associated with a lower risk of mucormycoses (37). Liver transplant recipients were more likely to have disseminated disease and developed infection significantly earlier after transplantation than other SOT recipients.

Mucormycosis can also develop in HIV-infected patients or intravenous drugs abusers. It can affect otherwise healthy individuals following cutaneous injuries with contaminated soil in almost 20% of the cases in some studies. Finally, it may present as community-acquired or as a health care-related disease. A careful review of cases published between 1970 and 2008 revealed a total of 169 individual (with 29% of them children) mucormycosis cases associated with health care, of which 72% were published after 1990 (21). Outbreaks of mucormycosis are rare but have been related to building construction and use of contaminated adhesive tape, ostomy bags, or wooden tongue depressors.

Clinical Manifestations

Localized and disseminated diseases need to be differentiated (40). The most frequent localized forms are sinusitis and pneumonia, representing 25 to 39% and 24 to 30% of clinical sites involved, respectively, in three recent series (17, 33, 50). Dissemination rates vary from 3% to more than 50% in patients with hematological malignancies (depending to some extent on the underlying diseases and the site of infection) (33, 40, 41).

Sinus involvement (isolated sinusitis, rhinocerebral, and sino-orbital forms) is the most common presentation in diabetes patients and intravenous drug abusers, while pulmonary infection is the second most common presentation, and the reverse is true in hematology patients (24, 33). Infection causes necrosis and hemorrhage and may be localized or associated with dissemination. Clinicoradiological presentation is similar to that of invasive aspergillosis in patients with hematological malignancies, although the presence of a reversed halo sign may be suggestive of (51) but not specific to (52, 53) mucormycosis. In a recent study of 58 SOT patients with mucormycosis, pulmonary localization was present in 31 (53%), including 23 with localized infection (54).

Localized cutaneous lesions are most often encountered in immunocompetent hosts (33) and follow injury and contamination with airborne spores or soil (55, 56, 154) and, less often, surgery or burns. Local extension and hematogenous dissemination may occur. In contrast, skin lesions resulting from dissemination from other sites are rare.

Cerebral infection can complicate sinusitis or occur independently, especially in intravenous drug abusers. Cognitive disturbances and focal neurological deficit are often present. Gastrointestinal lesions are described in 7% of cases and occur in low-birth-weight premature infants and malnourished individuals and after peritoneal dialysis (33). They are responsible for abdominal pain and digestive hemorrhage, and complications include digestive tract perforation, peritonitis, and dissemination to the liver.

Treatment

Prophylaxis

In immunocompromised patients, there is no benefit of the prophylactic use of azoles such as fluconazole or itraconazole due to of the lack of (major) activity against *Mucorales* (27, 41, 42). The best preventive measure is the reduction of environmental exposure, notably with the use of high-efficiency particulate air-filtered rooms (41). The specific role of posaconazole as a prophylactic agent against mucormycosis has not been demonstrated (57, 58), and there is no decreased incidence of the disease in the hematology population over recent years, although posaconazole has been available since 2007 (30).

Curative Treatment

In Roden et al.'s review, survival of untreated patients was 3% while 64% of patients who received antifungal therapy survived (33). A combined medical and radical surgical treatment, especially for rhinocerebral and skin localizations, in association with correction of the underlying disease wherever possible (i.e., acidosis, hyperglycemia, neutrophil recovery, and modulation of immunosuppressive therapy) offers the best chance of survival (59).

Lipid derivatives of amphotericin B (AMB) given early in the course of the disease are probably more effective and better tolerated than AMB deoxycholate (60). Clinical response has been obtained with a high dose of liposomal AMB in patients who failed with conventional dosages (61, 62); therefore, one of the two available options (lipid complex or liposomal formulation) should be first-line therapy (63, 64). Oral posaconazole in its current oral formulation can only be considered second-line therapy, iron chelators such as deferasirox should not be used, and echinocandins in combination with polyenes are considered a second-line therapy option. Global case fatality rates vary from 84 to 47% (33), with those of rhinocerebral forms ranging from 20 to 69% (26, 35). Survival is reduced in cases of hematological malignancies compared with diabetes mellitus (17).

Collection, Transport, and Storage of Specimens

Mucormycosis is one of the most rapidly progressing invasive fungal infections. The diagnosis of mucormycosis should be considered an emergency, since delaying therapy will impact outcome (65). Although clinical features are not specific, some early radiological features make the diagnosis of mucormycosis more probable compared to other invasive fungal infections, particularly invasive aspergillosis (53). A combination of well-known predisposing factors and clinical and/or radiological signs must alert the physician and prompt the institution of immediate diagnostic procedures. Since currently available methods developed for the diagnosis of invasive fungal infections are based on antigens that are not produced by *Mucorales* (66), the diagnosis relies mainly on direct examination and/or recovery of the fungus and/or detection of their nucleic acids in pathological tissues, body fluids, or exudates. Blood cultures are not appropriate for the diagnosis. In high-risk patients, specimens, preferentially from deep lesions and sterile sites, have to be rapidly and aseptically collected in sufficient quantity. Larger volumes will increase the likelihood of fungal recovery and will allow more diagnostic procedures (direct examination, culture, DNA extraction for PCR, histopathology) as well as storage of the sample for further analyses. For rhinocerebral localization, nasal discharge or scraping, sinus aspirate, or tissue specimen from abnormally vascularized areas should be obtained. For pulmonary localization, sputum and bronchoalveolar lavage fluids can be examined, taking into account the low sensitivity of these specimens (67, 68). If negative, transbronchial or percutaneous computerized tomography-guided biopsies of pulmonary lesions can be performed, keeping in mind the potential of induced morbidity (69).

Separate specimens must be sent for microbiological and histological analysis, since formalin used for histopathology inhibits fungal growth. Direct examination remains the most rapid diagnostic technique, and culture is essential to identify the isolate at the species level and to test susceptibility to antifungal drugs. The transport container should be sterile and humidified with a few drops of sterile saline. The specimen should not be refrigerated and should ideally be transported to the laboratory within 2 h after collection and processed rapidly, since *Mucorales* are easily damaged and sensitive to environmental stresses (70). Biopsy specimen grinding is deleterious for the *Mucorales* due the coenocytic nature of the hyphae. Biopsy specimens should be sliced into small pieces of tissue that are then dispatched for direct examination and culture.

Direct Examination and Histopathology

Demonstration of hyphae in clinical samples provides strong evidence of mucormycosis, since *Mucorales* are environmental airborne moulds that could be present in conidial form in nonsterile specimens, with false-positive cultures as a result. Direct examination is a key test for two reasons: (i) culture from clinical samples is frequently negative (71) and (ii) histology requires multiple steps that necessarily delay diagnosis.

Microscopy

Direct examination can be performed using wet mounts of the sample or after addition of chlorazol black. However, optical brightener (calcofluor white, Blankophor, or Uvitex 2B) that specifically stains the chitin contained in the cell wall, coupled with a clearing and dissociating agent, such as KOH, provides better sensitivity (67). It is always a good idea to examine the slides again the following day, especially in the presence of thick samples because of possible false negatives resulting from insufficient dissociation of tissues.

The morphological characteristics suggestive of *Mucorales* hyphae are specific and can be differentiated from those of *Aspergillus*, *Fusarium*, or *Scedosporium*. Hyphae are large (5 to 25 μm), irregular, hyaline, non- or pauciseptate, and thin walled with a ribbon-like morphology. A twisted or folded appearance of the hyphae is frequently observed. In contrast to hyalohyphomycetes for which an acute (45°) branching pattern is observed, wide branching angles (≥90°) are suggestive of *Mucorales*. If hyphae are fragmented, the typical features are missing, which makes it difficult to make a reliable diagnosis based solely on direct microscopy. However, the same characteristic features can be observed in tissue samples after histopathological preparation based on Gomori methenamine-silver or periodic acid-Schiff staining. Of note, the detection of *Mucorales* is difficult using hematoxylin-eosin stain. Sometimes, if only a few hyphae are present and the tissue section contains cross-sections through the hyphae, it can produce the appearance of yeast or vacuole-like structures, making the morphology difficult to interpret. Classically, *Mucorales* are angio-invasive, with invasion of venous and arterial walls frequently associated with infarction of the surrounding tissue (72). The inflammatory response is varied, ranging from none at all to a neutrophil infiltrate alone, granulomatous response alone, or both together. Perineural invasion can also be observed (72). Immunohistochemistry techniques based on commercially available kits can be used in difficult cases (73).

Antigen Detection

There are currently no specific antigen detection methods available for the diagnosis of mucormycosis. Moreover, testing for the presence of β-D-glucan is not helpful.

Isolation Procedures

Recovery of *Mucorales* from clinical specimens is difficult, with positive culture in only 15 to 25% cases (71). However, culture is suitable for the definite diagnosis of mucormycosis, especially in cases of negative direct examination, keeping in mind that the lack of galactomannan antigen detection may indirectly suggest the diagnosis of mucormycosis. Indeed, clinical or radiological features are not specific for invasive fungal infection, nor can they distinguish between mucormycosis and other invasive fungal infection due to *Aspergillus*, *Fusarium*, or *Scedosporium*, especially in the hematological setting. However, the therapeutic management is different and isolation of a fungus is sometimes the unique diagnostic element. Culture is also of prime importance for identification to the species level, especially since morphological structures are not species specific in tissues, and for antifungal susceptibility testing of the isolate (74). Primarily, culture is typically performed on rich medium such as Sabouraud dextrose agar with additional antibiotic to inhibit bacterial growth. Cycloheximide-containing media should not be used, since inhibition of fungal growth can be observed for some species. After inoculation, plates must be incubated between 25–30°C and 37°C to allow growth of thermotolerant and thermointolerant isolates (75). However, Sabouraud dextrose agar is often not appropriate for morphological identification purposes, and growth on potato dextrose agar (PDA) is more suitable.

Detection of Nucleic Acids in Clinical Materials

In many cases, histopathology and/or direct examination are positive but culture fails. Species identification is, however, important for epidemiological purposes and because of species-specific antifungal susceptibility profiles. PCR-based molecular identification can be performed using formalin-fixed, paraffin-embedded (76) frozen or fresh tissue specimens. In formalin-fixed, paraffin-embedded tissues, extracted DNA is often of poor quality due to fragmentation after fixation (77). Furthermore, extracted DNA usually contains small amounts of fungal DNA relative to the large quantity of human DNA. Consequently, PCR-based identification must rely on amplification of small fragments of DNA that should ideally allow sequence discrimination between species upon sequencing. PCR based on internal transcribed spacer 1 (ITS1) and ITS2 primers followed by sequencing has been proposed in formalin-fixed, paraffin-embedded organs recovered from mice with experimental mucormycosis (78). It has also been evaluated in formalin-fixed, paraffin-embedded human tissues with identification of *Mucorales* in 7/18 samples harboring nonseptate hyphae based on histopathological reporting (76). Seminested PCR technique based on the 18S ribosomal DNA loci has also been used with *Mucorales* identification in 13/23 samples for which nonseptate hyphae were observed by histology (79). This molecular tool has also been prospectively evaluated with identification of *Mucorales* in six samples, of which five were culture positive (80, 81). An approach based on real-time PCR has been developed based on cytochrome *b* locus amplification. It allowed discrimination between different genera of *Mucorales* using analysis of melting curves (identification of 35/62 formalin-fixed, paraffin-embedded tissue samples) (82). Multiplex PCR coupled with electrospray ionization in direct-positive/culture-negative unfixed clinical samples seems potentially interesting, allowing genus and species identification (A. Alanio and S. Bretagne, unpublished results).

Fluorescent dye-labeled oligonucleotide with direct hybridization (fluorescent *in situ* hybridization) on the pathologic tissue is a promising tool, since it does not require DNA extractions or PCR amplification and can be performed on formalin-fixed, paraffin-embedded tissue samples. It uses fluorescent DNA probes that are specific for 5.8S or 18S RNA of different groups of fungi. After hybridization, fluorescence of the microorganism can be observed directly on the slide (83, 84). Localization of the fluorescence can provide additional information that can reinforce the diagnosis. This tool seems particularly adapted when the yield of DNA extraction or the quality of the extracted DNA is poor. Indeed, PCR-negative fluorescent *in situ* hybridization-positive results have been observed (84).

PCR-Based Diagnosis in Serum: Mucormycosis Screening

In addition to the molecular methods developed for fungal identification from tissues, another strategy, inspired by the screening strategy for patients at risk of aspergillosis, is emerging for the diagnosis of mucormycosis. In a population with a relatively high prevalence of mucormycosis, one recent study proposed to screen sequential serum samples by PCR targeting of *Mucorales* genes (three genus-specific independent PCRs). Using this method, the authors were able to establish the diagnosis of mucormycosis between 68 and 3 days before that based on histopathology or culture (85). This strategy can be envisioned for use in the future in high-risk populations to shorten the current delay in diagnosis.

Identification

Phenotypic Identification

General Description

Routine identification of *Mucorales* to the species level is mainly based on the examination of their macroscopic and asexual microscopic characteristics, although some other criteria such as physiological tests (86) or maximum growth temperature can be used. The formation of zygospores is not useful in routine identification unless the species is homozygous. The use of media with high carbohydrate content favors production of an abundant mycelium which inhibits the production of asexual fruiting bodies by which the species can be identified. Therefore, media such as 2% malt, potato dextrose, and cherry decoction (acidic) agars are recommended for subculture of most of the *Mucorales* species, although some of these media are not commercially available. To trigger sporulation in genera such as *Apophysomyces* or *Saksenaea*, which are emerging pathogens (87–89), nutritionally deficient media containing a low percentage of yeast extract solution are advisable (90). Multiple factors such as light and temperature also influence the growth, the morphology, and the sporulation of these fungi. Several species are thermotolerant, capable of growing at temperature well over 40°C. Mycelial development of the *Mucorales* tends to be rapid (24 to 48 h) and extensive. Colony appearance varies according to the species, the age of the culture, and the mycological media used. It is recommended for an accurate morphological study of *Mucorales* to use subculture at 27 to 30°C. Macroscopic examination involves the description of the colonies (height, color, texture) and the sporangiophore spatial branching configuration that can be observed under a 10× objective or a binocular loupe. For microscopic characteristics, a detailed study of adhesive tape mounts or teased mounts (small piece of mycelium plus fruiting bodies placed in a water drop and gently needle teased) is essential for the description of the sporulating structures of the species. Fungi in the order *Mucorales* are characterized by branched, nonseptate, wide mycelia (10 to 20 μm) with chitinous walls. Sexual reproduction occurs by means of zygospore formation after fusion of hyphal branches from the same (homothallic) or from sexually differentiated mycelia (heterothallic) (91). The mature zygospore is often thick walled and undergoes an obligatory dormant period before germination. Asexual multiplication is by means of nonmotile sporangiospores (endospores) borne in closed sac-like structures named sporangia. They can exist as multispored sporangia or sporangiola (small sporangia) having few (to one) spores. Sporangia are supported by specialized hyphae named sporangiophores. In some species, they arise from a branched system of rhizoids, which anchor the sporangiophore to the substratum. These rhizoids are connected by a rooting branch called a stolon. Additional morphological structures are the columellae (central axis of the sporangium) and the apophysis (a swelling of the sporangiophore just below the columella). Some species can produce thick-walled and/or thin-walled swollen structures named chlamydospores and oidia, respectively. The liberation of sporangiospores occurs by breakage or deliquescence of the sporangial wall (Fig. 1). Structures of *Mucorales* used for routine identification include branching of sporangiophores, type of sporangia (merosporangia or sporangiola), shape, color, presence or absence of apophyses and columellae, and presence or absence of rhizoids or chlamydospores.

Several potentially useful websites are available: www.doctorfungus.org; www.mycology.adelaide.edu.au; www.cbs.knaw.nl; http://www.mycobank.org.

Description of Specific Genera and Species

Morphological (macroscopic and microscopic) characteristics of those species frequently implicated in human infections are described below. Only major features are mentioned based on our own experience and descriptions from specialized books (14, 20, 92). All colony morphology descriptions are made from subcultures on MEA (2% malt agar) at 30°C (unless otherwise specified). The colonies of *Mucorales* have mostly floccose textures with colors varying from white (*Saksenaea*) to yellow (*Mucor*), brownish (*Apophysomyces*), or gray (*Lichtheimia*, *Rhizomucor*). *Rhizopus* produces high aerial mycelium, whereas *Rhizomucor* produces a low aerial turf of 2- to 3-μm-high mycelium. The morphology of sporangiophores (height, branching) can vary depending on the genus. It can be branched as in *Lichtheimia* or *Mucor*, irregularly branched as in *Rhizomucor*, or mostly unbranched as in *Rhizopus*. In addition, the site from which the sporangiophore arises (between rhizoids or directly from the stolon) is a supplementary clue for the identification of the different genera (Fig. 2). A morphological key to the principal genera of *Mucorales* is illustrated in Fig. 3. The combination of asexual fruiting bodies and criteria such as maximum growth temperature are useful for the differentiation of these fungi to the species level. The phenotypic differences described below refer to species defined by nucleotide sequence analysis.

Family *Lichtheimiaceae*

Genus *Lichtheimia* Vuill. 1903 (formerly *Absidia* or *Mycocladus*). The genus *Absidia* has been revised on the basis of phylogenetic, physiological, and morphological characteristics (7). The thermotolerant species of *Absidia corymbifera*, *A. blakesleeana*, and *A. hyalospora* were placed in the new family of *Mycocladiaceae* and the genus *Mycocladus*. The same group suggested revision of the nomenclature rectification to create the family *Lichtheimiaceae* (instead of *Mycocladiaceae*) and the reassignation of the genus *Lichtheimia* in place of *Mycocladus* (93). The multigene sequence analysis of 38 isolates morphologically identified as *Lichtheimia corymbifera* uncovered the presence of a different species (named *L. ramosa*), which differs in morphology and nucleotide sequences from *L. corymbifera* (6). Of the five

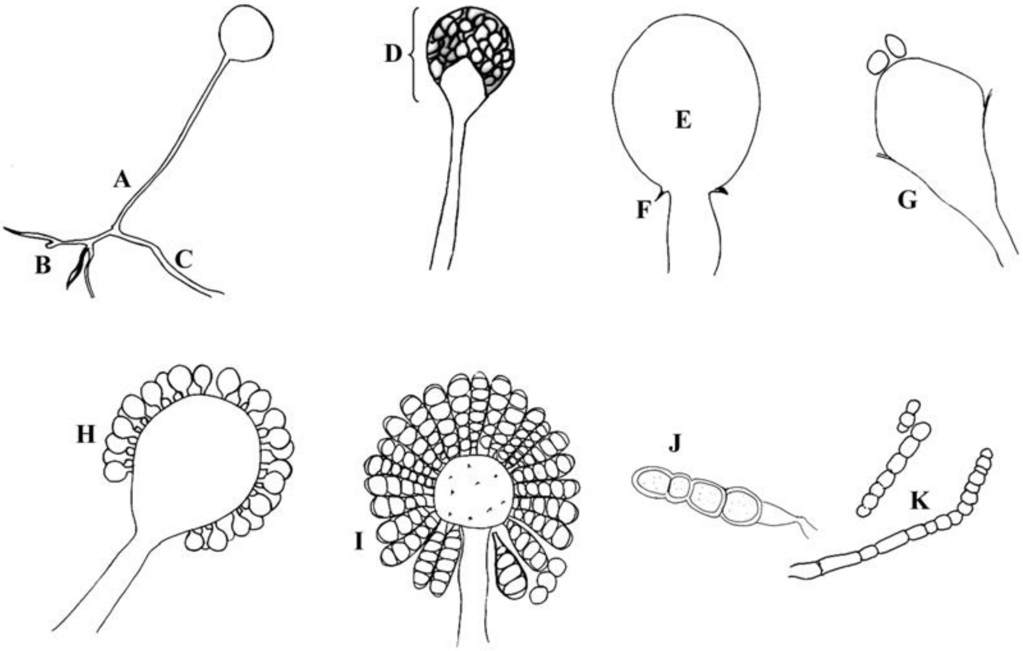

FIGURE 1 Schematic drawing of morphological structures observed in *Mucorales*. Sporangiophores (A) bear sporangia containing sporangiospores (D), can be anchored to the substrate by rhizoids (B), and expand by the means of stolons (C). The columella (E) is produced at the apex of the sporangiophore, and in some species, an apophysis (G) is present. For some species, after liberation of sporangiospores, a thin sporangium membrane may be visible (F). Sporangia with single or few spores are called sporangiola (H), and sporangia with few spores aligned in rows are called merosporangia (I). Thick-walled chlamydospores (J) and oidia (thin-walled swollen vesicles) (K) can be observed. Drawings by Dea Garcia-Hermoso. doi:10.1128/9781555817381.ch121.f1

recognized species of the genus *Lichtheimia*, only *L. corymbifera*, *L. ornata*, and *L. ramosa* are of clinical relevance (8). These species seem to have a worldwide distribution and have been isolated from diverse substrates including seeds, soil, and decaying vegetable debris. They produce white, fast-growing, woolly colonies, which become grayish brown with age. Maximum growth temperature is between 46°C and 52°C. *L. ramosa* differs from *L. corymbifera* and *L. ornata* by faster growth at high temperatures (8). Microscopically, sporangiophores are usually erect and highly branched and arise singly or in small corymbs from stolons but not opposite the rhizoids as in *Rhizopus*. Rhizoids are present but generally indistinguishable. Multispored sporangia are small and spherical to pyriform. Columellae are hemispherical or ellipsoidal with a marked conical apophysis, and sporangiospores are smooth, hyaline, and ellipsoidal, cylindrical, or subglobose (2.73 by 2.24 μm in diameter) (Fig. 4). Giant cells of irregular shape are frequently present. The morphologies of sporangiophores and sporangiospores are very similar for the three pathogenic species, even though significant differences in the lengths, widths, and length/width ratios of the sporangiospores were previously reported (41).

Genus *Rhizomucor* Lucet & Costantin 1900. *Rhizomucor pusillus* and *R. miehei* are the pathogenic species of the genus *Rhizomucor*. Species of the genus *Rhizomucor* are frequently isolated from composting or fermenting organic matter (94). Macroscopically, *Rhizomucor* produces rapidly growing gray (*R. miehei*) to brown (*R. pusillus*) woolly colonies. Sporangiophores are single or branched and arise from aerial mycelium or from stolons. Rhizoids are present but often difficult to recognize. Sporangia are multispored, apophyses are absent, and most species are thermophilic. Sporangiospores are round, hyaline, and smooth walled (Fig. 5). The species differ in the size of sporangia, sucrose assimilation, and the capacity to grow in presence of thiamine. *R. miehei* has smaller sporangia than *R. pusillus* (60 μm versus 100 μm in diameter) and fails to assimilate sucrose, and its growth depends on the presence of thiamine.

Family *Mucoraceae*

Genus *Mucor* Fresen. 1850. Four species are considered human pathogens: *Mucor circinelloides*, *M. irregularis* (formerly *R. variabilis* var. *variabilis*), *M. indicus*, and *M. ramosissimus* (10, 95).

Colonies of the genus *Mucor* are usually fast growing and white to yellow, becoming gray with time. Tall sporangiophores (they can reach several centimeters in height) are simple or branched, supporting multispored nonapophysate sporangia. Sporangiospores are hyaline and subspherical to ellipsoidal. In some species, residues of the sporangial wall (collarette) can be present, and some species can produce rhizoids and chlamydospores (Fig. 6).

Mucor circinelloides is a species complex comprising four varieties based mainly on differences in the shape of columellae and sporangiospores. Major phenotypic differences within the thermotolerant *Mucor* species involved in human infections are summarized in Table 2. Maximum growth temperatures for pathogenic *Mucor* species range from 35°C (*M. circinelloides*) to 42°C (*M. indicus*). The identification of these species can be complicated under unsuitable growth

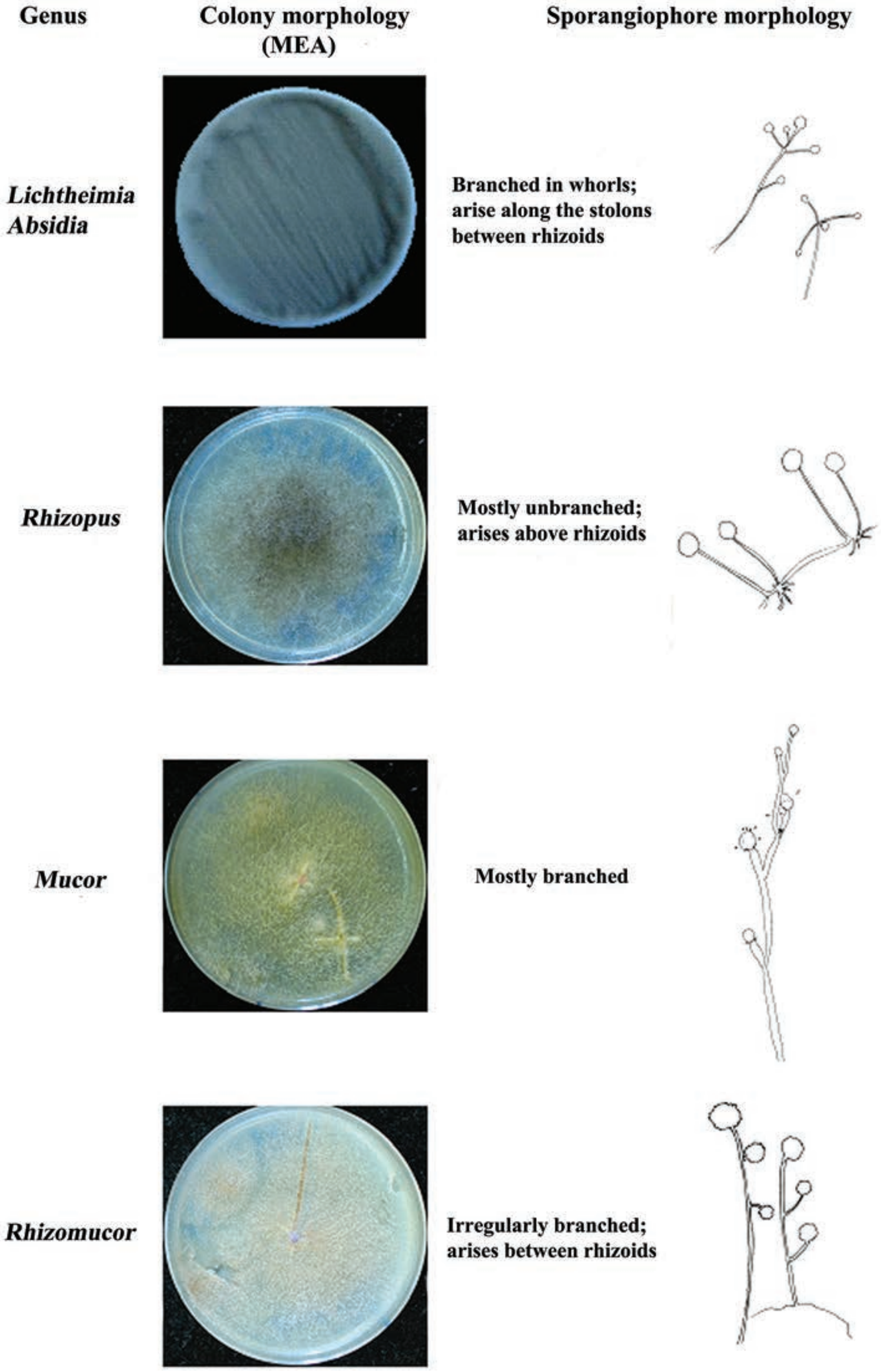
Genus
Colony morphology (MEA)
Sporangiophore morphology
Lichtheimia
Absidia
Branched in whorls; arise along the stolons between rhizoids
Rhizopus
Mostly unbranched; arises above rhizoids
Mucor
Mostly branched
Rhizomucor
Irregularly branched; arises between rhizoids

FIGURE 2 Culture variability of some of the genera of *Mucorales* and their branching development. doi:10.1128/9781555817381.ch121.f2

conditions which can induce morphological variations such as sterile sporangia, swelling in sporangiophores, and modifications in size and shape of sporangia, columellae, and sporangiospores (96).

Genus *Cokeromyces* Shanor 1950. *Cokeromyces recurvatus* remains a rare agent of mucormycosis. It has been mostly recovered from soil or from rabbit, rat, or lizard dung in North America (United States and Mexico). Colonies are slow growing (less than 1 mm high) and grayish to brown. Microscopic features include the presence of long, recurved, twisted stalks arising from terminal vesicles of unbranched sporangiophores, globose sporangiola (8 to 11 μm in diameter) borne on those stalks, and smooth-walled spherical sporangiospores 2 to 4 μm in size (Fig. 7). A yeast-like form can be obtained by subculturing on media such as brain heart infusion or yeast extract peptone agar. Yeast-like forms are thin to thick walled and spherical, measuring 10 to 100

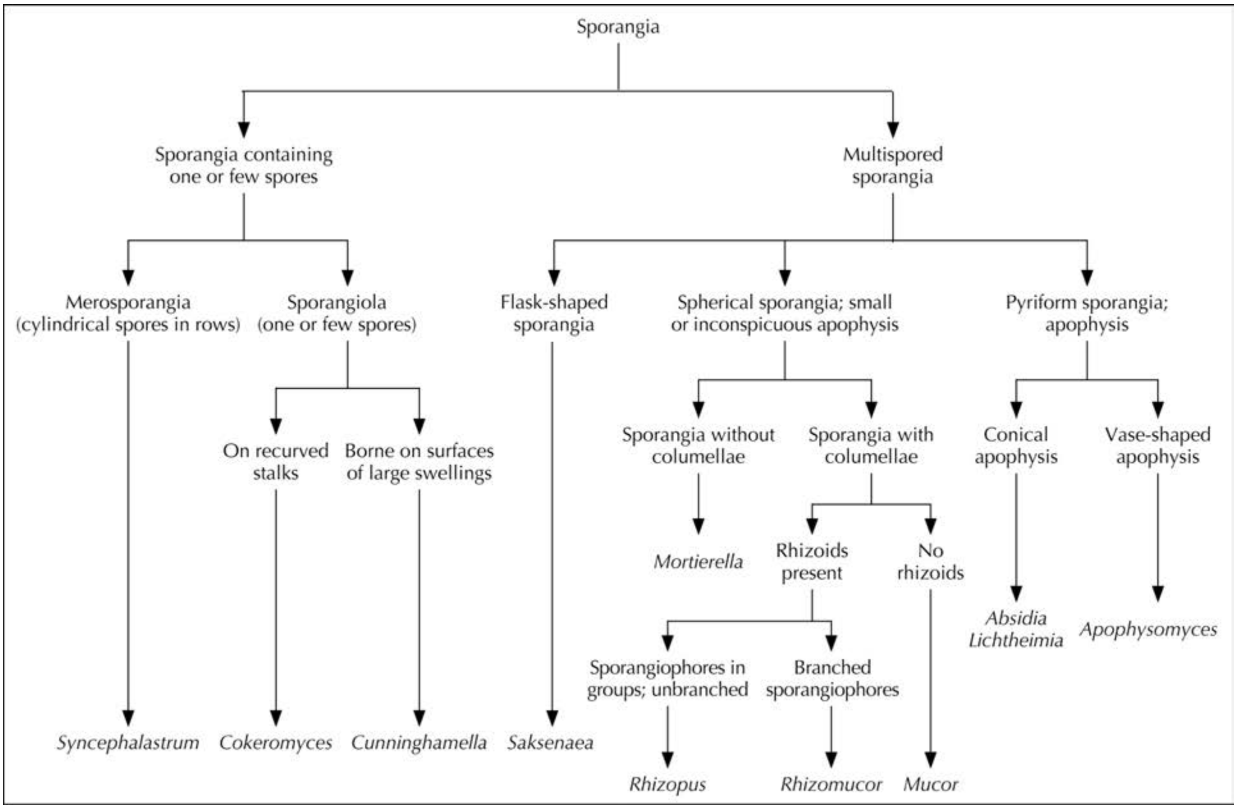
Sporangia
Sporangia containing one or few spores
Multispored sporangia
Merosporangia (cylindrical spores in rows)
Sporangiola (one or few spores)
Flask-shaped sporangia
Spherical sporangia; small or inconspicuous apophysis
Pyriform sporangia; apophysis
On recurved stalks
Borne on surfaces of large swellings
Sporangia without columellae
Sporangia with columellae
Conical apophysis
Vase-shaped apophysis
Mortierella
Rhizoids present
No rhizoids
Absidia
Lichtheimia
Apophysomyces
Sporangiophores in groups; unbranched
Branched sporangiophores
Syncephalastrum
Cokeromyces
Cunninghamella
Saksenaea
Rhizopus
Rhizomucor
Mucor

FIGURE 3 Flow diagram for the identification of the different genera of *Mucorales*.
doi:10.1128/9781555817381.ch121.f3

µm in diameter. Some cells produce single buds across their entire surface, producing a ship's wheel appearance similar to that of *Paracoccidioides brasiliensis* (20). The species is homothallic and produces lemon-shaped zygospores.

Family *Saksenaeaceae*

Genus *Apophysomyces* P.C. Misra 1979. The members of the genus *Apophysomyces* are soil fungi with a tropical to subtropical distribution. They produce fast-growing white to gray colonies. General features are erect sporangiophores, unbranched and single, bearing multispored sporangia, usually pyriform, of 20 to 60 µm in diameter. Columellae are hemispherical, cylindrical, trapezoidal, or ellipsoidal; the sporangiophores are smooth walled. The production of prominent vase-shaped, bell-shaped, or funnel-shaped apophyses is a distinctive feature of this species (Fig. 8). Good growth is observed at 42°C (92). The use of poor nutrient media (water-yeast extract medium) can improve sporulation often lacking on primary isolation media (90). Until recently, *Apophysomyces* was considered a monotypic genus with *A. elegans* as the only species. The molecular diversity study performed by Alvarez and collaborators revealed three additional species (*A. variabilis*, *A. trapeziformis*, and *A. ossiformis*) of *Apophysomyces* based on morphological, physiological, and phylogenetic differences (9).

Genus *Saksenaea* S.B. Saksena 1953. The genus *Saksenaea* is considered a complex of species. Alvarez et al. described two new species (*S. erythrospora* and *S. oblongispora*) different from *S. vasiformis*, long considered the only species in the genus (97). They are saprophytic fungi with a worldwide distribution first isolated from forest soil by Saksena (16). They produce white-grayish expanding colonies with a maximum growth temperature of 44°C. The use of specific poor nutrient media (water-yeast extract medium) can induce sporulation, as suggested by Padhye and Ajello (90). Czapek medium at 30 or 37°C also induces sporulation. Microscopically, sporangiophores are unbranched with melanized rhizoids. The length of sporangiophore and sporangia, the shape and size of sporangiospores, and the maximum temperature for growth are useful parameters for the recognition of the different species. Figure 9 shows the distinctive flask-shaped multispored sporangia, which is a key feature for the identification of this genus.

Family *Rhizopodaceae*

Genus *Rhizopus* Ehrenb. 1821. *Rhizopus arrhizus* (syn. *R. oryzae*) and *Rhizopus microsporus* are the most common pathogenic species. This genus commonly found in the air, soil, and compost is characterized by the rapid production of white cottony colonies, which turn brownish to black with time due to the presence of pigmented sporangiophores and sporangia. Table 3 indicates useful features allowing the distinction between the pathogenic species of the genus *Rhizopus*. The sporangiophores are unbranched, arising singly or in groups, with well-developed rhizoids at the base, which distinguishes the genus *Rhizopus* from the genera *Lichtheimia* and *Rhizomucor*.

The microscopic features of *R. arrhizus* include single or clustered brown sporangiophores of 1 to 2 mm in height bearing multispored sporangia (150 to 170 µm in diameter). Colu-

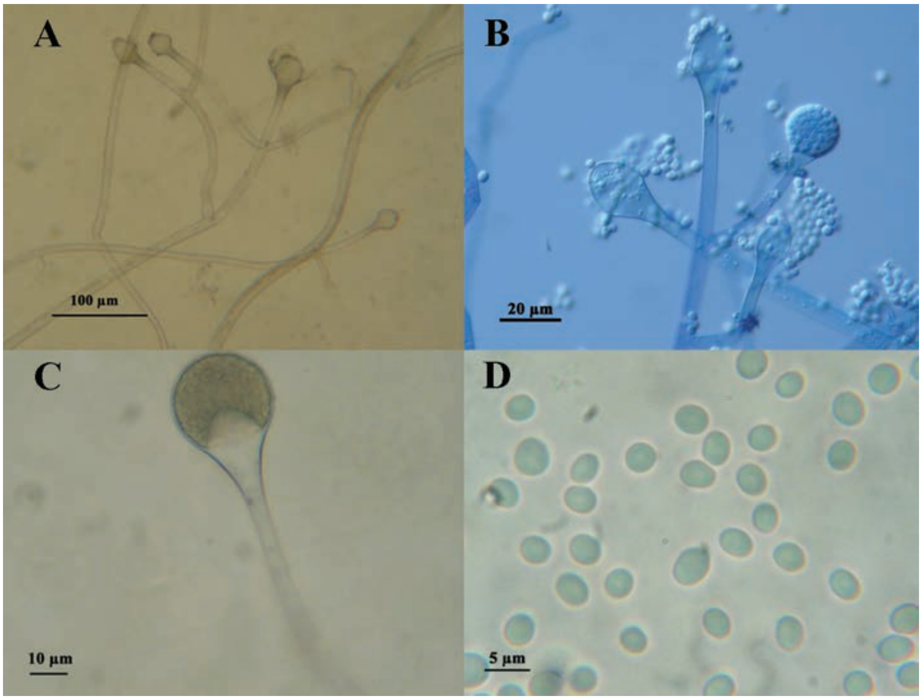

FIGURE 4 *Lichtheimia corymbifera*. (A) Branched sporangiophores; (B) hemispheric columellae and sporangiospores; (C) pyriform multispored sporangium and marked conical apophysis; (D) sporangiospores. doi:10.1128/9781555817381.ch121.f4

mellae are ellipsoidal and brown to gray, and they generally have a truncate base. Rhizoids are well developed and easily observed under the stereomicroscope. Sporangiospores are angular and round to ellipsoidal, with longitudinal ridges 6 to 8 µm by 4.5 to 5.0 µm in size. Chlamydospores (10 to 35 µm in diameter) can be present (Fig. 10). This thermotolerant species can grow at 40°C but not at 45°C. *R. arrhizus* differs from the nonpathogenic *R. stolonifer* which harbors longer sporangiophores (>2 mm) and larger columellae (up to 275 µm in diameter) and does not grow at 40°C (98).

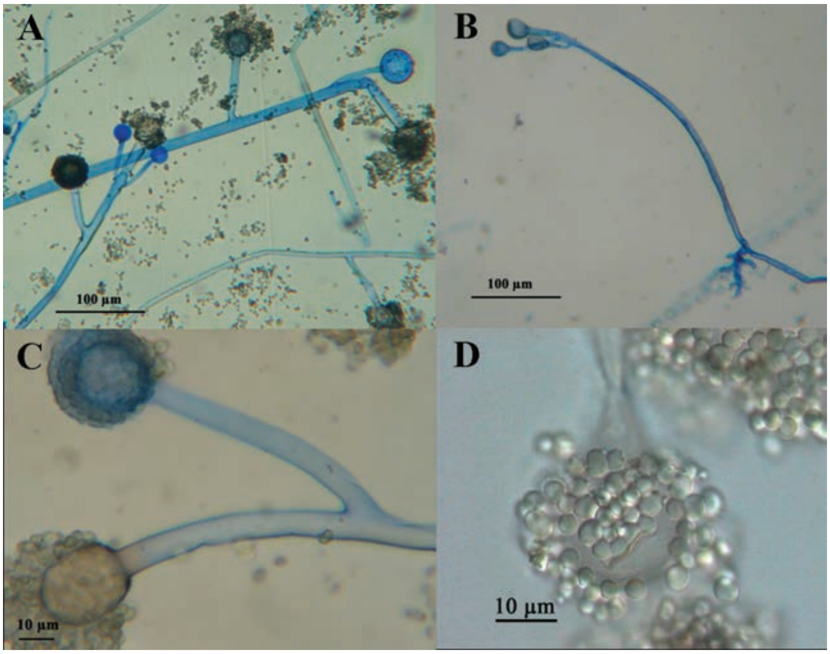

FIGURE 5 *Rhizomucor pusillus*. Sporangiophores with apical branching (A), simple rhizoids (B), and globose sporangia with round and smooth-walled sporangiospores (C and D) are shown. doi:10.1128/9781555817381.ch121.f5

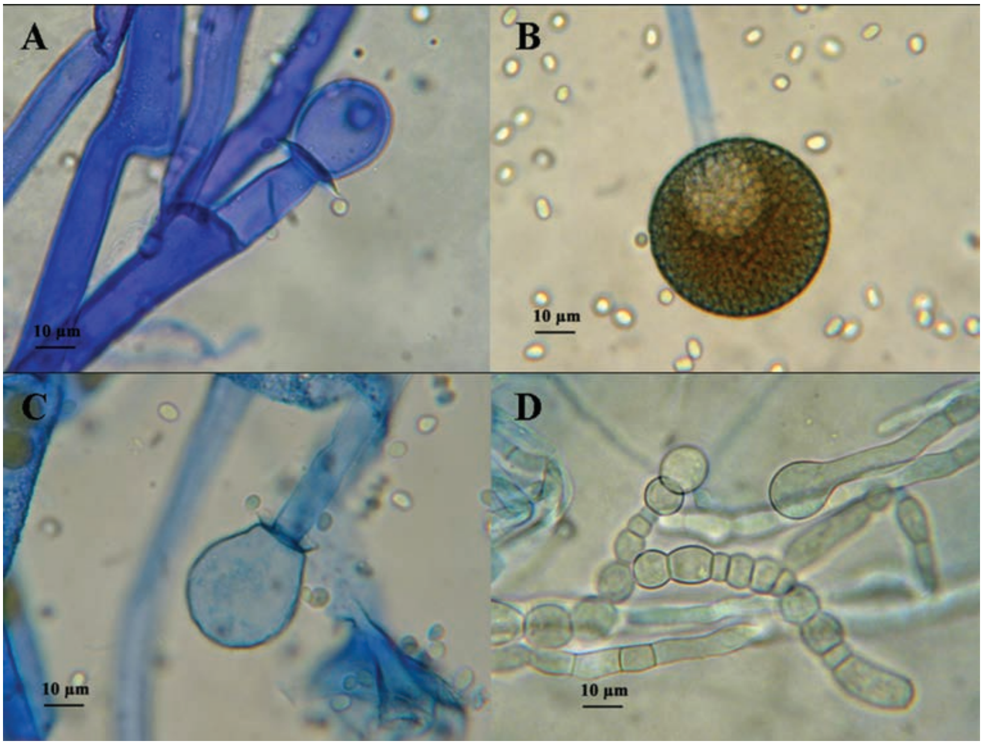

FIGURE 6 Micromorphology of *Mucor circinelloides*. (A) Sporangiophore; (B) sporangia; (B and C) ellipsoidal sporangiospores; (D) oidia. doi:10.1128/9781555817381.ch121.f6

According to one recent U.S. epidemiological survey, *R. microsporus* is the second most frequent agent of mucormycosis (99). It can be isolated from soil or wood products. Colonies are pale brownish gray with sporangiophores arising from stolons and measuring up to 400 µm in length (100). Sporangia are dark brown (up to 80 µm diameter), columellae are conical, and sporangiospores are striate and angular to ellipsoidal (Fig. 11). In addition to the typical *R. microsporus* var. *microsporus* there are three supplementary varieties: *R. microsporus* var. *chinensis*, *R. microsporus* var. *oligosporus*, and *R. microsporus* var. *rhizopodiformis*. They can be distinguished on the basis of the morphology of sporangiospores and temperature tolerance. These differences observed between varieties are not supported genetically (101). Table 3 indicates major differences among the species of the genus *Rhizopus*.

Family *Cunninghamellaceae*

Genus *Cunninghamella* Matr. 1903. Traditionally, *Cunninghamella bertholletiae* was considered the only clinically relevant species of the genus. Although rarely involved in human disease, *C. bertholletiae* has the capacity to infect immunocompetent individuals. Other species such as *C. elegans*, *C. echinulata* (102), and more recently, *C. blakesleeana* (103) have also been implicated in cases of human mucormycoses. *C. bertholletiae* is a thermotolerant fungus isolated from soil. It produces white to dark gray expanding colonies. Sporangiophores are erect with lateral branching at the apical zone. Each branch produces a globose vesicle (up to 40 µm in diameter), which bears 1-spored sporangiola. At maturity, each sporangiola will become a finely echinulate spherical sporangiospore (7 to 11 µm in diameter) (Fig. 12). Key features for the identification of *C. bertholletiae* are essentially its growth at 45°C (with a maximum of 50°C) and the presence of monosporic sporangiola borne on terminal vesicles.

Family *Syncephalastraceae*

Genus *Syncephalastrum* J. Schröt. 1886. Species from the genus *Syncephalastrum* have a worldwide distribution and have been isolated from soil, plants, and diverse foodstuffs (16). The only species associated with cases of human infection is *S. racemosum*, which is described below.

In culture, *S. racemosum* grows rapidly and produces expanding white grayish colonies, which turn darker due to the formation of sporangia and resemble the colonies formed by *Rhizopus* spp. The maximum temperature for growth is 40°C. Sporangiophores are short, erect, and mostly branched, arising from rhizoids and forming terminal globose vesicles. The whole surface of these vesicles is covered with cylindrical merosporangia containing (3 to 14) smooth-walled, spherical to ovoid merospores arranged in single rows (Fig. 13). This is a distinctive feature of the genus compared to other members of *Mucorales*.

Molecular Identification

The species identification of *Mucorales* using phenotypic methods remains a difficult task due to the common morphological features among the members of this group. In addition, some pathogenic species, such as members of *S. vasiformis* species complex and *A. elegans* species complex, regularly fail to sporulate on standard mycological media (104). Therefore, sequence-based identification is of interest as it provides fast and easily comparable data (105). In search of reliable DNA barcode markers for *Fungi*, Schoch and collaborators compared the performance of six markers: three nuclear ribosomal regions (ITS, ribosomal large subunit, ribosomal small subunit) and portions of three protein-coding genes on 17 fungal lineages. The ITS was finally proposed as the primary fungal

TABLE 2 Macroscopic and microscopic features in *Mucor* species involved in human infection

Species	Colony morphology; ht	Sporangiophores	Sporangia (diam)	Columellae (shape)	Sporangiospores (shape)	Maximum growth temp (°C)	Chlamydospores	Other characteristics
M. *circinelloides*	Light gray to brown; up to 6 mm	Repeatedly branched	Up to 80 µm	Obovoid to ellipsoidal	Ellipsoidal	36–40	Rare	Assimilation of ethanol
M. *velutinosus*	White-grayish; up to 2 mm	Sympodially branched	Up to 60 µm	Globose to conical	Verrucose, globose to subglobose	38	Abundant	Presence of rhizoids
M. *irregularis* (formerly *Rhizomucor variabilis* var. *variabilis*)	White to yellowish; up to 2 mm	Simple or once branched	Up to 100 µm	Irregular	Variable	38	Abundant	Presence of rhizoids
M. *ramosissimus*	Olive gray; up to 2 mm	Abundant swellings present	Up to 80 µm disposed on short lateral branches	Applanate	Smooth, subglobose to ellipsoidal	36	Absent	Presence of oidia in substrate mycelium
M. *indicus*	Deep yellow; up to 10 mm	Repeatedly branched	Up to 75 µm	Subglobose	Subglobose to cylindrical-ellipsoidal	42	Abundant	Light-dependent sporulation

barcode across all groups (106). Recently, a large phylogenetic study (more than 650 strains) validated the ITS marker as a useful barcode for the identification of *Mucorales*. Most of the accepted species that are currently taxonomically defined by morphology were well discriminated. However, the identity threshold of 99% defined by Balajee et al. in 2009 (107) did not cover the intraspecific variability of clinically important species tested (101). Additional phylogenetic studies to delimit species boundaries and the analysis of intraspecies and interspecies variations are needed to define a new identity threshold to establish a reliable ITS-based identification for these organisms.

Typing Systems

By definition, typing methods are developed to study the diversity and relatedness of isolates (environmental or clinical isolates) within a given species. Few methods have been evaluated for the typing of clinical isolates of *Mucorales* (24, 108, 109). These methods proved to be inefficient due to their low discriminatory power. Whole-genome sequencing may provide the key to designing efficient typing methods in the future (87).

Serologic Tests

No commercial assays are available at this time.

Antifungal Susceptibilities

In vitro antifungal susceptibility can be evaluated using the reference microdilution broth techniques from the CLSI (110) and the EUCAST (111) or, alternatively, using a commercially available test (Etest) (112). Although *Mucorales* share most antifungal susceptibility patterns, there is some specificity depending on genus and even species. AMB is the most active drug against the majority of *Mucorales* as shown both *in vitro* (74, 113–117) and in animal models of infection (118–122). Among the new azoles, voriconazole has poor activity, which is highlighted by breakthrough mucormycosis in patients treated with voriconazole (123) and in experimental models (124). In contrast, posaconazole has relatively low MICs (74, 114, 115, 122, 125), and the *in vivo* efficacy of posaconazole has been demonstrated as curative and prophylactic treatment in animal models (126, 127). Echinocandins have no significant *in vitro* activity (128, 129), despite the fact that *Rhizopus arrhizus* possesses the target enzyme for this class of compounds. *In vivo*, caspofungin alone exhibits modest efficacy (57) while it showed promising clinical efficacy in combination with AMB in some patients (96).

Evaluation, Interpretation, and Reporting of Results

Culture results should always be interpreted in light of the clinical presentation and along with the results of direct examination and/or histopathology. A positive culture of *Mucorales* can be due to contamination during collection of the sample or processing of the sample in the laboratory. A study in Spain showed that less than 8% of the *Mucorales* isolates recovered in the laboratory were from patients with invasive mucormycosis (32, 130). In the revised definitions of the EORTC/MSG study group for the diagnosis of invasive fungal infections, proven disease requires that "histopathologic, cytopathologic, or direct microscopic examination of a specimen obtained by needle aspiration or biopsy shows hyphae accompanied by evidence of associated tissue damage" or "the recovery of a mould by culture of a specimen obtained by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consistent with an infectious

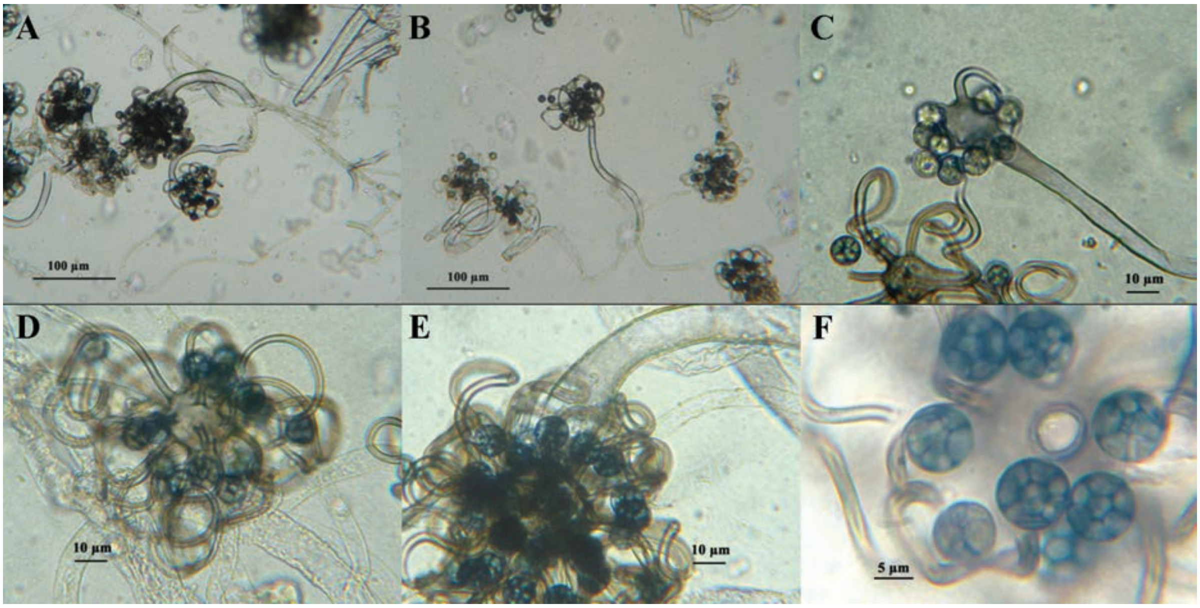

FIGURE 7 *Cokeromyces recurvatus*. (A and B) Mostly unbranched sporangiophores without rhizoids; (C) young sporangiolum; (D to F) recurved stalks arising from terminal vesicles and bearing few-spored sporangiola. doi:10.1128/9781555817381.ch121.f7

disease process, excluding bronchoalveolar lavage fluid, a cranial sinus cavity specimen, and urine" (131). However, "the failure to meet the criteria for invasive fungal infection does not mean that there is none, only that there is insufficient evidence to support the diagnosis. This is the most compelling reason for not employing these definitions in daily clinical practice." One may thus have proof of an invasive fungal infection and a high suspicion of mucormycosis without evidence of the latter in the absence of positive culture. As mentioned before, attempts to establish the definite diagnosis are important to offer the best management for the patient.

ENTOMOPHTHOROMYCOSIS

Epidemiology and Transmission

The majority of members of the *Entomophthoromycota* are pathogens of arthropods and other animals. They are present in soil, decaying vegetables, and dung worldwide but more abundantly in warm climates of Africa and Asia (20). Infections due to *Basidiobolus ranarum* are described in Asia (Indonesia, where the first cases were described; India; and Myanmar) and in several African countries (mostly Uganda and Nigeria) but rarely in South America. *B. ranarum* has recently

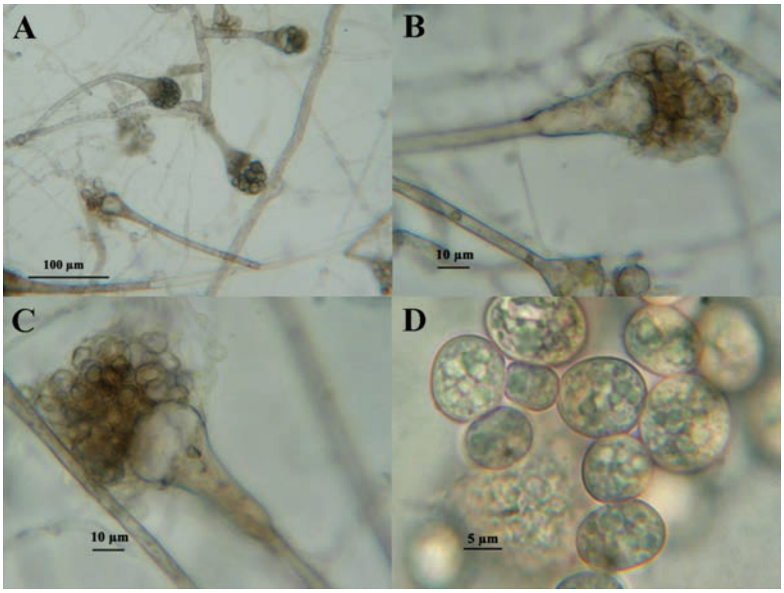

FIGURE 8 *Apophysomyces elegans*. (A) Unbranched sporangiophores bearing multispored sporangia with a vase-shaped apophysis (B and C); (D) cylindrical sporangiospores. doi:10.1128/9781555817381.ch121.f8

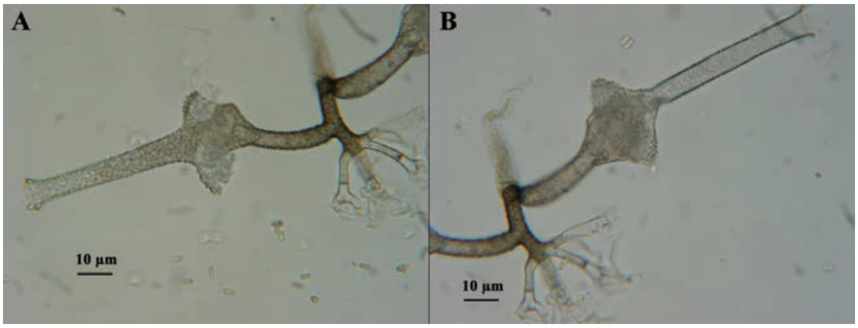

FIGURE 9 *Saksenaea vasiformis*. (A and B) Unbranched sporangiophores with dark rhizoids and typical multispored flask-shaped sporangia. doi:10.1128/9781555817381.ch121.f9

emerged with gastrointestinal presentations in the United States (132). Infections due to *Conidiobolus* spp. are described in Africa, Madagascar, Mayotte, India, China, Japan, and South America (133–135).

Clinical Manifestations

One of the major differences between mucormycosis and entomophthoromycosis is that the former occurs mainly in predisposed individuals, whereas the latter occurs mostly in apparently immunocompetent hosts.

B. ranarum is responsible for subcutaneous infections which mostly affect the limbs, buttocks, trunk, and perineum and less often the face and neck. The disease presents mostly in male children as a woody, hard, brawny, painless nodule that enlarges peripherally without affecting the overlying skin (136). Invasive infections with gastrointestinal involvement (132, 137) have been described in a small cluster in Arizona (138) and sporadically worldwide. Disseminated infections are extremely rare (139).

In contrast to basidiobolomycosis, infections due to *Conidiobolus* spp. affect adults (mostly males) and outdoor workers and are usually limited to the nose and face. The onset of the infection is thought to take place in the nasal mucosa after inoculation of spores following a minor trauma. Swelling extends locally to the nose, the nasolabial folds, cheeks, eyebrows, the upper lip, and even the palate and pharynx, producing the characteristic facies in severe forms (140). Conjunctival inoculation of the fungus has been documented once in Brazil (141). Rare cases of dissemination have been reported in immunosuppressed individuals (142). Tissue lesions caused by *C. coronatus* and *C. incongruus* are similar (143).

Treatment and Outcome

Therapeutic strategies for basidiobolomycosis and conidiobolomycosis are not standardized because of the lack of clinical trials for these rare infections. Surgical excision, potassium iodide, and prolonged azole therapy have been used successfully for infection due to *Basidiobolus* (26, 133). For infections due to *Conidiobolus* spp., potassium iodide was historically used with variable results. Prolonged oral azole therapy should now be used and is successful (134).

Collection, Transport, and Storage of Specimens

As several differential diagnoses are possible, a confirmation of the fungal agent should be obtained and tissue biopsy specimens of the affected area are the best diagnostic specimens. The diagnosis relies on classical procedures combining direct examination and culture in unfixed samples and/or histology in fixed samples. In the gastrointestinal form of basidiobolomycosis, biopsy specimens obtained during endoscopy are the

TABLE 3 Main characteristics allowing distinction among *Rhizopus* species

Species	Sporangiophores (ht)	Sporangiospores (shape and ornamentation)	Growth at temp (°C):				
			30	37	40	45	50
R. arrhizus (syn. *R. oryzae*)	Often higher than 1 mm	Angular, round to ellipsoidal; striate	+	+	+	Neg[a]	Neg
R. stolonifer			+	Neg	Neg	Neg	Neg
R. microsporus var. *microsporus*	Not exceeding 0.8 mm	Angular-ellipsoidal; striate	+	+	+	+	Neg
R. microsporus var. *oligosporus*		Large round; irregularly ornamented	+	+	+	Limited growth	Neg
R. microsporus var. *rhizopodiformis*		Small round; spinulose	+	+	+	+	+
R. microsporus var. *chinensis*		Angular, homogeneous	+	+	+	+ (immature sporangia)	Neg
R. microsporus var. *azygosporus*		Round; barely striate	+	+	+	+	+
R. schipperae		Round to ellipsoidal; striate	+	+	+	Very limited growth	Neg

[a] Neg, negative.

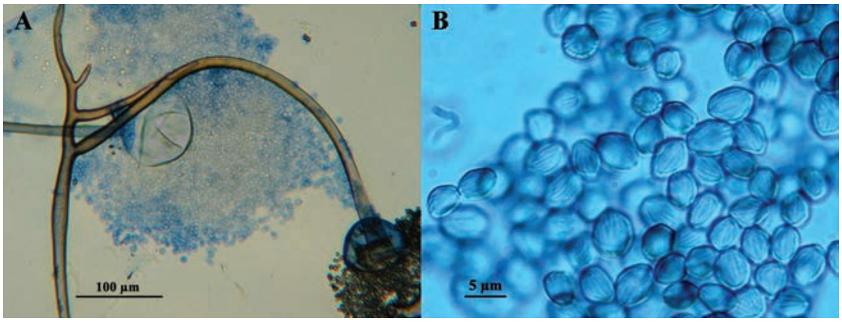

FIGURE 10 Typical microscopic features of *Rhizopus arrhizus*. (A) melanized sporangiophore arising opposite rhizoids; (B) striate angular sporangiospores. doi:10.1128/9781555817381.ch121.f10

gold standard. Specimens should be processed immediately and should not be refrigerated (136).

Direct Examination

A direct examination of fresh specimens can be performed after dissolution of the tissue sample in KOH and staining with calcofluor white. For histology, classical stains, including hematoxylin and eosin, periodic acid-Schiff, and Gomori Grocott, could be performed. Typically, hyphae are broad, thin walled, and generally more septate than those of the *Mucorales*. Simultaneous acute and chronic inflammatory reactions are observed in the affected tissue. Hyphae can be observed using routine staining procedures. The presence of a Splendore-Hoeppli reaction associated with typical hyphae in tissue sections stained with hematoxylin-eosin is highly suggestive of entomophthoromycosis. The pink structure corresponds to a sheath of amorphous eosinophilic material around hyphal fragments. Even if the presence of a Splendore-Hoeppli phenomenon is strongly suggestive of entomophthoromycosis, it can be observed in various bacterial, fungal, and parasitic infections as well as in noninfectious diseases (144). In contrast to mucormycosis, there is typically no necrosis and no invasion of blood vessels.

Antigen Detection

No antigen tests are currently available for the detection of entomophthoromycosis.

Detection of Nucleic Acids in Clinical Materials

A PCR-based technique targeting *Basidiobolus* has been developed for the diagnosis of entomophthoromycosis in human samples (145).

Isolation Procedures

Biopsy samples should be sliced or minced (not ground) and placed on Sabouraud agar or PDA. Media containing cycloheximide should be avoided because of inhibition of *Basidiobolus* and *Conidiobolus* growth. Cultures must be incubated at both 37°C and 25 to 30°C because of various optimum growth temperatures of these organisms. *Basidio-*

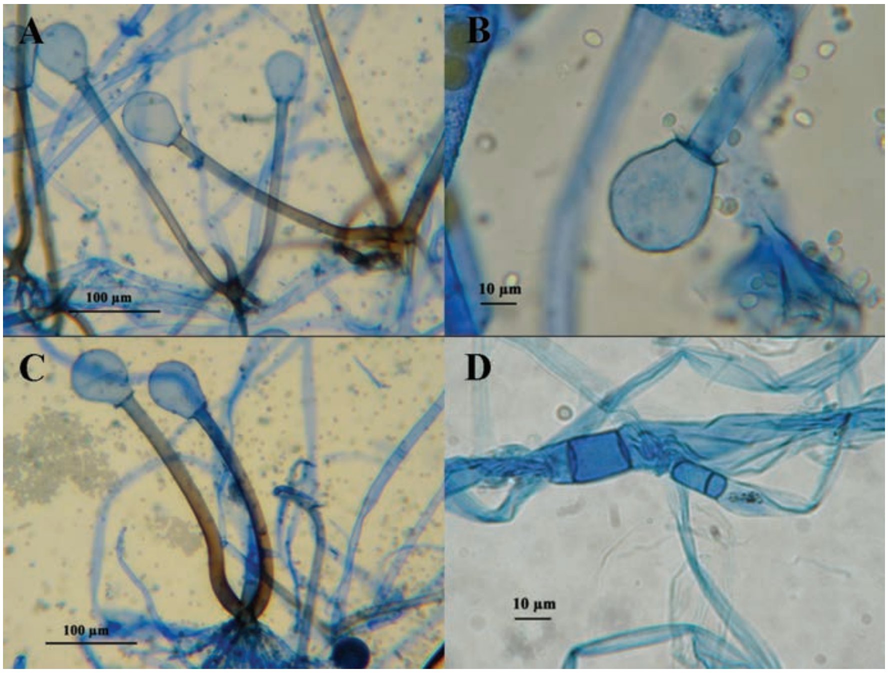

FIGURE 11 Short pigmented sporangiophores arising in pairs in *Rhizopus microsporus*. (A and C) Rhizoids; (B) conical columella; (D) chlamydospores. doi:10.1128/9781555817381.ch121.f11

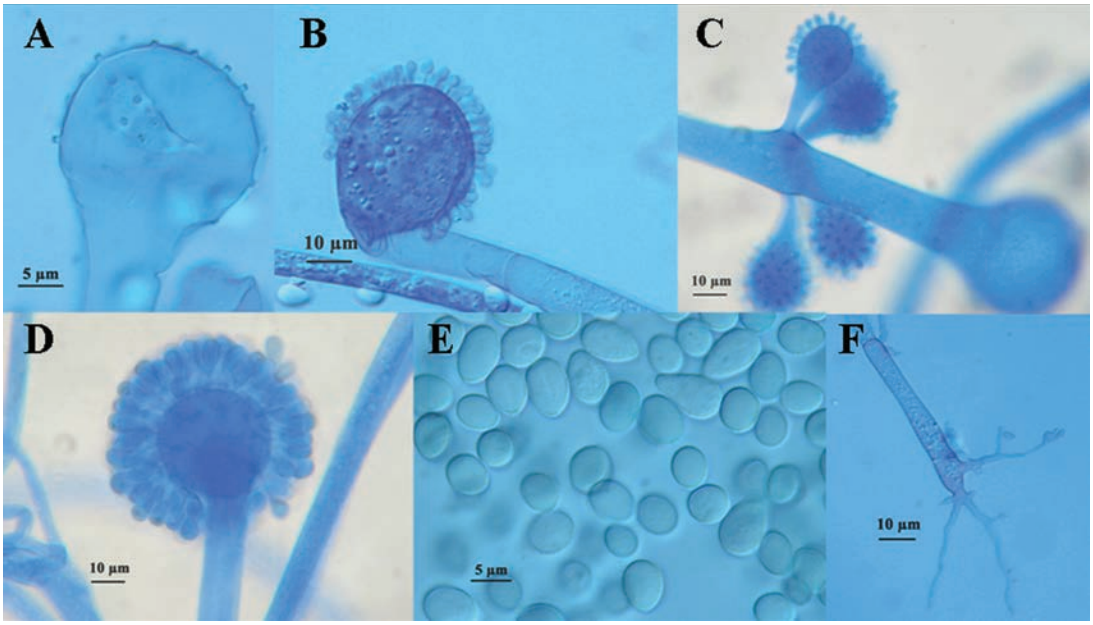

FIGURE 12 *Cunninghamella bertholletiae*. (A, B, and D) Development of single-spored sporangiola on the surface of globose vesicles, with frequent apical branching (C); (E) mature sporangiola grow to be single echinulate sporangiospores; (F) rhizoids. doi:10.1128/9781555817381.ch121.f12

bolus grows well at 30°C but less rapidly at 37°C, whereas *Conidiobolus* spp. grow rapidly at 37°C (16).

Identification

Phenotypic Identification

Members of *Entomophthoromycota* are characterized by the presence of coenocytic hyphae or short hyphal bodies and of primary and secondary conidia, which can be forcibly discharged at maturity. Primary conidia with papillate bases are produced straight from the thallus in repetitive cycles (14). They generate a germ tube or can produce smaller secondary conidia (similar in morphology to the primary conidia) in the presence of suitable substrate conditions. Villose conidia (old conidia with hair-like appendages) can also develop. Passive release of microconidia may also occur. In some species, thin

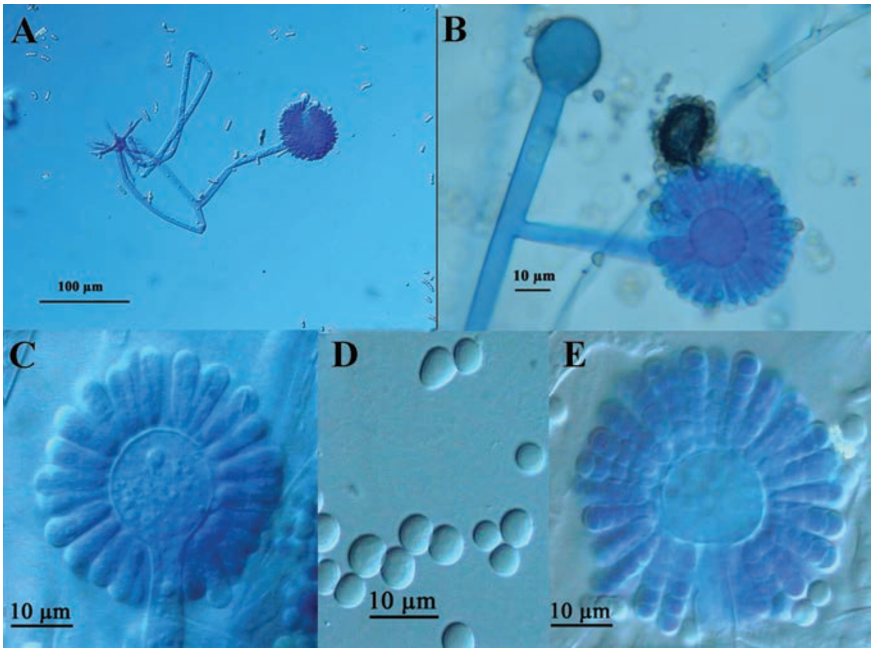

FIGURE 13 Short sporangiophores arising from rhizoids (A), terminal vesicles covered by merosporangia (B, C, and E), and merospores (D) characteristic of *Syncephalastrum racemosum*. doi:10.1128/9781555817381.ch121.f13

sporangiophores bear capilliconidia at their apex. These spores are characterized by an adhesive tip. Zygospores (thick, bilayered, walled spores) are produced after conjugation of undifferentiated gametangia and can have beak-like appendages coming from gametangial remains (14, 20, 92). Sporulation occurs after 3 to 10 days of culture. Colonies are usually waxy or powdery with radial folds and with colors ranging from white to tan-brown (14, 20). A key feature of these fungi is their capacity to forcibly discharge conidia. Therefore, placing a cover slide inside the Petri dish lid can be helpful to recover the papillate conidia. For a detailed description and additional information, see the taxonomic classification study by Ben-Ze'ev and Kenneth (146).

Class *Entomophthoromycetes,* Order *Entomophthorales*

Family *Ancylistaceae*

Genus *Conidiobolus* Bref. 1884. The genus *Conidiobolus* includes saprobes, facultative invertebrate, and vertebrate pathogens (13). The three species causing human conidiobolomycosis are *C. coronatus*, *C. incongruus*, and *C. lamprauges*.

Conidiobolus coronatus is a fast-growing fungus present in soil and on decaying leaves (92). It is most frequently isolated in the tropical forests of Africa (147). *C. coronatus* is an occasional pathogen of insects and has been recovered from diverse animals such as dolphins, chimpanzees, and horses (16).

Colonies are hyaline, radially folded, with an initially waxy appearance becoming powdery when mycelia become visible. The inside of the lid of the dish can be covered with conidia forcibly discharged by the conidiophores. These primary conidia are spherical (40 µm in diameter) and possess a prominent papilla. Villose conidia are present in older cultures (Fig. 14). Replicative passively discharged microconidia are regularly produced (14). *C. coronatus* can be differentiated from the two other pathogenic species by the absence of zygospores on PDA medium and the presence of villose conidia. Similarly, *C. incongruus* produces zygospores, which differ from those of *C. lamprauges* by the size and the formation of small globules inside the mature zygospores (148).

Order *Basidiobolales*

Family *Basidiobolaceae*

Genus *Basidiobolus*.

BASIDIOBOLUS RANARUM EIDEM. *Basidiobolus ranarum* is present in decaying fruit and vegetable matter (92). It can be present as a commensal in the intestinal tract of amphibians and reptiles (149). Colonies on PDA are yellowish and waxy, have radial folds, and do not form aerial mycelium when young. They grow well at 25 to 37°C. The microscopic examination after 7 to 10 days of culture reveals large asep-

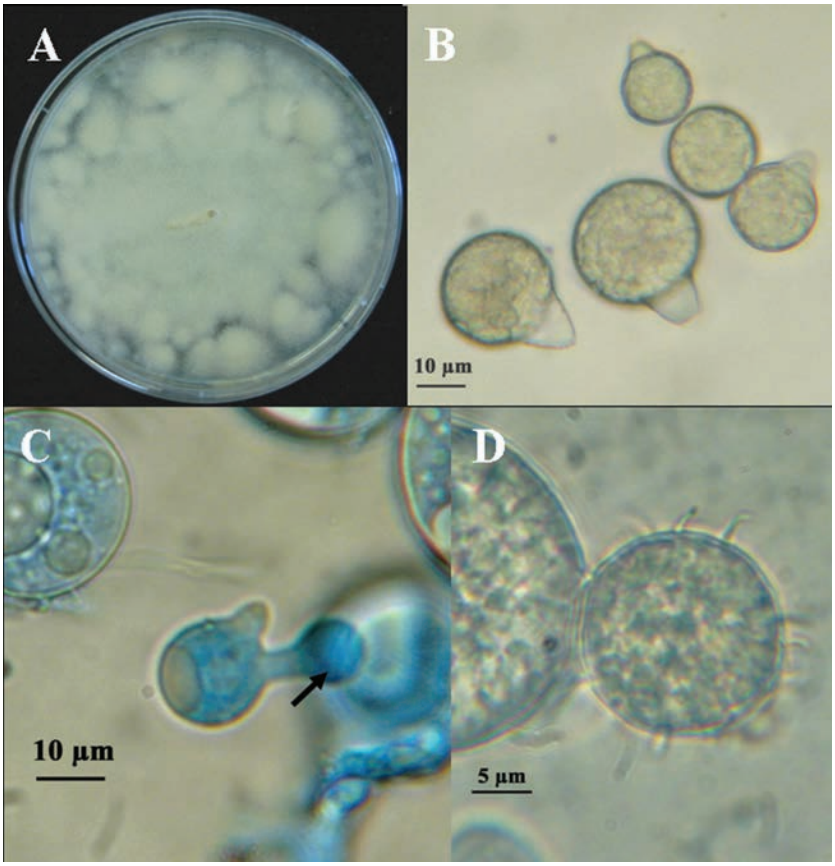

FIGURE 14 *Conidiobolus coronatus.* (A) Ten-day-old powdery colony on PDA medium; (B) primary conidia with papilla; (C) passively released secondary conidium (arrow); (D) villose conidium. doi:10.1128/9781555817381.ch121.f14

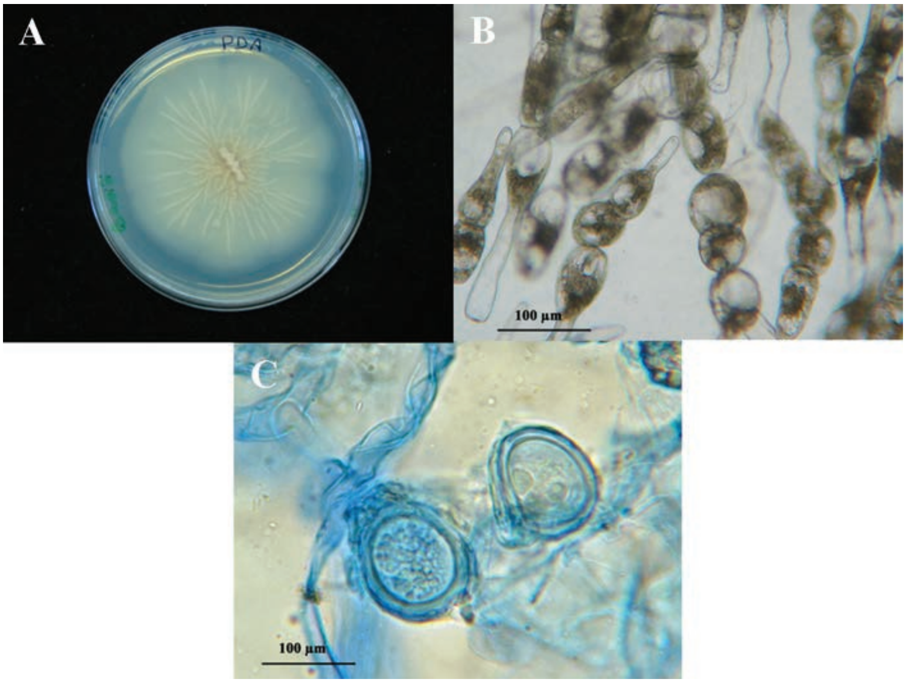

FIGURE 15 *Basidiobolus ranarum*. (A) Ten-day-old wrinkled colony on PDA medium (B); uninucleated hyphal elements; (C) young zygospores. doi:10.1128/9781555817381.ch121.f15

tate mycelia which can break up into free hyphal elements basically uninucleated. *B. ranarum* is homothallic. Sexual reproduction occurs by gametangial conjugation, producing thick-walled zygospores with lateral protuberances of gametangial remains (beaks) (Fig. 15). Primary conidiophores with swollen apices forcibly discharge spherical primary conidia. Secondary conidia are pyriform (clavate) and are passively released from the sporophore. These conidia possess a knob-like adhesive tip. Occasionally, elongated cells with a terminal adhesive tip (capilliconidia) are present (14, 147).

Molecular Identification

There is no technique published for the molecular identification of *Entomophthoromycota*.

Typing Systems

To date, there is no typing method developed for *Entomophthoromycota*.

Serologic Tests

There are no commercially available diagnostic tests.

Antifungal Susceptibilities

In vitro susceptibility data are scarce, and some technical issues (such as inoculum preparation) remain to be addressed. Potassium iodide shows no *in vitro* activity despite *in vivo* efficacy (150). Both *Conidiobolus* and *Basidiobolus* species exhibit relatively high MICs when tested with AMB, azoles, and echinocandins (151–153). Recently, Tondolo and colleagues reported terbinafine as an active drug against *C. lamprauges* (153). Overall, *Basidiobolus* spp. are more susceptible than *Conidiobolus* spp. to the different antifungals tested.

Evaluation, Interpretation, and Reporting of Results

In areas of endemicity, final diagnosis is based on the combination of direct examination showing broad thin-walled hyphae with a Splendore-Hoeppli phenomenon without necrosis or invasion of blood vessels. In case of positive cultures, results of antifungal susceptibility testing do not currently influence the therapeutic decision.

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Histoplasma, *Blastomyces*, *Coccidioides*, and Other Dimorphic Fungi Causing Systemic Mycoses[*]

GEORGE R. THOMPSON III AND BEATRIZ L. GÓMEZ

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TAXONOMY

The dimorphic fungi causing systemic disease belong to the class *Eurotiomycetes*, order *Onygenales*. The *Onygenales* share several general characteristics: their sexual stages (teleomorphs) form rudimentary asci surrounded by a network of hyphae, which may have complex appendages; and their asexual (anamorph) species generally possess one of two forms, either unicellular aleurioconidia or arthroconidia in chains of alternately viable and nonviable cells.

The order *Onygenales* contains the families *Ajellomycetaceae* and *Onygenaceae*. *Ajellomycetaceae* contains the species *Ajellomyces dermatitidis*, *Ajellomyces capsulatus*, *Ajellomyces duboisii*, and *Ajellomyces crescens*, the anamorphs of which are placed in the genera *Blastomyces*, *Histoplasma*, *Histoplasma*, and *Emmonsia*, respectively (1). The teleomorph of *Paracoccidioides brasiliensis* has yet to be discovered; however, DNA sequence data have placed *Paracoccidioides* in the *Ajellomycetaceae* family as well (2). *Onygenaceae* contains the species *Coccidioides immitis* and *Coccidioides posadasii*, and similarly, the teleomorphs of these genera have yet to be identified despite evidence that sexual reproduction and gene acquisition are ongoing in nature (3). Molecular phylogenetic studies have indicated that species of the *Onygenales* are divided into several clades (descendants of a common ancestor). The *Onygenaceae* and *Ajellomycetaceae* are well separated from the *Arthrodermataceae* (dermatophytes). In all of these phylogenetic trees, pathogenic organisms are interspersed with nonpathogenic relatives, which suggests that the capacity to infect humans has arisen numerous times during the evolution of the *Onygenales*.

This chapter covers the dimorphic members of the families *Onygenaceae* and *Ajellomycetaceae*, which include *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Histoplasma duboisii*, *P. brasiliensis*, and *C. immitis* and *C. posadasii* as well as *Emmonsia* species. Nonpathogenic species of *Chrysosporium*, *Uncinocarpus*, and other related genera are not covered here. It should be noted that taxonomic updates have taken effect as of 1 January 2013 and dual nomenclature for pleomorphic fungi has been discontinued (4). The ending of separate names for anamorphs and teleomorphs (one fungus now equals one name) has been a long-awaited change in mycology. The majority of fungi are likely to keep their more widely used anamorph name, however.

Histoplasma capsulatum

Historically, *H. capsulatum* was divided into three varieties: *H. capsulatum* var. *capsulatum*, a human pathogen found in North and South America; var. *duboisii*, a human pathogen found in Africa; and var. *farciminosum*, a pathogen of horses and mules found in parts of northern Africa and the Middle East. Phylogenetic studies have defined at least eight clades within *H. capsulatum*: North American class 1, North American class 2, Latin American group A, Latin American group B, Australian, Netherlands (Indonesian), Eurasian, and African clades (5). Seven of these eight clades comprise genetically and geographically distinct populations that can be regarded as phylogenetic species. The single exception, the Eurasian clade, originated from within the Latin American group A clade. *H. capsulatum* var. *farciminosum* was placed within the Eurasian clade. In addition to the seven phylogenetic species, another seven lineages represented by single isolates from Latin America were identified (5). These may represent additional phylogenetic species. Recent studies using multilocus sequence typing of *H. capsulatum* in formalin-fixed, paraffin-embedded tissues from cats living in regions where the pathogen is not endemic revealed a new phylogenetic clade. The *H. capsulatum* sequences recovered from the cats were most closely related to the North American class 1 clade but clustered separately outside this clade, suggesting that the *H. capsulatum* infecting the animals may represent a separate clade or phylogenetic species (6).

At this time, the disease African histoplasmosis is considered a distinct entity, but the taxonomic placement of *H. capsulatum* var. *duboisii* has been called into question by the finding of one var. *capsulatum* isolate from South Africa that was placed in the African (var. *duboisii*-containing) clade (5). This extends the results of earlier studies that had shown that var. *duboisii* had mitochondrial DNA restriction patterns identical to those of var. *capsulatum* strains.

The genome of *H. capsulatum* has an estimated size of between 28 and 39 Mbp, and currently sequence assemblies and annotations are available for four strains (http://www.broadinstitute.org/annotation/genome/histoplasma_capsulatum).

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[*] This chapter contains information presented by Mary E. Brandt, Beatriz L. Gomez, and David W. Warnock in chapter 120 of the 10th edition of this *Manual.*

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Blastomyces dermatitidis

The genus *Blastomyces* has previously been represented by a single species, *B. dermatitidis*, yet more recent work has challenged this notion and suggested that *B. dermatitidis* includes a cryptic subspecies or a separate species (7). Subsequent phylogenetic analysis using nuclear loci has placed *Blastomyces* isolates into one of two monophyletic clades: *B. dermatitidis* and the novel species *Blastomyces gilchristii* (8). Concordant with this work, genotyping studies of *B. dermatitidis* have revealed an association between clinical phenotype and genetic groups—findings suggesting that genotyping of isolates may help to predict patient clinical associations (9). Whole-genome sequence assemblies and annotations for four *B. dermatitidis* strains, including the highly virulent clinical strain SLH14081 and the relatively avirulent strain ER-3, can now be queried via the Broad Institute server (http://www.broadinstitute.org/annotation/genome/blastomyces_dermatitidis).

Coccidioides Species

Phylogenetic studies have led to the recognition of two species within the genus *Coccidioides*. The species name *C. immitis* is now restricted to isolates from California, while the name *C. posadasii*, in honor of Posadas, the author of the first description of coccidioidomycosis in Argentina in 1892 (10), has been proposed for all other isolates belonging to this genus. These two taxa were initially thought not to interbreed; however, more recent evidence has shown that genetic exchange between these species has occurred, with evidence of hybridization and genetic introgression (11). Later, a more extensive population sample, including isolates from Venezuela, Mexico, and Brazil, was studied by using a set of nine microsatellite markers (12). This study showed that two major clades could be distinguished. These clades, now named *C. posadasii* and *C. immitis*, correspond to the previous group I (non-CA [for non-California]) and group II (CA), respectively. Ongoing work using whole-genome sequencing will further assist our understanding of *Coccidioides* genotyping (13). Genome sequence assemblies and annotations of *C. immitis* and *C. posadasii* have been completed (14); the annotations of four strains of *C. immitis* and two strains of *C. posadasii* are now available for querying or downloading from http://www.broadinstitute.org/annotation/genome/coccidioides_group.

Paracoccidioides brasiliensis

Phylogenetic analysis of *P. brasiliensis* has shown that this fungus can be divided into at least three distinct species that appear to be confined to regions of endemicity: S1, PS2, and PS3 (15). These species have not been formally named. A proposal has also been made to name the highly divergent "Pb01-like" group "*Paracoccidioides lutzii*" (16, 17). Recent genomic and morphologic analyses strongly support the existence of a sexual cycle in species of the genus *Paracoccidioides* (18). Comparison of 18S and chitin synthetase sequences has indicated that *P. brasiliensis* is related to the uncultivable pathogen *Lacazia loboi*, the agent of lacaziosis (19) (chapter 127). However, these sequences demonstrated sufficient differences for *L. loboi* to be kept as an independent genus (19).

Since 2009, the Broad Institute has completed the sequencing, assembly, and annotation of the genomes of two strains of *P. brasiliensis* and one strain of *P. lutzii* (20): see http://www.broad.mit.edu/annotation/genome/paracoccidioides_brasiliensis.

Emmonsia Species

The genus *Emmonsia* currently includes three species: *E. crescens*, *E. parva*, and *E. pasteuriana* (21). The last has thus far only been recovered from a single case of disseminated cutaneous infection in a patient with AIDS (22), although a recent report from South Africa suggests that a new *Emmonsia* species related to *E. pasteuriana* may be more common than previously recognized (23).

E. crescens is known to form a sexual stage in the genus *Ajellomyces*, but *E. parva* has no known teleomorph. The assembly and annotation of *E. parva* and *E. crescens* genome sequences are currently under way (http://www.ncbi.nlm.nih.gov/bioproject/179100).

DESCRIPTION OF THE AGENTS

Histoplasma capsulatum

H. capsulatum is a thermally dimorphic fungus, displaying a filamentous mold form in the environment and in culture at temperatures below 35°C and a yeast phase in tissue and at temperatures above 35°C. The mold phase may contain two types of conidia (Fig. 1). Macroconidia are thick walled with a diameter of 8 to 15 µm and display characteristic tubercles or projections on their surfaces. Microconidia, smooth walled with a diameter of 2 to 4 µm, are the infectious particles (24, 25). The yeast phase develops as small, oval, budding cells with a diameter of 2 to 4 µm, often within macrophages (Fig. 2 and 3A). The yeast cell found in African histoplasmosis is thick walled and larger, 8 to 15 µm in diameter.

H. capsulatum is found in soils throughout the world. It grows best in soils with a high nitrogen content, particularly those enriched with bird or bat guano. Birds do not become colonized or infected with *H. capsulatum* (due to their high body temperatures), and their droppings are primarily a nutrient source. Soil samples from sites where birds have roosted have remained contaminated for at least 10 years after the roost has been cleared (26).

Histoplasmosis is the most common endemic mycosis in North America, but it is also found throughout Central and South America (24, 27). In the United States, the disease is most prevalent in states surrounding the Mississippi and Ohio Rivers, but foci of endemicity exist throughout the

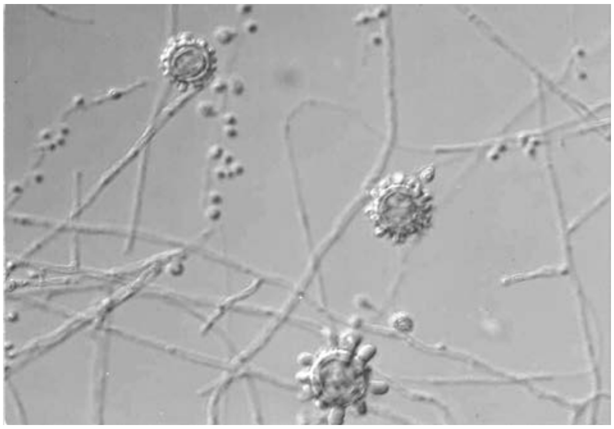

FIGURE 1 Mycelial phase of *H. capsulatum* showing tuberculate macroconidia and microconidia. Lactophenol cotton blue stain. Magnification, ~×245.
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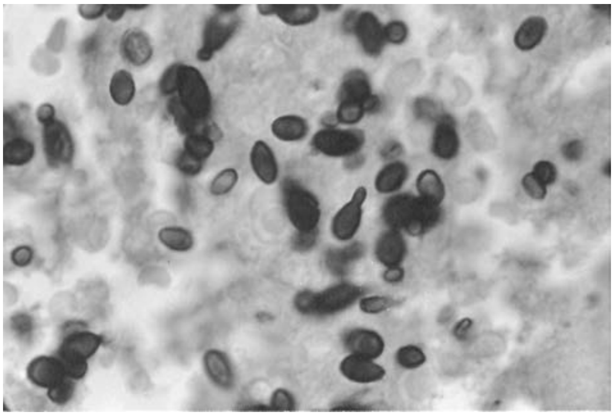

FIGURE 2 GMS stain showing blastoconidia of *H. capsulatum*. Magnification, ~×290.
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eastern half of the continent. Other regions of endemicity include parts of Africa, Australia, and eastern Asia, in particular India and Malaysia.

Blastomyces dermatitidis

B. dermatitidis is thermally dimorphic, converting from the mold phase to the yeast phase under appropriate conditions of temperature and nutrition. At room temperature, a floccose, white mold can be recovered (Fig. 4). The microconidia are oval or pyriform (pear shaped) with a diameter of 2 to 10 µm; no macroconidia are produced. Large, round, thick-walled yeast cells, 5 to 15 µm in size, with broad-based, budding daughter cells are found in tissue and on appropriate media at 37°C (Fig. 5). Yeast cells may occur inside or outside macrophages.

The natural habitat of *B. dermatitidis* is the soil, particularly near waterways, although the ecology of *Blastomyces* has not been completely defined due to the difficulty in isolating the organism in nature (28). It appears to survive best in moist acidic soils that contain a high nitrogen and organic content. Higher soil temperatures and recent rainfall facilitate growth of the fungus.

The largest number of cases of blastomycosis has been reported from North America, but the disease is also endemic in Africa and parts of Central and South America. In the United States, the organism is most commonly found in states surrounding the Mississippi and Ohio Rivers; in Canada, the disease occurs in the provinces that border the Great Lakes (29).

Coccidioides Species

In the environment and in culture at room temperature, the *Coccidioides* fungus exists as a mold producing septate hyphae and arthroconidia that usually develop in alternate hyphal cells (Fig. 6). As the arthroconidia mature, the alternating disjunctor cells undergo lytic degradation, releasing the barrel-shaped arthroconidia, ~2 to ~5 µm, which are the infectious particles. Inside the host and on special media, the arthroconidia transform into a structure called a spherule. A spherule is a large (up to 120 µm), thick-walled, spherical structure containing hundreds to thousands of endospores, each ~2 to ~4 µm, which can be released if the spherule ruptures (Fig. 7). Each endospore can develop into a spherule as well, continuing the process within the host.

Coccidioides is a soil-inhabiting fungus with a restricted geographical distribution (30). It is confined to regions of the Western Hemisphere that correspond to the Desert

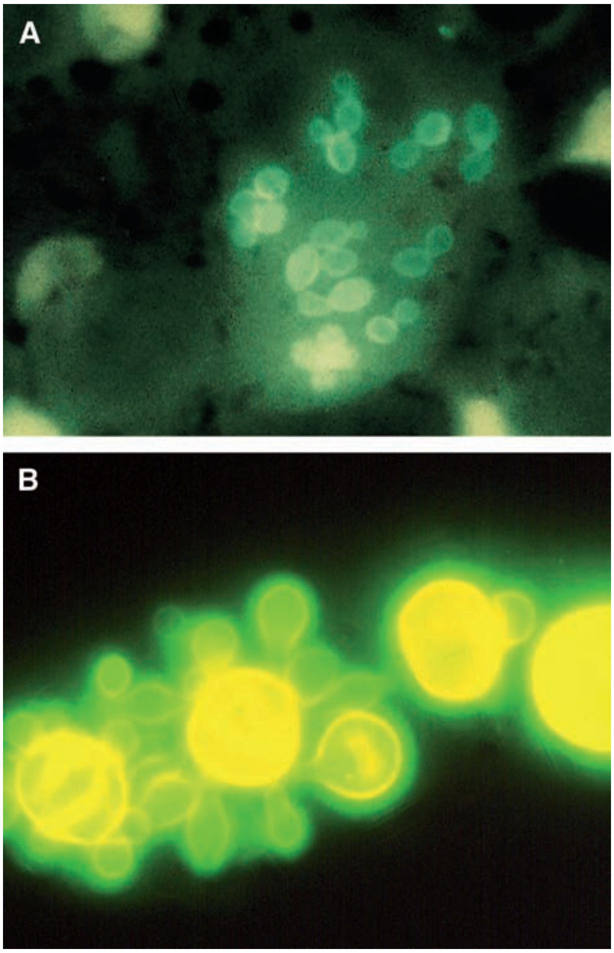

FIGURE 3 (A) Calcofluor white wet mount of sputum showing blastoconidia of *H. capsulatum*. Original magnification, ×475. Courtesy of the American Society of Clinical Pathology. (B) Calcofluor white wet mount of sputum showing blastoconidia of *P. brasiliensis*. Magnification, ~×255.
doi:10.1128/9781555817381.ch122.f3

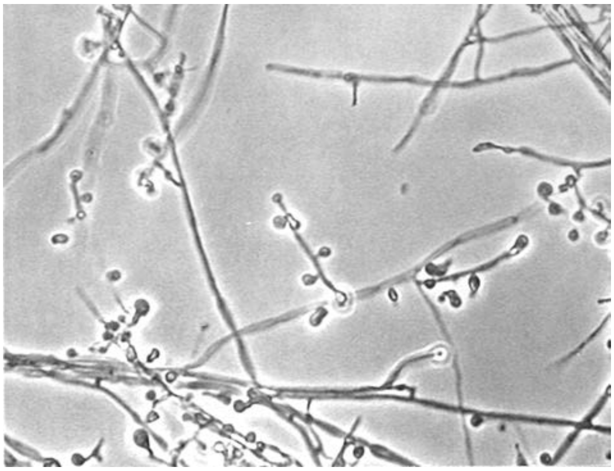

FIGURE 4 Mycelial phase of *B. dermatitidis*. Magnification, ~×240. doi:10.1128/9781555817381.ch122.f4

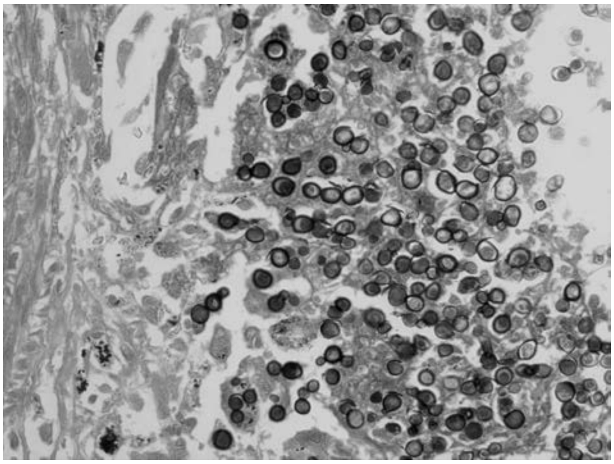

FIGURE 5 Blastoconidia of *B. dermatitidis*. GMS stain. Magnification, ~×290. doi:10.1128/9781555817381.ch122.f5

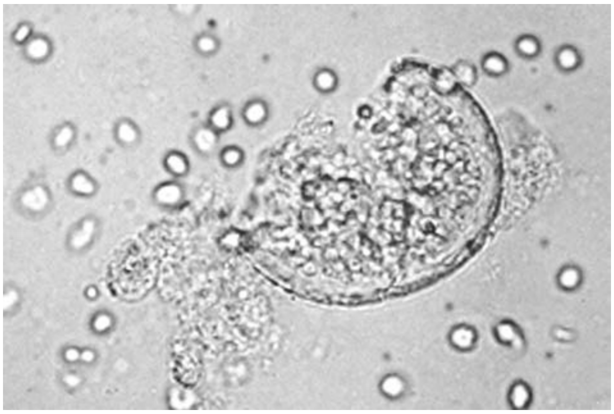

FIGURE 7 KOH preparation of pus from a lesion showing *Coccidioides* spherule and endospores. Magnification, ~×290. Reprinted with permission of Oxford University Press from reference 123. doi:10.1128/9781555817381.ch122.f7

Southwest, and although an animal reservoir has been postulated, this has yet to be definitively demonstrated (31). In the United States, the region of endemicity includes central and southern California, southern Arizona, southern New Mexico, part of Utah, and western Texas. The region of endemicity extends southwards into the desert regions of northern Mexico and parts of Central and South America.

Paracoccidioides brasiliensis

P. brasiliensis is a thermally dimorphic fungus. At room temperature, it grows as a mold. Growth requires a lengthy incubation, of up to 30 days. Isolates incubated on rich media produce thin, septate hyphae and occasional chlamydospores. Under conditions of nutritional deprivation, some isolates produce conidia, which vary in structure from arthroconidia to microconidia of <5 µm. Conidia respond to temperature changes, germinating into hyphae at 20 to 24°C or converting into yeasts at 36°C on appropriate media. Yeast cells are mostly oval and characteristically display a mother cell surrounded by multiple buds, a structure thought to resemble a ship's pilot wheel (Fig. 3B and 8).

Although *P. brasiliensis* has been isolated from soil, understanding of its precise environmental habitat remains limited. The region of endemicity extends from Mexico (23° N) to Argentina (34° S), sparing certain countries (Chile, Suriname, the Guyanas, Nicaragua, Belize, and most of the Caribbean islands) within these latitudes. Within countries where the disease is endemic, the mycosis is diagnosed only in areas with relatively well-defined ecologic characteristics (presence of tropical and subtropical forest, abundant watercourses, mild temperatures, high rainfall, and coffee/tobacco crops). The greatest numbers of reported cases have come from Brazil, Colombia, and Venezuela (32, 33). *P. brasiliensis* has been repeatedly recovered from human clinical samples; tissues of the nine-banded armadillo, *Dasypus novemcinctus*; and more rarely from the northern naked-tailed armadillo, *Cabassus centralis*. Occasionally *P. brasiliensis* has also been isolated from dogs and detected in other animal species (32).

Emmonsia Species

In the environment and in culture at room temperature, *Emmonsia* species exist as a mold that produces small, single-celled conidia (~4 µm in size) on the sides of the hyphae or on short side branches. Inside the host, the conidia of *E. crescens* and *E. parva* transform into structures termed adiaspores, which resemble the spherules of *Coccidioides* species (Fig. 9). Although adiaspores enlarge to become enormous, thick-walled structures, no endospores are produced and the spores

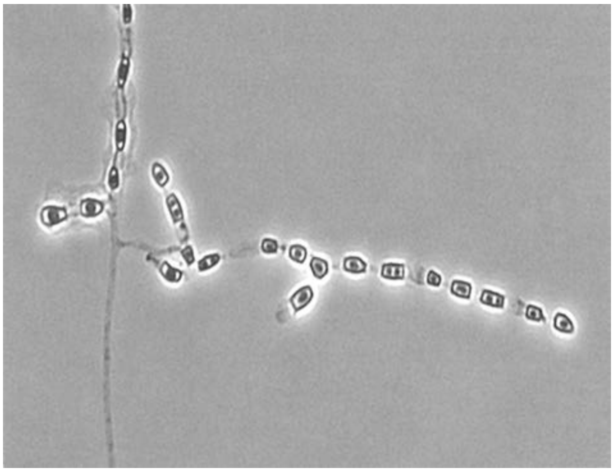

FIGURE 6 Mycelial phase of *C. immitis* showing alternating arthroconidia. Lactofuchsin stain. Magnification, ~×250. doi:10.1128/9781555817381.ch122.f6

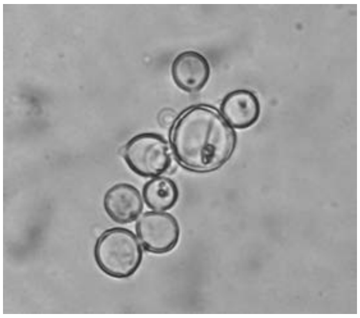

FIGURE 8 Wet mount showing blastoconidia of *P. brasiliensis*. Magnification, ~×290. doi:10.1128/9781555817381.ch122.f8

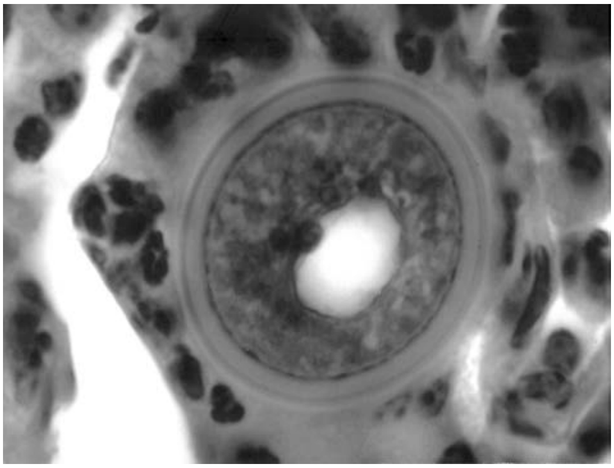

FIGURE 9 *E. parva* adiaspore in mouse tissue. PAS stain. Magnification, ~×280. doi:10.1128/9781555817381.ch122.f9

eventually die, failing to propagate *in vivo*, and thus probably do not disseminate from the lungs (34). Adiaspores can also be generated *in vitro* in rich media. *E. crescens* and *E. parva* are largely indistinguishable: the former produces large (25- to 400-µm), multinucleate adiaspores at 37°C, while the latter produces smaller (10- to 25-µm) adiaspores at 40°C (23). *E. pasteuriana* does not produce adiaspores in tissue; rather, at 37°C on rich media it produces structures that resemble budding yeast cells.

The natural habitat of *Emmonsia* species is the soil, and infection has occurred in numerous rodents, fossorial mammals, and their predators. Human cases are rare but have been reported from North, Central, and South America, as well as from several European and Asian countries and more recently from South Africa. The majority of recent cases had been reported from Brazil (22), yet following the report from South Africa, it is unclear if there are geographic differences between *Emmonsia* spp. or if a reporting bias exists.

EPIDEMIOLOGY AND TRANSMISSION

Histoplasmosis

Inhalation of microconidia is the usual mode of *H. capsulatum* infection in humans. The incubation period is 1 to 3 weeks. In cases of reinfection, the incubation period appears to be shorter (4 to 7 days after exposure). Histoplasmosis is not contagious, but there have been occasional reports of transmission from an organ donor to a recipient (35).

The major risk factor for infection is environmental exposure (25). The risk depends on several factors including the nature of the environmental site, the activities performed, and the duration and degree of dust or soil exposure. Longer and more intense exposures usually result in more severe pulmonary disease. Most reported outbreaks have been associated with exposures to sites contaminated with *H. capsulatum* or have followed activities that disturbed accumulations of bird or bat guano (26).

Persons with underlying illnesses are at increased risk for some forms of histoplasmosis. Disseminated infection is more common among individuals with underlying cell-mediated immunological defects, including those with HIV infection, transplant recipients, and individuals receiving tumor necrosis factor alpha inhibitors for rheumatoid arthritis. Immunocompromised persons with histoplasmosis have a higher mortality rate than those who are not immunosuppressed.

Blastomycosis

Inhalation of conidia is the usual mode of infection leading to blastomycosis. The incubation period has been estimated to be 4 to 6 weeks, although in some cases, disease manifests only months following exposure (36). Blastomycosis is not contagious.

Outbreaks have been associated with occupational and recreational activities, often along streams or rivers, and have resulted from exposures to moist soil enriched with decaying vegetation. Apart from outbreaks, blastomycosis is more commonly seen in adults than in children. More men than women are affected, and a disproportionate number of Asians compared with non-Asians were affected in a recent outbreak (37). The disease often occurs in individuals with an outdoor occupation or recreational interest.

B. dermatitidis is uncommon as an opportunistic pathogen, but it causes more aggressive disease in persons with underlying cell-mediated immunological defects, such as those with HIV infection and transplant recipients. Immunocompromised persons with blastomycosis have a higher mortality rate than those who are not immunosuppressed (38).

Coccidioidomycosis

Inhalation of arthroconidia is the usual mode of infection leading to coccidioidomycosis in humans. The incubation period is 1 to 3 weeks. In contrast to what is observed with histoplasmosis, once individuals have recovered from *Coccidioides* infection, they are usually immune to reinfection. The infection is not contagious, but occasional person-to-person spread has occurred via contaminated fomites (39) or by transmission from an organ donor to a recipient (40).

The major risk factor for infection is environmental exposure. The risk depends on a number of factors including the nature of the environmental site, the activities performed, and the duration and degree of dust or soil exposure. Infection has been associated with ground-disturbing activities, such as building construction, landscaping, farming, archaeological excavation, and numerous recreational pursuits (41). Natural events that result in the generation of dust clouds, such as earthquakes and windstorms, have been associated with an increased risk of infection and have resulted in large outbreaks (42).

Disseminated infection is more common among those of black, Asian, or Filipino race and among pregnant women in the third trimester. Individuals with underlying cell-mediated immunological defects, such as those with AIDS and those receiving immunosuppressive medications, are also at increased risk of disease dissemination (43).

Paracoccidioidomycosis

Inhalation of conidia is the usual mode of infection leading to paracoccidioidomycosis in humans. The incubation period is unknown, but it is clear that the fungus can remain dormant for very long periods in the lymph nodes following asymptomatic primary infection. Paracoccidioidomycosis is not contagious (32).

Paracoccidioidomycosis predominates in adults, who display 85 to 95% of cases, and in persons in agriculture-related occupations. The disease is more often diagnosed in males than in females (ratio of 15:1). Estrogen-mediated inhibition of the mold-to-yeast transformation could help to account for this (32). Sporadic cases have been reported in individuals with underlying immunosuppressive conditions, including HIV infection.

Adiaspiromycosis

Inhalation of conidia is the usual mode of infection leading to adiaspiromycosis in animals and humans, and even in cases with skin lesions, it is likely that this represents dissemination following inhalational exposure given the systemic manifestations (including liver function abnormalities) (44). The incubation period is unknown, although circumstantial evidence has suggested that symptoms may develop 1 to 3 weeks following exposure. No risk factors have been identified.

CLINICAL SIGNIFICANCE

Histoplasmosis

There is a wide spectrum of clinical manifestations of histoplasmosis, ranging from a transient pulmonary infection that subsides without treatment to chronic pulmonary infection or to more widespread disseminated disease (24, 25). Many healthy individuals develop no symptoms when exposed to *H. capsulatum* in a setting of endemicity. Higher levels of exposure result in an acute symptomatic and often severe flu-like illness. The symptoms, which include fever, chills, headache, nonproductive cough, myalgia, pleuritic chest pain, loss of appetite, and fatigue, usually disappear within a few weeks. The most severe form of this disease is disseminated histoplasmosis. The clinical manifestations range from an acute illness that is fatal within a few weeks if left untreated (often seen in infants, persons with AIDS, and solid-organ transplant recipients) to an indolent, chronic illness that can affect a wide range of sites.

Hepatic infection is common in nonimmunosuppressed individuals with disseminated histoplasmosis, and adrenal gland destruction is a frequent problem. Mucosal ulcers are found in >60% of these patients. The mouth and throat are often affected, but lesions also occur on the lip, nose, and other sites. Central nervous system disease occurs in 5 to 20% of patients, presenting as chronic meningitis or focal brain lesions. In persons with AIDS, disseminated histoplasmosis is usually associated with low CD4 T-lymphocyte counts and presents with nonspecific symptoms, such as fever and weight loss. Mucosal lesions are uncommon, but multiple cutaneous lesions may be present. Central nervous system involvement occurs in 10 to 20% of cases (24, 25).

African Histoplasmosis

The clinical manifestations of African histoplasmosis differ from those of classical histoplasmosis. The illness is indolent at onset, and the predominant sites affected are the skin and bones. Individuals with more widespread infection involving the liver, spleen, and other organs have a febrile wasting illness that is fatal within weeks or months if left untreated. Multiple cutaneous lesions often develop on the face and trunk. These lesions often enlarge and ulcerate. Osteomyelitis occurs in ~30% of patients. The infection may spread into contiguous joints, causing arthritis, or into adjacent soft tissue, causing a purulent subcutaneous abscess.

Blastomycosis

Blastomycosis encompasses a wide clinical spectrum, ranging from a transient pulmonary infection that subsides without treatment to chronic pulmonary infection or to widespread disseminated disease (45). Acute pulmonary blastomycosis usually presents as a nonspecific flu-like illness, similar to that seen with community-acquired pneumonia or primary histoplasmosis or coccidioidomycosis. Otherwise healthy persons generally recover after 2 to 12 weeks of symptoms, but some infected individuals return months later with infection of other sites. Other patients with acute blastomycosis fail to recover and develop chronic pulmonary disease or disseminated infection.

The skin and bones are the most common sites of extrapulmonary disease. The skin is involved in >70% of cases, the lesions presenting either as raised verrucous lesions with irregular borders or as ulcers. The latter can also appear on the mucosa of the nose, mouth, and throat. Osteomyelitis occurs in ~30% of patients with disseminated infection, with the spine, ribs, and long bones being the commonest sites of involvement. Arthritis occurs in ~10% of patients. Meningitis is rare, except in immunocompromised individuals. Other organs, such as the male genitourinary tract, adrenal glands, thyroid, liver, spleen, and gastrointestinal tract, are sometimes involved (36).

Coccidioidomycosis

Although a number of clinical manifestations may present after exposure, more than half of all coccidioidomycosis infections are thought to be subclinical. Apparent illness is most commonly a subacute process known as valley fever (primary coccidioidal infection). Respiratory symptoms such as cough, fever, chills, and fatigue are common and may last weeks to months. In regions of endemicity, primary coccidioidal pneumonia may account for 17 to 29% of all cases of community-acquired pneumonia (46, 47). Up to 50% of patients develop a mild, diffuse erythematous or maculopapular rash covering the trunk and limbs within the first few days of the onset of symptoms, a clinical sign that may be useful to differentiate the patient's symptoms from those caused by bacterial pneumonia (48). More dramatic and persistent is the rash of erythema nodosum or erythema multiforme, which occurs in ~5% of infected persons. Otherwise healthy persons recover without treatment, their symptoms disappearing in a few weeks to months.

Fewer than 3% of infected individuals develop disseminated coccidioidomycosis. This progressive disease usually develops within 3 to 12 months of the initial infection, although it can occur much later following reactivation of a quiescent infection in an immunosuppressed individual. The clinical manifestations range from a fulminant illness that is fatal within a few weeks if left untreated to an indolent chronic disease that persists for months or years. One or more sites may be involved, but the skin, soft tissue, bones, joints, and meninges are most commonly affected. Cutaneous and subcutaneous lesions are common. Osteomyelitis occurs in ~40% of patients, with the spine, ribs, cranial bones, and ends of the long bones being the most common sites. The disease may spread into contiguous joints, causing arthritis, or into adjacent soft tissue, causing subcutaneous abscess formation. Meningitis is the most serious complication of coccidioidomycosis, occurring in 30 to 50% of patients with disseminated disease. Without treatment, it is almost always fatal (49).

Paracoccidioidomycosis

Paracoccidioidomycosis encompasses a subclinical infection resulting from the initial contact with the fungus. The lungs are the usual initial site of *P. brasiliensis* infection, but the organism then spreads through the lymphatics to the regional lymph nodes. In most cases, the primary infection is asymptomatic. However, children and adolescents sometimes present with an acute disseminated form of infection in which superficial and/or visceral lymph node enlargement is the major manifestation. This presentation is also seen in immunocompromised patients. It has a poor prognosis.

The hallmarks of the chronic adult type of disease are significant lung involvement and extrapulmonary lesions. This is the predominant form, occurring in ~90% of cases (32). In 80% of cases, the disease involves the lungs. The disease is slowly progressive and may take months or even years to become established. Ulcerative mucocutaneous lesions of the face, mouth, and nose are the most obvious presenting sign. Other sites of infection include the small or large intestine, liver and spleen, adrenal glands, bones and joints, central nervous system, and male genitourinary tract. In 60 to 80% of cases, active pulmonary involvement and residual fibrotic lesions are observed. A residual form is also recognized in 50 to 80% of cases and is represented by fibrotic scarring occurring at the sites of previously active lesions, which can alter respiratory function and incapacitate the patient. *P. brasiliensis* infection may become dormant, to be reactivated later under the influence of ill-defined conditions prevalent in rural settings, such as chronic alcoholism, malnutrition, and smoking (32).

Adiaspiromycosis

In most cases, adiaspiromycosis is a self-limited, localized pulmonary infection with few or no symptoms. Because the adiaspores enlarge but do not reproduce, symptoms and clinical signs depend on the number of conidia inhaled. In some patients, the disease is discovered incidentally during the evaluation of other pulmonary conditions. However, nonproductive cough, dyspnea, and fever are not uncommon in those with adiaspiromycosis. Severe or even fatal cases are rare, although they have been reported (22), and, as recently reported (23, 50), can occur in immunocompromised individuals with cutaneous manifestations and elevated liver function tests. A single outbreak of ocular adiaspiromycosis has also been reported (51).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Clinical Specimens

Methods of collection, transport, and storage of specimens are detailed in chapter 114. Tissue samples should be obtained when appropriate and should be divided and submitted for microbiological and histopathological examination. If possible, special tissue stains such as the Grocott-Gomori methenamine silver (GMS) and periodic acid-Schiff (PAS) stains should be requested. Since colonization with the dimorphic fungal pathogens does not occur, their microscopic detection and/or isolation in culture is consistent with proven infection. Blood, urine, and cerebrospinal fluid (CSF) can also be collected as appropriate for antigen and/or antibody testing.

Histoplasma capsulatum

H. capsulatum organisms can be isolated from sputum or bronchoalveolar lavage fluid specimens in 60 to 85% of cases of chronic pulmonary histoplasmosis if multiple specimens are tested (52). In disseminated disease, useful specimens for culture include blood, urine, lymph node, and bone marrow samples. Bone marrow cultures are positive in >75% of cases. Blood cultures collected with the Isolator lysis-centrifugation system (Wampole Laboratories, Princeton, NJ) or Bactec Mycolytic bottles (BD Diagnostic Systems, Franklin Lakes, NJ) show the greatest sensitivity. Biopsy specimens of oral, cutaneous, and gastrointestinal lesions, adrenal glands, or liver and spleen have also provided a diagnosis. *Histoplasma* meningitis is difficult to diagnose, with CSF cultures being positive in no more than two-thirds of cases. The best results are obtained when large volumes of CSF (10 to 20 ml) are cultured on multiple occasions.

Blastomyces dermatitidis

Sputum samples, bronchoalveolar lavage fluid, or lung biopsy specimens may be submitted. Skin biopsy specimens are useful in the diagnosis of cutaneous disease. Collection of urine after prostatic massage may be helpful in the diagnosis of genitourinary blastomycosis.

Coccidioides Species

In addition to lower respiratory tract samples, material for microscopy and culture can be collected from suppurative cutaneous and soft tissue lesions. Organisms can be recovered only infrequently from CSF (~30% of patients), and usually only after culture of large volumes (10 to 20 ml) (53).

Paracoccidioides brasiliensis

In addition to lower respiratory samples, material can be collected from oral or pharyngeal lesions, cutaneous lesions, lymph nodes, adrenal glands, and the gastrointestinal tract. In these specimens, a simple wet mount suffices to reveal *P. brasiliensis* in >85% of patients.

Emmonsia Species

Sputum and bronchoalveolar lavage fluids are seldom culture positive in patients with disease. The diagnosis is usually based on observation of its unique histopathologic appearance, typically a single, large, thick-walled adiaspore within a granuloma, yet more-recent reports have found yeast in skin biopsy and blood smear specimens.

DIRECT EXAMINATION

Microscopy

Direct microscopic examination of clinical materials may provide a rapid presumptive diagnosis of a systemic fungal infection. However, it is important to appreciate that tissue-form cells of *H. capsulatum* and *B. dermatitidis* can appear similar to each other as well as to yeast cells of various *Candida* species, *Cryptococcus neoformans*, *Cryptococcus gattii*, and *Talaromyces marneffei* (formerly *Penicillium marneffei*) and to endospores of *Coccidioides* species. It is often helpful to stain fresh, wet preparations of sputum, bronchoalveolar lavage fluid, CSF, urine, pus, or other material with calcofluor white, a fluorescent compound that binds to the fungal cell wall, to assist with preliminary evaluation.

Histoplasma capsulatum

Giemsa and Wright's stains can be used to detect yeast cells of *H. capsulatum* in blood or bone marrow smears. These cells can also be seen in tissue sections stained with GMS or PAS but usually not with hematoxylin and eosin (H&E). Detection of the small (2- to 4-μm), oval, budding yeasts allows a presumptive diagnosis of histoplasmosis. Organisms can be found within macrophages or free in the tissues. It is unusual to find yeast cells on cytological examination of sputum or other respiratory tract fluids. The thick-walled, narrow-based, budding yeast cells causing African histoplasmosis, ~10 to ~15 µm in diameter, are about 4-fold larger than those of classical *H. capsulatum* in tissue sections and on occasion may be confused with *Blastomyces* spp.

Blastomyces dermatitidis

Direct calcofluor white or KOH mounts or Gram stains of sputum, tissues, and exudates often permit the detection of the large, round yeast cells of *Blastomyces* spp. The broad-based buds often attain the same size as the parent cells before becoming detached. On occasion, larger "giant" yeast forms (>40 µm) or filmentous forms may be found in tissue. Histopathological examination using PAS or GMS stains also can be of value.

Coccidioides Species

Tissue sections should be stained with PAS, GMS, or H&E to permit the detection of the characteristic large, thick-walled spherules of *Coccidioides* species. Microscopic examination of wet preparations of sputum, bronchoalveolar lavage fluid, pus, or other samples treated with KOH is also helpful but is less sensitive. *Prototheca wickerhamii* may resemble small spherules, and *Rhinosporidium seeberi* may simulate larger ones.

Paracoccidioides brasiliensis

The characteristic translucent-walled yeast cells of *P. brasiliensis* with multiple buds can often be found on direct microscopic examination of sputum, bronchoalveolar lavage fluid, pus from draining lymph nodes, or tissue biopsy specimens. Staining of wet preparations with lactophenol cotton blue, methylene blue, Gram stain, or calcofluor white can be helpful. Tissue sections can be stained with PAS, GMS, or H&E.

Emmonsia Species

Tissue sections stained with PAS or GMS are most helpful in demonstrating the characteristic adiaspores of *Emmonsia* species in lung tissue. It is important to appreciate that in the chronic stage the organism may collapse, forming various shapes that may resemble other fungi, helminths, or pollen grains. Adiaspores must also be distinguished from the spherules of *Coccidioides*. Adiaspores do not contain endospores, and adiaspores are typically much larger than empty *Coccidioides* spherules.

Antigen Detection

Histoplasma capsulatum

One well-known *Histoplasma* polysaccharide antigen (HPA) test is a microtiter plate-based double-antibody sandwich enzyme immunoassay (EIA) to detect antigen in urine, serum, or CSF in cases of disseminated histoplasmosis with a sensitivity of up to 95% (54). This test is also useful in the early diagnosis of acute pulmonary histoplasmosis and during treatment follow-up (55). Ten milliliters of urine, 5 ml of serum, or 1 ml of CSF is the preferred volume for the HPA test, although a minimum volume of 0.5 ml of any specimen is required for a single test. To obtain maximum test sensitivity, it is recommended that both serum and urine specimens be tested in parallel. Treatment success or failure may be assessed by collecting specimens at least 14 days after starting treatment and testing the newly acquired samples in parallel with the last specimen that was positive before initiation of treatment.

This test is performed on a fee-for-service basis by MiraVista Diagnostics (Indianapolis, IN) (http://miravistalabs.com). More recently, an antigen-capture enzyme-linked immunosorbent assay (ELISA) to detect *H. capsulatum* antigenuria in immunocompromised patients was described. The assay, which is not commercially available, uses polyclonal antibodies against *H. capsulatum* and has a reported sensitivity of 81% and specificity of 95% (56). Similar tests have also been developed for detection of antigenuria (Specialty Laboratories, Valencia, CA; and ImmunoMycologics [IMMY], Norman, OK) (57, 58), although varying results were reported when these tests were compared (59).

The detection of serum (1→3)-β-D-glucan, a component of the fungal cell wall, has undergone limited evaluation in histoplasmosis; however, preliminary evidence suggests that it is useful in the detection of histoplasmosis (87% sensitivity and 65% specificity) (60). A positive result must be confirmed with *Histoplasma*-specific testing.

It is worth mentioning that a commercial and widely used EIA, Platelia *Aspergillus*, showed cross-reactivity with *H. capsulatum*, *P. brasiliensis*, and *Cryptococcus* spp. (61). However, no similar false positives have been observed in any of the *Histoplasma* antigen assays described here.

Blastomyces dermatitidis

The *Blastomyces* antigen test is a microtiter plate-based double-antibody sandwich EIA to detect antigenuria and antigenemia in disseminated blastomycosis. The sensitivity is 89% in disseminated blastomycosis, with higher sensitivity in urine than in serum (62). However, specificity is modest, only 79% overall, due to cross-reactivity with other endemic mycoses (63). This test is performed on a fee-for-service basis by MiraVista Diagnostics.

The detection of serum (1→3)-β-D-glucan has been incompletely evaluated in blastomycosis; however, preliminary evidence suggests that it is not useful in the detection of *Blastomyces* (60).

Coccidioides Species

A coccidioidal urinary antigen test (MiraVista Diagnostics) has been developed and exhibited a sensitivity of 71% in a largely immunosuppressed population (64). In a veterinary population, the sensitivity has been shown to be much lower (<20%) (65). The findings call into question the usefulness of antigen testing in nonimmunosuppressed populations. The sample requirements are the same as those for the HPA test.

Serum (1→3)-β-D-glucan has undergone limited evaluation in the detection of coccidioidomycosis and has a limited role (44% sensitivity) in this population (66).

Paracoccidioides brasiliensis

At present, paracoccidioidomycosis antigen testing is not available as a routine diagnostic test. A 43-kDa glycoprotein and an 87-kDa heat shock protein have been described as useful targets for serum antigen detection (67, 68). Several reports have described the detection of *P. brasiliensis* antigen in urine, CSF, and bronchoalveolar fluid samples (68). Others have noted that antigen levels in serum diminished or even disappeared during successful treatment (69).

Emmonsia Species

No antigen test exists at this time for the diagnosis of adiaspiromycosis in humans.

Nucleic Acid Detection in Clinical Materials

No commercially available systems exist for detection of fungal nucleic acids in human clinical samples. However, a number of "in-house" methods remain under investigation, several of which are highlighted in this section. Conserved regions of rRNA genes have been used as targets in a number of PCR-based detection assays. It is important to appreciate that amplification of conserved genes can result in products derived both from pathogenic fungi and from genetically related nonpathogenic fungal species. The nonspecific nucleic acids could arise from colonization of the original sample with saprophytic organisms, from contamination during sample collection, or from contamination of PCR reagents with fungal DNA. It is very important that the identity of amplicons detected using conserved genes be verified by direct sequencing. In more-recent studies, genes specific for the fungus of interest have been chosen as PCR targets, thus eliminating this specificity problem.

Histoplasmosis

An increasing number of publications have described the use of PCR to detect *H. capsulatum* DNA in fixed, paraffin-embedded tissue samples, blood, bronchial lavage fluids, bone marrow, and ophthalmic samples. Early studies targeted the internal transcribed spacer (ITS) rRNA gene region, but the use of targets unique to *H. capsulatum* has provided better assay specificity. Bialek et al. designed a nested PCR that targeted the gene coding for a unique 100-kDa protein of *H. capsulatum* (70), which has successfully been used to detect *H. capsulatum* DNA in clinical samples from patients with histoplasmosis in French Guiana and Colombia (sensitivity, 100%; specificity, 95%) (71, 72). The original format of nested or seminested PCR has recently been adapted to real-time PCR with promising results (73). A consensus group recently started evaluation of the reproducibility of the most common in-house tests used (74).

Blastomycosis

PCR assays of ribosomal genes, the ITS region, repetitive sequences, and species- or genus-specific genes have all been evaluated for the molecular detection of *B. dermatitidis*. The majority of these tests have been evaluated in paraffin-embedded tissue, although a real-time PCR assay to identify *B. dermatitidis* in culture specimens, bronchial washings, bronchoalveolar lavage fluid, pleural fluid, sputum, and blood has been published (75, 76).

Coccidioidomycosis

Various PCR assays have proven extremely sensitive and specific on isolate material (77) and from sputum in active cases (unpublished data); however, direct detection from other clinical samples (serum, pleural fluid, CSF, and joint fluid) has not been as successful to date. Few studies have been published, although it appears that the sensitivity of PCR in clinical samples is similar to that of culture (~50%) (77, 78).

Paracoccidioidomycosis

The number of tests developed and evaluated so far for the detection of *P. brasiliensis* in clinical samples is very limited (79). The preferred target sequences have been *gp43* and rRNA genes. A real-time PCR assay targeting the ITS-1 region was developed to detect *P. brasiliensis* DNA in both cultures and clinical specimens. Although this molecular test was evaluated with a small number of patients, the authors reported 100% sensitivity and specificity (80). A nested-PCR assay targeting the immunogenic *gp43* gene was evaluated in the detection of *P. brasiliensis* DNA in lung homogenates from infected and uninfected mice, with 91% sensitivity (81). A test based on the 5′ nuclease assay using a fluorescent probe derived from the sequence of the gene coding for the gp43 antigen was used and could detect at least 10 copies of this DNA sequence (82).

Adiaspiromycosis

A panfungal PCR assay targeting the ribosomal ITS-1 and -2 regions identified *E. crescens* from a bronchoalveolar lavage fluid sample of a patient with confirmed adiaspiromycosis (83).

ISOLATION PROCEDURES

Biosafety

The mere lifting of a culture plate lid is often sufficient to release large numbers of conidia into the air, and screw-cap tubes or sealed plates should be used. Should a sporulating culture be dropped, millions of conidia may be dispersed. It is also important to note that local infections including granuloma formation have been reported following accidental percutaneous inoculation during injection of laboratory animals, or while performing autopsies of humans with histoplasmosis, coccidioidomycosis, or blastomycosis.

All procedures involving the manipulation of sporulating cultures of *Coccidioides* species, *B. dermatitidis*, and *H. capsulatum* and for processing soil or other environmental materials known or likely to contain these organisms should be performed inside a class II biological safety cabinet under conditions of biosafety level 3 containment. Biosafety level 2 practices and facilities are recommended for handling and processing clinical specimens and animal tissues (84). Hyaline molds of unknown identity should always be examined and manipulated inside a class II biosafety cabinet. Such molds should never be handled on an open laboratory bench. Recommendations for handling inadvertent exposure to *Coccidioides* in the laboratory have been published (85).

Biosecurity

In the United States, both species of *Coccidioides* were previously classified as select agents, although in 2012 they were removed from this list.

Culture for Mold Phase

In general, the organisms discussed in this chapter can be readily cultivated in the mold phase on general fungal media such as Sabouraud dextrose agar or potato dextrose agar incubated at 25°C. Incubation at 37°C is also helpful to recover the yeast phase of most dimorphic organisms. Media containing antibiotics such as chloramphenicol or gentamicin should be used when culturing clinical materials, such as sputum, that may be contaminated with bacteria. Media containing cycloheximide are useful to inhibit saprophytic fungi and to provide a useful differential tool in identification. Many unrelated saprophytic soil fungi fail to grow on media containing cycloheximide, fail to grow altogether at 37°C, or fail to convert to the yeast phase at 37°C. Screw-cap slants are preferable to plates for culturing dimorphic fungi. If plates are used, they should be sealed so that mold spores cannot escape into the ambient air. Seals that are permeable to air such as Shrink Seals (Scientific Device Laboratory, Des Plaines, IL) are useful for this purpose. In general, colonies develop within 3 to 7 days, but some strains of *H. capsulatum* and *P. brasiliensis* may require incubation for as much as 4 to 6 weeks.

Culture for Yeast Phase

H. capsulatum, *B. dermatitidis*, and *P. brasiliensis* can be recovered in the yeast phase by using appropriate media incubated at 37°C. *E. parva* and *E. crescens* produce adiaspores at elevated temperatures.

Histoplasma capsulatum

The yeast phase of *H. capsulatum* can be recovered in rich media such as brain heart infusion (BHI) agar or BHI with blood (BHIB). Plates or slants should be incubated at 37°C under aerobic conditions for at least 4 weeks.

Blastomyces dermatitidis

The yeast phase of *B. dermatitidis* can be recovered in rich media such as BHI agar, BHIB, Pine's medium, or Kelley's agar by incubation at 37°C under aerobic conditions. Yeasts are usually visible within 1 week, but media should be held for at least 4 weeks before being discarded.

Paracoccidioides brasiliensis

The yeast phase of *P. brasiliensis* can be recovered in media such as BHI agar, Pine's medium, or Kelley's agar by incubation at 37°C. The organism grows slowly, and plates or slants should be held for at least 4 weeks.

Emmonsia Species

E. parva and *E. crescens* produce adiaspores *in vitro* when cultivated on phytone yeast extract agar, BHI agar, or BHIB at 37 to 40°C depending on the species. *E. pasteuriana* produces yeast-like cells after culture on BHI agar at 37°C for ~10 days. The species recently recognized in South Africa does not produce adiaspores at 37 or 40°C, although the mycelial cultures were converted to the yeast phase by incubating streaked or single-colony subcultures on BHI agar at 37°C after 10 to 14 days.

IDENTIFICATION

In general, these organisms are identified by their characteristic morphologic features, by conversion to the yeast phase, by DNA probe testing if available, and/or by direct DNA sequencing. Slide cultures should not be performed on suspected dimorphic isolates due to the possibility of laboratory infection from accidental inhalation of infectious conidia.

Histoplasma capsulatum

The mold phase can be recovered after incubation at 25°C. The colony is initially white or buff-brown. Both types may be isolated from the same patient, and eventually the brown type may convert to the white type. The brown type generally produces more of the characteristic tuberculate macroconidia than the white type. On subculture, only ~30% of macroconidia show tubercles. Microconidia are abundant in fresh isolates of *H. capsulatum*. After multiple subcultures, the production of both macroconidia and microconidia may be diminished. The presence of both macroconidia and microconidia is not required for identification of *H. capsulatum*, as authentic *H. capsulatum* isolates that fail to produce either macroconidia or microconidia have been recognized. Macroconidia but no microconidia can also be seen in the saprophytic fungus *Sepedonium* as well as in the related fungus *Renispora flavissima*. Authentic *H. capsulatum* isolates can be recognized by their thermal dimorphism as well as by their growth on inhibitory mold agar.

Once the characteristic morphology has been recognized, mold-phase *H. capsulatum* isolates can be confirmed by conversion to the yeast phase. The isolate is transferred to BHI or BHIB agar and incubated at 37°C for at least 7 to 10 days. Hyphal cells may form buds directly or develop enlarged, transitional cells that subsequently begin to bud. The microconidia may also convert to budding yeast cells. Complete conversion rarely is achieved, and multiple transfers to fresh BHI or BHIB medium may be required. The colony develops a white, smooth, yeast-like appearance, and microscopic examination reveals oval, budding yeasts approximately 1 to 3 by 3 to 5 μm. The cells have a narrower base of attachment between the bud and parent cell than do those of *B. dermatitidis*.

The AccuProbe test (GenProbe, San Diego, CA) can also be used to confirm isolates as *H. capsulatum*. This test requires actively growing cultures: mold-phase cells not more than 4 weeks of age or yeast cells not older than 1 week. Isolates can be taken from solid media or broth cultures. In this assay, formation of specific DNA-RNA hybrids is quantitated in relative light units by use of a luminometer. Extracts that display relative light unit values of >50,000 are considered positive. The AccuProbe test has largely replaced exoantigen testing for identification of *H. capsulatum*. Several studies have shown that this test is sensitive and specific for identification of *H. capsulatum* (86–88), although false-positive results can be obtained when isolates that are genetically related to the *Ajellomycetaceae* are tested (89). An exoantigen testing kit to identify the organism is commercially available from IMMY. African *Histoplasma* isolates display colonial morphology similar to that of non-African isolates and yield positive results in the AccuProbe test for *H. capsulatum* (87). Histopathological examination of tissue forms is required to distinguish members of the African clade.

Blastomyces dermatitidis

At 25 to 30°C, isolates of *B. dermatitidis* produce a variety of forms ranging from a fluffy, white colony that is visible within 2 to 3 days to a glabrous (waxy), tan, nonconidiating colony that grows more slowly. Microscopic examination shows microconidia that are oval or pyriform, usually smooth walled, and formed on short lateral or terminal branches along the hyphae. *B. dermatitidis* also grows readily on inhibitory media containing cycloheximide. Conidia of the hyaline hyphomycete *Scedosporium apiospermum*, of some *Chrysosporium* species, and of the dermatophyte *Trichophyton rubrum* are morphologically similar and can be mistaken for *B. dermatitidis*. These species either fail to grow at 37°C (some *Chrysosporium* species) or grow as molds when incubated at 37°C (*S. apiospermum* and *T. rubrum*).

The identification can be confirmed by conversion to the yeast phase. Generally, isolates of *B. dermatitidis* convert readily to the yeast phase on BHI or BHIB agar or Pine's or Kelley's medium incubated at 37°C. Yeast cells are hyaline, smooth walled and thick walled, generally 8 to 15 μm in diameter, with the bud connected to the parent cell by a broad base of up to 4 to 5 μm in diameter. Conversion can be accomplished in 2 to 3 days, although occasional isolates may take several weeks.

Identification can also be confirmed using the AccuProbe test for *B. dermatitidis*. False-positive *B. dermatitidis* GenProbe results were obtained with *P. brasiliensis* (90) and with *Gymnascella hyalinospora*, so care must be taken to distinguish these organisms. An exoantigen testing kit to identify the organism is commercially available from IMMY.

Coccidioides Species

At 25 to 30°C, isolates of *Coccidioides* species display considerable variation in colony morphology. Colonies can range from moist, glabrous, and grayish to abundant, floccose, and white. Colonies may become tan and even red with age (91). Microscopic examination shows hyphae that are thin and septate, with fertile (spore-producing) hyphae usually arising at right angles. Arthroconidia are hyaline, one-celled, short, cylindrical to barrel shaped, moderately thick walled, smooth walled, and 2 to 8 by 3 to 5 μm. Arthroconidia alternate with thin-walled empty disjunctor cells. At maturity, the disjunctor cells undergo lytic degradation, releasing the arthroconidia. After this fragmentation, the arthroconidia may display frill-like remains of the disjunctor cells on each end. Isolates of *Coccidioides* grow well on inhibitory mold agar containing cycloheximide, which helps to distinguish this organism from similar soil saprophytes such as *Malbranchea* species. It is also important to distinguish true alternate arthroconidia from aging mycelia that, due to cytoplasmic shrinkage, display an appearance that can be misinterpreted as arthroconidia. True arthroconidia eventually fragment and disperse; aging mycelia do not. The two species of *Coccidioides* cannot be readily distinguished morphologically.

When incubated at 37 to 40°C *in vitro* in appropriate media and increased CO_2 tension, the organism can produce spherules, but this procedure is rarely performed on routine clinical isolates.

The AccuProbe for *Coccidioides* may be used for confirmation of unknown isolates as *Coccidioides* species. This test is generally sensitive and specific, although pretreatment of isolates with formaldehyde leads to false-negative results. The test does not distinguish between the two species of *Coccidioides*. An exoantigen testing kit to identify the organism is commercially available from IMMY (92).

Paracoccidioides brasiliensis

When incubated at 25 to 30°C, isolates display slow growth and produce a variety of forms ranging from glabrous, leathery, brownish, flat colonies with a few tufts of aerial mycelium to wrinkled, folded, floccose colonies to velvety, white-to-beige forms. The colonies are very similar in appearance to those of *B. dermatitidis*. Most strains grow for long periods of time without the production of conidia.

When cultures are transferred to 37°C on rich media such as BHI, Pine's, or Kelley's agar, the resulting yeast colonies are generally folded (cerebriform) in appearance. Mycelial elements may be seen mixed with yeast cells. Conversion to the yeast phase also is quite slow. Yeasts are 2 to 30 μm in diameter and are oval or irregular in shape, displaying the characteristic "pilot's wheel" appearance of multiple thin-necked, round buds developing around the parent cell. The walls are thinner than those of *B. dermatitidis* yeast cells, and the buds are not broad based.

Identification is usually confirmed by demonstrating thermal dimorphism. There is no commercial DNA probe test for *P. brasiliensis*. The AccuProbe test for *B. dermatitidis* cross-reacts with *P. brasiliensis* (90), so care must be taken when employing this test on isolates that may actually be *P. brasiliensis*. Slow-growing, nonsporulating isolates are more characteristic of *P. brasiliensis* than of *B. dermatitidis*.

Emmonsia Species

At 25°C, *Emmonsia* species organisms grow as glabrous, colorless colonies, which produce yellowish white aerial mycelia in time. Some strains display pale orange to grayish orange aerial mycelia. The colonies often have areas that alternate between a tufted mycelium and a glabrous consistency. Reverse pigmentation is pale gray to grayish brown. On microscopic examination, the hyphae are septate and branching. Sporulation is enhanced on potato dextrose or Pablum cereal agar. Numerous conidia are produced either directly from the sides of the hyphae or on short stalks that branch at right angles from the hyphae. Each stalk bears a single terminal conidium. Sometimes the swollen end may bear one to three secondary spine-like pegs, which in turn form a secondary conidium in a flower-like arrangement (21). The conidia are round, oval, or pyriform and measure 2 to 4 μm by 3 to 5 μm. The conidial wall is smooth but may roughen with age. *E. parva* and *E. crescens* are indistinguishable in morphology and appearance at 25°C.

E. crescens displays no hyphal growth at 37°C and forms larger adiaspores (20 to 140 μm in diameter) on BHI or phytone yeast extract agar at this temperature. *E. parva* produces hyphae at 37°C and produces smaller adiaspores (8 to 20 μm in diameter) at 40°C. *E. pasteuriana* displays features at 25°C that are similar to those of *E. crescens* and *E. parva*. At 37°C on BHIB, this species produces yeast-like cells that are oval or lemon shaped, budding on a narrow base, and 2 to 4 μm. The colonies are creamy and smooth. This species does not produce adiaspores *in vitro* or *in vivo* (93).

TYPING SYSTEMS

In general, typing systems have been used to show geographic differences among isolates and cryptic species. In some genera, diversity can be shown among isolates collected from a single geographic area.

Histoplasma capsulatum

Isolates can be divided into at least eight clades as described earlier (5). This typing system can be used to place an unknown isolate into one of the major worldwide geographic groupings. For further delineation, restriction fragment length polymorphism (RFLP) typing with the yeast phase-specific nuclear gene *yps-3* and/or mitochondrial DNA probes and random amplified polymorphic DNA-based and ITS-based typing methods have been used in several studies (reviewed in reference 94). These studies have shown that considerable polymorphisms can be demonstrated among individual patient isolates from a particular geographic location (94); that animal and soil strains from Brazil display indistinguishable subtypes (95); and that strains from patients in Brazil, where mucocutaneous histoplasmosis is much more common than in the United States, display distinct ITS and *yps-3* subtypes not seen in strains from U.S. patients (96). Recently a study compared multiple typing methods that have been developed to study *H. capsulatum* epidemiology in 51 environmental, animal, and human isolates from Brazil. The M13 PCR fingerprinting and PCR-RFLP analyses produced very similar results and separated the *H. capsulatum* isolates into three major groups (97).

Blastomyces dermatitidis

In a study using RFLP with several rRNA gene probes to study 59 isolates from the United States, India, and Africa, three major groups were defined. These groups were further divided using random amplified polymorphic DNA fingerprinting into 5, 15, and 12 types, respectively, that correlated with the geographic origin of the isolate (98). Interestingly, these studies showed that soil isolates collected from an outbreak of blastomycosis in Eagle River, WI, were not responsible for the majority of cases of disease in that outbreak. A study exploring polymorphisms in the promoter region upstream of the *BAD-1* virulence gene revealed further genetic diversity, with large insertions in the promoter region (99). Further work examining microsatellite markers revealed two genetically distinct groups, suggesting a possible new species (7), and recent evidence has confirmed the separation of *Blastomyces* spp. into *B. dermatitidis* and *B. gilchristii* (8).

Coccidioides Species

Two major clades, corresponding to the two species *C. immitis* and *C. posadasii*, were defined when an extensive population sample, including isolates from Venezuela, Mexico, and Brazil, was studied by using a set of nine microsatellite markers (12). Typing of isolates to *C. immitis* (CA) or *C. posadasii* (non-CA) species can be accomplished by examining any of 17 sites fixed for alternate alleles (100). Microsatellite typing conducted with 121 clinical isolates from Arizona concluded that this disease in Arizona could not be linked to a dominant strain of *C. posadasii* (101). Ongoing work will further clarify the population genetics of this organism.

Paracoccidioides brasiliensis

Multilocus sequence typing at eight loci demonstrated the existence of at least three distinct species within this organism (15). The *gp43* gene encoding a dominant glycoprotein antigen has been studied by several groups as a useful target for subtyping. In an earlier study, microsatellite sequences were compared as markers to discriminate among a set of *P. brasiliensis* human isolates causing either chronic or acute disease (102). These authors did not observe any clustering of isolates associated with either acute or chronic disease.

Emmonsia Species

Based on ITS sequences, isolates of *E. crescens* fall into two phylogenetic groups, North American and Eurasian, depending on the continents from which the isolates were obtained (1). Isolates of *E. parva* separate into two groups as well, one group isolated from the North American prairies and the second group from the desert southwest of the United States or from Italy.

SEROLOGIC TESTS

Histoplasmosis

Serologic tests have an important role in the rapid diagnosis of several forms of *H. capsulatum* infection but are most useful for persons with chronic pulmonary or disseminated histoplasmosis (103). Of the different methods that have been developed, the immunodiffusion (ID), complement fixation (CF), and latex agglutination (LA) tests are the most popular. The principal antigen used in these tests is histoplasmin, a soluble filtrate of mycelial-phase broth cultures. Histoplasmin contains *H. capsulatum* species-specific H and M antigens as well as C antigen. The H antigen is a β-glucosidase against which antibodies are formed during acute histoplasmosis. The M antigen is a catalase against which antibodies are produced during all phases of the disease. The H and M antigens were once thought to be specific proteins for the detection of anti-*H. capsulatum* antibodies. The M antigen, however, was found to be not specific unless used in a deglycosylated form.

The ID test is a qualitative method that detects precipitins to the H and M glycoprotein antigens of *H. capsulatum* present in histoplasmin. Both serum and CSF can be used. Patients with negative serum reactions during the acute phase of infection should have additional samples taken 3 to 4 weeks later. ID test kits and reagents are available from Gibson Laboratories (Lexington, KY), IMMY, and Meridian Bioscience (Cincinnati, OH). Commercial kits include mycelial-phase culture filtrates containing *H. capsulatum* H and M antigens, positive-control sera containing antibodies against both H and M antigens, and ID plates. Positive-control sera must be included each time the test is performed and must react with both the H and M antigens. The ID test is a useful screening procedure or can be used as an adjunct to the CF test. It is more specific but less sensitive than the CF test.

The CF test is a quantitative procedure in which two antigens are employed: histoplasmin and a suspension of intact, merthiolate-killed *H. capsulatum* yeast-phase cells. The latter is more sensitive (~80% versus ~20%) but less specific (~90% versus ~99%) than histoplasmin. Serum, peritoneal fluid, or CSF can be used in the CF test. Patients with negative serum reactions during the acute phase of infection should have additional samples taken 3 to 4 weeks later. No commercial kits are available, but antigens, antisera, and other reagents can be purchased from several commercial sources (e.g., IMMY and Meridian). Negative-control serum and positive-control serum from human histoplasmosis cases demonstrating a CF titer of 1:32 or greater with the homologous antigen should be tested each time the CF test is performed.

More recently, an ELISA for the detection of antibodies to *H. capsulatum* using metaperiodate-treated purified histoplasmin was reported, with sensitivities of 100% in acute disease, 90% in chronic disease, 89% in disseminated infection in individuals without HIV infection, 86% in disseminated disease in the setting of HIV infection, and 100% in mediastinal histoplasmosis (104). This test is, however, not commercially available.

An LA test using histoplasmin as antigen is commercially available (LA-Histo antibody system; IMMY). This semiquantitative test detects IgM antibodies and is used primarily for the presumptive diagnosis of acute histoplasmosis. It is less helpful for the detection of chronic infection.

Blastomycosis

Substantial improvement in the performance of serologic tests for blastomycosis has been achieved by the use of two purified surface antigens of *B. dermatitidis*, one termed the A antigen and the other the WI-1 antigen. Both molecules are released from the yeast phase of *B. dermatitidis* by autolysis and can be recovered from culture filtrates. Immunological comparison of the two antigens has shown that they are very similar, but WI-1 is a 120-kDa protein that is not glycosylated while the A antigen is a 135-kDa glycosylated protein (105).

However, serologic testing for blastomycosis still lacks both sensitivity and specificity. The most useful serologic test is the ID test, which measures the antibody to *B. dermatitidis* A antigen. This test is relatively specific, yet sensitivity ranges from 28 to 64% (106). Patients with negative serum reactions during the acute phase of infection should have additional samples taken 3 to 4 weeks later.

CF testing has also been evaluated, although this test has shown even lower sensitivity and specificity (9 to 43%) (107). Newer EIAs have shown improved sensitivity; however, it has been at the expensive of specificity (108).

ID test kits and reagents are available from Gibson, IMMY, and Meridian. Commercial kits include purified *B. dermatitidis* A antigen, positive-control serum containing antibodies against A antigen, and ID plates. The positive-control serum must be included each time the test is performed and must react with the homologous reference antigen to form the A precipitin line.

Coccidioidomycosis

Despite the fact that sensitive procedures such as EIA have been developed, the ID and CF tests remain the most reliable methods for the serologic diagnosis of coccidioidomycosis. The principal antigen used in these tests is coccidioidin, a soluble filtrate of mycelial-phase broth cultures.

The simultaneous use of heated and unheated antigens permits the ID test to be employed to detect either IgM or IgG antibodies on a single plate. The ID test for tube precipitin (TP) antibodies (IDTP) utilizes heated coccidioidin as antigen, detects IgM, and gives results comparable to those obtained with the classical tube precipitin test. It is most useful for diagnosing recent infection. The IDCF test utilizes unheated coccidioidin, detects IgG antibodies, and gives results comparable to those obtained by the CF method (see below). It is less sensitive but more specific than the CF test (109). Commercial kits are available (Gibson, IMMY, and Meridian) and include unheated and heat-treated coccidioidin antigens, positive-control sera containing IgM or IgG antibodies, and ID plates. The sensitivity of the IDTP and IDCF tests can be improved by 10-fold concentration of serum prior to testing. Positive-control sera must be included each time the test is performed and must react with the homologous reference antigen to form a precipitin line. The ID test is useful for initial screening of specimens and can be followed by other tests if positive.

The CF test is a sensitive quantitative method in which unheated coccidioidin is used to detect IgG antibodies. The major disadvantage of CF is that it is a laborious and time-consuming procedure that requires experienced personnel for optimum performance. No commercial kits are available, but reagents for in-house use can be obtained from several commercial sources (IMMY and Meridian). Negative-control serum and a positive-control serum from a human case of coccidioidomycosis (with a titer of ≥1:32) should be included each time the test is performed. Anticomplementary activity in serum samples can occur and may be resolved by subsequent ID testing. In addition to serum, the CF test can be performed with CSF, pleural, or joint fluid samples.

A qualitative LA test using heat-treated coccidioidin as antigen is available from several commercial sources (e.g., LA-Cocci antibody system from IMMY and Coccidioides latex agglutination system from Meridian). This test is simple and rapid to perform; however, the false-positive rate is higher than that observed with the IDTP and/or CF methods. It is not recommended for screening CSF specimens, because false-positive reactions can occur (105).

The Premier Coccidioides EIA (Meridian) is a qualitative test for detection of IgM and IgG antibodies in serum or CSF specimens. The antigen used in this test is a mixture of purified TP and CF antigens. False-positive reactions have been obtained with sera from some patients with blastomycosis and in patients with alternative diagnoses, and this test is now often used for “screening,” with positive EIA results evaluated by IDTP and IDCF tests for confirmation (110).

Paracoccidioidomycosis

The most popular serologic methods for diagnosis of paracoccidioidomycosis are ID and CF, but other tests, such as ELISAs, counterimmunoelectrophoresis, dot blot, and immunoblotting, have also been employed (32). The ID test demonstrates circulating antibodies in >90% of cases. A reactive result allows a diagnosis to be made. The CF test allows a more precise evaluation of the patient’s response to treatment, but cross-reactions with *H. capsulatum* antigens can occur. The principal antigens used in these tests are derived from culture filtrates of mycelial-phase or yeast-phase broth cultures of *P. brasiliensis*. The major diagnostic antigen found in these preparations is a 43-kDa glycoprotein. Cell wall antigens have proved less useful than culture filtrate antigens, largely because cell wall antigens are dominated by cross-reactive galactomannan (32). Commercial mycelial-form culture filtrate antigen can be obtained for in-house use from IMMY. No commercial kits are available for this test. The CF test is performed with *P. brasiliensis* yeast-form culture filtrate antigen. No commercial kits or reagents are available.

Improvements in serodiagnosis include the detection of antibodies against chemically characterized and/or recombinant *P. brasiliensis* antigens, notably gp43, pb27, and the 87-kDa heat shock protein. A combination of two recombinant products has resulted in increased sensitivity (92%) and specificity (88%) (111, 112).

Adiaspiromycosis

No serologic tests are available for diagnosis of adiaspiromycosis.

ANTIMICROBIAL SUSCEPTIBILITIES

Established treatment options for *H. capsulatum*, *B. dermatitidis*, *Coccidioides* species, and *P. brasiliensis* include amphotericin B and the azoles voriconazole, posaconazole, itraconazole, ketoconazole, and fluconazole. The echinocandins have a limited, if any, role in the treatment of endemic fungi. *In vitro* antifungal susceptibility testing for dimorphic fungi remains unstandardized, and no susceptibility breakpoints have been determined for these organisms (113). Table 1 lists the *in vitro* susceptibilities of these organisms to established and investigational antifungal agents as reported in studies that were performed in accordance with Clinical and Laboratory Standards Institute documents M38 (for filamentous fungi) (113) and M27 (for yeast) (114). Fluconazole treatment failures have been reported in some cases of histoplasmosis and coccidioidomycosis, partially attributed to organisms that demonstrated drug MICs of ≥64 μg/ml, although they remained responsive to itraconazole (115, 116).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Histoplasmosis

The definitive diagnosis of histoplasmosis can be accomplished by direct microscopic detection of *H. capsulatum* in clinical specimens or its isolation in culture. However, isolation and identification may take 2 to 4 weeks.

Antigen detection complements other diagnostic methods for histoplasmosis and is particularly useful in immunocompromised patients with more extensive disease, often providing a rapid diagnosis before positive cultures can be identified. HPA has been detected in serum, urine, CSF, and bronchoalveolar lavage fluid specimens obtained from individuals with disseminated histoplasmosis. The sensitivity of antigen detection in disseminated histoplasmosis is higher in immunocompromised patients than in immunocompetent patients and in patients with more severe illness. Antigen levels are higher in urine than in serum. For patients with AIDS and disseminated histoplasmosis, sensitivity is between 81 and 95% in urine and 86% in serum

TABLE 1 *In vitro* susceptibilities of dimorphic fungi to antifungal agents

Fungus	Antifungal agent	MIC range (μg/ml)	MIC_{90} range (μg/ml)	Reference(s)
B. dermatitidis	Amphotericin B	≤0.03–1	0.5	117, 118
	Fluconazole	1–64	NR[a]	113
	Itraconazole	≤0.03–4	0.125	117–119
	Posaconazole	≤0.03–1	NR	119, 120
	Voriconazole	≤0.03–16	0.25	117, 118
	Isavuconazole	0.5–4	NR	119
	Anidulafungin	2–8	NR	120
	Caspofungin	0.5–8	NR	120
Coccidioides spp.	Amphotericin B	0.125–2	0.5–1	117, 118, 121
	Fluconazole	2–64	64	118, 121
	Itraconazole	0.125–2	1	117, 118
	Posaconazole	0.06–1	1	119, 122
	Voriconazole	≤0.03–1	0.25	117–119
	Isavuconazole	0.125–1	0.5	119
	Caspofungin	8–64	32	121
H. capsulatum	Amphotericin B	≤0.03–2	0.25	117, 118
	Fluconazole	≤0.125–64	NR	118
	Itraconazole	≤0.03–8	0.06	117, 118
	Posaconazole	≤0.03–2	NR	119, 120
	Voriconazole	≤0.03–2	0.25	117
	Isavuconazole	0.125–2	2	119
	Anidulafungin	2–4	NR	120
	Caspofungin	0.5–4	NR	120
P. brasiliensis	Amphotericin B	0.125–4	NR	118
	Fluconazole	≤0.125–64	NR	118
	Itraconazole	≤0.03–1	NR	118
	Voriconazole	≤0.03–2	NR	118
	Isavuconazole	NR	NR	

[a] NR, not reported.

(54, 56). Specificity is ~98%. Antigen has been detected in the CSF of patients with *Histoplasma* meningitis.

Antigen levels in the urine and serum decline with effective treatment, becoming undetectable in most patients (55). Failure of antigen concentrations to fall during treatment suggests therapeutic failure. In patients who have responded to treatment and in whom antigen levels have previously fallen, an increase in antigen levels in the urine or serum is suggestive of relapse.

The LA test for *Histoplasma* antibodies is most useful for the diagnosis of acute infection, positive results being obtained within 2 to 3 weeks after exposure. An LA titer of 1:16 is presumptive evidence of infection, and a titer of ≥1:32 is considered strong presumptive evidence of active or recent infection (103). Because false-positive reactions can occur, the results should be confirmed by the ID test. Low-titer results from single specimens should be interpreted with caution. In such cases, the test should be performed on another specimen collected 4 to 6 weeks later.

In the ID test, precipitins to the M antigen of *H. capsulatum* are the first to appear (4 to 8 weeks after exposure) and can be detected in up to 75% of persons with acute histoplasmosis. However, they can also be found in nearly all individuals with chronic pulmonary infection, as well as in those who have undergone a recent skin test with histoplasmin. Precipitins to the H antigen are specific for active disease but occur in <20% of cases. They usually disappear within the first 6 months of infection and are seldom, if ever, found in the absence of M precipitins. The presence of precipitins to both H and M antigens is highly suggestive of active histoplasmosis, regardless of other serologic test results.

The CF test is useful in the diagnosis of acute, chronic, disseminated, and meningeal forms of histoplasmosis. In acute infections, antibodies to the yeast antigen are the first to appear (~4 weeks after exposure) and the last to disappear after resolution of the infection. Antibodies to histoplasmin appear later and reach lower titers than those observed for the yeast antigen. In contrast, histoplasmin titers are usually higher in persons with chronic histoplasmosis. CF test results can be difficult to interpret because cross-reactions can occur with sera from persons with blastomycosis, coccidioidomycosis, and other fungal infections. In such instances, titers usually range between 1:8 and 1:32 and occur mainly against the yeast-form antigen. However, many serum samples from culture-confirmed cases of disseminated histoplasmosis yield titers in the same range. CF titers of ≥1:8 with either antigen are considered presumptive evidence of histoplasmosis. Titers of ≥1:32 and rising titers in serial samples offer stronger evidence of infection.

Titers of CF antibodies to *H. capsulatum* decrease following resolution of the infection but increase in individuals with chronic progressive disease. However, clinical and microbiological findings should also be considered in assessing the patient's prognosis or making treatment decisions. In some patients, positive CF titers decline slowly and persist long after the disease has been cured. The significance of persistently elevated or fluctuating CF titers is unclear, as is the effect of antifungal treatment on antibody clearance (25).

Serologic tests are particularly useful in patients with *Histoplasma* meningitis. The detection of precipitins to H and M antigens in CSF specimens is sufficient to make a diagnosis in the appropriate clinical setting and often is the only positive diagnostic test.

Blastomycosis

Although microscopic examination and culture remain the most sensitive means of establishing the diagnosis of blastomycosis, serologic tests can also provide useful information. A positive reaction in an ID test using the A antigen of *B. dermatitidis* is specific and diagnostic for blastomycosis (106). However, a negative ID test does not rule out the diagnosis because the sensitivity of this method has been reported to range from ~30% for cases of localized infection to ~90% for cases of disseminated blastomycosis. In established cases of the disease, a decline in the number or the disappearance of precipitin lines is evidence of a favorable prognosis.

With urine specimens, the *Blastomyces* antigen test has been reported to have a sensitivity of 89% for disseminated infection and 100% for pulmonary disease. However, cross-reactive antigens occurred in urine from all patients with paracoccidioidomycosis and from 96% of patients with histoplasmosis (62).

Coccidioidomycosis

Although the definitive laboratory diagnosis of coccidioidomycosis depends on microscopic examination and culture, serologic tests are of proven usefulness in diagnosis and management. A positive IDTP test result is indicative of acute coccidioidomycosis. IDTP-reactive IgM antibodies can be detected in up to 75% of cases within 1 week of symptom onset, and ~90% are positive within 3 weeks. Although infrequent, a positive IDTP test result with CSF is indicative of acute meningitis. In cases where the IDTP test is negative but the CF test is positive, patients should be investigated for microbiological or histopathological evidence of histoplasmosis or blastomycosis. In addition, sera should be obtained at 3-week intervals and examined by CF and ID tests for coccidioidomycosis, histoplasmosis, and blastomycosis. False-positive IDTP reactions have been reported to occur in 15% of sera obtained from cystic fibrosis patients in the absence of a positive culture (109).

A positive IDCF test result is presumptive evidence of recent or chronic infection. IDCF-reactive IgG antibodies can usually be detected within 2 to 6 weeks after onset of symptoms. Although the IDCF test is generally not performed as a quantitative test, it can be used in this manner. Titers obtained using the quantitative IDCF are not identical to titers obtained from the CF test, but the observed trends are comparable.

The LA test is more sensitive than the IDTP test in detecting acute infection but is less specific. For this reason, a positive test result with undiluted serum should be confirmed by the ID and/or CF test.

The CF test does not become positive until about 4 to 12 weeks after infection, but CF antibodies persist for long periods in individuals with chronic pulmonary or disseminated coccidioidomycosis. Testing of serial specimens to detect rising or falling titers can reveal the progression or regression of illness and the response to antifungal treatment. A CF titer to coccidioidin at any dilution should be considered presumptive evidence of coccidioidomycosis. In most instances, the titer is proportional to the extent of the infection, and failure of the CF titer to fall during treatment of disseminated coccidioidomycosis is an ominous sign (30). Titers of 1:2 or 1:4 are usually indicative of early, residual, or meningeal disease. CF titers of >1:16 should lead to a careful assessment of the patient for possible spread of the disease beyond the respiratory tract. More than 60% of patients with disseminated coccidioidomycosis have CF titers of >1:32. However, false-negative results can occur in immunocompromised individuals, such as persons with AIDS. Patients with clinical presentations consistent with coccidioidomycosis but with negative or low serum CF titers should be retested at 3- to 4-week intervals.

The detection of CF antibodies in the CSF is usually diagnostic of coccidioidal meningitis and remains the single most useful test for diagnosis of that infection.

Paracoccidioidomycosis

The definitive diagnosis of paracoccidioidomycosis depends on microscopic examination and culture. However, isolation and identification of *P. brasiliensis* from clinical specimens may take up to 4 weeks.

Serologic tests are useful for the rapid presumptive diagnosis of paracoccidioidomycosis, particularly in cases of disseminated infection (32). The ID test with yeast-form culture filtrate antigen is highly specific and is positive in 65 to 100% of cases of acute or chronic pulmonary infection or disseminated paracoccidioidomycosis. The CF test with yeast-form culture filtrate antigen is less specific than the ID test, and cross-reactions can occur with cases of histoplasmosis. However, CF titers of ≥1:8 are considered presumptive evidence of paracoccidioidomycosis. Low CF titers are usually associated with localized infection, while higher titers are found in those with multifocal disease. Falling CF titers are often predictive of successful treatment, and high or fluctuating CF titers are suggestive of a poor prognosis. Some reports, however, have indicated that ID and CF results do not correlate well with the clinical status of the patient (69).

Adiaspiromycosis

The definitive diagnosis of adiaspiromycosis can be accomplished by direct microscopic detection of *Emmonsia* species in clinical specimens or their isolation in culture.

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Trichophyton, *Microsporum*, *Epidermophyton*, and Agents of Superficial Mycoses

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TAXONOMY

The etiologic agents of dermatophytosis are classified, along with some nonpathogenic relatives, in three genera: *Trichophyton*, *Microsporum*, and *Epidermophyton*. These generic names are historically based on anamorphic (asexual state) names. Any dermatophytes capable of reproducing sexually, i.e., producing ascomata with asci and ascospores, also had historical teleomorph names in the genus *Arthroderma* (1). After current "one fungus, one name" nomenclature (2) came into force on 1 January 2013, it appeared likely that the clinically familiar anamorph names would be chosen over *Arthroderma* for ongoing use in this group of fungi. The redisposition of a few species that only have valid names in *Arthroderma* has not yet been effected. Despite these nomenclatural changes, the dermatophytes currently remain in the family *Arthrodermataceae* of the order *Onygenales* (3), phylum *Ascomycota*. The clinically recorded species of the dermatophytes as well as the dermatophytoids (i.e., the soilborne *Trichophyton* and *Microsporum* species that are best not called "dermatophytes" [Greek for "skin plants"] because they are not pathogenic [4, 5]) are given in Table 1. The long-used *Arthroderma* names are included in the table to facilitate comparison with earlier literature.

As part of the ongoing molecular revolution in biology, fungal taxonomy is ever more strongly influenced by our greatly increased understanding of population genetics (6). Dermatophytes show two population genetics patterns differing among species having "population hosts" (7) in different zoological families, orders, or classes (8, 9). (A population host, which is the normal epidemiological reservoir of the species, is distinguished from "occasional host" species that may acquire infection but that do not support ongoing populations; for example, *Microsporum canis* has mostly feline population hosts, and while humans are frequently infected by feline carriers, the species is seldom directly transmitted from human to human, making our species only an occasional host.) Some dermatophyte species, including pathogens with population hosts in the rodent, rabbit, pig, dog, and cat families, are potentially sexual, with sexual reproduction occurring only off the host, i.e., on hair or other keratinous debris in contact with soil. Other species, particularly those with human, ungulate, equine, or avian population hosts, have no access to a soil-based location suitable for sexual reproduction and host reinfection; not surprisingly, these species are found on investigation to be asexual and clonal. They consist, in all known cases, of genetically highly uniform isolates (10–12) sharing, where known, a single mating type factor (8, 13). Most appear to have evolved from a single strain of a sexual ancestral species that was able to make the rare, successful switch to ongoing contagious infection of a new animal host. A few epidemiological and phenotypic characters in the clonal species appear to have undergone accelerated evolution due to strong selection for increased compatibility with the new host. This process has tended to produce differences allowing relatively easy laboratory identification of these species. At the same time, the basic cellular "housekeeping" genes investigated in phylogenetic taxonomic studies have evolved at a normal rate and thus strongly tend to resemble forms seen in ancestral species complexes or in sibling species. This has led to considerable recent confusion about species concepts. Although most recent phylogenetic studies continue to support the ongoing classification of dermatophytes in the three traditional anamorph genera mentioned above, long-recognized dermatophyte species, such as *Trichophyton equinum* and *Microsporum gallinae*, that have distinct epidemiologies and that are easily identified in the routine diagnostic laboratory have nonetheless been suggested not to be distinct species as traditionally conceived (14, 15), since their ribosomal internal transcribed spacer (ITS) region sequences and some other sequences are strongly similar to those of related species (e.g., *Trichophyton tonsurans* and *Arthroderma grubyi*, respectively, for the examples mentioned). The ITS sequences of the dermatophytes proposed for synonymy, however, generally show a small number of consistent sequence polymorphisms compared with sequences of the species with which synonymy has been proposed. This suggests that further genetic study will tend to support the lineages in question as recently diverged but nonetheless separate at the species level. *T. equinum*, for example, was reinstated as a recognized distinct species after more detailed genetic study (16, 17). In this respect, it is important to note that recent proteomic approaches support essentially the same phylogenies as those previously proposed based on phenotypic, clinical, and epidemiological characters (18). Thus, the present chapter retains a relatively cautious approach to the ongoing debates in this area (19), synonymizing traditionally recognized species only when there is unequivocal, multigene molecular evidence that they are not supported at the species level. The list of recognized species is in Table 1.

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TABLE 1 Important characteristics of clinically isolated dermatophytes and dermatophytoids

Dermatophyte species and abundance (teleomorph name[s] if formation of sexual state is known)	Growth	Macromorphology	Micromorphology	BCPMSG medium pH results (for 7–10 days)	Urea test (7 days, broth)	Hair perforation	Other comments
Epidermophyton floccosum[a]	Moderately rapid	Flat, slightly granular at first, soon developing white puffs of degeneration; sandy to olive-brown (Fig. 18e); reverse pale to yellowish	Macroconidia abundant, club shaped with broadly rounded apex, usually with <6 cells (Fig. 12iiiii); no microconidia formed; many chlamydospores in primary isolates	Alkaline	Pos	Neg	Invades skin and nails, rarely hair; no microconidia
Microsporum audouinii[b]	Moderately rapid	Flat to velvety, thin, pale salmon to pale brownish reverse	Rare, deformed macroconidia, often with beak, constricted midregion, and at least trace granulation (Fig. 13); drop-shaped microconidia and aerial arthroconidia may be present; pectinate branching, apiculate terminal chlamydospores often seen	No pH change or alkaline	Neg	Neg	Poor growth and no or brownish pigment on polished rice medium; usually connected with patient or index patient in or recently from Africa; only children typically infected
M. *canis*[a] (*Arthroderma otae*)	Rapid	Flat to velvety, thin, pale to yellow (Fig. 18g), with yellow (rarely pale) reverse	Macroconidia thick walled, roughened, and beaked (Fig. 14); microconidia drop shaped	No pH change; macroconidia often abundant	Pos	Pos	Good growth and yellow pigment on polished rice medium; human infection usually from cat or dog; M. *canis* "distortum" phenotype has macroconidia distorted, bizarrely shaped; "M. *equinum*" phenotype from horses has few, short macroconidia
M. *cookei* complex[b] (includes M. *cookei* [*A. cajetani*], M. *mirabile* [*A. mirabile*])	Moderately rapid	Granular to velvety; reverse wine red	Macroconidia rough, thick walled with cellular compartments rather than true cross walls (Fig. 15); microconidia drop shaped	No pH change	Pos	Pos	Probably nonpathogenic; existing case reports poorly substantiated
M. *ferrugineum*[c]	Slow	Flat or folded, waxy to slightly velvety; surface and reverse yellow, rusty, or pale	No conidia; coarse, straight "bamboo" hyphae with prominent septa may be present	No pH change	Neg	Neg	Yellow colony on Lowenstein-Jensen medium (compare *T. soudanense*); geographically restricted to parts of Africa, Asia, and eastern Europe

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TABLE 1 Important characteristics of clinically isolated dermatophytes and dermatophytoids *(Continued)*

Dermatophyte species and abundance (teleomorph name[s] if formation of sexual state is known)	Growth	Macromorphology	Micromorphology	BCPMSG medium pH results (for 7–10 days)	Urea test (7 days, broth)	Hair perforation	Other comments
M. gallinae[c]	Moderately rapid	Flat to velvety; surface white tinged with pink; reverse red; red pigment diffuses into agar	Macroconidia smooth to slightly rough, often bent and with thickest cells near the apex, sometimes slightly rough; microconidia drop shaped	No data	Neg	Neg	Rare; human infection usually from chicken; species may be an asexual phenotype within *A. grubyi* according to Gräser et al. (14)
M. gypseum complex[a] (*A. gypseum*, *A. incurvatum*, *A. fulvum*, *Microsporum duboisii*)	Rapid	Granular, sandy in color, or occasionally light cinnamon or rosy buff; reverse usually pale to brownish	Macroconidia abundant, thin walled, fusoid (tapered at both ends), roughened, with up to 6 septa (Fig. 16); microconidia drop shaped, mostly formed along sparsely branched hyphae (a feature only noted if *M. racemosum* is queried)	No pH change	Pos	Pos	Human infection usually from soil contact
M. nanum[b] (*A. obtusum*)	Moderately rapid	Powdery, sandy in color; reverse often reddish brown	Macroconidia rough, usually only 1–3 cells long, egg shaped to ellipsoidal	No data	Pos	Pos	Human infection usually from pig; now rare
M. persicolor[b] (*A. persicolor*)	Rapid	Powdery, sandy in color; reverse pale to yellowish, sometimes with rosy tones	Macroconidia fusoid (tapered on both ends), often absent or smooth walled on Sabouraud agar but usually common and rough walled on Sabouraud with added salt (3 or 5% NaCl) (Fig. 17); microconidia formed on pedicels (must be checked within 5 days)	No pH change	Pos	Pos	Usually poor growth at 37°C *in vitro*; rose to wine-red reverse on sugar-free media, e.g., glucose-free Sabouraud agar; human infection usually from soil (fomites from voles)
M. praecox[b]	Rapid	Powdery, sandy in color, reverse yellow	As *M. gypseum*	No pH change	Pos	Neg	Uncommon
M. racemosum[c] (*A. racemosum*)	Rapid	Powdery, sandy in color, reverse red	Macroconidia as *M. gypseum*; microconidia mostly formed in densely branched formations structured like grape clusters (racemes)	No pH change	Pos	Pos	Rare
Trichophyton ajelloi[b] (*A. uncinatum*)	Moderately rapid	Powdery, rich tan to medium orange-brown in color; reverse pale, brownish, or with purple-black pigment	Macroconidia smooth, thick walled, cylindrical, often >7 cells long, with cellular compartments rather than true cross walls (Fig. 5)	No data	Pos	Pos	Nonpathogenic in humans
M. vanbreuseghemii[c] (*A. grubyi*)	Rapid	Powdery, pinkish or buff; pale to yellow reverse	Macroconidia rough, thick walled, cylindrical, often >8 cells long, with cellular compartments rather than true cross walls	No data	Pos	Pos	Rare; anamorph may be conspecific with phenotypically and epidemiologically different *M. gallinae* according to Gräser et al. (14)

T. concentricum[c]	Slow	Folded, honey brown to reddish brown, glabrous or slightly velvety colony	No conidia	No data	Pos or neg	Neg	Only from indigenous Asian Austronesian/Melanesian or indigenous Central and South American people with distinct tinea imbricata infection
T. equinum[b]	Moderately rapid	Flat to velvety colony with cream-colored surface and yellow to red-brown reverse	Macroconidia uncommon, cylindrical to club shaped, smooth; microconidia abundant, on small pedicels (examine before 5 days)	Alkaline	Pos	Usually neg, sometimes pos	Human infection usually from horse; has a nicotinic acid requirement except in autotrophic variant from Australia and New Zealand
T. erinacei hedgehog form[b] (*A. benhamiae*)	Rapid	Granular to powdery, yellow-cream to buff surface, yellow reverse	Macroconidia uncommon, club shaped, smooth; microconidia nearly spherical, abundant, mostly produced in dense tufts; spiral appendages present	Alkaline	Neg (European/New Zealand form); pos (African form)	Pos	Human infection usually from hedgehog or its fomites; therefore, mostly restricted to regions with wild hedgehogs or to pet hedgehog owners
T. megninii[b]	Moderately rapid	Cottony, with white down sometimes suffused with rosy pigment; reverse red to red-brown	Macroconidia seldom seen, pencil shaped; microconidia drop shaped	Alkaline	Pos or weak pos	Neg	Region of endemicity in Portugal and nearby areas; requires histidine; considered a subtype of *T. rubrum* by Gräser et al. (14)
T. mentagrophytes complex (zoophilic)[a] inclusive of *T. mentagrophytes sensu stricto* (formerly *T. mentagrophytes* var. *quinckeanum*) plus animal-adapted forms of *T. interdigitale* and the *Trichophyton* anamorph of *A. benhamiae* (*A. vanbreuseghemii*, *A. benhamiae*); also includes the rare species *T. eriotrephon* and probably *T. bullosum*	Rapid	Granular to powdery, yellow-cream to buff surface (Fig. 18b), pale to red-brown reverse	Macroconidia uncommon, club shaped, smooth; microconidia nearly spherical, abundant, mostly produced in dense tufts; spiral appendages present	Alkaline (rarely weak)	Pos	Pos	Human infection usually from rodent or rabbit; macroconidia induced on SGA + 3 or 5% NaCl (Fig. 6)

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TABLE 1 Important characteristics of clinically isolated dermatophytes and dermatophytoids *(Continued)*

Dermatophyte species and abundance (teleomorph name[s] if formation of sexual state is known)	Growth	Macromorphology	Micromorphology	BCPMSG medium pH results (for 7–10 days)	Urea test (7 days, broth)	Hair perforation	Other comments
T. mentagrophytes complex (anthropophilic)[a] inclusive of human-adapted forms of *T. interdigitale* and possibly of the *Trichophyton* anamorph of *A. benhamiae* (*A. vanbreuseghemii*, *A. benhamiae*)	Rapid	Powdery to cottony, yellow-cream to buff or white surface, pale to red-brown reverse	Macroconidia uncommon, club shaped, smooth; microconidia nearly spherical or drop shaped, abundant, produced mainly in dense tufts when round and on sparsely branched hyphae when drop shaped; spiral appendages present but rare in very cottony isolates	Alkaline	Pos	Pos	Macroconidia often induced on SGA + 3 or 5% NaCl
T. mentagrophytes ("nodular" variant[b] formerly called *T. krajdenii*, now known to be a distinct morph of *T. interdigitale*)	Moderately slow	Cottony, cream to white surface often with yellow marginal zone (Fig. 18a), intense yellow reverse	Macroconidia rare; microconidia usually drop shaped, sometimes also round; coiled, yellow "nodular bodies" and yellow pigment granules present in submerged mycelium; spiral appendages seldom seen	Alkaline	Pos	Pos	Although usually very different in morphology, this variant so far is not genetically distinguishable from other anthropophilic isolates of *T. interdigitale*
T. rubrum[a] (cosmopolitan variant)	Moderately slow	Cottony to velvety, white to reddish surface (Fig. 18c), typically wine-red reverse (Fig. 18d) but yellow variants occasional; red color poorly formed in presence of common bacterial contamination	Macroconidia seldom seen, pencil shaped (Fig. 8); microconidia drop shaped, abundant, scanty or not formed; lateral hyphal projections often present	No pH change (alkalinity after 14 days)	Neg (rarely weak)	Neg	Melanoid variants secreting brown pigment rarely seen
T. rubrum (Afro-Asiatic variant[b] formerly called *T. raubitschekii*, *T. fluviomuniense*, or, when microconidia absent, *T. kanei*)	Moderately slow	Powdery to low velvety, cream to deep red; reverse wine red	Macroconidia abundant (Fig. 7), club shaped, sometimes with "rat-tail" extension; microconidia drop shaped to round; many chlamydospores in primary isolates	No pH change (alkalinity after 14 days)	Pos	Neg	Although usually very different in morphology, this variant so far is genetically distinguished from typical *T. rubrum* only at microsatellite markers; it is often from upper body infection (tinea corporis, tinea cruris)

T. schoenleinii[c]	Slow	Convoluted, slightly velvety whitish colony	No conidia seen; "favic chandeliers" or "nailhead hyphae" present	Alkaline	Variable	Neg	Very rare; associated with clinically recognizable "favus" lesions; now extirpated except in rural central Asia and rural Africa
T. simii[b] (*A. simii*)	Rapid	Granular to powdery, yellow-cream to buff surface, pale to red-brown reverse	Macroconidia abundant, often with some cells swollen as chlamydospores; microconidia drop shaped	Alkaline	Pos	Pos	Endemic to India and Africa; similar to zoophilic *T. mentagrophytes* but macroconidial number and shape are atypical; reference distinction is by mating or molecular study
T. soudanense[b]	Moderately slow	Flat, bright yellow to (less commonly) wine-red colony with radial striations and star-like margin; uncommonly cottony; reverse yellow to wine red	Macroconidia not seen; microconidia drop shaped, scarce or absent; reflexive hyphal branches in radial striations	Alkaline, with small zone of clearing	Usually neg, occasionally pos	Neg	Endemic to sub-Saharan Africa but widely disseminated in cosmopolitan parts of Europe and Americas; dark colony on Lowenstein-Jensen medium (compare *M. ferrugineum*); may or may not grow on growth factor test media; considered a subtype of *T. rubrum* by Gräser and colleagues (108, 109)
T. terrestre complex[b] (*A. lenticulare*, *A. quadrifidum*, *A. insingulare*); also similar, *T. eboreum* (*A. olidum*), *T. thuringiense*	Moderately rapid	Powdery white to cream or pinkish surface; pale or rarely yellow to red reverse	Macroconidia numerous, mostly small (5 or fewer cells) intergrading with large club-shaped microconidia in a continuous series (Fig. 9), so that 3-, 2-, and 1-celled conidia are present	Alkaline	Pos	Pos	No growth at 37°C *in vitro*; nonpathogenic
T. tonsurans[a]	Moderately slow	Powdery to velvety, white to yellowish or red-brown surface; reverse chestnut red-brown (Fig. 18f) and/or sulfur yellow, rarely pale	Macroconidia uncommon, small, pencil or club shaped; microconidia abundant (Fig. 10), often on broad "matchstick" pedicels; "balloon forms" and "filiform branches" may be seen	Alkaline, sometimes weak	Pos	Usually neg, sometimes pos	Stimulated by thiamine; a form producing only macroconidia has been described
T. vanbreuseghemii[c] (*A. gertleri*)	Moderately slow	Buff colony, leathery, finely grainy; reverse whitish or pale yellow	Abundant macroconidia with cells of uneven length, tending to fragment into single cells; microconidia small, boxy	No data	Pos	Pos	Geophilic; very rare in clinical laboratory

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TABLE 1 Important characteristics of clinically isolated dermatophytes and dermatophytoids *(Continued)*

Dermatophyte species and abundance (teleomorph name[s] if formation of sexual state is known)	Growth	Macromorphology	Micromorphology	BCPMSG medium pH results (for 7–10 days)	Urea test (7 days, broth)	Hair perforation	Other comments
T. verrucosum[a]	Slow	Convoluted, slightly velvety whitish or less commonly tan to ochraceous colony	Macroconidia seldom seen, with "rat-tail" extension; microconidia round to drop shaped; chains of symmetrical chlamydospores seen on milk solids media at 37°C (Fig. 11)	Alkaline (may be weak) with broad zone of clearing	Neg	Neg	Human infection usually from cattle; growth stimulated at 37°C
T. violaceum[b]	Slow	Glabrous, smooth or convoluted colony; purple-red, sometimes with white sectors; some east African isolates purely whitish	Macroconidia seldom seen; microconidia drop shaped, formed mostly on thiamine medium or on sporulation media; chains of asymmetrical chlamydospores seen on milk solids media at 37°C	No pH change or weak alkaline with small to broad zone of clearing (always broad after 14 days)	Pos or weak	Neg	Endemic to north Africa and Middle East but widely disseminated in cosmopolitan parts of Europe, Americas, and South Africa

[a] Common.

[b] Uncommon but likely to be seen by large labs in Americas and Europe.

[c] Unlikely to be seen except in region where endemic or proficiency test or soil isolation experiment.

EPIDEMIOLOGY AND TRANSMISSION

Dermatophytes are keratinophilic fungi that are capable of invading the keratinous tissues of living animals. They are grouped into three categories based on host preference and natural habitat (Table 2) (20). Anthropophilic species almost exclusively infect humans; animals are rarely infected. Geophilic species are soil-associated organisms, and soil per se or soilborne keratinous debris (e.g., shed hairs or molted feathers) is a source of infection for humans as well as other animals. Zoophilic species are essentially pathogens of nonhuman mammals or, rarely, birds; however, animal-to-human transmission is not uncommon. Understanding this ecological classification for case isolates may be helpful in determining the source of infection; e.g., human infections caused by *M. canis* are often the result of contact between susceptible children and newly acquired or stray kittens (21). Clinical species identification of dermatophytes assists in controlling infections that may have a family pet or other domesticated animal as an ongoing source of inoculum. Moreover, scalp infections with *M. canis* require a treatment regimen differing from that used for scalp infections caused by the more common agents of tinea capitis. This underscores the importance of correct identification of the etiologic agent.

Some dermatophytes, e.g., *Trichophyton rubrum*, are cosmopolitan, whereas others, e.g., *Trichophyton concentricum*, are geographically limited (22). *T. concentricum* is found only in the Pacific Islands and regions in Southeast Asia and Central and South America. However, the ability of dermatophytes to spread to new geographical niches in conjunction with population movements should not be underestimated. In the United Kingdom, the large-scale introduction of *T. rubrum* and the emergence of *Microsporum audouinii* in rural areas can be correlated respectively with troop repatriation from the Far East and evacuation of children from major cities during the First and Second World Wars. Similarly, the reemergence of *T. tonsurans* as the preponderant agent of tinea capitis in British cities has been driven by large-scale immigration from the Caribbean (23). Thus, care should be taken against complacent assumptions based on historical epidemiological patterns.

Anthropophilic fungi are usually transmitted either directly through close human contact or indirectly through sharing of clothes, combs, brushes, towels, bed sheets, etc. Tinea capitis is highly contagious and may spread rapidly within a family, institution, or school. Transmission of tinea cruris is associated with shared clothing, towels, and sanitary facilities. The transmission of tinea pedis and tinea unguium often involves communal showers, baths, or other aquatic facilities but may depend on both environmental and host factors (24, 25). Acquisition of chronic *T. rubrum* tinea pedis has been suggested to require a dominant autosomal susceptibility gene (26).

Infections with geophilic dermatophytes involve transmission of soilborne inoculum to humans or other mammals. Outbreaks originating from infected soil with secondary human-to-human transmission have been reported (27). Infections by zoophilic species result from animal-to-human contact (cats, dogs, cattle, laboratory animals, etc.) or from indirect transmission involving fomites. The fungi may then be transmitted among humans to a limited extent, especially in institutions (28).

CLINICAL SIGNIFICANCE

The dermatophytoses (tinea or ringworm) generally manifest as infections of the keratinized tissues (hair, nails, skin, etc.) of humans, other mammals, and birds. Cutaneous infections resembling dermatophytoses may occasionally be caused by yeasts or by unrelated filamentous fungi that are normally saprobes or plant pathogens; these infections are referred to as opportunistic dermatomycoses (25).

Dermatophytes are among the very few fungal species that cause contagious, directly host-to-host-transmissible diseases of humans and animals. The transmission of these fungi is usually carried out by arthroconidia that have formed in or on infected host tissue. These conidia may be spread by direct skin-to-skin contact or via fomites containing free arthroconidia, shed skin scales, or hairs. Typical fomites include such divergent materials as hats, shoes, shower room floors, bedding, clothing of nursing staff in chronic care institutes, animal bedding and nesting material, and farm fence posts used by animals for scratching. Tissue invasion is normally cutaneous; dermatophytes are usually unable to penetrate deeper tissues as a result of nonspecific inhibitory factors in serum (29), inhibition of fungal keratinases (30), a barrier formed of epidermal keratinocytes (31), and other immunological barriers (32, 33). In acute cases, there is a strong Th1 reaction mediated in part by CD4$^+$ lymphocytes (34), while in chronic cases, there is an immediate hypersensitivity-type reaction characterized by high levels of IgE and IgG4 antibodies and production of Th2 cytokines by mononucleocytes (35).

TABLE 2 Grouping of dermatophytes on the basis of host preference and natural habitat[a]

Anthropophilic	Zoophilic	Geophilic
Epidermophyton floccosum	*Microsporum* spp.	*Microsporum* spp.
Microsporum spp.	*M. canis*	*M. gypseum* complex
M. audouinii	*M. gallinae*	*M. praecox*
M. ferrugineum	*M. nanum*	*M. racemosum*
Trichophyton spp.	*M. persicolor*	*M. vanbreuseghemii*
T. concentricum	*Trichophyton* spp.	*Trichophyton vanbreuseghemii*
T. megninii	*T. equinum*	
T. mentagrophytes complex (velvety and cottony isolates)	*T. erinacei*	
T. rubrum	*T. mentagrophytes* complex (granular isolates)	
T. schoenleinii	*T. simii*	
T. soudanense	*T. verrucosum*	
T. tonsurans		
T. violaceum		

[a] Normally nonpathogenic, soil-associated dermatophytoids such as *T. terrestre* and *M. cookei* are not included in this table.

Dermatophytes tend to grow in an annular fashion on most affected skin regions, producing a "ringworm" infection form with a more or less raised and erythematous active area at the periphery and a relatively scaly inactive zone at the center of established lesions. The organism can often only be isolated from the active, peripheral ring. Infection may range from mild to severe, partly as a consequence of the reaction of the host to the metabolic products of the fungus. Also important in determining the severity of infection are the virulence of the infecting strain, the anatomic location of the infection, the status of the host's immune system, and local environmental factors. Occasionally, especially in immunocompromised patients, subcutaneous tissue may be invaded, e.g., in Majocchi's granuloma, kerion, mycetoma-like processes (36, 37), or, more rarely, a generalized systemic infection (38). A *T. rubrum* infection suggestive of cutaneous blastomycosis has been reported in an immunocompromised patient (39), and a fungal culture-negative chronic ulcerated foot lesion was diagnosed by PCR and DNA sequencing as *T. rubrum* tinea pedis in a chronic granulomatous disease patient (40).

In synopsis, the principal current risk factors for common forms of dermatophytosis are age (youth for tinea capitis or advanced age for onychomycosis); family history of chronic dermatophytosis; participation in athletics featuring extensive body contact (e.g., wrestling and judo) or foot maceration (e.g., marathon running); barefoot use of communal aquatic facilities (showers and swimming areas); exchange of headgear, footwear, or inadequately cleaned bedding; contact with feral domestic animals (especially street kittens) or animals recently supplied by en masse breeding operations (especially cats, guinea pigs, rabbits, laboratory rats, and cattle); inhabitation of rodent-infested dwellings; and, especially for children in developing countries, contact with livestock suffering from untreated dermatophytosis and contact with barbering instruments that have not been effectively disinfected (26, 33, 41–48).

Anatomic Specificity

Infections caused by dermatophytes are named according to the anatomic location involved, e.g., tinea barbae (beard and moustache), tinea capitis (scalp, eyebrows, and eyelashes), tinea corporis (face, trunk, and major limbs), tinea cruris (groin, perineal, and perianal areas), tinea pedis (soles and toe webs), tinea manuum (palms), and tinea unguium (nails). Different dermatophyte species may produce clinically identical lesions; conversely, a single species may infect many anatomic sites.

Tinea barbae, usually caused by zoophilic fungi, e.g., *Trichophyton verrucosum* and granular, zoophilic forms of the *Trichophyton mentagrophytes* complex, is typically highly inflamed and may present as acute pustular folliculitis that can progress to suppurative, boggy lesions (kerion). A less severe form that appears as dry, erythematous, scaly lesions also occurs. Tinea capitis may vary from highly erythematous, patchy, scaly areas with dull gray hair stumps to highly inflamed lesions with folliculitis, kerion formation, alopecia, and scarring. *T. tonsurans* and *M. canis* are the most common agents, depending on the precise geographic location (22, 23). Favus (tinea favosa), usually caused by *Trichophyton schoenleinii* but also potentially caused by *Trichophyton violaceum* or *Microsporum gypseum*, is a now very rare, chronic infection of the scalp and glabrous (bald-looking) skin characterized by the formation of cup-shaped crusts (scutula) resembling honeycombs. Tinea corporis, which can be caused by any dermatophyte but is often associated with zoophiles, classically manifests as circular, erythematous lesions with scaly, raised, active, and often vesicular borders. Chronic lesions on the trunk and extremities usually are caused by *T. rubrum*, in particular by Afro-Asiatic forms of this fungus (formerly often called *Trichophyton raubitschekii*). Tinea cruris ("jock itch"), usually caused by *T. rubrum* or *Epidermophyton floccosum*, typically appears as scaly, erythematous to tawny brown, bilateral, and asymmetric lesions extending down to the inner thigh and exhibiting a sharply marginated border frequently studded with small vesicles. Tinea pedis varies in appearance: the most common manifestation is maceration, peeling, itching, and painful fissuring between the fourth and fifth toes, but an acute inflammatory condition with vesicles and pustules can also occur, as can a hyperkeratotic chronic infection of the sole ("moccasin foot"). Members of the *T. mentagrophytes* complex frequently cause the more inflammatory type of infections, whereas *T. rubrum* usually causes the more chronic type. Infection of the sole by human-adapted forms of *Trichophyton interdigitale*, one of the members of the *T. mentagrophytes* complex, can be recognized by the formation of bullous vesicles in the thin skin of the plantar arch and along the sides of the feet and heel adjacent to the thick plantar stratum corneum (49). Tinea unguium, or nail infection by dermatophytes, is a subcategory of the more general phenomenon of onychomycosis, fungal nail infection. It is most often caused by *T. rubrum* and usually appears as thickened, deformed, friable, discolored nails with accumulated subungual debris. This type of presentation results from invasion of the underside of the distal nail and is therefore termed distal-subungual onychomycosis. A less common infection type usually caused by *T. interdigitale* typically manifests, especially in its earlier stages, as "superficial white onychomycosis," i.e., white patches in the superficial portions of the nail. "Proximal-subungual tinea unguium" may also occur. This infection, in which the nail is subungually infected beginning near its point of origin in the area of the lunula, is usually caused by *T. rubrum* and often signals immunosuppression, e.g., AIDS (50).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Preliminary Patient Examination

In areas where tinea capitis caused by *M. canis* is common, patients may be examined with a Wood's lamp (filtered UV light with a wavelength of 365 nm) in a darkened room for the presence of bright green fluorescent hairs. These are ideal for collection as laboratory specimens, though in some cases diagnosis may be done by Wood's light alone. The fluorescent hairs, considered "Wood's light positive," typically show a small-arthroconidial, ectothrix type of hair invasion in direct microscopy. Apart from *M. canis*, *M. audouinii* and *Microsporum ferrugineum* also cause this type of fluorescent ectothrix infection. Hairs infected with *T. schoenleinii* may show a dull green color (48). The Wood's lamp can also be used to differentiate between dermatophytosis and nonfungal skin conditions that may be similar clinically, e.g., erythrasma. In erythrasma, the skin fluoresces orange to coral red, whereas in the dermatophytosis, the skin is not fluorescent.

Sampling Preparations and Practice

Sufficient clinical material should be collected for both direct microscopic examination and culture. However, if quantities of clinical material are limited, direct microscopic examination should always be preferred over fungal culture. It is the more sensitive technique (23), and the microscopic detection of fungal elements in dermatology specimens is diagnostic of fungal infection and should be sufficient to elicit antifungal therapy. Whenever feasible, aseptic technique should be used to minimize contamination during the collection and transport of specimens. The following equipment should be available: forceps for epilating hairs; sterile no. 15 scalpel blades or sharp curette; sterile nail clippers; scissors; sterile gauze squares; 70% alcohol for disinfection; sterile water for cleansing painful areas; and clean pill packets or clean paper envelopes to contain and transport the clinical specimens, such as hairs, skin scrapings, or nail clippings. Black photographic paper or strong black paper may be used for collecting and better visualizing scrapings. After collection, the paper is folded, tightly taped in the corners, and placed in an envelope for transport. There are several commercial transport package systems available; the MycoTrans Specimen Transport System (MycoTrans, Biggar, Lanarkshire, United Kingdom) and Dermapak (Dermaco, Bedford, Bedfordshire, United Kingdom) are two of these. Closed tubes are not recommended for specimens, since they retain moisture, which may result in an overgrowth of bacterial as well as fungal contaminants. Disposable sterilized brushes have been recommended for collection of specimens from the scalp or from the fur of animals (51). Indeed, in the United States, disposable toothbrushes are often used to sample skin, with the bristle portion being pushed into the culture media in several locations after sampling. If histopathological processing is done, the nail plate may be placed in a 4% formaldehyde solution (52). Culture media (detailed below) may be inoculated directly on collection.

Hairs from the scalp should be epilated with sterile forceps. If the specimen is Wood's light positive, epilate only fluorescent hairs. Nonfluorescent hairs, especially those infected with endothrix fungi such as *T. tonsurans*, may need to be dug out with the tip of a sterile scalpel blade because the hairs often break off at scalp level and are thus difficult to grasp with a forceps. Rubbing with a sterile moistened swab has been successful with pediatric patients (53). In the rare event that favus is seen, the scutulum at the mouth of the hair follicle is suitable for culture and microscopic examination. In lower-body dermatophytoses, lesions with defined borders should be preliminarily disinfected with alcohol or cleansed with sterile water and then active border areas should be scraped with a scalpel (or toothbrush; see above) to collect epidermal scales. Where borders are not visible to indicate the area of maximal fungal activity, as in tinea manuum, the preliminarily cleansed infected area can be broadly scraped to obtain specimens from a variety of areas that may imperceptibly differ in current fungal activity. In vesicular tinea pedis, the tops of the vesicles can be removed with sterile scissors for direct examination and culture. Culture of the vesicle fluid is not recommended.

Nails should be disinfected with alcohol gauze squares. The most desirable material for culture in typical subungual onychomycosis is the waxy subungual debris, which contains the fungal elements. The highest proportion of viable elements for culturing is often found close to the juncture of the nail bed. To remove contaminating saprobic fungi and bacteria, the crumbly debris directly underneath the nail near the tips is removed with the scalpel before material is collected for culture. Some investigators clip the nail short first and perform this scraping-away of contaminated material on the clipping. If the dorsal nail plate is diseased (superficial white onychomycosis), scrape and discard the outer surface before underlying material is removed for culture. In rare cases with a presentation consistent with *Trichophyton soudanense* endonyx nail infection (54), in which the internal strata of the nail plate are milky white but the upper and lower nail surfaces appear relatively unaffected, clippings may be taken or the milky area may be exposed and scraped.

Any specimen needing to be transported, or for any other reason not processed immediately, should be retained in the paper packets described above. Closed tubes are not recommended for specimens since they may retain moisture, resulting in an overgrowth of contaminants. Dermatophytes in dry specimens of skin, hair, and nails may be stored for years in viable condition, provided the material is not subjected to temperature extremes.

LABORATORY TESTING OF SPECIMENS

Direct Microscopic Examination

Direct microscopic examination of skin, hair, and nails is the most rapid method of determining fungal etiology and is traditionally accomplished by examining the clinical material in 10% potassium hydroxide (KOH) (freely interchangeable with the cheaper sodium hydroxide [NaOH]) (55, 56). Another common procedure is to use 25% potassium or sodium hydroxide mixed with 5% glycerin to impede desiccation (55). Addition of fluorescent brighteners such as calcofluor white (Sigma-Aldrich, St. Louis, MO) or Blankophor (Blankophor GmbH, Leverkusen, Germany) significantly increases accuracy, especially where staff are not highly experienced in visual detection of fungal elements (57, 58). In nails, histopathology based on staining nail biopsy material with periodic acid-Schiff stain has been shown to have potential for generating results more accurate than those afforded by hydroxide-based direct microscopy (59). The suggestion, however, that biopsy/periodic acid-Schiff can also replace culturing (59) is unsound, in that this technique does not permit reliable distinction of dermatophytes from other nail-invading species (60).

Nail clippings should be aseptically cut into smaller fragments and, where possible, either pounded with a heavy object inside their collection packet or scraped with a sterile scalpel blade to release friable, flaky material containing the greatest amount of dermatophyte inoculum. Skin or nail scrapings, nail fragments, or hair roots are placed in 1 or 2 drops of one of the above-mentioned KOH or NaOH solutions on a clean glass slide. A coverslip is placed on top, and the preparation is heated gently (short of boiling) by being passed rapidly over a Bunsen burner or other heat source three or four times and then allowed to sit at room temperature for a few minutes for clearing. The exact time needed depends on the concentration of hydroxide used, thickness of specimen fragments, and exact amount of heat imparted by contact with flame. A slide warmer set at 51 to 54°C may also be used to heat the slides for 1 h (55). Alternatively, samples may be incubated with alkali in closed sterile microtubes at room temperature for 1 to 2 h, prior to addition of fluorescent enhancer. Clearing is evident to the naked eye as a pronounced decrease in the opacity of the scraping. Laboratories using a 10% KOH or NaOH solution for skin may find that nail scrapings may require a stronger alkali solution (up to 25% KOH or NaOH). Demonstration of fungal elements may be facilitated by use of glucan-binding fluorescent brighteners such as calcofluor white (56) or Congo red (55). These require use of a fluorescence microscope set up to visualize the specific fluorescence obtained. Calcofluor white is added directly to the KOH drop on the slide as an approximately equal drop of 0.1%

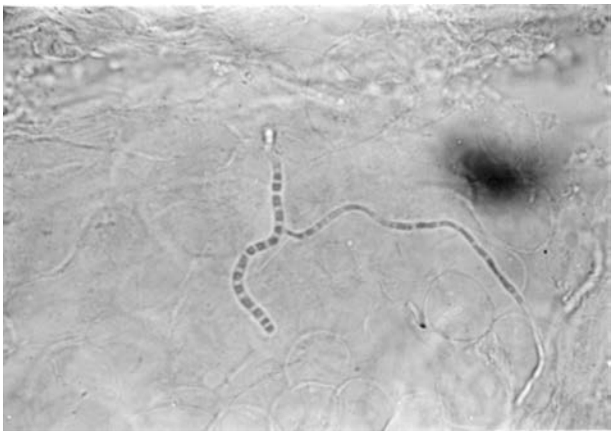

FIGURE 1 Dermatophyte hyphae in skin scraping. NaOH mount. Magnification, ×400.
doi:10.1128/9781555817381.ch123.f1

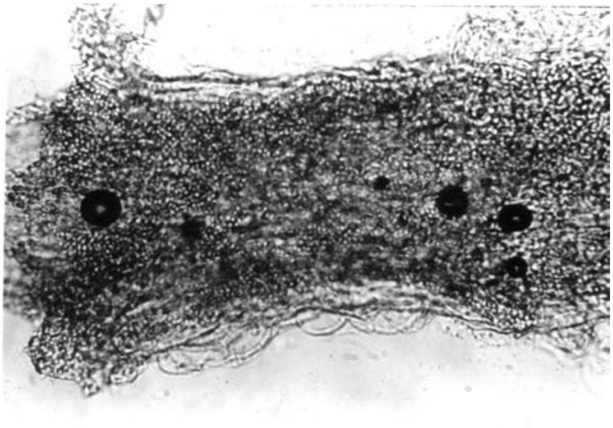

FIGURE 2 *Microsporum audouinii*, ectothrix type of hair invasion. Magnification, ×400.
doi:10.1128/9781555817381.ch123.f2

solution (61). All preparations should be examined under low power and confirmed under high power.

Skin and nails infected by dermatophytes may reveal one or more of the following: hyaline hyphal fragments; septate, often branched hyphae; and chains of arthroconidia (Fig. 1). The appearance of infected hairs depends on the invading dermatophyte species. Hyphae invade the hairs, and arthroconidia are formed by fragmentation of these hyphae. The appearances and locations of the arthroconidia may suggest the infecting genera or species (Table 3), as may the sizes (48). Three main types of colonizations (ectothrix, endothrix, and favic) are observed by direct microscopic examination. The terms "ectothrix" and "endothrix" refer to the location of the arthroconidia in relation to the hair shaft ("ecto-" and "endo-" meaning "outside" and "inside," respectively), while "favic" refers to the distinctive infection caused by *T. schoenleinii*, a fungus that is now extremely rare except in some parts of central Asia and the African Sahel.

In ectothrix colonization, arthroconidia appear as a mosaic sheath around the hair or as chains on the surface of the hair shaft (Fig. 2). In *M. canis*, *M. audouinii*, and *M. ferrugineum* infections, colonized hairs fluoresce green under Wood's lamp; other ectothrix infections (Table 3) are nonfluorescent. Endothrix hair invasion is observed as chains of arthroconidia filling the insides of shortened hair stubs (Fig. 3). Hairs are Wood's lamp negative. In favic hairs, hyphae, air bubbles, or tunnels and fat droplets are observed within the hair (Fig. 4). These hairs are dull green under the Wood's lamp. In general, infected hairs from all infection types show hyphae within the hair shaft at some time during the course of infection, usually during the early stages.

TABLE 3 Hair invasion by dermatophytes on the human host

Ectothrix	Endothrix	Favic
Microsporum audouinii	*Trichophyton soudanense*	*Trichophyton schoenleinii*
M. canis	*T. tonsurans*	
M. ferrugineum	*T. violaceum*	
M. gypseum complex		
M. praecox		
Trichophyton megninii		
T. mentagrophytes complex		
T. verrucosum		

Isolation Procedures

Scrapings, hairs, and other materials collected as outlined above for direct examination are plated on selected isolation media and incubated at 32°C for optimal growth; temperatures between 24 and 32°C are also acceptable if the total incubation time is suitably adjusted to compensate for slower outgrowth expected at lower temperatures. Generally from 5 to 15 skin or nail fragments are planted per plate or tube used for isolation, and these fragments are separated so that antibiotic-resistant mould or bacterial contaminants from one piece cannot overgrow the others. Hairs are also well separated. Cultures on primary isolation medium are routinely incubated at 25 to 30°C and examined weekly for up to 4 weeks.

The most common medium used for the isolation of dermatophytes is Sabouraud glucose agar (SGA) (original formulation with 4% glucose or Emmons' modification with 2% glucose), amended with chloramphenicol and cycloheximide to inhibit bacterial and saprobic fungal contamination. This type of medium is available commercially as,

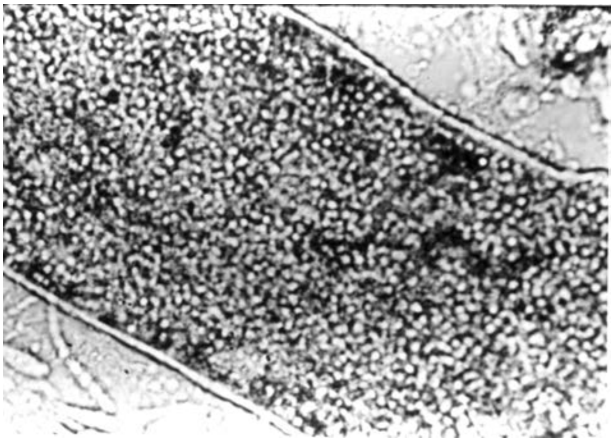

FIGURE 3 *Trichophyton tonsurans*, endothrix type of hair invasion. Magnification, ×1,000.
doi:10.1128/9781555817381.ch123.f3

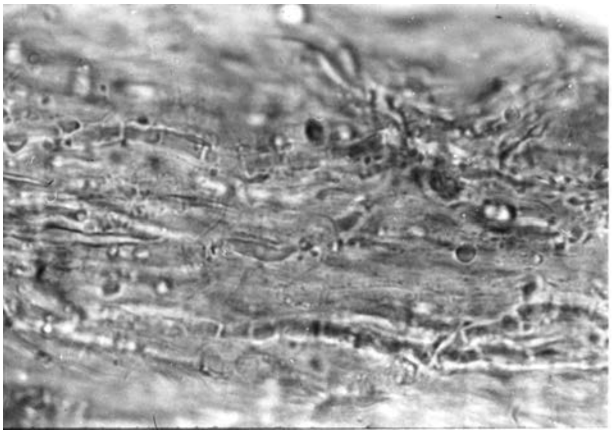

FIGURE 4 Hair infected by *Trichophyton schoenleinii* from a patient with favus. Magnification, ×1,000.
doi:10.1128/9781555817381.ch123.f4

for example, Mycobiotic agar (Acumedia Manufacturers, Lansing, MI; Remel, Lenexa, KS; Delasco, Council Bluffs, IA), Mycosel (BD Diagnostic Systems, Sparks, MD), or Dermasel Selective Supplement (Oxoid, Basingstoke, United Kingdom; note that Remel is currently the U.S. distributor for Oxoid). An alternative medium promoting more rapid conidiation and colony pigmentation development is potato flake agar amended with cycloheximide and chloramphenicol (Hardy Diagnostics, Santa Maria, CA). For the isolation of cycloheximide-susceptible fungi that cause clinical infections resembling dermatophytosis, any of SGA with chloramphenicol (Remel or BD), inhibitory mould agar (BD, Remel, or Hardy), or Littman oxgall agar (BD) may be recommended (56). Inhibitory mould agar and Littman oxgall agar have the advantage of restricting contaminant colony diameters. For all media, the addition of gentamicin is recommended for specimens heavily contaminated by bacteria (62). SGA with cycloheximide, chloramphenicol, and gentamicin is routinely used as an isolation medium in some laboratories (55).

Additional and alternative media may be used in special circumstances. For example, vitamin-free Casamino Acids (BD)-erythritol-albumin agar medium plus cycloheximide, chloramphenicol, and gentamicin may be used for filament-positive skin and nail specimens, especially from body sites where *Candida* overgrowth may be a problem (e.g., groin and fingernails). This medium (currently not commercially available, to our knowledge) prevents the common suppression of dermatophyte outgrowth by heavy inoculum of *Candida albicans*, *Candida parapsilosis*, and related biotin-requiring yeasts (63). Dermatophyte species with vitamin requirements (very uncommon in these types of cases) may grow poorly on it, and it is always used in combination with a cycloheximide-containing SGA.

Another primary isolation medium that may be used is dermatophyte test medium (DTM; available commercially from BD, Hardy, and Remel, among others [see chapter 115]). This selective medium screens for the presence of dermatophytes in heavily contaminated material (nails, etc.). The growth of dermatophytes causes a rise in pH, thus changing the phenol red indicator from yellow to red (64). The use of DTM should be combined with morphological study, since dermatophytoids such as *Trichophyton terrestre*, as well as various *Chrysosporium* species and other nondermatophytic fungi, can grow and turn the medium red (55, 65, 66). Rapid sporulation medium, mentioned above for potato flake agar, contains a pH indicator that works on a similar principle but turns from yellow to blue-green, leaving the red reverse pigment of typical *T. rubrum* visible (67). DTM may uncommonly give false-negative results with some *Microsporum* isolates (68).

Nucleic Acid-Based Direct Detection Techniques

Numerous techniques have been published for directly detecting dermatophyte DNA in tissue, but as yet none is established as a routine diagnostic procedure. This is due mainly to the relatively low cost of traditional procedures. However, in terms of accuracy, traditional procedures are by no means optimal; in onychomycosis, for example, they disclose at best ~85% of true-positive cases from the initial patient specimen, and this percentage is much lower in many laboratories (60). This has given strong incentive for development of molecular detection techniques. At present, a large number of primary studies related to rapid PCR of dermatological specimens for dermatophytosis and related mycoses (e.g., nondermatophyte filamentous fungal onychomycosis) have been published. Techniques employed include PCR–enzyme-linked immunosorbent assay (69), PCR-reverse line blotting (70), PCR coupled with restriction fragment length polymorphism study (71), multiplex PCR (72), nested PCR (73), PCR based in part on a microsatellite locus permitting strain typing of *T. rubrum* (74), and direct PCR with sequencing (54). Also, related techniques, such as real-time PCR (75–77), have been shown to work well in trial studies but also need to prove themselves as consistently practical and cost-effective in interlaboratory study related to routine diagnosis. Many of these techniques have been extensively discussed in the excellent review by Jensen and Arendrup (78). In unusual situations, molecular direct detection methods may be invaluable: for example, in a deep dermatophytosis case in which all conventional tests had given negative results, *T. rubrum* was identified by means of a nested PCR study directed at amplifying a portion of the ITS region from paraffin-embedded sections (79); and real-time PCR and sequencing unambiguously identified *T. rubrum* from biopsy samples in a culture-negative case of tinea pedis in a chronic granulomatous disease patient (40).

IDENTIFICATION

At present, the great majority of dermatophytes are identified phenotypically. Identification is often based on (i) colony characteristics in pure culture on SGA and (ii) microscopic morphology. These criteria alone, however, may be insufficient, since colonial appearance may vary or be similar for different species. Characteristic pigmentation may fail to appear, and isolates, especially *Trichophyton* spp., may not sporulate. Special media may be required to stimulate pigment production; it may be necessary to use sporulation and physiological tests in conjunction with morphology to identify the species correctly. The majority of isolates are easily identified when visual examination is combined with any needed testing for characteristic growth factor requirements; the consequence of this is that phenotypic studies remain the least expensive option for routine identification. However, although seldom practically applicable except in high-level reference laboratories, molecular identification strategies involving PCR amplification and sequencing of ITS regions (currently the gold standard for mould identification [80]) identify unusual isolates that are misbehaving phenotypically. Moreover, several recent studies have demonstrated that rapid and robust identification of most common dermatophytes is possible by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry. Indeed, identifications with this technique correlated much more closely with the results of ITS sequencing than with identifications based on conventional phenotypic approaches (18, 81).

Colony Characteristics

In observing gross colony morphology, note the color of the surface and the reverse of the colony, texture of the surface (powdery, granular, woolly, cottony, velvety, or glabrous), topography (elevation, folding, margins, etc.), and rate of growth.

Microscopic Morphology

Microscopic morphology, especially the appearance and arrangement of the conidia (macroconidia or microconidia) and other structures, may be determined by teased mounts, sticky tape mounts, or slide culture preparations mounted in lactophenol cotton blue (LCB), in lactophenol aniline blue (phenol, an ingredient of LCB and lactophenol aniline blue, is listed as a hazardous chemical; therefore, solutions containing phenol should be prepared, stored, and used in an approved chemical safety cabinet), in lacto-fuchsin (55), or in more-permanent mounting fluids (82). Sometimes a special medium, such as cornmeal or cornmeal glucose agar, potato glucose agar, SGA plus 3 to 5% NaCl (83, 84), Pablum cereal agar (55), rapid sporulation medium (67), or lactrimel agar (85, 86 [see chapter 115]), may be required to stimulate sporulation.

Physiological Tests

In Vitro Hair Perforation Test

The *in vitro* hair perforation test distinguishes between atypical isolates of the *T. mentagrophytes* complex and *T. rubrum* (87). It may also be used to assist in making other distinctions such as *M. canis* versus *M. audouinii* and *Microsporum praecox* versus *M. gypseum* (88). Hairs exposed to *T. mentagrophytes* complex members, *M. canis*, and *M. gypseum* show wedge-shaped perforations perpendicular to the hair shaft (a positive test result), whereas *T. rubrum*, *M. audouinii*, and *M. praecox* do not form these perforating structures.

Place short strands of human hair (ideally hair from a child <18 months old) in petri dishes, and autoclave the dishes at 121°C for 10 min; add 25 ml of sterile distilled water and 2 or 3 drops of 10% sterilized yeast extract. Inoculate these plates with several fragments of the test fungus that has been grown on SGA, incubate the plates at 25°C, and examine them at regular intervals over a period of 21 days. Hairs may be examined microscopically for perforations by removing a few segments and placing them in a drop of LCB mounting fluid. Gently heating the mounts aids in the detection of the fungus. A positive-control test should always be run with a known perforating species; *M. canis* is recommended. Some hair samples may prove unsuitable for unknown reasons, e.g., possible prior contact with shampoos containing antifungal inhibitors.

Special Nutritional Requirements

Nutritional tests aid in the routine identification of *Trichophyton* species that seldom produce conidia or that resemble each other morphologically (89). Certain species have distinctive nutritional requirements, whereas others do not. The method employs a Casamino Acids basal medium that is vitamin free (*Trichophyton* agar 1 [T1]) and to which various vitamins are added, i.e., inositol (T2), thiamine plus inositol (T3), thiamine (T4), and nicotinic acid (T5). In addition, the series includes an ammonium nitrate basal medium (T6) to which histidine is added (T7). These media are available commercially in dehydrated form from BD Biosciences and in prepared form from Remel. A small fragment (about the size of the head of a pin) from the culture to be tested is placed on the surface of the basal medium (controls) and the media containing the vitamin and amino acid additives. Care must be taken to avoid transferring agar from the fungal inoculum to the nutritional media. Cultures are incubated at room temperature (or 37°C if *T. verrucosum* is suspected) and read after 7 and 14 days. The amount of growth is graded from 0 to 4+. Commonly observed reactions are summarized in Table 4.

TABLE 4 Dermatophyte nutritional response as elucidated by *Trichophyton* agars[a]

Species	Response in vitamin tests					Response in amino acid tests	
	1 Vitamin free	2 Inositol	3 Thiamine + inositol	4 Thiamine	5 Nicotinic acid	6 Amino acid free	7 Histidine
Microsporum gallinae						4	4
Trichophyton concentricum, 50%	4	4	4	4	4		
T. concentricum, 50%	2	2	4	4	2		
T. equinum var. *equinum*	0	0	0	0	4		
T. equinum var. *autotrophicum*	4	4	4	4	4		
T. megninii						0	4
T. soudanense	v	v	v	v	v	v	v
T. tonsurans	1	1	4	4	1		
T. verrucosum, 84%	1	2	4	2	1		
T. verrucosum, 16%	1	1	4	4	1		
T. violaceum (typical)	1	1	4	4	1		
T. violaceum (rare "*T. yaoundei*" form)	4	4	4	4	4		

[a] Only the growth responses for organisms with growth factor requirements and the selected organisms that must be most closely compared with them are included in this table. The numbers in the table body indicate the relative degree of growth according to traditional 1+ to 4+ visually approximated scale: 0, no growth; 1, slight growth, strongly nutrient-deprived colony morphology (very sparse, subsurface colonial growth only or colony diameter strongly reduced compared to Sabouraud agar control); 2, partially stimulated growth but still significantly suppressed compared to that of the control; 4, growth comparable to that of the control (the table includes no 3+ reactions); v, variable. Blank spaces in the table indicate growth responses that are not customarily examined but that are insignificantly different from control growth responses on Sabouraud agar.

Urea Hydrolysis

The ability to hydrolyze urea provides additional data to aid in distinguishing the typical, cosmopolitan form of *T. rubrum* (urease negative) from members of the *T. mentagrophytes* complex (typically urease positive), and from the urease-positive Afro-Asiatic or "granular" form of *T. rubrum*, formerly often called *T. raubitschekii* (90, 91). Christensen urea agar and broth may both be used; the broth appears to be the more sensitive of these alternatives (92). After the urea medium is inoculated, it is incubated at 25 to 30°C up to 7 days. The tubes should be examined every 2 to 3 days for the color change from orange or pale pink to purple-red that indicates the presence of urease, a positive test result. Negative and positive controls should always be done on new batches of these media. See Table 1.

Growth on BCPMSG

Bromcresol purple (BCP)-milk solids-glucose (BCPMSG) is available commercially as Dermatophyte Milk Agar (Hardy). Type of growth (profuse versus restricted) and a change in the pH indicator (BCP) indicating alkalinity are especially useful for distinguishing *T. rubrum* from the *T. mentagrophytes* complex, and *T. mentagrophytes* from *Microsporum persicolor* (55, 84, 93). *T. rubrum* shows restricted growth and produces no alkaline reaction on BCPMSG, whereas members of the *T. mentagrophytes* complex typically show profuse growth and an alkaline reaction. Although *M. persicolor* shows profuse growth, it does not result in an alkaline reaction. Other tests for distinguishing *T. mentagrophytes* and close relatives from *M. persicolor* are described elsewhere (94).

Cultures to be tested are inoculated onto slants of BCPMSG and examined for pH change and growth characteristics at the end of a 7-day incubation at 25°C. A color change from pale blue to violet-purple indicates an alkaline reaction.

Growth on Polished Rice Grains

Unlike most dermatophytes, *M. audouinii* grows poorly on rice grains and produces a brownish discoloration of the rice (55, 94). This is a useful test for differentiating this species from *M. canis* and from other dermatophytes that typically grow and sporulate on rice grains. It may be especially useful in areas where *M. audouinii* is still endemic, e.g., sub-Saharan Africa, or where immigrants or travelers from such areas are reintroducing it.

The medium is prepared in 12-ml flasks by mixing 1 part raw unfortified rice grains and 3 parts water (95) or 8.0 g of rice grains and 125 ml of distilled water. Autoclave at 15 lb/in^2 for 15 min. Inoculate the surface of the rice, and incubate the sample for 2 weeks at 25 to 30°C.

Temperature Tolerance and Temperature Enhancement

Tests for temperature tolerance and enhancement are useful for distinguishing the *T. mentagrophytes* complex from *T. terrestre* (96), *T. mentagrophytes* from *M. persicolor* (84), *T. verrucosum* from *T. schoenleinii* (48), and *T. soudanense* from *M. ferrugineum* (97). At 37°C, members of the *T. mentagrophytes* complex show good growth, whereas *T. terrestre* does not grow and *M. persicolor* generally grows poorly or not at all (a single atypical isolate with good growth has been observed); growth of *T. verrucosum* and *T. soudanense* is enhanced, but that of *T. schoenleinii* and *M. ferrugineum* is not.

Inoculate two slants of SGA with an equivalent fragment of the culture. Incubate one slant at room temperature (25 to 30°C) and one at 37°C. Compare the growth at the two temperatures when mature colonies appear at room temperature. Appropriate controls are recommended and should be compared first.

Molecular Identification Techniques

The recommendation of standard DNA barcodes (17) is in progress, with the ITS-1 region emerging as a key panfungal region for the molecular identification of moulds, including dermatophytes (80, 98). However, although PCR amplification and sequencing of ITS-1 have proven efficacy for dermatophyte identification (98), several caveats remain. First, caution is required when comparing the sequences obtained with those in public synchronized databases, which are strewn with erroneously identified fungi. Second, relatively few conserved nucleotide positions separate the different *Trichophyton* spp., principally due to the relatively slow evolution rate of the nuclear ribosomal repeat region. Many other nucleic acid-based identification techniques for dermatophytes were published in previous years but have not been widely adopted. Some recent contenders for rapid, state-of-the-art species identification include PCR using a $(GACA)_4$ primer (99) and restriction fragment length polymorphism of the ITS-2 gene region (100).

DESCRIPTION OF ETIOLOGIC AGENTS

Characteristic features of dermatophyte species are presented in Table 1. The table also includes data on some similar but rarely or never pathogenic *Microsporum* and *Trichophyton* species that must be distinguished from pathogenic species.

Two types of conidia may be produced on the aerial mycelium of the dermatophytes: large, multicellular, smooth or rough, thin- or thick-walled macroconidia and smaller, unicellular, smooth-walled microconidia. The three genera are classically grouped according to the presence or absence of these two types of conidia and the appearance of the surface of the macroconidia, i.e., rough versus smooth. In reality, when atypical isolates and species are taken into account, the genera show considerable overlap in morphology (101). For this reason, it is often more convenient to identify isolates directly at the species level than it is to try to identify them at the genus level first. Identification of species is based on the microscopic appearance and arrangement of the conidia (Fig. 5 through 17), colonial morphology on SGA (Fig. 18), and physiological tests (Table 5).

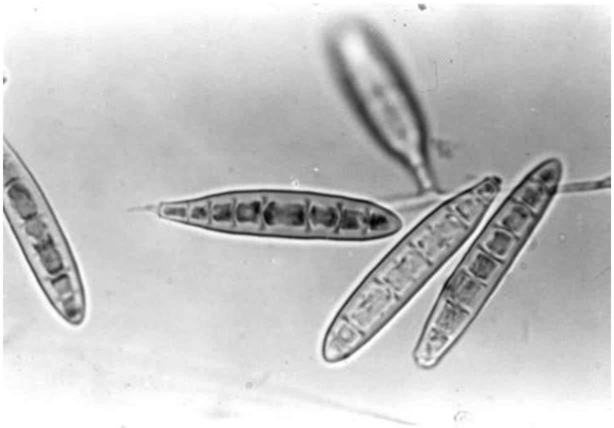

FIGURE 5 Smooth-walled macroconidia of *Trichophyton ajelloi*. Magnification, ×400.
doi:10.1128/9781555817381.ch123.f5

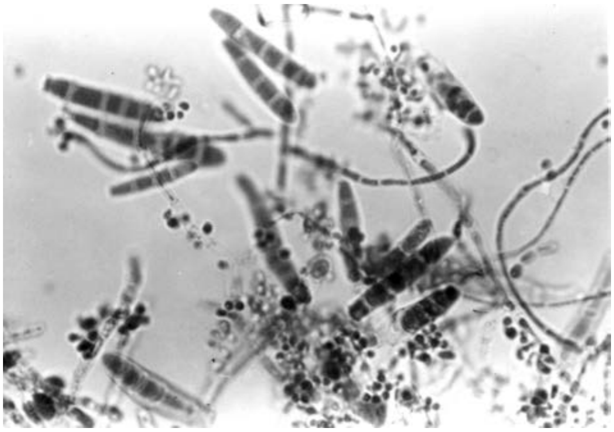

FIGURE 6 Macroconidia and microconidia of *Trichophyton mentagrophytes* complex on SGA with 5% NaCl. Magnification, ×400. doi:10.1128/9781555817381.ch123.f6

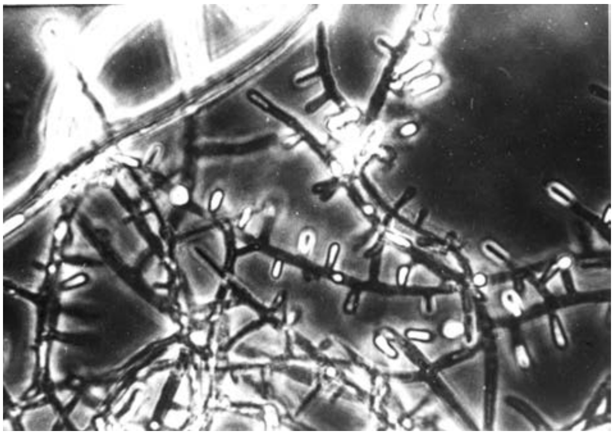

FIGURE 9 Clavate macroconidium, microconidia, and intermediate conidia of *Trichophyton terrestre*. Phase contrast; magnification, ×400. doi:10.1128/9781555817381.ch123.f9

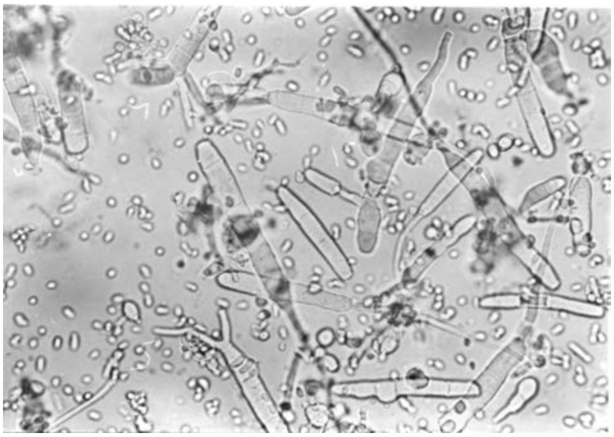

FIGURE 7 Smooth-walled macroconidia of Afro-Asiatic type *Trichophyton rubrum* (*T. raubitschekii*) from primary isolate on SGA. Magnification, ×1,000. doi:10.1128/9781555817381.ch123.f7

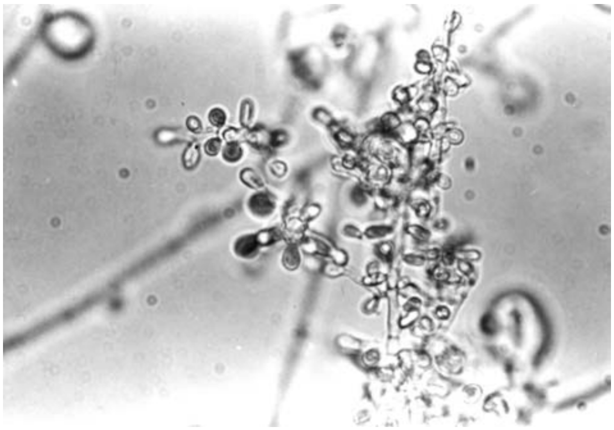

FIGURE 10 Microconidia with typical refractile cytoplasm of *Trichophyton tonsurans*. Magnification, ×400. doi:10.1128/9781555817381.ch123.f10

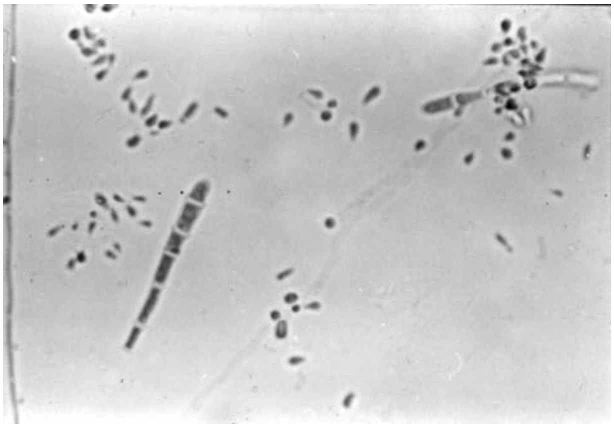

FIGURE 8 Long, narrow macroconidium and clavate to pyriform microconidia of *Trichophyton rubrum*. Magnification, ×400. doi:10.1128/9781555817381.ch123.f8

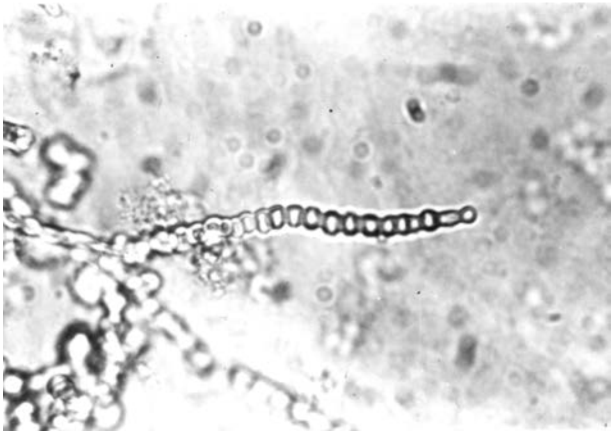

FIGURE 11 Characteristic chlamydospores produced by *Trichophyton verrucosum* or BCP-milk solids-yeast extract agar. Magnification, ×400. doi:10.1128/9781555817381.ch123.f11

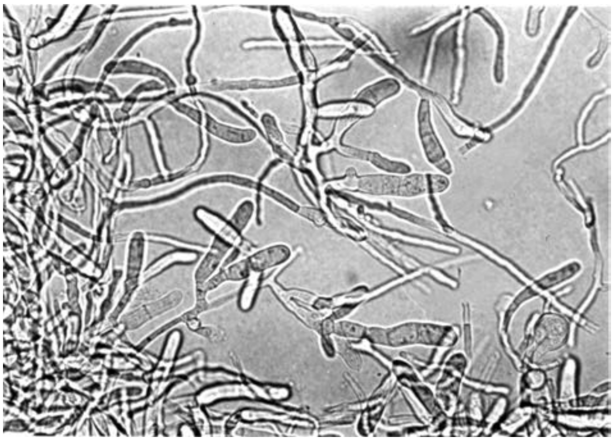

FIGURE 12 Macroconidia of *Epidermophyton floccosum* on SGA. Note the absence of microconidia. Magnification, ×400. doi:10.1128/9781555817381.ch123.f12

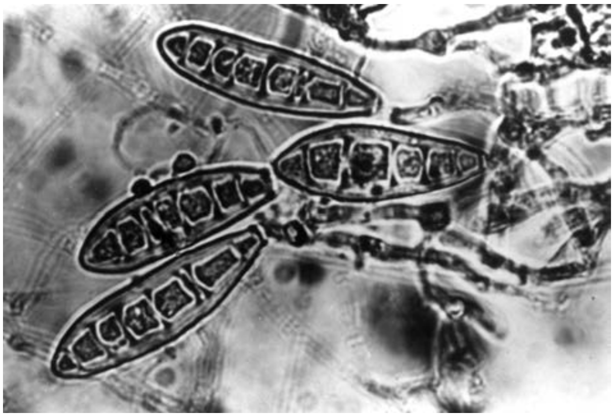

FIGURE 15 Macroconidia of *Microsporum cookei*, showing thick walls and pseudosepta. Magnification, ×400. doi:10.1128/9781555817381.ch123.f15

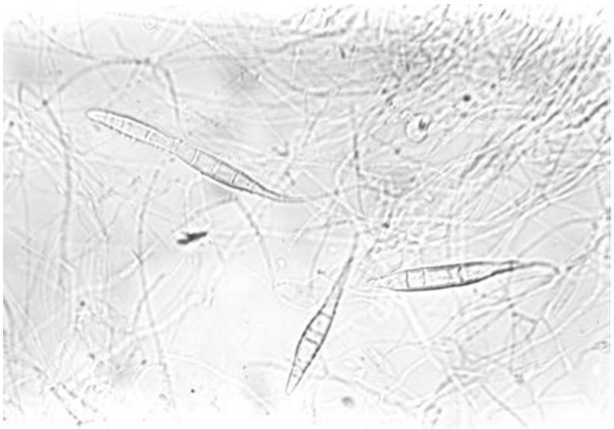

FIGURE 13 Macroconidia of *Microsporum audouinii* on SGA with 3% NaCl. Magnification, ×400. doi:10.1128/9781555817381.ch123.f13

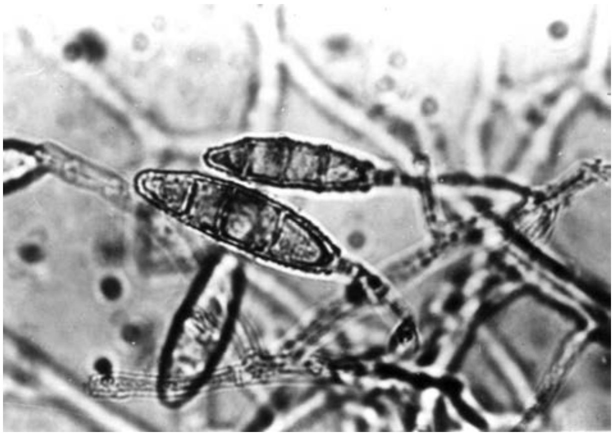

FIGURE 16 Macroconidia of *Microsporum gypseum*. Magnification, ×400. doi:10.1128/9781555817381.ch123.f16

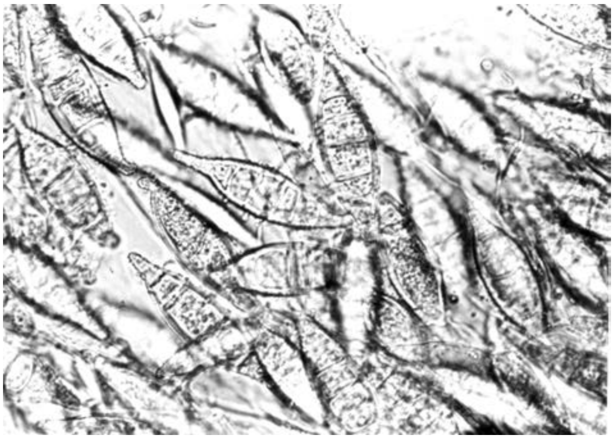

FIGURE 14 Macroconidia of *Microsporum canis* with rough, thick walls. Magnification, ×400. doi:10.1128/9781555817381.ch123.f14

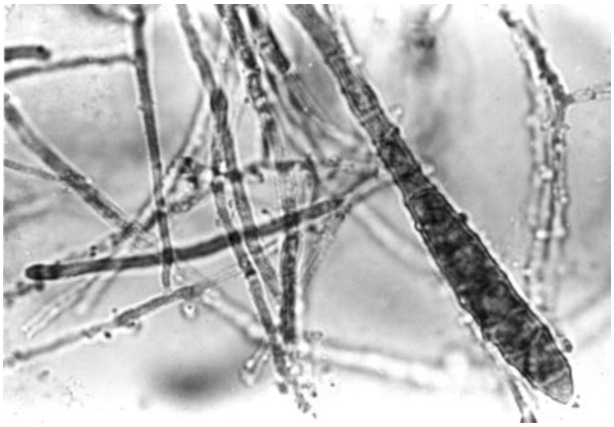

FIGURE 17 Rough-walled macroconidium of *Microsporum persicolor* on SGA with 3% NaCl. Magnification, ×1,000. doi:10.1128/9781555817381.ch123.f17

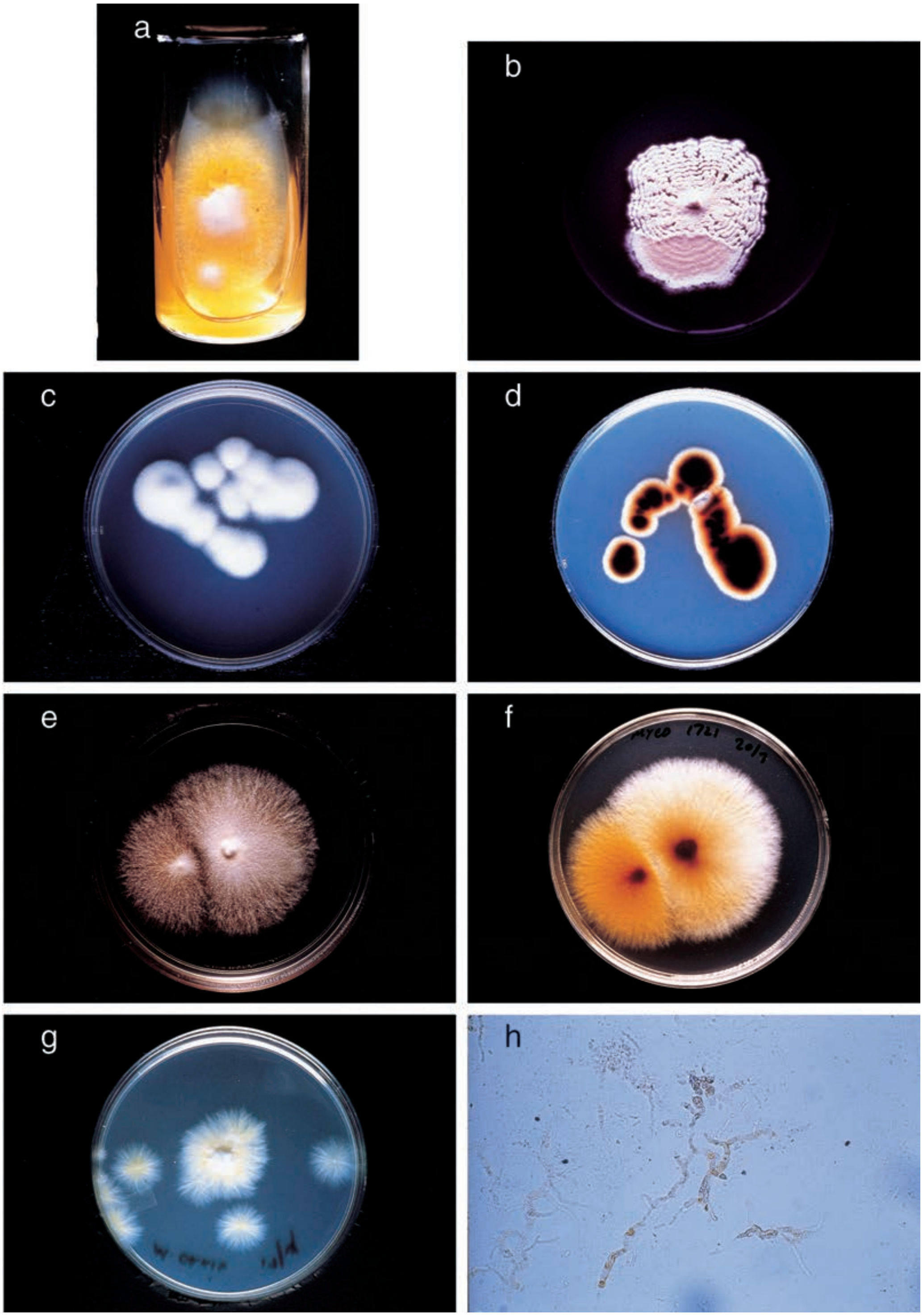

FIGURE 18 (a) *Trichophyton mentagrophytes* complex: “nodular” variant of *Trichophyton interdigitale* (formerly *Trichophyton krajdenii*), SGA, 12 days, showing typical bright yellow pigmentation. (b) *Trichophyton mentagrophytes* complex: granular, zoophilic type *Trichophyton interdigitale* (mating tester strain of *Arthroderma vanbreuseghemii*), 14 days. (c) *Trichophyton rubrum*, SGA, 10 days, surface showing cottony white mycelium. (d) *Trichophyton rubrum*, SGA, 10 days, reverse showing typical red pigment. (e) *Trichophyton tonsurans*, SGA, 14 days, surface showing low velvety texture, mixed white and brownish mycelium. (f) *Trichophyton tonsurans*, SGA, 14 days, reverse showing mixture of mahogany red-brown and sulfur yellow coloration. (g) *Microsporum canis*, SGA, 10 days, relatively flat colony showing pale striate margin and yellowish pigment near colony center. (h) Brown filaments of *Hortaea werneckii* in NaOH mount of scraping from tinea nigra. Magnification, ×400. doi:10.1128/9781555817381.ch123.f18

TABLE 5 Sequence of procedures for phenotypic identification of dermatophytes in pure culture[a]

1. Examine colony at day 7 and, if necessary, day 14, for colors of surface and reverse, topography, texture, and rate of growth. Proceed to step 2.
2. Prepare tease mounts and search for identifying microscopic morphology, especially presence, appearance, and arrangement of macroconidia and microconidia (consult Fig. 5 to 18 and Table 1). If the results are inconclusive, proceed to step 3.
3. Prepare slide cultures or transparent tape mounts and examine for characteristic morphology as indicated above if tease mounts do not provide sufficient information. Consider special media if sporulation is absent (potato glucose agar, lactrimel, BCPMSG, SGA with 3–5% NaCl). At the same time, proceed to step 4.
4. Perform as many of the following physiological and other special tests as necessary for identification.
    a. Urease (ensure culture is bacteria free!)
    b. Nutritional requirements if *Trichophyton* is suspected
    c. Growth on rice grains if an unusual *Microsporum* sp. is suspected
    d. Elevated temperature response (37°C on SGA or, if *T. verrucosum* is suspected, BCPMSG)
    e. Special differentiation media: e.g., BCPMSG to distinguish *T. mentagrophytes* complex from *T. rubrum* and *M. persicolor*; Lowenstein-Jensen or BCPMSG to distinguish *T. soudanense* from *M. ferrugineum*; SGA + 3–5% NaCl to distinguish *T. mentagrophytes* complex from *M. persicolor* and atypical *T. rubrum*; DTM to distinguish nonsporulating dermatophytes from most other nonsporulating hyaline fungi
    f. *In vitro* hair perforation test
    g. Molecular or mating studies (to be performed in reference laboratories)

[a] It may be necessary to incubate cultures on brain heart infusion agar or BCPMSG to ensure absence of antibiotic-resistant bacterial contamination before proceeding to step 4. Procedures are adapted from Weitzman and colleagues (25) and Kane et al. (55).

Trichophyton Species

Macroconidia have smooth, thin to thick walls; are variable in shape (clavate, fusiform to cylindrical); vary in number of septa (1 to 12) and in size (8 to 86 by 4 to 14 µm); and are borne singly or in clusters. Microconidia, which are usually present and more numerous than macroconidia, may be globose, pyriform, or clavate and are borne singly along the sides of hyphae or in grape-like clusters. Though species in this genus generally produce microconidia more readily than macroconidia, two lineages producing macroconidia predominantly or exclusively have been described: (i) "*Trichophyton kanei*" (102), now known to be a variant of the Afro-Asiatic genotype of *T. rubrum*, and (ii) a variant all-macroconidial form of *T. tonsurans* (101). Fresh isolates of the dermatophytoid *T. ajelloi* may also produce many macroconidia and few or no microconidia. Some species such as *T. schoenleinii* rarely produce conidia of any kind in culture, and nonsporulating isolates of normally conidial species, especially *T. rubrum*, may also be frequently encountered, especially in primary cultures.

Microsporum Species

Microsporum species produce macroconidia and microconidia that may be rare or numerous, depending on the species and the substrate. The distinguishing characteristic is the macroconidium, which is typically rough walled (varying from minutely to conspicuously roughened). Macroconidia also vary in shape (obovate, fusiform to cylindrofusiform), number of septa (1 to 15), size (6 to 160 by 6 to 25 µm), and width of the cell wall. Microconidia are pyriform or clavate and usually are arranged singly along the sides of the hyphae. *Microsporum* species invade skin, hair, and, rarely, nails. Biological (teleomorphic) species within the *M. gypseum* complex can be provisionally recognized on the basis of colonial and microscopic features on Takashio's medium (103), but these species are best distinguished by mating or molecular testing.

Epidermophyton floccosum

In *E. floccosum*, microconidia are lacking; only smooth-walled, broadly clavate macroconidia are produced, often with an abundance of intercalary and terminal chlamydospores in primary cultures. The macroconidia have one to six septa, are 20 to 40 µm long by 7 to 12 µm wide, and are borne singly or in clusters of two or three. *E. floccosum* is currently the only recognized *Epidermophyton* species; the former *E. stockdaleae* is now considered a synonym of *Trichophyton ajelloi* (14).

STRAIN TYPING SYSTEMS

Dermatophyte strains within anthropophilic species tend to be very closely related, hindering the development of useful techniques for epidemiological analysis, but strains of *T. rubrum* were eventually distinguished by polymorphisms in the numbers of subrepeat elements in the ribosomal non-transcribed spacer region (104–107). In addition, the Afro-Asiatic lineage of *T. rubrum* was distinguished from the now-cosmopolitan epidemic *T. rubrum* form by means of a microsatellite marker designated T1 (108). More recently, an elegant system involving multiple microsatellite markers has been developed (109). Nontranscribed spacer polymorphisms can also be applied to distinguish *T. interdigitale* and *T. tonsurans* isolates (104, 110, 111). While it is beyond the scope of this chapter to review all current molecular differentiation techniques for dermatophytes or their applications in outbreaks, the multilocus genotyping system of Abdel-Rahman et al. (112) should be cited as an example of how such methods can foster the development of epidemiological insight.

ANTIMICROBIAL SUSCEPTIBILITIES

Dermatophytes can in principle be tested for susceptibility to antifungal drugs using the Clinical and Laboratory Standards Institute M38-A2 standard procedure for moulds (113). A trial has shown that this type of methodology can be applied with good inter- and intralaboratory reproducibility to the commonly used drugs ciclopirox, fluconazole, griseofulvin, itraconazole, posaconazole, terbinafine, and voriconazole (114). However, drug resistance is very rarely encountered in dermatophytes, and treatment failures are almost always due to factors other than resistance (115). Thus, potentially burdensome requests for dermatophyte susceptibility testing should be closely screened for scientific and clinical appropriateness.

EVALUATION, INTERPRETATION, AND REPORTING OF LABORATORY RESULTS

For nonimmunocompromised patients, positive direct microscopy compatible with dermatophytosis is conventionally interpreted as presumptively indicating this condition in hair and in specimens other than those from nails, soles, and palms. The positive microscopic report itself conveys this information; it should be issued within 2 working days of specimen receipt. With soles and palms, persons who have lived in tropical areas or who are of south Asian heritage may have dermatophytosis-like *Neoscytalidium* (116) (previously called *Scytalidium*, *Nattrassia*, and *Hendersonula*; see chapter 120) infections, which are similar to dermatophytoses both clinically and in direct microscopy (55); no presumptive diagnosis can be inferred until the culture result is available. The direct microscopic result, however, is still reported immediately. For patients without risk factors for *Neoscytalidium*, qualified physicians may make presumptive diagnoses of dermatophytosis for sole and palm skin as for other skin sites. In onychomycosis, >35 different mould species may be involved in producing conditions clinically and microscopically resembling tinea unguium (55), particularly in geriatric patients. The presence of a nondermatophyte mould may be suspected upon direct microscopic examination, which often reveals intact distorted hyphae (as opposed to arthrospores) with pronounced terminal fronding or hyphal swellings. Microscopic results are still reported promptly, but culture results are of high interest. At the same time, the outgrowth of known onychomycosis-causing nondermatophytes may or may not be significant, excepting the nail-infecting *Neoscytalidium* species, which are always considered significant when grown. The complexities of accurately reporting nondermatophytes from nail specimens are beyond the scope of this chapter but are discussed in light of rigorous validation studies on this topic by Summerbell et al. (60).

Isolation of a dermatophyte culture from lesional skin, hair, or nails is interpreted as diagnostic whether or not fungal elements are seen in the initial direct microscopy examination. Note that dermatophytoids such as *Microsporum cookei* and *T. terrestre* are presumed to be contaminants until proven otherwise; they are, however, reported along with the comment "normally nonpathogenic" when grown. These fungi have been the subjects of numerous false and questionable case reports in the literature, and there may be some confusion about their status. If such a fungus were to infect human epidermis, the gold standard for scientifically evidencing the case would be three successive, consistent repeat isolations of the fungus from the lesion in specimens collected on different days, plus demonstration of compatible fungal elements in direct microscopy of the affected tissue. No inferential diagnoses from lower-quality evidence should be accepted as conclusive, even if the patient is said to have been successfully treated. A common dermatophyte such as *T. rubrum* that failed to grow out in initial culture(s) is very likely to be the actual etiologic agent in such cases (60). In onychomycosis in particular, true causal dermatophytes have only at best an ~75% chance of being grown from the initial specimen taken from any given patient; another ~15% will be recovered from a second specimen, while ~10% can be detected only by doing three or more serial specimens, something very seldom done in practice (60). Cycloheximide-tolerant dermatophytoids that grow (in any quantity whatsoever) from single, otherwise unproductive specimens should not be assumed to be causal simply because they share a genus name with dermatophyte species.

SUPERFICIAL MYCOSES

In the superficial mycoses, the causative fungi colonize the cornified layers of the epidermis or the suprafollicular portion of the hair. There is little tissue damage, and cellular response from the host generally is lacking. The diseases are largely cosmetic in impact, involving changes in the pigmentation of the skin (tinea versicolor or tinea nigra) or formation of nodules along the distal hair shaft (black piedra and white piedra).

In contrast to agents of the dermatophytoses, the etiologic agents are diverse and unrelated.

Tinea Versicolor (Pityriasis Versicolor)

Tinea versicolor is an infection of the stratum corneum caused by a group of closely similar lipophilic yeast species of the *Malassezia furfur* complex. Members of this complex infecting human skin were often treated in former times as a single species but were then shown by molecular, physiological, and serotyping studies to be separate (117, 118). The complex includes *M. furfur* (synonyms: *Pityrosporum furfur* and *Pityrosporum ovale pro parte*), *M. sympodialis*, *M. globosa* (probable synonym: *Pityrosporum orbiculare*), *M. restricta*, *M. slooffiae*, *M. obtusa*, *M. dermatis*, *M. japonica*, and *M. yamatoensis* (9, 119). Some additional species have been reported for animals. In routine clinical reporting, referring to these organisms as members of the *M. furfur* complex is normally sufficient. *Malassezia pachydermatis*, which causes animal ear infections and occasional human iatrogenic fungemias, is not considered a member of the *M. furfur* complex and, if reported, is reported under its individual species name. It is easily distinguished from *M. furfur* complex members by its ability to grow on ordinary laboratory media such as Sabouraud agar (see comments on *Malassezia* culture below). It is insignificant when grown from human skin except where investigations of catheter-related problems are involved.

Tinea versicolor lesions appear as scaly, discrete or concrescent, hypopigmented or hyperpigmented (fawn, yellow-brown, brown, or red) patches chiefly on the neck, torso, and limbs. The infection is largely cosmetic, becoming apparent when the skin fails to tan normally. The disease has a worldwide distribution: in tropical climates, 30 to 35% of the population may be affected, while incidence in areas of temperate climate is much lower, with only 1.0 to 4.0% of the population affected. *M. furfur* and related yeasts are found on the normal skin and elicit disease only under conditions, local or systemic, that favor the overgrowth of the organism.

The *M. furfur* complex has been associated with folliculitis (120), obstructive dacryocystitis (22), systemic infections in patients receiving intralipid therapy (121), and seborrheic dermatitis, especially in patients with AIDS (122). Excellent reviews are available on human infections caused by *Malassezia* spp. and on the characteristics of the genus (123–126). Additional information is also found in chapter 117 of this *Manual*.

Direct Examination

The fungi are observed readily when scrapings are mounted in 10% KOH plus ink (127), 25% NaOH plus 5% glycerin, calcofluor white, or Kane's formulation (glycerol, 10 ml; Tween 80, 10 ml; phenol, 2.5 g; methylene blue, 1.0 g; distilled water, 480 ml). In cases in which skin scrapings

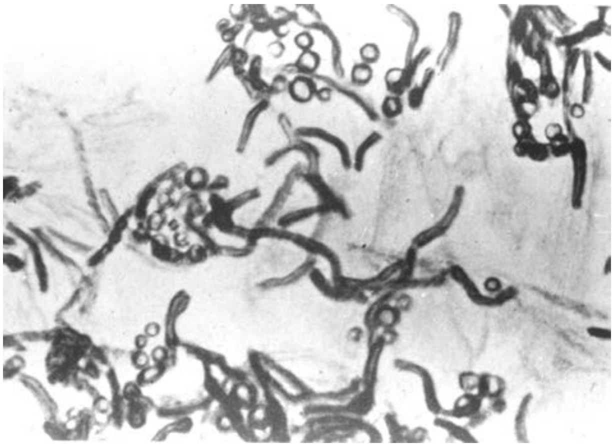

FIGURE 19 *Malassezia furfur* in skin scrapings from a lesion of tinea versicolor (Kane's stain). Magnification, ×1,000. doi:10.1128/9781555817381.ch123.f19

from unspecified body sites are examined, Kane's formulation has the advantage of vividly staining both fungi and the differential-diagnostic organisms causing erythrasma and pitted keratolysis. (These two bacterial infections, often confused with superficial mycosis, are mainly from intertriginous sites and foot soles, respectively.) Tinea versicolor is signaled in microscopy by the presence of "spaghetti and meatballs," i.e., short, septate, occasionally branching filaments 2.5 to 4 µm in diameter and of variable lengths intermingled with clusters of small, unicellular, oval or round, budding yeast cells (Fig. 19). The yeasts show the presence of a collarette between mother and daughter cells (budding is phialidic and unipolar) and average 4 µm (up to 8 µm) in size.

Isolation and Culture

Culture is not essential for identification unless the findings of direct microscopic examination are atypical or unless full species identification is desired for research purposes. Also, *M. furfur* complex members are part of the normal flora of the skin, and positive culture does not indicate infection. The species require exogenous lipid and do not grow on routine mycology media. If culture is desired, scrapings may be inoculated on Leeming-Notman medium, which uses whole milk as a major lipid source (128); on Dixon agar (129); or on modified Dixon agar, consisting of malt extract (3.6%), mycological peptone (Oxoid) (0.6%), desiccated ox bile (bile salts; Oxoid) (2%), Tween 40 (1%), glycerol (0.2%), oleic acid (0.2%), and agar (1.2%). Growth of the yeasts is slow; colonies are cream colored, glossy or rough, and raised (Fig. 20), later becoming dull, dry, and tan to brownish. Only budding yeast cells generally appear in culture (Fig. 21).

Tinea Nigra

Tinea nigra is characterized by the appearance, primarily on the palms of the hands and less commonly on the dorsa of the feet, of flat, sharply marginated, brownish black, nonscaly macules that may resemble melanoma (126).

The disease, almost always caused by *Hortaea werneckii* (*Phaeoannellomyces werneckii*, *Exophiala werneckii*, or *Cladosporium werneckii*), is most common in tropical areas (130) but has been contracted occasionally in coastal areas in and near the southeastern United States (126, 131). Cases diagnosed outside the area of endemicity have mostly resulted from travel to the American tropics or the Caribbean islands (130). *H. werneckii* is related to the ascomycetous order *Dothideales* (132).

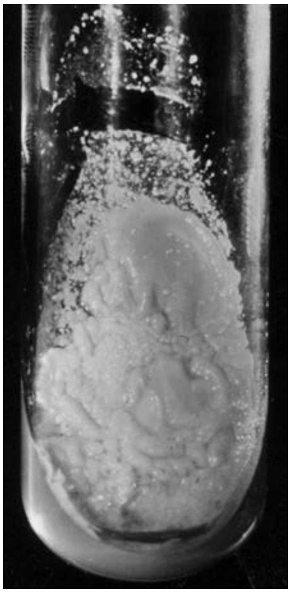

FIGURE 20 Culture of *Malassezia furfur* on Littman oxgall agar overlaid with oil. doi:10.1128/9781555817381.ch123.f20

Direct Microscopic Examination

Microscopic examination of skin scrapings in KOH or NaOH reveals numerous light brown, frequently branching septate filaments 1.5 to 5 µm in diameter (Fig. 18h); short, sinuous filaments; and budding cells, some septate.

Isolation

On SGA with or without antibiotics, *H. werneckii* grows slowly and usually appears within 2 to 3 weeks as moist, shiny olive to greenish black yeast-like colonies. The yeast-like cells are usually two-celled when reproductive and, instead of budding, produce new yeast cells from thick (up to 2 µm in diameter), distinctly annellated (multiply ringed

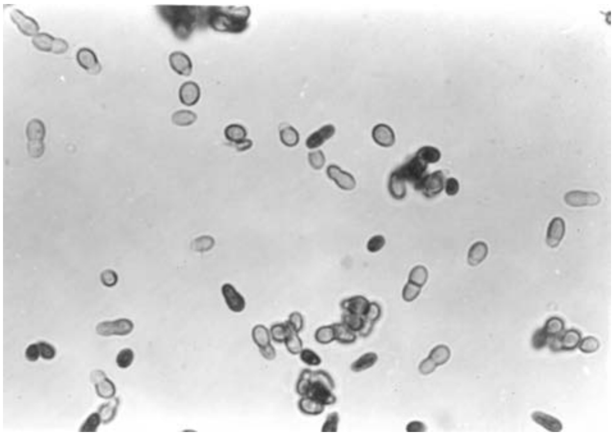

FIGURE 21 Microscopic appearance of *Malassezia furfur* yeast cells on Littman oxgall overlaid with olive oil. Magnification, ×400. doi:10.1128/9781555817381.ch123.f21

as if wearing several bracelets) pegs. After 7 or more days, colonies may develop a fringe of thick, dark, conspicuously septate hyphae that also bear annellated fertile structures. These produce conidia that are indistinguishable from young yeast cells. The micromorphology of *H. werneckii* is described in more detail in chapter 124.

Black Piedra

Black piedra is a fungal infection of the scalp hair, less commonly of the beard or moustache, and rarely of axillary or pubic hairs. The disease is characterized by the presence of discrete, hard, gritty, dark brown to black nodules adhering firmly to the hair shaft (Fig. 22). It is found mostly in tropical regions in Africa, Asia, and Central and South America. Humans as well as other primates are infected (126, 130, 133).

The etiologic agent in humans is *Piedraia hortae*, an ascomycete (order *Dothideales*) forming nodules that serve as ascostromata containing locules that harbor the asci and ascospores.

Direct Microscopic Examination

Hair fragments containing one or more black nodules are placed in 25% KOH or NaOH with 5% glycerol. The preparation is heated gently and carefully squashed so as not to break the coverslip, as the nodules are very hard. A squashed preparation of a mature nodule should reveal compact masses of dark, septate hyphae and round or oval asci containing two to eight hyaline, aseptate, banana-shaped (fusiform) ascospores that bear one or more appendages. The preparation should first be observed under the low-power objective to reveal the dark mass of compacted hyphae around the surface of the hair, and then examined under the high-power objective to observe the asci and ascospores.

Isolation, Culture, and Identification

When ascospores are seen in direct specimen microscopy, culture is unnecessary. Otherwise, SGA with chloramphenicol and SGA with chloramphenicol and cycloheximide may be used for isolation. Some reports have indicated that cycloheximide may be inhibitory; however, others have used this antibiotic successfully. SGA amended with chloramphenicol alone may be used for successful isolation.

Colonies are very slow growing; appear dark brown to black; and are glabrous at first and later covered with short, dark brown to black aerial mycelium. They tend to be heaped in the center with a flat periphery. Some colonies produce a reddish brown, diffusible pigment on the agar. Microscopic examination reveals only highly septate, dark hyphae and swollen intercalary cells. Conidia and ascospores are usually not found on routine mycological media.

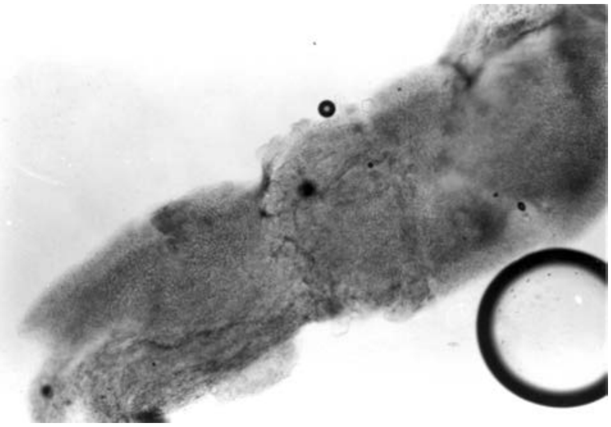

FIGURE 23 White piedra nodule on hair from the groin. Magnification, ×1,000. doi:10.1128/9781555817381.ch123.f23

White Piedra

White piedra is a fungal infection of the hair shaft characterized by the presence of soft white, yellowish, beige, or greenish nodules found chiefly on facial, axillary, or genital hairs (Fig. 23) and less commonly on scalp, eyebrows, and eyelashes. Nodules may be discrete or more often coalescent, forming an irregular transparent sheath.

The infection occurs sporadically in North America and Europe and more commonly in South America, Africa, and parts of Asia (130). Although white piedra is an uncommon infection, genital white piedra is occasionally but regularly seen in certain populations (126, 134).

Microscopic examination of hairs containing the adherent nodules mounted in 10% KOH or 25% NaOH-5% glycerin and squashed under a coverslip will reveal intertwined hyaline septate hyphae, hyphae breaking up into oval or rectangular arthroconidia 2 to 4 μm in diameter (Fig. 24), occasional blastoconidia, and bacteria that may surround the nodule as a zooglea (jelly-like mass).

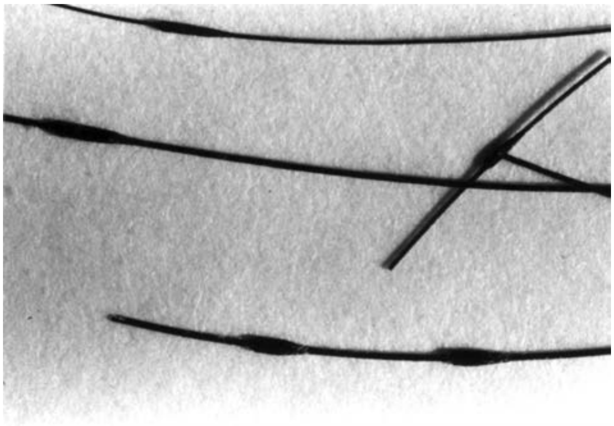

FIGURE 22 Black piedra nodules on scalp hair. NaOH mount. Magnification, ×100. doi:10.1128/9781555817381.ch123.f22

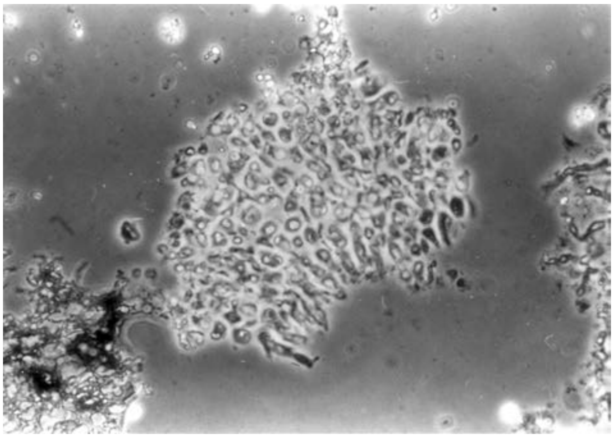

FIGURE 24 Arthroconidia from a crushed nodule of white piedra. Magnification, ×1,000. doi:10.1128/9781555817381.ch123.f24

The isolates were formerly described as *Trichosporon beigelii* or *Trichosporon cutaneum* but are now correctly identified in most cases as *Trichosporon ovoides* (causes scalp hair white piedra), *Trichosporon inkin* (causes most cases of pubic white piedra), and *Trichosporon asahii* (135, 136). As with the M. *furfur* complex, these species form a complex of difficult-to-identify species whose distinction, except in demonstrated cases of piedra, is not known to have strong clinical implications in dermatologic mycology. They are also very common skin contaminants. Ordinarily, they may be reported for skin, hair, and nails of nonneutropenic patients simply as members of the genus *Trichosporon*, based on production of budding yeast cells, arthroconidia, and a positive urease test, unless a research-level identification of a proven etiologic agent is attempted. The causal agents of white piedra may be readily isolated on SGA with chloramphenicol or other isolation media containing antibacterial antibiotics. The isolation medium should not contain cycloheximide, since this drug is inhibitory to some of the species. Growth is rapid, yielding white to cream-colored colonies that exhibit a variety of colonial morphologies depending on the species. A description of the genus and characteristics of the species involved in white piedra are given in papers by Guého et al. (136, 137). More information about *Trichosporon* is found in chapter 117 of this *Manual*.

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Curvularia, *Exophiala*, *Scedosporium*, *Sporothrix*, and Other Melanized Fungi

JOSEP GUARRO AND SYBREN DE HOOG

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This chapter covers most of the agents of phaeohyphomycosis, chromoblastomycosis, and sporotrichosis, as well as a number of agents of superficial and cutaneous disease. The genera discussed in this chapter belong to the ascomycetous orders Botryosphaeriales (*Lasiodiplodia* and *Neoscytalidium*), Chaetothyriales (*Cladophialophora*, *Exophiala*, *Fonsecaea*, *Knufia*, *Phialophora*, *Rhinocladiella*, and *Veronaea*), Calosphaeriales (*Pleurostomophora*), Diaporthales (*Phaeoacremonium*), Dothideales (*Aureobasidium* and *Hormonema*), Microascales (*Scedosporium*), Ophiostomatales (*Sporothrix*), Sordariales (*Phialemonium*, discussed in chapter 120), Pleosporales (*Alternaria*, *Curvularia*, and *Exserohilum*) and Venturiales (*Verruconis*, formerly *Ochroconis*) In this chapter, the genera are treated according to their ordinal relationships (Table 1). Definitions of mycological terms are provided in chapter 113.

The term dematiaceous applies to fungi with black (containing melanin) hyphae in general (1), but it has been recommended in medical mycology to reserve this term for the rapidly growing members of Pleosporales only (2), as these are very different from Chaetothyriales in all respects. Also, the term phaeoid indicates brown hyphae; "phaeohyphomycosis" is an umbrella term for infection caused by moulds that display brownish yeastlike cells, pseudohyphae, or hyphae or a combination of these forms in host tissue. "Black yeasts and relatives" is preferably used to indicate members of Chaetothyriales and Dothideales only (2). The black yeasts are not a formal taxonomic group, but the term is applied to a wide range of unrelated ascomycetous and basidiomycetous fungi that are able to produce budding cells at some stage in their life cycle.

Pleoanamorphism (multiple morphological forms) is particularly striking in members of the black yeasts and in the genus *Scedosporium*. These fungi are frequently seen in the clinical laboratory to produce more than one asexual form of propagation (anamorphs). Many species still bear separate names when the connection is not known. At this moment a nomenclatural transition is taking place, which should lead to a single name for a particular fungal species. Independently propagating asexual forms, then, no longer are assigned formal names, but the former generic names are used as descriptive nouns. Only a small percentage of fungal species have the ability to produce a sexual form (teleomorph), characterized by the formation of fruiting bodies with meiotic ascospores, under routine laboratory conditions. For each organism only a single teleomorph can be produced.

TAXONOMY AND DESCRIPTION OF THE AGENTS

Botryosphaeriales

Lasiodiplodia

Lasiodiplodia is a coelomycete genus characterized by spherical fruit bodies filled with asexual conidia. The conidia initially are ellipsoidal and hyaline but gradually become brown and develop a median septum at maturation. *Lasiodiplodia theobromae* (Fig. 1) is the only species involved in human infection.

Neoscytalidium

Neoscytalidium dimidiatum (formerly known as *Scytalidium dimidiatum*) is a plant pathogen that produces arthroconidia in culture, and some isolates also produce pycnidia (flask-shaped structures containing conidiogenous cells) under appropriate growth conditions. The coelomycetous synanamorph had been given the separate name *Nattrassia mangiferae*, previously known as *Hendersonula toruloidea*. However, molecular studies have demonstrated that *N. dimidiatum* and *N. mangiferae* are two different species and that the latter, which is not pathogenic to humans, must be accommodated in the recently described genus *Neofusicoccum* (3).

Inside multilocular fruit bodies hyaline, ellipsoidal conidia develop, which in part become brownish and have one or two septa. In culture, usually only a rapidly growing, jet black, floccose anamorph with dark arthroconidia (*Neoscytalidium dimidiatum* [Fig. 1]) is seen; the pycnidia are only produced after 2 months' growth on a moistened plant leaf. Melaninless mutants (4), which also show reduced conidiation, were until recently referred to as *Scytalidium hyalinum*. The fungus is a common plant pathogen in the tropics.

Calosphaeriales

Recently, some melanized phialidic fungi were segregated from *Phialophora* on molecular grounds; these now constitute small islands of clinical significance in the order

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TABLE 1 Overview of the clinically most relevant species and their attribution to the ordinal level

Order, genus, and species	Synanamorph	Teleomorph	Obsolete name(s)
Botryosphaeriales			
Lasiodiplodia theobromae		*Botryosphaeria theobromae*	*Botryodiplodia theobromae*
Neoscytalidium dimidiatum			*Nattrassia mangiferae*, *Scytalidium hyalinum*, *Hendersonula toruloidea*, *Scytalidium dimidiatum*
Calosphaeriales			
Pleurostomophora			
repens			*Phialophora repens*
richardsiae			*Phialophora richardsiae*
ochracea			
Capnodiales			
Cladosporium			
cladosporioides			
oxysporum			
Hortaea werneckii			*Exophiala werneckii*, *Phaeoannellomyces werneckii*
Chaetothyriales			
Cladophialophora			
arxii			
bantiana			*Xylohypha bantiana*, *Cladosporium trichoides*
boppii			
carrionii	Unnamed *Phialophora*		
devriesii			
emmonsii			
modesta			
mycetomatis			
samoënsis			
saturnica			
Knufia epidermidis			*Coniosporium epidermidis*
Cyphellophora			
laciniata			*Pseudomicrodochium suttonii*, *Pseudomicrodochium fusarioides*
pluriseptata			
Exophiala			
asiatica			
attenuata			
bergeri			
dermatitidis	Unnamed *Phialophora*		*Wangiella dermatitidis*
jeanselmei			
oligosperma			
phaeomuriformis	*Sarcinomyces phaeomuriformis*		
spinifera	Unnamed *Phialophora*		
xenobiotica			
Fonsecaea			
monophora			
nubica			
multimorphosa			
pedrosoi	Unnamed *Phialophora*		*Fonsecaea compacta*
Phialophora			
americana		*Capronia semiimmersa*	
europaea			
verrucosa			
Rhinocladiella			
mackenziei			*Ramichloridium mackenziei*
basitona			
aquaspersa			
similis	Unnamed *Exophiala*		
Diaporthales			
Phaeoacremonium			
alvesii			

(Continued on next page)

TABLE 1 *(Continued)*

Order, genus, and species	Synanamorph	Teleomorph	Obsolete name(s)
griseobrunneum			
krajdenii			
parasiticum			*Phialophora parasitica*
rubrigenum			
venezuelense			
Dothideales			
Aureobasidium pullulans		*Discosphaerina fulvida*	
Hormonema dematioides		*Sydowia polyspora*	
Microascales			
Scedosporium			
apiospermum	Unnamed *Graphium*	*Pseudallescheria apiosperma*	
aurantiacum	Unnamed *Graphium*		
boydii	Unnamed *Graphium*	*Pseudallescheria boydii*	
prolificans			*Scedosporium inflatum*
Ophiostomatales			
Sporothrix			
brasiliensis			
globosa			
luriei			
schenckii			
Pleosporales			
Alternaria			
alternata			*Alternaria tenuissima*
infectoria		*Lewia infectoria*	
Curvularia			
australiensis		*Cochliobolus australiensis*	*Drechslera australiensis, Bipolaris australiensis*
hawaiiensis		*Cochliobolus hawaiiensis*	*Drechslera hawaiiensis, Dissitimurus exedrus, Bipolaris hawaiiensis*
spicifera		*Cochliobolus spicifera*	*Drechslera spicifera, Bipolaris spicifera*
geniculata		*Cochliobolus geniculatus*	
lunata		*Cochliobolus lunatus*	
Exserohilum rostratum		*Setosphaeria rostrata*	*Exserohilum longirostratum, Exserohilum macginnisii*
Sordariales			
Achaetomium strumarium			*Chaetomium strumarium*
Chaetomium			
globosum			
perlucidum			
Phialemonium obovatum			
Incertae sedis (Sordariomycetes class)			
Phialemoniopsis curvata			*Phialemonium curvatum*
Venturiales			
Verruconis gallopava			*Ochroconis gallopava, Dactylaria gallopava, D. constricta* var. *gallopava*

Calosphaeriales, which otherwise contains plant-associated moulds. Infections are mostly of traumatic nature and are supposed to originate directly from the woody plant material of the fungal habitat.

Pleurostomophora

Pleurostomophora is a genus of mainly wood-inhabiting fungi. Hyphae are dark and bear pale, tapering phialides that may be single or aggregated in dense brushes; hyaline, slimy conidia are produced through small or large collarettes. *Pleurostomophora repens* was occasionally reported from subcutaneous infections in humans. The second species of the genus involved also in human infections is *Pleurostomophora richardsiae* (Fig. 2) (syn. *Phialophora richardsiae*), a soft-rot fungus on wood. The recently published species *Pleurostomophora ochracea* is exceptional in producing yellow grains in mycetoma, which previously had been interpreted as being of bacterial nature (5).

Capnodiales

Cladosporium

Species of *Cladosporium*, especially *Cladosporium cladosporioides* (Fig. 3) and *C. oxysporum*, are extremely common contaminants in the clinical laboratory but have also been described as occasional human opportunists. Such cases need thorough evaluation, as infection by *Cladosporium* is highly unlikely.

Hortaea

Members of the genus *Hortaea* have rather wide hyphae which become profusely septate during growth of the

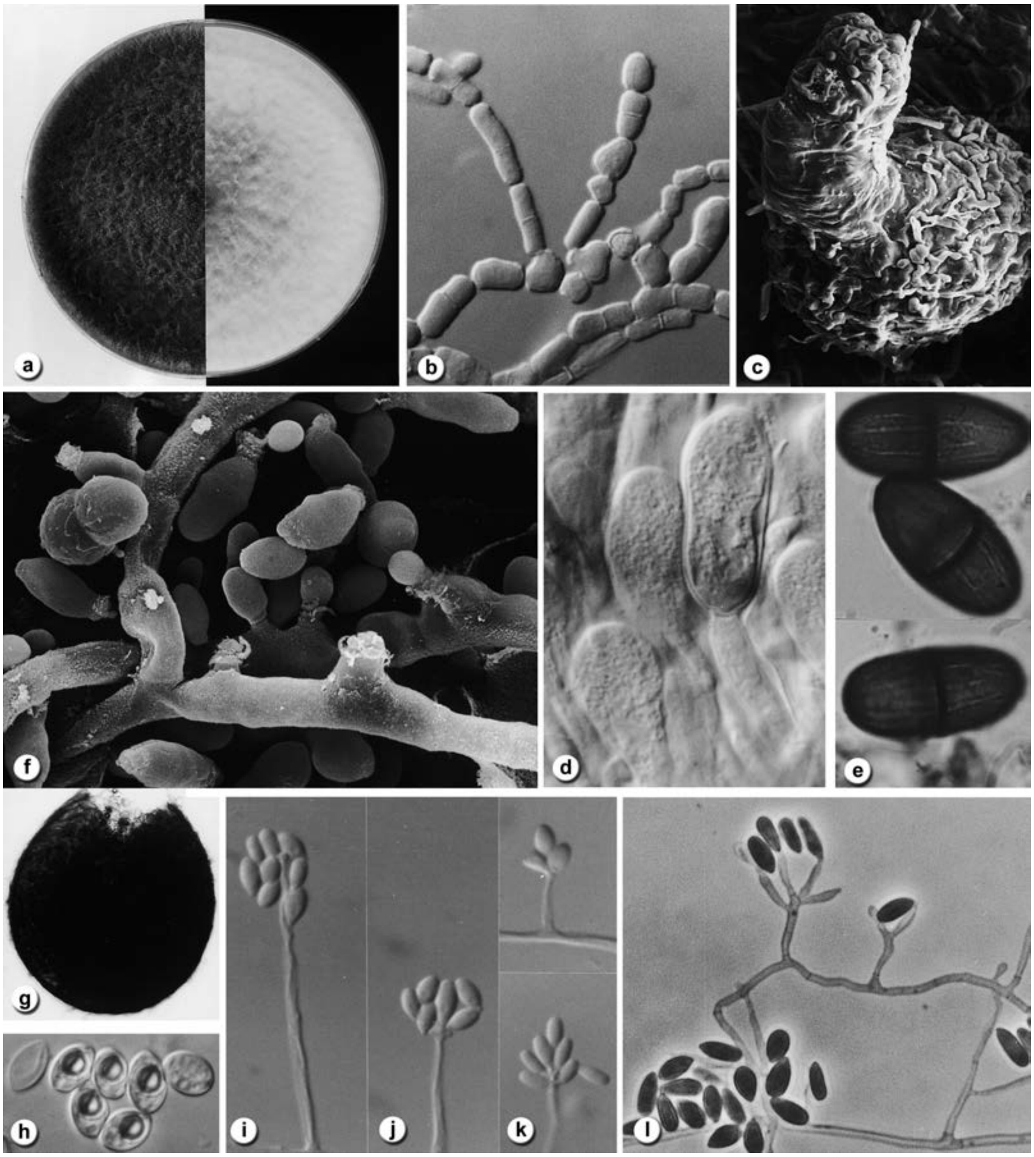

FIGURE 1 (a and b) *Neoscytalidium dimidiatum*; (c to e) *Lasiodiplodia theobromae*; (f) *Hortaea werneckii*; (g and h) *Pseudallescheria boydii*; (i to l) *Scedosporium boydii*. Reproduced from reference 2. doi:10.1128/9781555817381.ch124.f1

fungus, and they have annellidic conidiogenesis from broad scars. The halophilic species *H. werneckii* (Fig. 1) lives in evaporation ponds at the subtropical seashore and causes superficial infections. The clinical picture is known as tinea nigra.

Chaetothyriales

The rather small order *Chaetothyriales* is clinically highly relevant, because about half of the species known to date are able to cause infections in humans.

Cladophialophora

Catenate (in chains), dry conidia and an absence of differentiated conidiophores characterize *Cladophialophora*. This genus contains 10 pathogenic species (Table 1), 7 of which are almost exclusively known from humans and other warm-blooded animals. The most significant species are *Cladophialophora bantiana* and *C. carrionii*. *C. bantiana*, a remarkable neurotropic mould, is recognizable by very long, coherent, poorly branched conidial chains and by an ability to grow at 40°C (Fig. 2; see also Fig. 5). It has a characteristic 558-bp intron at position 1768 of the small-subunit (SSU) ribosomal operon (6). *C. carrionii* is a common agent of chromoblastomycosis, with small conidia in profusely branched chains.

Cyphellophora

Cyphellophora (including clinical species of *Pseudomicrodochium* [7]) is a rare group of infective agents characterized by slender, curved, mostly 1- to 3-septate conidia. Cultures are evenly melanized and show limited expansion growth; budding cells are absent. Conidia are produced from poorly developed collarettes alongside the hyphae. *Cyphellophora laciniata* and *C. pluriseptata* (Fig. 2) are rare agents of superficial mycoses. The incidence of these fungi is probably low, but they are likely to be underdiagnosed.

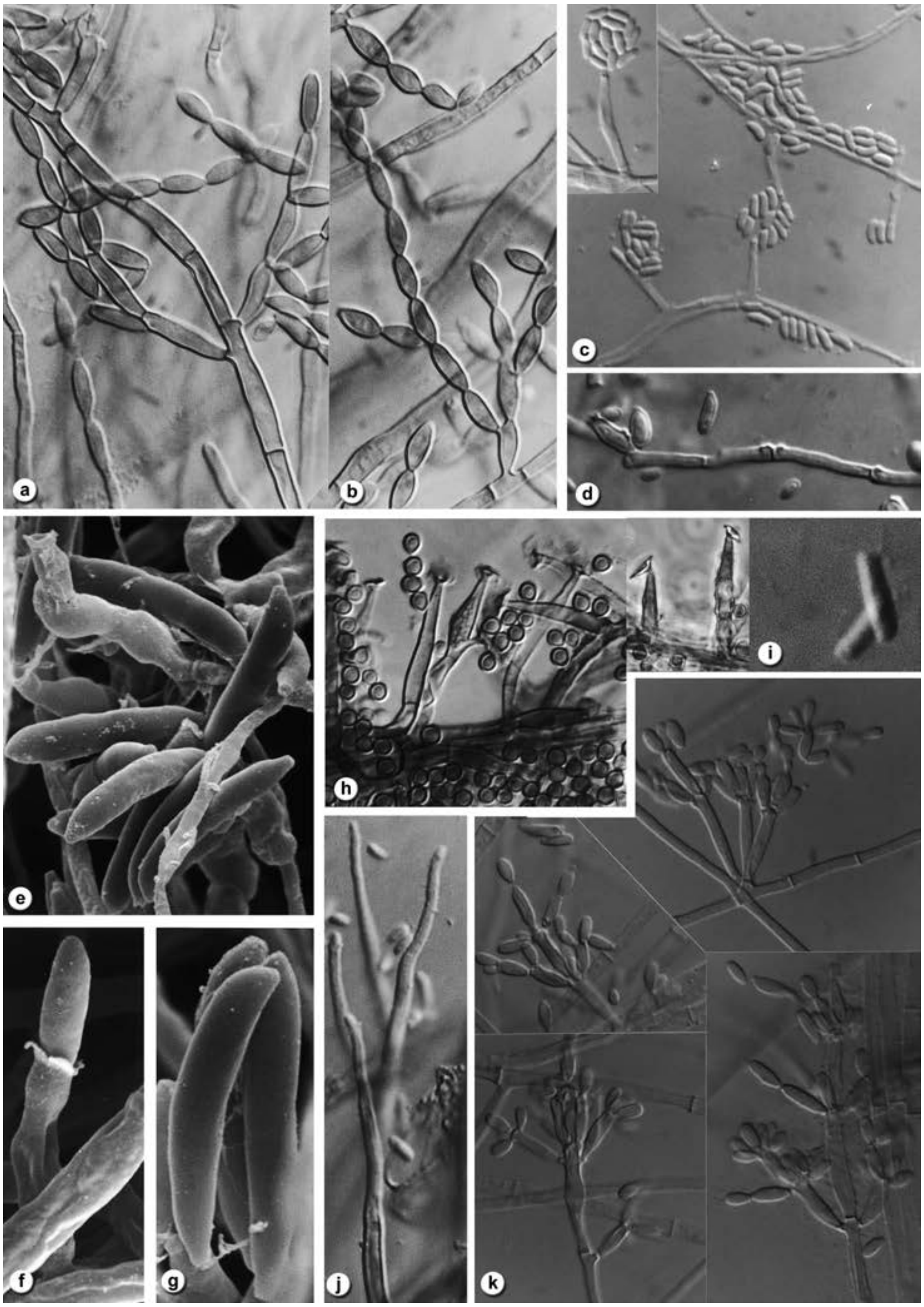

FIGURE 2 (a and b) *Cladophialophora bantiana*; (c) *Phialemoniopsis curvata*; (d) *Exophiala dermatitidis*; (e to g) *Cyphellophora pluriseptata*; (h and i) *Pleurostomophora richardsiae*; (j) *Phaeoacremonium parasiticum*; (k) various pictures of *Fonsecaea pedrosoi*. Reproduced from reference 2.
doi:10.1128/9781555817381.ch124.f2

Exophiala

Exophiala is the main genus of clinically relevant black yeasts. Strains show high degrees of morphological diversity. Most isolates initially grow in a yeast form that is succeeded by a hyphal anamorph. As a result, colonies are moist and slimy at first, becoming velvety to woolly with age. The process of conidium production is annellidic, from narrow, inconspicuous scars or extensions. Occasionally a very slowly growing, meristematic morphology ("*Sarcinomyces*") is preponderant. Fresh isolates or strains cultivated on nutritionally deficient media frequently produce conidia in chains and may produce scattered or compacted phialides with huge collarettes (8). The most frequently identified species are *Exophiala dermatitidis* (Fig. 2) and *E. jeanselmei*. The latter has been subdivided into a number of taxa, such as *E. oligosperma*, *E. bergeri*, and *E. xenobiotica*, on molecular grounds; they are morphologically indistinguishable (9). *E. dermatitidis* is recognizable morphologically by phialides without collarettes, which are wide, scar-like, very short annellated zones. In addition, growth at 40°C and the inability to assimilate nitrate are characteristic

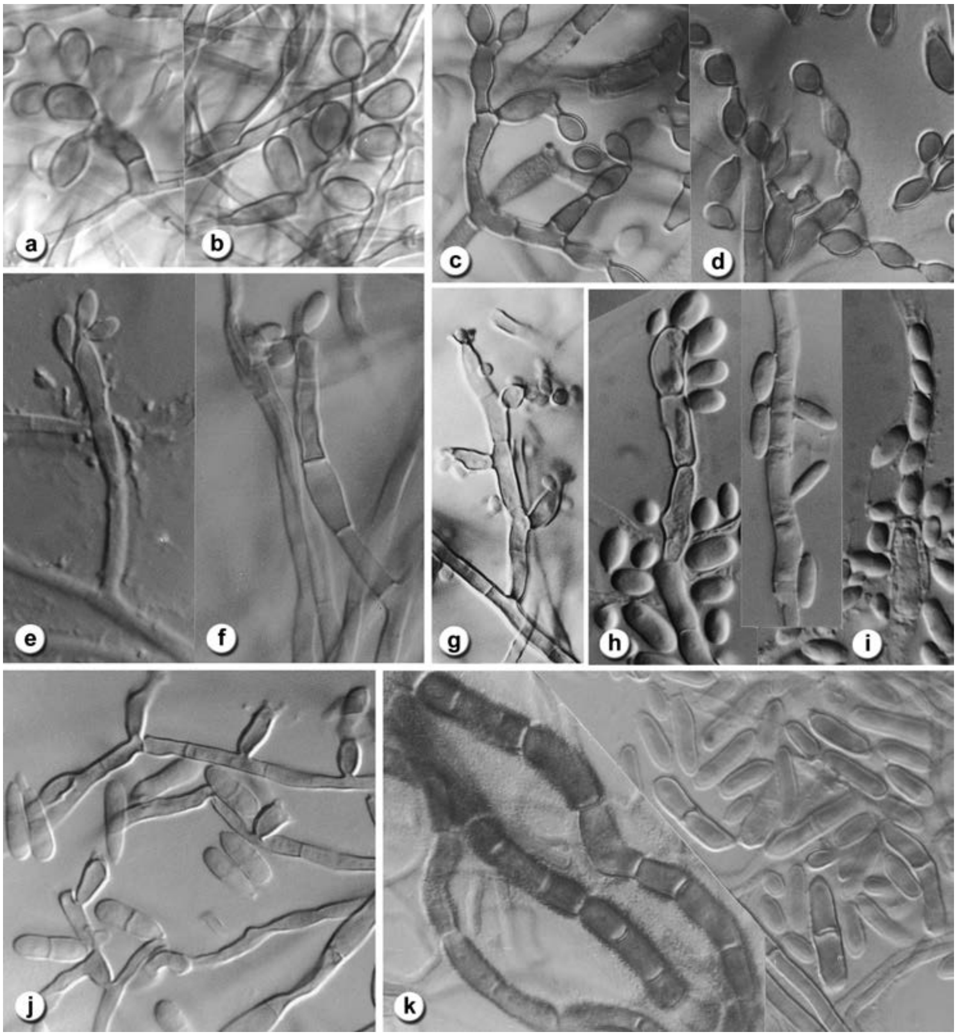

FIGURE 3 (a and b) *Rhinocladiella mackenziei*; (c and d) *Cladosporium cladosporioides*; (e and f) *Rhinocladiella aquaspersa;* (g) *Phialophora verrucosa;* (h and i) *Aureobasidium pullulans*; (j) *Verruconis gallopava*; (k) *Hormonema dematioides*. Reproduced from reference 2.
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(10). The related species *E. phaeomuriformis* differs by having a growth maximum at 38°C (11). The less frequent species *E. spinifera* can be recognized by the presence of large, stiff conidiophores. Recently, a similar species with reduced conidiogenous cells, *E. attenuata*, was described (9). Other *Exophiala* species, such as *E. asiatica* (12), have occasionally been described from severe cases in otherwise healthy individuals.

Fonsecaea

The four species known in the genus *Fonsecaea*, characterized by conidia produced in chains of maximally four, are all human pathogens. In culture, *Fonsecaea* species mostly have one morphological form, but they may produce additional phialides with collarettes releasing balls of one-celled conidia. No budding cells are produced on routine media. *Fonsecaea pedrosoi* (Fig. 2, including the mutant *F. compacta*) is one of the etiologic agents of human chromoblastomycosis.

The other agents of this disease, *F. monophora* (13) and *F. nubica* (14), are morphologically indistinguishable. *F. multimorphosa* was described from a cerebral infection in a cat (15).

Knufia

Although most species of *Knufia* have been described as colonizers of rock and marble building walls, they are occasionally found as colonizers of human skin. *Knufia epidermidis* has exclusively been recovered from human skin, causing very mild infections (16).

Phialophora

When phialides are the only form of propagation in chaetothyrialean fungi, the generic name *Phialophora* is applied. Dematiaceous phialidic fungi belonging to other orders are now classified in genera such as *Cadophora*, *Phaeoacremonium*, and *Pleurostomophora* (17). No budding cells are produced in *Phialophora*. *Phialophora verrucosa*, with darkened, funnel-shaped collarettes (Fig. 3), is one of the commonest species of this genus. *P. americana* differs mainly by having vase-shaped collarettes (18). Given the close molecular similarity, there is no consensus as to whether *P. verrucosa* and *P. americana* comprise two species or one (18).

Rhinocladiella

Rhinocladiella is morphologically characterized by pale brown conidiophores bearing noncatenate conidia on denticles and occasionally shows exophiala-like budding cells in culture (19). It comprises three pathogenic species, *Rhinocladiella mackenziei* (Fig. 3), *R. aquaspersa* (Fig. 3), and *R. basitona*, which, however, are polyphyletic within the Chaetothyriales. *R. atrovirens* has been reported to cause cerebral phaeohyphomycosis in an AIDS patient (20), but as this species in a modern, molecular circumscription is limited to isolates growing on conifer wood in northern countries, these cases probably concerned the sibling species *R. similis* (21).

The neurotropic species *R. mackenziei* is morphologically similar to *Pleurothecium obovoideum* (formerly *Ramichloridium obovoideum*) and for a long time was thought to be conspecific, but phylogenetically it does not even belong to the same order (the latter species belongs to the Sordariales) (19).

Diaporthales

Phaeoacremonium

Phaeoacremonium (22) is morphologically characterized by slender, tubular, and often tapering or inflated brown phialides. Hyaline, slimy conidia are produced through inconspicuous collarettes. Six species are known from opportunistic infections in humans (Table 1), *Phaeoacremonium parasiticum* (Fig. 2) being the most common. The prevalent type of infection is mycetoma.

Dothideales

The order Dothideales comprises numerous saprobes which thrive well under conditions of decreased water activity, such as on sugary leaf surfaces, rock, glass, and moist medical devices.

Aureobasidium and *Hormonema*

The genus *Aureobasidium* contains a single, ubiquitous, and highly polymorphic species, *A. pullulans* (Fig. 3). Colonies *in vitro* are often pink due to the preponderance of wide, hyaline, oligokaryotic (having 3 to 10 nuclei) hyphae bearing synchronously produced conidia and containing scattered endoconidia (conidia formed inside a hypha). *Aureobasidium* is often confused with *Hormonema* (Fig. 3), which, however, produces conidia in a basipetal succession with the youngest at the base (23). In addition, the internal transcribed spacer (ITS) ribosomal DNA (rDNA) sequences of the two species are clearly different (24).

Microascales

The order Microascales comprises *Scopulariopsis* with its *Microascus* teleomorphs (see chapter 120) but also *Scedosporium*, linked to *Pseudallescheria* and *Petriella* teleomorphs.

Pseudallescheria/Scedosporium

The genus *Pseudallescheria* (anamorph *Scedosporium*) comprises several species of clinical interest (25, 26). This fungus has become one of the most frequently misidentified organisms in medical mycology, largely due to the differential abundance of each of the phenotypes of *Pseudallescheria boydii*. This has led to a large number of new introductions in totally unrelated genera (2). However, it is currently accepted that *P. boydii* and *Scedosporium apiospermum* are two different species, i.e., *P. boydii* (anamorph *Scedosporium boydii*) and *Pseudallescheria apiosperma* (anamorph *Scedosporium apiospermum*) (27). The former species is homothallic, and the teleomorph (Fig. 1) is frequently found in clinical samples. The latter species is heterothallic, and the teleomorph is not present in cultures of clinical samples. The anamorphs of these two species are morphologically difficult to distinguish from each other and are characterized by hyaline, Fontana-Masson-negative hyphae from which slimy, pale brown conidia are produced in basipetal succession from inconspicuous annellides. In addition, some isolates are able to produce a *Graphium* synanamorph. A closely related species with a high incidence in Australia is *S. aurantiacum*, which is characterized by the production of a yellow to orange pigment (27). Another clinically important species that does not belong to the *P. boydii* species complex is *S. prolificans*. However, the latter fungus has flask-shaped conidiogenous cells and is monomorphic (see Fig. 6). *Scedosporium* species have hyaline hyphae, but conidia and synnemata are melanized; colonies are usually gray to black.

Ophiostomatales

Ophiostomatales is a large order of plant-pathogenic fungi. Most are blue-stain fungi in wood and live in association with bark beetles. Only a few species are human or animal pathogens. The main teleomorph genus is *Ophiostoma*.

Sporothrix

Sporothrix schenckii has colorless hyphae that bear a cluster of thin denticles with hyaline, tear-shaped conidia at their tips. Additional spherical and sessile conidia may or may not be present; this is the only melanized part of the fungus. The mould can be transformed *in vitro* to a stable yeast at 37°C on enriched media such as chocolate agar or brain heart infusion agar. Occasional isolates are difficult to convert and may require multiple subcultures and extended incubation. Transition is observed in environmental as well as clinical isolates.

Recent molecular studies have demonstrated that *S. schenckii sensu lato* is a complex of numerous phylogenetic species (28–30). The combination of phenotypic (morphology of the sessile pigmented conidia; growth at 30, 35, and 37°C; and assimilation of sucrose, raffinose, and ribitol) and genetic (analysis of the calmodulin gene sequences) approaches allows differentiation of some of these species. Apart from *S. schenckii sensu stricto*, the species *S. brasiliensis*, *S. globosa*, and *S. luriei* are also involved in human infections. *S. brasiliensis* is a clonal species with high virulence (31) emerging from large epidemics in Brazil; transmission occurs particularly through stray cats. *S. globosa* is the prevalent species in Asia (32), where particularly children are infected. *S. luriei* is a rare species that has only been reported from a single case (29).

Pleosporales

Most members of the order Pleosporales have rapidly expanding, floccose colonies, which show optimal conidium production on media poor in nutrients, such as potato carrot agar. The large conidia are then mostly visible with the aid of a stereomicroscope. Conidia are generally produced on septate, dark brown, erect conidiophores. Conidia consist of several compartments. True septa are found in those conidia where the outer wall and septum are continuous; such conidia are euseptate (in *Alternaria* and *Curvularia*). False septa are observed in those conidia where only the inner wall layers are involved in septation and the outer wall forms a sac-like structure around the individual cells; such conidia are distoseptate (in *Bipolaris* and *Exserohilum*).

Alternaria

Since clinical strains are very likely to belong to only two species of *Alternaria*, *Alternaria infectoria* and *A. alternata*,

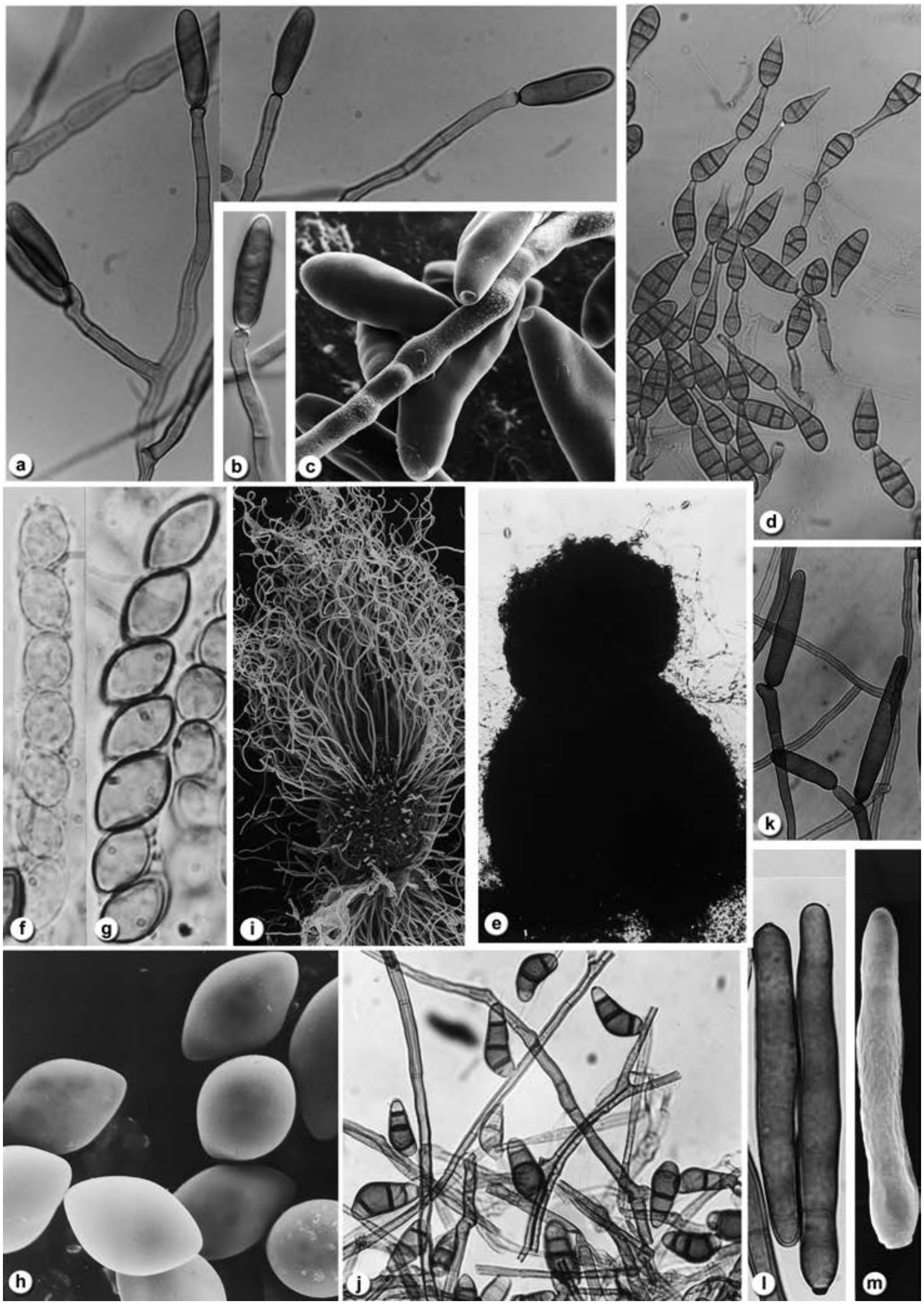

FIGURE 4 (a to c) *Curvularia hawaiiensis*; (d) *Alternaria alternata*; (e to h) *Achaetomium strumarium*; (i) *Chaetomium globosum*; (j) *Curvularia lunata*; (k to m) *Exserohilum rostratum*. Reproduced from reference 2. doi:10.1128/9781555817381.ch124.f4

isolates are easily identified down to the species level. Available criteria comprise characteristics of growth, sporulation, and morphology, and very large differences in the ITS rDNA, which can be displayed by sequencing or by restriction fragment length polymorphisms (33). *Alternaria infectoria* in tissue may present with hyaline yeast cells rather than melanized hyphae. Cultures of *A. infectoria*, in contrast to those of *A. alternata*, frequently exhibit creamish patches and show reduced sporulation. Conidia of *A. infectoria* bear long apical beaks which serve as secondary conidiophores. *A. alternata* displays conidia in chains.

Bipolaris

The preponderant *Bipolaris* species in human infections are *Bipolaris australiensis*, *B. hawaiiensis* (Fig. 4), and *B. spicifera*. They are characterized by large, ellipsoidal, straight or curved conidia. Recently, these species have been transferred to *Curvularia* (34).

Curvularia

Most species of the genus *Curvularia* are easily recognizable on the basis of the characteristics of their conidia, which are ellipsoidal, often curved, and with dark and flat scars

(1). Saprobic members of *Curvularia*, particularly *Curvularia geniculata* and *C. lunata* (Fig. 4), have been identified as potential agents of human infections.

Exserohilum

Exserohilum (35, 36) is characterized by very long, distoseptate conidia with a distinct, protruding basal hilum. Three species have been recognized as opportunistic agents (*Exserohilum longirostratum*, *E. macginnisii*, and *E. rostratum* [Fig. 4]), but given their close molecular similarities, they may be morphological variants of a single species (2, 37).

Sordariales

Sordariales is a large and diverse order of ascomycetes. *Madurella mycetomatis* was recently proven to be another member of Sordariales (38); this species is treated in chapter 125. The genera *Phialemonium*, *Chaetomium*, and *Achaetomium* are treated in chapter 120. Currently, *Phialemoniopsis curvata* (formerly *Phialemonium curvatum*) is *incertae sedis* in the Sordariomycetes class.

Venturiales

Ochroconis (Verruconis)

The genus *Ochroconis* is paraphyletic (group of fungi that includes some, but not all, of the descendants from a common ancestor) to but at significant distance from the Venturiales. All species have rust brown to olivaceous colonies and produce 1- to 3-septate conidia from small, open denticles inserted in low numbers on sympodial cells. Members of *Ochroconis* are occasional agents of mild skin infections. Molecular distances within the Ochroconiales are remarkably large; for this reason, the neurotropic species *O. gallopava* (Fig. 3), with hyaline, clavate conidia and growing well at 40°C, was reallocated to a separate genus, *Verruconis* (39).

EPIDEMIOLOGY AND TRANSMISSION

Phaeohyphomycoses are usually subcutaneous but can also be systemic or involving the central nervous system. Infections of the central nervous system are frequently manifested as brain abscesses or meningitis, which are usually fatal. In these infections fungi can be present in the cerebrospinal fluid. Most cases are produced in immunocompetent patients. Typical symptoms include headache, neurologic deficits, and seizures. Common neurotropic species are *Cladopialophora bantiana*, *Ramichloridium mackenziei*, *Verruconis gallopava*, and *Exophiala dermatitidis* (40, 41). The fungi more commonly involved in phaeohyphomycosis are *Exophiala jeanselmei*, *E. dermatitidis*, *E. spinifera*, *Cladophialophora bantiana*, and *Curvularia* spp. Superficial infections are mostly caused by *Exophiala oligosperma*, *Phialophora europaea*, *Hortaea werneckii*, and other black yeast-like species (42).

The route of (sub)cutaneous infection is likely to be traumatic implantation, with the fungus causing a reaction leading to the formation of an inflammatory abscess with hyphae. In contrast, chromoblastomycosis leads to excessive proliferation of skin layers, with muriform cells the invasive form. The development of either phaeohyphomycosis or chromoblastomycosis in black yeast-like fungi is largely species specific. The species that cause subcutaneous infections are generally uncommon in the environment and occupy hitherto-unrevealed microhabitats. Some thermotolerant species seem to be associated with environmental xenobiotics or occur in animal feces (43); others are found on slightly osmotic surfaces such as fruit (44). Psychrophilic species that cause infections in cold-blooded vertebrates are frequent in water, either ocean water (45), municipal drinking water (46), or hospital water (47). In the indoor environment, *Exophiala* has been found in steam baths (44) and dishwashers (48). Members of the Pleosporales that rarely cause infections are common saprobes on decaying plant material. They may be encountered in allergic sinusitis of human and cattle (49). Many members of the Dothideales are tolerant of extreme growth conditions and are found in Antarctic or Mediterranean rock (50) or in hypersaline ponds (51). These species have the ability to transition morphologically to a tolerant, meristematic ecotype consisting of clumps of amorphous, thick-walled, highly melanized cells. *Aureobasidium pullulans* has a yeast phase that colonizes moist surfaces; i.e., it forms isodiametrically expanding cells that become subdivided in all directions and may eventually fall apart into small cell clumps.

Chromoblastomycosis

Chromoblastomycosis is one of the most frequently found subcutaneous mycoses. It occurs usually in the tropical and subtropical regions, affecting mainly adult males working in agriculture or related activities. The fact that males are predominantly affected has been related to a possible role of human sex hormones (52, 53). The fungus usually penetrates the cutaneous barrier through puncture wounds, usually by a thorn or a splinter. The fungal agents causing these infections are occasionally found on rotting plant material. Several reports have associated some of these fungi with palm trees and xerophyte plants, but recent sequencing data have proven that mostly other, strictly saprobic relatives are concerned. For example, cactus plant thorns carry *Cladophialophora yegresii*, while human cases are caused by *C. carrionii* (54). *C. yegresii* produces muriform cells in the spines morphologically similar to those known as the invasive form of *C. carrionii* in chromoblastomycosis. These cells may thus be regarded as an extremotolerant survival phase and are likely to play an essential role in the natural life cycle of these organisms (54). The ecology of the fungi causing chromoblastomycosis is clearly different. *Fonsecaea* species are isolated in the evergreen forests of tropical areas, whereas *C. carrionii* is identified only in desert areas under arid climatic conditions (55).

Sporotrichosis

Sporothrix schenckii can be found in soil and thorny plants. Infection is acquired through traumatic inoculation of fungi from contaminated soil or plants. *Sporothrix brasiliensis*, however, is particularly transmitted from bites or scratches from stray cats which are considered a primary host of this fungus (31). Occasional outbreaks occur in different populations, such as gardeners, rural workers, armadillo hunters, and persons in contact with domestic cats. In Brazil a large epidemic occurs due to expansion of *S. brasiliensis* (56). In the United States several outbreaks of sporotrichosis, associated with occupational or recreational exposure to sphagnum moss, have been reported; the largest occurred in 1988 and involved 84 patients (56). Most infections occur in otherwise healthy individuals, but *Sporothrix* infections have also been recognized as opportunistic in immunocompromised individuals.

CLINICAL SIGNIFICANCE

Disorders caused by the fungi described in this chapter are mainly localized and occur in otherwise healthy hosts.

The infections arise mostly after traumatic inoculation of contaminated material from the environment; less frequently, e.g., in the case of sinusitis, otitis, or growth in the mucus of lungs of patients with cystic fibrosis, asymptomatic or mildly symptomatic colonization of cavities is observed. A number of the fungi treated in this chapter are typical opportunists, causing infections increasing in severity in individuals with impaired innate immunity and metabolic diseases such as diabetes. Such patients may have cutaneous infections by black yeasts that are otherwise found on cold-blooded animals (57), but the clinical course of such infections is usually mild (58). Infections also arise in patients with severe immunological disorders, such as neutropenic patients, solid-organ transplant recipients, or patients undergoing long-term corticosteroid therapy. This is particularly noted with *Scedosporium* species. Some species of Chaetothyriales (such as *C. bantiana*) are able to cause deep or disseminated infections in hosts with no known immune disorder. If untreated, such infections may take a chronic, fatal course after a destructive disease process. The frequency of these infections is low, but given the potentially severe course of the disease, as well as the sometimes very high degrees of resistance to antifungal drugs, attentiveness to these fungi is mandatory. Chromoblastomycosis is a relatively frequent infection with significant morbidity in rural subtropical regions. *Sporothrix* (order Ophiostomatales) is the only agent of infection that increases in severity with defects of acquired cellular immunity, such as AIDS (59).

Among the specific disease entities caused by the melanized fungi are the following.

Phaeohyphomycosis

Superficial

Botryosphaeriales

Neoscytalidium dimidiatum is a common plant pathogen in the tropics and is regularly involved in syndromes very similar to dermatophytosis on skin and nails, usually leading to extensive hyperkeratosis (60).

Capnodiales

The halophilic species *Hortaea werneckii* can adhere to exceptionally salty human hands (46), causing a syndrome in the dead keratin layers known as tinea nigra.

Chaetothyriales

Some species of the order Chaetothyriales cause occasional superficial infections in humans. Recently described was *Knufia epidermidis*, which so far has almost exclusively been found on human skin (16). The species may cause asymptomatic infections, may be found in association with dermatophyte infections, or may cause mild cutaneous infections. *Cyphellophora laciniata* and *C. pluriseptata* are occasionally isolated from human skin and nails (7), but their etiology has not unambiguously been proven. *Phialophora europaea* is fairly commonly involved in mild skin and nail infections (58), but due to its slow growth, it is frequently overlooked.

Cutaneous and Corneal

Botryosphaeriales

Lasiodiplodia theobromae is occasionally found to cause ocular infections following injury to the cornea (61, 62).

Capnodiales

Species of *Cladosporium*, especially *C. cladosporioides* and *C. oxysporum*, are associated with allergic disease in the indoor environment, with numerous (sub)cutaneous (63, 64) and even deep (65) infections, but there remains some doubt about their pathogenic role.

Chaetothyriales

Cladophialophora emmonsii, *Cladophialophora boppii*, and *Cladophialophora saturnica* are rare agents of mild cutaneous infections (66, 67); also, several *Exophiala* species may cause skin infections with various degrees of severity (42).

Pleosporales

One of the main recognized clinical entities associated with Pleosporales is cutaneous infection in immunosuppressed patients caused by *Alternaria* and mainly affecting patients on long-term steroid usage, tacrolimus, or other immunosuppressive agents (68). Nearly all cases were caused by *Alternaria infectoria* (69, 70) and *A. alternata* (71, 72), the two main saprobic species of the genus (33). Infections attributed to *A. chlamydospora* (73, 74) probably concerned meristematic segregants of *A. alternata*. Several cutaneous infections have been attributed to *A. tenuissima* (75), which is only doubtfully separate from *A. alternata* (33). *Exserohilum rostratum* can occasionally cause infections of the skin and cornea (36).

Subcutaneous

Botryosphaeriales

Rare cases of subcutaneous infection by *Lasiodiplodia theobromae* (76) and *Neoscytalidium dimidiatum* (77) have been reported.

Calosphaeriales

Members of the order Calosphaeriales are typical agents of subcutaneous infections. Most of the six pathogenic species of *Phaeoacremonium* cause this type of infection, *P. parasiticum* being the most common (78, 79). Human infections by *Pleurostomophora richardsiae* mostly involve subcutaneous cysts (80), occasionally with bone involvement (81). Most patients have some underlying condition such as diabetes or transplantation (82). A case of yellow-grain mycetoma was caused by *P. ochracea* (5).

Chaetothyriales

Exophiala dermatitidis is the most commonly encountered species in clinical settings, causing infections of cutaneous and subcutaneous tissues (83) in mostly immunocompromised patients. *E. oligosperma* is a common etiologic agent of subcutaneous infections in immunosuppressed, elderly, diabetic, or otherwise debilitated individuals (84, 85). *Exophiala jeansemei* is considered an agent of mycetoma (86), often in otherwise healthy individuals.

Microascales

Several species of *Scedosporium* are common causes of subcutaneous infections and mycetoma in temperate regions, especially in North America (87, 88), having a predilection for the joints.

Pleosporales

Members of the order Pleosporales (most commonly *Curvularia*, *Exserohilum*, and *Alternaria*) are able to cause subcutaneous infections, although more commonly they produce allergic sinusitis with occasional cerebral involvement in otherwise healthy individuals (36, 41, 68, 89).

Systemic

Botryosphaeriales

Lasiodiplodia theobromae has been involved in a case of pneumonia (90). *Neoscytalidium dimidiatum* caused deep infections in immunocompromised patients (91, 92).

Calosphaeriales

Phaeoacremonium parasiticum is able to cause disseminated infections in debilitated patients (93).

Chaetothyriales

A disease entity that is largely confined to Chaetothyriales is primary cerebral infection in immunocompromised or immunocompetent individuals, i.e., cerebritis in which the first symptoms of disease are of a neurologic nature. Hyphal elements that show melanization either directly or after Fontana-Masson staining are observed in abscesses in the brain parenchyma. The portal of entry may be the lung, but frequently symptoms are confined to the brain. Five species account for most nontraumatic brain infections. *Cladophialophora bantiana* has caused about one-third of the cases in otherwise healthy individuals (40, 82, 94, 95). If untreated, the infections are fatal within 1 to 6 months. *Exophiala dermatitidis* is responsible for a striking number of fatal, neurotropic infections in young, healthy individuals, all in Asia (96). The other three species are *Cladophialophora modesta*, *Fonsecaea monophora*, and *Rhinocladiella mackenziei*. *Rhinocladiella mackenziei* is a remarkable fungus because it is exclusively known from fatal brain infections in the Middle East or from immigrants from that region (41). Several patients had no known immune disorder; the environmental niche of the species is unknown. Occasionally systemic dissemination is observed in patients with or without proven immune disorder; these infections are fatal if they go untreated. Secondary cutaneous lesions often lead to marked eruptions with high morbidity. The disorder has been observed repeatedly in *Cladophialophora devriesii*, *C. modesta*, *C. arxii*, *Exophiala dermatitidis*, and *E. spinifera* (41, 97, 98). The last two species have capsule-like extracellular polysaccharides around yeast cells (99) and have a high virulence in humans. The rare infections by *E. spinifera* can be localized, but in about one-half of the cases, fatal, disseminated mycoses are observed in adolescents (reviewed by de Hoog et al. [100]). *Exophiala dermatitidis* and, to a lesser extent, *E. phaeomuriformis* are regular pulmonary colonizers of patients with cystic fibrosis (101).

The etiologic agents in cases of phaeohyphomycosis published under the name *F. pedrosoi* should be reconsidered, since it has been observed that the second species of the genus, *F. monophora*, has a more diverse clinical spectrum which includes brain infection (41). Occasionally, opportunistic infections including endocarditis and osteomyelitis caused by *Phialophora verrucosa* have been reported (102, 103). It is possible that some of these infections were attributable to *P. americana*.

Dothideales

Aureobasidium pullulans has been implicated as an agent of catheter-related septicemia, peritonitis, and disseminated infection (41, 104), and *Hormonema dematioides* has been implicated in a case of peritonitis (105).

Microascales

Relatively common deep and disseminated infections caused by *Scedosporium* are noted for immunosuppressed or otherwise debilitated patients. The reported clinical spectrum of *S. apiospermum*/*P. boydii* has changed over time, from prevalently chronic mycetoma in otherwise healthy patients between 1911 and 1980 to systemic opportunistic infection after 1980 (87, 88). Systemic infections after solid-organ transplantation are relatively frequent. Osteomyelitis and arthritis are also relatively common (87, 88). Fisher et al. (106) were the first to describe an association of *S. apiospermum* with a near-drowning syndrome, which characteristically leads to delayed, potentially fatal brain infection after the patient has recovered from the primary effects of aspiration of polluted water. In severely compromised patients, cerebral dissemination may take place from local foci. *P. boydii* shows a high incidence in cystic fibrosis patients (87, 88).

Despite its rather frequent occurrence in clinical settings, the history of *Scedosporium prolificans*, one of the most virulent species of Microascales, is remarkably short (41). Malloch and Salkin described the first clinical cases in 1984 (107). Since then, numerous strains have been recovered, mostly from clinical cases with major immunosuppression, and since no older reports of cases in immunocompetent patients are known, the species is a truly emerging opportunist (108–110). The species has been reported from bone and soft tissue infections (87, 88) as well as from (secondary) cutaneous infection, fungemia, and endocarditis (87). The fungus may disseminate to visceral organs in immunocompromised individuals (81). A nosocomial outbreak has been described (111).

Venturiales

Verruconis gallopava is also the cause of epizootic encephalitis in flocks of turkeys and chickens (86) and is able to cause pulmonary infections in immunocompetent individuals (112, 113).

Pleosporales

A wide spectrum of opportunistic infections has been attributed to *Curvularia*, including endocarditis, pulmonary infection, cerebral infection, and peritonitis (114–116). *Exserohilum rostratum* caused a large iatrogenic outbreak due to injection with contaminated methylprednisolone preparations leading to numerous fatal cases of meningitis and central nervous system vasculitis (117–120).

Chromoblastomycosis

Chromoblastomycosis occurs in otherwise healthy patients; it is characterized by chronic, cutaneous to subcutaneous lesions and frequently with marked hyperplasia. A primary lesion is represented by a papula at the site of inoculation that slowly enlarges over time, becoming a tumoral (cauliflower-like) lesion that can spread via the lymphatic system, although hematogenous dissemination has also been proposed. Lesions contain the typical and resistant spherical and mostly cruciately septate muriform (sclerotic) bodies, indicative of chromoblastomycosis.

Three fungal species of Chaetothyriales account for virtually all cases of chromoblastomycosis: *Cladophialophora carrionii*, *Fonsecaea pedrosoi*, and *Phialophora verrucosa*. Infections by *C. carrionii* mainly occur in arid climates (121, 122), probably acquired via traumatic inoculation of cactus spines (123). Occasionally *Cladophialophora boppii* (124) and, more recently, the new species *Cladophialophora samoënsis* have also been reported as causes of chromoblastomycosis (66). Infections by *P. verrucosa* mainly occur in tropical climatic zones (125, 126). *Rhinocladiella aquaspersa* is a rare agent of chromoblastomycosis in South America (127–129).

Sporotrichosis

Sporotrichosis is a cutaneous to subcutaneous chronic infection that can undergo lymphatic spread. Musculoskeletal involvement and disseminated infection are relatively rare, as are the nasal and pulmonary infections that may arise from inhalation of conidia (46, 130, 131). Most infections originate from traumatic implantation of the fungus. Some disseminated cases were observed in AIDS patients (132, 133). In addition, infections have been described as being transmitted by animals (134, 135).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Collection, transport, and storage of specimens are described in chapter 114. Appropriate collection of specimens is essential for mycological study. There should be sufficient quantity for direct microscopic examination as well as for isolation.

DIRECT EXAMINATION

Microscopy

Melanization of vegetative cells or conidia, which results in colony coloration ranging from olive or gray to black, is caused by the deposition of dihydroxynaphthalene melanin in cell walls. This property occurs commonly among species classified in very different parts of the fungal kingdom. The amount of melanin expressed in host tissue may be very small and difficult to observe using traditional histologic stains. The use of the Fontana-Masson stain (136) (see chapter 115) for demonstrating the presence of melanin is therefore recommended as a routine to distinguish fungi with melanized hyphae from those causing "hyalohyphomycosis," e.g., *Aspergillus*. This does not apply for *Sporothrix* and *Scedosporium*, which are not melanized but are able to produce melanized conidia either *in vitro* or *in vivo* or both. *Sporothrix* has a characteristic yeast form in tissue; the recognition of *Scedosporium* rests upon methods other than histopathology.

There are numerous procedures for treating the specimen to enhance fungal detection, including treatment with potassium hydroxide or calcofluor white and Giemsa, Wright, or Gram stain (see chapter 115). Histopathology procedures using periodic acid-Schiff (PAS), hematoxylin and eosin, or methenamine silver enhance observation. Fontana-Masson staining is useful to detect melanization in cells that appear hyaline with light microscopy. To summarize, some characteristic features include the following.

Skin Infections

Pigmented hyphal fragments, occasionally melanized yeast-like cells, are visible in infected skin tissue (Fig. 5).

Sinusitis

In sinusitis, the sinuses are occluded by amorphous fungus balls. Histologic examination reveals the presence of dense masses of pigmented, branched, and septate hyphae not invading the mucosa.

Cerebral Infection

In cases of cerebral infection, the hyphae are often poorly colored and scantily branched. In advanced cases, abscesses are formed (Fig. 5).

Disseminated Infection

Pigmented or poorly colored fungal elements such as hyphae or yeast-like cells can be observed in cases of disseminated infection (Fig. 6).

Chromoblastomycosis

The hallmark of chromoblastomycosis is the presence of muriform or sclerotic cells in tissue sections or wet preparations of pus, scrapings, or biopsy samples. Muriform cells are swollen, spherical, dark brown, thick-walled cells which often develop a septum and may finally become divided by intersecting septa in more than one plane (Fig. 6).

Sporotrichosis

In sporotrichosis, hyaline yeast-like cells are occasionally present, bearing slender daughter cells at very narrow bases. They are usually few in number and may easily be missed during microscopy, so Gomori methenamine-silver (GMS) or PAS staining is therefore recommended; Fontana-Masson staining is negative (Fig. 6).

Antigen and Nucleic Acid Detection

There are few data for detection of antigens or nucleic acids in human clinical samples for diagnosis of the mycoses included in this chapter. A panfungal PCR assay has been developed that targets the ITS1 region of the rDNA gene cluster for detecting fungal DNA in fresh and paraffin-embedded tissue specimens. This method was useful for the identifying species of *Scedosporium*, *Exophiala*, and *Exserohilum*. PCR products were sequenced and compared with sequences in the GenBank database (137). Linked to the recent outbreak of *Exserohilum rostratum* infections due to injections of contaminated steroids in United States, several molecular tests were developed for rapid identification of the fungus. Gade et al. developed a test based on the detection of free-circulating fungal DNA from human fluids and subsequent PCR amplification, using panfungal and *Exserohilum*-specific primers which target the ITS2 region, and sequencing. This test proved to be more specific than cultures (138). A very sensitive *Exserohilum rostratum*-specific real-time PCR for rapid detection and quantification of the fungus in cerebrospinal fluid or brain tissues of patients with meningitis has also been developed (139).

ISOLATION PROCEDURES

The use of nutritionally minimal media such as 2% water agar, moistened sterile wooden sticks, or moistened sterile filter paper stimulates the formation of conidia. In the case of infection due to more than one agent, the strain that grows in a more limited manner may pass unnoticed for a long period. Therefore, a loopful of cells suspended in 0.1% Tween and streaked onto a fresh culture plate is useful to select an individual colony for identification.

IDENTIFICATION

For the identification of most of the species included in this chapter, the classical method is to grow the fungi in culture and to examine the relevant morphological characteristics described above. This will mostly provide

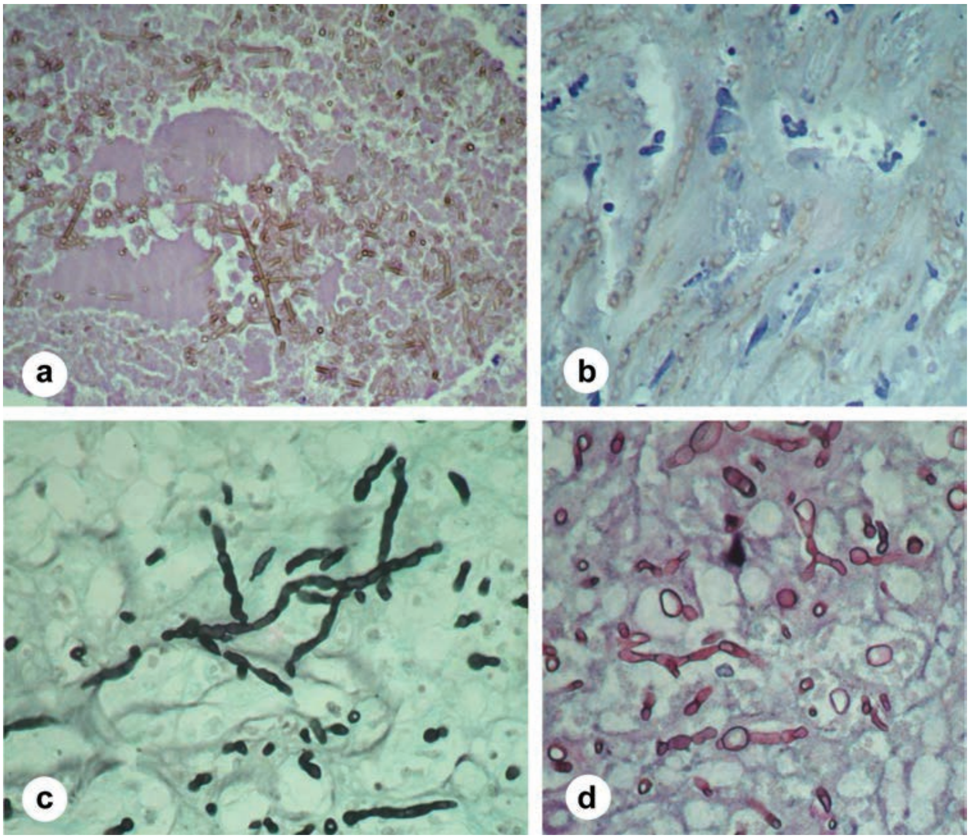

FIGURE 5 (a) Hematoxylin and eosin stain of subcutaneous nodule biopsy sample showing branching hyphae of *Fonseacea monophora*; (b to d) brain tissue showing irregular, branching, and septate hyphae of *Cladophialophora bantiana*, stained with hematoxylin and eosin stain (b), GMS stain (c), and PAS stain (d). Magnifications, ×250 (a) and ×400 (b to d). (Courtesy of E. Mayayo, Universitat Rovira i Virgili.) doi:10.1128/9781555817381.ch124.f5

an identification at the generic level. Appropriate culture media are described in chapter 115. Slide culture preparations (36) using potato dextrose agar or cornmeal dextrose agar, to be handled only within a biological safety cabinet, are ideal for determining conidiogenesis. For morphological features of the fungi treated in this chapter, the reader is referred to the work of de Hoog et al. (2).

Molecular identification of most species is currently performed by sequencing of ribosomal genes and comparison with dedicated databases (140). Care should be taken when the GenBank database is used for the purpose of identification in less known fungal groups, because over 10% of the sequences may be incorrect (33). Sequences should be evaluated not only from a technical point of view but also nomenclaturally, i.e., by comparison with ex-type strains.

When ribosomal genes are used, the phylogenetic position of taxa can be established by sequencing the nuclear SSU (18S) or partial large subunit (26S) rRNA gene. This gene is mostly invariant between closely related species. In most fungal groups, species diagnostics is possible with ITS sequences. But application of this technique as a "gold standard" for melanized fungi is still in dispute. For some genera, such as *Alternaria*, the numerous species described on the basis of morphology prove to be invariant in the ITS region. In this case the question as to whether ITS shows insufficient polymorphism or whether simply too many species have been introduced remains. In contrast, ITS-based species distinction with black yeasts provides satisfactory results. For some genera, such as *Sporothrix*, sequencing of the calmodulin gene is necessary to gain resolution of species within the complex (30). In *Scedosporium* (26) and *Phaeoacremonium* (79), β-tubulin gene sequencing is used for this purpose.

TYPING SYSTEMS

A diversity of high-resolution molecular typing systems has been developed in recent years. These have been applied mainly to epidemiological tracking of fungal pathogens in the hospital and the community but also to show geographical differences among isolates and to detect cryptic species.

Chaetothyriales

On the basis of a multigene phylogeny, it has been demonstrated that important phenotypic features have evolved independently several times in the order Chaetothyriales and that most of the species of *Cladophialophora* belong to a monophyletic group comprising two main clades (*carrionii* and *bantiana* clades) (66).

In a study that investigated the molecular diversity of oligotrophic and neurotropic members of the genus *Exophiala* using ITS sequences and M-13 fingerprint and SSU intron data, two main groups could be distinguished within *E. dermatitidis*. The environmental strains were mainly placed in one of these groups, while the clinical strains were

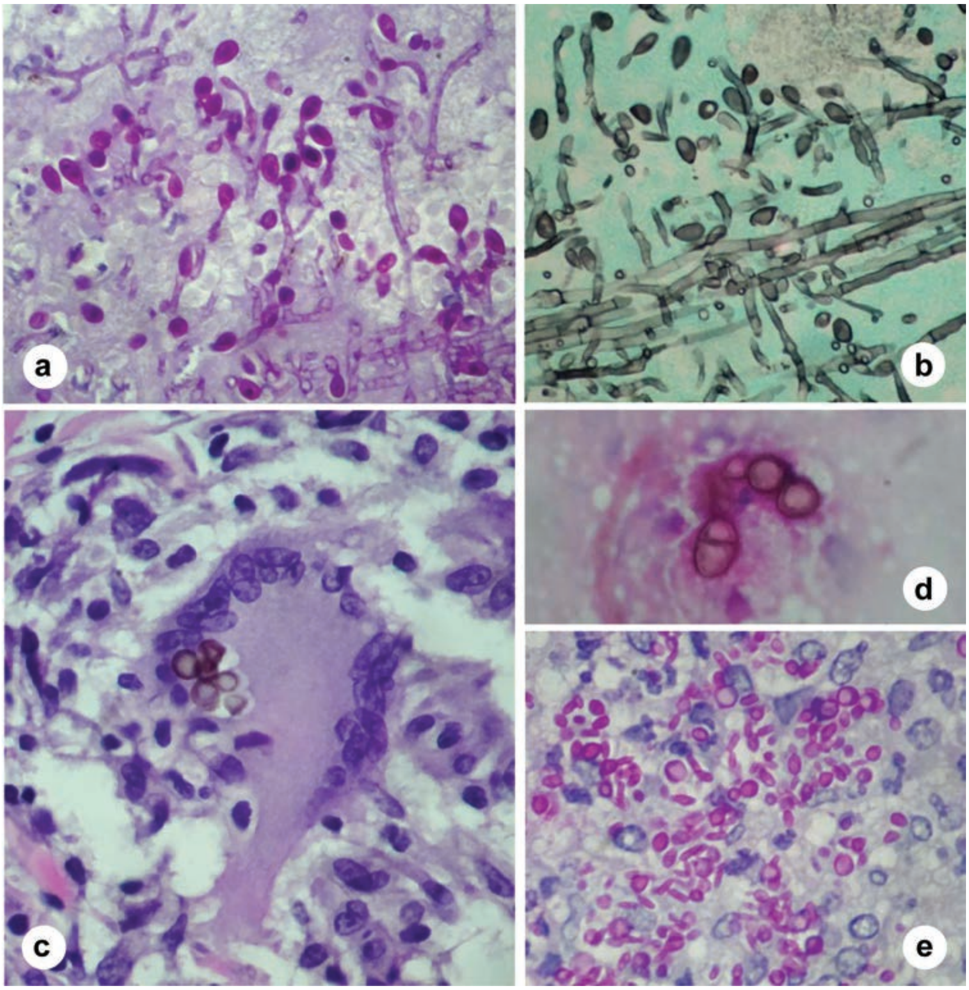

FIGURE 6 (a and b) Lung tissue with numerous conidia and hyphae of *Scedosporium prolificans* stained with PAS (a) and GMS (b); (c) PAS stain of a subcutaneous nodule biopsy sample showing sclerotic cells of *Fonsecaea pedrosoi* within a giant cell; (d) PAS stain of a fine-needle aspirate of a subcutaneous nodule showing sclerotic cells of *Fonsecaea pedrosoi*; (e) PAS stain of a hepatic vessel showing a massive embolization by *Sporothrix schenckii* showing characteristic elongate cells. Magnifications, ×400. (Courtesy of E. Mayayo, Universitat Rovira i Virgili.)
doi:10.1128/9781555817381.ch124.f6

in the second one. Interestingly, strains from East Asia that clustered in the clinical group caused severe brain and disseminated infections, and strains of the same group recovered from outside East Asia caused only a relatively mild fungemia (11).

The natural niche of *Fonsecaea*, one of the most common agents causing chromoblastomycosis, remains uncertain. To elucidate where and how patients acquire the infection, probably through traumatic inoculation, numerous isolates with *Fonsecaea*-like morphology from environmental sources were typed using random amplified polymorphic DNA methodology. The results revealed a high degree of strain diversity and showed that most strains isolated from environments to which symptomatic human patients were exposed were found to be more closely related to species of *Cladophialophora* than to *Fonsecaea* (13).

Dothideales

Multilocus typing at four loci demonstrated the existence of at least five different sequence types in isolates of *Neoscytalidium dimidiatum*, of which two were detected exclusively in isolates from plants, two were found only in clinical isolates, and one was observed in isolates from humans and from a mango tree. This has been proposed as the possible source of infection in a case of mycetoma in an agricultural field worker and should be considered as a potential reservoir of pathogenic strains of the fungus (141).

Microascales

In numerous molecular studies there has been a high genetic diversity among isolates of *Scedosporium* from different origins (reviewed in reference 87). However, recent multilocus sequence analysis has shown that such variability could be explained by the existence of numerous cryptic species in the *Pseudallescheria boydii* complex (25, 26). In contrast, in *S. prolificans* genetic variation seems to be low to absent.

Ophiostomatales

With *Sporothrix*, as with *Scedosporium*, numerous molecular studies (reviewed in reference 30) have proven the existence of a high level of intraspecific variability, with isolates mainly grouped according to their geographical origins. But recent multilocus studies have demonstrated that *S. schenckii sensu lato* comprises several phylogenetic species and morphospecies (28, 29) with a marked geographical distribution; i.e., *S. brasiliensis* isolates are mainly found in Brazil, and all the isolates from India tested molecularly belong to *S. globosa*. This likely correlates with the genetic and morphological diversity shown within *S. schenckii* by many authors.

SEROLOGIC TESTS

A latex agglutination test is commercially available (Immuno-Mycologics Inc., Norman, OK) for detecting antibodies against *S. schenckii*, particularly in disseminated cases, but despite a study (142) reporting a sensitivity of 90 to 94% and a specificity of 95 to 100%, the test has not been used widely. However, the detection of antibodies was demonstrated to be useful in the diagnosis of central nervous system sporotrichosis in several patients when culture-based diagnosis had failed (143).

ANTIFUNGAL SUSCEPTIBILITIES

In Vitro

The available *in vitro* data for dematiaceous fungi are increasing every day, and in general the antifungal susceptibilities of the most clinically relevant species are known. However, interpretive breakpoints have not been defined, and clinical correlation data are practically nonexistent.

Amphotericin B generally has good *in vitro* activity against most clinically important dematiaceous fungi, such as *Exophiala* (144) and *Alternaria* (145). However, some species have been consistently resistant *in vitro*, including *Scedosporium* spp. and *Scopulariopsis brumptii* (88, 146–148). The azoles, in general, have demonstrated the most consistent *in vitro* activity against dematiaceous fungi (reviewed in reference 88) apart from *S. prolificans* (146).

The newer triazoles posaconazole and voriconazole have a broad spectrum of activity, being active against most of the fungi included in this chapter. The activities of these triazoles were similar against agents of chromoblastomycosis (149) and *Pseudallescheria boydii* complex (147). The activity of posaconazole is higher than that of voriconazole against *Alternaria* spp. (145), *Exophiala* spp. (144), and *Sporothrix* spp. (150), although against the last the activity of both drugs was very poor. Terbinafine showed a clear fungicidal activity *in vitro* against filamentous fungi. Studies of *in vitro* activity against dematiaceous fungi are emerging and fairly broad-spectrum activity is seen, including against *Alternaria* and *Curvularia* (41). Echinocandins appear to have variable and species-dependent fungistatic activities for the dematiaceous fungi (151). Micafungin has demonstrated some moderate activity against *Scedosporium* spp. (148). The *in vitro* activities of the available antifungal drugs against selected dematiaceous fungi have been reviewed recently (41).

In Vivo in Animal Models

Based on the small number of clinical cases produced by most of the fungi discussed in this chapter, the ideal treatment regimen against the infections that most of them produce is not yet known. Therefore, studies using different animal models have been designed to evaluate efficacy and/or corroborate the results obtained *in vitro*. Animal studies have demonstrated that posaconazole is generally the most effective against infections by less common moulds, including some of those included in this chapter (reviewed in reference 152).

Chromoblastomycosis

Recent studies using animal models of chromoblatomycosis testing athymic mice demonstrated that posaconazole showed higher efficacy than the recommended drugs, itraconazole and terbinafine, against infections by *F. pedrosoi* (153) and *Cladophialophora carrionii* (154); voriconazole did not work against the former species.

Scedosporiosis

Voriconazole and posaconazole, but not amphotericin B, showed efficacy that correlated with *in vitro* MICs against *Scedosporium boydii*, *S. apiospermum*, and *S. aurantiacum*. No drug has shown efficacy against *Scedosporium prolificans* infections. The effects of double or triple antifungal drugs combinations were tested against *S. prolificans* murine infections, and micafungin plus voriconazole or amphotericin B was the most effective, being able to prolong mouse survival and to reduce fungal load in the kidneys and brain. The combination of all three mentioned drugs was ineffective (155). In a few clinical cases, the combination of terbinafine and voriconazole demonstrated synergy and favorable results (156–158).

Phaeohyphomycosis

Different animal studies have evaluated the most useful antifungal drugs for treating disseminated infections produced by some clinically relevant fungi. Against *Fonsecaea monophora* (159) and *Exophiala dermatitidis* (160) posaconazole was better than amphotericin B and itraconazole. In an experimental treatment of *Neoscytalidium dimidiatum* amphotericin B worked better than voriconazole and posaconazole (161). For the treatment of *Cladophialophora bantiana* murine studies tested amphotericin B, micafungin, voriconazole, flucytosine, and posaconazole alone and in double and triple combinations. The only therapeutic regimen that was able to prolong animal survival for at least 10 months was the combination of three drugs (posaconazole, micafungin, and flucytosine) (162).

Sporotrichosis

Posaconazole showed excellent efficacy in experimental infections against the two most common species causing sporotrichosis, i.e., *S. schenckii* and *S. brasiliensis* (163).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

More than 100 species of melanized fungi have caused infection in humans and animals, and many of these are relatively rare as etiologic agents. As a result, few clinicians are familiar with these fungi and they are frequently overlooked. Infections caused by dematiaceous fungi are being diagnosed increasingly among healthy as well as compromised patients. Concurrently, the expanding diversity of etiology within this group of fungi is becoming apparent.

Determining whether a particular dematiaceous fungus is involved in a disease process can be difficult because most of these fungi are occasionally recovered as contaminants from clinical specimens. Repeated recovery of a suspected etiologic agent is significant, while DNA sequence identity of clinical material and the isolate is highly supportive. Isolation of a dematiaceous fungus from a normally sterile body site should not be dismissed as contamination, particularly if colonies are numerous or more than one culture plate shows growth. If isolated from a nonsterile pulmonary specimen such as skin, sputum, or bronchial lavage fluid, well-documented opportunists from genera such as *Cladophialophora*, *Fonsecaea*, *Verruconis* (*Ochroconis*), or *Scedosporium*, which are not seen as contaminants, also are highly indicative. Correlation between culture and histopathology results should also be determined.

Failure to order fungus culture when tissues are collected during surgical procedures is an increasing problem in the management of these infections, and clinicians should order fungus culture whenever warranted. In the future, direct identification of fungal genera from tissue blocks using immunohistochemistry, *in situ* DNA hybridization, or DNA sequencing will be a promising approach to rapid detection and identification of these agents.

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Fungi Causing Eumycotic Mycetoma*

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TAXONOMY AND DESCRIPTION OF THE AGENTS

All fungi known to cause eumycetoma probably belong to the phylum Ascomycota, although for many species no ascus-producing state is known (1). Ordinal relationships are hypothesized on the basis of ribosomal small subunit (2) and internal transcribed spacer (ITS) DNA sequences (3) (Tables 1 and 2; see also listing by order below). The etiologic agents described in this chapter are species that are isolated most commonly from human or lower-animal mycetoma.

Order Chaetothyriales

Exophiala jeanselmei

E. jeanselmei is one of the black yeasts, which belong to the ascomycete order Chaetothyriales. Members of this order are frequently involved in black grain mycetoma in the tropics (1, 4). *E. jeanselmei* (see Fig. 4) has been reported as an agent of eumycetoma in India (5, 6), Malaysia, Thailand (7), Argentina (8), and the United States (9, 10), but only a few cases (11, 12) have been confirmed by molecular data (13).

The related fungus *Cladophialophora bantiana*, a species that in humans is linked mainly with brain infections (14), was recently reported to cause mycetoma in humans (15, 16) and dogs (17, 18).

Order Diaporthales

Phaeoacremonium Species

Phaeoacremonium is the anamorph filamentous fungus of the teleomorph genus *Togninia*, which belongs to the order Diaporthales in the class Ascomycetes (19). *Phaeoacremonium* contains many species associated with plants and human infections (19). Members of the genus *Phaeoacremonium* are occasionally reported as agents of mycetoma. de Albornoz (20) described a case of infection caused by *Phaeoacremonium inflatipes*, Rowland and Farrar (21) and Hemashettar et al. (22) described cases of infection by *Phaeoacremonium krajdenii*, and Hood et al. (23) described a case of infection by *Phaeoacremonium parasiticum*. A mycetoma with yellow grains caused by *Pleurostomophora ochracea* was published by Mhmoud et al. (24).

Order Dothideales

Neotestudina rosatii

N. rosatii, which has been isolated from soil from tropical countries, is classified in the class Ascomycetes, order Dothideales (1, 2). Mycetomata caused by *N. rosatii* have been described in Australia, Cameroon, Guinea, Senegal, and Somalia (25, 26).

Order Hypocreales

Fusarium falciforme

F. falciforme is the filamentous fungus previously known as *Acremonium falciforme* (27). The genus *Fusarium* is classified in the order Hypocreales in the class Ascomycetes (see chapter 120). *F. falciforme* (see Fig. 5) is occasionally found as a cause of mycetoma in the United States and Argentina (28–30). It was also reported as the cause of an opportunistic mycetoma infection in a renal transplant recipient (31).

Order Microascales

Pseudallescheria/Scedosporium Species

The genus *Scedosporium* comprises a larger number of species than supposed originally (32–34). The most clinically significant species among *Scedosporium* species is *Scedosporium apiospermum*. Until recently, *S. apiospermum* has been considered the anamorph of *Pseudallescheria boydii* (the currently accepted anamorph is *Scedosporium boydii*). However, it becomes evident that they are phylogenetically different species (see chapter 124) (32, 33). Therefore, previously reported mycetoma cases attributed to *S. boydii* might actually have been caused by a different species.

S. boydii is associated with manure and polluted environments (35, 36) and has been known as an agent of human mycetoma since the 1920s (37). *S. boydii* is the most common agent of mycetoma in humans as well as lower animals in temperate climates (38), occurring mostly in the limbs (39).

Order Pleosporales

Biatriospora mackinnonii (Previously Known as *Pyrenochaeta mackinnonii*)

Molecular analysis of the rRNA genes also revealed that *Pyrenochaeta mackinnonii* is not similar to the type species

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[*] Some of the material in this chapter was presented in chapter 123 by Abdalla O. A. Ahmed and G. Sybren de Hoog in the 10th edition of this *Manual*.

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TABLE 1 Overview of main species causing eumycotic mycetoma

Order	Species	Geographic distribution	Color of grains	Size of grains (mm)
Chaetothyriales	*Exophiala jeanselmei*	Worldwide	Black	0.5–1.0
Diaporthales	*Phaeoacremonium* spp.	South America, India	White or black	0.5–2.0
Diaporthales	*Pleurostomophora ochracea*	Sudan	Yellow	0.5–1.0
Dothideales	*Neotestudina rosatii*	Central Africa	Whitish	0.5–1.0
Hypocreales	*Fusarium falciforme*	Worldwide	Whitish	0.2–0.5
Microascales	*Scedosporium boydii*	North and South America	Whitish	0.2–2.0
Pleosporales	*Biatriospora mackinnonii*	Central and South America	Black	0.3–1.0
Pleosporales	*Curvularia geniculata*	Worldwide	Black	0.5–1.0
Pleosporales	*Curvularia lunata*	Worldwide	Black	0.5–1.0
Pleosporales	*Falciformispora senegalensis*	West Africa, India	Black	0.5–2.0
Pleosporales	*Medicopsis romeroi*	Arid subtropics	Black	0.3–1.0
Pleosporales	*Trematosphaeria grisea*	South America, India	Black	0.3–0.6
Sordariales	*Madurella mycetomatis*	East Africa, Middle East	Black	Up to 5.0 or more

of *Pyrenochaeta* (*Pyrenochaeta nobilis*) (40, 41). Furthermore, *Medicopsis romeroi* (formerly *Pyrenochaeta romeroi*) and *Pyrenochaeta mackinnonii* were found to be phylogenetically remote from each other. Therefore, *Pyrenochaeta mackinnonii* was assigned to the genus *Nigrograna* and renamed *Nigrograna mackinnonii* by de Gruyter et al. in 2012 (41). Later, after more-extensive phylogenetic analyses, it was reassigned to *Biatriospora mackinnonii* by Ahmed et al. (S. A. Ahmed, W. W. J. van de Sande, D. A. Stevens, A. H. Fahal, and G. S. de Hoog, unpublished data) (41). *B. mackinnonii* has been reported as the causative agent of mycetoma in patients originating from Mexico and Venezuela.

Curvularia Species

Numerous species of *Curvularia* are known, mostly occurring in decaying vegetations. *Curvularia* is a hyphomycete dematiaceous mold, with some known teleomorphs belonging to the class Euascomycetes in the phylum Ascomycota (1). The ubiquitous saprobe *Curvularia lunata* (Fig. 1 and 2) has been described as an etiologic agent of mycetoma in humans in Senegal and Sudan (42, 43), and the common saprobe *Curvularia geniculata* has occasionally been reported as an etiologic agent of mycetoma in dogs in the United States (44).

Falciformispora senegalensis (Previously Known as *Leptosphaeria senegalensis*)

Based on the combined DNA sequence data set of the 18S small ribosomal subunit (SSU), the 26S large ribosomal subunit (LSU), RNA polymerase second largest subunit (*RPB2*), and translation elongation factor 1-alpha (*TEF1*) genes, it was demonstrated that *Leptosphaeria senegalensis* and *Leptosphaeria tompkinsii* were found to cluster in the pleosporalean order *Trematosphaeriaceae*, with *Falciformispora lignatilis* as its closest relative. Therefore, *Leptosphaeria senegalensis* was renamed *Falciformispora senegalensis* and *Leptosphaeria tompkinsii* as *Falciformispora tompkinsii* (Ahmed et al., unpublished). *F. senegalensis* is a dematiaceous filamentous fungus with a known teleomorph belonging to the class Euascomycetes in the phylum Ascomycota (1). *F. senegalensis* and the related species *F. tompkinsii* cause mycetomata in the northern tropical portion of Africa, especially in Senegal and Mauritania, and in India (45, 46).

TABLE 2 Current and outdated names of some of species that cause eumycotic mycetoma

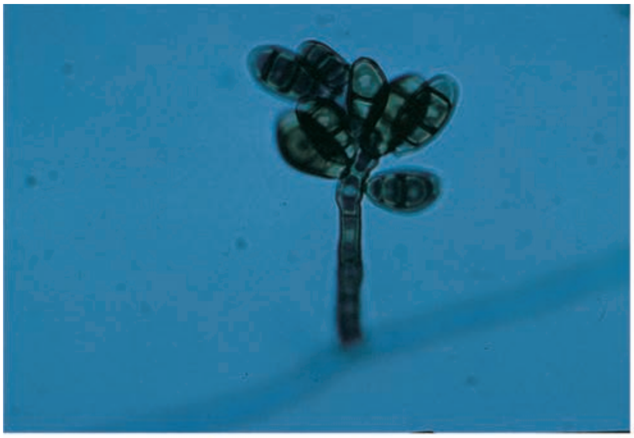
Order	Current species name	Former species name
Hypocreales	*Fusarium falciforme*	*Acremonium falciforme*
Pleosporales	*Biatriospora mackinnonii*	*Pyrenochaeta mackinnonii*
Pleosporales	*Falciformispora senegalensis*	*Leptosphaeria senegalensis*
Pleosporales	*Falciformispora tompkinsii*	*Leptosphaeria tompkinsii*
Pleosporales	*Medicopsis romeroi*	*Pyrenochaeta romeroi*
Pleosporales	*Trematosphaeria grisea*	*Madurella grisea*

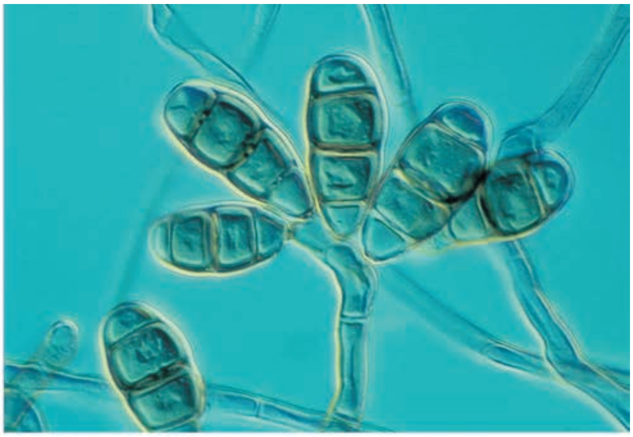
Medicopsis romeroi (Previously Known as *Pyrenochaeta romeroi*)

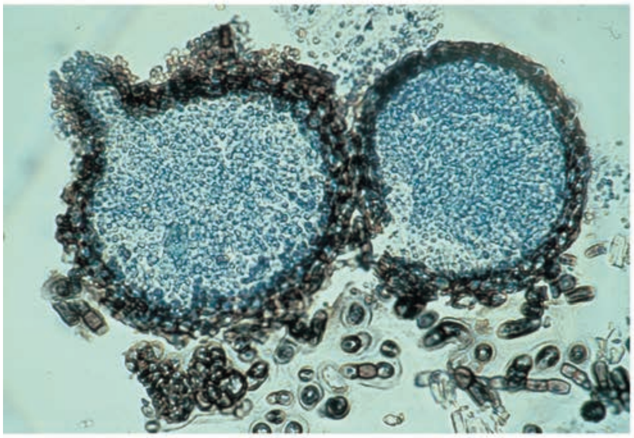
Based on sequences of the 18S ribosomal DNA (rDNA) and the 28S rDNA, it has become clear that *Pyrenochaeta romeroi* is distantly related to the type species of *Pyrenochaeta* (*Pyrenochaeta nobilis*) (40). Therefore, *Pyrenochaeta romeroi* was renamed *Medicopsis romeroi* by de Gruyter et al. in 2012 (41). *M. romeroi* is a coelomycete anamorphic fungus. *M. romeroi* might be misidentified as *T. grisea* when cultures are lacking pycnidia (1). Mycetomata caused by *M. romeroi* (Fig. 3) have been reported from Somalia, India, and South America (10, 25, 47). The color of the colonies and close resemblance of the granules produced by *M. romeroi* to those of *T. grisea* may suggest that the latter species could be a nonsporulating counterpart of *M. romeroi*.

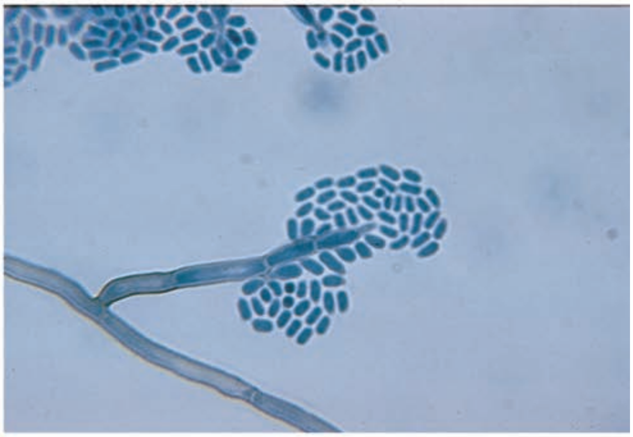
Trematosphaeria grisea (Previously Known as *Madurella grisea*)

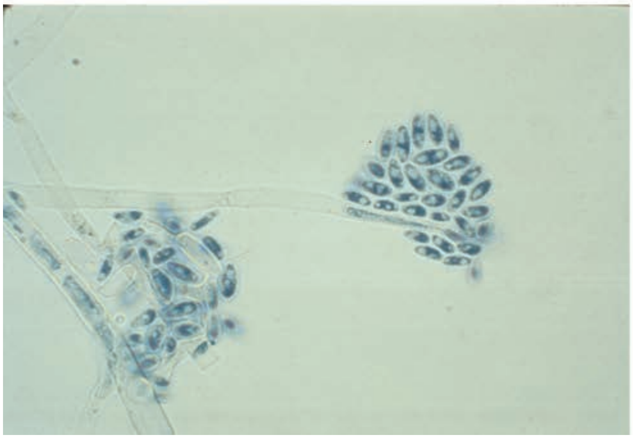
In 2004, it was demonstrated by molecular phylogeny and diagnostics that the members of the genus *Madurella* encompass a hidden diversity beyond the currently recognized species (2). An rRNA restriction fragment length polymorphism was used for species distinction by Ahmed et al. (48, 49), and a specific ITS PCR was used by Ahmed et al. (49). Based on the rDNA sequence, the taxonomic position of *M. grisea* was changed to the order Pleosporales, and therefore it should no longer be considered a sister species to *Madurella mycetomatis*. Using the combined molecular data set of SSU, LSU, *RPB2*, and *TEF1*, it was demonstrated that *M. grisea* formed a well-supported clade with *Trematosphaeria pertusa*. This finding was also confirmed with an ITS tree constructed for the family *Trematosphaeriaceae*. Therefore, it was suggested that the name of *M. grisea* should be changed to *Trematosphaeria grisea* (Ahmed et al., unpublished). *T. grisea* occurs as an etiologic agent of black grain mycetoma in South America, India, Africa, and North and Central America (42, 50–52). The fungus is a coelomycete, but











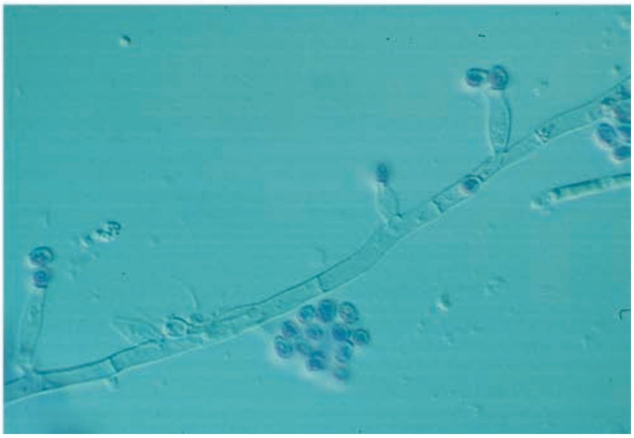


FIGURE 1 (Top left) *C. lunata*. Slide culture on potato dextrose agar showing geniculate conidiogenous cell and septate, curved conidia. Magnification, ×160. doi:10.1128/9781555817381.ch125.f1

FIGURE 2 (Top right) *C. lunata*. Geniculate conidiogenous cell bearing smooth, predominantly four-celled conidia. Magnification, ×160. doi:10.1128/9781555817381.ch125.f2

FIGURE 3 (Middle left) *M. romeroi*. Ostiolate conidiomata (pycnidia) containing numerous cylindric conidia. Magnification, ×250. doi:10.1128/9781555817381.ch125.f3

FIGURE 4 (Middle right) *E. jeanselmei*. Lateral, septate conidiophore bearing closely annellated conidiogenous cell producing smooth, nonseptate, ellipsoidal to cylindric conidia. Magnification, ×160. doi:10.1128/9781555817381.ch125.f4

FIGURE 5 (Bottom left) *F. falciforme*. Slide culture on SDA showing erect, septate conidiophore, phialidic conidiogenous cell, and slightly curved conidia. Magnification, ×160. doi:10.1128/9781555817381.ch125.f5

FIGURE 6 (Bottom right) *M. mycetomatis*. Slide culture on soil extract agar showing lateral phialides and globose conidia. Magnification, ×250. doi:10.1128/9781555817381.ch125.f6 pycnidia (asexual fruiting bodies containing conidia) may remain absent. With strictly sterile cultures, molecular confirmation is required for identification.

Order Sordariales

Madurella mycetomatis

Madurella mycetomatis belongs to the ascomycete order Sordariales. The taxonomy of *Madurella mycetomatis* was determined by a number of phylogenetic studies in which the DNA sequences of the mitochondrial genome, rDNA ITS, beta-tubulin gene (BT2), and RNA polymerase II subunit gene (*RPB2*) have been determined (2, 53, 54). *Madurella mycetomatis* is the most common fungal causative agent of mycetoma. It is common in the arid climatic zone of Africa (Sudan, Mali, and Djibouti) and is occasionally encountered in the Middle East and India.

Madurella pseudomycetomatis, Madurella tropicana, and *Madurella fahalii*

Based on the ITS, *BT2*, and *RPB2* sequences, it appeared that several other species previously misidentified as *M. mycetomatis* belong to different *Madurella* species. These new *Madurella* species include *Madurella pseudomycetomatis*, *Madurella tropicana*, and *Madurella fahalii* (54, 55). Of these species, *M. fahalii* seemed to be the most resistant to antifungal agents. Since these species can be differentiated only by molecular identification, it is not certain how many of the previous reported mycetoma cases attributed to *M. mycetomatis* might actually have been caused by a different species.

EPIDEMIOLOGY AND TRANSMISSION

The number of species involved in mycetoma is increasing, but little is known about the epidemiology and mode of transmission of these species. Case reports are indicative of the presence of certain species in particular geographical locations but do not provide a clear understanding of the environmental distribution of species and the possible ecological niches.

The causal agents of eumycetoma are largely saprobes that live on hard plant materials, such as various types of thorns and spines, associated with soil (56–59). Segretain and coworkers (26, 43), using specific isolation media and techniques, showed that *F. senegalensis* and *F. tompkinsii* could be recovered from about 50% of the dry thorns of *Acacia* trees that they examined, particularly those that had been stained by mud during the rainy season. *N. rosatii* was reportedly isolated from sandy ground (25), and *M. mycetomatis* was isolated from soil and anthills (60, 61). It should be noted, however, that the identity of these fungi has not been verified with molecular methods; the possibility that environmental strains do not always belong to the same species as the clinical strains cannot be excluded. Using ITS sequencing, Badali et al. (4) reidentified a collection of *E. jeanselmei* clinical and environmental strains. *E. jeanselmei* identification was confirmed in all mycetoma or mycetoma-like infections, while other environmental strains were found to belong to other *Exophiala* species, suggesting some predilection for human invasion with particular species. Another example of possible human predilection is *M. mycetomatis*, which is a common agent of black grain mycetoma in the arid climate zones of East Africa. The direct isolation of this species from the environment is difficult (62), but its DNA could be found in both soil and thorns. Recently, phylogenetic findings even suggested dung as a possible new habitat for *M. mycetomatis* (63).

Borelli (64) isolated sterile fungus, reported as *Madurella grisea* (now known as *Trematosphaeria grisea*), from soil in Venezuela; the ecological niche and clinical potential of this rare species have not yet been determined.

However, in the case of other relatively common species, such as *M. mycetomatis* and *Scedosporium* species, phenotypic identification only is not enough to determine the exact epidemiology, since these apparently similar entities might hide great species diversity (see “Taxonomy and Description of the Agents”).

Mycetoma may infect all people living in areas of endemicity but develops more commonly among persons who are in contact with contaminated materials, such as field workers, farmers, and fishermen. There is no obvious immune defect in mycetoma patients; however, some studies showed some genetic association with some defects in immune genes in patients with mycetoma (65–67). It appeared that there were some associations with single nucleotide polymorphisms in genes involved in the functioning of the host immune system, such as those coding for complement receptor 1 (CR1), the CC chemokine ligand 5 (CCR5), chemokines interleukin-8 (CXCL8) and interleukin-10 (IL-10), the interleukin receptor CXCR2, nitric oxide synthase NOS2, and trombospondin-4, and in genes involved in the sex hormone synthesis, such as those coding for cytochrome P450 subfamily 19 (CYP19) and catechol-O-methyltransferase (COMT) (65–67). The latter set could help explain the epidemiological finding that mycetoma is more common in males than in females.

Areas of endemicity are located in tropical climate zones particularly. *M. mycetomatis* is limited to semiarid to arid climates, while *Falciformispora* species are found in the rainforest. Locally acquired mycetomata in temperate climates invariably are caused by *S. boydii*. Cases observed in the United States and Europe caused by species other than *S. boydii* are imported by immigrants from tropical countries. For example, de Hoog et al. (68) reported cases in the Netherlands originating from Indonesia and Suriname, and Ahmed et al. (48) reported *M. mycetomatis* mycetoma cases seen in France but originating in Mali.

Climate has a definite influence on the prevalence and distribution of mycetoma. Rivers that flood each year during the wet season in many countries of Africa and Asia influence the distribution of the causal agents. Rainfall also aids the spread of the etiologic agents on organic matter (69).

CLINICAL SIGNIFICANCE

A mycetoma (plural, mycetomata) (70) is a localized, chronic, noncontiguous, granulomatous infection involving cutaneous and subcutaneous tissues and eventually, in some cases, bones. Mycetomata are generally confined to either the feet or the hands but occasionally affect sites such as the back, shoulders, and buttocks. Rarely, other body sites can be involved such as scalp, eye, jaw, and oral cavity (55, 71–73). The characteristic triad of a painless subcutaneous mass, sinuses, and discharge containing grains (masses of fungal organisms) is characteristic of mycetoma; however, a similar condition (actinomycetoma) may be caused by aerobic actinomycete bacteria.

The disease is more commonly seen in humans than in lower animals. Only a few cases of mycetoma involving such animals as cats, dogs, horses, and goats have been described in the literature (18, 42, 44, 74–76). Most cases occur in otherwise healthy patients. Cases of development of mycetoma after an accidental implantation of the etiologic agent following surgery (77) and in a renal transplant recipient (31) have been reported.

A mycetoma develops after a traumatic injury by microbe-contaminated thorns, splinters, fish scales or fins, snake bites, insect bites, farm implements, and knives. The initial lesion is often characterized by a feeling of discomfort and pain at the point of inoculation. Weeks or months later, the subcutaneous tissue at the site of inoculation becomes indurated, abscesses develop, and fistulae may drain to the surface. As described above, mycetomata are characterized by swelling, granulomas, abscesses, and sinuses from which serosanguinous fluid containing fungal grains are discharged. Grains, which are not seen with other subcutaneous mycoses, can vary from approximately 0.2 to over 5 mm in diameter. The size, color, shape, and internal architecture of the grains vary depending on the species of the etiologic agent. Some of the host material, especially at the periphery of the grain, provides a protective barrier for the fungus against antifungal agents and humoral immune responses such as antibodies. In mycetoma due to *S. boydii*, subcutaneous infections frequently lack the formation of grains when the patients' immunity is impaired (78).

A mycetoma develops slowly beneath thick fibrosclerous tissue. The subsequent phase of proliferation involves the invasion of muscles and intramuscular layers by portions of the sclerotium that break free from their parent structure. The granulomatous lesions can extend as deep as bone, where severe bone destruction, formation of small cavities, and complete remodeling may occur. Early osteolytic damage includes loss of the cortical margin and external erosion of the bone. As the infection progresses, blood, lymphatic vessels, and nerves may be damaged. Frequently, secondary bacterial infections and osteomyelitis producing total bone destruction occur. Pain in mycetoma is often associated with bone involvement and secondary bacterial infection. In mycetoma caused by *S. boydii*, arthritis is frequently observed, due to the predilection of this species for cartilaginous tissues (79–82).

Fungus balls in preexisting lung cavities are sometimes inappropriately called mycetomata (83, 84). In the absence of well-organized grains, they should be referred to as aspergillomas, or simply as fungus balls, depending on the etiologic agent (85). Similarly, mycelial aggregates formed by dermatophytes in cutaneous or subcutaneous tissues differ from grains of mycetomata by lacking granule ontogeny, a distinct Splendore-Höeppli reaction (see "Microscopy" below) surrounding the mycelial aggregates, and the entry of the fungus from the hair follicles into deeper tissue following the rupture of the follicular epithelium. Such infections caused by dermatophytes have been referred to as pseudomycetomata (86).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Methods of collection, transport, and storage of specimens are described in detail in chapter 114. Different specimens can be obtained from patients with eumycetoma, but direct examination and culture of fungi causing mycetoma always require surgical tissue biopsies and/or bone curettage. Tissue materials should be divided and submitted for both microbiology and histopathology examination. It should be noted that tissue biopsy specimens without visible grains usually result in negative cultures. The best specimen for fungal cultures is usually collected during surgery as excisional or incisional biopsy. Grains are not always present in tissue specimens obtained by a cutaneous biopsy procedure with local anesthesia. However, incisional biopsy specimens obtained under local anesthesia, when containing grains, can also result in positive cultures. Grains collected with cotton swabs from the sinus tracts are not recommended, because such grains can be contaminated or dead.

Several grains are usually needed for direct examination and culture, and therefore, as much tissue as possible should be obtained. Tissue should be collected in sterile, dry, leakproof containers and transported to the laboratory as soon as possible at room temperature. If transport is delayed for more than 2 h, specimens can be refrigerated. Small biopsy samples can be covered with 2 to 3 ml of sterile saline to prevent drying during transportation to the laboratory.

DIRECT EXAMINATION

Microscopy

The grains of eumycetomata are composed of septate mycelial filaments at least 2 to 5 μm in diameter. The mycelium may be distorted and unusual in form and size, and the cell walls of the fungi, especially toward the periphery of the grains, are thickened. Vesicles are frequently present, especially at the periphery of the grain. The mycelium of the grains may be embedded in a cementlike substance, depending on the species involved. Wethered et al. (87) described the cement surrounding grains of *M. mycetomatis* as being an amorphous, electron-dense material with areas containing different-sized membrane-bound vesicular inclusions. Recently, Ibrahim et al. studied the composition of *M. mycetomatis* grains, which were found to contain melanin, heavy metals, proteins, and lipids (88). It was suggested that the elements composing the grains in mycetoma may play a role in pathogenicity and resistance to antifungal agents (88, 89). Often, the sclerotia elicit an immune response, known as the Splendore-Höeppli reaction (70, 86), seen histologically in the form of an eosinophilic deposit of amorphous material around the grain. A similar tissue reaction in experimental eumycetoma in mice has been described (90).

Grains are white or yellow-brown ("white grain mycetoma") when the agent is producing mostly hyaline mycelia, and they are black ("black grain mycetoma") in the case of melanized fungi. A single proven yellow-grain fungal mycetoma has been reported thus far, but earlier cases may have been incorrectly diagnosed as bacterial mycetomata (24). Melanin is a high-molecular-weight compound that is anchored to extracellular proteins. A precursor for production of melanin in ascomycetous fungi is 1,8-dihydroxynaphthalene (DHN-melanin) (70, 91). In *Madurella mycetomatis*, melanin was shown to be produced through the DHN pathway and offers the fungus protection against strong oxidants and antifungal drugs (89). Reports of hyaline fungi such as *Sarocladium kiliense* (formerly *Acremonium kiliense*) and *Fusarium solani* var. *coeruleum* forming black grains (92) are probably erroneous. Etiologic agents with melanized hyphae or conidia such as *P. inflatipes* and *S. boydii* produce whitish grains in tissue (8, 20, 59, 93). Melanin production in *Phaeoacremonium* species is facultative, while in *S. boydii* dark pigmentation is limited to the conidia.

Curvularia geniculata

C. geniculata grains are black to dark brown, firm, and 0.5 to 1.0 mm or more in size. In tissue sections, grains are spherical, ovoid, or irregularly shaped and are often surrounded by a zone of epithelioid cells. The periphery of the grain is a dense, interwoven mass of dematiaceous mycelium and thick-walled, chlamydosporelike cells embedded in a cementlike substance. The interior of the grains is vacuolar and consists of a loose network of septate, hyphal filaments.

Curvularia lunata

The grains of *C. lunata* resemble those of *C. geniculata* in their morphologic characteristics.

Falciformispora Species

The grains of the two *Falciformispora* species that cause mycetoma are indistinguishable from each other. They are black, 0.5 to 2 mm in size, and firm to hard. In tissue sections, the grains are round to polylobulated, with large vesicles. At the periphery, the mycelium is embedded in a black, cementlike substance. The central portion of each grain consists of a loose network of hyphae.

T. grisea

Grains of *T. grisea* are black, 0.3 to 0.6 mm in diameter, and soft to firm. In tissue sections, the grains are oval, lobulated, or reniform (kidney shaped), and sometimes vermiform (worm shaped). They are composed of a dense network of hyphae weakly pigmented in the center and brown to blackish brown in the peripheral region as the result of the presence of a brown, cementlike interstitial material.

M. romeroi

M. romeroi produces soft to firm black sclerotia that are oval, lobulated, sometimes vermiform, and about 1.0 mm in diameter. They resemble those of *T. grisea* (94).

E. jeanselmei

In host tissue, *E. jeanselmei* produces dark sclerotia that are brown to black, irregular in shape, and fragile. Detached portions or fragments of the sclerotia often are found within giant cells. When extruded through fistulae, the grains often look like worms (vermiform) because of their elongated shapes and irregular surfaces. In tissue sections, grains appear as hollow structures or as sinuous bands that are vermiform. The external surface is composed of brown, thick-walled hyphae and thick-walled chlamydosporelike cells. The grains are cement free. Within the hollow grains, smaller, degenerated hyphal fragments with leukocytes and giant cells may be seen.

M. mycetomatis

The grains produced by *M. mycetomatis* are reddish brown to black. They may reach 5 mm or more in diameter and are firm to hard. In tissue sections, the sclerotia are compact, variable in size and shape, and frequently multilobulated. They are composed of hyphae 1.2 to 5 μm in diameter that terminate in enlarged hyphal cells at the periphery of the grains, which measure 12 to 15 μm in diameter. The cell wall pigment is minimal, but hyphal cells contain brown particles. The hyphae are embedded in a conspicuous brown matrix that is characteristic of *M. mycetomatis*. Some grains are vesicular and more regular in size and shape. The vesicles are predominantly visible in the peripheral zone in a dense, brown, cementlike matrix.

N. rosatii

The sclerotia of *N. rosatii* are white to brownish white, 0.5 to 1.0 mm in diameter, and soft. In tissue sections, the sclerotia appear to be polyhedral to subregular and consist of hyphae that are embedded in the peripheral cementing material. The sclerotia demonstrate an eosinophilic border. The central portion of each sclerotium consists of more or less disintegrated mycelium and chlamydospores.

Phaeoacremonium Species

Although *Phaeoacremonium* is a dematiaceous fungus and the well-known agent of phaeohyphomycosis, it seems that melanin production is facultative in eumycetoma. White grain mycetoma caused by *P. krajdenii* has been reported by Hemashettar et al. (22).

F. falciforme

The grains of *F. falciforme* are white to pale yellow, soft, and 0.2 to 0.5 mm in diameter. They are composed of slender, polymorphic, septate hyphae 1.5 to 2.0 μm in diameter with irregular bulbous swellings and peripheral cementing material.

Pseudallescheria/Scedosporium

Grains of *Pseudallescheria/Scedosporium* species in tissue are white to yellowish white and soft to firm; they vary from globose to subglobose or lobulated and are 0.2 to 2.0 mm in diameter. They are composed of hyaline hyphae 1.5 to 5.0 μm in diameter that radiate from the center into terminal thick-walled cells 15 to 20 μm in diameter at the peripheries. The central portion of each grain consists of loosely interwoven hyaline mycelium.

Antigen Detection

Serological techniques are important noninvasive, non-culture-based tools for diagnosis of infectious diseases. Since obtaining a pure culture of fungi that cause mycetoma is not always easy, the availability of good antigen or antibody tests (see "Serologic Tests") would be helpful in the diagnosis and follow-up of patients under treatment. Unfortunately, there are no commercially available antigen tests for these agents.

Nucleic Acid Detection Techniques

Molecular diagnostics have been developed for selected agents. However, most of these molecular assays have been used for culture identification (see below) and have not been validated for direct detection from clinical specimens. PCR-based assays for rapid diagnosis of *Scedosporium apiospermum* infections from infected tissue are useful (95, 96). Willinger et al. (97) used molecular techniques for detection of *S. apiospermum* and similar organisms from fungus balls in the maxillary sinus. A multiplex PCR for direct detection of *Scedosporium* species in respiratory specimens has also been developed (98).

ISOLATION PROCEDURES

To maximize the chances of obtaining pure cultures of the etiologic agents, grains from the eumycotic mycetomata should be washed several times with saline containing antibacterial antibiotics such as penicillin and streptomycin. The grains are then cultured on Sabouraud dextrose agar (SDA) containing chloramphenicol (50 mg/liter) and SDA containing chloramphenicol and cycloheximide (500 mg/liter) in petri dishes. Plates should be incubated at 25 and 37°C. Because many of the fungi that cause mycetoma grow slowly, culture plates should be incubated for 4 weeks before being discarded as negative. Identification of the isolated fungus is based on gross morphology of fruit bodies and conidia, if present.

IDENTIFICATION

Morphological Identification

The mycological identification of most fungi causing mycetoma can be achieved by studying the morphological characteristics described below. For additional morphological features and simplified identification keys, readers are referred to specialized references such as the *Atlas of Clinical Fungi* (1).

C. geniculata

In culture, *C. geniculata* develops a rapidly growing, floccose to downy, olive gray to black colony. Microscopically, the melanized, septate hyphae bear solitary, geniculate (bent like a knee) conidiogenous cells. The conidia are without protruding hila. They are smooth walled, predominantly five celled, curved, with the swollen median cell pale to dark brown, in contrast to the lighter end cells.

C. lunata

In culture, *C. lunata* produces geniculate conidiophores that are sympodial in their development. Conidia are without protruding hila. They are smooth and predominantly four celled, with the penultimate cell being pale to dark brown.

Falciformispora Species

In culture, *F. senegalensis* and *F. tompkinsii* grow rapidly and produce gray-brown colonies. On cornmeal agar, both species produce ascostromata that are nonostiolate (without a natural opening), scattered, immersed or superficial, globose to subglobose, black, and covered with brown, smoothly bent hyphae. The asci, produced after prolonged incubation on plant stems, are eight spored, clavate to cylindrical, and double walled. The major difference between the two species is found in the ascospores, which differ in size, shape, septation, and the nature of the gelatinous sheath that surrounds them (46, 99).

T. grisea

In culture, *T. grisea* forms slow-growing, velvety colonies that are cerebriform, radially furrowed or smooth, and dark gray to olive brown to black. The reverses of the colonies are black. Microscopically, the hyphae are septate, light to dark brown, 1 to 3 µm in diameter, and nonsporulating. Chlamydospores are rare. Large moniliform hyphae, 3 to 5 µm in diameter, are often present. Some isolates of *T. grisea* have been described as producing abortive or fertile pycnidia (94, 100). Such isolates are morphologically indistinguishable from *B. mackinnonii* (101, 102), but rRNA ITS sequence data (2) show large differences between these species.

M. romeroi

In culture, colonies of *M. romeroi* are fast growing and floccose to velvety, with a gray surface and whitish margin. The reverse of the colony is black, with no diffusible pigment. On nutritionally deficient media such as oatmeal agar under near-UV light, pycnidia develop after 3 to 4 weeks. The pycnidia are subglobose, 80 to 160 µm in diameter, and dark brick to fawn, later becoming dark brown with short necks. They are ostiolate (with a natural opening), and setae around ostioles are septate and roughened and measure 80 to 100 by 3 µm. The conidiophores inside the conidiomata are sparse and have lateral branches. Conidiogenous cells are hyaline and are borne on branches or arise directly from cells lining the conidiomatal cavity, which produce hyaline to yellowish, shortly cylindrical conidia measuring 1 to 2 by 1.0 µm.

E. jeanselmei

Initially, the colonies of *E. jeanselmei* may be yeastlike and black, gradually spreading, becoming raised or dome shaped, with limited expansion growth. After 2 weeks on SDA, the colonies are covered with short aerial hyphae and appear olive gray with an olive black reverse. Microscopically, annellidic, rocket-shaped conidiogenous cells are inserted at right angles on undifferentiated hyphae. The septate mycelium is sometimes toruloid, branched, and pale brown. The conidia, which aggregate in masses at the tips of annellides, tend to slide down the conidiophore or along the hyphae. The smooth conidia are exogenous, nonseptate, subspherical, and ellipsoidal to cylindrical, measuring 1.5 to 2.8 µm.

M. mycetomatis

In culture, *M. mycetomatis* shows wide variation. Colonies are slow growing and white at first, becoming olivaceous, yellow, or brown; they are flat or dome shaped and velvety to glabrous, often with a rust brown diffusible pigment. On nutritionally deficient media, sclerotial bodies 750 µm in diameter develop. These are black and consist of undifferentiated polygonal cells. On SDA, the mycelium is sterile. On nutritionally poor media, such as soil extract or hay infusion agar, about 50% of the isolates produce round to pyriform conidia 3 to 4 µm in diameter at the tips of phialides. The phialides are tapering, ranging from 3 to 15 µm in length, often with an inconspicuous collarette (Fig. 6).

M. mycetomatis grows better at 37 than at 30°C, with a maximum of 40°C, whereas *T. grisea* grows better at 30 than at 37°C. *M. mycetomatis* is slowly proteolytic and utilizes glucose, maltose, and galactose but not sucrose. It utilizes potassium nitrate, ammonium sulfate, asparagine, and urea, and it hydrolyzes starch. *T. grisea*, on the other hand, is weakly proteolytic and assimilates glucose, maltose, and sucrose but not lactose.

N. rosatii

In culture, colonies of *N. rosatii* are slow growing, attaining diameters of 25 to 28 mm in 2 weeks, and have an aerial mycelium that is grayish black to brownish black. On potato-carrot or cornmeal agar incubated at 30°C, most of the ascomata are submerged. The ascostromal walls are smooth and are surrounded by interwoven brown to hyaline hyphae. The eight-spored asci, 12 to 35 by 10 to 25 µm, are scattered in the central part of the ascostroma (Fig. 7) and are globose to subglobose, thick walled, and bitunicate, becoming evanescent as the ascospores mature. The ascospores vary in size (9 to 12.5 by 4.5 to 8.0 µm) and shape, ranging from ellipsoidal to rhomboidal, asymmetrical, or slightly curved; they are constricted at the median transverse septum and have brown smooth walls.

Phaeoacremonium Species

Members of the genus *Phaeoacremonium* are morphologically characterized by slender, gradually tapering, melanized conidiophores with narrow phialide openings producing ellipsoidal to reniform conidia.

F. falciforme

Colonies of *F. falciforme* on SDA are slow growing, reaching 60 to 65 mm in diameter in 2 weeks. They are downy and gray-brown, becoming gray-violet. The reverse of the colony develops a violet-purple pigment. The hyphae are hyaline, septate, smooth, branched, and 1.5 to 2.5 µm in diameter. They bear erect, undifferentiated, unbranched, repeatedly septate conidiophores. The conidia are borne at the tip of

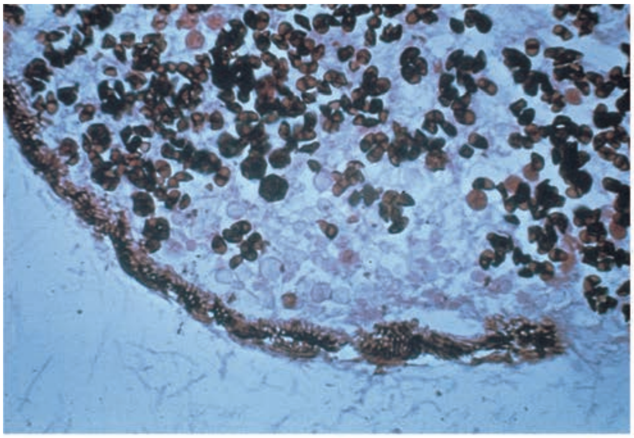
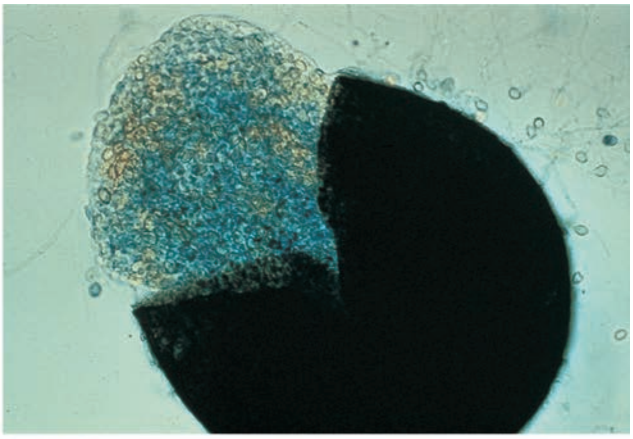
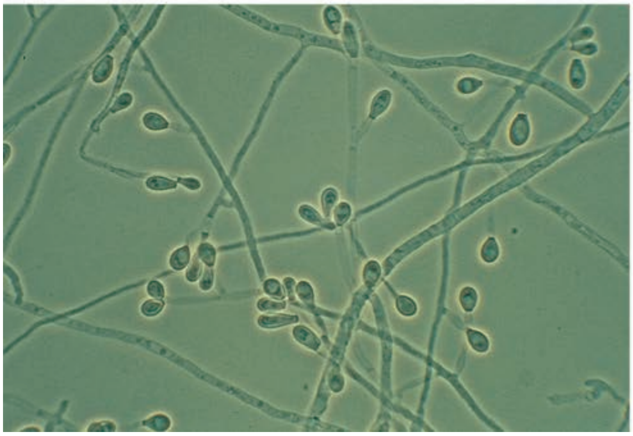
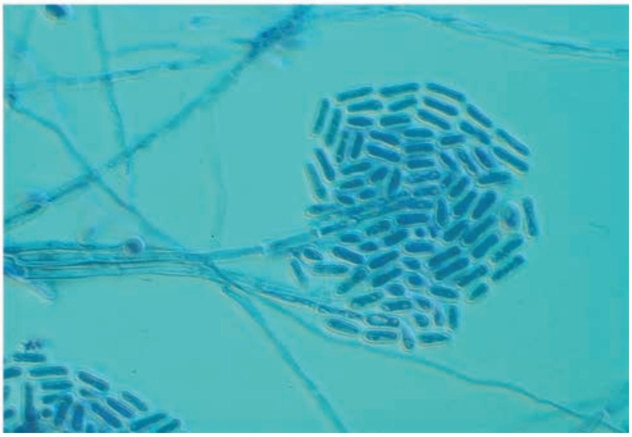





FIGURE 7 (Top left) *N. rosatii*. Cross section of an ascocarp showing the ascomatal wall of interwoven hyphae and containing dark asci and ascospores. Magnification, ×250. doi:10.1128/9781555817381.ch125.f7

FIGURE 8 (Top right) *S. boydii*. Globose, nonostiolate, ruptured cleistothecium containing ellipsoid to oblate ascospores. Magnification, ×160. doi:10.1128/9781555817381.ch125.f8

FIGURE 9 (Bottom left) *S. boydii*. Slide culture on potato dextrose agar showing lateral, single, egg-shaped to clavate, truncate conidia. Magnification, ×160. doi:10.1128/9781555817381.ch125.f9

FIGURE 10 (Bottom right) *Graphium* synanamorph of *S. boydii*. Slide culture on potato dextrose agar showing ropelike bundles of hyphae producing cylindric, smooth conidia. Magnification, ×160. doi:10.1128/9781555817381.ch125.f10

phialidic conidiogenous cells, where they accumulate in mucoid clusters. The conidia are allantoid (sausage shaped), slightly curved, and nonseptate to monoseptate and measure 7 to 8.5 by 2.7 to 3.2 µm. The intercalary or, rarely, terminal chlamydospores are smooth, thick walled, and 5 to 8 µm in diameter.

Pseudallescheria/Scedosporium

Pseudallescheria/Scedosporium colonies grow rapidly and are floccose and white at first, becoming gray as conidia are produced. With age, the colonies become dark grayish brown. Submerged ascocarps (cleistothecia) (Fig. 8) may be produced when isolates are grown on cornmeal agar and are visible macroscopically as small black dots. They are globose, nonostiolate, 140 to 200 µm in diameter, and often covered with brown, thick-walled, septate hyphae 2 to 3 µm wide. They have a wall 4 to 6 µm thick that is composed of two or three layers of interwoven, flattened, dark brown cells, each 2 to 6 µm wide. The cleistothecia open at maturity by an irregular rupture of the wall. The eight-spored asci are ellipsoidal to nearly spherical and 12 to 18 by 9 to 13 µm. The ascospores are ellipsoidal to oblate, symmetrical or slightly flattened, measure 6 to 7 by 3.5 to 4.0 µm, are straw colored, and have two germ pores.

The anamorph *Scedosporium boydii* (Fig. 9) produces conidia that are oval to clavate, truncate, and subhyaline, becoming pale gray to pale brown in mass. Conidia are produced singly. They remain attached at the tips of annellides. Annellations can be detected at the tips of conidiogenous cells as swollen rings. Some isolates also produce a *Graphium* synanamorph of *S. boydii* (Fig. 10), which is characterized by ropelike bundles of hyphae with annellidic conidiogenesis. The hyphae are fused into long stalks known as synnemata. The conidia produced are hyaline, cylindric to clavate, and truncate at the base. A fungus consisting of elongate, multicellular conidia and described as *Polycytella hominis* (103) was recently proven to be a degenerate anamorph of *S. boydii* (104).

Molecular Identification

Certain species (*T. grisea*, *M. mycetomatis*, *N. rosatii*, *B. mackinnonii*, and *M. romeroi*) do not sporulate readily but can be recognized by molecular techniques (49, 105). Borman and coworkers developed a rapid protocol for identifying agents of black grain mycetoma by sequence data of the rRNA ITS region (106). The black yeast *E. jeanselmei* (4), *M. mycetomatis* (48), *Curvularia lunata*, *Medicocopsis romeroi*, *Biatriospora mackinnonii* (107), and agents of the genus *Phaeoacremonium* can be identified by using the same marker (19, 108). Wedde et al. (105) also developed specific primers based on rRNA ITS sequences for the identification of *Pseudallescheria* and *Scedosporium* species. Given the large infraspecific variability of the species, a less variable region, such as the 26S rRNA operon (109), might also provide successful detection of *S. apiospermum*-like genotypes. Recently, Lu et al. developed and evaluated three molecular methods targeting partial beta-tubulin gene for the identification of six closely related species of the *S. apiospermum* complex using quantitative real-time PCR, PCR-based reverse line blot, and loop-mediated isothermal amplification (110). Restriction fragment length polymorphism of the beta-tubulin gene and ITS base rolling-circle amplification was also used for identification of *Scedosporium* and *Pseudallescheria* (111, 112).

TYPING SYSTEMS

Over the years, many molecular typing techniques have been established for a number of fungal species, including those causing mycetoma. Such techniques are useful not only in genotyping but also in accurate species identification (49). Genotyping enables differentiation of environmental and clinical isolates, and therefore it is an important tool in understanding the environmental distribution of species and source of infection. Harun et al. (113) provided a comprehensive review of the methods used in the genotyping of *Scedosporium* species. van de Sande et al. (114) in an amplified fragment length polymorphism study revealed clones of *Madurella* species differing in virulence and geographic distribution. Subspecific typing of *S. apiospermum* at the population level is unproblematic because of the large degree of heterogeneity. Rainer et al. (115) found with M-13 fingerprinting that nearly all strains analyzed belonged to different genotypes; several genotypes could be recovered from a single sampling site. The high degree of polymorphism enabled Zouhair et al. (116) to monitor the dissemination of a strain colonizing the lungs of a patient with cystic fibrosis to cutaneous locations by using multilocus enzyme electrophoresis and randomly amplified polymorphic DNA. In a longitudinal study, Defontaine et al. (117) analyzed nine patients with cystic fibrosis, none of which were found to harbor strains that shared the same genotype.

SEROLOGIC TESTS

Currently, there are no commercially available assays for antibody detection of these agents, but some in-house-developed antibody detection tests have been used in some areas of endemicity. The clinical value of these tests is hampered by a lack of standardized antigen preparation, sensitivity, and specificity. The most widely used assays for antibody detection in the area of endemicity are immunodiffusion and counter-immuno-electrophoresis (CIE) using crude antigens prepared from fungal hyphae (118). Using immunodiffusion and CIE, it was possible to differentiate patients with eumycetoma due to *M. mycetomatis* from patients with actinomycetoma, but negative results in confirmed cases were frequent (118–120). Next to immunodiffusion and CIE, several enzyme-linked immunosorbent assays (ELISAs) have been developed. The antigens used were either whole fungus cytoplasmatic extracts or recombinant proteins such as the *M. mycetomatis* translationally controlled tumor protein, fructose bisphosphate aldolase or pyruvate kinase (102, 121–123). For most of the ELISAs, no clear distinction between patients and healthy controls could be demonstrated (121–123). Furthermore, sera from patients infected with *M. mycetomatis* also cross-reacted with antigens prepared from *S. boydii* (123). Only the ELISA developed by Wethered et al. in 1988 for *M. mycetomatis* cytoplasmatic antigens could clearly differentiate between patients and healthy controls based on IgM antibody responses, but when this ELISA was repeated by Zaini et al., again no differentiation between patients and healthy controls was obtained (123, 124).

ANTIMICROBIAL SUSCEPTIBILITIES

Limited antifungal susceptibility data are available for most fungi causing eumycotic mycetoma. However, for some species such as *M. mycetomatis*, *S. boydii*, and *E. jeanselmei*, a reasonable number of strains have been tested.

With the exception of *S. boydii* and *Fusarium* species, most fungi causing eumycotic mycetoma are susceptible *in vitro* to ketoconazole, itraconazole, voriconazole, and posaconazole. However, it should be noted that *in vitro* activities and clinical responses to these agents are variable (4, 125–130). Ketoconazole and itraconazole are the most common agents used for the treatment of mycetoma, but their clinical response is poor (125, 126, 131, 132). The newer triazoles, such as voriconazole and posaconazole, have the highest *in vitro* and *in vivo* efficacy (126); however, treatment failure has also been reported, especially with voriconazole (130). As shown in Table 3, most of the mycetoma-causative agents have relatively high MICs for amphotericin B, fluconazole, terbinafine, flucytosine, caspofungin, anidulafungin, and micafungin. In contrast, most species have relatively lower MICs for the azoles, with the lowest MICs for all species for posaconazole. However, it should be noted that antifungal susceptibilities of different azoles vary among different causative agents (Table 3), which makes species identification essential for the proper selection of an antifungal agent.

Only limited patient-based studies have been performed to determine the outcome of antifungal treatment, and studies comparing different antifungal agents with clinical outcome are missing.

Nevertheless, as a summary of the few available studies, we can draw some conclusions. In the case of *M. mycetomatis* mycetoma, treatment with ketoconazole resulted in the cure of only 5 of 13 patients (131), while treatment with itraconazole resulted in the cure of only 1 of 13 patients (132). Although the cure rates were not promising, all patients receiving regular treatments were partially improved or had stable lesions. However, with the newer azoles, such as posaconazole, a better outcome has been achieved (133). Negroni and his group treated 6 mycetoma patients with posaconazole and were able to cure 4 patients, and the two other patients were either clinically improved or had stable mycetoma lesions (133). The only nonazole drug that was evaluated clinically for treatment of mycetoma is terbinafine. In the study of N'diaye et al., 20 patients were treated with terbinafine, of which 4 were cured, 12 showed clinical improvement, and 4 showed no effect (134). Based on these limited observations and the *in vitro* data summarized in Table 3, posaconazole seems to be a promising choice for the treatment of mycetoma, but properly designed clinical trials are needed to confirm this assumption.

TABLE 3 *In vitro* susceptibilities of mycetoma-causative fungi

Antifungal agent	*Madurella mycetomatis*		*Trematosphaeria grisea*		*Scedosporium boydii*		*Falciformispora senegalensis*		*Medicopsis romeroi*		*Exophiala jeanselmei*	
	MIC_{50}[a] (range)	No. of strains	MIC_{50} (range)	No. of strains	MIC_{50} (range)	No. of strains	MIC_{50} (range)	No. of strains	MIC_{50} (range)	No. of strains	MIC_{50} (range)	No. of strains
Amphotericin B	0.5 (<0.016–4)	34	8 (2 to >16)	3	1 (0.25–2)	21	2 (2)	4	0.125 (0.125)	5	1 (0.25–2)	9
Ketoconazole	0.06 (<0.03–4)	38	1 (0.125–8)	11			0.5 (0.5–1)	4	8 (0.125–8)	7	1 (1–8)	2
Itraconazole	0.03 (<0.03–0.5)	38	0.5 (0.03–4)	11	0.25 (<0.03–4)	21	0.125 (0.125–0.25)	4	16 (0.125 to >16)	7	0.125 (0.03–0.25)	11
Posaconazole	<0.03 (<0.03–0.125)	34	0.03 (0.03–0.25)	3	0.5 (ND[b])	30	0.06 (0.06–0.25)	4	0.5 (0.25–1)	5	0.03 (0.016–0.063)	9
Fluconazole	4 (0.25 to >128)	34	64 (16–64)	3	16 (8–32)	21	64 (64–128)	4	>256 (>256)	5	16 (8–32)	9
Voriconazole	0.06 (<0.016–1)	34	0.25 (0.25–0.5)	3			0.25 (0.25–0.5)	4	0.125 (0.125–0.25)	5	1 (0.25–2)	9
Isavuconazole	0.03 (<0.016–0.25)	22									ND	
Terbinafine	8 (1 to >16)	34									ND	
Flucytosine	>64 (>64)	34	64 (16 to >64)	3			32 (8–32)	4	8 (8–32)	5	ND	
Caspofungin	64 (16 to >128)	17	>16 (8 to >16)	3			16 (16 to >16)	4	>16 (16 to >16)	5	4 (2–8)	9
Anidulafungin	>128 (0.5 to >128)	17									0.5 (0.063–4)	9
Micafungin	> 128 (8 to >128)	17									ND	
Reference(s)	128, 136–141		141; Ahmed et al, unpublished		142, 143		Ahmed et al., unpublished		141; Ahmed et al, unpublished		141, 144	

[a] MIC values are in μg/ml.

[b] ND, data not available.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Because agents of eumycotic mycetoma are soil or plant saprobes, their etiologic role in mycetoma must be carefully established. A definitive diagnosis is based on the demonstration of grains in tissue, which are expelled through draining sinuses. Grains may become entangled on gauze bandages placed over fistulae. Pus, exudate, or biopsy material should be examined for the presence of grains that are detectable with the naked eye. Their color, internal architecture, size, and shape give a fair indication of the identity of the possible etiologic agents.

Actinomycotic mycetomata are differentiated from eumycotic mycetomata by the examination of crushed, Gram-stained grains. Actinomycotic grains, as well as coccoid and bacillary forms, are composed of Gram-positive, interwoven, thin filaments, 0.5 to 1.0 μm in diameter. Grains of the eumycotic agents, on the other hand, are composed of broader, interwoven, septate hyphae, 2 to 5 μm in diameter, with many unusually shaped, swollen cells up to 15 μm in diameter, especially at the periphery of the grains. In many species, the grains are embedded in cementlike material.

Although the gross and microscopic characteristics of the grains provide insight into the identity of the etiologic agent or a particular group to which it belongs (135), definitive identification of the etiologic agent should be based on isolation of the same fungus from several grains. Clinical microbiology laboratories should avoid reporting patients' specimens as "black grain mycetoma" or "pale grain mycetoma," and isolation and identification of species involved should be attempted. Careful evaluation of culture results is important, especially when new or uncommon fast-growing species are reported, since some of these species might represent contamination during collection of specimens or isolation. On the other hand, physicians should avoid planning a patient's therapy based on grain morphology in clinical materials alone; rather, results of laboratory diagnosis with species identification should be awaited.

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Mycotoxins*

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Mycotoxins are secondary metabolites produced by many different species of ubiquitous fungi having adverse effects on humans and animals. Secondary metabolites, also referred to as natural products, are not required for growth under laboratory conditions but afford protective roles for the producing fungi. These roles range from protection from predators to exclusion of other microbes for niche securement (1, 2). Accordingly, many secondary metabolites are bioactive and cause damage when ingested by humans and animals. Not all toxic compounds produced by fungi are referred to as mycotoxins; for example, yeast and mushroom poisons are excluded by convention, compounds inhibitory mainly to bacteria are termed antibiotics, and those toxic to plants are called phytotoxins, although there can be overlap in toxicity to several kingdoms (3). In this chapter, we review the most common mycotoxins and their relevance to sick building syndrome (SBS), bioterrorism, and food safety. We do not describe the impacts of mycotoxins on health, health costs, or the economy, as these topics have been reviewed extensively elsewhere (4–7). The reader is also referred to other recent reviews on mycotoxins (3, 8).

CHEMICAL CLASSIFICATION AND BIOSYNTHESIS OF MYCOTOXINS

The vast majority of mycotoxins, and indeed all fungal secondary metabolites, arise from a few well-known chemical precursors. Polyketides, like fatty acids, are synthesized from acyl coenzyme A (acyl-CoA), nonribosomal peptides from amino acids, alkaloids from prenylated aromatic amino acids, and terpenes from isoprene. Figure 1 provides a list of some of the most commonly encountered mycotoxins, their chemical class and structure, and the fungi that produce them.

The structure and in some cases the biochemistry of the most common mycotoxins were elucidated earlier than the genetics. However, within the last decade, the genes encoding the enzymes required to synthesize many common mycotoxins have been found (8). The first mycotoxin gene clusters to be characterized were the aflatoxin cluster in both *Aspergillus flavus* and *Aspergillus parasiticus* (9, 10) and the sterigmatocystin cluster in *Aspergillus nidulans* (11). The finding that the genes were clustered in a single genetic locus has turned out to be a hallmark of fungal secondary metabolites (12). The molecular genetics of fungal secondary metabolite clusters has been the subject of many recent reviews (13).

Aflatoxins

The aflatoxin class of mycotoxins was the first to be discovered and studied. Some of the most common aflatoxins (Fig. 1) are aflatoxins B_1, B_2, G_1, and G_2, named for their blue or green fluorescence under UV light (14), with aflatoxin B_1 being the most potent natural carcinogen known (15). There have been several recent reviews of aflatoxins and the fungi producing them (16).

Aflatoxins are produced by several species of *Aspergillus*, in particular, *A. flavus* and *A. parasiticus*. *A. flavus* is the most common contaminant of agricultural products, including cereals, rice, figs, nuts, and tobacco (17, 18). Contamination of crops can occur in the fields before harvest, especially in times of drought (18, 19), or during storage, depending upon the moisture content of the substrate and the humidity of the storage conditions (17, 20). Aflatoxin contamination can be the cause of a variety of economic and health problems and is particularly problematic in developing countries (21). For instance, the presence of aflatoxin in grain significantly lowers the grain's value, as feed or an export commodity, because of the toxin's link to increased mortality in farm animals (22). Further, ingestion of aflatoxin by dairy cows can lead to the presence of aflatoxin M_1, a hydroxylated form of B_1, in their milk (23).

There are substantial differences in the susceptibilities of vertebrate species to aflatoxin exposure. One of the first indications of the effects of aflatoxins was observed in 1960, when more than 100,000 turkey poults died from aflatoxin-contaminated feed, an outbreak named "turkey X disease" (24). Other outbreaks have occurred in ducklings and chickens (25), swine (26), and calves (27), due mostly to contaminated Brazilian peanut meal used as feed. The most recent notable outbreak of acute aflatoxicosis in humans was in Kenya in 2004 (28), followed by lesser yet significant outbreaks in 2005 and 2006 (29). These outbreaks illustrate the need for monitoring and regulation of the amount of mycotoxins in foods meant for human consumption, a luxury not normally available to developing countries. Aflatoxin contamination of pet food, particularly dog food, is a recurring problem in the United States and other countries (30).

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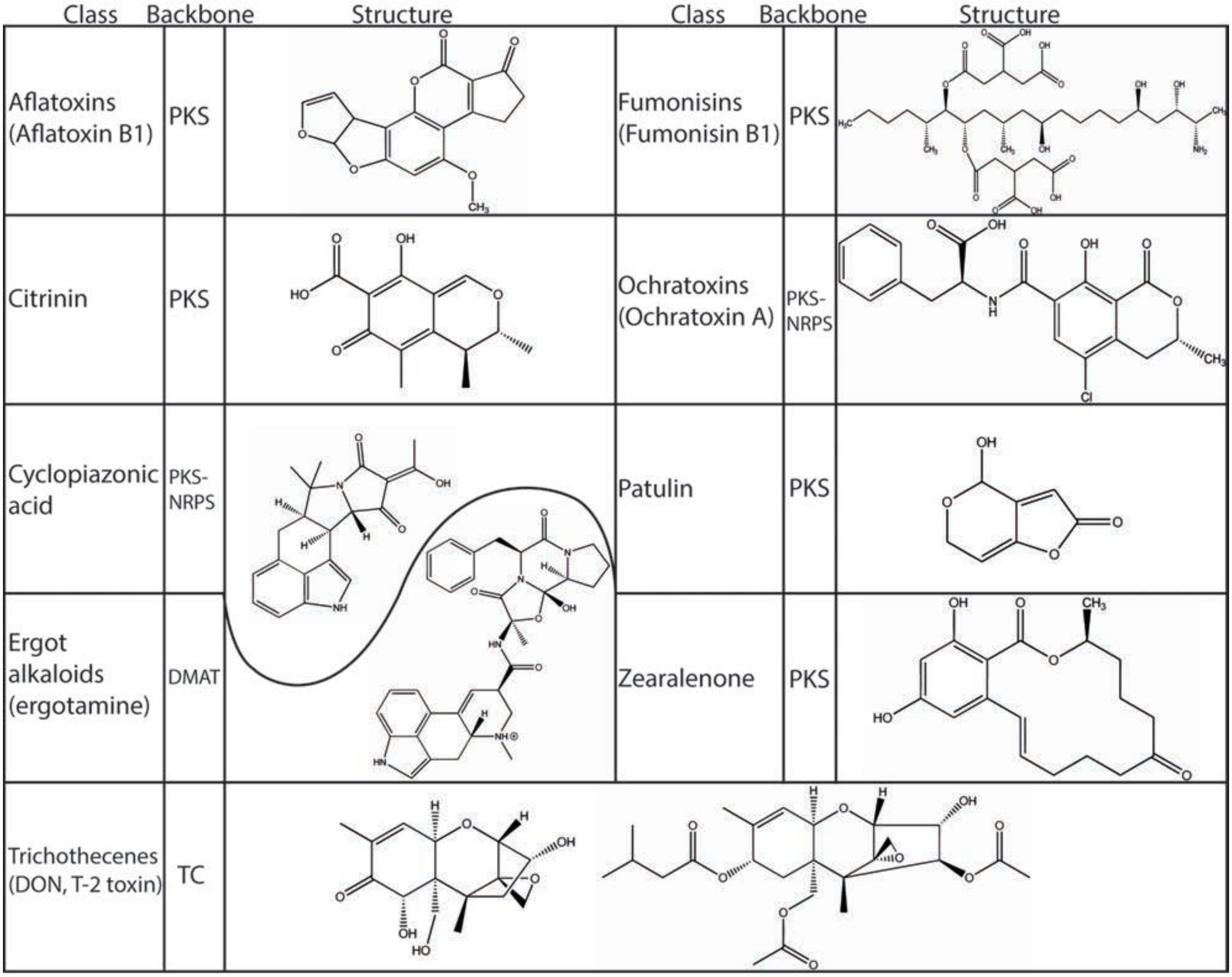
Class
Backbone
Structure
Class
Backbone
Structure
Aflatoxins (Aflatoxin B1)
PKS
Fumonisins (Fumonisin B1)
PKS
Citrinin
PKS
Ochratoxins (Ochratoxin A)
PKS-NRPS
Cyclopiazonic acid
PKS-NRPS
Patulin
PKS
Ergot alkaloids (ergotamine)
DMAT
Zearalenone
PKS
Trichothecenes (DON, T-2 toxin)
TC

FIGURE 1 Classes of mycotoxins, the central enzyme in their biosyntheses, and the structures of examples of each. PKS, polyketide synthase; NRPS, nonribosomal peptide synthetase; DMAT, dimethylallyltryptophan synthase; TC, terpene cyclase; DON, deoxynivalenol (3).
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Citrinin

Citrinin (Fig. 1) is a simple, low-molecular-weight compound that crystallizes as lemon-colored needles. It was tested as an antibiotic and a treatment for ulcers (31) before the discovery of its mycotoxic effects. The most common organisms producing citrinin are the *Penicillium* species *P. citrinum*, *P. expansum*, *P. aurantiogriseum*, and *P. camemberti* (used to produce cheese), *Aspergillus neoniveus* and *Aspergillus oryzae* (used to produce sake and soy sauce), *Aspergillus terreus*, and *Monascus* spp., used to produce red food dyes (32).

Citrinin, first isolated from *P. citrinum*, has been associated with Japanese yellow rice disease (33). It has also been found in various grains, peanuts, and fruits, and there is limited evidence of its surviving unchanged in cereal products (3). Citrinin has demonstrated nephrotoxic effects on all animal species tested (34) and was shown to inhibit dehydrogenase activity in rats' kidneys, liver, and brain (35). However, there have been no reported outbreaks of human citrinin poisoning, and its relevance to human health is unknown.

Cyclopiazonic Acid

Cyclopiazonic acid (CPA) (Fig. 1) is an indole-tetramic acid mycotoxin produced by several species of *Penicillium* and *Aspergillus* (36, 37). It has demonstrated severe toxicity on all species tested, including rats (38), chickens (39), dogs (40), and pigs (41). Contamination of various food products, including grains, meats, and cheese, with CPA is sometimes coincident with aflatoxin contamination (42). As such, it is suspected that some of the symptoms of turkey X disease may have been due to the toxicity of cocontaminant CPA (43).

Interestingly, the CPA gene cluster is present in *A. flavus* but truncated in its close relative *A. oryzae* (44); this is reminiscent of repeated findings of truncated or mutated aflatoxin clusters in this species (45). *A. oryzae*, a species used in the manufacture of fermented rice products, does not produce CPA due to a deletion in the backbone gene of the cluster; or, in some *A. oryzae* strains where the backbone gene is present, CPA is converted to a less toxic compound, 2-oxoCPA, by an enzyme not present in *A. flavus* (46). Little is known about its toxicity to humans, but it may affect the heart (47) and liver and is thought to cause "kodo poisoning" (48).

Ergot Alkaloids

Ergot alkaloids, produced by the ergot fungus *Claviceps purpurea*, are the causative agent of ergotism, or St. Anthony's fire, which can manifest either as a gangrenous or convulsive condition following ingestion of contaminated grains, especially rye (49). Two main classes of ergot alkaloids exist: lysergic acid derivatives and clavines. Both are indole alkaloids derived from tetracyclic ergoline. Lysergic acid, common to all ergot alkaloids, often forms amide, amino acid, or peptide derivatives, e.g., ergine, ergonovine, lysergic acid diethylamide (LSD), and ergovaline. The clavines contain the ergoline structure but do not have amino acid or peptide components, e.g., pergolide and lisuride (49). Semisynthetic ergot alkaloids have been developed through decades of research, which began with the series of lysergic acid derivatives that included the infamous hallucinogen LSD (50). Most recently, ergot alkaloids have been investigated for their anticancer potential (51) and their effect on serotonin and serotonin receptors (52, 53). A representative compound, ergotamine, is shown in Fig. 1.

Aspergillus, *Balansia*, *Claviceps*, *Epichloë*, and *Neotyphodium* spp. have been found to produce ergot alkaloids. *A. fumigatus* has been shown to produce fumigaclavines A, B, and C and festuclavine, first described for *C. purpurea* and later for *Neotyphodium* (54). Modern methods of cleaning grains have all but eliminated the threat to the human food chain. However, ergotism remains an important veterinary concern, as symptoms of gangrene, convulsions, and abortion in cattle, sheep, pigs, and chickens mimic those in humans (55).

Fumonisins

Fumonisins (Fig. 1) are among the most recently discovered mycotoxins. In the late 1970s and early 1980s, it was determined that fumonisins produced by *Gibberella fujikuroi* were the cause of leukoencephalomalacia in horses (56) and hepatocarcinoma in rats (57). By 2002, there were 28 known fumonisin analogs, separated into groups A, B, C, and P (58). *Fusarium proliferatum*, *Gibberella nygamai*, *Aspergillus niger*, and *Alternaria alternata* also produce these mycotoxins (59, 60).

Besides the syndromes mentioned above, fumonisins have been shown to cause pulmonary edema and hydrothorax in swine (61). *G. fujikuroi*, the major producer of fumonisins, can have an important effect on the supply of corn, as it causes a variety of blights and rots depending on environmental conditions (62). There is considerable concern and evidence that fumonisins cause several human diseases, particularly esophageal cancer (63). Likely due to these concerns, international monitoring systems are now in place for this metabolite (64), which, unfortunately, is often found in food and feed products, cocontaminated with aflatoxin (65).

Ochratoxins

Ochratoxins A and B (Fig. 1) are produced by *Penicillium verrucosum* and *Aspergillus* spp., especially *A. ochraceus*. These toxins can be carcinogenic, immunosuppressive, and nephrotoxic (22, 66). Ochratoxin A, first discovered to be toxic to animals in 1965 (67), is more common than ochratoxin B and can be metabolized by cytochrome P450 in the liver (68). Ochratoxins are most often found contaminating barley but may be present in oats, rye, wheat, and coffee beans (3, 67). They are of particular concern because they can be carried through the food chain, especially in milk and pork (69). Scandinavian countries, particularly Denmark, have had high incidence of porcine nephropathy and high levels of ochratoxin A contamination (70). Some studies speculate that ochratoxin A may contribute to Parkinson's disease (71).

Patulin

Patulin (Fig. 1) was first isolated in the 1940s from *Penicillium griseofulvum*, and efforts were made to mass-produce it for its antibiotic effects (72). However, patulin was reclassified as a mycotoxin in the 1960s following discovery of its toxicity to plants and animals (3). Patulin occurs commonly in unfermented juice made from *P. expansum*-contaminated fruit (73). Though toxic at high concentrations *in vitro*, natural patulin poisoning in humans has yet to be proven (3). Nevertheless, due to concern of its prevalence in apple juice and other items preferentially consumed by children, several countries limit patulin levels in certain foods and beverages (74). The reader is referred to a recent review on the biosynthesis and toxicity of patulin (75).

Trichothecenes

Trichothecene mycotoxins are secondary metabolites of a variety of *Fusarium*, *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma*, and *Trichothecium* spp., among others. Some of the most common and well-studied *Fusarium* trichothecenes (Fig. 1) are T-2 toxin, HT-2 toxin, diacetoxyscirpenol, nivalenol, and deoxynivalenol, also called DON or vomitoxin. DON is one of the most common mycotoxins found in grain, including barley, oats, rye, and wheat. When ingested in large quantities by livestock, it can cause nausea, vomiting, and diarrhea. Ingestion of smaller quantities results in weight loss and feed refusal (76). DON can also suppress bovine and porcine neutrophil function *in vitro* (77). *Stachybotrys chartarum* produces several trichothecenes, including verrucarins B and J, roridin E, satratoxins F, G, and H, and isosatratoxins F, G, and H (78, 79). *Stachybotrys*-contaminated straw was first described as causing a highly fatal equine disease (80) and has since become better known as a factor in SBS, discussed below.

Zearalenone

Zearalenone (Fig. 1) is a nonsteroidal estrogen or phytoestrogen produced by various species of *Gibberella*, including *G. pulicaris* and *G. tricincta* (81). Zearalenone is a common contaminant of cereal crops worldwide and has been implicated in hyperestrogenism of farm animals as a result of digestion of moldy corn and grains (82). Some of the symptoms of hyperestrogenism are enlargement of the uterus and nipples, vaginal prolapse, and infertility (83). Several outbreaks of zearalenone poisoning of swine have been reported (84).

FOOD SAFETY

Consumption of moldy food products is the leading cause of mycotoxicoses in agricultural animals and humans alike. Contamination can occur at any point, ranging from the crop field through storage and shipping. The economic consequences of mycotoxin contamination are immense, and mycotoxins pose a higher chronic dietary risk than synthetic contaminants, plant toxins, food additives, or

TABLE 1 Simplified taxonomy of mycotoxin-producing fungi[a]

Mycotoxin class	Genera	Representative species
Aflatoxin	*Aspergillus*, *Penicillium*	*A. fumigatus*, *A. flavus*, *A. parasiticus*, *P. aurantiogriseum*
Citrinin	*Penicillium*	*P. citrinin*, *P. expansum*
Cyclopiazonic acid	*Aspergillus*, *Penicillium*	*P. griseofulvum*, *A. flavus*
Ergot alkaloid	*Aspergillus*, *Balansia*, *Claviceps*, *Epichloë*, *Neotyphodium*	*A. fumigatus*, *C. purpurea*
Fumonisin	*Gibberella*, *Fusarium*	*G. fujikuroi*, *F. proliferatum*
Ochratoxin	*Aspergillus*, *Penicillium*	*A. ochraceus*, *P. verrucosum*
Patulin	*Aspergillus*, *Paecilomyces*, *Penicillium*	*A. clavatus*, *Paecilomyces fulvus*, *Paecilomyces niveus*, *P. griseofulvum*, *P. expansum*
Trichothecene	*Stachybotrys*	*S. chartarum*
Zearalenone	*Gibberella*, *Fusarium*	*F. crookwellense*, *F. culmorum*, *G. zeae*, *F. incarnatum*

[a] Data from reference 113.

pesticide residues (85). Tables 1 and 2 list the mycotoxins discussed in this chapter and their common substrates. The reader should note that neither table is exhaustive, and other fungi and substrates can be involved in mycotoxin poisonings.

Detection

Since it would be impossible to prevent all mycotoxin contamination, methods of monitoring food and feed must be established. The regulatory guidelines for limiting mycotoxins differ between countries but have been summarized in compendia published by the Food and Agriculture Organization of the United Nations and more recently reviewed (74, 86). Mycotoxins as a group are comprised of diverse chemical structures, which are often present in smaller amounts than other interfering substances, requiring that each be separated from its substrate and studied by a unique assay. Generally, sample preparation consists of an extraction step and a purification step. Though this method is the most definitive, it is very expensive and time-consuming. Thus, newer screening methods for the presence of mycotoxins have been developed over the past decade. On the other hand, many of these screening methods are still plagued by cross-reactivity and require confirmation with more-selective validated methods.

Several reviews on current detection methodologies exist (87). Confirmatory tests and novel screening techniques include immunoassays such as enzyme-linked immunosorbent assay, fluorescence polarization immunoassay, surface plasmon resonance, and other conductometric measurements (88). High-performance liquid chromatography (HPLC) and gas chromatography (GC) are widely used, and since the introduction of atmospheric pressure ionization, liquid chromatography-mass spectrometry has become a routine technique for detecting the presence of mycotoxins, including tricothecenes, ochratoxins, zearalenone, fumonisins, and aflatoxins (89). The latest official methods, validated by the Association of Official Analytical Chemists International, are based on immunoaffinity column cleanup of conventional extracts, followed by fluorescently labeled HPLC (22). The reader is referred to comprehensive reviews of recent developments in analytical protocols (88, 90).

Effects of Climate Change

It is estimated that one-quarter of the world's crops are contaminated to some extent with mycotoxins (91); this proportion varies from year to year based on environmental factors. There is much evidence of the influence of environmental factors, mainly temperature, humidity, drought, insect attack, and other plant stressors, on mycotoxin production by molds. Further, the pathogenicity of different molds may be additive, and competition between mold species may be temperature dependent (83). Each species and its ability to produce mycotoxins must be evaluated independently under its optimum growth conditions. For wheat species, infection of the cereal ears by *Gibberella* spp., particularly *G. zeae*, a predominant producer of DON, is enhanced by warm, humid weather (92). At the other extreme, production of fumonisins and aflatoxins is greater under drought conditions. Warmer temperatures and fewer frost days result in more insect and plant pathogens surviving the winter and causing plants to be more susceptible to mold infestation. Recent studies have shown increases in mycotoxin contamination of multiple crops (93). In summary, climate changes in any particular region may result in radical changes in the amount and type of crop damage and mycotoxin contamination.

TABLE 2 Mycotoxins and their common food substrates[a]

Mycotoxin class	Barley	Cheese	Coffee beans	Corn	Fruits	Oats	Peanuts	Rice	Rye	Wheat
Aflatoxin				X			X	X		X
Citrinin	X			X		X		X	X	X
Cyclopiazonic acid		X		X			X			
Ergot alkaloid	X					X			X	X
Fumonisin				X						
Ochratoxin	X		X			X			X	X
Patulin					X					
Trichothecene				X						
Zearalenone				X						

[a] Data from reference 113.

Current Practices and the Future of Prevention Amelioration of Mycotoxin Contamination

Several different approaches are currently being developed to protect the food supply from mycotoxin contamination, including measures taken before, during, and after cultivation of crops, yet the most reliable preventative treatments are still cultural practices and humidity control in postharvest facilities. Though most breeding successes have been reported for wheat and barley, it is also essential to breed for resistance in other crops, such as corn and oats (94). Several labs have attempted to create transgenic lines resistant to various mycotoxigenic fungi, but none are in production. However, several reports suggest that Bt crops, i.e., those transgenically expressing insecticidal *Bacillus thuringiensis* toxins, are less susceptible to mycotoxin contamination due to decreased insect infestation (95). Indeed, insecticides have been shown to reduce the growth of *Aspergillus* and the resultant accumulation of ochratoxin A (96).

One biological control discussed but not implemented to any degree is the application of mycoviruses, which are typically easily spread through asexual fungal spores. Though many mycoviruses have minimal effects on their host fungi, they have the potential to be used as vectors for resistance genes. This approach may expand with the increasing availability of virus-specific molecular detection methods (5). Additionally, a recent study shows that the application of nontoxigenic strains of *A. flavus* to corn plant whorls prior to tasseling significantly reduced aflatoxin accumulation, presumably through competition with toxigenic strains (97). This method appears promising and is being assessed in many different climates worldwide (98).

Finally, recent studies have concentrated on degradation of mycotoxins following crop harvest. Many microorganisms, such as soil and water bacteria, fungi, protozoa, and specific enzymes isolated from microbial systems, degrade mycotoxins, specifically aflatoxins (99). The addition of sodium carbonate and other feed additives has demonstrated success, lowering mortality and reducing adverse effects of mycotoxin-contaminated feed in livestock (58, 100).

BIOTERRORISM

In response to concerns about the possible use of mycotoxins in bioterrorism, the Committee on Protection from Mycotoxins was formed by the National Research Council in 1982 (101). Following years of research, Ciegler addressed the possibility of use of mycotoxins as chemical weapons in 1986 (102). However, the choice of aflatoxin as a weapon of mass destruction is odd at best; the effects of aflatoxicosis, such as liver cancer, are too slow-acting to be effective during war.

Trichothecenes are much more suited for warfare than aflatoxins; they act immediately upon contact, and several milligrams can be lethal. Of historical note is the "yellow rain" incident of 1981 (103). The United States accused the Soviet Union of using nivalenol, DON, and T-2 toxin against Hmong tribespeople in Cambodia and Laos. However, it was later concluded that this "yellow rain" was not a weapon but the excreta of swarms of wild Asian honeybees (104).

The use of mycotoxins in bioterrorism has been assessed by several investigators in recent years (3, 105, 106). Aflatoxins and trichothecenes have been tested for combined toxicity and were found to have additive effects in most cases. The combination of these mycotoxins resulted in a synergistic effect in human bronchial epithelial cells (107). Interestingly, chlorine dioxide may be effective in the detoxification of trichothecenes, roridin A, and verrucarin A (108) in the case of widespread exposure. T-2 toxin, along with aflatoxin, received some notoriety as a possible biological weapon used in the Gulf War (109). However, there is no clear data to support a role for either mycotoxin in ensuing illnesses afflicting veterans of this war (110). Much of the information addressing mycotoxins in biological warfare remains classified, and thus the question remains unresolved. In general, it is accepted that while the presence of mycotoxin weapons may cause terror, the actual use of such weapons would not be effective or reliable in a time of war.

SICK BUILDING SYNDROME

SBS is a loosely defined term that applies to indoor environments suspected of causing a variety of health problems for occupants. The buildings usually have many issues, including water damage, improper heating, ventilation, and air conditioning systems, poor construction, and bacterial, fungal, and/or insect infestation. Symptoms may include eye, nose, and throat irritation, fatigue, headache lack of concentration, frequent respiratory tract infections, shortness of breath, dizziness, and nausea (111). Mycotoxins are just one of many factors considered to be possible contributors to SBS. Several mycotoxin-producing fungi have been implicated, including *Alternaria*, *Aspergillus*, *Cladosporium*, *Chaetomium*, and *Penicillium* spp. One of the most widely investigated species is *S. chartarum*. For comprehensive reviews, see references 4 and 112.

It has not, however, been proven that occupants develop illnesses either from "sick" buildings or the molds inside them. Recent reviews of SBS conclude that there is no evidence that mold presents a threat to life for healthy members of the general population in typical exposures; indoor airborne microorganisms are only weakly correlated with human disease, and a causal relationship has not been established; and the concentration of mold required to create infective doses is inconsistent with the reported spore concentrations in buildings (4, 111).

A further argument against mycotoxins as the cause of SBS symptoms is that most mycotoxins are not volatile, and therefore, widespread exposure is unlikely. Moreover, the conditions conducive for mold growth are not necessarily optimal for mycotoxin production, and fungi differ in their abilities to produce mycotoxins. To conclude that mold found within a sick building is associated with occupants' symptoms, the existence of the mold indoors, the mode of transmission, either by contact or proximity to spores and related byproducts, and the portal of entry, whether by inhalation, ingestion, or skin absorption, must be established. The mere presence of fungi in an indoor environment is insufficient evidence to establish a causative relationship and does not prove that mycotoxin is also present.

CONCLUSIONS

There are several classes of mycotoxins, produced by a wide range of fungal species, that have been linked to various environmental issues such as SBS, veterinary problems, bioterrorism, and food safety. Over the past decade, there have been many developments in the processes for identifying mycotoxin contamination and advances in methods to control its production. Much research is yet needed to reduce the threat of mycotoxin-producing fungal species to the health of human, livestock, and plant populations and its resultant effects on the international economy.

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Lacazia, *Lagenidium*, *Pythium*, and *Rhinosporidium*

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In the past 100 years the microbial pathogens described in this chapter have been classified as fungal and/or parafungal protistan pathogens (1–4). Based on their apparent epidemiological connection with water, they were at one point also placed in a new category of hydrophilic infectious agents (1). However, based on taxonomic and other morphological characteristics, these three anomalous species were not well understood (1, 4). This frustrating situation fueled a strong controversy that has only recently been solved with the advent of molecular methodologies (5, 6). Despite the recent finding that both *Pythium insidiosum* and *Rhinosporidium seeberi* are protistan pathogens, they are still studied by medical mycologists, continuing a historical tradition. More recently, the finding of an oomycete in the genus *Lagenidium* affecting mammalian hosts alerted the medical community to the presence of a novel pathogen phenotypically similar to the fungi and indistinguishable from the clinical and pathological features displayed by *P. insidiosum* during infection. Based on rDNA phylogenetic analysis, the evolutionary location of the microbial pathogens discussed in this chapter is illustrated in Fig. 1. Table 1 summarizes the most prominent features of the pathogens covered in this section.

LACAZIA LOBOI

Taxonomy

Jorge de Oliveira Lobo first described lacaziosis in a patient from the state of Pernambuco, Brazil (7). He stated that the new pathogen was phenotypically similar to the parasitic stages of the genera *Blastomyces* and *Paracoccidioides*. The earliest attempts to isolate this pathogen in culture met with failure and soon it became clear that this pathogen cannot be cultured (4, 8). This fact led to great controversy regarding its epidemiological and taxonomic affinities, reflected by the numerous names under which this pathogen has been known. Taborda et al. in 1999 (9) suggested the binomial *Lacazia loboi* to end more than 70 years of taxonomic ambiguity. Because the epithet lobomycosis was derived from the obsolete genus *Lobomyces* (4), Vilela et al. (10) suggested the name lacaziosis as a more suitable term. Their suggestion was based on the proposal by Taborda et al. (9) and the placement of *L. loboi* as a separate taxon from the genus *Paracoccidioides* (10–12). Initial phylogenetic studies placed *L. loboi* as the sister taxon to *P. brasiliensis* (5, 11), but besides the long branches described in phylogenetic analysis between these pathogens, there were not sufficient DNA sequences to support this claim. Vilela et al. (13), using at least five different loci and 20 *L. loboi* human strains, presented a strong argument for the phylogenetic placement of this pathogen as an independent taxon. This proposal was supported by other studies indicating that an ancestor of both pathogens gave origin to two *Paracoccidioides* species (*P. brasiliensis* and *P. lutzii*) and to *L. loboi* (10, 12). Currently *L. loboi* is classified within the Ascomycota in the order Onygenales, family Ajellomycetaceae.

Description of the Agent

Because *L. loboi* cannot be cultured, our knowledge of this pathogen is based on the morphological features observed in its parasitic stage. Its *in vivo* phenotype is characterized by the development of unicellular, thick-walled, lemon-shaped, yeast-like cells that can be found forming one, two, or more cells in chains with occasional branching and characteristically connected by short tubules (Fig. 2). The yeast-like cells of *L. loboi* measure ~5 to 12 µm in diameter and can be easily observed with most stains. Interestingly, *L. loboi*'s *in vivo* phenotype is very similar to the parasitic stage of *P. brasiliensis*. The morphological features of *L. loboi* and the cross-reaction observed in serological tests of patient sera with lacaziosis and the antigens of *P. brasiliensis* led Almeida and Lacaz (4) to classify this pathogen in the genus *Paracoccidioides*. Electron microscopic analysis of *L. loboi* showed thick chitinous yeast-like cell walls and an amorphous cytoplasmic content. Approximately 60% of the yeast-like cells observed lacked a defined cytoplasmic region, a finding in agreement with the result of viability studies.

Epidemiology and Transmission

Lacaziosis (Jorge Lobo's disease) is known to occur only in patients inhabiting the tropical areas of the Americas (7). In addition to the many human cases, reports of the disease in species of dolphins around the coasts of South America, Florida, and the Gulf of Mexico have also been found (6). Although the majority of patients with lacaziosis have been from Brazil, cases from Mexico, Central America, Colombia, Suriname, Venezuela, and other nearby countries have also been reported (4). Two European human patients apparently acquired the infection after contact with an infected bottle-nosed dolphin in an aquarium. In addition, American and Canadian patients were reported with the infection after visiting or working in the areas of endemicity (14, 15).

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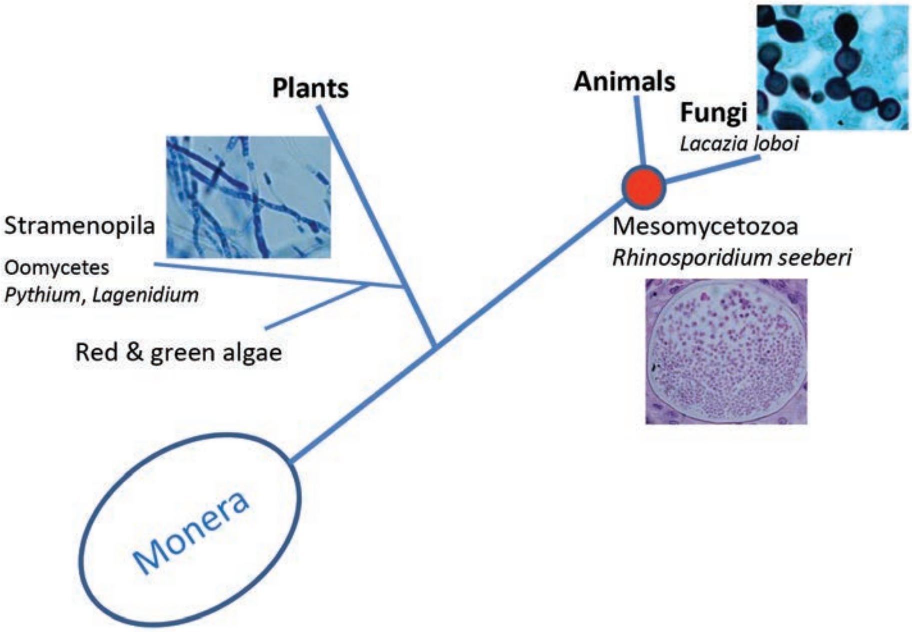

FIGURE 1 Phylogenetic location of the four microbial pathogens studied in this chapter using small-subunit ribosomal DNA sequences. Forming a sister group, the algae and stramenopilans are placed basal to the plants. The latter includes the mammalian pathogenic Oomycetes *Pythium* spp. and *Lagenidium* spp. They develop hyphal-like elements, vesicles with biflagellate zoospores, and oogonia. *R. seeberi* is placed at the point where the animals and fungi diverge (red circle). This uncultivated protist is characterized by the development of spherical phenotypes with endoconidia. *L. loboi* is an anomalous, uncultivated, ascomycetous fungus developing yeast-like cells in chains. doi:10.1128/9781555817381.ch127.f1

Interestingly, an apparent lacaziosis case in a Greek woman with no history of traveling to the Americas was recently reported (16). The diagnosis was based on histological morphology. However, the yeast-like cells depicted in this particular patient lacked some of the morphological features observed in Fig. 2B and C. Moreover, no molecular testing was conducted to further authenticate the case.

It is believed that the infection is acquired through small traumatic skin lesions after contact with the pathogen near aquatic ecological niches. The report of dolphins with lacaziosis tends to support this assumption. However, the real ecological niche of this pathogen is unknown. The disease seems to occur in apparently healthy hosts. It is quite possible that *L. loboi* cells possess hyphae with propagules (perhaps conidia) similar to those in *P. brasiliensis* that may make contact with hosts through trauma, thus causing lacaziosis. Transmission of the disease from one patient to another is rare, but some cases have been reported of autoinoculation and accidental infection by physicians in contact with infected patients (4, 17) or with infected dolphins (18). In addition, the disease can be experimentally reproduced in mice (19).

Clinical Significance

Infections caused by *L. loboi* are rarely observed in the endemic tropical areas of the Americas. Recent reports indicate that human cases of the disease are sporadic in the areas of endemicity and occur mainly in males. The number of cases so far reported is around 600, but its real occurrence could be higher. The disease is diagnosed in apparently healthy hosts inhabiting such areas. A genetic predisposition theory was abandoned after the finding that the number of cases in a Brazilian tribe with a high occurrence of the disease dramatically decreased when the tribe was moved to a new location. One common problem in the areas of endemicity is that patients with the disease do not seek medical attention until years after infection, when the lesions have increased in size and spread to other skin areas. One explanation may be that the affected population is usually poor with no access to health care and that the lesions are rarely painful. The disease has been also reported in dolphins inhabiting coastal areas of regions of endemicity as well as areas of nonendemicity (18, 20).

Frequently affected anatomical areas are the arms, ears, back, chest, face, and lower limbs. Some investigators believe that the distribution of lesions on cooler areas of skin in humans and in dolphins may indicate that the pathogen does not tolerate well a temperature of 37°C. Initially, a single small (0.5 to 1.0 cm in diameter), smooth, parakeloidal skin lesion develops. Some patients complain of slight pruritus at this stage. The infection is not life-threatening and usually evolves very slowly, sometimes over 20 or more years. Usually, when patients seek medical attention they have already developed more than one lesion. In the chronic phase, the lesions are polymorphic. At least five clinical manifestations are recognized, including the typical parakeloidal type and the infiltrative, gummatous, ulcerated, and verrucous forms. However, Lacaz et al. (4) pointed out that

TABLE 1 Taxonomic, epidemiological, clinical, and mycological features of the unusual microbes *Lacazia loboi*, *Lagenidium* spp., *Pythium insidiosum*, and *Rhinosporidium seeberi*

Pathogen	Taxonomy/phylogeny	Epidemiology	Clinic	*In vivo* form	*In vitro* form
Lacazia loboi	Ascomycete fungus located with the dimorphic onygenales	Restricted to South America. Cases in dolphins. Acquired by trauma. Hydrophilic.	Parakeloidal skin lesions in head, arms, chest, low limbs	Lemon-shaped, yeast-like cells in chains, connected small tubes	Uncultivated
Lagenidium spp. (two species have been mentioned, but they are awaiting official description)	Kingdom Straminipila (Protista), Oomycetes (Peronosporomycetes), known as the "water molds"	Tropical, subtropical, and temperate areas. Acquired in aquatic or terrestrial environments by trauma. Hydrophilic.	Keratitis and ulcerated skin lesions, dissemination to large vessels and lungs has been mentioned.	Sparsely septate hyphae ~8–20 μm. Splendore-Hoeppli phenomenon. Prominent eosinophilia.	Submerged colonies. Sparsely septate hyphae ~10–25 μm. Some with large spherical structures. Biflagellate zoospores in water.
Pythium insidiosum	Kingdom Straminipila (Protista), Oomycetes (Peronosporomycetes), known as the "water molds"	Tropical, subtropical, and temperate areas. Acquired in aquatic or terrestrial environments by trauma. Hydrophilic.	Keratitis, cutaneous, intestinal, vascular, and dissemination to other organs, including lungs	Sparsely septate hyphae ~4–10 μm. Splendore-Hoeppli phenomenon. Prominent eosinophilia.	Submerged colonies. Sparsely septate hyphae ~5–12 μm without fruiting bodies. Biflagellate zoospores in water.
Rhinosporidium seeberi	Kingdom Protista, class Mesomycetozoea, located at the animal-fungal divergence	All continents except Australia. Endemic in India and Sri Lanka. Acquired by trauma. Hydrophilic.	Polypoidal mucosal and skin lesions. Is common in mouth, nares, and eyes.	Spherical sporangia, with or without endospores, at different stages of development	Uncultivated

two or more forms, including a macular type, could be found in a single patient. The differential diagnosis includes chromoblastomycosis, leishmaniasis, leprosy, neoplasia, paracoccidioidomycosis, and similar skin conditions.

Collection, Transport, and Storage of Specimens

The guidelines for the collection, processing, storage, and examination of specimens are provided in chapter 114. Clinical specimens collected in cases of lacaziosis are mostly biopsied tissues from the infected sites. *L. loboi* cannot be cultured; thus, clinical specimens from the infected areas are usually fixed in formaldehyde for histopathologic evaluation.

Direct Examination

Microscopy

In contrast to the systemic spread of *P. brasiliensis* infections, *L. loboi* is typically confined to cutaneous and subcutaneous tissues. The lesions are characterized by fibrosis and granulomatous reaction with numerous histiocytes and giant cells containing numerous yeast-like cells of *L. loboi*. Areas of necrosis and the presence of other inflammatory cells have been reported. When tissue is stained with hematoxylin and eosin (H&E), the typical yeast-like cell arrangement in chains is not observed. Instead, *L. loboi* appears as oval to spherical, poorly stained cells or as round empty spaces with thick cell walls (Fig. 2A). Special fungal stains such as Gomori methenamine silver (GMS) and periodic acid-Schiff (PAS) should be used for the histopathological diagnosis of lacaziosis. With GMS, *L. loboi* yeast-like cells are dark or have the appearance of empty cells (Fig. 2B). The yeast-like cells are in chains of two or more cells and form branches, similar to the yeast cells observed in the infected tissue of patients with paracoccidioidomycosis. The cells are typically connected with small tubules, a feature also observed with other fungal stains such as PAS (Fig. 2B and C).

For the diagnosis of lacaziosis using direct microscopy, biopsy specimens should be cut into pieces of 2 to 5 mm in diameter. One or more pieces are then placed with 1 or 2 drops of 10% potassium hydroxide (KOH) on a glass slide with a coverslip. The slide should be heated without boiling and then held for about 15 min at room temperature before microscopic evaluation. In 10% KOH, *L. loboi* yeast-like cells appear in great quantities (Fig. 2C). Cells ~2 to 12 μm in diameter and uniform in size can be found as single yeast cells or as chains connected by tubules of three or more cells. In wet mounts, moving protoplasmic granules can also be detected within some of the yeast-like cells (Fig. 2C).

Nucleic Acid Detection Techniques

So far, there are no commercially available nucleic acid probes for the detection of this pathogen in clinical samples.

Isolation Procedures, Identification, and Typing Systems

Claims that *L. loboi* has been isolated in pure culture have not yet been validated. Most of the recovered organisms were fungal contaminants or *P. brasiliensis* (4, 8). The identi-

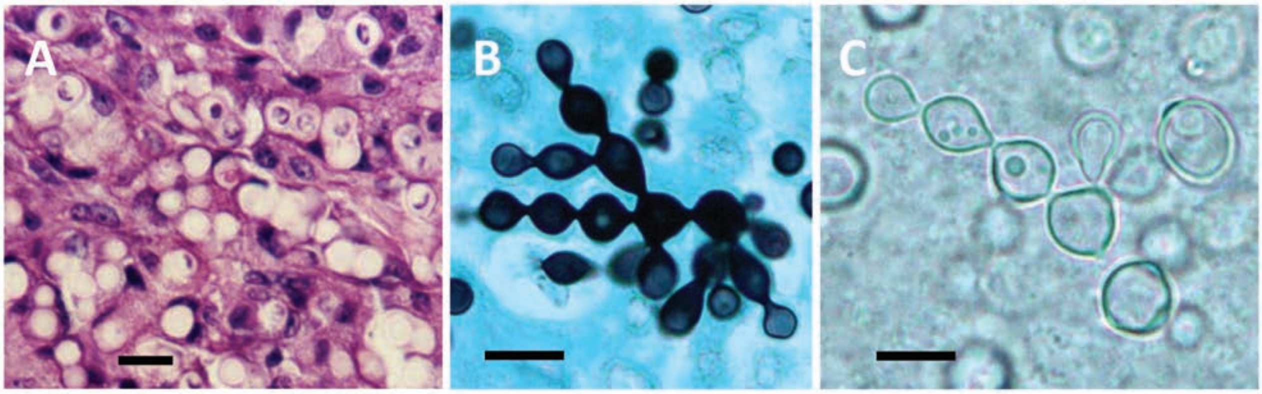

FIGURE 2 *L. loboi*. Histological sections stained with H&E (A) and silver stain (B) show the typical morphological features found in patients with cutaneous lacaziosis. (A) *L. loboi* yeast-like cells are poorly stained with H&E and are observed as empty round structures surrounded by an area of granulomatous reaction (bar, 8 µm). (B) The presence of abundant lemon-shaped yeast-like branching cells in chains connected with slender tubules is the main feature of *L. loboi* in silver-stained sections (bar, 13 µm). (C) A wet mount preparation in 10% KOH shows numerous *L. loboi* yeast-like cells in chains, some of them containing small dancing bodies in their cytoplasm (bar, 10 µm). doi:10.1128/9781555817381.ch127.f2

fication of the etiologic agent is based on the clinical features and the phenotypic characteristics on wet-mount preparations and/or histopathological analyses of the infected tissues. No typing systems for this organism exist at this time. However, in-house molecular procedures from suspected tissue, using primers previously used by others, could be of help to authenticate histopathological findings (8).

Serologic Tests

One major obstacle to developing serologic assays for lacaziosis has been the lack of cultures. Thus, most of the serologic approaches have been carried out using parasitic forms in infected tissues as antigen or using cultures of closely related pathogens (4). These tests showed that *L. loboi* possesses cross-reactive antigens in common with fungal pathogens such as *Blastomyces dermatitidis*, *Histoplasma capsulatum*, and *P. brasiliensis* (4). Mendoza et al. (21), using antigenic proteins derived from the yeast-like cells of *L. loboi* from experimentally infected mice, found that the antibodies in the sera of humans, dolphins, and mice with lacaziosis detected a 193-kDa *L. loboi* immunodominant protein. Although antibodies in these sera did react with a *P. brasiliensis* purified gp43 antigen (21), they did not detect the corresponding gp43 antigen of the *L. loboi* parasitic stage. This finding suggests that the 193-kDa protein of *L. loboi* may have epitopes in common with the gp43 of *P. brasiliensis*. It also implies that, although the 193-kDa antigen expressed during lacaziosis infection cross-reacts with the antibodies against the gp43 of *P. brasiliensis*, it might be an entirely different antigenic protein. The role of this *L. loboi* antigenic protein in the pathogenesis of lacaziosis is under investigation. The use of *L. loboi* antigens in serological surveys to study the epidemiology of lacaziosis has been suggested (21). Although serology is not commercially available, Biomedical Laboratory Diagnostics, Michigan State University, have developed a Western blot assay that can be requested at https://bld.natsci.msu.edu/research/pythium-insidiosum/.

Antimicrobial Susceptibilities

Although antifungal therapy has cured some cases of lacaziosis, *L. loboi* is well known for its resistance to antimicrobial drugs, including most antifungals (4). This fact has left clinicians with only one choice: surgery. Due to the intractability of this organism to culture, susceptibility testing is not possible.

Evaluation, Interpretation, and Reporting of Results

Clinical samples from patients suspected of lacaziosis submitted to the laboratory comprise deep skin scrapings and tissue biopsies. The samples have to be processed as described above ("Direct Examination" and "Microscopy") and evaluated for the presence of uniform yeast-like cells connected by small tubules forming short chains. Because *P. brasiliensis* in the parasitic phase can also form yeast cells connected by tubules, the significant variation in size could be used to separate *L. loboi* from *P. brasiliensis*. The latter pathogen tends to develop large and small yeast cells in the infected tissues, whereas *L. loboi* yeast-like cells have uniform size. In addition, the lack of fungal growth in culture could also be used as an aid in the diagnosis of this uncultivated pathogen. After laboratory evaluation of the clinical material, the presence of uniform yeast-like cells that yielded no fungal growth in culture is used to confirm lacaziosis. This is particularly important for patients who have visited areas where the disease is endemic. The report of the results should include the finding of uniform yeast-like cells connected by tubules, the lack of fungal growth in culture, and the histopathological results of the evaluated tissue samples.

PYTHIUM INSIDIOSUM

Taxonomy

P. insidiosum was first reported more than 150 years ago in Indonesian equines (3), but successful isolation of this organism in pure culture was not possible until the beginning of the 20th century. Later, a similar organism was isolated again from horses and the name *Hyphomyces destruens* was introduced. The finding that a strain from New Guinea (22) developed zoospores suggested that this pathogen was an organism in the genus *Pythium*. De Cock et al. (3) introduced the binomial *P. insidiosum*, placing this pathogen within the protist

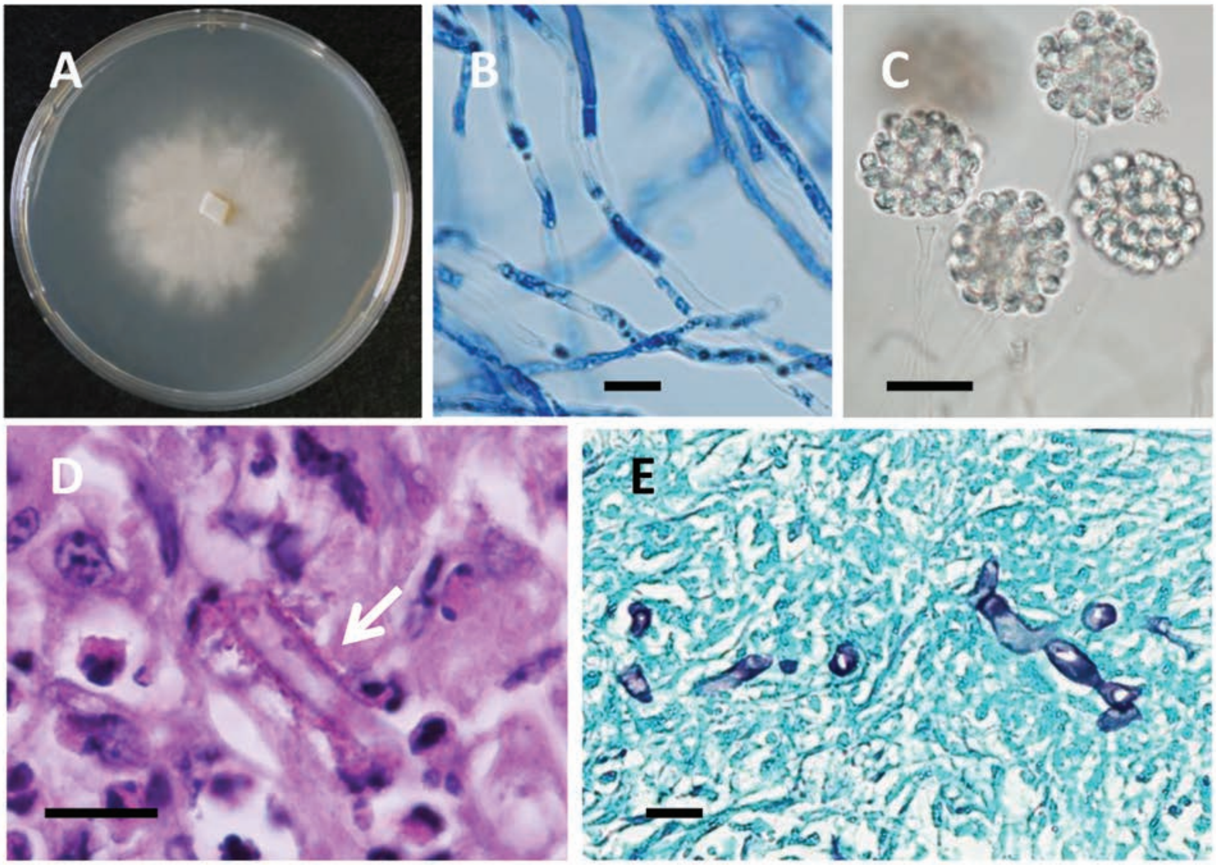

FIGURE 3 *P. insidiosum*. (A) Colony of *Pythium insidiosum* on SDA showing a submerged colony with few aerial mycelia. (B) Slender sparsely septate hyphae without fruiting bodies. (C) Four vesicles of *P. insidiosum* containing numerous unhatched zoospores. (D) Several eosinophils are observed around the stained hyphae of *P. insidiosum* from a case of human subcutaneous pythiosis. Note the eosinophilic reaction (Splendore-Hoeppli phenomenon) around the hypha (arrow). (E) Silver-stained hyphae showing the typical feature of *P. insidiosum* in infected hosts. Bars: (B) 40 µm; (C) 50 µm; (D) 25 µm; (E) 20 µm. doi:10.1128/9781555817381.ch127.f3

kingdom Stramenopila, class Oomycota, order Pythiales, and family Pythiaceae (Fig. 1). Molecular studies (23, 24) showed that strains of *P. insidiosum* cluster phylogenetically according to their geographic origin. Thus, it is likely that *P. insidiosum* could be a phylogenetic complex of closely related species, or perhaps a complex of different strains belonging to one single species, *P. insidiosum* (25).

Description of the Agent

P. insidiosum can be readily isolated on most mycological media (3). This pathogen develops white submerged colonies with a characteristic radiate pattern and few to no aerial hyphae, very similar to that in the mammalian pathogenic *Lagenidium* spp. (Fig. 3A). The hyphae have perpendicular lateral branches measuring 4 to 10 µm in diameter and possess few cross-septa (Fig. 3B). Zoosporogenesis (development of zoospores) is only possible in water cultures containing various ions, including Ca^{2+} (3, 26, 27). The structures in which the zoospores develop are termed vesicles (Fig. 3C).

Zoospores form by progressive cleavage and mature inside large vesicles (20 to 60 µm in diameter) (Fig. 3C). Once fully formed, the biflagellate zoospores mechanically break the sporangial wall, swim, and then encyst. It has been proposed that the zoospores may be the infectious units due to their motility. Zoospores are kidney-like in shape, and two unequal flagella arise from inside a lateral groove. Upon encystment, the zoospores lose their flagella and become spherical. Under the right conditions, the encysted zoospores develop a germ tube and form long filaments (26). *P. insidiosum* oogonia (sexual stage) have been rarely observed and are believed to represent a resistant stage in nature.

Epidemiology and Transmission

Infections caused by this hydrophilic pathogen have been recorded in tropical, subtropical, and some temperate areas of the world. In the Americas, pythiosis is common in tropical Central, North, and South America, with most cases reported in Brazil, Colombia, Costa Rica, the United States, and Venezuela (3, 26). In the United States, infections are more prevalent in animals and humans inhabiting southern states such as Alabama, Georgia, Florida, Louisiana, Mississippi, North Carolina, South Carolina, and Texas. However, cases of the disease have also been reported in other states, including California, Illinois, Indiana, Kansas, New Jersey, Missouri, Tennessee, Virginia, and as far north as Wisconsin and New York. In Asia, pythiosis has been reported in Japan, India, Indonesia, Pacific islands, South Korea, and Thailand and also in nearby areas such as Australia, New Guinea, and New Zealand. Tropical Africa is ideal for pythiosis; however, only one case in a dog with the cutaneous form was reported in the northwest African country of Mali. This might suggest that the disease has been misdiagnosed in this geographic region (23).

Until recently, infections caused by *P. insidiosum* were considered exotic. The disease was believed to be restricted to animals in tropical regions with few or no occurrences in other geographical areas. Interestingly, in the last 10 years the number of pythiosis cases has increased on all continents (3, 28). The finding that humans (28–31), dogs (32, 33), and other animals can be infected indicates that previously pythiosis was erroneously diagnosed as a fungal infection. Most cases of pythiosis occur in apparently healthy humans and animals. In Thailand, however, the disease in humans is associated with thalassemia or similar blood disorders (29).

The infection is acquired after *P. insidiosum* propagules enter the skin or the intestinal tract through traumatic lesions (26, 33). The infection is believed to be acquired from wet environments. Supabandhu et al. (34) cultured and identified *P. insidiosum* from environmental samples, confirming the presence of this oomycete in wet agricultural environments in Thailand. In addition, cases of pythiosis in the absence of water suggest that *P. insidiosum* can cause infection after contact with resting spores from terrestrial environments. The transmission of *P. insidiosum* from one individual to another (human or animal or *vice versa*) has not been recorded. Only rabbits seem to be susceptible to experimental inoculation (35).

Clinical Significance

The clinical manifestations of pythiosis vary according to the infection site. Most patients with the disease state that they had a skin injury prior to the infection. The lesions caused by *P. insidiosum* in humans can be classified into superficial infections (keratitis); cutaneous and subcutaneous forms, including orbital pythiosis; and vascular forms that usually lead to systemic infection and death. Keratitis caused by *P. insidiosum* is similar to that caused by fungi and other etiologic agents (36–38). It begins with trauma to the superficial layers of the eye, followed by the development of conjunctivitis, photophobia, corneal ulcers, and hypopyon (pus in the interior chamber of the eye). Cutaneous and subcutaneous infection is characterized by the formation of granulomatous plaques and/or ulcerated swellings that remain localized. Once the pathogen has reached the subcutaneous tissues, itchy papules may develop. The orbital form of subcutaneous pythiosis is rare and has been observed mainly in children in Australia and the United States (39). Vascular pythiosis starts with a traumatic lesion, usually on the lower limbs, followed by dissemination of the pathogen to the nearby arteries (37, 46). Initially, the infected skin shows signs of dry gangrene, and the formation of painful necrotic ulcers in some patients has also been reported (28). Clinical symptoms are claudication (limping) of the affected limb, local ischemia, swelling, pain, and the absence of the dorsalis pedis pulse. As the infection progresses, an ascending arteritis, with the formation of thrombi and aneurysms of the large arteries, is the main feature. If not treated, *P. insidiosum* may spread through the arteries and reach the iliac and renal arteries and abdominal aorta, causing disseminated pythiosis. This form of pythiosis is more common in Thailand among patients with thalassemia, and it is usually life-threatening (28, 30, 31). Human pythiosis should be differentiated from arteriosclerosis, diabetes mellitus, *Lagenidium* spp. cutaneous leishmaniasis, mycotic keratitis, subcutaneous tuberculosis, subcutaneous mucormycosis, and other mycoses associated with filamentous fungi.

Collection, Transport, and Storage of Specimens

The clinical specimens required for diagnosis of pythiosis vary according to the infected host and the clinical form (25, 28). The infections caused by *P. insidiosum* share identical methodologies for the collection, transport, and storage of the clinical samples with that in *Lagenidium* spp. A portion of the small tissue samples used for microscopy also can be used to inoculate Sabouraud dextrose agar (SDA) plates. The pieces have to be submerged into the agar and incubated at 37°C for 24 to 48 hours. *Lagenidium* spp. that are pathogenic for dogs, cats, and humans develop white-yellowish, flat, glabrous colonies (Fig. 4A), similar to that recovered in cases of pythiosis. The presence of broad, 9- to 18-μm, coenocytic hyphae displaying large spherical structures connected by short strands of hyphae is the main feature of strains recovered from dogs (Fig. 4B). A strain of *Lagenidium* sp. recovered recently in a case of human keratitis showed only broad, sparsely septate hyphae (40). Strains of *Lagenidium* displaying spherical structures connected by short strands of hyphae can be used to differentiate *P. insidiosum* from *Lagenidium* sp. (Fig. 4B); however, some mammalian pathogenic *Lagenidium* spp. could develop only hyphae. Those cases have to be investigated using molecular methodologies (see below). So far, there is no typing system available for *Lagenidium* spp.

For patients with keratitis, swabs or scrapings of the affected eyes should be collected. Dryness of clinical specimens from keratitis patients could prevent the microscopic detection of the hyphal elements and subsequent culture of *Lagenidium* spp. Swabs must be immediately transported at room temperature in tubes with high humidity (or containing small quantities of sterile distilled water) to the laboratory and should be processed upon arrival. The collected eye scrapings should be placed in small aseptic petri dishes and immediately transported at room temperature to be processed promptly upon arrival. Biopsy or necropsy samples are usually collected in sterile distilled water and transported at room temperature to the laboratory. Specimens collected from patients with cutaneous, subcutaneous, vascular, and disseminated lagenidiosis can also be placed in tubes containing sterile distilled water plus 100 U of penicillin/ml and 0.25 mg of streptomycin or 0.4 mg of chloramphenicol per ml. If these antibiotics are not available, the use of sterile distilled water is encouraged. Although some studies indicate that transportation or storage at 4°C of specimens from patients suspected of lagenidiosis did not interfere with the isolation of the pathogen in the laboratory, others report a considerable reduction in the number of positive cultures from samples stored at 4°C (26). The reader should consult chapter 114 for more information on collection.

Direct Examination

Microscopy

Clinical specimens of *P. insidiosum* in 10% KOH characteristically show the presence of long (4.0 to 9.0 μm diameter), hyaline, sparsely septate hyphal structures (Fig. 3B). Some hyphal elements may reach more than 15-μm lateral branches at a 90° angle, a typical feature of this oomycete.

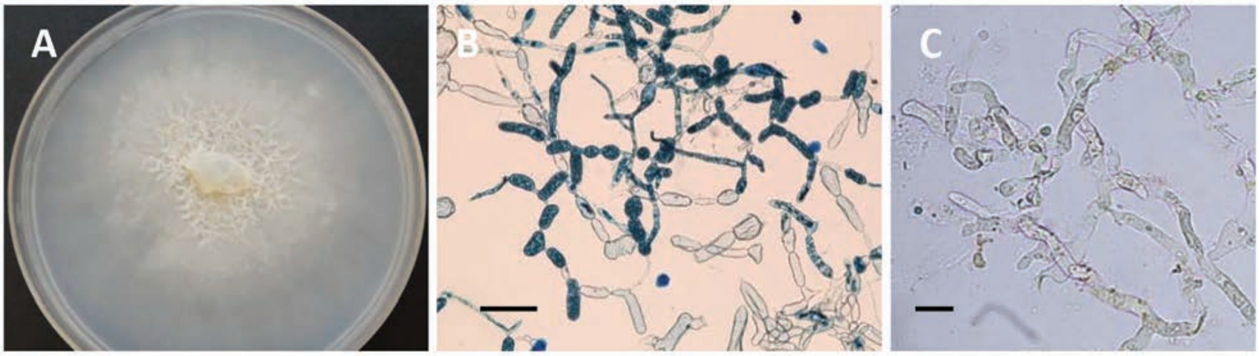

FIGURE 4 *Lagenidium* sp. (A) Colony of *Lagenidium* sp. on SDA isolated from biopsied dog tissue. *Lagenidium* sp. produces cream to white submerged colonies at 25 and 37°C. (B) Microscopically, ribbon-type hyphae are found in samples collected from agar. In lactophenol blue stain, the presence of large spherical and oval structures is common in some isolates (bar, 60 µm). However, some strains may develop only hyphae. (C) 10% KOH preparation from biopsied tissue depicting several broader hyphal elements without septa (bar, 18 µm). doi:10.1128/9781555817381.ch127.f4

In histopathological preparations stained with H&E, *P. insidiosum* appears as short or long, hyaline, coenocytic (without septa) hyphae 6 to 10 µm in diameter (Fig. 3D). The fact that *in vivo* structures of *P. insidiosum* are detectable in H&E has been used to differentiate this oomycete from the hyphal elements developed by members of the order Enthomophthorales (genera *Basidiobolus* and *Conidiobolus*). In infected tissues *P. insidiosum* triggers eosinophilic granulomas with giant cells, mast cells, and other inflammatory cells (Fig. 3D). The hyphal elements of *P. insidiosum* are found in the center of microabscesses with numerous eosinophils that usually degranulate over the organism's hyphae (Splendore-Hoeppli phenomenon) (Fig. 3D, arrow). The activation of an eosinophilic inflammatory response with the Splendore-Hoeppli phenomenon is a feature in common with the order Entomophthorales, from which it must be differentiated. Although most experts in the diagnosis of pythiosis agree that it is difficult to distinguish between the hyphal structures of *P. insidiosum* and those features of the Entomophthorales, such as the poor staining capabilities in H&E, the ribbon-type morphology and the bigger size of the zygomycetes hyphal filaments sometimes help to distinguish these fungi from *P. insidiosum*. *P. insidiosum*'s hyphal elements are well stained by GMS and appear as short or long, sparsely septate, tubular dark structures (Fig. 3E). Transversely sectioned, poorly stained (with GMS or PAS) hyphae can be also found as ring-shaped bodies.

The most important immunohistological tests for the specific identification of the hyphal elements of *P. insidiosum* in infected tissues have been the peroxidase and the immunofluorescence tests (41). Because the hyphal structures of *P. insidiosum* are difficult to differentiate from those in the fungi, especially the mucormycetes and entomophthorales (formerly zygomycetes), these assays have been of paramount importance for the accurate identification of this oomycete in the absence of culture. Most of these assays are available through reference laboratories for pythiosis in the United States at Michigan State University (https://bld.natsci.msu.edu/research/pythium-insidiosum/) and Pan American Veterinary Laboratories (http://pavlab.com) and in other countries such as Brazil and Thailand.

Nucleic Acid Detection Techniques

The first molecular approach for the diagnosis of *P. insidiosum* from clinical specimens was carried out on a patient with keratitis (36). The hyphal elements present in the specimen were identified by sequencing part of the 18S ribosomal DNA region using the NS1 and NS2 and ITS universal primers. This approach has been successful on clinical specimens (36, 30). Grooters and Gee (42) introduced a PCR technique for the identification of *P. insidiosum* from cultures and from clinical specimens. A set of primers (PI-1 and PI-2) that amplified 105 bp of the ITS-1 region of *P. insidiosum* were tested. These primers have been used by several laboratories (30) and were entirely specific when tested against several filamentous fungi. Schurko et al. (43) introduced a dot-blot hybridization technique by constructing a 530-bp species-specific DNA probe for the detection of *P. insidiosum*. This DNA probe specifically binds to the intergenetic spacer 1 (IGS1) of this pathogen, and it did not hybridize with the genomic DNA from 23 other *Pythium* species, *Lagenidium gigantum*, or several pathogenic fungi, including the entomophthoramycetes fungi *Conidiobolus coronatus* and *Basidiobolus ranarum*. This probe may be ideal for the detection of *P. insidiosum* from environmental samples and for the specific diagnosis of pythiosis from clinical specimens from susceptible hosts. Other similar in-house molecular assays have been suggested, but they have limited availability.

Isolation Procedures, Identification, and Typing Systems

In contrast with the other hydrophilic pathogens covered in this chapter, *Lagenidium* spp. (see below) and *P. insidiosum* can be cultured on various media. The most common media used for the isolation of *P. insidiosum* are 2% SDA or broth with or without antibiotics (see "Collection, Transport, and Storage of Specimens"), blood agar, cornmeal agar (Difco), potato dextrose agar, and nutritive agar (Difco). Biopsy or necropsy tissues and kunkers (stony hard masses found only in horses with pythiosis) from patients suspected of pythiosis are usually cut into small fragments 2 to 5 mm in diameter, placed into tubes containing sterile distilled water, and vigorously washed two to three times before plating. The small fragments are then physically pushed into the agar and the plates are incubated at 25 and 37°C for 2 or more days. The relative humidity of the incubator should be enhanced by placing a beaker of water inside the chamber. Specimens that have been transported for more than 24 h can also be inoculated into tubes containing broth and then incubated at 37°C. Usually, cottony colonies surrounding the clinical specimens are detectable after 24 to 48 h of incubation (25). On solid medium, *P. insidiosum* develops only sparsely septate hyphae (Fig. 3B). The appearance of *P. insidiosum* on solid medium was previously described.

P. insidiosum can be identified definitively only if the isolate develops the characteristic oogonia (sexual stage) on culture plates. The formation of oogonia, however, is extremely rare, which further complicates the final identification of this pathogen in the clinical setting. Given that *Lagenidium* spp. and *P. insidiosum* display identical lesions in infected mammals, the development of zoospores in water as a definitive characteristic for the identification of *P. insidiosum* needs to be revisited. The identification of *P. insidiosum* using molecular methods with isolates and clinical specimens as described above is recommended (see "Nucleic Acid Detection Techniques," above). A drawback to this approach is that only a few laboratories possess these capabilities. Currently, there are no typing systems available for *P. insidiosum*.

Serologic Tests

Early serological tests showed that anti-*P. insidiosum* antibodies could be detected in the sera of infected hosts and, thus, could be used for the diagnosis of pythiosis. The most common assays for pythiosis are agglutination, enzyme-linked immunosorbent assay (ELISA), immunodiffusion, and Western blotting. Immunodiffusion has proven to be a very specific test, with several precipitin bands, but it is too insensitive and yields many false negatives, especially when performed with sera from humans and dogs with pythiosis (44). To overcome this drawback, an ELISA and a Western blot assay were later introduced (30, 32, 44–46). These assays are extremely sensitive in detecting anti-*P. insidiosum* IgG. However, it may cross-react with anti-*Lagenidium* spp. antibodies (47). Recently, an agglutination test was used for the rapid diagnosis of pythiosis. Although it proved to be a good screening test, it had a high rate of false positives and false negatives (48). The finding that antibodies in the sera of different hosts recognize different antigenic proteins when evaluated with several geographically divergent strains of *P. insidiosum* suggests frequent subclinical infections with multiple *P. insidiosum* strains (49).

Antimicrobial Susceptibility

Pythium insidiosum, like the other oomycetes (including *Lagenidium* spp.), does not possess ergosterol in its cytoplasmic membrane. Despite this obvious contraindication, amphotericin B and other antifungal drugs that target ergosterol have been used with mixed results. For instance, two children with orbital pythiosis were successfully treated with amphotericin B in Australia (39). However, this antifungal did not have an effect in humans from Thailand with the disease (29, 31). Recently, susceptibility testing on a strain isolated from a child in Tennessee with orbital pythiosis showed the strain to have low MICs of terbinafine and itraconazole (50). Although this combination of drugs was successfully used in this case, the same combination was less effective when tested in other humans and animals. One explanation for this contradiction is the possibility that these antifungal drugs may be affecting pathways other than sterols. The inconsistent results with most antifungal drugs for the treatment of pythiosis have led to the use of unconventional treatments such as immunotherapy (31). Immunotherapy has been found effective in 55% of humans and dogs and in 70% of equines with the disease (48). In addition, some investigators have evaluated *in vitro* combinations of several antifungals against *P. insidiosum* with promising results (41, 51–53). These studies indicate that terbinafine plus fluconazole or ketoconazole (52), or terbinafine plus amphotericin B (53), substantially reduced the *in vitro* growth of *P. insidiosum*. However, these combinations of antifungal drugs have yet to be tested in clinical cases.

Evaluation, Interpretation, and Reporting of Results

Culture is the gold-standard test for pythiosis. Because *P. insidiosum* morphological features in infected tissues are identical to those displayed by the fungal entomophthoromycetes *Conidiobolus* and *Basidiobolus*, wet mounts, histopathological examination of tissue sections, serological assays, and culture have to be evaluated as a whole for a proper interpretation and identification of this pathogen in the laboratory. For instance, the finding of sparsely septated hyaline hyphae in a wet mount preparation must be confirmed by culture. Moreover, serological assays such as ELISA and Western blot could detect anti-*P. insidiosum* antibodies in hosts with putative clinical diagnosis of pythiosis, but they should be interpreted with caution. *P. insidiosum* can cause subclinical infections in human and animals inhabiting areas of endemicity. Thus, false positives have been found in apparently healthy individuals (48). When culture is not possible, the use of molecular approaches and/or immunohistochemical (peroxidase and immunofluorescence) tests could be of help. The presence of hyaline hyphae in wet mount preparations and in histopathology should be reported as suggestive of pythiosis, whereas positive results in culture (development of zoospores in water cultures) or positive reactions in molecular assays and/or in immunohistochemical staining tests are reported as confirmatory tests of disease. In such cases the report of results should include the following: "pythiosis caused by *P. insidiosum* confirmed by culture and supported by serological and/or molecular assays."

LAGENIDIUM SPP.

Taxonomy

Until recently, *P. insidiosum* was considered the only oomycete causing disease in mammalian hosts (3, 25). However, in 1999 several cases of novel mammalian pathogenic oomycete species causing skin infection with dissemination to blood vessels were reported (47). Although a complete description of the species causing lagenidiosis in mammalian hosts was still pending, unpublished molecular data indicated that at least two *Lagenidium* species were involved (51, 54). The genus *Lagenidium* comprises oomyceteous species affecting lower animals such as crabs, mosquito larvae, nematodes, and others (55). Because members of the oomycetes develop hyphal-like elements, they were for a long time known as the "aquatic fungi" (water molds). However, they are not true fungi. They are currently classified within the kingdom Stramenopila, phylum Heterokonta, class Oomycota, order Lagenidiales, and family Lagenidiaceae (Fig. 1). Although Dick (56), using phenotypic characteristics, recently questioned the validity of this genus, current phylogenetic studies have found *Lagenidium* species clustered with *L. giganteum*, a mosquito larval pathogen with strong statistical support (R. Vilela and L. Mendoza, unpublished data). This finding suggests that the classification of *Lagenidium* species based on morphology alone is problematic.

Description of the Agent

Lagenidium species can be readily isolated in media used to recover fungi in the clinical laboratory (47, 55). This is in contrast with some of the other pathogens covered in this chapter. On SDA, *Lagenidium* species develop well at 37°C and slower at 25°C. At 37°C they develop white-yellow submerged colonies without aerial mycelia in less than 24 hours (Fig. 4A). Based on cases studied at Michigan State University, *Lagenidium* spp. developed 8- to 15-µm ribbon-type hyphae and spherical structures 20 to 45 µm in diameter (unpublished data) (Fig. 4B). In liquid media, undifferentiated hyphae can develop vesicles at the tip of these structures. The vesicle increases in size as more protoplasmic material enters the vesicle, and zoospores are formed within the following 20 minutes. The presence of sexual structures (oogonia) has not yet been reported. The two species mentioned so far in the literature, *L. caninum* and *L. karlingii*, were introduced without a formal description (54). Thus, the microscopic and phylogenetic features of these two novel species are still awaiting description.

Epidemiology and Transmission

So far, most cases of lagenidiosis in mammalian hosts have been reported in the United States (54, 55). A case of keratitis caused by a *Lagenidium* sp. in a Thai human patient was recently reported (40). In addition, an Australian dog case of lagenidiosis was also studied by us (unpublished data). In the U.S., lagenidiosis occurred in the same areas reporting *P. insidiosum* infection (see below) (51, 54, 55). This includes the states bordering the Gulf of Mexico and Arkansas, Georgia, Illinois, Indiana, Maryland, North Carolina, South Carolina, Tennessee, and Virginia, among others. *Lagenidium* spp. complete their life cycle in wet environments, possibly using lower animal hosts or plants. Mammals with open skin injuries entering contaminated environments can be exposed to the zoospores developed by these species and could develop lagenidiosis. Transmission from one infected host to another has not been observed. Experimental infection in mice has been unsuccessful (47).

Clinical Significance

The genus *Lagenidium* and *P. insidiosum* have been recognized as emerging pathogenic oomycetes causing invasive superficial, cutaneous, subcutaneous, and arterial infections (25). These pathogens are rarely observed causing systemic infection. The common sites of infection in humans and animals are cornea, gastrointestinal tract, limbs, and other anatomical areas (40, 55). Mammalian-pathogenic *Lagenidium* species have been reported in only three geographical areas (see above), but other tropical and subtropical regions may contain these pathogenic species as well.

Collection, Transport, and Storage of Specimens

See the corresponding text in the section on *Pythium insidiosum*.

Direct Examination

Microscopy

Small pieces of biopsied tissue cut in 5-mm blocks, scrapings of lesions in wet mounts, and cytological samples stained with Giemsa reveal the hyphal structures of *Lagenidium* spp. in infected tissues. In wet mount, broad 9- to 18-µm, branched, sparsely septate hyphae (broader than those in *P. insidiosum*) are observed in cases of lagenidiosis (Fig. 4C). Differentiation in histopathological sections between *Lagenidium* and *P. insidiosum* hyphae is difficult. Both trigger an eosinophilc granuloma, and although *Lagenidium* hyphae are larger than *P. insidiosum*, a definitive diagnosis can only be made by culture (see the section on *P. insidiosum*) or by using molecular tools. The microscopic features of some *Lagenidium* sp. causing lagenidiosis in dogs in culture display the presence of oval and spherical structures, a morphological feature that could be used to separate these mammalian-pathogenic oomycetes in the laboratory (Fig. 4B). However, there are some other *Lagenidium* strains pathogenic for mammals that lack these features and, thus, morphology alone would not provide an unequivocal distinction between these two organisms.

Isolation Procedures, Identification, and Typing Systems

See the corresponding text in the section on *Pythium insidiosum*.

Serologic and DNA Tests

ELISA and Western blot assays have been tested in cases of lagenidiosis in both humans and lower animals (15, 47). Although both assays detected the presence of antibodies in cases of lagenidiosis, the antigenic banding patterns observed in Western blot were very difficult to interpret and ELISA had a strong cross-reaction with *P. insidiosum* antigens. Despite this limitation, the ELISA has been suggested to monitor the response to treatment because a decline in anti-*Lagenidium* antibodies has been demonstrate in cured cases (47). Specific primers for the identification of isolates and *Lagenidium* hyphae in biopsied tissue section have been described (47). However, their specificity is not known.

Antimicrobial Susceptibilities

As previously mentioned, the oomycetes differ from the true fungi in several aspects, one of which is that oomycetes lack sterols in their cytoplasmic membranes. Thus, *Pythium* spp. and *Lagenidium* spp. are intrinsically resistant to most antifungal drugs targeting this pathway. Thus, an early and accurate diagnosis is essential for a good response in infected hosts. Several antifungals have been tested *in vitro* using *Lagenidium* isolated from humans and lower animals (51). Sadly, the *in vivo* response has shown contradictory results.

Evaluation, Interpretation, and Reporting of Results

Cases of lagenidiosis with positive culture have to be evaluated by microscopy looking for the typical structures encountered in some strains (Fig. 4B). If the strain does not display fruiting bodies, the induction of zoospores must be performed (25, 27). The development of sporangia and biflagellate zoospores could be interpreted as proof that the isolate is an oomycete. So far, there are no reports of oomycetes as normal laboratory contaminants or normal microbiota of humans or lower animals. In addition, genomic DNA from the strain under investigation should be tested using molecular methodologies (see above) to further confirm the findings. The report of the case should include the following: (i) the presence of broad coenocytic hyphae in wet mounts and stained preparations from clinical samples, (ii) the development of zoospores from positive cultures, and (iii) the identification of the strain by morphology and DNA tools.

RHINOSPORIDIUM SEEBERI

Taxonomy

The first two cases of rhinosporidiosis were reported in 1900 by Guillermo Rodolfo Seeber in his M.D. thesis in Argentina (1, 2). He stated that in 1896 he had found two patients with nasal polyps containing an organism similar to that reported by Posadas in 1892 (coccidioidomycosis). He also mentioned that, in 1892, Malbran had studied a case of a nasal polyp showing a spherical microbe with identical morphological features. A nasal case of rhinosporidiosis observed by O'Kinealy in 1903 in India was studied in detail by Minchin and Fanthan in 1905 (2). These investigators suggested the binomial *Rhinosporidium kinealyi* to identify this pathogen. Seeber in 1912 introduced the name *Rhinosporidium seeberi* and called attention to its priority over *R. kinealyi*. Ashworth in 1923 (2) stated that the genus *Rhinosporidium* proposed by Minchin and Fanthan should be adopted and that, based on the description of Seeber (2) and the name *Coccidioides seeberia* reintroduced by Belou, the binomial *R. seeberi* has priority.

The fact that this pathogen has not been cultured led some investigators to extreme hypotheses that were critically reviewed by Vilela and Mendoza (57). The suggestion that *R. seeberi* is a cyanobacterium in the genus *Microcystis* is the most recent in a long list of similar views. This and other views have been challenged by several groups showing that morphological, cell cycle, and phylogenetic analyses all linked this pathogen with the protistal eukaryotes (57). The placement of *R. seeberi* within the Mezomycetozoa came as a surprise. This group comprises orphan aquatic fish and amphibian parasites with spherical forms and endospores strikingly similar to those of *R. seeberi* (5, 6). Using the ribosomal DNA internal transcriber spacer region (ITS), it was recently found that *R. seeberi* may include several species-specific strains that could represent new species (58).

Description of the Agent

This anomalous pathogen has resisted culture; thus, its morphological features are only known through *in vivo* microscopic and ultramicroscopic studies (1, 2). In infected tissues, *R. seeberi* has a complex parasitic cell cycle. It appears as multiple spherical structures known as sporangia (cysts in most mesomycetozoans) in different stages of development (Fig. 5A and B). The *in vivo* life cycle starts with the release of hundreds of oval or spherical endospores, 7 to 15 μm in diameter, from a pore developed only in mature sporangia. The endospores increase in size and progressively develop from juvenile (JS) (10 to 100 μm), to intermediate (IS) (100 to 150 μm), then to mature sporangia (MS) (150 to more than 450 μm). The endospores are released from MS and then the *in vivo* cycle is somehow reinitiated (Fig. 5C). Although the diameter of the sporangium has been used to identify the *in vivo* stages, the mature sporangium differs from other stages by the presence of well-developed endospores (Fig. 5D). The finding of mitotic figures within some sporangia in histological preparations has been mentioned (59). These authors found that the nuclei within IS sporangia synchronously divide without cytokinesis. A cell wall is developed around each endospore in large sporangia with thousands of nuclei, becoming an MS.

Epidemiology and Transmission

Rhinosporidiosis usually occurs in most tropical and subtropical areas of the world except Australia (1). Although the infection was first recognized in Argentina, India and Sri Lanka show the highest occurrence of the disease (54). Rhinosporidiosis occurs sporadically in other geographical areas such as the Americas, Africa, Europe, and Asian countries including the Middle East. Because some cases of rhinosporidiosis occur in dry areas, especially after sand storms, the hydrophilic nature of this pathogen has been long questioned (1, 2, 60). Based on accounts from patients with the disease, it is believed that rhinosporidiosis is acquired through contact with aquatic environments contaminated with *R. seeberi*, but the precise mechanism of infection from natural sources is unknown.

The finding linking *R. seeberi* to aquatic pathogens of fish and amphibians (5, 6, 61), tends to confirm its hydrophilic nature. Most probably, this pathogen evolved from an aquatic niche, in which it still can be found, to terrestrial environments by the development of resistant spores. This is a very likely scenario since the formation of zoospores, as in the other members of the Dermocystida among which *R. seeberi* is phylogenetically located, has not been found (1, 2, 6, 60). The disease tends to occur as single cases, but two outbreaks of rhinosporidiosis in humans in Serbia (62) and in swans from Florida, United States (63), have also been recorded. The resistant spores present in water and soil may gain entry through small cutaneous or mucocutaneous wounds and establish infection. Although the disease occurs in apparently normal hosts, some investigators have suggested associations with particular occupational and social conditions (1, 60). Little is known about the predisposing factors leading to the disease. The disease has not been induced in experimental animals, and transmission from one host to another has yet to be reported.

Clinical Significance

In addition to humans, rhinosporidiosis around the facial areas has been also reported in several animal species including cattle, cats, dogs, goats, horses, river dolphins, and birds (1, 6). In humans, the most common clinical manifestation is the formation of painless polyps usually located on mucosal areas of the nose, eye, larynx, genitalia, and rectum. Multicentric skin lesions have also been recorded (60). The disease is not life-threatening, but it can cause breathing difficulties when the polyps obstruct the nose or laryngeal passages. Rhinorrhea and bleeding are common with polyps located in the nose. The slow-growing polipoidal masses are usually found as single or multiple, pedunculate (attached to the skin), sessile, red lesions that bleed easily. Pruritus of the affected areas is also common. The differential diagnosis includes bacterial and fungal infection, neoplasia, and other similar mucosal and skin conditions.

Collection, Transport, and Storage of Specimens

The guidelines for the collection, processing, storage, and examination of specimens are provided in chapter 114. Clinical specimens collected in cases of rhinosporidiosis are usually biopsy tissues from infected sites. Since *R. seeberi* cannot be cultured, clinical specimens are usually fixed in formaldehyde upon collection to be histopathologically evaluated later. Nonetheless, fresh samples should also be examined in the laboratory to rule out other etiologic agents and to confirm the histopathological findings. In these cases, biopsy specimens should be aseptically collected and transported immediately to the laboratory. For samples collected far from the laboratory, cooling (−80 to 4°C) of collected specimens for shipping or storage purposes may be necessary.

Direct Examination

Microscopy

Wet mount preparations from clinical specimens from cases of rhinosporidiosis usually show the presence of mature and immature spherical sporangia and numerous endospores. Mature sporangia with endospores have thin cell walls and measure more than 400 μm in diameter. JS and IS sporangia are smaller and may have thicker cell walls. In fresh specimens treated only with water, the release of endospores from MS sporangia has been reported (6). A purification system to study *R. seeberi* phenotypes was recently proposed (1). The presence of *R. seeberi* sporangia and endospores can also be

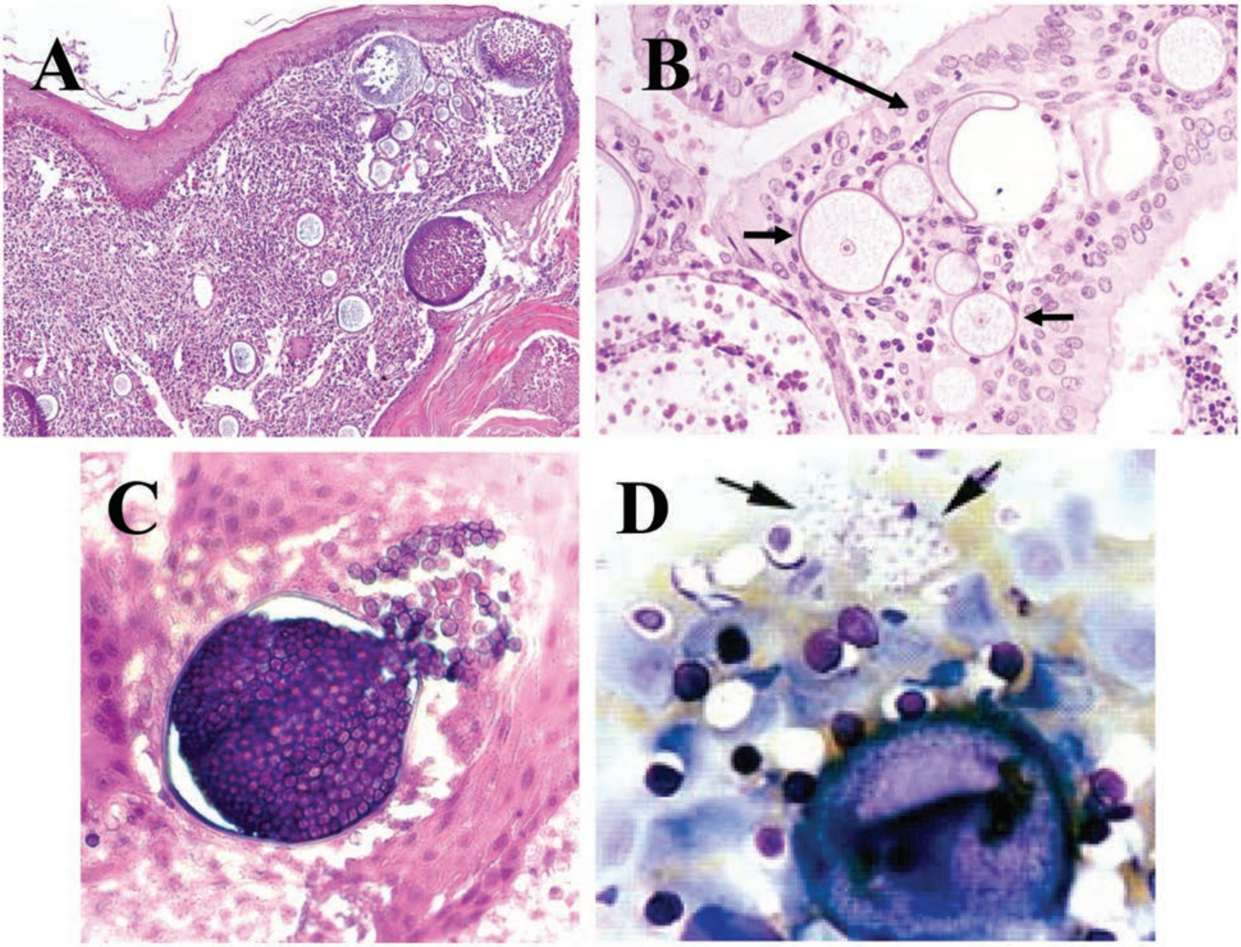

FIGURE 5 *R. seeberi.* (A) H&E-stained mature sporangia with endospores and numerous juvenile sporangia of different sizes. Magnification, ×10. (B) A collapsed sporangium in U-shaped and juvenile sporangia with prominent nuclei and nucleoli (long and short arrows, respectively). Magnification, ×30. (C) Mature sporangium releasing endospores through a cell wall pore. Magnification, ×30. (D) Wright-Giemsa impression smear from a dog with nasal rhinosporidiosis. An immature collapsed sporangium may be observed in the lower section. Numerous endospores surrounded by a clear halo are shown near the top. Magnification, ×70. Panel D is courtesy of W. A. Meier. doi:10.1128/9781555817381.ch127.f5

found on smears stained with Giemsa or Gram stain (Fig. 5D). The spherical unstained elements of *R. seeberi* develop autofluorescence when viewed with a fluorescence microscope.

The parasitic spherical structures of this mesomycetozoan pathogen stain very well with H&E, but they also stain with GMS and PAS, a feature used by many in the past to suggest a link with the members of the kingdom Fungi. Biopsy tissue from polypoidal lesions stained with H&E is characterized by the presence of numerous sporangia at different stages of development (Fig. 5A and B). Hyperplasia of the mucous membranes and/or skin with fibrovascular and fibromyxomatous connective tissue containing numerous sporangia is the main feature of the infection. Inflammatory infiltrates of lymphocytes, neutrophils, plasma cells, and more rarely giant cells and eosinophils are usually observed in cases of nasal, ocular, and skin infections. JS and IS possess a central nucleus with a prominent nucleolus (Fig. 5B). The collapse of defective sporangia may cause the formation of U-shaped structures. Mature sporangia possessing several thousand endospores are usually found near the mucosal epithelium, where they are transported from the internal infected areas by a transepidermal elimination phenomenon (Fig. 5A and C) (60). The cell wall of the mature sporangia is usually thin and the enclosed endospores may contain clusters of 1- to 3-μm reddish spherical vesicles. Endospores have a mucoid capsule that does not stain in H&E preparations. The presence of a pore on the sporangial wall is also observed, depending on the plane of the sectioned tissue (Fig. 5C).

Nucleic Acid Detection in Clinical Materials

Currently there are no available DNA-based techniques for the diagnosis of *R. seeberi.*

Isolation Procedures, Identification, and Typing Systems

Despite numerous reports claiming that *R. seeberi* has been isolated in pure culture, such claims have not yet been validated. Most of the organisms recovered from cases of rhinosporidiosis have proven to be fungal or bacterial contaminants (1, 60). Because *R. seeberi* has not yet been cultured, the identification of this pathogen is based on its phenotypic characteristics on wet mount preparations and/or histopathological analyses. The morphological characteristics of *R. seeberi* in infected tissues are almost pathognomonic. Nonetheless, the organism's epidemiological features and the clinical signs of disease should be taken into consideration for a final diagnosis. Morphologically, the parasitic (spherule) stages of *Coccidioides immitis* and *C. posadasii* mimic the *R. seeberi* sporangia with endospores. However, the sporangia size range of *R. seeberi* (<400 to 4 µm), the epidemiological, clinical, and phylogenetic features of coccidioidomycosis, and the fact that *Coccidioides* species can be readily cultured categorically separate this pathogen from *R. seeberi*.

Serologic Tests

Although early investigators did not detect *R. seeberi* antibodies in the sera of infected hosts using endospores or sporangia as antigens, others suggested that the reason for the failure was the use of insensitive assays such as immunodiffusion. Using an immunoelectronmicroscopic approach, it was shown for the first time that anti-*R. seeberi* antibodies reacted against a specific antigen in mature sporangia in the sera of patients with rhinosporidiosis (60). Despite these efforts, there are no available serological diagnostic assays for rhinosporidiosis in the clinical setting.

Antimicrobial Susceptibility

R. seeberi is resistant to most antifungal drugs (1, 60); however, the use of dapsone was found helpful to control some cases (1). Because this pathogen cannot be isolated in culture, susceptibility testing is not possible. Treatment is carried out by surgical removal of the infected tissues.

Evaluation, Interpretation, and Reporting of Results

The finding of spherical structures at different stages of development, some of them containing numerous endospores on histopathology, in cytological samples stained with Giemsa, Gram, and wet mount preparations, is suggestive of rhinosporidiosis. Since the *R. seeberi* spherical structures with endospores mimic the parasitic stage of *Coccidioides* species, a differential diagnosis is required, especially in the endemic areas of coccidioidomycosis. The finding of >300-µm spherical sporangia with endospores and negative cultures could be confirmatory of rhinosporidiosis. The report of the results should contain the finding of typical spherical structures that resisted culture.

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Microsporidia

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TAXONOMY

Microsporidia are obligate intracellular, unicellular, spore-forming eukaryotes. More than 160 microsporidial genera and 1,300 species that are pathogenic in every major animal group have been identified (1–5). To date, nine genera (*Anncaliia*, *Encephalitozoon*, *Endoreticulatus*, *Enterocytozoon*, *Nosema*, *Pleistophora*, *Vittaforma*, *Tubulinosema*, and *Trachipleistophora*), as well as unclassified microsporidia, assigned to the collective group *Microsporidium*, have been implicated in human infections (Table 1).

Microsporidia develop intracellularly exclusively and have no metabolically active stages outside the host cell. A life cycle (Fig. 1) involving a proliferative merogonic sequence followed by a sporogonic sequence results in environmentally resistant spores of unique structure. Mature spores contain a tubular extrusion apparatus (polar tube) for injecting infective spore contents (sporoplasm) into the host cell.

Microsporidia are true eukaryotes because they have a membrane-bound nucleus, an intracytoplasmic membrane system, and chromosome separation on mitotic spindles, but they are unusual eukaryotes in that they have bacterium-like ribosomes, no recognizable mitochondria, no peroxisomes, and simple vesicular Golgi membranes. Compared with those of other eukaryotes, the genomes of microsporidia are reduced in size and complexity (6, 7). The genome sizes of different microsporidia vary between 2.3 and 19.5 Mbp, and the numbers of chromosomes range from 7 to 16 (8). The compactness of the microsporidial genomes results from the loss of genes and from the reduction of coding and noncoding elements. The small-subunit rRNA genes of microsporidia, for example, are significantly shorter than those of other eukaryotes.

Due to the absence of mitochondria in microscopical studies, it had been postulated that microsporidia are ancient protists that diverged before the mitochondrial endosymbiosis (9). However, genes related to mitochondrial functions were identified in *Encephalitozoon cuniculi* (6), and immunolocalization of the mitochondrial heat shock protein 70 in the microsporidian *Trachipleistophora hominis* revealed tiny organelles with double membranes, named mitosomes (10, 11). Recent genome-wide sequence and synteny analyses indicate that the organisms of the phylum Microsporidia belong to the kingdom of the Fungi (12), being derived from an endoparasitic chytrid ancestor on the earliest diverging branch of the fungal phylogenetic tree (13, 14). Also, structural features of the organisms such as the presence of chitin in the spore wall, diplokaryotic nuclei, and electron-dense spindle plaques associated with the nuclear envelope suggest a possible relationship between fungi and microsporidia, whereas the life cycle of microsporidia is unique and dissimilar to that of other fungal species.

DESCRIPTION OF THE GENERA AND SPECIES

The species are illustrated in Fig. 1 and listed in Table 1.

Anncaliia spp. have diplokaryotic nuclei and develop in direct contact with host cell cytoplasm. Additionally, the organisms produce electron-dense extracellular secretions and vesiculotubular appendages. Three former members of the microsporidial genus *Brachiola* that are pathogenic in humans were transferred to the genus *Anncaliia* based on novel ultrastructural and molecular data (15). Disporoblastic sporogony of *Anncaliia vesicularum* (formerly *Brachiola vesicularum*) produces 2.5- by 2-µm diplokaryotic spores containing 7 to 10 anisofilar coils of the polar filament arranged in one to three rows, usually two (16). On the basis of diplokaryotic nuclei, disporoblastic sporogony, and the formation of vesiculotubular secretions, *Nosema connori* and *Nosema algerae*, also discovered in human infections, have been reclassified as species of *Anncaliia*. Spores of *Anncaliia connori* measure 2.0 to 2.5 by 4.0 to 4.5 µm and contain polar tubes with 10 to 12 coils (17). Spores of *Anncaliia algerae* measure 3.7 to 5.4 by 2.3 to 3.9 µm and have 8 to 11 coils of the polar tube (18).

Encephalitozoon spp. develop intracellularly in parasitophorous vacuoles bounded by a membrane of presumed host-cell origin. Nuclei of all stages are unpaired. Meronts divide repeatedly by binary fission and lie close to the vacuolar membrane. Sporonts appear free in the center of the vacuole and divide into two or four sporoblasts, which mature into spores. The spores measure 1.0 to 1.5 by 2.0 to 3.0 µm, and the polar tube has four to eight isofilar coils.

Encephalitozoon cuniculi was isolated from a range of animals before human infections were identified. Human isolates of three *Encephalitozoon* spp. and animal isolates of *E. cuniculi* are morphologically identical. In 1991, *Encephalitozoon hellem* was distinguished from *E. cuniculi* on the basis of different protein patterns (19). In 1993, *Septata intestinalis* was described and named on the basis of the unique morphological findings that the organisms are contained in intracellular vacuoles and that a pathogen-secreted fibrillar network

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TABLE 1 Microsporidial species pathogenic in humans, and clinical manifestations

Microsporidial species	Clinical manifestations	
	Immunocompromised patients	Immunocompetent persons
Anncaliia algerae[a]	Myositis, nodular cutaneous lesions	Keratitis
Anncaliia connori[b]	Disseminated infection	Not described
Anncaliia vesicularum[c]	Myositis	Not described
Encephalitozoon cuniculi	Disseminated infection, keratoconjunctivitis, sinusitis, bronchitis, pneumonia, nephritis, hepatitis, peritonitis, intestinal infection, encephalitis	Encephalitis
Encephalitozoon hellem	Disseminated infection, keratoconjunctivitis, sinusitis, bronchitis, pneumonia, urogenital infection	Possibly diarrhea
Encephalitozoon intestinalis[d]	Chronic diarrhea, cholangiopathy, sinusitis, bronchitis, pneumonitis, nephritis, bone infection, nodular cutaneous lesions	Self-limiting diarrhea, asymptomatic carriers
Endoreticulatus spp.	Not described	Myositis, disseminated infection
Enterocytozoon bieneusi	Chronic diarrhea, wasting syndrome, "AIDS-cholangiopathy," cholangitis, acalculous cholecystitis, chronic sinusitis, pneumonitis	Self-limiting diarrhea in adults and children, traveler's diarrhea, asymptomatic carriers
Microsporidium africanum, *M. ceylonensis*[e]	Not described	Corneal ulcer, keratitis
Nosema ocularum	Not described	Keratitis
Pleistophora sp., *P. ronneafiei*	Myositis	Not described
Trachipleistophora anthropophthera	Disseminated infection, keratitis	Not described
Trachipleistophora hominis	Myositis, myocarditis, keratoconjunctivitis, sinusitis	Keratitis
Tubulinosema acridophagus	Disseminated infection, myositis, hepatitis, pulmonary, peritoneal and skin infection	Not described
Vittaforma corneae[f]	Disseminated infection, urinary tract infection	Keratoconjunctivitis

[a] Formerly *Brachiola algerae*, *Nosema algerae*.
[b] Formerly *Brachiola connori*, *Nosema connori*.
[c] Formerly *Brachiola vesicularum*.
[d] Formerly *Septata intestinalis*.
[e] *Microsporidium* is a collective generic name for microsporidia that cannot be classified because available information is not sufficient.
[f] Formerly *Nosema corneum*.

surrounds the developing organisms, giving the vacuoles a septate appearance (20). Subsequently, on the basis of phylogenetic analyses, *S. intestinalis* was reclassified as *Encephalitozoon intestinalis*. Finally, so far four genotypes of *E. cuniculi* have been identified that partially differ in animal-host preferences and geographic distributions (21, 22).

Endoreticulatus spp. was proposed in a single case based on genetic findings; ultrastructural results are not available (23).

Enterocytozoon bieneusi develops in direct contact with host-cell cytoplasm (Fig. 2). The proliferative and sporogonial forms are rounded multinucleate plasmodia with unpaired nuclei measuring up to 6 µm in diameter. The oval spores measure 0.7 to 1.0 by 1.1 to 1.6 µm. The polar tube, derived from electron-dense disks in sporonts, has five to seven isofilar coils that appear in two rows when seen in transverse section by transmission electron microscopy (24).

Nosema spp. develop in direct contact with host-cell cytoplasm, nuclei are paired (diplokaryotic), divisions are by binary fission, and sporonts are disporoblastic. Only one species of human origin, *Nosema ocularum*, has been retained in this genus. It will probably require reclassification, but insufficient information is currently available for a new generic assignment. Spores measure 3 by 5 µm and have polar tubes with 9 to 12 coils.

Pleistophora spp., including *P. ronneafiei*, which is pathogenic in humans, have unpaired nuclei, and all stages are multinucleate plasmodia, which divide into smaller multinucleate segments. Meronts have a thick, amorphous coat, which separates from the surface in sporogony to form a sporophorous vesicle. Sporogonic divisions give rise to a large and variable number of spores, packaged in the persistent sporophorous vesicle. The spores contain polar tubes with 9 to 12 coils and measure 2 to 2.8 by 3.3 to 4.0 µm (25).

Trachipleistophora hominis forms spores in sporophorous vesicles; these arise from repeated binary fissions and not from multinucleate plasmodia. The vesicles, which contain 2 to more than 32 spores, enlarge as the number of spores increases. The nuclei are unpaired in all stages of development. The pear-shaped spores measure 2.4 by 4.0 µm and have about 11 anisofilar coils (26). *Trachipleistophora anthropophthera* is similar to *T. hominis* but appears to be dimorphic, as two different forms of sporophorous vesicles and spores have been observed (27).

Tubulinosema acridophagus was proposed based on genetic analyses. Ultrastructurally, all developing stages are in direct

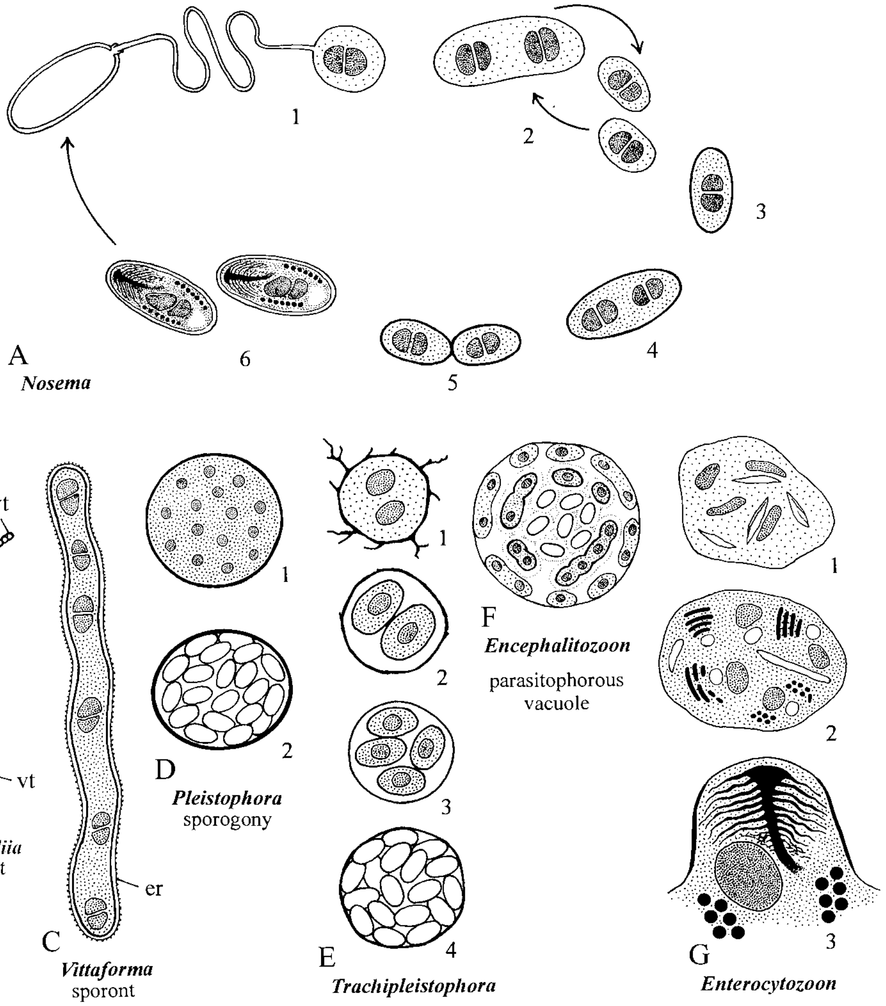

FIGURE 1 Generalized life cycle of microsporidia and identifying characteristics of the genera known to infect humans. Light stippling indicates merogonic stages; heavy stippling indicates sporogonic stages; no stippling indicates spores and sporoplasm. (A) Basic life cycle, illustrated by *Nosema*. Development may occur in direct contact with host-cell cytoplasm (*Nosema*, *Anncaliia*, and *Enterocytozoon*) or in isolation by host cell membranes (*Vittaforma* and *Encephalitozoon*) or a cyst-like sporophorous vesicle of pathogen origin (*Pleistophora* and *Trachipleistophora*). 1, Sporoplasm: the infective stage emergent from the spore. It may have unpaired nuclei (monokaryotic) or two nuclei in close apposition (diplokaryotic), depending on the genus. 2, Merogony: proliferative stage. It may have a simple plasma membrane, but a surface coat is present in *Anncaliia*, *Pleistophora*, and *Trachipleistophora*. Division can be by binary or multiple fission into two or more individuals. 3, Sporont: the first stage of sporogony. If not already present, a surface coat is added (this step is delayed in *Enterocytozoon*). 4, Sporogony: divisions culminating in spore production. Binary or multiple fissions give rise to sporoblasts. 5, Sporoblasts: end products of sporogony, which mature into spores. 6, Spores: resistant stages for transmission. Spores are characterized by an extrusion apparatus (polar tube), which serves to conduct the sporoplasm into a host cell. The polar tube may be of uniform diameter (isofilar) or show a sharp decrease in diameter in the most posterior coils (anisofilar). (B to G) Identifying characteristics of genera. (B) *Anncaliia*. Members of this genus are diplokaryotic and disporoblastic; the life cycle is like that of *Nosema*, but meronts are of bizarre shapes and possess a surface coat with vesiculotubular structures (vt) embedded in it and extended from it. (C) *Vittaforma*. Members of this genus are diplokaryotic and polysporoblastic; all stages, including spores, are isolated in a close-fitting, ribosome-studded cisterna of host endoplasmic reticulum (er). (D) *Pleistophora*. Members of this genus are monokaryotic and polysporoblastic; meronts and sporonts are multinucleate stages called plasmodia; a thick surface coat is already present on meronts and sporonts (1); this coat separates from the sporogonial plasmodium to form a cyst-like vesicle, the sporophorous vesicle; the plasmodium divides within it to produce numerous spores (2). (E) *Trachipleistophora*. Members of this genus are monokaryotic and polysporoblastic. The meront surface coat has branched extensions (1); these are withdrawn when the coat separates to form the sporophorous vesicle around a uninucleate sporont; the sporont undergoes a series of binary fissions (2 and 3) and finally encloses numerous spores (4). (F) *Encephalitozoon*. Members of this genus are monokaryotic and di- or tetrasporoblastic; all stages of the life cycle, developing from the sporoplasm, occur concurrently within a host cell vacuole (parasitophorous vacuole); merogonic stages are appressed against the vacuole wall; sporogonic stages are free; the vacuole is finally packed with spores, so that it superficially resembles a sporophorous vesicle. (G) *Enterocytozoon*. Members of this genus are monokaryotic and polysporoblastic; meronts (1) have irregular nuclei and lucent clefts; sporonts (2) are multinucleate with rounded nuclei and have highly characteristic electron-dense disks, which are polar tube precursors; all spore organelles are formed prematurely, so that constriction around the sets of organelles and a nucleus (3) gives rise directly to almost mature spores. The surface coat is deposited only during constriction. doi:10.1128/9781555817381.ch128.f1

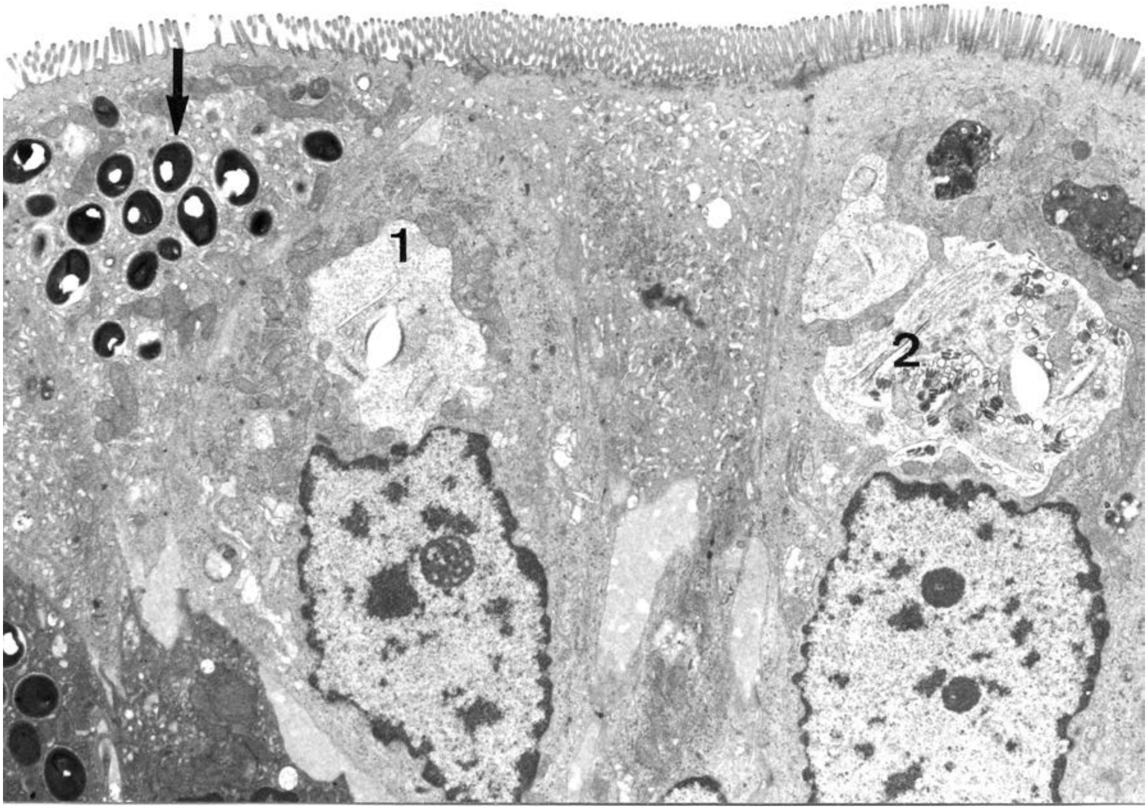

FIGURE 2 Transmission electron micrograph showing the duodenal epithelium of a HIV-infected patient infected with *Enterocytozoon bieneusi*. The different developmental stages between the enterocyte nuclei and the microvillus border include a proliferative plasmodium (1), late sporogonial plasmodia (2), and mature spores (arrow). Magnification, ×5,370. (Courtesy of M. A. Spycher, University Hospital, Zurich, Switzerland.) doi:10.1128/9781555817381.ch128.f2

contact with the host-cell cytoplasm. Spore size ranges from 1.4 to 2.4 µm in length, nuclei are diplokaryotic, and polar filaments are anisofilar and contain 11 coils arranged mostly in single rows, although in some spores double rows are present (28, 29).

Vittaforma corneae, originally classified as *Nosema corneum*, was transferred to a new genus on the basis of ultrastructural features (30). Nuclei are diplokaryotic, sporogony is polysporoblastic, sporonts are ribbon-shaped, and all stages, including spores, are individually enveloped by a cisterna of host endoplasmic reticulum studded with ribosomes. The spores contain polar tubes with five to seven coils and measure 1.2 by 3.8 µm.

Microsporidium is a collective generic name for microsporidia that cannot be classified because available information is not sufficient. *Microsporidium ceylonensis* and *Microsporidium africanum* have been assigned to the group name (31).

EPIDEMIOLOGY AND TRANSMISSION

Human microsporidial infections have been documented globally. The sources of microsporidia infecting humans and their modes of transmission are uncertain but ingestion of the environmentally highly resistant spores is probably the most important mode of transmission. Transmission by dust or aerosol has also been considered based on respiratory or ocular infections (32, 33). Direct contact with water during bathing in hot spring spas was associated with microsporidial keratitis (34). Water contact was found to be a risk factor for microsporidiosis. *E. bieneusi*, *E. intestinalis*, and *V. corneae* homologous DNA has been detected in sewage effluent, groundwater, and surface water (35, 36), also suggesting anthroponotic transmission. Furthermore, foodborne outbreaks associated with microsporidia have been reported (37), and microsporidia pathogenic in humans have been detected in fresh food produce (38) and in milk specimens from dairy cows (39). Studies with mammals suggest that *Encephalitozoon* spp. can be transmitted transplacentally from mother to offspring, but no congenitally acquired human infections have been reported.

Direct zoonotic transmission of microsporidia has not been verified but appears likely because many microsporidial species can infect both humans and animals (3). *E. cuniculi* is considered to be a zoonotic pathogen; two of its four genotypes that were detected in natural infections in rabbits, dogs, and tamarins have been isolated from human immunodeficiency virus (HIV)-infected patients (21, 40). In addition to documentation in humans, *Encephalitozoon* spp. have been detected in psittacines kept in aviaries, in a variety of wild birds, including in feces of urban feral pigeons (41), in domestic animals in Mexico (42), and in gorillas in Uganda. *E. bieneusi*, discovered in humans, is increasingly being recognized in animals (wildlife, livestock, and companion animals). Genetic analyses of this pathogen revealed two major groups, one comprising anthroponotic genotypes, and the other containing multiple genotypes with zoonotic potential (43). Also, an insect-pathogenic microsporidian, *A. algerae*, was isolated from three patients (44, 45).

CLINICAL SIGNIFICANCE

Although microsporidiosis appears to occur most frequently in persons infected with HIV, it is emerging in otherwise immunocompromised but also in immunocompetent persons (Table 1) (1, 4). In the non-HIV-infected immunocompromised host, microsporidial infections were described in organ-transplant recipients (46), in patients with hematologic malignancies receiving monoclonal antibody therapy (47), in patients with rheumatic disease undergoing anti-tumor necrosis factor therapy (48), in the elderly (49), and in malnourished children (50). In immunocompetent persons, microsporidia have been associated with keratoconjunctivitis (51), self-limiting diarrhea (52), and rarely cerebral infection (53–55). Microsporidia are also found in asymptomatic persons (56).

Microsporidiosis has been associated with abnormalities in structures and functions of infected organs, but the mechanisms of pathogenicity of the different microsporidial species are not sufficiently understood. Patients with severe cellular immunodeficiency appear to be at the highest risk for developing microsporidial disease. Unfortunately, little is known about immunity to this infection, although the importance of T cells has been demonstrated in experiments with athymic mice (57). It is not understood whether microsporidiosis in immunocompromised patients is primarily a reactivation of latent infection acquired prior to the state of suppressed immunity or whether microsporidial disease is caused by recently acquired infection.

Enterocytozoon bieneusi

E. bieneusi infects the enterocytes of the small intestine and epithelial cells of the biliary tree and respiratory tract. Clinical disease is probably caused by the continuous excess loss of epithelial cells. *Enterocytozoon* infection may be accompanied by alterations in small bowel physiology such as decreased brush border sucrase, lactase, and maltase activities, as well as malabsorption.

E. bieneusi is estimated to be one of the most important HIV-associated intestinal pathogens, present in 5 to 30% of patients with chronic diarrhea, weight loss, or cholangiopathy, particularly when CD4 lymphocyte counts are below 100/μl (58). Upper or lower respiratory tract infections have been detected in a few patients (59), but systemic infection due to *E. bieneusi* has not been documented. Case reports have documented that the organisms are also a cause of diarrhea in organ transplant recipients (46).

In immunocompetent adults and in children, *E. bieneusi* is associated with self-limited watery diarrhea (lasting up to 2 to 3 weeks), particularly among persons who reside or have traveled in tropical countries (60). Furthermore, the organisms have been identified among malnourished children in tropical areas (50), and elderly persons (residing in resource-rich countries) with acute or chronic diarrhea (49).

Encephalitozoon spp.

Encephalitozoon spp. infect epithelial and endothelial cells, fibroblasts, macrophages, and possibly other cell types, as reported for humans and mammals. Human encephalitozoonosis was first described in two children with a seizure disorder (53, 54). Cerebral infections have subsequently been substantiated (55, 61, 62). Unexpectedly, *E. cuniculi* endocarditis was diagnosed in a non-immunocompromised patient with a dual-chamber pacemaker (63).

The spectrum of recognized *E. cuniculi*- and *E. hellem*-associated disease in patients with AIDS, organ transplant recipients, and otherwise immunocompromised patients includes keratoconjunctivitis, intraocular infection, sinusitis, bronchiolitis, pneumonitis, nephritis, ureteritis, cystitis, prostatitis, urethritis, hepatitis, sclerosing cholangitis, peritonitis, diarrhea, and encephalitis (1, 19, 40, 61). Clinical manifestations may vary substantially, ranging from an asymptomatic carrier state to organ failure.

E. intestinalis infects primarily enterocytes, but the organism is also found in intestinal lamina propria, and dissemination to the kidneys, airways, and biliary tract appears to occur via infected macrophages (20). Chronic diarrhea and disseminated disease due to *E. intestinalis* have been diagnosed mainly in immunodeficient patients. A case of nodular cutaneous *E. intestinalis* infection was observed in a patient with AIDS (64). Furthermore, *E. intestinalis* has been detected in stool specimens from healthy children and adults, with or without diarrhea, living in Mexico (65), and among travelers with diarrhea returning from tropical countries (66).

Other Microsporidia

Different microsporidial species have been isolated from immunocompetent persons (1, 30, 67) and from immunodeficient persons (68) with keratoconjunctivitis, severe keratitis, or corneal ulcers. Also, keratoconjunctivitis due to microsporidia has been diagnosed in immunocompetent contact lens wearers (Table 1).

In immunocompromised patients, disseminated infections and myositis have been linked to infections with different microsporidia (Table 1), including *Pleistophora* sp. (69), *P. ronneafiei* sp. (25), *T. hominis* (70), *A. vesicularum* (16), *A. algerae* (44), and *T. acridophagus* (28, 29). *A. algerae* has also been isolated from erythematous skin nodules from a boy with acute lymphocytic leukemia (71). Myositis due to *Endoreticulatus* spp. was diagnosed in an otherwise healthy man in Thailand (23).

Trachipleisthophora anthropophthera has been identified at autopsy in cerebral, cardiac, renal, pancreatic, thyroid, hepatic, splenic, lymphoid, and bone marrow tissue of patients with AIDS who initially presented with seizures (72). Disseminated infection due to *A. connori* was found at autopsy in a 4-month-old athymic male infant (17).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Spores of enteropathogenic microsporidia can be detected in stool specimens or duodenal aspirates that have been fixed in 10% formalin or in sodium acetate-acetic acid-formalin, fresh stool samples, or biopsy specimens. The spores of microsporidia causing disseminated infection can usually be detected in fresh or fixed urine sediments, other body fluids (including sputum, bronchoalveolar lavage fluid, nasal secretions, cerebrospinal fluid, and conjunctival smears), corneal scrapings, or tissue. For histologic examination, tissue specimens are fixed in formalin. For electron microscopy, fixation of tissue with glutaraldehyde is preferred. Collection of fresh material (without fixative) may be useful for cell culture and for molecular identification. Microsporidial spores are environmentally resistant and, if prevented from drying, can remain infectious for periods of up to several years (73).

DETECTION PROCEDURES

The most robust technique for the diagnosis of microsporidial infection is light-microscopic detection of the organisms themselves. Spores, the stages of microsporidia pathogenic in humans that are usually identified, are small, ranging in size from 1 to 4 µm. Evaluation of patients with suspected intestinal microsporidiosis should begin with light-microscopic examination of stool specimens, and microsporidia which cause systemic infection are best detected in urine sediments or other body fluids. Definitive species identification of microsporidia is made by electron microscopy and, preferably, genetic analysis (73).

Examination of Stool Specimens

Preparation of Smears, Staining, and Microscopic Examination

Smears are prepared using 10 to 20 µl of unconcentrated stool that is very thinly spread onto slides. Most of the coprological procedures that have been adapted for the concentration of ova and parasites fail to concentrate microsporidial spores. The formalin-ethyl acetate concentration and different flotation methods remove significant amounts of fecal debris, and smears prepared from these concentrates appear to be easier to read by light microscopy, but are less sensitive (74).

The most commonly used stains are chromotrope-based stains (74) and chemofluorescent optical brightening agents (75, 76), including calcofluor white and other chemofluorescent stains. Regardless of which staining technique is utilized, the use of positive-control material is essential. The detection of microsporidial spores requires adequate illumination and magnification, i.e., magnification of ×630 or ×1,000 (oil immersion). The differences in the sizes of *Enterocytozoon* spores (1 to 1.5 µm) and *Encephalitozoon* spores (2 to 3 µm) often permit a tentative identification of the genus from light-microscopic examination of stool specimens.

Chromotrope-based staining procedures

Microsporidial spores are ovoid and have a specific appearance when stained with chromotrope stains (Fig. 3 and 4, right panel) (74). The spore wall stains bright pinkish red, some spores appear transparent, and other spores show a distinct pinkish red-stained belt-like stripe that girds the spores diagonally or equatorially. Most background debris in stool specimens counterstains a faint green (or blue, depending on the staining technique). Some other fecal elements, such as parasite cysts (e.g., *Cyclospora*), yeasts, and some bacteria, may also stain reddish, but they are distinguished from microsporidial spores by their sizes, shapes, and staining patterns.

Several modifications of the original chromotrope-staining solution (74) have been proposed, including modifications of the counterstain and changes in the temperature of the standard chromotrope-staining solution and in the staining time (77). An acid-fast trichrome stain (78), that permits visualization of acid-fast cryptosporidial oocysts as well as microsporidial spores on the same slide, and a "quick-hot" Gram-chromotrope-staining technique (79) have also been developed.

Chemofluorescent Agents

Chemofluorescent optical brightening agents are chitin stains, which require examination with a fluorescent microscope. With the correct wavelength illumination, the chitinous wall of the microsporidial spores fluoresces brightly, facilitating the detection of spores. However, staining is not specific because small yeast cells, which may be present in fecal material, and other fecal elements may also be brightened. Some experience is necessary to distinguish the microsporidia.

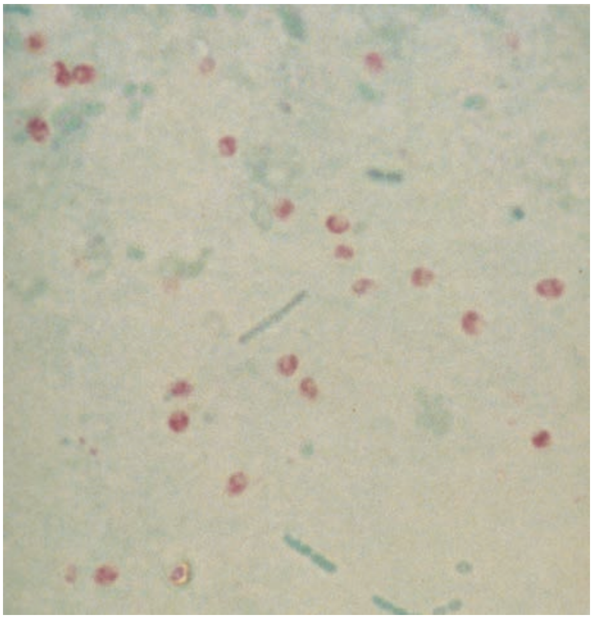

FIGURE 3 Smear of a stool specimen from a patient with AIDS and chronic diarrhea, showing pinkish red-stained spores of *Enterocytozoon bieneusi* that measure 0.7 to 1.0 by 1.1 to 1.6 µm. Chromotrope staining was used. Magnification, ×1,000 (oil immersion). doi:10.1128/9781555817381.ch128.f3

Epidemiological comparisons of the chromotrope staining technique with methods that use chemofluorescent optical brighteners indicate that these tests are robust for routine use and that the sensitivities of both methods are similarly high. Some laboratories use both staining techniques because the chromotrope stains result in a highly specific visualization of spores, whereas the chemofluorescent agents may be more sensitive but may produce false-positive results.

Immunofluorescent-Antibody Tests

Monoclonal antibodies against *Encephalitozoon* spp. (80) and *E. bieneusi* (81) have been generated, of which some have been evaluated for diagnostic purposes with stool specimens. However, immunofluorescence procedures for the detection of *Encephalitozoon* or *Enterocytozoon* microsporidial spores are not commercially available.

Cytological Diagnosis

Microsporidial spores have been detected in sediments obtained by centrifugation of body fluids for 10 min at 500 × *g*, including duodenal aspirates, bile, biliary aspirates, urine (Fig. 4, right panel), bronchoalveolar lavage fluid (Fig. 4, middle panel), and cerebrospinal fluid, and in smears of conjunctival swabs, sputum, and nasal discharge. Microscopic examination of stained smears of centrifuged duodenal aspirate obtained during endoscopy is a highly sensitive technique for the diagnosis of intestinal microsporidiosis. Because microsporidial infection often involves multiple organs, the detection of microsporidia in virtually any tissue or body fluid should prompt a thorough search of other sites. Particularly

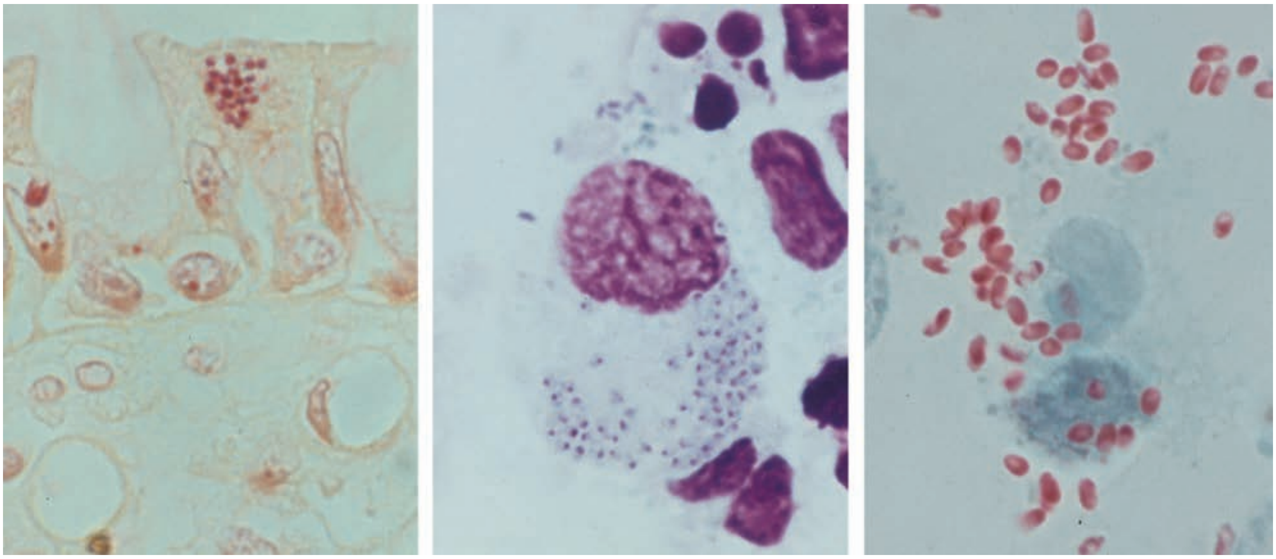

FIGURE 4 (Left) Terminal ileal tissue obtained by ileocolonoscopy from a patient with AIDS and chronic diarrhea due to *Enterocytozoon bieneusi* infection. Gram-labile microsporidial spores, measuring 0.7 to 1.0 by 1.1 to 1.6 µm, are found at a supranuclear location within small intestinal enterocytes. Brown-Brenn stain was used. Magnification ×1,000. (Middle) Cytospin preparation of bronchoalveolar-lavage fluid from a patient with AIDS and intestinal *E. bieneusi* infection, showing intracellular microsporidia. Giemsa stain was used. Magnification ×1,000 (oil immersion). (Right) Urine sediment from a patient with AIDS and disseminated *Encephalitozoon cuniculi* infection, showing pinkish red-stained microsporidial spores measuring 1.0 to 1.5 by 2.0 to 3.0 µm. Chromotrope stain was used. Magnification, ×1,000 (oil immersion). doi:10.1128/9781555817381.ch128.f4

for patients with suspected disseminated microsporidiosis, urine specimens should be examined (1, 73).

Examination of Biopsy Specimens and Corneal Scrapings

Examination of duodenal and terminal ileal tissue has resulted in the detection of intestinal microsporidia, but the pathogens are rarely found in colonic tissue sections. Microsporidial species causing disseminated infection have been found in almost every organ system (1).

Only highly experienced pathologists have reliably and consistently identified microsporidia in tissue sections by using routine techniques such as hematoxylin and eosin staining. Ultrathin plastic sections stained with methylene blue-azure II-basic fuchsin or with toluidine blue may facilitate detection. In our experience, tissue Gram stains (Brown-Brenn or Brown-Hopps) have proved to be the most useful for the rapid and reliable identification of HIV-associated microsporidia in routine paraffin-embedded tissue sections (Fig. 4, left panel) and corneal scrapings (1, 32). The microsporidial spores are Gram variable, and they are readily identified because of the contrasting dark blue or reddish staining against a faint brown-yellow background. Others prefer a silver stain (Warthin-Starry stain) (82), the chromotrope-based staining technique, or chemofluorescent agents.

Molecular Techniques

Universal panmicrosporidian and genus- or species-specific primer pairs that target the rRNA genes and their application in the diagnosis of intestinal microsporidial infection have been described (5). The detection and identification of *E. bieneusi* and the different *Encephalitozoon* spp. have been successfully performed with fresh stool specimens, formalin-fixed stool specimens, intestinal tissue obtained by endoscopic biopsy, urine specimens, and other body fluids. A real-time PCR method was developed for quantitation of *E. bieneusi* DNA (83) and *E. intestinalis* DNA (84) in stool specimens. Furthermore, *in situ* hybridization to visualize *E. bieneusi* in tissue sections has been developed (85).

ISOLATION OF MICROSPORIDIA

Microsporidia cannot be grown axenically. *Encephalitozoon* spp., *T. hominis*, *V. corneae*, and *A. algerae* have been isolated with different cell culture systems, including RK-13 (rabbit kidney), MDCK (Madin-Darby canine kidney), MRC-5 (human embryonic lung fibroblast) cells, and other cells (31, 40, 86). Only short-term *in vitro* propagation has been accomplished with *E. bieneusi*. The isolation of microsporidia has no relevance for diagnostic purposes but is an important research tool.

IDENTIFICATION

The identification of microsporidia and their taxonomy have been based primarily upon ultrastructural characteristics. Microsporidial ultrastructure is unique and pathognomonic for the phylum, and ultrastructural features can distinguish all microsporidial genera (Fig. 1) (31). Nevertheless, morphologic features alone do not sufficiently characterize all microsporidial species pathogenic for humans. The characterization of the three *Encephalitozoon* spp., which share most of their morphologic features, requires antigenic or genetic analyses, which may also reveal subtype-specific variation (19, 21). Identification of novel microsporidial species can be achieved by phylogenetic analyses of gene sequences derived from PCRs using panmicrosporidian primers.

SEROLOGIC TESTS

Serologic assays (including the carbon immunoassay, indirect immunofluorescence test, enzyme-linked immunosorbent assay, and Western blot immunodetection) have been useful in detecting specific antibodies to *E. cuniculi* in several species of animals. However, the value of such tests for humans has controversially been discussed because of possible cross-reactivity of the spore wall antigens of the *Encephalitozoon* species. Furthermore, results of serologic studies with humans were not substantiated by the detection of organisms in individuals with antibody responses. By employing recombinant antigens of the polar tube of *E. cuniculi*, improved specificity was demonstrated, and the development of serodiagnostic tools seems feasible (87).

ANTIMICROBIAL SUSCEPTIBILITIES

Albendazole has been found to cause growth deformities of *Encephalitozoon* and *Vittaforma* and to reduce or eradicate the organisms propagated in cell cultures but does not destroy mature microsporidial spores; thus, these may sustain infection (88). Fumagillin, its analog TNP-470, nikkomycin Z, and fluoroquinolones have been shown *in vitro* to inhibit completely or partially the replication or spore germination of *Encephalitozoon* and *Vittaforma* (88, 89). *In vitro* systems to investigate *E. bieneusi* are not available.

Treatment studies with humans are limited, and only two randomized controlled trials have been conducted. Albendazole can result in clinical cure of HIV-associated encephalitozoonosis in parallel with the cessation of spore excretion (90). In contrast, albendazole is not effective against *Enterocytozoon* infection and does not reduce pathogen load, although previous observations had suggested that clinical improvement may occur in some patients. Orally applied fumagillin appeared to eradicate *E. bieneusi* in severely immunodeficient HIV-infected patients (91) and in renal transplant recipients (92), but serious adverse events have also been reported (91, 93).

Enterocytozoon bieneusi-associated diarrhea as well as systemic infection due to *Encephalitozoon* spp. is mainly observed in severely immunodeficient patients. Improvement of immune functions by antiretroviral therapy results in complete clinical response and normalization of intestinal architecture, which parallel the clearance of intestinal microsporidia (94). Microsporidial keratoconjunctivitis may be a self-limiting disease (95, 96), or may be cleared by repeated corneal swabbing (97). Successful systemic albendazole treatment (98) or response to topical voriconazole application (99) have been reported.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Microsporidia are predominantly opportunistic pathogens capable of causing disease in severely immunodeficient HIV-infected persons (with CD4 cell counts below 200/µl) and otherwise immunocompromised patients, including organ transplant recipients. Therefore, stool examination or examination of other specimens is particularly indicated for these patient groups, and it is prudent to consider microsporidia as the etiologic agents when they are detected in clinical specimens from such patients. Furthermore, various microsporidial species may cause self-limited diarrhea or keratoconjunctivitis in immunocompetent and otherwise healthy persons.

Routine diagnosis of microsporidiosis is based upon microscopic detection of microsporidial spores. Although sensitive and specific, light-microscopic examination does not allow the identification of the organisms to the genus and species level. This can be achieved in most cases by electron microscopy, which is relatively insensitive for the detection of microsporidia because only small samples are examined and sampling errors may occur, or by PCR assays and further sequence analyses. Immunocompetent patients may excrete lower numbers of microsporidial spores in feces or urine, and therefore, the threshold of the current light-microscopic detection procedures may not be sufficient for the reliable detection of microsporidia in this group.

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section VII

ANTIFUNGAL AGENTS AND SUSCEPTIBILITY TEST METHODS

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Antifungal Agents*

SHAWN R. LOCKHART AND DAVID W. WARNOCK

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After a long period of slow development, the past decade has seen the introduction of an important new class of antifungal agents (the echinocandins), expansion of the spectrum of an established class of agents through chemical modification (the triazoles), and the development of novel methods for delivering established agents (lipid-based formulations of amphotericin B). These developments have changed the standards of care for the treatment of many invasive fungal infections, particularly aspergillosis and candidiasis. This chapter reviews the four major families of antifungal drugs that are currently available for systemic administration: the allylamines, the azoles, the echinocandins, and the polyenes. The comparative activities of the major systemic antifungal agents against important groups of fungi are summarized in Table 1. This chapter also discusses the characteristics of several other agents that can be used for the oral or parenteral treatment of superficial, subcutaneous, or systemic fungal infections. Novel agents that are currently in clinical trials are briefly reviewed.

ALLYLAMINES

The allylamines are a group of synthetic antifungal compounds effective in the topical and oral treatment of dermatophytoses. Two drugs, terbinafine and naftifine, are licensed for clinical use. Naftifine is only available as a topical preparation.

Mechanism of Action

The allylamines inhibit squalene epoxidase, a critical enzyme in the formation of ergosterol, the principal sterol in the membrane of susceptible fungal cells. The consequent accumulation of squalene leads to membrane disruption and cell death (1).

Terbinafine

Terbinafine (Lamisil; Novartis Pharmaceuticals) is a lipophilic drug that is available for oral or topical administration. It is widely used for the treatment of superficial fungal infections caused by dermatophytes.

Spectrum of activity

Terbinafine is effective against several groups of pathogenic fungi, including dermatophytes (*Epidermophyton*, *Microsporum*, and *Trichophyton* spp.) (2–4) and dematiaceous fungi (5). It also has some activity against *Aspergillus* spp. (6), *Candida* spp. (6), *Blastomyces dermatitidis* and *Histoplasma capsulatum* (7), *Paracoccidioides brasiliensis* (8), *Talaromyces* (*Penicillium*) *marneffei* (9), and *Sporothrix schenckii* (6, 8).

Acquired Resistance

The development of resistance to terbinafine among dermatophytes, even after prolonged exposure, is rare.

Pharmacokinetics

Terbinafine is well absorbed after oral administration and is then rapidly and extensively distributed to body tissues (10). It reaches the stratum corneum as a result of diffusion through the dermis and epidermis, and secretion in sebum. Diffusion from the nail bed is the major factor in its rapid penetration of nails. Terbinafine has been found to persist in nails for long periods after cessation of treatment. It is extensively metabolized by the human hepatic cytochrome P-450 enzyme system, and the inactive metabolites are mostly excreted in the urine (11).

Clinical Use

Terbinafine is the drug of choice for dermatophyte infections of the skin and nails where topical treatment is considered inappropriate or has failed (12, 13). It is not as effective as itraconazole for treatment of fungal nail infections (onychomycosis) involving nondermatophytes. Terbinafine has also proven effective in some patients with aspergillosis, chromoblastomycosis, and sporotrichosis (14), but it is not licensed for these indications. Anecdotal evidence suggests that the use of terbinafine in combination with voriconazole may be beneficial in the treatment of infections with *Scedosporium prolificans* (15).

Drug Interactions

Although terbinafine is metabolized by the human hepatic cytochrome P-450 enzyme system, it does not inhibit most CYP enzymes at clinically relevant concentrations (11). Blood concentrations of terbinafine are reduced when it is given together with drugs, such as rifampin, that induce the hepatic cytochrome P-450 system.

Toxicity and Adverse Effects

Terbinafine produces few adverse reactions. These include abdominal discomfort, nausea, diarrhea, impairment of taste,

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*This chapter contains information presented in chapter 126 by David Warnock in the 10th edition of this *Manual*.

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TABLE 1 Spectrum and extent of activity of commonly used systemic antifungal agents[a]

Organism	Activity[b] of antifungal agent							
	Amphotericin	Fluconazole	Itraconazole	Posaconazole	Voriconazole	Anidulafungin	Caspofungin	Micafungin
Aspergillus spp.	+++	−	++	+++	+++	++	++	++
B. dermatitidis	+++	+	+++	+++	+++	−	−	−
Candida spp.								
C. albicans	+++	+++	+++	+++	+++	+++	+++	+++
C. glabrata	+++	++	++	++	++	+++	+++	+++
C. krusei	++	−	+++	+++	+++	+++	+++	+++
C. lusitaniae	++	+++	+++	+++	+++	++	++	++
C. parapsilosis	+++	+++	+++	+++	+++	++	++	++
C. tropicalis	+++	+++	+++	+++	+++	+++	+++	+++
Coccidioides spp.	+++	+++	+++	+++	+++	−	−	−
Cryptococcus spp.	+++	+++	++	+++	+++	−	−	−
Fusarium spp.	++	−	+	++	++	−	−	−
H. capsulatum	+++	++	+++	+++	+++	−	−	−
Mucoraceous molds	++	−	−	++	−	−	−	−
P. brasiliensis	+++	++	+++	+++	+++	−	−	−

[a] This table is a general overview for comparison of the activities of some systemic drugs against various fungi. Readers are recommended to refer to the text for more detailed information.

[b] −, no meaningful activity; +, occasional activity; ++, moderate activity but resistance is noted; +++, reliable activity with occasional resistance

and transient skin rashes (16). Rare, but serious, side effects include Stevens-Johnson syndrome and hepatotoxic reactions, including cholestasis and hepatitis.

AZOLES

This large group of synthetic agents contains many compounds that are effective in the topical treatment of dermatophyte infections and superficial forms of candidiasis; a number are suitable for systemic administration. Members of this group have in common an imidazole or triazole ring with *N*-carbon substitution. The systemic-use antifungals are the triazoles.

Mechanism of Action

Azole compounds inhibit a fungal cytochrome P-450-dependent enzyme, lanosterol 14 α-demethylase, which is responsible for the conversion of lanosterol to ergosterol, the principal sterol in the membrane of susceptible fungal cells. This results in the accumulation of various toxic methylated sterols and the depletion of ergosterol with subsequent disruption of membrane structure and function. The activity is essentially fungistatic, although voriconazole and itraconazole can exert fungicidal effects against *Aspergillus* and some other mold species at concentrations achieved with recommended dosages (17).

Several mechanisms of resistance have been described (see chapter 130). These include upregulation of multidrug efflux-transporter genes, upregulation of the *ERG11* gene that encodes the target enzyme, lanosterol 14 α-demethylase, and decreased affinity of this enzyme for azole agents due to either intrinsic or acquired amino acid substitutions (18). Changes in other enzymes involved in the ergosterol biosynthesis pathway, such as loss of $\Delta^{5,6}$ sterol desaturase activity, may also contribute to azole resistance (18).

Pharmacokinetics

With the exception of fluconazole, food has a significant effect on the absorption of azole antifungals. Administration with lipid-rich food improves the absorption of ketoconazole, posaconazole, and the capsule formulation of itraconazole (19–22). In contrast, absorption of voriconazole and the oral solution formulation of itraconazole is reduced when given with a high-fat meal (23, 24).

Peak blood concentrations of azoles are typically reached within 2 to 3 h after oral administration. With fluconazole and posaconazole, blood levels increase in proportion to dosage (25). In contrast, increases in itraconazole dosage produce disproportionate changes in peak blood concentrations due to saturable first-pass metabolism in the liver (26). In adults, there is a disproportionate increase in blood levels of voriconazole with increasing oral and parenteral dosage (27). In children, however, increases in dosage produce proportional changes in drug levels, and clearance of the drug is more rapid (28).

Due to its low protein binding (about 12%), fluconazole attains high concentrations in most tissues and body fluids. Levels of the drug in cerebrospinal fluid (CSF) usually exceed 50% of the simultaneous blood concentration (25). Likewise, voriconazole is extensively distributed into tissues (29), with CSF levels that are around 30 to 60% of the simultaneous blood concentration (30). Voriconazole and fluconazole concentrations in vitreous and aqueous fluids are around 40 to 50% of the simultaneous blood level, which makes them useful for treating enophthalmitis (31). Levels of itraconazole in the CSF are minimal (22).

Levels of itraconazole in tissues such as lung, liver, brain, and bone are two to three times higher than in serum. High concentrations are also found in the stratum corneum as a result of drug secretion in sebum (32). Itraconazole has been found to persist in the skin and nails for weeks to months after the end of a course of treatment, thereby allowing intermittent pulse regimens for dermatophyte infections and onychomycosis (32, 33).

With the exception of fluconazole and posaconazole, the azoles are extensively metabolized by the human hepatic cytochrome P-450 enzyme system, and are eliminated as inactive metabolites in the bile or urine. More than 90% of a dose of fluconazole is eliminated in the urine, predominantly as unchanged drug, which makes it useful for treating urinary tract infections caused by susceptible species (34). More than 75% of a dose of posaconazole is eliminated in the feces, predominantly as unchanged drug, with the remainder being excreted as glucuronidated derivatives in the urine (35). Itraconazole is unusual because its major metabolite, hydroxyitraconazole, is bioactive and has a similar spectrum of activity as the parent compound (36). This metabolite is found at serum concentrations about 2-fold higher than those of the parent drug (22).

Voriconazole is metabolized by several different hepatic cytochrome P-450 enzymes, primarily CYP-2C19, with more than 80% of a dose being eliminated as inactive metabolites in the urine (34). However, as a result of a point mutation in the gene encoding this enzyme, some persons are poor metabolizers while others are extensive metabolizers. About 3 to 5% of Caucasians and 15 to 20% of non-Indian Asians are poor metabolizers (29). Voriconazole blood concentrations are as much as 4-fold lower in individuals who metabolize the drug more extensively and can reach toxic levels in poor metabolizers.

Drug Interactions

Most azole antifungal agents are extensively metabolized by the human hepatic cytochrome P-450 enzyme system and are potent inhibitors of CYP-3A4; some also inhibit CYP-2C9 and CYP-2C19. Their coadministration with other drugs that are metabolized by these enzymes can result in increased blood concentrations of the azole, the interacting drug, or both (37). When an azole agent is discontinued, the change in metabolism that occurs may necessitate upward or downward adjustment of the dosage of the other drugs. Administration of azoles with drugs that are potent inducers of the human cytochrome P-450 enzyme system, such as rifampin, results in a marked reduction in blood concentrations, especially with itraconazole and ketoconazole (37).

Fluconazole

Fluconazole (Diflucan; Pfizer) is a water-soluble bis-triazole available in both oral and parenteral formulations. It is extensively used as prophylaxis and in treating yeast infections, particularly in the treatment of candidiasis and cryptococcosis.

Spectrum of Activity

Fluconazole possesses the narrowest spectrum of all the azole antifungals currently available for systemic use. It is active against most *Candida* spp. and *Cryptococcus neoformans* (38, 39). However, isolates of *Candida krusei* are intrinsically resistant and *Candida glabrata* has decreased susceptibility (39, 40). The spectrum of activity also includes several dimorphic fungi (*B. dermatitidis*, *Coccidioides* spp., and *H. capsulatum*) (41). Fluconazole has no activity against *Aspergillus* spp., *Fusarium* spp. (42), or mucoraceous moulds (43).

Acquired Resistance

There have been few reports of resistance developing in *C. albicans* during short-term fluconazole treatment in patients with mucosal or deep-seated forms of candidiasis (18). In contrast, many strains of *C. glabrata* rapidly become resistant to fluconazole during treatment (44). In persons with AIDS, resistant strains of *C. albicans* have appeared following repeated courses of low-dose fluconazole treatment for oral or esophageal infection. However, with the widespread use of highly active antiretroviral treatment for HIV infection, resistant strains are now rarely encountered (45). There are a few reports of resistant strains of *C. neoformans* from AIDS patients with relapsed infection following long-term maintenance treatment with fluconazole, but those cases are thought to be rare (46, 47).

Clinical Use

Fluconazole is widely used in the treatment of mucosal and systemic candidiasis (48), coccidioidomycosis (49), and cryptococcosis (50). It is also widely used for the prevention of candidiasis in neutropenic patients (48), as well as for the prevention of relapse of cryptococcal meningitis in persons with AIDS (50). Fluconazole is an alternative for the treatment of histoplasmosis and sporotrichosis, but is less effective than itraconazole (51, 52).

Therapeutic Drug Monitoring

Serum concentrations of fluconazole are predictable from dosing and organ function, and routine monitoring of drug levels is not required (53–55).

Toxicity and Adverse Effects

Fluconazole is one of the least toxic and best-tolerated azole drugs and side effects during treatment are rare. The most common patient complaints include headache, hair loss, and loss of appetite. Transient abnormalities of liver enzymes and rare serious skin reactions, including Stevens-Johnson syndrome, have been reported.

Itraconazole

Itraconazole (Sporanox; Ortho-McNeil-Janssen Pharmaceuticals) is a lipophilic triazole drug available for oral or parenteral administration. It is extensively used, particularly in the treatment of superficial fungal infections, as well as in a range of subcutaneous and systemic infections.

Spectrum of Activity

Itraconazole has good activity against a broad spectrum of pathogenic fungi, including *Aspergillus* spp. (56–60), *Candida* spp. (39, 56, 59), many dematiaceous moulds (5, 61), dermatophytes (2–4), and dimorphic fungi (*B. dermatitidis*, *Coccidioides* spp., *H. capsulatum*, *P. brasiliensis*, *T. marneffei*, and *S. schenckii*) (8, 9, 41, 62–64). Itraconazole has modest activity against *C. neoformans* (39, 59), but is ineffective against *Pseudallescheria boydii* (65) and most mucoraceous moulds (43).

Acquired Resistance

Itraconazole-resistant strains of *A. fumigatus* have been reported following treatment (66, 67). Of more concern are increasing reports of itraconazole resistance due to an environmentally acquired mutation in *cyp51a*, TR/L94H (68). This mutation has now been identified in isolates from Europe, China, and India and has become a serious problem in parts of Europe (66, 69–72).

Clinical Use

Itraconazole has been widely used to treat various superficial fungal infections, including the dermatophytoses, onychomycosis, pityriasis versicolor, and mucosal and cutaneous forms of candidiasis. It is also effective in patients with paracoccidioidomycosis, chromoblastomycosis, sporotrichosis, and certain forms of phaeohyphomycosis (51, 73, 74). Despite its limitations, itraconazole continues to be a drug of choice in the management of mild to moderate forms of blastomycosis and histoplasmosis (52, 75). It was the first orally active drug for aspergillosis, but its use in seriously ill patients with life-threatening forms of this disease is not recommended (76). Itraconazole is the drug of choice for long-term maintenance treatment to prevent relapse in AIDS patients with histoplasmosis (52), but it is less effective than fluconazole as maintenance treatment in AIDS patients with cryptococcosis (50).

Therapeutic Drug Monitoring

Absorption of the capsule formulation of itraconazole after oral administration shows marked variation between individuals. Because low serum concentrations are often predictive of treatment failure, measurement of blood levels is advisable in situations where the drug is used to treat or prevent serious invasive fungal infections (53–55). For prophylaxis, a target trough concentration of >0.5 μg/ml has been proposed; for treatment, a trough of >1 to 2 μg/ml has been recommended (53).

Toxicity and Adverse Effects

Most side effects associated with itraconazole are mild and reversible. The most frequently reported adverse events are headache, loss of appetite, nausea, abdominal discomfort, diarrhea, skin rashes, and transient elevations of liver enzymes. Gastrointestinal intolerance is more common with itraconazole oral solution and is sometimes severe enough to necessitate discontinuation of treatment (77, 78). Rare, but serious, side effects include Stevens-Johnson syndrome, hepatitis, and congestive heart failure.

Ketoconazole

Ketoconazole (Nizoral; Ortho-McNeil-Janssen Pharmaceuticals) is a lipophilic drug formulated for oral or topical use. It is the only antifungal imidazole still available for systemic administration, but its main use is now as a topical agent.

Spectrum of Activity

Ketoconazole has useful activity against dermatophytes (2–4) and dimorphic fungi (*B. dermatitidis*, *Coccidioides* spp., *H. capsulatum*, *P. brasiliensis*, and *S. schenckii*) (62, 79). It is also active against *Candida* spp. and *C. neoformans*, although it is less effective than the newer triazoles.

Acquired Resistance

This is rare, but several instances were documented in the 1980s among patients given long-term treatment for chronic mucocutaneous candidiasis due to *C. albicans*.

Clinical Use

Due to the availability of less toxic, more efficacious alternatives, ketoconazole is now little used, except in resource-limited environments. Ketoconazole remains a useful topical agent for dermatophytosis, cutaneous candidiasis, pityriasis versicolor, and seborrheic dermatitis (80).

Toxicity and Adverse Effects

Unwanted effects include loss of appetite, abdominal pain, nausea, and vomiting. Transient elevations of liver enzymes are common with oral ketoconazole, and fatal hepatitis is a rare but well-recognized adverse event. High doses of ketoconazole inhibit human adrenal and testicular steroid synthesis, with clinical consequences such as alopecia, gynecomastia, and impotence.

Posaconazole

Posaconazole (Noxafil; Merck) is a second-generation, broad-spectrum triazole compound which is currently only available as an oral suspension. An intravenous formulation has passed a phase I clinical trial. A new tablet with better bioavailability, release in the small bowel, and absorption is also in development. Posaconazole is highly lipophilic and has a chemical structure similar to that of itraconazole.

Spectrum of Activity

Posaconazole is highly active against most *Aspergillus* spp. (56, 58, 60), as well as *Candida* spp., *C. neoformans*, and *Trichosporon* spp. (39, 56, 64, 81). It has potent activity against a number of dimorphic fungi, including *B. dermatitidis*, *Coccidioides* spp., *H. capsulatum*, *T. marneffei*, and *S. schenckii* (41, 64). It is less active against *Fusarium* spp. and *P. boydii*, but appears to be effective against dematiaceous fungi (41, 42, 64, 65). Unlike other azole antifungals, posaconazole has significant activity against some mucoraceous moulds (43, 56, 64, 82).

Acquired Resistance

Posaconazole sometimes has activity against strains of *Aspergillus* and *Candida* spp. that show resistance to itraconazole, fluconazole, and/or voriconazole (64, 68). Posaconazole resistance in *A. fumigatus* isolates harboring the TR/L98H mutation has been shown (66, 69, 72).

Clinical Use

The lack of an intravenous formulation of posaconazole is a major disadvantage when treating seriously ill patients. In the United States, the drug has been approved for the treatment of oropharyngeal candidiasis (including infections refractory to itraconazole and/or fluconazole), as well as for prophylaxis of invasive aspergillosis and candidiasis in high-risk patients, such as hematopoietic stem cell transplant (HSCT) recipients with graft-versus-host disease and neutropenic cancer patients. In the European Union, posaconazole has been licensed for similar indications, as well as for salvage treatment of invasive aspergillosis, coccidioidomycosis, chromoblastomycosis, *Fusarium* infections, and mycetoma. Other indications for which posaconazole has proved effective but is not currently licensed include histoplasmosis (83), coccidioidomycosis (84), and infections caused by mucoraceous moulds (82, 85).

Therapeutic Drug Monitoring

Similarly to itraconazole and voriconazole, there appears to be a relationship between posaconazole trough serum concentrations and clinical response, and measurement of drug levels may therefore be useful (86). For prophylaxis, a target trough concentration of >0.5 μg/ml has been proposed; for treatment, a trough of >0.5 to 1.5 μg/ml has been suggested (53).

Toxicity and Adverse Effects

Posaconazole is well tolerated, even among patients receiving the drug for longer than 6 months (87). The most frequently reported side effects have been gastrointestinal symptoms and headache. Transient transaminase abnormalities have also been reported. Rare cases of cholestasis or hepatic failure have occurred during treatment with posaconazole.

Voriconazole

Voriconazole (Vfend; Pfizer) is a second-generation broad-spectrum triazole compound available for oral or intravenous administration. Its chemical structure is similar to that of fluconazole.

Spectrum of Activity

Voriconazole is highly active against most *Aspergillus* spp., *Fusarium* spp., and *P. boydii* (42, 56, 58, 60, 61, 65), as well as *Candida* spp., *C. neoformans*, and *Trichosporon* spp. (64, 81). Voriconazole has potent activity against a number of dimorphic fungi, including *B. dermatitidis*, *Coccidioides* spp., *H. capsulatum*, and *T. marneffei* (41, 63), as well as dematiaceous moulds (61). Voriconazole is ineffective against mucoraceous moulds (43, 61, 64).

Acquired Resistance

Some fluconazole-resistant strains of *Candida* spp. have shown reduced susceptibility to voriconazole, and essentially all *C. glabrata* isolates that are resistant to fluconazole are nonsusceptible to voriconazole (64). *Aspergillus* isolates may acquire voriconazole resistance during long-term azole therapy (66, 88). Environmentally acquired resistance to voriconazole in *A. fumigatus* due to a TR/L98H mutation, as well as a newly described *cyp51a* mutation, is occurring more frequently (69–71, 88, 89).

Clinical Use

The availability of both an intravenous formulation and a well-absorbed oral formulation of voriconazole is a distinct advantage when treating seriously ill patients. In the U.S., the drug has been approved for the treatment of invasive aspergillosis and has become the drug of choice for these infections (76). It is also licensed for the treatment of candidemia in nonneutropenic patients, for disseminated infections caused by *Candida* spp., and for esophageal candidiasis, as well as for salvage treatment of *Fusarium* and *Scedosporium* infections. In the European Union, voriconazole has been approved for similar indications. Voriconazole has no activity against mucoraceous moulds, and its use in immunocompromised patients has sometimes been associated with breakthrough infections caused by these organisms.

Therapeutic Drug Monitoring

Voriconazole serum concentrations are highly variable, largely due to differences in the rate of metabolism between individuals, and it may be beneficial to monitor drug levels (53–55). For prophylaxis, a target trough concentration of >0.5 μg/ml has been proposed; for treatment, a trough of >1 to 2 μg/ml has been recommended (53). To avoid toxicity, trough concentrations of 6 μg/ml of voriconazole should not be exceeded.

Toxicity and Adverse Effects

Voriconazole is generally well tolerated. About 30% of patients experience transient visual disturbances and hallucinations, usually during the first week of treatment (29). Other side effects include skin rashes and transient abnormalities of liver enzymes. Rare, but serious, adverse effects include Stevens-Johnson syndrome, hepatic failure, and cardiovascular events.

ECHINOCANDINS

The echinocandins are a new class of semisynthetic lipopeptide antifungal agents that target the fungal cell wall. Three echinocandins have been approved for the treatment of serious fungal infections: anidulafungin, caspofungin, and micafungin. Due to their high molecular weight and low oral bioavailability, these drugs are only available as intravenous preparations. They are now widely used, particularly in the treatment of candidiasis.

Mechanism of Action

The echinocandins disrupt fungal cell-wall synthesis by inhibiting the enzyme 1,3-β-D-glucan synthase. This results in inhibition of the formation of 1,3-β-D-glucan, an essential polysaccharide component of the cell wall of susceptible fungi. Inhibition leads to osmotic lysis of the cell and eventual cell death. Echinocandin drugs bind to Fksp, the major subunit of 1,3-β-D-glucan synthase, which is encoded by three *FKS* genes in *Candida* spp. (90). The echinocandins are fungicidal for *Candida* spp., but fungistatic for *Aspergillus* spp. (91), where they block the growth of the apical tips of the hyphae.

Resistance to echinocandin drugs among clinical isolates has been associated with mutations in the *Candida* sp. *FKS1* gene that lead to amino acid substitutions in the Fks1p subunit of 1,3-β-D-glucan synthase (90, 92). These changes result in altered drug binding and confer cross-resistance to all echinocandin drugs. Mutations in the *FKS1* and *FKS2* genes are responsible for reduced susceptibility to caspofungin, micafungin, and anidulafungin in *C. glabrata* (93, 94).

Spectrum of Activity

The echinocandins have a limited spectrum of activity. They are highly active against a broad range of *Candida* spp., including fluconazole-resistant strains (91, 95–98). *C. parapsilosis*, *C. lusitaniae*, and *C. guilliermondii* have higher MIC values to the echinocandins, but the clinical implications of these values are not yet clear (95–98). The echinocandins are also active against *Aspergillus* spp., including those that are intrinsically resistant to amphotericin B (58, 91, 96, 99, 100).

The echinocandins are ineffective against fungi that lack a significant amount of 1,3-β-D-glucan in their cell wall, including *C. neoformans* and *Trichosporon* spp., as well as *Fusarium* spp. and the mucoraceous moulds (43, 56, 58, 91, 96, 99, 101). Micafungin has been reported to be active against the mycelial forms of several dimorphic fungi, including *B. dermatitidis* and *H. capsulatum*, but is ineffective against the tissue forms of these pathogens (102).

Acquired Resistance

This is rare at present, but resistant strains of several *Candida* spp. have been recovered from patients failing echinocandin treatment (103–106). Resistance has been associated with acquisition of mutations in the *FKS1* and/or *FKS2* genes that led to amino acid substitutions within the FKS1p and FKS2p subunits of 1,3-β-D-glucan synthase (93, 94, 103–105). The highest rates of resistance have been detected in *C. glabrata*, especially in strains already resistant to fluconazole (103, 107).

Pharmacokinetics

Blood concentrations of all three echinocandins increase in proportion to dosage (108–110). These drugs are extensively distributed to body tissues, but levels in the CSF are negligible. The predominant differences among these agents lie in their metabolism and half-life. Caspofungin and micafungin are largely metabolized by the liver and eliminated as inactive metabolites in the feces and urine (111–114). Anidulafungin is not eliminated by hepatic metabolism, but undergoes slow nonenzymatic degradation in the blood to an inactive open-ring peptide (115). Less than 1 to 3% of an echinocandin dose is excreted unchanged in the urine (108, 112, 114, 115). In adults, the half-life of caspofungin is about 9 to 10 h (110), while that of micafungin is 13 h (112) and that of anidulafungin is 18 to 27 h (108, 115). The three echinocandins have a shorter half-life in children (116–118). The 24-hour area under the concentration-time curve (AUC)/MIC is a good indicator of the exposure-response relationship with a ratio that should exceed 10 to 20 (119).

Drug Interactions

The echinocandins do not interact with the human hepatic cytochrome P-450 system and their use has been associated with very few significant drug interactions.

Therapeutic Drug Monitoring

At this time, there is no established relationship between efficacy or toxicity of the echinocandins and serum concentrations (54). Routine monitoring of serum levels during treatment with these drugs is not required.

Toxicity and Adverse Effects

As a class, the echinocandins are well tolerated and their use is associated with very few significant adverse effects (112, 116, 117). The most common side effects are gastrointestinal in nature, but only occur in around 5% of patients. Occasional cases of infusion-related pain and phlebitis have been noted with anidulafungin and micafungin, but these are less common with caspofungin. Transient elevations of liver enzymes have been reported in a few patients.

Anidulafungin

Anidulafungin (Ecalta or Eraxis; Pfizer) was the first echinocandin to go into development and the most recent to be licensed for clinical use. It differs from caspofungin and micafungin in that it is insoluble in water. Anidulafungin is derived from a fermentation product of *Aspergillus nidulans* and is formulated for intravenous infusion.

Clinical Use

In the U.S., anidulafungin is currently approved for the treatment of esophageal candidiasis, candidemia, and two invasive forms of candidiasis (abdominal abscesses and peritonitis). In the European Union, anidulafungin is approved for the treatment of invasive candidiasis in nonneutropenic patients. Anidulafungin has not been evaluated in sufficient numbers of neutropenic patients to determine its effectiveness in that group.

Caspofungin

Caspofungin (Cancidas; Merck) is a water-soluble lipopeptide derived from a fermentation product of *Glarea lozoyensis*. It is formulated for intravenous infusion.

Clinical Use

In the U.S., caspofungin is currently approved for the treatment of esophageal candidiasis, candidemia, and certain invasive forms of candidiasis, including abdominal abscesses, peritonitis, and pleural-space infections. Caspofungin is also licensed for the salvage treatment of invasive aspergillosis in patients who have failed to respond to or are intolerant of other antifungal agents. Caspofungin is approved for the empiric treatment of presumed fungal infections in febrile neutropenic patients. It has similar indications in the European Union, with a license for the treatment of invasive candidiasis in adult and pediatric patients, salvage treatment of aspergillosis, and empiric treatment of febrile neutropenia in adult or pediatric patients.

Micafungin

Micafungin (Mycamine; Astellas Pharma, Fujisawa Healthcare) is a water-soluble antifungal agent derived from a fermentation product of *Coleophoma empetri*. It is formulated for intravenous administration.

Clinical Use

In the U.S., micafungin is currently approved in adults for the treatment of esophageal candidiasis, candidemia, and several invasive forms of candidiasis, including abdominal abscesses and peritonitis, and for prophylaxis of *Candida* infections in HSCT patients. In the European Union, the drug is approved for the treatment of esophageal candidiasis in adults and for invasive candidiasis in adults and children, including neonates. In addition, micafungin is licensed as prophylactic treatment to prevent *Candida* infections in HSCT recipients in the U.S. and European Union. In Japan, the license includes respiratory and gastrointestinal mycosis due to *Aspergillus* spp.

POLYENES

Around 100 polyene antibiotics have been described, but few have been developed for clinical use. Amphotericin B and its lipid formulations are used for the treatment of systemic fungal infections. Nystatin, natamycin, and mepartricin are topical polyene agents used in the treatment of oral, vaginal, and ocular fungal infections. A liposomal formulation of nystatin entered clinical trials, but its development has ceased. The polyenes are large molecules that consist of a closed macrolide lactone ring. One side of the ring is composed of a rigid lipophilic chain with a variable number of conjugated double bonds, and on the opposite side there are a similar number of hydroxyl groups. Thus, the molecule is amphipathic and this feature of its structure is believed to be important in its mechanism of action.

Mechanism of Action

The polyenes bind to sterols, principally ergosterol, in the membranes of susceptible fungal cells, causing impairment of membrane barrier function, leakage of cell constituents, metabolic disruption, and cell death (120). In addition to its membrane-permeabilizing effects, amphotericin B can cause oxidative damage to fungal cells through a cascade of oxidative reactions linked to lipoperoxidation of the cell membrane.

Amphotericin B

Amphotericin B (Fungizone; Apothecon) is a fermentation product of *Streptomyces nodosus* available for intravenous infusion. The conventional micellar suspension formulation of this drug (amphotericin B deoxycholate) is often associated with serious toxic side effects, particularly renal damage. During the 1990s, three new lipid-associated formulations of amphotericin B were developed in an effort to alleviate the toxicity of the agent. These are liposomal amphotericin B (AmBisome; Astellas Pharma, Gilead Sciences), in which the drug is encapsulated in phospholipid-containing liposomes; amphotericin B lipid complex (ABLC) (Abelcet; Enzon Pharmaceuticals), in which the drug is complexed with phospholipids to produce ribbon-like structures; and amphotericin B colloidal dispersion (ABCD) (Amphotec; Three Rivers Pharmaceuticals), in which the drug is packaged into small lipid disks containing cholesterol sulfate. These formulations possess the same broad spectrum of activity as the micellar suspension, but are less nephrotoxic.

Spectrum of Activity

Amphotericin B is active against a broad spectrum of pathogenic fungi including most *Aspergillus* spp., *Candida* spp., *C. neoformans*, and the mucoraceous moulds (43, 56–58, 60, 64, 96). However, most isolates of *A. terreus* are resistant to amphotericin B (57, 58, 60, 121), as are isolates of *Aspergillus lentulus*, a new sibling species of *A. fumigatus* (122). *Candida krusei* also demonstrates reduced susceptibility to amphotericin B (123). Amphotericin B is effective against the dimorphic fungi (*B. dermatitidis*, *Coccidioides spp*., *H. capsulatum*, and *P. brasiliensis*) and many dematiaceous fungi (61, 63, 64). Strains of *P. boydii*, *S. prolificans*, *Fusarium* spp., and

Trichosporon spp. are often intrinsically resistant to amphotericin B (42, 64, 65, 81).

Acquired Resistance

This is rare, but amphotericin B-resistant strains of *C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. tropicalis*, *Cryptococcus neoformans*, and especially *C. lusitaniae* with alterations in the cell membrane, including reduced amounts of ergosterol, have been reported following prolonged treatment (124, 125).

Pharmacokinetics

Amphotericin B is poorly absorbed after oral administration and must be administered as a slow intravenous infusion. The drug is widely distributed to many tissues, with the highest concentrations being found in the liver, spleen, and kidneys. Levels in the CSF are less than 5% of the simultaneous blood concentration. Amphotericin B is mostly excreted as unchanged drug in the urine (21%) and feces (42%) (126). No metabolites have been identified. The drug is cleared very slowly, with the conventional deoxycholate formulation having a terminal half-life of around 127 h.

The pharmacokinetics of lipid-based formulations of amphotericin B are quite diverse. Maximal serum concentrations of the liposomal formulation are much higher than those of the deoxycholate formulation, while levels of ABCD and ABLC are lower due to more rapid distribution of the drug to tissue (127). Administration of lipid-associated formulations of amphotericin B results in higher drug concentrations in the liver and spleen than are achieved with the conventional formulation (128). Renal concentrations of the drug are lower and the nephrotoxic side effects are greatly reduced.

Clinical Use

Although other agents have subsequently been introduced, amphotericin B remains the treatment of choice for many serious fungal infections, including blastomycosis, coccidioidomycosis, histoplasmosis, sporotrichosis, cryptococcosis, and mucormycosis (49–52, 75). However, with the advent of voriconazole and the echinocandins, amphotericin B is no longer regarded as the drug of first choice for many cases of aspergillosis or candidiasis (48, 76). The three lipid-based formulations of amphotericin B are currently licensed for treatment of invasive fungal infections in patients who are refractory to, or intolerant of, conventional amphotericin B. In addition, liposomal amphotericin B is licensed for the treatment of cryptococcal meningitis in persons with AIDS, as well as for the empirical treatment of presumed fungal infection in febrile neutropenic patients. Clinical experience with these preparations has demonstrated that they are safer and no less active than the conventional formulation and, for some infections, they are more effective, especially since the reduced toxicity allows them to be used at a higher dose.

Drug Interactions

Amphotericin B can augment the nephrotoxicity of many other agents, including aminoglycoside antibiotics and cyclosporine.

Therapeutic Drug Monitoring

Serum and tissue concentrations of amphotericin B show marked variation with formulation, especially among the lipid-based products, and there are few data relating either efficacy or toxicity to blood levels. Therefore, there is no need to monitor serum concentrations of amphotericin B during therapy (53). However, due to the risk of toxicity, kidney function should be monitored.

Toxicity and Adverse Effects

Amphotericin B deoxycholate causes infusion-related reactions, including hypotension, fever, rigors, and chills, in approximately 70% of patients (129). The major adverse effect of the drug is nephrotoxicity. This is dose related and may occur in more than 80% of patients receiving treatment. The lipid-associated formulations all lower the risk of amphotericin B-induced renal failure (130). However, infusion-related side effects, such as hypoxia and chills, are more common in patients treated with ABCD. In contrast, infusion-related reactions are uncommon in patients receiving liposomal amphotericin B or ABLC (131).

OTHER MISCELLANEOUS AGENTS

Flucytosine

Flucytosine (5-fluorocytosine) (Ancobon; Valeant Pharmaceuticals) is a synthetic fluorinated analogue of cytosine and the only available antifungal agent acting as an antimetabolite. In the U.S., flucytosine is available as oral tablets; elsewhere it is also available as an infusion for parenteral administration.

Mechanism of Action

Flucytosine disrupts pyrimidine metabolism and thus the synthesis of DNA, RNA, and proteins within susceptible fungal cells. Flucytosine is transported into these cells by the enzyme cytosine permease and there converted by cytosine deaminase to 5-fluorouracil (5-FU). Two mechanisms then account for the antifungal activity. The first involves the conversion of 5-FU into 5-fluoruridine triphosphate, which is incorporated into fungal RNA in place of uridylic acid, with resulting inhibition of protein synthesis. The second mechanism involves the conversion of 5-FU to 5-fluorodeoxyuridine monophosphate, which blocks the enzyme thymidylate synthetase, causing inhibition of fungal DNA synthesis. Fungi lacking cytosine deaminase are intrinsically resistant to flucytosine.

Spectrum of Activity

Flucytosine has a narrow spectrum of activity. It includes *Candida* spp., *C. neoformans*, and some dematiaceous fungi causing chromoblastomycosis (56, 96). Primary resistance to flucytosine is very uncommon among *Candida* spp., occurring in around 2 to 3% of isolates (96, 132).

Acquired Resistance

Monotherapy with flucytosine often leads to the induction of resistance among *Candida* spp. and *C. neoformans* (133).

Pharmacokinetics

Flucytosine is rapidly and almost completely absorbed after oral administration. The drug is widely distributed, with levels in most body tissues and fluids usually exceeding 50% of the simultaneous blood concentration (134). Flucytosine is primarily eliminated by renal excretion of unchanged drug. The serum half-life is between 3 and 6 h, but may be greatly extended in renal failure, necessitating modification of the dosage regimen.

Clinical Use

Due to the risk of resistance, flucytosine is rarely administered as a single agent. It is most commonly used in combination with amphotericin B in the treatment of candidiasis and cryptococcosis (48, 50). Combination treatment with fluconazole has also been shown to be effective in AIDS-associated cryptococcal meningitis (50).

Drug Interactions

The antifungal activity of flucytosine is competitively inhibited by cytarabine (cytosine arabinoside), and the two drugs should not be administered together (134). Nephrotoxic drugs, such as amphotericin B, decrease the elimination of flucytosine, and serum concentrations of the latter should be monitored when these agents are administered together. Flucytosine is myelosuppressive (see below) and should be used with caution in patients receiving other drugs, such as zidovudine, that could enhance its immunosuppressive side effects.

Therapeutic Drug Monitoring

Regular monitoring of serum drug concentrations of flucytosine is advisable to reduce the risk of hepatotoxicity and hematological toxicity; this is essential when there is renal impairment. To avoid toxicity, a peak concentration of 100 μg/ml of flucytosine should not be exceeded (54). In contrast to toxicity, there are few data relating efficacy to blood levels for flucytosine. A reasonable goal is to maintain a post-dose concentration of >25 μg/ml but <100 μg/ml (53, 54).

Toxicity and Adverse Effects

The most common, and least harmful, side effects of flucytosine are gastrointestinal, and include nausea, diarrhea, vomiting, and abdominal pain. The most severe adverse effects include bone marrow depression and hepatotoxicity (134). These complications are more likely to occur if excessively high blood concentrations are maintained.

Griseofulvin

Griseofulvin is an antifungal antibiotic derived from a number of *Penicillium* species, including *P. griseofulvum*. Introduced in 1958, oral griseofulvin transformed the treatment of dermatophytosis.

Mechanism of Action

Griseofulvin is a fungistatic drug that binds to microtubular proteins and inhibits fungal cell mitosis (135).

Spectrum of Activity

The spectrum of useful activity is restricted to dermatophytes causing skin, nail, and hair infections (*Epidermophyton*, *Microsporum*, and *Trichophyton* spp.) (2, 4). Resistance has rarely been reported.

Pharmacokinetics

Absorption of griseofulvin from the gastrointestinal tract differs between individuals, but is improved if the drug is given with a high-fat meal. Griseofulvin appears in the stratum corneum within a few hours of ingestion, as a result of secretion in perspiration. However, levels begin to fall soon after the drug is discontinued, and within 48 to 72 h it can no longer be detected. Griseofulvin is metabolized by the liver to 6-desmethyl griseofulvin, which is excreted in the urine.

Clinical Use

Newer oral agents, such as terbinafine or itraconazole, are often preferred for nail infections, but griseofulvin remains a useful second-line agent for moderate to severe dermatophytoses of the skin and scalp hair, where topical treatment is considered inappropriate or has failed.

Drug Interactions

Absorption of griseofulvin is reduced in persons receiving concomitant treatment with barbiturates. Griseofulvin may decrease the effectiveness of oral anticoagulants, oral contraceptives, and cyclosporine.

Toxicity and Adverse Effects

In most cases, prolonged courses and high doses are well tolerated. Adverse effects occur in around 15% of patients and include headache, nausea, vomiting and abdominal discomfort, and rashes.

NOVEL ANTIFUNGAL AGENTS IN DEVELOPMENT

Two promising broad-spectrum triazole compounds, isavuconazole and albaconazole, are currently in clinical trials (136). Isavuconazole (Basilea Pharmaceutica, Astellas Pharma) is a water-soluble compound that can be administered orally or intravenously. The drug has predictable and dose-proportional pharmacokinetics and has completed phase III clinical trials for the treatment of invasive candidiasis and candidemia, treatment of invasive aspergillosis, and treatment of rare mould infections (137, 138).

Isavuconazole has shown good *in vitro* activity against both *Candida* and other yeast species, as well as *Aspergillus* sp. other than *A. niger* (139–141), and has been granted orphan drug status by the U.S. Food and Drug Administration for the treatment of invasive aspergillosis. Albaconazole (Stiefel) is an oral agent that has demonstrated high levels of bioavailability and antifungal activity. It was evaluated in a phase I trial for tinea pedis and has completed a phase II trial for the treatment of toenail onychomycosis (142).

CONCLUSION

The recent surge in development of new antifungal agents has greatly increased the number of drugs available to combat the growing number of serious fungal infections. There are now few life-threatening conditions for which there is no effective treatment, and there are many for which there are several therapeutic options. With judicious use of the available agents, antifungal drug resistance should continue to be a minor clinical problem. As more compounds have become licensed, the number of novel antifungal drugs entering preclinical development appears to have diminished. It remains to be seen which, if any, of these will reach the marketplace.

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Mechanisms of Resistance to Antifungal Agents*

DAVID S. PERLIN

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Globally, 300 million people of all ages suffer from serious fungal infections, resulting in over 1,350,000 deaths anually. Some fungal diseases are acute and severe (e.g., cryptococcal meningitis, invasive aspergillosis, and fungal eye infection), while others are recurrent (e.g., *Candida* vaginitis or oral candidiasis in AIDS). The most serious fungal infections occur as a consequence of other serious health problems such as asthma, AIDS, cancer, organ transplantation, and corticosteroid therapies (1). All require specialized testing for diagnosis and most require antifungal therapy.

More than 90% of all reported fungal-related deaths result from species that belong to one of three genera: *Cryptococcus*, *Candida*, and *Aspergillus* (1). Failure to treat effectively, because of either diagnostic delays or missed diagnosis, often leads to death, serious chronic illness, or blindness. This global recognition of the importance of fungal infections has led to a dramatic rise in the use of antifungal agents for treatment and prevention. Unfortunately, treatment options for invasive fungal infections are limited, as there are few chemical classes represented by existing antifungal drugs.

Classes of antifungal drugs include polyenes, azoles, allylamines, flucytosine, and echinocandins. The azoles (e.g., fluconazole, voriconazole, and posaconazole) and allylamines (e.g., terbinafine) target ergosterol biosynthesis. Like cholesterol in mammalian cells, ergosterol is the major sterol in the fungal plasma membrane. Polyenes, like amphotericin B, bind to ergosterol in the plasma membrane, where they form large pores that disrupt cell function. Flucytosine (5-fluorocytosine) inhibits pyrimidine metabolism and DNA synthesis. Finally, the echinocandins (caspofungin, anidulafungin, and micafungin) are cell wall-active agents that inhibit the biosynthesis of β-1,3-glucan, a major component of the fungal cell wall.

The expanding use of antifungal agents has led to rising drug resistance. The emergence of acquired drug resistance among prevalent fungal pathogens constrains or eliminates treatment options, with a devastating impact on patient management. A greater understanding of biological factors that contribute to mechanism-specific resistance and that promote strategies to overcome resistance is critical to the field of medical mycology. The detailed and complex biological nature of antifungal drug resistance mechanisms is the topic of this chapter.

DEFINING CLINICAL AND MICROBIOLOGICAL RESISTANCE

The development of antifungal resistance is complex and relies on multiple host and microbial factors. Any discussion of drug resistance must distinguish between multifaceted clinical resistance and microbial resistance to antifungal agents.

"Clinical resistance" refers to therapeutic failure, when a patient inadequately responds to an antifungal drug following administration of a standard dose. A variety of host, drug, and microbial factors contribute to therapeutic failure. A major factor is the immune status of the host. Antifungal drug action and the host immune system often must work synergistically to control and clear an infection. Patients with severe immune dysfunction are more refractory to treatment, as the antifungal drug must combat the infection without the positive benefit of the immune response. The immune system has a large dynamic range that can help eliminate or enhance the clinical manifestations of fungal diseases (1). The presence of indwelling catheters, artificial heart valves, and other surgical devices may also contribute to refractory infections, as the infecting fungus attaches to these objects, creating resilient biofilms that provide protection from drug therapy. The site of the infection also contributes to clinical resistance, since it may be inaccessible to drug therapy. Drug penetration is a major factor for many infections, as it is difficult to deliver drugs at an adequate concentration in certain infected tissues and organs. Appropriate therapy requires that the drug reach its microbial target at a suitable concentration to inhibit growth or kill the organism, and the pharmacokinetics (PK) of many antifungal drugs are known. Yet, we still do not have a good understanding of drug penetration at all sites of infection, with the result that some microorganisms are exposed to drug at inadequate levels. Finally, patient compliance with prescribed drug regimens is critical for effective treatment, as poor adherence reduces the effectiveness of the drug, contributing to development of persistent drug-tolerant cell populations. The selection of strains that fail to respond to drugs forms a significant component of drug failures during therapy. Overall, there remains a strong relationship between drug exposure and the emergence of resistance.

"Microbiological resistance" refers to decreased susceptibility of a fungal strain to an antifungal agent in standardized *in vitro* susceptibility testing relative to a susceptible standard

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reference strain. (The details of antifungal susceptibility testing according to guidelines established by the Clinical and Laboratory Standards Institute [CLSI] and the European Committee on Antimicrobial Susceptibility Testing [EUCAST] are addressed in chapter 131.) Susceptibility testing provides a measure of the MIC. MIC resistance breakpoints refer to those drug concentrations that separate strains where there is a high likelihood of treatment success from those organisms where treatment is more likely to fail (2). Yet, interpreting the results of *in vitro* antifungal susceptibility testing can be problematic, as MIC values above the breakpoint do not always directly associate with response to antifungal therapy. This apparent discordance between *in vivo* and *in vitro* data is illustrated by the "90–60 rule," which maintains that infections due to susceptible strains respond to appropriate therapy in ~90% of cases, whereas infections due to resistant strains respond in ~60% of cases (3). Microbiological resistance can be primary (intrinsic) or secondary (acquired). Primary resistance is found naturally among certain fungi without prior drug exposure. It may involve the same mechanism responsible for acquired resistance, or unknown mechanisms.

Primary Resistance

Antimicrobial agents are generally developed for efficacy against the most prominent pathogens causing disease. Universal broad-spectrum activity (e.g., panfungal), while desirable, is rarely achieved, and there will always be a subset of species or naturally occurring variants of mostly susceptible species that are inherently resistant. Presently, there are more than 200 species of *Candida*, and while fewer than 40 are known to cause human infections (4), there is great genetic diversity that can affect drug action. The extended application of antifungal agents against a wide spectrum of mycoses can result in the selection of naturally occurring species with inherent resistance (5). Nevertheless, the overall selection of resistant species, subspecies, or less susceptible variants from the environment or from patient reservoirs occurs uncommonly (6, 7). The common feature of intrinsic resistance is that the underlying resistance mechanism is inherent and not acquired during therapy.

Polyenes

The polyene drug amphotericin is fungicidal and resistance to it rarely occurs. When it does, it is almost always due to selection of strains with high intrinsic reduced susceptibility (MIC, >2 µg/ml) (8), which correlates with the amphotericin B epidemiological cutoff values (ECV) for most *Candida* species (9). The organisms *Scedosporium apiospermum*, *Fusarium* spp., *Trichosporon* spp., and *Sporothrix schenckii* are frequently resistant to amphotericin (10). Breakthrough infections have been reported for *Candida rugosa* (11), *Candida lusitaniae* (12), and *Candida tropicalis* (13). Among the *Aspergillus* spp., primary resistance to amphotericin B has been reported for strains of *A. terreus* (14, 15), *A. flavus* (16), and *A. ustus* (17).

Azoles

The azole antifungal agents are the most prominent example of drug selection for less susceptible species (10). Numerous global epidemiological studies have documented the impact of widespread triazole use on the distribution and shift of *Candida* species toward less susceptible strains like *C. glabrata* and *C. krusei*. In many regions where azole use (e.g., fluconazole) is prevalent, there has been a shift away from *Candida albicans* as the predominant cause of invasive infections toward less susceptible non-*C. albicans* species (18). *C. glabrata* has inherent reduced susceptibility to fluconazole, and it is the species whose incidence has increased the most to account for a decrease in the prevalence of *C. albicans* (18, 19). Similarly, fluconazole use is linked to emergence of the highly resistant *C. krusei* (20) and *Candida guilliermondii* (21) as significant causes of candidemia. In many cases, inherent resistance in *Candida* species to fluconazole also carries with it resistance to more highly active triazoles like voriconazole. This is not true for *Aspergillus* and other moulds that are resistant to fluconazole but susceptible to more highly active triazoles. Yet, breakthrough infections against highly active triazole drugs have been reported for *A. ustus* (22) and *Aspergillus fumigatus*-like species such as *A. lentulus*, which show pleiotropic resistance to multiple antifungal drugs (23, 24).

Sometimes, a susceptible species develops a prevalent variant that is the source of resistant infections. In the bacterial world, the regional and global spread of drug-resistant strains from a common progenitor is commonly observed. Such transmission is not typically observed for fungal drug resistance. A notable exception occurred with the recent emergence of a multidrug-resistant variant of *A. fumigatus* in the Netherlands (25, 26). This resistant strain was encountered in patients who failed therapy for invasive aspergillosis despite having no prior azole exposure (27). This highly azole-resistant strain variant was selected in the environment as a consequence of the prevalent use of agricultural azoles. The resistance mechanism unique to these isolates is discussed later in the chapter; such resistant strains are spreading through Europe and into parts of Asia (28).

Echinocandins

Finally, the echinocandins are highly active against most *Candida* spp., but they have inherent reduced susceptibility against the *Candida parapsilosis* group (*C. parapsilosis sensu stricto*, *C. orthopsilosis*, and *C. metapsilosis*) and *C. guilliermondii* (29). Some breakthrough infections have been reported during therapy and have been attributed to the inherent reduced susceptibility of these strains (30). Resistance to echinocandin drugs has also been described for *A. lentulus* (24). In some filamentous fungi, such as *A. lentulus*, *A. ustus*, and *Fusarium* spp., there is coresistance that confers resistance to azoles and echinocandin drugs, and occasionally resistance to polyenes.

In summary, drug pressure is a powerful selection tool that results in infections of uncommon fungi with inherent reduced susceptibility to antifungal drugs. In a majority of cases, the nature of drug insensitivity is a reflection of underlying resistance mechanisms that emerge in susceptible strains in response to drug action.

RESISTANCE TO AZOLES

Mechanism of Action of Azoles

Azoles inhibit the biosynthesis of ergosterol, the principal sterol in fungal cell membranes, by interfering with the action of lanosterol 14α-demethylase, which is encoded by *ERG11* (*Cyp51A* in *Aspergillus*). There are many licensed azole antifungal drugs (imidazoles, triazoles), yet the triazole drugs fluconazole, voriconazole, itraconazole, and posaconazole are the most commonly prescribed drugs for prophylaxis or treatment of systemic and mucosal fungal infections. Triazoles are chemically characterized by having a five-member ring moiety of two carbons and three nitrogen atoms. The drugs differ in their target affinities, which influences their spectrum of activity. Fluconazole has the weakest interaction with its target and shows the narrowest spectrum of activity. It is active against many yeasts, but it has poor activity against moulds like *A. fumigatus*. The more highly active triazoles, like voriconazole and posaconazole, interact more strongly with the demethylase target and show broad-spectrum activity against yeasts and moulds, as well as activity on some fluconazole-resistant strains. Fluconazole and its chemical analogue voriconazole are structurally similar, while posaconazole and itraconazole are more closely related. It is this moderate chemical diversity around a core unit that promotes cross-reactivity, and at times differential susceptibility, which ultimately depends upon the nature of the resistance mechanism.

Epidemiology of Azole Resistance

The widespread use of fluconazole in the pre-antiretroviral therapy era as a safe and effective antimycotic agent to treat HIV-infected patients with oropharyngeal or esophageal candidiasis led to the emergence of resistance among susceptible strains like *C. albicans*. A multitude of sentinel and population-based surveillance programs from more than 40 countries have contributed to our understanding of azole resistance (6, 31–38). Overall, the studies confirm that acquired resistance among susceptible species is low, while resistance is more significant in non-*albicans Candida* species. For example, the ARTEMIS DISK Global Antifungal Surveillance Study evaluated clinical isolates of *Candida* spp. from 142 sites in 41 countries over a 10-year period from 1997 to 2007. For all *Candida* spp. isolates, 90.2% were susceptible to fluconazole, while 95.0% were sensitive to voriconazole. Among 128,625 isolates of *C. albicans*, resistance was 1.4 and 1.2% for fluconazole and voriconazole, respectively. In 23,305 clinical isolates of *C. glabrata*, there was a higher population of resistant isolates: 15.7 and 10% for fluconazole and voriconazole, respectively. Importantly, there was a yearly trend upward for increased azole resistance among *C. glabrata* (39), and resistance to fluconazole varied by region (6, 34–37). Increases in resistance are notable with *C. guilliermondii* and *C. krusei*, and an overall increase in fluconazole resistance over time was seen with *C. parapsilosis*, *C. guilliermondii*, *C. lusitaniae*, and *Candida pelliculosa* (40). In contrast to the increase in azole resistance seen with *C. glabrata*, the trend has reversed for *Cryptococcus neoformans* in developed countries (29, 33, 41). In developing countries where AIDS is more prevalent, fluconazole resistance is low but more significant (42). The details of antifungal susceptibility testing according to guidelines established by the CLSI and EUCAST are addressed in chapter 131. Triazole-resistant strains of *A. fumigatus* were first reported in 1997 from patients in California treated with itraconazole (43). Azole-resistant *A. fumigatus* has now been reported in China, Canada, the United States, and several European countries. High rates in the United Kingdom (15 to 20%) (44, 45) and in the Netherlands (5 to 7%) have been observed (26, 46). Approximately 5% of *Aspergillus* isolates were found to be azole resistant in studies involving respiratory colonization of cystic fibrosis patients (47). The ARTEMIS global surveillance program reported a 5.8% resistance rate in 2008 and 2009 (48). Azole resistance among *A. fumigatus* is notably high due to both acquired resistance during therapy and acquisition of resistant environmental variants (28, 49, 50). Yet, the rates may underestimate the global prevalence of *Aspergillus* drug resistance. The true frequency of triazole resistance is unknown because *A. fumigatus* is cultured from less than 30% of infected patients. PCR can more efficiently identify the presence of *Aspergillus* in respiratory specimens that are otherwise culture negative, and the simultaneous detection of characteristic drug-resistance markers in these assays indicates that resistant strains are more prevalent than reported by culture (51).

MECHANISMS OF AZOLE RESISTANCE

The underlying molecular mechanisms responsible for acquired azole resistance have been extensively studied over the past several decades, especially in the model organism *Saccharomyces cerevisiae* and in laboratory and clinical strains of *C. albicans*. Early studies elucidating the biological machinery central to resistance have evolved into eloquent descriptions of cellular regulatory mechanisms and circuitry that help modulate azole resistance mechanisms following exposure of a susceptible strain to drug. Many excellent reviews have covered this topic (52–55). The general mechanisms and effectors of drug resistance are summarized in Table 1 and include:

1. reduced drug-target interaction;
2. increase in target copy number;
3. reduction of intracellular drug concentration mediated via drug efflux transporters and reduced uptake;
4. modification of other ergosterol biosynthesis pathway elements; and
5. biofilms and persister cells.

The biological responses revealing these resistance mechanisms involve adaptive cellular responses and modification of genetic regulatory elements. The relative contribution of individual mechanisms to development of resistance varies by genus and species. In some clinical strains, a single dominant mechanism may prevail, while in others, stepwise development of high-level resistance involves a combination of resistance mechanisms that may act additively or synergistically. The contribution of specific resistance mechanisms is strain dependent and generally falls into both multicomponent mechanisms and single dominant mechanisms represented by the major pathogens *C. albicans*, *C. glabrata*, and *A. fumigatus*.

Drug Target Modification

Genetic modification of the target Erg11p resulting in reduced affinity for drug is one of the most direct mechanisms of resistance. At least 60 amino acid substitutions in Erg11p from azole-resistant clinical isolates have been described (56–61). The impact of individual substitutions determines the relative degree of resistance and cross-reactivity within the class. For example, Y132H, G450E, G464S, R467K, and S405F reduce susceptibility to fluconazole and voriconazole, but not to the longer-chain drugs itraconazole or posaconazole. A limited number of mutations are predicted to strongly affect drug-target interactions, which would confer prominent cross-reactivity among the class. The overall contribution of many *ERG11* mutations to fluconazole resistance is weak, as some have a modest impact on azole resistance phenotypes. Furthermore, in diploid *C. albicans*, nucleotide mutations may occur as homozygous (both alleles) or heterozygous (single allele) substitutions, which can alter the resistance phenotype. In *C. neoformans*, strains resistant to fluconazole may contain characteristic mutations in the *ERG11*

TABLE 1 Antifungal targets, drug resistance mechanisms, and effectors

Antifungal agent	Cellular target	Mode of action	Mechanism of resistance	Effectors of resistance
Triazoles				
Fluconazole Voriconazole Itraconazole Posaconazole Ravuconazole	Lanosterol 14α-demethylase (Erg11, Cyp51A) Sterol Δ^{22} desaturase (ERG5)	Blocks ergosterol biosynthesis	Altered drug affinity by Erg11 (Cyp51A) target	
			Upregulation of ERG11 (CYP51A)	Upc2 LOH Aneuploidy
			Upregulation of CDR and MDR multidrug transporters	Tac1, Mrr1, biofilms, *HSP90*, *CRZ1* LOH Aneuploidy
			Other ergosterol biosynthesis genes (ERG3)	
			Biofilms	HSP90, SMI1 Adh5, Csh1, Gca1, Gca2, Rlmp, Fks1
			Drug uptake	
Polyenes				
Amphotericin B Nystatin	Binds ergosterol and forms aqueous pores	Forms large aqueous pores	Sterol depletion Biofilms	
Echinocandins				
Caspofungin Micafungin Anidulafungin	β-1,3 glucan synthase (Fks1, Fks2)	Alters cell wall integrity by blocking β-1,3 glucan biosynthesis	Modification of Fks1 and/or Fks2 subunits of glucan synthase Biofilms	*HSP90* *CRZ1* Chitin Sphingolipids
Allylamines				
Naftifine Terbinafine	Squalene epoxidase (Erg1)	Blocks sterol biosynthesis	Modification of Erg1	
5-Fluorocytosine (5FC)	Inhibits nucleic acid synthesis	Inhibits nucleic acid and protein synthesis	Uracil pyrophosphorylase (Fur1) Cytosine deaminase (Fcy1) Cytosine permease (Fcy2)	

gene (62, 63). In *A. fumigatus*, modification of the azole target lanosterol 14α-demethylase is the predominant mechanism underlying clinical resistance. A limited number of acquired amino acid substitutions in Cyp51A have been described, which confer a variety of triazole resistance phenotypes (45, 49, 64). All modifications confer resistance to itraconazole, while others also confer some cross-resistance to voriconazole and posaconazole (Table 2). The most prominent modifications occur at amino acids Gly54, Leu98, Gly138, F219, M220, and Gly448. The acquisition of resistance-conferring mutations in Cyp51A requires drug pressure, as there appears to be a fitness cost that affects relative virulence (65).

Environmentally Acquired Resistance Is a Major Factor

In the Netherlands, most resistance (>90%) in *A. fumigatus* is due to a mutation in Cyp51A at the codon for Leu98 and a tandem repeat (TR) of 34 bp in the promoter region (25, 46, 66–68), which confers pan-azole resistance (Table 2). The TR/L98H resistant isolates arose as a consequence of azole use in the agricultural world (26). This singular mechanism (25) has been identified in many parts of Europe, India, and parts of Asia (69–71), but it has not been observed in patients who evolve resistance during therapy. Recently, a new TR46/Y121F/T289A mechanism has been observed that further suggests an environmental route of resistance selection (72).

Structural Modeling of Resistance

Nonsynonymous mutations in *CYP51A* (and *ERG11*) result in structural alterations to lanosterol 14-demethylase that reduce drug binding. Homology overlay modeling of Cyp51A and Erg11 using related high-resolution structural models has been used to describe the impact of specific amino acid substitutions on the interaction of triazole drugs with the target enzyme (73). Such models predict interactions based on different chemical structural properties of the various triazole drugs (e.g., voriconazole versus posaconazole) (73). Most mutations are clustered in three hot-spot mutation regions (74). The most prominent resistance-conferring substitutions alter the apparent interaction of drug with the heme cofactor. Yet, many Erg11 amino acid replacements from fluconazole-resistant *C. albicans* strains are peripheral and are predicted to exert a relatively weak action and resulting phenotype. In *A. fumigatus* Cyp51A, amino acids Gly54, Gly138, Pro216 and Phe219, Met220, and Gly448 conferring triazole resistance are predicted to lie in close proximity to the opening of one of two ligand access channels, which would allow azole compounds to enter the enzyme active site (75–77) (Fig. 1). Modification of the channel openings is presumed to disturb the docking of azole molecules. The model helps predict why modification of Gly54 yields itraconazole and posaconazole resistance, yet retains voriconazole susceptibility. Leu98 is located on a loop that partly forms an arch-like structure highly conserved among the members of the Cyp51 family of proteins (68, 75).

TABLE 2 Amino acid substitutions in A. *fumigatus* Cyp51A conferring triazole resistance and associated MIC values[a]

Cyp51A locus	Amino acid substitutions	MIC (mg/liter)		
		Itraconazole	Voriconazole	Posaconazole
F46	Y	>8	2–4	0.125–0.5
G54	E, R, V	>8	0.125–1	1–>8
TR34-L98	H	>8	8	1–2
TR46-Y121/T289	F/A	>4	>16	1
H147	Y	>8	>8	0.5
M172	V	>8	2–4	0.125–0.5
P216	L	>8	1	1
F219	C	>8	1	1
M220	K, T	>8	1–4	0.5–>8
N248	T	>8	2	0.25
D255	E	>8	2	0.25
E427	G, K	>8	2–4	0.125–0.5
Y431	C	>8	4	1
G434	C	>8	4	1
G448	S	>8	>8	0.5–1

[a] Adapted from Howard and Arendrup (49) and Bader et al. (70)

[b] TR#, Tandem repeat promoter (number of bases).

Increasing the Target Abundance

Lanosterol 14C-demethylase is a key enzyme in the ergosterol biosynthesis pathway. In response to azole antifungals and/or sterol depletion, *Candida* strains may overexpress *ERG11* (78) and other genes involved in ergosterol biosynthesis (79). Elevated abundance of target proteins decreases the effectiveness of azole drugs at a standard dosage. Increased *ERG11* expression arises from *cis*-acting gain-of-function (GOF) mutations within the promoter region or from alterations in a *trans*-acting factor, Upc2p (80). The C. *albicans* Upc2p is a Zn_2Cys_6 cluster transcription factor homologous to S. *cerevisiae* paralogues *UPC2* and *ECM22* (81, 82). Upc2p is required for upregulation of *ERG11* (83) and other sterol biosynthesis genes in response to sterol depletion (81, 82). Mutations in *UPC2* result in increased expression of *ERG11* and decreased fluconazole susceptibility (80, 82). The Upc2p activates transcription of target genes by binding to a conserved core sequence known as the sterol response element (81). An 11-bp sterol response element was identified in the promoters of *ERG2* and *ERG3*, and Upc2 and Ecm22 directly bind to this element. Induced overexpression of *ERG11* or *UPC2* increases resistance to azoles, while mutants lacking *UPC2* show no induction of *ERG* genes and they are hypersusceptible to these drugs (82). *CaUPC2* activity can increase up to 100-fold with prolonged azole exposure. The *CaUPC2* promoter contains a putative sterol response element (81), and transcriptional

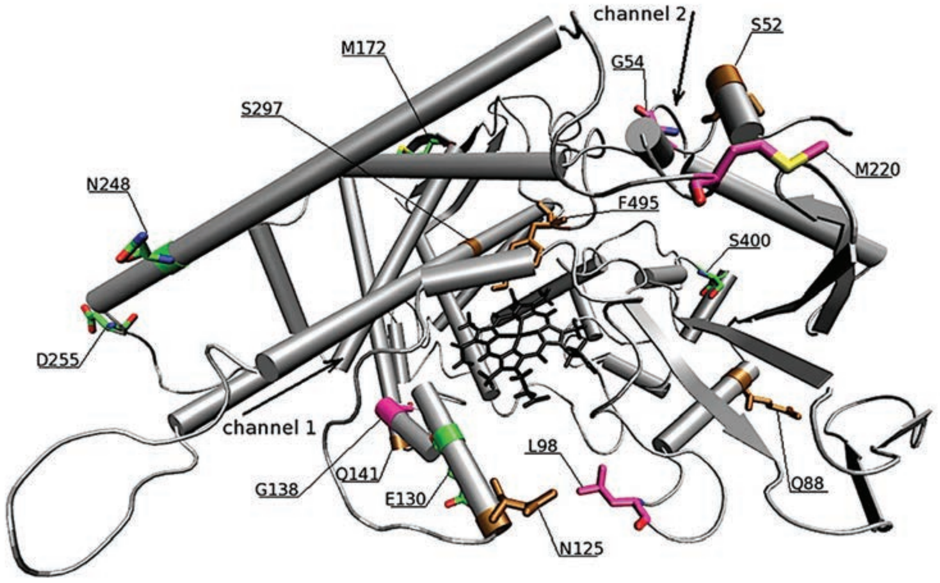

FIGURE 1 Homology structure model for Cyp51A from A. *fumigatus* showing amino acid positions of substitutions conferring azole resistance (from Snelders et al. [75]).
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regulation of *UPC2* expression occurs through Upc2p-dependent and independent mechanisms (84). *UPC2* mutations may also potentiate resistance by influencing expression of other genes, including *MDR1*, which encodes a multiple drug resistance protein linked to resistance (80, 85). Stepwise enhancement of fluconazole resistance can involve constitutive high-level expression of *ERG11* and overexpression of drug transporters (86, 87). Less is known about the regulation of Cyp51A in *A. fumigatus*, although upregulation has been described for itraconazole-resistant mutants (65, 88).

Reducing Cellular Drug Levels: Drug Efflux Transporters

Fungi encode numerous putative drug efflux transporters that have the potential to influence susceptibility to azole-class antifungal agents, which serve as transport substrates. These high-capacity efflux systems, present in all higher eukaryotes, reduce the effective cellular concentration of drugs below their target inhibitory threshold. Overexpression of multidrug efflux transporters is well recognized as an important mechanistic component conferring resistance to azole antifungal drugs (89, 90). There are two main classes of efflux pumps that contribute to azole resistance phenotypes in fungi. They include ATP-binding cassette (ABC) transporters (e.g., CaCdr1, CaCdr2), which use energy derived from ATP hydrolysis to transport drugs, and the major facilitator superfamily (MFS) transporters (e.g., CaMdr1), which utilize the plasma membrane electrochemical gradient to translocate substrates.

The ABC superfamily is one of the largest protein families known. These proteins transport a wide variety of substrates across extracellular and intracellular membranes, including metabolic products, lipids and sterols, and drugs. A common feature of all ABC transporters is that they consist of two distinct domains, the transmembrane domain and the nucleotide-binding domain composed typically of Walker A and Walker B motifs. The *C. albicans* genome contains at least 27 genes with ABC domains that include these topologies (91). In *C. albicans*, Cdr1 is an integral plasma membrane protein of 1,501 amino acids (92). Cdr1 was first identified as a protein capable of transporting a variety of unrelated cytotoxic drugs and was shown to be one of the major determinants of fluconazole resistance in clinical isolates of *C. albicans* (93). The closely related transporter Cdr2 exhibits 84% amino acid sequence identity with Cdr1. Cdr2 confers resistance to azole antifungal agents, other antifungals (e.g., terbinafine), and a variety of metabolic inhibitors. However, *CDR2* overexpression is also observed in resistant mutants that revert spontaneously to wild-type levels of susceptibility (94), which suggests that its impact on clinical azole resistance is less pronounced than that of *CDR1*. In *C. glabrata*, azole resistance is almost exclusively due to enhanced drug efflux, which is a consequence of overexpression of the ABC transporter family. The genes encoding these transporters, *CgCDR1* (95), *CgCDR2* (*PDH1*) (96), and *CgSNQ2* (97), have been well described in laboratory and clinical isolates. In *C. glabrata*, petite mutants displaying a loss of mature mitochondria can become azole resistant through increased expression of *CgCDR1* and, to a lesser extent, *CgCDR2* (98). Similarly, in *C. neoformans*, an ABC transporter-encoding gene, CnAFR1, can confer resistance to fluconazole (99). In *C. krusei*, *ABC1* is strongly upregulated in response to azole exposure, where it may play a role in intrinsic resistance (100).

MFS transporters account for nearly half of the solute transporters encoded within the genomes of microorganisms. The *C. albicans* genome contains at least 95 open reading frames that may encode an MFS transporter (101). MFS transporters are single-polypeptide secondary carriers capable only of transporting small solutes in response to electrochemical ion gradients. In *C. albicans*, Mdr1 (formerly BEN^r) (102) was originally identified because it was highly overexpressed in an azole-resistant clinical isolate (89). CaMdr1 confers intermediate-level resistance to fluconazole, in contrast to CaCdr1, which is associated with efflux of a wider range of substrates at higher capacity. In most *C. albicans* strains, *MDR1* is not significantly expressed, but it is induced in the presence of certain chemicals like benomyl, H_2O_2, or azoles (103). Another MFS transporter, Flu1, confers resistance to fluconazole and is closely related to CaMDR1. However, *FLU1* expression varies among azole-susceptible and azole-resistant *C. albicans* clinical isolates, and therefore it does not appear to be an important factor in clinical resistance (104).

In *A. fumigatus*, overexpression of ABC and MFS drug transporters has also been described, as they may confer resistance to highly active triazole antifungal agents (105). Multiple mechanisms can occur in resistant strains whereby both target-site mutations and upregulation of drug efflux transporters like *AfuMDR3* and *AfuMDR4* may occur (88, 106), as well as newly described Cdr1b (107). Overexpression of *Cyp51A* confers reduced susceptibility (108), as does *Cyp51B* (109). Finally, in a significant percentage of *A. fumigatus* isolates, the mechanism of triazole resistance is unclear and may be novel (44).

It is important to note that resistance to azoles in all fungi is highly dependent on the level of *CDR* or *MDR* overexpression (110). This raises the question of whether reporting overexpression of any one or all CDR or MDR genes is a sufficient indicator of resistance, and what threshold level of overexpression is required for resistance. Overall, high-level azole resistance most often correlates with overexpression of *CDR1* and to a lesser extent *CDR2*. However, in some isolates, *MDR1* is the only pump gene overexpressed (89). Finally, clinical isolates of azole-resistant *C. albicans* and other *Candida* species show overexpression of multiple efflux pump classes, including CaCdr1p, CaCdr2p, and CaMdr1p (87, 111–113). It has been demonstrated in serial isolates from patients exposed to azole antifungal agents that multifactorial resistance develops stepwise with drug exposure and involves CDR and/or MDR gene expression (86, 87, 112) and other mechanisms (e.g., *ERG11* overexpression).

Regulation of Azole Resistance

The underlying mechanism regulating overexpression is an important component for development of azole resistance phenotypes. A major emphasis in recent years has been directed at understanding regulatory circuits controlling the expression of these genes. It is well established for *C. albicans* and other *Candida* spp. that activating GOF mutations in the zinc cluster transcription factors Tac1 and Mrr1 regulates the expression of *CDR* and *MDR* genes, respectively (114–118). Specific mutations in *TAC1* mediate the overexpression of the genes encoding ABC transporters CDR1 and CDR2 (114, 119), while Mrr1 regulates the MFS transporter Mdr1 in azole-resistant isolates (118, 120, 121). Mutants lacking these transcription factors cannot upregulate their target genes in response to drugs, and mutations render the transcription factors constitutively active even under noninducing conditions. In *C. glabrata*, CgPdr1p is a Zn_2-Cys_6 transcription factor involved in the regulation of the

ABC transporter genes *CgCDR1*, *CgCDR2*, and *CgSNQ2* (122, 123). GOF mutations in *CgPDR1* are responsible for intrinsic high expression of ABC transporters (122, 124), and a salient feature is their extraordinarily high diversity among *CgPDR1* alleles from azole-resistant clinical isolates, with more than 65 nonsynonymous substitutions identified (122, 124). Finally, mRNA stability is an important factor as it contributes to message stability and sustained overexpression of *CDR1* in azole-resistant *C. albicans* isolates (125).

Loss of Heterozygosity and Other Chromosomal Abnormalities

High-level azole resistance may occur when *C. albicans* upregulates and/or carries mutations in *ERG11* and *TAC1*. An increase in copy number can occur for these genes as a consequence of loss of heterozygosity (LOH). *TAC1* is located on chromosome 5 (Chr 5), along with *ERG11*. Chr 5 rearrangements lead to *TAC1* homozygosity but also to LOH in other Chr 5 regions, including the mating type locus (*MTL*) and *ERG11* loci. Finally, azole resistance can result from segmental aneuploidy on Chr 5, which increases copies of *TAC1*, *MTL*, and *ERG11* via formation of isochromosome 5L [i(5L)] (119, 126). This genetic plasticity provides *C. albicans* with a dynamic response to azole drugs that is prevalent in resistant clinical isolates (127). In *C. neoformans*, heteroresistance to fluconazole is an adaptive mode of azole resistance (128). Heteroresistant cell populations respond differentially to drug and adapt in a stepwise manner to higher concentrations of the drug. Strains adapting to fluconazole levels above the MIC often contain disomies of Chr 1. The duplication of Chr 1 is associated with both the target encoding gene *ERG11* and the major transporter Afr1 (63, 129, 130). In the absence of drug, the strains return to their basal level of susceptibility by losing the extra copy of Chr 1, followed by loss of the extra copies of the remaining disomic chromosomes.

Azole Resistance and Virulence

It is generally anticipated for most pathogens that acquisition of drug resistance may be associated with a loss of fitness or virulence. In *C. albicans*, GOF mutations in Mrr1, Tac1, and Upc2 that result in resistance appear to have a fitness cost, suggesting that regulation of drug pumps must be tightly controlled by the cell (131). In *A. fumigatus*, acquisition of azole resistance involving modification of Cyp51A can alter virulence (132). Furthermore, acquisition of azole resistance in the absence of known *Cyp51A* mutations can also lead to reduced virulence (65). Virulence defects suggest that resistant strains should be self-limiting in a vast reservoir of highly fit wild-type strains. Yet, this does not appear to be the case for L98H/TR or other environmentally driven strains, since they maintain cellular fitness and virulence which is manifested as widespread transmission of infecting strains. Similarly, *C. glabrata* GOF mutations in *CgPDR1* modulate host interactions in ways that promote increased virulence in murine models of disseminated infection (122). Several host-based mechanisms appear to play a role in this phenomenon. For example, GOF *CgPDR1* mutations decrease adherence and uptake by macrophages, which may allow evasion from the host's innate cellular immune response (133). Similarly, enhanced virulence is observed for azole-resistant strains of *C. glabrata* with mitochondrial DNA deficiency (petite mutants), which upregulate the ABC transporter genes *CDR1* (*CgCDR1*), *CgCDR2*, and *CgSNQ2* (134).

Other ERG Genes

ERG3 encodes sterol $\Delta^{5,6}$-desaturase, which contributes to sterol biosynthesis. Azole exposure results in the accumulation of 14α-methylated sterols and 14α-methylergosta-8,24(28)-dien-3,6-diol, and formation of the latter sterol metabolite is catalyzed by sterol $\Delta^{5,6}$-desaturase. Inactivation of *ERG3* suppresses toxicity, which facilitates azole resistance (135). Clinical isolates of *C. albicans* resistant to both azoles and amphotericin B have been shown to have sterol depletion and defects in *ERG3* (136, 137). Yet, inactivation of *ERG3* does not always result in azole resistance since, in *C. glabrata*, a null *ERG3* mutant was not azole resistant (138).

Biofilms

Biofilms are one of the most prevalent forms of microbial growth in nature, and a characteristic property of biofilms is that they are often refractory to common antifungal agents. *Candida* species are among the most common etiologic agents of yeast-related biofilm infections (139), although *C. albicans* is the most prominent fungal biofilm producer (140). Other yeasts and filamentous fungi are important biofilm producers, including other *Candida* spp., *Cryptococcus*, *Blastoschizomyces*, *Trichosporon*, *Pneumocystis*, *Saccharomyces*, *Aspergillus*, and *Coccidioides* (140). The mature *C. albicans* biofilm displays an organized three-dimensional structure composed of a dense network of yeast and filamentous cells embedded in an exopolymeric matrix consisting of carbohydrates, proteins, and nucleic acids. The complex organization of the extracellular biofilm matrix is a major feature that distinguishes biofilms from planktonic cells (Fig. 2).

Candida biofilms are intrinsically resistant to azoles like fluconazole. The mechanisms for this resistance against azoles and other drugs in *Candida* biofilms are multifactorial, involving both induction of drug efflux transporters and drug sequestration within the extensive matrix structure (139–143) (Fig. 2). In biofilms, drug efflux transporter genes *CDR* and *MDR* are upregulated during biofilm development (143, 144). Yet, a larger component of the multidrug resistance phenotype is imparted by drug sequestration within the extracellular matrix (145). The complex matrix provides infrastructure for biofilm accumulation, conferring protection from antimicrobial peptides and environmental antagonists (146, 147). A key constituent of the *C. albicans* matrix is β-1,3-glucan produced by glucan synthase, which is encoded by *FKS* genes in *Candida*. This glucan is modified and incorporated into both the cell wall and the extracellular matrix, and a variety of genes are responsible for the delivery and arrangement of β-1,3-glucan in the matrix (145, 148–150). Glucan synthesis by Fks1 is critical for biofilm-specific drug resistance in *C. albicans*, which helps to sequester drug, thus reducing the effective concentration encountered by the cell (148) (Fig. 2). Other cellular proteins, such as alcohol dehydrogenases Adh5, Csh1, and Ifd6 and glucoamylases CaGCA1 and CaGCA2, have roles in matrix production and resistance phenotypes (151). Heat shock protein 90 (Hsp90) is a conserved and essential chaperone that regulates cellular signaling by stabilizing numerous client proteins involved in signal transduction (152). It is important for biofilm health, as its depletion in *C. albicans* reduces biofilm growth and maturation and causes impaired dispersal and diminished resistance to azole antifungals (153). Impairment of Hsp90 function, genetically or pharmacologically, enables fluconazole to be effective in eradicating azole-insensitive biofilms *in vivo* (153). Collec-

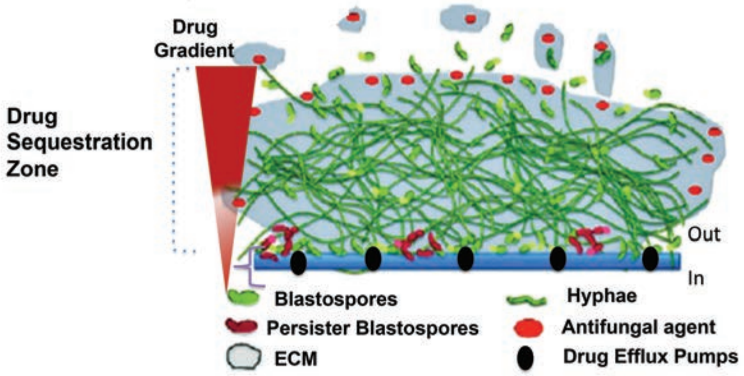


FIGURE 2 Schematic of biofilm composition and mechanisms of resistance showing effects on drug levels (adapted from Ramage et al. [55]). doi:10.1128/9781555817381.ch130.f2

tively, C. *albicans* matrix production is highly regulated and is a key resistance factor. It is also a factor in a number of other *Candida* spp., including C. *glabrata*, C. *parapsilosis*, C. *tropicalis*, and C. *dubliniensis* (154).

Persister Cells: Stress Adaptation

Persister cells that are tolerant to drug are an important mechanism of resistance for infections requiring prolonged antifungal therapy, and biofilms provide an important source of such drug-tolerant cells (55). In C. *albicans*, C. *krusei*, and C. *parapsilosis*, persistence was observed in a small subset of yeast cells in biofilms exposed to drug that displayed resistance to amphotericin B and were independent of upregulation of efflux pumps and cell membrane composition (140, 155, 156). Cellular stress-response mechanisms are critical for persistence. Hsp90, as a cellular stress modulator, plays a critical role in promoting cell adaptation. Loss of Hsp90 function reduces tolerance of *Candida* species to azoles (157). Inhibition of Hsp90 can also block the development of azole resistance and mitigate azole resistance upon loss of function of Erg3 or host-induced factors (158). Overall, Hsp90 helps regulate the cellular circuitry that is involved in stress responses and promotes the emergence of resistance to azoles (157, 159, 160).

MECHANISMS OF POLYENE RESISTANCE

The polyenes nystatin and amphotericin B are among the oldest antimicrobial drug class. Amphotericin B deoxycholate was the first antifungal agent approved in the United States (1957) for human use to treat potentially life-threatening invasive fungal infections (161). Polyene drugs interact with the fungal-specific sterol ergosterol in the cellular membrane to create concentration-dependent high-conductance channels that allow ions and other cellular components to flow from the cell, causing cell death. For decades, amphotericin B was a mainstay therapy for invasive fungal infections including invasive aspergillosis, cryptococcosis, blastomycosis, candidemia, coccidioidomycosis, histoplasmosis, and mucormycosis. However, its avidity for sterols such as cholesterol in animal cell membranes contributes to renal toxicity, and it has been relegated to largely a second-line antifungal option. Lipid formulations of amphotericin B overcome the most serious limitations but still suffer from issues of cost and potential toxicity.

Epidemiology of Resistance

Refractive therapeutic response to polyenes generally involves inherently insensitive moulds such as A. *terreus*, *Pseudallescheria boydii*, *Scedosporium* spp., *Purpureocillium lilacinum*, and *Fusarium* spp. (14, 23, 162). In recent years, the prevalence of polyene resistance in *Aspergillus* species has increased, with only 11.5% of A. *fumigatus* isolates inhibited at 1 μg/ml (163, 164). Less common *Aspergillus* species, such as A. *ustus* and A. *lentulus* (23, 24), are highly resistant to amphotericin B as well as other antifungal agents. For yeasts, acquired resistance to polyenes among *Cryptococcus* and *Candida* strains is rare, although reports of high MICs to amphotericin B and/or poor therapeutic outcome have been reported for C. *albicans*, C. *krusei*, C. *rugosa* (11), C. *lusitaniae* (12), and C. *glabrata* (165, 166).

Mechanisms of Polyene Resistance

The mechanism of resistance to amphotericin B in yeasts and moulds typically involves a reduced content of ergosterol in the cell membrane. Acquired resistance to amphotericin B is rare, even among patients with long-term exposure who ultimately fail therapy (8). In this context, prior exposure to an azole, which lowers cellular sterol levels, can confer stable polyene resistance (167). Resistant mutants of A. *fumigatus* can be selected *in vitro* in response to amphotericin B (168), and occasionally high-MIC isolates are identified from patient failures. However, acquired resistance to amphotericin B has been most extensively evaluated in yeasts. Mutant yeast strains with defects in sterol pathway genes *ERG1*, *ERG2*, *ERG3*, *ERG4*, *ERG6*, and *ERG11* are largely depleted of ergosterol, which confers varying levels of resistance to polyene agents (137, 165, 169). In C. *neoformans*, strains with defective C8-isomerase activity and diminished sterol content exhibit reduced susceptibility to polyenes (170). In C. *albicans*, clinical and laboratory strains resistant to both azole antifungals and amphotericin B were found to be defective in *ERG3* (136, 138). In A. *fumigatus*, however, deletion of *ERG3* did not alter amphotericin B susceptibility, even though total sterol content was reduced (171). Resistance to amphotericin B appears to be more complex in *Aspergillus* spp. and most likely involves other mechanisms (172). Biofilm formation is an important factor that restricts drug entry, as highly hydrophobic drugs get trapped in the extensive glucan matrix. Other mechanisms may involve cell stress adaptation to drug exposure.

MECHANISMS OF ECHINOCANDIN RESISTANCE

Echinocandin Antifungal Drugs

The echinocandin drugs anidulafungin, caspofungin, and micafungin are lipopeptides that target and inhibit glucan synthase, which is responsible for the biosynthesis of β-1,3-D-glucan, a central building block of most fungal cell walls (173). Echinocandins are now the preferred antifungal for the treatment of invasive candidiasis (174). The fungal cell wall consists of three sugar polymers (glucan, chitin, and mannan) and is a layered matrix consisting of an outer layer of glycoproteins and an inner layer of carbohydrate polymers. The cell wall is a dynamic structure, as polymers are modified and rearranged during cell wall biosynthesis (175). Consistent with the observation that cell wall integrity is essential for cell survival, echinocandin drugs show *in vitro* fungicidal activity against susceptible *Candida* spp. (176). Yet they are only fungistatic against moulds, where they can lyse the tips of some growing hyphae but do not completely block cell growth (177, 178). Echinocandin drugs are generally impotent against invasive *Zygomycetes*, *Cryptococcus* spp., or *Fusarium* spp. Importantly, isolates resistant to echinocandins do not exhibit cross-resistance to other antifungal agents, which enables them to be effective against azole-resistant yeasts (179) and some *Candida* biofilms (180, 181). The expanding use of these agents has resulted in growing echinocandin resistance among *Candida* spp. (182, 183)

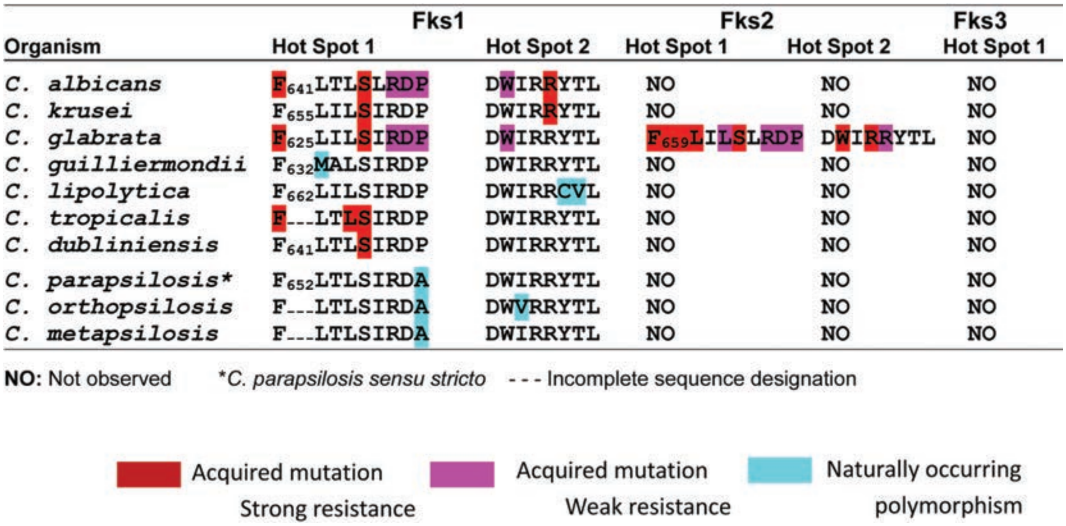
The echinocandin target β-1,3-D-glucan synthase is a multisubunit enzyme complex that catalyzes the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds. The enzyme complex consists of at least two subunit components, Rho and Fks. The GTP-binding protein Rho helps regulate the overall activity of glucan synthase (184). The catalytic subunit Fks is encoded by three related genes, *FKS1*, *FKS2*, and *FKS3*. The *FKS1* gene is essential in *C. albicans* (185) and other *Candida* spp., but in *C. glabrata*, *FKS1* and *FKS2* are functionally redundant (186).

Epidemiology of Echinocandin Resistance

Candida spp. isolates resistant to echinocandin drugs are increasingly encountered (187), although the frequency remains relatively low (<2 to 3%) with *C. albicans* and most other *Candida* spp. (188–190). The exception is *C. glabrata*, in which resistance is growing more severe. The widespread use of echinocandins and azoles has resulted in an epidemiologic shift with *C. glabrata* emerging as the most dominant fungal bloodstream pathogen in some health care centers (191). Drug resistance may occur after prolonged therapy (192) or it may be rapid, even within the initial days of therapy (193). The SENTRY Antimicrobial Surveillance Program from 2006–2010 reported echinocandin resistance of 8.0 to 9.3% among 1,669 bloodstream infections of *C. glabrata* (39). Furthermore, in a 10-year study involving 293 unique episodes of *C. glabrata* bloodstream infections, echinocandin resistance of *C. glabrata* rose from 2 or 3% during 2001–2006 to >13% in 2009–2010 (194).

Fks Mechanism of Resistance

Clinical resistance resulting in breakthrough infections involves modification of Fks subunits of glucan synthase, and clinical breakpoints reflect this underlying mechanism (195). Unlike azole antifungal agents, echinocandins are not substrates for multidrug transporters (196, 197). Echinocandin resistance is conferred by characteristic amino acid substitutions in Fks subunits (198), which induce elevated MIC values (0.5 to 2 log) and reduce the sensitivity of glucan synthase (IC_{50}) to drug by 50- to 3,000-fold (199–201). The most prominent *FKS1* mutations in *C. albicans*, resulting in amino acid substitutions at positions Ser641 or Ser645, are effectively insensitive to drug and respond poorly to all three echinocandins in pharmacodynamic model studies (202–204). In *C. albicans* and most other *Candida* spp., mutations occur in two highly conserved "hot-spot" regions of *FKS1* and/or *FKS2* of *C. glabrata* only (Fig. 3). These limited regions encompass residues Phe641–Pro649 and Arg1361 (205). For *C. albicans*, amino acid changes at Ser645 are the most abundant and cause the most pronounced resistance phenotype (183, 198, 200). In *C. glabrata*, comparable mutations conferring resistance occur in



	Fks1		Fks2		Fks3
Organism	Hot Spot 1	Hot Spot 2	Hot Spot 1	Hot Spot 2	Hot Spot 1
C. albicans	F$_{641}$LTLSLRDP	DWIRRYTL	NO	NO	NO
C. krusei	F$_{655}$LILSIRDP	DWIRRYTL	NO	NO	NO
C. glabrata	F$_{625}$LILSIRDP	DWIRRYTL	F$_{659}$LILSLRDP	DWIRRYTL	NO
C. guilliermondii	F$_{632}$MALSIRDP	DWIRRYTL	NO	NO	NO
C. lipolytica	F$_{662}$LILSIRDP	DWIRRCVL	NO	NO	NO
C. tropicalis	F---LTLSIRDP	DWIRRYTL	NO	NO	NO
C. dubliniensis	F$_{641}$LTLSIRDP	DWIRRYTL	NO	NO	NO
*C. parapsilosis**	F$_{652}$LTLSIRDA	DWIRRYTL	NO	NO	NO
C. orthopsilosis	F---LTLSIRDA	DWVRRYTL	NO	NO	NO
C. metapsilosis	F---LTLSIRDA	DWIRRYTL	NO	NO	NO

NO: Not observed **C. parapsilosis sensu stricto* - - - Incomplete sequence designation

🟥 Acquired mutation Strong resistance

🟪 Acquired mutation Weak resistance

🟦 Naturally occurring polymorphism

FIGURE 3 Fks amino acid substitutions and polymorphisms in hot-spot regions conferring reduced susceptibility to echinocandin drugs among clinical isolates of *Candida* spp. doi:10.1128/9781555817381.ch130.f3

both *FKS1* and *FKS2*. S663P, -F, and -Y (equivalent to C. *albicans* S645) in Fks2 are the most prominent amino acid substitutions (>50%) (199). Other high-frequency resistance substitutions include S629P in Fks1 and F659V,Y in Fks2. Resistance-conferring mutations occur in *FKS2* of C. *glabrata* clinical isolates at twice the frequency of *FKS1* mutations (183, 198, 199).

The echinocandin resistance level conferred by hot-spot mutations in *FKS1* or *FKS2* may also depend on the relative expression of their genes, which can vary more than 20-fold (186, 199). *FKS2* expression is calcineurin dependent and downregulated by FK506 (tacrolimus) (206). Resistance conferred by FKS2 but not FKS1 was reversed following treatment with the calcineurin inhibitor FK506 (186).

Recently, a third hot-spot region was identified that confers *in vitro* resistance. It is defined by W695 of *S. cerevisiae* Fks1 and equivalent residues F695 and W760 from *Scedosporium* species and *Schizosaccharomyces pombe*, respectively (207) Hot-spot 3 is well conserved among both yeasts and moulds (207). Finally, amino acid substitutions in Fks1 of C. *albicans* are more likely to confer reduced fitness (186, 199), as they often result in a decrease in the catalytic reaction rate maximum for glucan biosynthesis (199, 200), resulting in altered cell wall morphology (208). The consequence of reduced fitness is that echinocandin-resistant strains compete poorly with their wild-type counterpart (208), which may help explain why resistance is associated with acquired *de novo* resistance and not transmission.

Hot-Spot Polymorphisms and Inherent Reduced Susceptibility

Candida parapsilosis complex (C. *parapsilosis sensu stricto*, C. *orthopsilosis*, and C. *metapsilosis*) and C. *guilliermondii* display higher echinocandin antifungal MIC values (MIC 0.5 to 8 μg/ml) relative to other highly susceptible *Candida* species (209, 210), and resistance breakpoints reflect this inherent reduced susceptibility (211). The clinical significance of this intrinsic reduced susceptibility is unclear since patients with these infecting strains are often successfully treated with echinocandin drugs at standard dosages (212, 213), although clinical efficacy may vary with patient population (30, 214). The mechanism underlying reduced echinocandin susceptibility appears to be naturally occurring polymorphisms in FKS hot-spot regions, which confer moderate reduced sensitivity of glucan synthase to drug (215). In C. *parapsilosis* complex, the highly conserved proline at the distal edge of hot-spot 1 (Pro 660) is present as an alanine (Fig. 3). Overall, it appears that naturally occurring Fks1 polymorphisms in hot-spot 1, hot-spot 2, and hot-spot 3 of non-*albicans Candida* spp. and other fungi account for reduced susceptibility to echinocandin drugs.

Biofilms

As with azole resistance, the glucan matrix acts to sequester echinocandin drugs, preventing them from reaching the cell membrane. Disruption of this process by genetic or chemical modification of μ-1,3-glucan synthase decreases drug sequestration in the matrix, rendering biofilms susceptible to antifungal agents (150). This mechanism accounts for a large fraction of the drug resistance phenotype during biofilm growth (149), and it is a factor in the observation of persister cells that may be encountered in the presence of drug. A role has been proposed for *SMI1* in biofilm matrix glucan production and development of the associated drug resistance phenotype. This pathway appears to act through transcription factor Rlm and glucan synthase Fks1p. Smi1 appears to function in conjunction with Rlm1 and Fks1 to produce drug-sequestering biofilm β-glucan (150).

Cellular Stress, Tolerance, and Resistance Emergence

Fungi possess adaptive mechanisms (e.g., Hsp90, cell wall integrity pathway, chitin biosynthesis) that help protect against cellular stresses such as those encountered following inhibition of glucan synthase by echinocandin drugs (216, 217). These stress adaptation responses may result in elevated *in vitro* MIC values to echinocandins, but they are not typically associated with clinical failures (218, 219). This is because glucan synthase remains sensitive to drug and treated cells are attenuated. Fungal cells acutely sense cell wall stress. Inhibition of glucan biosynthesis by the echinocandins induces stress-tolerance pathways including cell wall integrity, *PKC*, Ca^{2+}/calcineurin/Crz1, and *HOG* (220, 221) (Fig. 4). Furthermore, modulation of sphingolipid biosynthesis can lead to a mixed phenotype in which strains are resistant to caspofungin and hypersensitive to micafungin (222). Cowen and colleagues have shown that Hsp90 helps orchestrate tolerance to echinocandin drugs through its principal client protein calcineurin and its downstream effector Crz1 (157, 159, 223). Pharmacological or genetic impairment of Hsp90 function reduces tolerance and resistance of laboratory and clinical isolates of C. *albicans* and C. *glabrata* to caspofungin (223, 224). Echinocandin action also results in compensatory increases in chitin synthesis, which are induced by cell wall stress/adaptation and play an increasing role in predisposing cells for high-level resistance. Cell wall mutants with higher basal chitin contents are less susceptible to caspofungin (220, 221, 225) and can confer stable albeit low-level resistance *in vivo* (226). Paradoxical growth at very high drug levels (227) is also linked to compensatory responses in chitin biosynthesis (228).

These adaptive responses are important in stabilizing cells in the presence of drug. Ultimately, they likely predispose cells for resistance even though they are not sufficient to induce therapeutic failure themselves. The consequence of drug tolerance is a physiological state that allows cells to break through drug action by forming stable FKS mutations.

MECHANISMS OF ALLYLAMINE RESISTANCE

Dermatophytosis is a common infection of the keratinized tissues (skin, hair, and nails) caused by dermatophytes. Among the three known genera of dermatophytes—*Epidermophyton*, *Microsporum*, and *Trichophyton*—*Trichophyton* species, especially *T. mentagrophytes*, *T. tonsurans*, and *T. rubrum*, are the most common pathogens, with *T. rubrum* being the most frequently isolated organism. Treatment often involves the fungicidal allylamine terbinafine, which is the most frequently prescribed oral antifungal agent in the U.S. and Canada for onychomycosis. Antifungal resistance in dermatophytes is rare, despite the high worldwide incidence of dermatophytosis and required long-term therapy for some infections. Nevertheless, resistance is associated with mutations in yeast *ERG1*, which encodes the squalene epoxidase target (229, 230). Mutations in the equivalent ErgA of *Aspergillus* spp. confer resistance *in vitro* (231), but this has not been observed in clinical isolates.

MECHANISMS OF FLUCYTOSINE RESISTANCE

Flucytosine (5-fluorocytosine or 5FC) is a prodrug that, when taken up by cells, is metabolized by organisms expressing cyto-

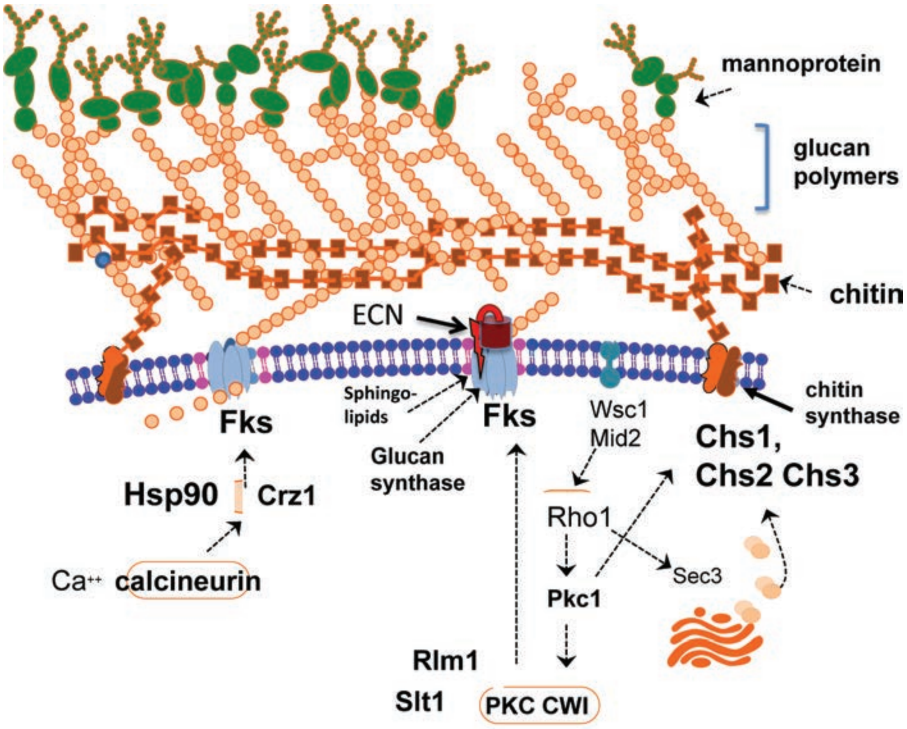


FIGURE 4 Schematic overview of major cellular processes induced in response to inhibition of glucan synthase (Fks) by echinocandin drugs resulting in cell wall stress and drug tolerance. doi:10.1128/9781555817381.ch130.f4

sine deaminase to a toxic form, 5-fluorouracil, which disrupts DNA and protein synthesis. 5FC is generally administered in combination with other antifungal agents (e.g., amphotericin or fluconazole) because of a high propensity for fungal cells to develop resistance, since it targets a nonessential salvage pathway. In yeasts, decreased activity of either cytosine deaminase or uracil phosphoribosyltransferase uracil (UPRTase) plays a major role. In *C. albicans*, nucleotide polymorphism in the *FUR1* gene (uracil pyrophosphorylase) is responsible for resistance to 5FC and plays a major role in clinical resistance (232, 233). In *C. lusitaniae*, inactivation of *FCY2* (cytosine permease), *FCY1* (cytosine deaminase), and *FUR1* genes yielded differential resistance to 5FC (234). In *C. glabrata*, high-level 5FC resistance is conferred by a wide array of mutations conferring null phenotypes for *FCY1* or *FUR1*, while low-level resistance is conferred by mutations in one or more *FCY2* permeases (235).

SUMMARY AND PERSPECTIVE

As the global burden of fungal infections rises, antifungal therapy will continue to be an important element of patient management. Yet, treatment choices remain restricted due to the limited classes of antifungal agents and the emergence of antifungal resistance. The mechanisms contributing to resistance are well characterized. They include reducing the drug-target interaction by either modifying the target (drug affinity changes or target abundance) or reducing the effective cellular content of drug (ABC or MFS drug pumps, biofilm glucan trap). *C. albicans* shows a full complement of resistance mechanisms, yet not all mechanisms are present in all fungal strains despite the genetic potential. For example, the expression of CDR pump genes is more important for azole resistance in *C. glabrata* than is the development of target site mutations. In contrast, echinocandin resistance in all *Candida* strains is dependent on development of target site mutations, while drug pumps play no role. Azole resistance in *Aspergillus* is mostly influenced by target site mutations, while in some strains drug pumps and unknown mechanisms may play a significant role.

Enormous progress has been made in understanding the importance of biofilms, as they convert normally susceptible planktonic cells into highly resistant cell communities and create persister cells that seed resistance. Similarly, a great deal has been learned about the genetic regulatory elements that influence overexpression of *ERG11 and FKS*, as well as prominent ABC and MFS transporters. In some strains, point mutations in transcription factors are sufficient to upregulate expression, while in others, large-scale changes in chromosomes alter the transcriptional profiles. Fungal cells are highly dynamic and adapt to environmental challenges, including antifungal agents. Compensatory mechanisms, such as enhanced chitin biosynthesis following inhibition of glucan synthase by echinocandin action, help ensure cell wall integrity. Furthermore, a wide range of cellular stress response pathways contribute to cell stability. Critical cellular factors such as Hsp90 stabilize enzymes during stress, which promotes cell survival. All of the components contribute to the development of persister cells that can transiently adapt to drug exposure with the potential to break through therapy by induction of more permanent resistance mechanisms.

A detailed understanding of the principal resistance mechanisms and the factors that contribute to their evolution is important for developing new diagnostic approaches to more easily identify drug resistance and create new strategies for therapeutic intervention that prevent and overcome resistance. Advances in molecular diagnostic tools now make it highly feasible to rapidly determine genus and species while simultaneously assessing mutations conferring drug resistance. Furthermore, by interfering with adaptive responses, it may be possible to develop combination drug regimens that both overcome and prevent resistance.

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Susceptibility Test Methods: Yeasts and Filamentous Fungi*

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ANTIFUNGAL SUSCEPTIBILITY TESTING

Rationale

The groups of antifungal agents licensed for the systemic treatment of invasive fungal infection include the polyene amphotericin B and its three lipid formulations; the azoles fluconazole, itraconazole, ketoconazole, posaconazole, and voriconazole; the echinocandins anidulafungin, caspofungin, and micafungin; the pyrimidine flucytosine; and the allylamine terbinafine (see chapter 129). With their increasing use has come the recognition of innate resistance to one or more agents in some isolates of yeast and mould (1–9) and the emergence of resistance during therapy (10–17). Moreover, a recent worrying development has been the emergence of strains of *Aspergillus fumigatus* with cross-resistance to therapeutic triazole drugs due to environmental exposure to agricultural azoles (18–23). As a result, clinical laboratories are now asked to assume a greater role in the selection and monitoring of antifungal chemotherapy. Thus, accurate and predictive antifungal susceptibility testing has become imperative and is now so widely accepted as a useful tool for informing decision making during the management of patients with invasive fungal infections that recommendations for testing are starting to appear in management guidelines (24, 25).

In order to be clinically useful, the requirements of *in vitro* susceptibility tests are that they should (i) provide a reproducible and reliable indication of the activities of antifungal agents, (ii) provide results that correlate with *in vivo* activity and therefore help to predict the likely outcome of therapy, (iii) provide a means with which to detect the development of resistance during therapy by applying clinical breakpoints, (iv) act as a surveillance tool for monitoring the development of resistance mechanisms among a normally susceptible wild-type population of organisms by adopting species-specific epidemiological cutoff values (ECV/ECOFF), and (v) have value as a screening tool to predict the therapeutic potential of newly discovered investigational agents. In order to provide this information, there has to be a careful analysis of the pharmacokinetic and pharmacodynamic interactions of the drug, as well as host-and-organism interactions.

The basic methodology of reference antifungal susceptibility testing has remained stable since the introduction in the early 1990s of the first standardized broth dilution method (National Committee for Clinical Laboratory Standards [NCCLS] M27-P) (26), which has since been modified to include a microdilution method conducted in a microtiter plate (CLSI M27-A3) (27). The accepted reference methods published by the Committee for Clinical and Laboratory Standards (CLSI) Subcommittee on Antifungal Susceptibility Testing and the Subcommittee on Antifungal Susceptibility Testing (AFST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) European Committee for Antimicrobial Susceptibility Testing (EUCAST) are now well established (27–32). The CLSI Subcommittee has developed reference methods for broth macro- and microdilution susceptibility testing of yeasts (CLSI M27-A3 document) and moulds (CLSI M38-A2 document), as well as disk diffusion methods for yeasts (CLSI M44-A2 document) and moulds (CLSI M51-A document) (27, 28, 31, 32), while EUCAST has developed broth microdilution methods for fermentative yeast (EDef 7.2 document) and for conidia-forming moulds (EDef 9.1 document) (29, 30). The main differences between the EUCAST and CLSI reference methods were the EUCAST recommendation of a higher glucose and inoculum concentration, and for yeast, a shorter incubation time and a spectrophotometer reading rather than 2 days of incubation and visual reading as recommended by CLSI. However, as experience with these methods has become more widespread and their impact better understood, there have been changes. Thus, recent evaluation of the CLSI M27 method (27) for yeast susceptibility testing suggests that, in agreement with the EUCAST reference method (30) for most yeast species, results with fluconazole, amphotericin B, and the echinocandins can be evaluated after 24 h of incubation, thus reducing the total time to result from 72 to 48 h (27, 33–35). Studies have also suggested that, in line with EUCAST methodology, posaconazole and voriconazole MICs for *Candida* spp. can also be read at 24 h (36).

The most significant recent development has been the introduction of species-specific breakpoints for the systemically active antifungal agents, together with revision of the CLSI breakpoints based on the 24-h reading that has brought them in line with those proposed by EUCAST

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*This chapter contains information presented by Elizabeth M. Johnson, Ana V. Espinel-Ingroff, and Michael A. Pfaller in chapter 128 of the 10th edition of this *Manual*.

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TABLE 1 Clinical breakpoints for *Candida* spp. according to CLSI (previous and revised breakpoints) and EUCAST[a]

Drug	CLSI			EUCAST Species-specific breakpoints (mg/liter)	
	M27-S3 Non-species-specific breakpoints (mg/liter)	M27-S4 Species-specific breakpoints (mg/liter)			
Amphotericin				≤1	
Anidulafungin	≤2	≤0.25; >0.5	(*C. albicans*, *C. krusei*, *C. tropicalis*)	≤0.03; >0.03	(*C. albicans*)
		≤0.125; >0.25	(*C. glabrata*)	≤0.06; >0.06	(*C. glabrata*, *C. krusei*, *C. tropicalis*)
		≤2; >4	(*C. parapsilosis*)	≤0.002; 4	(*C. parapsilosis*)[b]
Caspofungin	≤2			–[c]	
Micafungin	≤2	≤0.25; >0.5	(*C. albicans*, *C. krusei*, *C. tropicalis*)	≤0.016; >0.03	(*C. albicans*)
				≤0.03; >0.03	(*C. glabrata*)
		≤0.06; >0.125	(*C. glabrata*)	≤0.002; 2	(*C. parapsilosis*)[b]
		≤2; >4	(*C. parapsilosis*)		
Fluconazole	≤8; >32	≤2; >4	(*C. albicans*, *C. parapsilosis*, *C. tropicalis*)	≤2; >4	(*C. albicans*, *C. parapsilosis*, *C. tropicalis*)
		SDD: ≤32; >32	(*C. glabrata*)[d]	≤0.02; 32	(*C. glabrata*)[b]
Itraconazole	≤0.125; >0.5			–	
Posaconazole	–	–		≤0.06; >0.06	(*C. albicans*, *C. parapsilosis*, *C. tropicalis*)
Voriconazole	≤1; >2	≤0.125; >0.5	(*C. albicans*, *C. parapsilosis*, *C. tropicalis*)	≤0.125; >0.125	(*C. albicans*, *C. parapsilosis*, *C. tropicalis*)
		≤0.5; >1	(*C. krusei*)		

[a] Breakpoints are presented as S ≤ X, R > Y (except for the revised fluconazole breakpoint for *C. glabrata*). The "I" category (if present) is readily interpreted as the values between the S and the R breakpoints. Data from references 37–40 and 100.

[b] The entire wild-type population of *C. parapsilosis* is classified by EUCAST as intermediate "I" to anidulafungin and micafungin, and the wild-type population of *C. glabrata* is likewise classified as "I" to fluconazole. This is in order to accommodate use of these compounds in some clinical situations.

[c] EUCAST breakpoints for caspofungin have not been established due to an unacceptable variation of MIC ranges obtained using different lots of pure substance and across laboratories and time. However, as there is substantial evidence suggesting cross-resistance between anidulafungin and caspofungin, EUCAST recommends that isolates categorized as anidulafungin-susceptible can be regarded as susceptible to caspofungin until drug-specific EUCAST breakpoints are available for caspofungin.

[d] The wild-type population of *C. glabrata* is classified by CLSI as susceptible dose dependent (SDD) to fluconazole in order to accommodate use of fluconazole at elevated doses in some clinical situations.

and published in document CLSI M27-S4 (35, 37–40) and Table 1.

Basic Test Principles

Minimum Inhibitory Concentration

In its simplest form, susceptibility testing is a measure of the potency of an agent to inhibit the growth *in vitro* of an organism, and the MIC is the lowest concentration of the agent able to inhibit growth to a predetermined degree. Growth inhibition may be determined in a broth format, usually in microtiter plates, or on solid agar by either incorporation of the agent into the agar base or impregnation into a disk or strip applied to the surface of a previously inoculated plate. Solid agar incorporation is particularly useful in the high-throughput testing required for surveillance screening of environmental isolates. Commercial plates with four wells containing three different antifungal agents—itraconazole (4 mg/liter), posaconazole (0.5 mg/liter), and voriconazole (1 mg/liter)—and a control well have been designed for this purpose (made to order by Balis Laboratorium VOF, Boven Leeuwen, the Netherlands) (41) (Fig. 1). However, *in vitro* susceptibility testing is heavily influenced by a number of technical variables, including inoculum size and preparation, medium formulation and pH, duration and temperature of incubation, and the criterion used for MIC endpoint determination, all of which have to be considered when developing and interpreting test methods (42–46). In addition, antifungal susceptibility testing is complicated by problems unique to fungi, such as slow growth rates (relative to bacteria) and the ability of certain fungi (dimorphic) to grow either as a unicellular yeast form that produces blastoconidia or as a hyphal or filamentous fungal form that may have the ability to produce conidia or sporangiospores. Finally, the basic properties of the antifungal agents themselves, such as solubility, chemical stability, modes of action, and the tendency to produce partial inhibition of growth over a wide range of concentrations above the MIC, must be taken into account.

Minimum Fungicidal/Lethal Concentration

Many of the patients contracting invasive fungal infections are immunocompromised and thus unable to mount a significant immune response. Therefore, it has been postulated that antifungal agents that demonstrate fungicidal activity at concentrations that can be achieved *in vivo* may provide better outcomes than those agents that are fungistatic and thus rely on some host phagocytic cell activity to remove the remaining viable pathogens. It has thus been reported that the clinical outcome is significantly better for patients with invasive candidiasis due to *Candida albicans* and treated with an echinocandin (fungicidal) compared to those receiving fluconazole (fungistatic) despite the fact that *C. albicans* is susceptible to both (47, 48). Standard testing parameters are not yet available for evaluation of the fungicidal activity of antifungal agents. The determination of minimum fungicidal concentrations (MFCs) requires the subculturing onto an agar medium of fixed volumes from each MIC tube or well that shows complete inhibition of growth. The criteria for MFC determination vary in different publications, and the MFC has been described as the lowest drug concentration resulting in either no growth or three to five

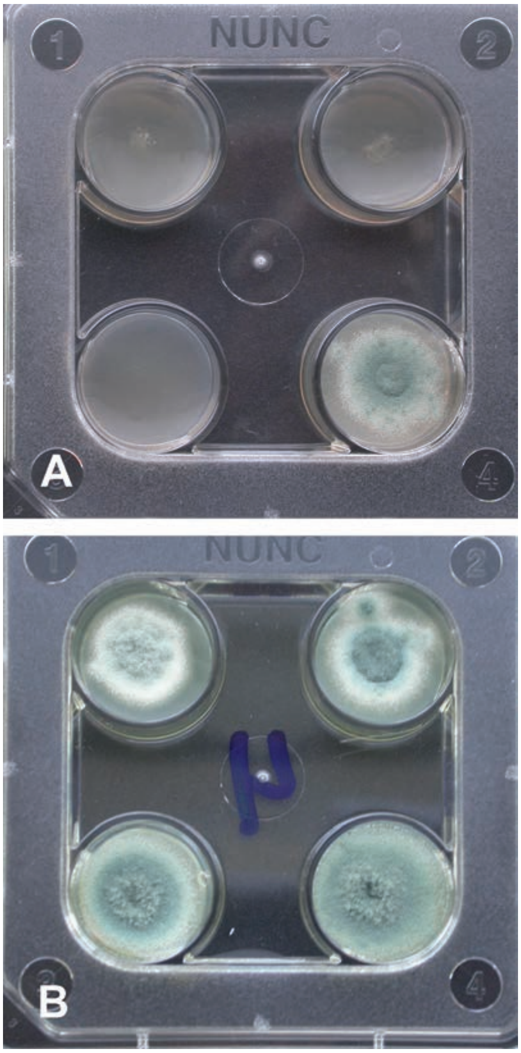

FIGURE 1 Azole agar screening plate. The four wells contain itraconazole, 4 mg/liter (ITR) (top left well); voriconazole, 1 mg/liter (VRC) (top right well); posaconazole, 0.5 mg/liter (PSC) (bottom left well); or no antifungal compound (control) (bottom left well). The upper agar multidish (A) is inoculated with a wild-type azole-susceptible *A. fumigatus* isolate, whereas the lower agar multidish (B) is inoculated with a multiazole-resistant *A. fumigatus* isolate. (Plates have been incubated 2 days.) doi:10.1128/9781555817381.ch131.f1

colonies (49–51). The clinical relevance and the development of standard guidelines for MFC determination still need to be addressed (52).

Clinical Resistance and Clinical Breakpoint Setting

Wild-Type Distribution

Within any group of organisms tested by a defined method with any given drug there will usually be a distribution of MIC results that may be modal, bimodal, or occasionally appearing to be quite random. Often, further taxonomic studies of those species complexes that appear to have a random MIC distribution will further delineate taxonomic groupings within the complex that each display separate susceptibility profiles, such as *Scedosporium aurantiacum* within the *S. apiospermum* complex (53) and *Aspergillus lentulus* within the *A. fumigatus* complex (3). Within most fungal species, the MICs of the majority of organisms are concentrated within a small number of doubling dilutions, and rules have been defined to predict less susceptible outliers. Susceptibility characteristics of a population can be described by defining the range of MIC values encountered and calculating the value at which 50% (MIC_{50}) and that at which 90% (MIC_{90}) of the population is inhibited. However, when constructing wild-type distribution curves, one must always be aware of the inherent variability of even well-standardized and controlled testing. Arendrup and colleagues (54) tested a single isolate of *Candida glabrata* with a fluconazole MIC of 2.0 mg/liter, as assessed by the EUCAST susceptibility testing method, a total of 45 times. They found MIC results spanning three 2-fold dilutions ranging from 2.0 to 8.0 mg/liter, almost directly mirroring those results produced when 35 different *C. glabrata* isolates were tested. It was postulated that variation within the wild-type population could be explained solely by test variation allowing for a doubling dilution on each occasion. Therefore, in a normal distribution the MIC_{50} reflects the susceptibility of the entire wild-type population and is a useful predictor of species susceptibility.

Microbiological Resistance and Epidemiological Cutoff Values

In the absence of interpretable clinical outcome data to predict clinical breakpoints, the wild-type distributions can be used to define ECOFF/ECV. This provides an indication of the normal susceptibility patterns encountered with a given drug-organism combination and thus confirms whether a given isolate conforms to the predicted wild-type susceptibility profile or is displaying a less susceptible or non-wild-type phenotype. An MIC above the ECOFF/ECV cannot be explained by inherent test variation. Such outlier organisms can most often be found to harbor resistance mechanisms (5, 8). Clinical breakpoints should therefore not be set higher than ECOFF/ECV unless there is supporting clinical evidence to demonstrate that such isolates do respond to standard therapy (55). In some cases clinical breakpoints can reflect the need to achieve higher than normal blood concentrations for a given drug in order to achieve good outcomes. CLSI has used the term "susceptible dose dependent" (SDD) for such isolates. Such categorization has been used with itraconazole and fluconazole where there is clear clinical evidence that higher doses, and therefore higher blood concentrations, can influence outcome in isolates with MICs above the usual susceptible range (56). When this supporting clinical evidence is lacking, isolates with MIC results only just in excess of the normal wild-type MIC_{50} may be described as having intermediate susceptibility, which really means that the data currently available do not allow the organism to be categorized as either fully susceptible or resistant. EUCAST has a slightly different terminology and has not adopted the SDD category. In line with the antibacterial classification of susceptibility (S), I and R are used. The "I" category is assigned for isolates/species that may respond to treatment under certain conditions (e.g., high drug concentration at the target site) in order to accommodate use of the antifungal compound in some clinical situations. Another important point to consider when setting breakpoints is that they should not divide wild-type distributions of important target species (54); species-specific breakpoints have been introduced to prevent this occurrence while not incorrectly classifying other groups. Table 1 shows a comparison of the species-specific breakpoints for yeast isolates accepted by the CLSI and EUCAST.

Clinical Breakpoints and Determinants Associated with Outcome

The following parameters are considered when setting breakpoints: (i) standard dosing, (ii) species-specific MIC distributions, (iii) ECOFFs/ECVs, (iv) pharmacokinetic-pharmacodynamic analyses, and (v) clinical experience and MIC-outcome relationships. In the ideal situation, all of these data would be available; however, more often, breakpoints are selected upon less perfect datasets usually comprising MIC distributions, some pharmacokinetic and pharmacodynamic information, and clinical experience from infections involving wild-type isolates. Breakpoint setting therefore is not an exact science, and classification of isolates into S, I, and R based on clinical breakpoints does not *per se* predict outcome because other factors, including severity of the infection and underlying disease, timing of therapy, and virulence of the organisms all influence the outcome. Susceptibility classification, however, predicts the likelihood of a successful outcome. It has been suggested that for antifungal therapy the "90-60 rule" should be applied, which suggests that infections due to susceptible organisms will respond 90% of the time while those due to resistant organisms will respond in 60% of cases (57). The lack of adjunctive host response in the neutropenic patient may have an additional impact on the likely outcome, and it is well recognized that removal of intravenous catheters also influences outcome irrespective of choice of antifungal therapy (47, 58, 59). For systemic therapy, dose, dosing interval, route of administration, and effect of coadministration of other drugs will have a direct impact on achievable blood levels, and it is usually considered that blood levels should be several times in excess of the MIC for successful outcome. For example, a dose/MIC ratio of 25 (using the higher MICs obtained with 48-h CLSI reading) or of 100 (using the lower MICs obtained with EUCAST and CLSI with 24-h reading) was found to correlate well with successful outcome of treatment with fluconazole and was supportive of the suggested breakpoint (38, 60). It is becoming increasingly recognized that monitoring of blood levels for some drugs is therefore an important part of the management of patients treated particularly with the triazole agents itraconazole, posaconazole, and voriconazole (61–63).

When agents are used topically, such as in the treatment of cutaneous and mucosal forms of candidiasis, otomycosis, and mycotic keratitis, different, higher breakpoints may be more applicable as local concentrations of the agent may be manyfold in excess of the MIC of the infecting organism. An example of this is in the topical treatment of mycotic keratitis, where topical agents such as 0.15% (1,500 mg/liter) amphotericin B, 1% (10,000 mg/liter) natamycin, 1% (10,000 mg/liter) voriconazole, and 2% (20,000 mg/liter) econazole would greatly exceed the *in vitro* susceptibility cutoffs for most organisms. However, although local concentrations may be high, there are other complexities and consideration has to be given to issues such as tissue penetration and duration of contact so, to date, breakpoint setting for topical therapy has not been formally addressed.

STANDARDIZED BROTH DILUTION METHODS FOR YEASTS

Currently, there are two internationally recognized broth dilution methods for testing yeasts such as *Candida* spp. Broth microdilution testing (Table 2) has become the most widely used reference technique for antifungal susceptibility testing; this approach is described in the M27-A3 document (27) and the EUCAST EDef 7.2 document (29) and is also outlined below. Although the antifungal broth macrodilution test was the first method proposed by the CLSI Subcommittee, this test is cumbersome for use in the clinical laboratory. The microdilution test provides consistent MIC results, and interlaboratory agreement of the microdilution MICs can be higher than that of the macrodilution MICs for some drugs (64).

Standard Medium

The test medium recommended for both established reference methods is the Roswell Park Memorial Institute (RPMI) 1640 broth medium with L-glutamine and a pH indicator and without sodium bicarbonate (04-525Y from BioWhittaker, Walkersville, MD, and American Biorganics, Inc., Niagara Falls, NY; and R-6504 from Sigma Chemical Co., St. Louis, MO). The medium should be buffered to a pH of 7.0 at 25°C with morpholinepropanesulfonic acid (MOPS; final molarity at pH 7.0, 0.165). This medium is suitable for testing most fungi (27, 28, 64, 65), but it may not be adequate to support the growth of some strains of *Cryptococcus neoformans* or to determine amphotericin B MICs (66). RPMI medium containing a higher concentration of 2% dextrose (67, 68) is the basal medium used in EUCAST testing as it allows faster growth of the yeast, thus facilitating the determination of MICs at 24 h (29). This modification to include glucose at a final concentration of 20 g/liter is also included in the CLSI M27-S4 as a suggestion to simplify endpoint determination (37).

Drug Stock Solutions

Antifungal powders can be obtained directly from the drug manufacturers or from reputable commercial sources. Clinical intravenous or oral preparations should not be used. Antifungal stock solutions should be prepared at concentrations at least 10 times the highest concentration to be tested (e.g., 1,280 µg/ml for fluconazole and flucytosine). Solutions of standard powders of hydrophilic substances are prepared in distilled water. For testing non-water-soluble agents, sufficient drug standard should be weighed to prepare a solution of 1,600 µg/ml. EUCAST and CLSI methodology specifies the use of dimethyl sulfoxide (DMSO) for all non-water-soluble agents. Following a study of MICs obtained with caspofungin and micafungin dissolved in water or DMSO that suggested that the latter solvent yielded lower MICs and tighter ranges for a panel of known echinocandin-resistant or -susceptible isolates, it is recommended that all the echinocandins should be dissolved in DMSO (69). The use of DMSO has also been confirmed to allow storage of prepared plates for up to 6 months without loss of echinocandin potency (70). The actual amount to be weighed must be adjusted according to the specific biological activity of each standard. Amphotericin B solutions must be protected from light, and drug stock solutions prepared with solvents should be allowed to stand for 30 min before use.

The sterile stock solutions may be stored in small volumes in sealed, sterile polypropylene or polyethylene vials, ideally at −70°C or below and certainly no higher than −20°C; caspofungin can be stored at −80°C for 3 to 6 months without significant loss of activity. Vials should be removed as needed and used on the same day. The use of quality

TABLE 2 CLSI M27-A3 document and EUCAST EDef 7.2 broth microdilution guidelines for antifungal susceptibility testing of yeasts[a]

Parameter	CLSI M27-A3 microdilution modification	EUCAST EDef 7.2
Test organism	Yeast	Yeast
Broth medium	RPMI 1640 broth buffered with MOPS buffer (0.165 M) and 0.2% dextrose to pH 7.0 at 25°C	RPMI 1640 broth buffered with MOPS buffer (0.165 M) and 2.0% dextrose to pH 7.0 at 25°C
Microdilution plates	Sterile plastic, disposable 96-well plates with 300-µl-capacity round-bottomed wells	Sterile plastic, disposable 96-well plates with 300-µl-capacity flat-bottomed wells
Medium modifications	(i) Yeast nitrogen base broth (pH 7.0) with MOPS provides better growth for *C. neoformans*, and (ii) RPMI 1640 with 2% dextrose	*Cryptococcus* spp. tested as for other yeast and read if optical density value above 0.2. If not, repeat test but incubate at 30°C.
Drug dilutions	Additive 2× 2-fold drug dilutions with medium (fluconazole and flucytosine [5FC]), or 100× with solvent (amphotericin B, other azoles, anidulafungin, caspofungin, and micafungin)	Prepared according to ISO recommendations
Drug dilution ranges:		
5FC and fluconazole	0.12–64 µg/ml	0.12–64 µg/ml
Other azoles	0.03–16 µg/ml	0.015–8 µg/ml
Amphotericin B and echinocandins	0.03–16 µg/ml	0.03–16 µg/ml
Storage of prepared plates	Sealed in plastic bags and stored at −70°C or below for up to 6 mo	Sealed in plastic bags or aluminum foil and stored at −70°C or below for up to 6 mo or −20°C for not more than 1 mo
Inoculum preparation	Five colonies from 24-h (*Candida* spp.) or 48-h (*C. neoformans*) cultures on Sabouraud dextrose agar or potato dextrose agar	Five colonies (>1 mm) from 18- to 24-h cultures suspended in 5 ml sterile distilled water
Stock inoculum suspension	Adjusted by spectrophotometer at 530 nm by addition of sterile distilled water to match the turbidity of a 0.5 McFarland standard (1 to 5 × 10^6 CFU/ml)	Adjusted by spectrophotometer at 530 nm by addition of sterile distilled water to match the turbidity of a 0.5 McFarland standard (1 to 5 × 10^6 CFU/ml)
Test inoculum	Mix stock inoculum on a vortex for 15 s then 1:1,000 dilution (1:20 followed by 1:50 dilution) with medium of the stock inoculum suspension.	1:10 dilution in sterile distilled water of the stock inoculum suspension
Plate inoculation	100 µl of diluted test inoculum plus 100 µl of 2× drug concn; final concn, 0.5 × 10^3 to 2.5 × 10^3 CFU/ml	100 µl of diluted test inoculum plus 100 µl of 2× drug concn; final concn, 0.5–2.5 × 10^5 CFU/ml
Growth control(s)	100 µl of diluted inoculum plus 100 µl of drug-free medium (or plus 2% of solvent)	100 µl of diluted inoculum plus 100 µl of drug-free medium (or plus 2% of solvent)
Sterility control	Column 12 of the plate can be used to perform the sterility control (drug-free medium only, no inoculum).	Column 12 of the plate can be used to perform the sterility control (drug-free medium only, no inoculum + 100 µl of sterile distilled water used to make inocula).
QC strains	Select QC strains that have MICs that fall near the midrange for all drugs tested. Rules have been established for QC testing based on frequency of testing.	At least two QC strains with results close to the middle range
Incubation	Incubate at 35°C	Without agitation in ambient air at 35 ± 2°C
Time of reading	Amphotericin B, 24 or 48 h; fluconazole, 24 or 48 h; echinocandins, 24 h only; 5FC and other azoles, 48 h	24 ± 2 h all drugs, 48 h for *Cryptococcus* spp. If plates show absorbance ≤0.2 indicating poor growth, reincubate for further 12–24 h. Failure to reach absorbance of 0.2 after this time is a failed test. For *Cryptococcus*, repeat test at 30°C.
MIC determination	Visual assessment with the aid of a reading mirror	Spectrophotometric assessment at 530 nm (or alternatively 405 or 450 nm). The value of the inoculum-free control should be subtracted from the readings of the other wells.
Amphotericin B	Lowest drug concn that prevents any discernible growth (100% inhibition)	≥90% inhibition as compared to drug-free control
5FC, azoles, caspofungin, and other echinocandins	Lowest drug concn that shows prominent (~50%) decrease in turbidity	≥50% inhibition as compared to drug-free control
Reading modification[b]	*C. neoformans* MICs determined spectrophotometrically at 492 nm after 48 h of incubation	

[a] Data from references 28 and 30.

[b] M27-A3 document includes this reading modification when using the broth microdilution method with yeast nitrogen base broth and a 10^4 CFU/ml inoculum.

control (QC) strains such as those listed in Table 3 is mandatory in evaluating drug activity (27, 29).

Preparation of Inocula

Inocula should be prepared by the spectrophotometric method (71) as outlined in Table 2. The inoculum suspension is prepared by picking five colonies, each at least 1 mm in diameter, ideally from 24-h-old cultures of *Candida* spp. or 48-h-old cultures of *C. neoformans*. These are then suspended in 5 ml of sterile 0.85% NaCl or sterile distilled water for the EUCAST method and vigorously shaken on a vortex mixer. The turbidity of the cell suspension measured at 530 nm is adjusted with the appropriate suspension medium to match the transmittance produced by a 0.5 McFarland barium sulfate standard. This procedure produces a cell suspension containing 1×10^6 to 5×10^6 CFU/ml. For the CLSI method, this is then diluted 1:1,000 with RPMI medium to provide the 2× test inoculum (1×10^3 to 5×10^3 CFU/ml). The 2× inoculum is diluted 1:1 when the wells are inoculated to achieve the desired final inoculum size (0.5×10^3 to 2.5×10^3 CFU/ml). For the EUCAST method, which utilizes a higher final inoculum, a working suspension is prepared from a 1:10 dilution of the standardized suspension in sterile distilled water to produce 1×10^5 to 5×10^5 CFU/ml.

Drug Dilutions and Performance of Microdilution Test for Yeasts

For hydrophobic drugs dissolved in solvents other than water (e.g., the polyenes, itraconazole, posaconazole, voriconazole, anidulafungin, caspofungin, and micafungin), intermediate test drug dilutions are prepared from stock solutions to be 100 times the strength of the final drug concentration, with 100% DMSO used as a diluent (e.g., 1,600 to 3 µg/ml for amphotericin B, echinocandins, and all azoles except fluconazole). Dilutions should be prepared according to ISO recommendations (72). This procedure prevents precipitation of agents with low solubility in aqueous media. Alternative dilution schemes may be used if they are shown to perform as well as the reference method (73). Despite careful procedures, itraconazole and some other agents do not remain completely solubilized upon dilution into aqueous media, which makes the use of QC procedures vital to minimize inaccuracies (74). For water-soluble drugs, such as flucytosine and some formulations of fluconazole, dilutions are prepared from the stock to be 10 times the final test drug concentrations directly in RPMI medium according to the additive, 2-fold drug dilution schema (27) (e.g., 640 to 1.2 µg/ml for fluconazole and flucytosine). The 10× and 100× drug concentrations should be diluted 1:5 and 1:50, respectively, with RPMI to achieve the 2× drug concentrations needed for the microdilution test; after the inoculation step the drug concentrations are 16 to 0.03 µg/ml for amphotericin B and triazoles and 64 to 0.12 µg/ml for fluconazole and flucytosine. As the echinocandins are potent at lower concentrations and to encompass the EUCAST breakpoints, drug dilution series from 8 to 0.015 µg/ml or lower are recommended for these compounds.

The broth microdilution test is performed by using sterile, disposable, multiwell microdilution plates (96 U-shaped wells or flat-bottom plates for EUCAST) (e.g., Dynatech Laboratories, Inc., Alexandria, VA). A multichannel pipette (or a large dispensing instrument for 96-well trays) is used to dispense the 2× drug concentrations in 100-µl volumes into the wells of columns 1 to 10 of the microdilution plates. Column 1 contains the highest drug concentration and column 10 contains the lowest drug concentration. Microdilution trays can be sealed in plastic bags and stored frozen at −70°C (or −80°C) for up to 6 months or at −20°C for not more than 1 month (27, 29, 70). Each well is inoculated on the day of the test with 100 µl of the corresponding 2× inoculum, which brings the drug dilutions and inoculum densities to the final test concentrations (final volume in each well, 200 µl). The growth control wells (column 11) contain 100 µl of sterile drug-free medium (for water-soluble agents) or 100 µl of sterile drug-free medium with 2% solvent (for non-water-soluble agents) and are inoculated with 100 µl of the corresponding 2× inoculum. The QC yeasts are tested in the same manner as the other isolates and are included each time an isolate is tested. Row 12 of the microdilution plate can be used for the sterility control (drug-free medium only).

Incubation and Determination of Microdilution MICs for Yeasts

The microdilution plates are incubated at 35°C in ambient air. For the EUCAST method, 24 h of incubation is recommended. For the CLSI method and in most instances when testing *Candida* spp., results with fluconazole, posaconazole, voriconazole, amphotericin B, and the echinocandins can also be evaluated after 24 h (27, 33–36). The breakpoints suggested for fluconazole, voriconazole, and the echinocandins are based on a 24-h reading (37).

The determination of MIC endpoints is a critical step in antifungal susceptibility testing, especially with the azoles (for yeasts) and echinocandins (for yeasts and moulds). For the CLSI methodology, the endpoint reading is undertaken by eye, whereas the flat-bottom plates used for EUCAST testing allow a nonsubjective spectrophotometric reading and cutoff defined by a fixed percentage of the optical density achieved in the corresponding control well. The recommended absorbance for reading the plates is 530 nm, although others can be used (e.g., 405 or 450 nm), and the value of the blank background should be deducted from the readings for the other wells. For the CLSI methodology, the growth in each well is compared with that in the growth control (drug-free) well with the aid of a reading mirror (e.g., Cooke Engineering Co., Alexandria, VA). The MIC for amphotericin B is defined as the lowest concentration at which complete absence of growth (optically clear) is observed, i.e., 100% inhibition or ≥90% inhibition if read with a spectrophotometer for the EUCAST method.

The partial inhibition or trailing that is observed with fungistatic compounds such as the azoles prevents adoption of a complete absence-of-growth endpoint. Moreover, the highest degree of reproducibility is obtained if the steepest part of the growth inhibition curve is taken as the endpoint. Therefore, the MIC of the azoles, echinocandins, and flucytosine is defined as the lowest concentration at which prominent growth inhibition is observed (≥50% inhibition as compared to the growth control well) for both standards. For visual interpretation, agitation of the microdilution trays is highly recommended prior to MIC determination; this step facilitates the visual estimate of prominent growth inhibition (74).

Heavily trailing endpoints are seen with about 5% of isolates when reading fluconazole MICs at 48 h, but studies of the *in vivo* response of such isolates in animal models of infection and in patients with oropharyngeal candidiasis suggest that they respond in the same way to low-dose fluconazole therapy as fully susceptible strains (56, 75).

TABLE 3 MIC ranges for commonly used QC and reference isolates for CLSI and EUCAST broth microdilution methods[a]

QC or reference isolates	Antifungal agent	MIC range (μg/ml)		
		EUCAST EDef 7.2 and 9.1	CLSI M27-A3 and M38-A2 24 h, microdilution	CLSI M27-A3 and M38-A2 48 h, microdilution
QC isolates				
Candida parapsilosis	Amphotericin B	0.12–1.0	0.25–2.0	0.5–4.0
ATCC 22019	Anidulafungin	0.25–1.0	0.25–2.0	0.5–2.0
	Caspofungin	NA	0.25–1.0	0.5–4.0
	Flucytosine (5FC)	0.12–0.5	0.06–0.25	0.12–0.5
	Fluconazole	0.5–2.0	0.5–4.0	1.0–4.0
	Itraconazole	0.03–0.12	0.06–0.5	0.06–0.5
	Ketoconazole	NA	0.03–0.25	0.06–0.5
	Micafungin	NA	0.5–2.0	0.5–4.0
	Posaconazole	0.015–0.06	0.03–0.25	0.03–0.25
	Voriconazole	0.015–0.06	0.016–0.12	0.03–0.25
Candida krusei	Amphotericin B	0.12–1.0	0.5–2.0	1.0–4.0
ATCC 6258	Anidulafungin	≤0.06	0.03–0.12	0.03–0.12
	Caspofungin	NA	0.12–1.0[b]	0.25–1.0[b]
	Flucytosine (5FC)	1.0–4.0	4.0–16	8.0–32
	Fluconazole	16–64	8.0–64	16–128
	Itraconazole	0.03–0.12	0.12–1.0	0.25–1.0
	Micafungin	NA	0.5–2.0	0.12–0.5
	Ketoconazole	NA	0.12–1.0	0.25–1.0
	Posaconazole	0.015–0.06	0.06–0.5	0.12–1.0
	Voriconazole	0.03–0.25	0.06–0.5	0.12–1.0
Paecilomyces variotti	Amphotericin B	NA	NA	1.0–4.0
ATCC MYA-3630	Anidulafungin (MEC)	NA	≤0.015	NA
	Itraconazole	NA	NA	0.06–0.5
	Posaconazole	NA	NA	0.03–0.25
	Voriconazole	NA	NA	0.015–0.12
Reference isolates				
Aspergillus flavus	Amphotericin B	0.5–2.0	NA	0.5–4.0
ATCC 204304	Itraconazole	0.12–0.5	NA	0.25–0.5
	Posaconazole	0.12–0.5	NA	0.06–0.5
	Ravuconazole	NA	NA	0.5-4.0
	Voriconazole	0.5–2.0	NA	0.5–4
Aspergillus flavus	Amphotericin B	NA	NA	1.0–8.0
ATCC MYA-3631	Posaconazole	NA	NA	0.12–1.0
	Voriconazole	NA	NA	0.5–2.0
Aspergillus fumigatus	Amphotericin B	NA	NA	0.5–4.0
ATCC MYA-3626	Anidulafungin (MEC)	NA	≤0.015	NA
	Posaconazole	NA	NA	0.25–2.0
	Voriconazole	NA	NA	0.25–1.0
Aspergillus fumigatus	Amphotericin B	NA	NA	0.5–4.0
ATCC MYA-3627	Itraconazole	NA	NA	>16
	Voriconazole	NA	NA	0.25–1.0
Aspergillus terreus	Amphotericin B	NA	NA	2.0–8.0
ATCC MYA-3633	Anidulafungin (MEC)	NA	≤0.015	NA
	Voriconazole	NA	NA	0.25–1.0
Fusarium moniliforme	Amphotericin B	NA	NA	2.0–8.0
ATCC MYA-3629	Anidulafungin (MIC)	NA	≥8.0	NA
	Itraconazole	NA	NA	>16
	Posaconazole	NA	NA	0.5–2.0
	Voriconazole	NA	NA	1.0–4.0
Fusarium solani ATCC 3636	Anidulafungin (MIC)	NA	≥8.0	NA

(*Continued on next page*)

TABLE 3 MIC ranges for commonly used QC and reference isolates for CLSI and EUCAST broth microdilution methods[a] *(Continued)*

QC or reference isolates	Antifungal agent	MIC range (μg/ml)		
		EUCAST EDef 7.2 and 9.1	CLSI M27-A3 and M38-A2 24 h, microdilution	CLSI M27-A3 and M38-A2 48 h, microdilution
Scedosporium apiospermum ATCC MYA-3635	Amphotericin B	NA	NA	4.0–16
	Posaconazole	NA	NA	1.0–4.0
	Voriconazole	NA	NA	0.5–2.0
Scedosporium apiospermum ATCC MYA-3634	Anidulafungin (MEC)	NA	NA	1.0-4.0
Trichophyton mentagrophytes MRL 1957 ATCC MYA-4439	Ciclopirox	NA	NA	0.5–2.0 (4 days)
	Griseofulvin	NA	NA	0.12–0.5 (4 days)
	Itraconazole	NA	NA	0.03–0.25 (4 days)
	Posaconazole	NA	NA	0.03–0.25 (4 days)
	Terbinafine	NA	NA	0.002–0.008 (4 days)
	Voriconazole	NA	NA	0.03–0.25 (4 days)
Trichophyton rubrum MRL 666 ATCC MYA-4438	Ciclopirox	NA	NA	0.5–2.0 (4 days)
	Fluconazole	NA	NA	0.5–4.0 (4 days)
	Voriconazole	NA	NA	0.008–0.06 (4 days)

[a] Data from EUCAST EDef 7.2 and 9.1, M27-S3, M38-A2, and M27-S4 (27–30, 100, 102). NA, not available. Data for further strains are available in these publications.

[b] The QC range for caspofungin and C. *krusei* ATCC 6258 was established using data generated in 2010 from 15 reference laboratories. Since then, caspofungin susceptibility testing has been associated with significant variation; the reason for which has not yet been fully elucidated. The clinical breakpoints approved in the M27-S4 document are based on the use of high-potency caspofungin powder for which the 24-h MIC range (mode) for the C. *krusei* ATCC 6258 was 0.06–0.25 μg/ml (0.125 μg/ml). Misclassification of susceptible isolates may therefore occur despite acceptable performance of the QC strain according to the range above (100).

Macrodilution

Broth macrodilution tests are adequate for the testing of all antifungal agents and are suitable for small laboratories in which the volume of these tests is low. Only the steps and testing conditions that are relevant to the macrodilution test are discussed in detail here (Table 2). Each intermediate drug concentration solution is further diluted (1:10) in RPMI medium to obtain 10 times the final strength. This step reduces the final solvent concentration to 10%. The 10× drug dilutions are dispensed in 0.1-ml volumes into round-bottom, snap-cap, sterile polystyrene tubes (12 by 75 mm; e.g., Falcon 2054; Becton Dickinson Labware, Lincoln Park, NJ); these tubes can be stored at −60°C or lower for 3 to 6 months. On the day of the test, each tube is inoculated with a 0.9-ml volume of the corresponding diluted yeast inoculum suspension. This step brings the drug dilutions to the final test drug concentrations mentioned above and the corresponding solvent to 1% in each MIC tube. The stock inoculum suspensions are prepared and adjusted as described above for the microdilution test and are then diluted 1:2,000 with RPMI to provide an inoculum of 0.5×10^3 to 2.5×10^3 CFU/ml. The growth control tube(s) is inoculated with a 0.9-ml volume of the inoculum suspension(s) and a 0.1-ml volume(s) of drug-free medium with 1% of the corresponding solvent. The QC yeasts are tested in the same manner as the other isolates and are included each time an isolate is tested. In addition, 1 ml of uninoculated drug-free medium (for water-soluble agents) or drug-free medium with 1% of the corresponding solvent is included as a sterility control.

Incubation and Determination of Macrodilution MICs for Yeasts

The MIC tubes are incubated at 35°C without agitation for 24 to 48 h (*Candida*) or 70 to 74 h (*C. neoformans*) in ambient air; the turbidity or growth in each tube is visually graded. For amphotericin B, the MIC is read as the lowest concentration that prevents any discernible growth. For azoles and flucytosine, the MIC is defined as the lowest drug concentration that causes a prominent decrease in turbidity to about 50% relative to that of the growth control (Table 2) (27).

Quality Control for Yeast Testing

QC of MIC tests is essential to good laboratory practice. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 are frequently selected as the QC strains according to the CLSI and EUCAST guidelines for such selection. However, the EUCAST guidelines caution against use of these as QC strains when testing caspofungin as they are not sufficiently sensitive in detecting variation in caspofungin potency, so for this drug *C. albicans* ATCC 64548 or ATCC 64550 is preferred (29, 70). Table 3 summarizes the expected MIC ranges of 10 antifungal agents for these QC isolates (27, 37). Each new batch of medium and lot of macrodilution tubes and microdilution trays should be checked with one of the two QC strains to determine if the MICs are within these ranges. In addition, the overall performance of the test system should be monitored by testing either or both QC isolates each day on which a test is performed for each drug. Details regarding corrective measures when the MICs for the QC isolates are not within the expected ranges are found in the M27-A3 document (27) and the EUCAST 7.2 document (29). A selection of potentially useful reference strains has been deposited with the American Type Culture Collection (ATCC) (Table 3).

YEAST GENERA OTHER THAN *CANDIDA*

Although the CLSI and EUCAST methods only provide suggested breakpoints for the most common human pathogenic *Candida* spp. (see Table 1), with certain caveats these methods are broadly suitable for most fermentative yeast. However, there are certain issues with nonfermentative yeast species such as *Cryptococcus* spp., *Malassezia* spp., *Pichia* spp., *Yarrowia lipolytica*, *Geotrichum* spp., *Rhodotorula* spp., *Sporobolomyces* spp., and *Trichosporon* spp. The CLSI method (27) and EUCAST method (29) do encompass the testing of *Cryptococcus* spp. As they are slower growing than *Candida* spp., a 72-h incubation period is advised (27), or with EUCAST methodology the plates can be read when the optical density exceeds 0.2. It is suggested that if this value is not reached then tests should be repeated with incubation of the trays at 30°C. Moreover, there is a suggestion in CLSI M27-S4 (37) for the use of yeast nitrogen base glucose (YNBG) broth, which may enhance the growth of *C. neoformans*, thus facilitating the determination of MICs (76–78). A recent study examined a number of different parameters for testing a range of nonfermentative yeast species. These included growth medium (RPMI versus YNBG); glucose at 0.2 or 2%; shaken or static; a different nitrogen source; incubation temperature (30 versus 35°C); and inoculum size (10^3, 10^4, or 10^5 CFU/ml). It concluded that the use of YNBG medium, shaking, and a lower incubation temperature enhanced the growth rate of *Cryptococcus* spp. and most of the other nonfermentative yeast species tested. This allowed reading after 24 h and more consistent endpoint determination; however, there were no significant differences in MIC obtained by the different methods (66). Two large global studies utilized the CLSI M27-A3 method in RPMI medium read at 72 h to define ECVs for fluconazole, itraconazole, posaconazole, and voriconazole (79, 80). The largest of these (80) reported susceptibility patterns for more than 3000 isolates of *C. neoformans* and more than 700 isolates of *Cryptococcus gattii*. A much smaller subset of isolates was tested in YNBG medium, and the modal MIC was higher for fluconazole as has been noted previously (81), which suggests some variability of susceptibility test results using different methodologies.

Most *Malassezia* spp. will not grow in RPMI medium as they require a lipid-rich environment, and to date there is not a standard method validated through a consensus procedure. *Malassezia pachydermatis* will grow in RPMI medium but is slower growing than most fermentative yeast species, although microdilution testing as well as a number of other procedures have been reported in the literature. However, this has resulted in conflicting results in terms of both absolute MIC values and interpretive results, and there are no established breakpoints for this genus. Recent publications have utilized the CLSI M27-A3 methodology and applied the breakpoints developed for *Candida* spp. (82, 83). To date, susceptibility testing of *Malassezia* is still investigational and not recommended for clinical practice (84). Moreover, this method is not validated for the yeast form of dimorphic endemic pathogens.

SPECIAL CONSIDERATIONS

Amphotericin B

Amphotericin B MICs determined by the microdilution methods are clustered between 0.25 and 1.0 μg/ml for 94% of clinical yeast isolates and are >2 μg/ml for the other 6%. An amphotericin B MIC of 2.0 μg/ml (or greater) suggests probable clinical resistance and is often used as a breakpoint for this drug. The difference in amphotericin B MICs for susceptible and potentially resistant isolates is therefore probably very small, so caution should be exercised in the interpretation of results. Although it has been suggested that antibiotic medium 3 provides reliable detection of resistant isolates, lot-to-lot variability has been documented (85). In addition, this medium did not improve the detection of potentially amphotericin B-resistant isolates recovered from patients with candidemia, who had failed amphotericin B therapy (microbiological failure) (86). Application of Etest methodology may more readily detect amphotericin B resistance *in vitro* (87). Further optimization is needed but is difficult due to the lack of isolates with confirmed resistance mechanisms.

Caspofungin

The validity of performing microdilution testing with caspofungin by either the CLSI or EUCAST methodology has recently been cast in doubt (88, 89). In a multicenter study of data from 17 laboratories analyzing up to 11,550 *Candida* isolates, wide discrepancies were found for most species in modal values, as well as truncated and bimodal distributions. Despite examination of many different testing parameters, including caspofungin powder source, storage time and temperature, solvent, and MIC determination, no single source of this variability could be established (89). In general, the efficacy of the three echinocandins against *Candida* isolates is uniform in the sense that resistance mutations confer resistance across all three compounds. Therefore both anidulafungin and micafungin have been evaluated as markers for caspofungin resistance (40, 90, 91). However, differential activity has been demonstrated for some *C. glabrata* strains where *FKS* mutations have conferred resistance to anidulafungin and caspofungin but not to micafungin *in vitro* and in animal models (8). Therefore, the recommendation at this time is to use anidulafungin as a marker of echinocandin susceptibility or resistance, and to retest *C. glabrata* isolates for susceptibility to micafungin if found anidulafungin resistant (89). EUCAST has only proposed interpretative breakpoints for anidulafungin and micafungin (55, 92).

CLSI AND EUCAST BREAKPOINTS FOR *CANDIDA* SPECIES

There is now a considerable body of data indicating that standardized antifungal susceptibility testing (27, 29) for *Candida* spp. and some triazoles, amphotericin B, as well as the echinocandins provides results that have predictive utility consistent with the 90-60 rule. Interpretive MIC breakpoints (Table 1) have been established for isolates tested by the CLSI M27-A3 method for fluconazole, voriconazole, anidulafungin, caspofungin, and micafungin following some correlation with clinical data predominantly from patients with oropharyngeal candidiasis, candidemia in nonneutropenic patients, and some more invasive infections but also taking into account epidemiological cutoff values (37, 93, 94). Breakpoints for yeast isolates tested by the EUCAST EDef 7.2 method are available for amphotericin B, fluconazole, posaconazole, voriconazole, anidulafungin, and micafungin (55, 92, 95–97). Considerable effort has been expended on rationalization of the discrepant CLSI and EUCAST breakpoints for yeast isolates, as there was concern that the results of two very similar methods should be interpreted with different breakpoint criteria (35, 36, 39, 98–100). This is especially true since the publication of CLSI M27-A3, which, like EUCAST EDef 7.2, allows the reading of results at 24 h, which has led to lower CLSI MICs for most drug-organism combinations (as illustrated also by the lower QC ranges for the 24-h reading) (27). Changes to the CLSI breakpoints have now been published in a supplement to the reference method, document M27-S4 (37), and these, together with the EUCAST breakpoints, are presented in Table 1. There are for the first time species-specific breakpoints, which makes accurate species identification a vital part of breakpoint interpretation. Harmonization has been achieved for almost all drug-organism combinations; where differences remain is mainly dependent on differences in the endpoints achieved by the two methods (e.g., for anidulafungin).

Fluconazole inhibits the majority (~90%) of *Candida* spp. at concentrations of ≤2 µg/ml, so this is the breakpoint for susceptibility for the majority of species; 4.0 µg/ml is considered SDD, and ≥8 µg/ml is considered resistant. The SDD designation for fluconazole encompasses isolates in which susceptibility is dependent on achievable peak levels in serum of 40 to 60 µg/ml at fluconazole dosages of 800 mg/day versus the expected peak levels of ≤30 µg/ml at lower dosages. The pharmacodynamic parameter that predicts efficacy for fluconazole is ~100 (area under the concentration-time curve [AUC]/MIC ratio). Fluconazole MICs for *C. glabrata* and *C. krusei* are generally higher than for other species, at 4 to 16 and 16 to ≥64 µg/ml, respectively (101). The EUCAST recommendation is that these species are not good targets for fluconazole, but because *C. glabrata* is not fully resistant, the *C. glabrata* wild-type population is classified as intermediate in order to accommodate use of these compounds in some clinical situations (29, 55). The CLSI also states that *C. krusei* is innately resistant but suggests that isolates of *C. glabrata* with MICs of ≤32 mg/liter may respond to high-dose fluconazole (37).

Breakpoints for itraconazole are not addressed in M27-S4, but non-species-specific breakpoints for this agent were included in M27-S3 (102). Itraconazole is generally quite active *in vitro*, with MICs of 0.01 to 1.0 µg/ml or less for most yeast isolates, except for *C. glabrata* (0.06 to 8 µg/ml) and *C. krusei* (0.5 to 2 µg/ml). Overall, ≥99% of isolates of *Candida* spp. are inhibited by ≤1 µg/ml, so this was the suggested resistance breakpoint (103). Isolates with an MIC of ≤0.125 µg/ml were considered susceptible, while those with an MIC of 0.5 µg/ml were classified as SDD. For itraconazole, an MIC within the SDD range indicates the need for higher serum concentrations for an optimal response. The need for species-specific breakpoints has been recognized, and species-specific ECOFFs/ECVs have recently been published that may assist in detecting and monitoring any acquired resistance development (93).

For voriconazole, a consensus susceptible breakpoint of ≤0.12 µg/ml has been agreed upon by CLSI and EUCAST groups for most *Candida* spp. documented, but whereas the CLSI document specifies an SDD of 0.5 µg/ml and a resistant breakpoint of ≥1.0 µg/ml (Table 1), EUCAST classifies isolates with MICs of >0.12 µg/ml as resistant. Isolates for which the voriconazole MIC is ≥1.0 µg/ml (CLSI resistant endpoint) are mostly *C. glabrata*, for which there are no accepted breakpoints, but also include non-*Candida* genera such as *Sporobolomyces salmonicolor* and *Rhodotorula rubra*, as well as some *C. albicans* isolates (101). Pharmacokinetics and pharmacodynamic parameters indicate that 24-h free-drug AUC/MIC ratios of 24 and 75 to 100 are predictive of the 50% effective dose and a 2-log CFU reduction, respectively. Recommended doses would produce free-drug AUCs of ~20 µg h/ml (104). However, voriconazole has nonlinear and variable pharmacokinetics, and the coefficient of variation of the AUC has been estimated to be 74 to 100%. Monte Carlo simulations showed that a target free-drug AUC/MIC of 24 would inhibit 99% of isolates with an MIC of ≤0.5 mg/liter if treatment were given intravenously and 99% of isolates with MICs of ≤0.25 mg/liter if treatment were given orally (97).

The EUCAST group has also addressed breakpoints for posaconazole for *Candida* species and suggests a susceptible breakpoint of ≤0.06 µg/ml and resistant breakpoint of >0.12 µg/ml, again based on careful evaluation of known wild-type distributions and the pharmacokinetic parameters of the drug (96). A recent multilaboratory analysis of ECVs of eight *Candida* spp. to fluconazole, posaconazole, and voriconazole suggests that breakpoints should be higher if assessed by CLSI methodology (105).

Reevaluation of the clinical CLSI breakpoints for the echinocandins following the analysis of accumulated data suggested that the previous CLSI breakpoint threshold of ≤2.0 µg/ml as susceptible and ≥2.0 µg/ml as nonsusceptible were too high for most *Candida* species. This led to the misclassification as susceptible of a significant number of isolates from infections that were refractory to echinocandin therapy and had known *FKS* mutations, which are recognized as a marker for echinocandin resistance (99). Thus, with the exception of *C. parapsilosis* and *C. guilliermondii*, lower drug- and species-specific epidemiological cutoff values have been suggested for the echinocandins (see Table 1). The CLSI breakpoints differ between the three echinocandins and are lower for *C. glabrata* than for most other *Candida* species. Applying these new lower breakpoints to 15,269 isolates of *Candida* spp. from 100 centers worldwide collected over an 8-year period from 2001 to 2009, Pfaller and colleagues detected a significant number of isolates with non-wild-type resistance patterns that would have been missed by applying the higher clinical breakpoints (40). At present, EUCAST has refrained from setting breakpoints for caspofungin due to significant variation associated with *in vitro* testing of this compound (89). The EUCAST breakpoints for anidulafungin and micafungin are lower than those proposed by the CLSI because the EUCAST method provides lower MICs than are obtained by CLSI. Hence, for both methods, the breakpoints basically mirror the ECOFFs/ECVs and the differences are method driven. Anidulafungin is considered a good marker for caspofungin susceptibility until the reproducibility issue has been solved and breakpoints can be established for this agent (93). Differential activity has, however, been demonstrated for micafungin and *C. glabrata*, as some weaker mutations elevate the anidulafungin and caspofungin MIC but not the micafungin MIC and are not associated with efficacy loss in an animal model (8). Hence anidulafungin is the preferred testing agent for caspofungin and anidulafungin susceptibility.

The breakpoints suggested for amphotericin B are ≤1.0 µg/ml (susceptible) and >1.0 µg/ml (resistant) (95). These have been applied historically and numerous publications support their application, although very little clinical experience exists for *Candida* isolates with MICs above 1 mg/liter.

ALTERNATIVE APPROACHES FOR YEASTS

Although the CLSI and EUCAST methods for *in vitro* susceptibility testing were essential for standardization and for improving interlaboratory reproducibility, they may not be the best methods for testing all organisms or all drugs or for routine use in clinical laboratories. Once reference methods become established, this allows the introduction of commercially available methods that produce comparative results. The methods that have been most frequently applied to antifungal susceptibility testing are listed in Table 4.

Colorimetric Methods

Colorimetric indicators or fluorescent dyes can facilitate determination of MIC endpoints. Commercial (Sensititre

TABLE 4 Methods used for antifungal susceptibility testing[a]

Test method	Means of endpoint determination
Broth macrodilution (yeasts)	Visual comparison of turbidity (≥50% inhibition) with that of growth control (M27-A3 document)
Broth microdilution (yeasts)	Visual comparison of turbidity (≥50% inhibition) with that of growth control (M27-A3 document)
Colorimetric microdilution (yeasts [YeastOne] and moulds)	Visual observation of color change
Spectrophotometric microdilution (yeasts)	Turbidimetric MIC determination by spectrophotometer (EUCAST EDef 7.2)
Macro- and microdilution (filamentous fungi)	Visual comparison of growth (50% inhibition or more [nondermatophytes] or 80% or more [dermatophytes] or MEC) with that of growth control (M38-A2 document, EUCAST EDef 9.1)
Agar macrodilution (yeasts and moulds, standard dishes)	Visual
Agar diffusion (yeasts and moulds)	Zone diameter (visual)
Disk (yeasts and moulds)	Zone diameter (visual) (M44-A2, M51-A1)
Antifungal gradient strip (e.g., Etest) (yeast and moulds)	Ellipse of inhibition (visual)

[a] Data from references 27–32.

YeastOne, ASTY, and Fungitest) and noncommercial (tetrazolium salt methods and substrate uptake indicators) procedures have been adapted for antifungal susceptibility testing (106–109). The Sensititre YeastOne YO2IVD plate (TREK Diagnostic Systems, Inc., Cleveland, OH) follows the same microdilution format as the CLSI reference method; it has been approved by the U.S. Food and Drug Administration (FDA) and is CE-marked for the testing of nonfastidious yeast species with fluconazole, itraconazole, voriconazole, flucytosine, and caspofungin, with the facility to include a QC organism on the same plate. Other systemic antifungal agents included on the Sensititre YeastOne YO9 plate, such as amphotericin B, posaconazole, anidulafungin, and micafungin, are available for nondiagnostic use in the United States, but the same range of antifungals on Sensititre YeastOne Y10 has been CE marked for use in Europe. Reading of endpoints is enhanced by the inclusion of Alamar Blue as the oxidation-reduction colorimetric indicator. If wells remain blue, there is no growth; pink wells indicate growth, and purple wells indicate partial inhibition. Agreement to within 2 doubling dilutions with reference broth microdilution MICs has been excellent with posaconazole and voriconazole (95.4%) and with anidulafungin, caspofungin, and micafungin (100%), all read after 24-h incubation (107, 109). Recent evaluation against the CLSI microdilution method using the new clinical breakpoints and epidemiological cutoff values for the echinocandins with 404 isolates of *Candida* spp. showed excellent (100%) essential agreement and close agreement with categorical values. For *C. albicans*, categorical agreement ranged from 93.6% (caspofungin) to 99.6% (micafungin) with less than 1% very major or major errors (110). For *C. glabrata* and *C. krusei* and caspofungin, it was somewhat less optimal (87.9 and 69.1%, respectively). However, an important caveat is that virtually no isolates with acquired resistance were included in any of the evaluation studies of the Sensititre test (and most others), and therefore the performance as to detection of such isolates remains largely unknown.

Vitek 2 Yeast Susceptibility Testing

In an effort to automate yeast susceptibility testing, bioMérieux (Hazelwood, MO) developed the Vitek 2 yeast susceptibility test, a commercial test system based on spectrophotometric analysis and incorporating a card-based miniaturized version of the doubling-dilution reference method. This was shown to produce reproducible, rapid, and accurate results consistent with those produced by the CLSI broth microdilution method for amphotericin B, flucytosine, fluconazole, and voriconazole with several hundred isolates of *Candida* spp. (111, 112). One study investigated the potential of the Vitek 2 system to specifically detect resistance to fluconazole and voriconazole in 36 isolates of *C. albicans* and 86 isolates of *C. glabrata* with well-characterized resistance mechanisms (113). The Vitek 2 system exhibited excellent agreement with the reference broth microdilution method for detecting resistance, with overall categorical agreement of 97.5% for both fluconazole and voriconazole. In a study of 154 isolates, including some resistant to the azoles and amphotericin B, Cuenca-Estrella and colleagues (114) compared the Vitek 2 antifungal susceptibility testing system with the CLSI and EUCAST broth dilution reference methods and with the Sensititre YeastOne and Etest techniques. With essential agreement to within 2 doubling dilutions of >95% for *Candida* spp. and 92% for *Cryptococcus neoformans*, they concluded that the Vitek 2 was a reliable technique to determine antifungal susceptibility of yeast species and, moreover, was a more rapid and easier alternative to the reference procedures. The average time to reading was 15.5 h for *Candida* spp. and 34 h for *Cryptococcus*. Recently, caspofungin has been included in the panel. A drawback is that the MIC range that can be reported is ≤0.25 to ≥4 mg/liter and thus does not include the revised breakpoint for *C. glabrata* (S: ≤0.125 mg/liter), and hence susceptible and intermediate isolates cannot be discriminated. A recent study evaluated the performance of the Vitek 2 for caspofungin testing using a well-defined panel of wild-type and resistant mutants of the five most common *Candida* spp. If *C. glabrata* isolates with an MIC of ≤0.25 were considered susceptible, there were no misclassifications of susceptible wild-type isolates. However, 19.4% (6/31) of isolates harboring *FKS* hot-spot resistance mutations were misclassified as susceptible (115). The AST-YS05 card for use with the Vitek 2 system, which contains fluconazole, voriconazole, and caspofungin, is available in the United States and Europe.

Flow Cytometry

Flow cytometric methods also have been adapted for antifungal susceptibility testing by introducing DNA-binding vital dyes into the culture to detect fungal cell damage after exposure to an antifungal agent. MICs determined by this approach have been comparable to those obtained by the M27-A3 methods (116, 117). Although these methods produce faster results (4 to 6 h), the need for a flow cytometer for MIC determination would preclude their use in small laboratories; moreover, they are not FDA approved or CE marked.

Standardized Disk Diffusion Method for Yeasts

Worldwide, the most commonly used technique for antibacterial susceptibility testing is the disk diffusion test, which yields a quantitative result (zones of inhibition) and a qualitative interpretive category (e.g., susceptible or resistant) based on correlation of zone sizes obtained with organisms with known MICs. Agar disk diffusion testing is a simple, flexible, and cost-effective alternative to broth dilution testing. The CLSI Subcommittee on Antifungal Susceptibility Testing has developed a disk diffusion method (Table 5) for testing *Candida* spp. with caspofungin, fluconazole, posaconazole, and voriconazole, although interpretive criteria are only available for caspofungin, fluconazole, and voriconazole, and as yet there are no commercially available FDA-approved disks (CLSI M44-A2 document) (118). One significant advantage of the M44-A2 disk diffusion method is that results can be obtained after 20 to 24 h of incubation. Extensive worldwide testing with fluconazole and voriconazole as part of a global survey suggests this method performs very well (119). It has also demonstrated good performance with caspofungin (120). The M44 disk test method has also been shown to be a useful approach for determining the susceptibility of *C. neoformans* and other genera of yeast (119, 121). Zone interpretive criteria have been approved for fluconazole, voriconazole, caspofungin, and micafungin (99) (CLSI M44-S3) (122).

Standard Medium

The CLSI M44-A2 method uses Mueller-Hinton agar supplemented with 2% glucose to improve growth and 0.5 μg/ml methylene blue to produce sharper zone definition (118). The pH of the medium should be 7.2 to 7.4 at room temperature after gelling, and the surface of the agar should be moist but without moisture droplets. The medium can be prepared and poured with the two supplements, or the supplements can be added to commercially prepared Mueller-Hinton agar plates; the latter enables the use of routine agar plates from the bacteriology laboratory. Detailed instructions for the preparation of the agar plates are found in the M44-A2 document (118).

Preparation of Inocula

The M44-A2 method employs an inoculum suspension adjusted to the turbidity of a 0.5 McFarland standard by the spectrophotometer, as described above for broth dilution standard methods (Table 2) (27).

Performance of Disk Diffusion Method for Yeasts

The agar plates are inoculated within 15 min of adjusting the inoculum suspension, as follows (Table 5). Briefly, a sterile cotton swab is dipped into the undiluted inoculum suspension, rotated several times, and pressed firmly against the inside wall of the tube above the fluid level to remove excess fluid. The entire dried agar surface is evenly streaked in three different directions, swabbing the rim of the plate as the final step. The lid of the plate should be left ajar to allow the agar surface to dry for no more than 15 min. Fluconazole (25 μg), posaconazole (5 μg), voriconazole (1 μg), caspofungin (5 μg), and micafungin (10 μg) disks are dispensed onto the inoculated agar surface. Disks must be pressed down to ensure complete contact with the agar and distributed evenly so they are not closer than 24 mm from center to center. After the disks are placed, they cannot be moved because drug diffusion is almost instantaneous. Plates should be incubated within 15 min after disks have been placed (118).

Incubation and Determination of Disk Diffusion Zone Diameters for Yeasts

After 20 to 24 h of incubation at 35°C, the resulting inhibition zones should be uniformly circular and a confluent lawn of growth should be present. The plates are read above a black, nonreflecting background illuminated with reflected light (118). The zone diameters surrounding the disks are measured to the nearest whole millimeter at the point at which there is prominent reduction in growth. Pinpoint microcolonies at the zone edge or large colonies within a zone are encountered frequently and should be ignored. If growth is insufficient, the plates should be read at 48 h (Table 5) (118). QC zone diameter limits have been defined for fluconazole, posaconazole, voriconazole, and caspofungin when testing *Candida* spp. (122).

Agar-Based Alternative Approaches for Yeasts

NeoSensitabs Tablets

A commercial agar diffusion test from Rosco Diagnostica (Rosco Laboratory, Taastrup, Denmark; distributor, Key Scientific Products, Stamford, TX) is available for antifungal susceptibility testing of yeasts. Tablets of established and some of the new antifungal agents (e.g., voriconazole, caspofungin, and posaconazole) are available. Preliminary comparisons with both reference broth dilution and disk diffusion methods have provided promising results and they are CE marked (123).

TABLE 5 CLSI M44-A2 document guidelines for antifungal disk diffusion susceptibility testing of *Candida* spp.[a]

Parameter	Description
Agar medium	Mueller-Hinton agar + 2% dextrose and 0.5 μg of methylene blue dye/ml
Inoculum preparation	From 24-h cultures on Sabouraud dextrose agar as described in Table 2 for broth micro- and macrodilution methods
Test medium	Stock inoculum suspension, adjusted by spectrophotometer at 530 nm to match the turbidity of a 0.5 McFarland standard: 1×10^6 to 5×10^6 CFU/ml
Disk contents	Caspofungin, 5 μg; fluconazole, 25 μg; posaconazole, 5 μg; voriconazole, 1 μg
Incubation conditions	20–24 h at 35°C
Reading zone diameter	To the nearest whole millimeter at the point at which there is prominent reduction in growth. Pinpoint microcolonies at the zone edge or large colonies within the zone should be ignored.

[a] Data from CLSI M44-A2 (31).

Gradient Strip Testing

The Etest (bioMérieux; Marcy l'Etoile, France, and Durham, NC) was the first antifungal gradient strip test to be produced, but others are now commercially available. Such tests are based on the diffusion of a stable concentration gradient of an antimicrobial agent from a plastic strip onto an agar medium. There are commercially available gradient strips for amphotericin B, fluconazole, flucytosine, ketoconazole, itraconazole, posaconazole, voriconazole, anidulafungin, caspofungin, and micafungin. For clinical use, the FDA has approved fluconazole, itraconazole, voriconazole, and flucytosine Etest strips; all are CE marked. Agreement of Etest and reference MICs has been species and medium dependent. The medium that provides the best performance for Etest MICs is solidified RPMI medium supplemented with 2% glucose, and reading requires expertise and close adherence to the manufacturer's instructions. If a clear zone is seen, the MIC can easily be read where the zone of inhibition intersects the strip, and false susceptibility has not been reported. Problems can arise when inexperienced readers incorrectly interpret faint background growth of small colonies within the zone as resistance. This is most often seen with fluconazole, and if an isolate is unexpectedly found to be fluconazole resistant by Etest, it should be retested by a reference method. In this regard, it is crucial to ensure that a pure culture is used; for example, with a mixture of C. *albicans* and C. *glabrata*, the susceptibility of the more susceptible C. *albicans* would be read, and the smaller C. *glabrata* colonies in the C. *albicans* inhibition zone could be mistaken as trailing that should be ignored. The Etest may be useful in testing yeasts suspected of being potentially resistant to amphotericin B (87, 124). Amphotericin B MICs of ≥0.38 μg/ml determined by Etest for *Candida* spp. have been associated with therapeutic failure in patients treated with amphotericin B for candidemia (125). However, there are conflicting reports suggesting that even when isolates are tested by this methodology, susceptibility data for *Candida* spp. do not appear to correlate with treatment failure or success. This suggests that factors other than MIC may have a greater impact on the outcome of invasive candidiasis (126). This method has also been evaluated and found to correlate well with CLSI methodology for testing the susceptibilities of *Candida* spp. to triazoles and echinocandins (127–129). It is clear that the breakpoints developed for the reference tests cannot necessarily be applied to results obtained with the commercial methods. A recent study suggests that if the modified CLSI breakpoints for caspofungin are applied to Etest results for some isolates of C. *glabrata* and C. *krusei*, a significant number will be misclassified as intermediate (130). This finding has been confirmed for C. *glabrata* when evaluating the clinical response for patients with infections involving isolates with MICs just above the CLSI breakpoint (131).

Direct Susceptibility Testing

Although most published susceptibility data have been derived following subculture of organisms, there have been attempts to perform tests directly on blood samples from patients with fungemia, thus still further reducing the incubation time required to complete the test (132, 133). In a recent study of 195 prospectively collected and 133 laboratory-simulated specimens of a wide range of clinically relevant yeast species, Guinea and colleagues were able to show high rates of agreement between direct Etest results obtained within 24 h and the reference CLSI M27-A3 method obtained in 48 to 72 h for fluconazole, voriconazole, isavuconazole, and caspofungin (133).

Proteomics and Antifungal Susceptibility Testing

Early work suggests that proteomic methodology can be used to detect the presence of resistance to several classes of antifungal drugs (134, 135). The protein composition of yeast cells varies in relation to the inhibition of cell growth in varying drug concentrations, and analysis of such changes could be exploited to herald a new generation of antifungal susceptibility testing. Marinach and colleagues (134) studied the effect of different fluconazole concentrations on the spectral profiles produced by one reference and 16 other strains of C. *albicans* with different susceptibilities to fluconazole and different resistance mechanisms. They analyzed deviations from the control spectra that were quantifiable in order to produce minimal profile change concentration endpoints representing the lowest drug concentration at which mass spectrum profile changes can be detected. There was a high degree of correlation (100% ± 2 doubling dilutions, 94% ± 1 doubling dilution) between minimal profile change concentration results and MIC results obtained with the standard CLSI methodology, and only one isolate would have been in a different susceptibility category (minor error). This methodology has also been applied to an analysis of caspofungin susceptibility testing of 34 yeast and 10 mould isolates with and without known resistance-associated *FKS* mutations (135). As in earlier tests, minimal profile change concentration results were compared with MIC (or minimum effective concentration [MEC] for the moulds) results obtained by the appropriate CLSI methodology. There was 100% essential agreement (± 2 dilutions) for both yeast and mould isolates and 94.1% categorical agreement.

Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry-based methods appear to be a reliable and reproducible tool for antifungal susceptibility testing with results analyzed after 15 h incubation as compared to 24 h for CLSI and EUCAST methods; moreover, objective analyses are produced for both yeast and mould isolates. Although currently as labor-intensive and time-consuming as a full susceptibility testing method, such a process has the potential to be adapted to breakpoint testing to provide an early surveillance test for antifungal drug resistance in a given isolate or as a screening tool for emerging resistance within a population.

Molecular Methods

There are multiple mechanisms that lead to reduced susceptibility or overt resistance to azole antifungal drugs in *Candida* species; these include changes in cell wall composition leading to reduced uptake, increased efflux, upregulation of target gene production, and mutation in the target enzymes (see chapter 130). One or more of these may be present in a cell, and stepwise acquisition appears to be common (136, 137). Thus, any method for molecular determination of resistance must be a multiplex system and must also be capable of determining not just presence but upregulation of housekeeping genes. For this reason, such methods are currently the province of research laboratories. Resistance to echinocandins usually centers on mutations of two hot-spot regions of the FSK1 gene, although other mutations have been induced in the laboratory setting (55). These are easier to detect by simple sequencing, including pyrosequencing (5, 138). A recent study attests to the utility of a Luminex-based multiplex assay that can be used for high-throughput screening and proved to be 100% concordant with DNA sequencing results on 102 isolates of C. *glabrata* with mutations in the FKS1 and FKS2 hot-spot domains (139). However, molecular techniques will be unable to detect previously uncharacterized mechanisms of resistance so should always be confirmed by phenotypic testing.

STANDARDIZED BROTH DILUTION METHODS FOR MOULDS

Antifungal susceptibility testing of moulds is becoming increasingly important due to the range of emerging opportunistic pathogens and the reporting of both innate and emergent resistance among the most common mould pathogens (1). Both CLSI (M38-A2) (28) and EUCAST (EDef 9.1) (30) have developed broth microdilution methods for filamentous fungi that are similar to those for yeast and are summarized in Table 6.

Microdilution

Standard Medium and Drug Stock Solutions

The test medium employed by the CLSI method is the same MOPS-buffered standard RPMI recommended for yeast testing and for the EUCAST method supplemented with 2% glucose. Drug stock solutions are prepared as described above for yeast testing (27, 29) and in the CLSI M38-A3 and EUCAST EDef 9.1 documents (28, 30).

Preparation of Inocula

Since nongerminated conidia are easier to prepare and standardize, this is the method of inoculum preparation described and is broadly similar in both documents. For the CLSI method, the inoculum for each isolate is prepared by first growing the mould on potato dextrose agar slants (oatmeal agar for *T. rubrum*) for 7 days at 35°C or until good sporulation is obtained, which may be 48 h for some genera. Each slant is then flooded with 1 ml of sterile 0.85% saline (addition of 0.01 ml of Tween 20 will help for *Aspergillus* spp.), and the surface of the colony is gently probed with the tip of the pipette. The resulting mixture is withdrawn, and the heavy particles are allowed to settle for 3 to 5 min. The upper homogeneous suspension, containing the mixture of nongerminated conidia or sporangiospores and hyphal fragments, is aliquoted and mixed for 15 s with a vortex. The turbidity is measured with a spectrophotometer at 530 nm, and the optical density adjusted by addition of sterile saline to 0.09 to 0.13 for *Aspergillus* spp., *Paecilomyces* spp., *Exophiala dermatitidis*, and *S. schenckii*; to 0.15 to 0.17 for *Fusarium* spp., *Scedosporium apiospermum*, *Scolecobasidium gallopavum* (formerly *Ochroconis gallopava*), *Cladophialophora bantiana*, and mucoraceous moulds; and to 0.25 to 0.3 for *Bipolaris* spp. and *Alternaria* spp. Stock inoculum suspensions are then diluted 1:50 in medium to obtain 2× the final inoculum of 0.4×10^4 to 5×10^4 CFU/ml. Certain genera (e.g., *Scedosporium*, *Bipolaris*, and *Alternaria*) may require a lower (50%) dilution factor (28).

The main difference with the EUCAST method is that the inoculum is higher and is prepared by counting in a hemocytometer as opposed to spectrophotometric determination. Conidia are harvested in 5 ml of sterile water + 0.1% Tween 20 by rubbing 2- to 5-day growth at 35°C on potato dextrose agar slants with a sterile cotton swab and then transferring the suspension to a sterile tube and homogenizing on a vortex mixer for 15 s. The resulting suspension is diluted as appropriate for hemocytometer counting. Suspensions containing clumps should be vortexed for a further 15 s and if hyphae are present should be filtered through an 11-μm pore size filter. The inoculum is adjusted with sterile distilled water to give a 2×10^6 to 5×10^6 CFU/ml suspension, which is diluted 1:10 with sterile distilled water to obtain a final working inoculum of 2×10^5 to 5×10^5 CFU/ml, which must be used within 30 min.

Drug Dilutions and Performance of Microdilution Test for Moulds

Drug dilutions are prepared and dispensed in sterile, disposable, multiwell microdilution trays, as described above for yeast testing. On the day of the test, each well is inoculated with 100-μl volumes of the diluted conidial or sporangiospore suspensions.

Incubation and Determination of Broth Microdilution MICs for Moulds

All microdilution trays are incubated at 35°C without agitation, and MICs are determined visually after 21 to 74 h. Most mucoraceous moulds should be read at 24 h and most other opportunistic fungi at 48 h; occasionally, 72 to 74 h may be required to allow sufficient growth in the control wells. Testing of the dimorphic fungi may require 5 to 7 days of incubation. The MIC endpoint criterion for moulds is the lowest drug concentration that shows complete growth inhibition when testing amphotericin B, itraconazole, voriconazole, and posaconazole. Endpoint determination with the echinocandin agents is difficult to assess and requires microscopic evaluation (see below) of the MEC, which for most moulds is read at 21 to 26 h and at 46 to 72 h for *Scedosporium* spp.

Echinocandins: MEC

When testing the echinocandins, most *Aspergillus* isolates show trailing growth, and conventional MIC determination could categorize these trailing isolates as resistant to caspofungin. A more careful examination of the microdilution wells reveals the presence of compact, round microcolonies. Under microscopic examination, these microcolonies correspond to significant morphologic alterations. The hyphae grow abnormally as short, highly branched filaments with swollen germ tubes. Kurtz and colleagues (140) defined the concentration of drug producing these morphologic changes as the MEC to distinguish it from conventional MICs. A multicenter study demonstrated that caspofungin MECs were reliable endpoints in 14 of 17 laboratories (141), and in another study in eight of these laboratories, evaluating anidulafungin MECs against a variety of mould species provided reliable endpoints (142). More recently, an international study involving five laboratories was able to determine wild-type distributions and epidemiological cutoff points for caspofungin against *Aspergillus* spp. (143). However, it is notable that broth microdilution testing failed to identify an animal-model-confirmed resistant isolate from a patient failing therapy as non-wild type even though this isolate failed to produce an inhibition ellipse with a caspofungin Etest strip (6). Consequently, because conflicting results have been reported from some laboratories, caution and further refinement of this testing approach are needed (141).

Macrodilution

There is good agreement between results obtained by both micro- and macrodilution methods for moulds (144). This test format may be more suitable for testing those organisms that require longer incubation periods due to problems with evaporation from the microtiter plates on prolonged incubation. Macrodilution testing conditions are described in the

TABLE 6 CLSI M38-A2 document for filamentous fungi and EUCAST broth microdilution guidelines for antifungal susceptibility testing of conidia-forming moulds[a]

Parameter	CLSI M38-A2	EUCAST EDef.9.1N
Test organism	Filamentous fungi	Conidia-forming moulds
Broth medium	RPMI-1640 broth buffered with MOPS buffer (0.165 M) and 0.2% dextrose to pH 7.0 at 25°C	RPMI-1640 broth buffered with MOPS buffer (0.165 M) and 2.0% glucose to pH 7.0 at 25°C
Microdilution plates	Sterile plastic, disposable 96-well plates with 300-µl-capacity round-bottomed wells	Sterile plastic, disposable 96-well plates with 300-µl-capacity flat-bottomed wells
Drug dilutions	Additive 2× 2-fold drug dilutions with solvent	Additive 2× 2-fold drug dilutions with solvent in double-strength RPMI to allow for dilution factor when inoculum is added
Drug dilution ranges:		
Azoles	0.03–16 µg/ml	0.015–8 µg/ml
Amphotericin B	0.03–16 µg/ml	0.03–16 µg/ml
Echinocandins	0.015–8.0 µg/ml	0.03–16 µg/ml
Dermatophyte testing:		
Ciclopirox	0.06–32 µg/ml	
Griseofulvin, fluconazole	0.125–64 µg/ml	
Itraconazole, voriconazole, terbinafine	0.001–0.5 µg/ml	
Posaconazole	0.004–8.0 µg/ml	
Storage of prepared plates	Sealed in plastic bags and stored at −70°C or below for up to 6 mo	Sealed in plastic bags or aluminum foil and stored at −70°C or below for up to 6 mo (echinocandins no more than 2 mo) or at −20°C for not more than 1 mo (not echinocandins)
Inoculum preparation	Incubate test isolates on potato dextrose agar (oatmeal agar for *Trichophyton rubrum*) for 7 days at 35°C or until good sporulation is obtained, which may be 48 h for some genera. Flood with 1 ml of sterile 0.85% saline (addition of 0.01 ml Tween 20 will help for *Aspergillus* spp.); gently probe the colony with the tip of the pipette. Allow to settle for 3–5 min, then withdraw upper homogeneous suspension to sterile tube. Vortex mix for 15 s.	Incubate test organisms on potato dextrose agar slants (or other sporulation-enhancing medium) at 35°C for 2 to 5 days or longer if required. Cover colonies with 5 ml sterile water + 0.1% Tween 20, rub with sterile cotton swab, then transfer suspension to sterile tube. Homogenize on a vortex mixer for 15 s, then dilute as appropriate for hemocytometer counting. If suspension contains hyphae, filter through 11-µm filter, or if clumps remain, revortex for further 15 s.
Stock inoculum suspension	Optical density adjusted by spectrophotometer at 530 nm by addition of sterile saline to 0.09–0.13 for *Aspergillus* spp., *Paecilomyces* spp., *Exophiala dermatitidis*, and *Sporothrix schenckii*; 0.15–0.17 for *Fusarium* spp., *Scedosporium apiospermum*, *Ochroconis gallopava*, *Cladophialophora bantiana*, and mucoraceous moulds; and 0.25–0.3 for *Bipolaris* spp. and *Alternaria* spp.	Count conidia on a hemocytometer and adjust with sterile distilled water to give 2×10^6 to 5×10^6 CFU/ml.
Test inoculum	Dilute suspension 1:50 in medium to produce 2× final inoculum 0.4×10^4 to 5×10^4 CFU/ml (NB: *Scedopsorium*, *Bipolaris*, and *Alternaria* may require a lower [50%] dilution factor).	Dilute suspension 1:10 with sterile distilled water to obtain a final working inoculum of 2×10^5 to 5×10^5 CFU/ml, use within 30 min
Plate inoculation	100 µl of diluted test inoculum plus 100 µl of 2× drug concn; final concn	100 µl of diluted test inoculum plus 100 µl of 2× drug concn; final concn
Inoculum verification	Plate 0.01 ml of a 1:10 dilution, or neat for dermatophytes, onto Sabouraud dextrose agar and incubate at 28−30°C; observe daily and count when colonies become visible up to 5 days.	Remove 20 µl from the growth control well immediately after inoculating, dilute in 2 ml sterile distilled water with 0.1% Tween, homogenize with vortex mixer, spread 100 µl on suitable agar plate, and incubate for 24–48 h, after which there should be 50–250 colonies.
Growth control(s)	100 µl of diluted inoculum plus 100 µl of drug-free medium (or plus 2% of solvent)	100 µl of diluted inoculum plus 100 µl of drug-free medium (or plus solvent)

(Continued on next page)

TABLE 6 CLSI M38-A2 document for filamentous fungi and EUCAST broth microdilution guidelines for antifungal susceptibility testing of conidia-forming moulds[a] *(Continued)*

Parameter	CLSI M38-A2	EUCAST EDef.9.1N
Sterility control	Column 12 of the plate can be used to perform the sterility control (drug-free medium only, no inoculum).	Column 12 of the plate can be used to perform the sterility control (drug-free medium only, no inoculum).
QC strains	Select QC strains that have MICs that fall near the midrange for all drugs tested. Rules have been established for QC testing based on frequency of testing.	At least two QC strains with results close to the middle range
Incubation	Incubate at 35°C without agitation or 30°C if more suitable for the species tested.	Without agitation in ambient air at 35 ± 2°C
Time of reading	21–74 h depending on species; echinocandins, 24 h or as soon as there is confluent growth in the control well	24–48 h; Mucorales should be read at 24 h; other moulds at 48 h, occasionally 72 h to allow sufficient growth in control well
MIC or MEC determination	Visual assessment with the aid of a reading mirror	Visual assessment with the aid of a reading mirror
Amphotericin B, itraconazole, posaconazole, ravuconazole, voriconazole	Lowest drug concentration that prevents any discernible growth (100% inhibition)	Lowest drug concn that prevents any discernible growth (100% inhibition). Ignore single colonies on the surface and "skipped wells."
Echinocandins	MEC, which is the lowest concentration of drug that leads to the growth of small, round, compact hyphal forms as compared to confluent growth in the control	MEC, which is the lowest concentration in which abnormal, short, and branched hyphal clusters are observed in contrast to the long, unbranched hyphal elements seen in the growth control
Ciclopirox, griseofulvin, terbinafine	80% growth reduction as compared to control	
Colorimetric modification	Addition of 2× concn of colorimetric indicator, modified resazurin, to 2× concn RPMI medium when preparing the plates may help end-point interpretation with itraconazole.	

[a] Data from references 28 and 30.

CLSI M38-A2 document (28). Briefly, inoculum stock suspensions and drug dilutions are prepared as for the microdilution test. The 100-fold drug dilutions should be diluted 1:10 with RPMI to achieve 10 times the strength needed for the macrodilution test. The stock inoculum suspensions are diluted 1:100 with medium to obtain 0.4×10^4 to 5×10^4 CFU/ml. The 10× drug concentrations are dispensed into 12- by 75-mm sterile tubes in 0.1-ml volumes. Each tube is inoculated on the day of the test with 0.9 ml of the corresponding suspension. Tubes are incubated at 35°C without agitation and observed for the presence or absence of visible growth. The MICs are determined as described above for the microdilution method for moulds.

Quality Control for Mould Testing

Either one of the QC yeast organisms or the QC *Paecilomyces variotii* ATCC MYA-3630 isolate may be tested in the same manner as the other mould isolates or as described above for yeasts and should be included each time an isolate is evaluated with any antifungal agent. In addition, other moulds have been selected as reference isolates by CLSI and EUCAST (Table 3) (28, 30).

Expected Results and Interpretation of Breakpoints for Moulds

EUCAST has published clinical breakpoints for selected *Aspergillus* species with some drugs (Table 7), but few corre-

TABLE 7 EUCAST breakpoints for *Aspergillus* species

Antifungal compound	Breakpoint[a]				
	A. flavus	*A. fumigatus*	*A. nidulans*	*A. niger*	*A. terreus*
Amphotericin	IE[b]	1/2	Note	1/2	Poor target
Itraconazole	1/2	1/2	1/2	IE[b]	1/2
Posaconazole	IE[b]	0.125/0.25[c]	IE[b]	IE[b]	Note[d]
Voriconazole	Note[d]	1/2	Note[d]	Note[d]	Note[d]

[a] Breakpoints are presented as S ≤ X, R >Y (except for the revised fluconazole breakpoint for *C. glabrata*). The "I" category (if present) is readily interpreted as the values between the S and the R breakpoints.

[b] IE (insufficient evidence)–MICs are higher than for *A. fumigatus*. There is insufficient evidence that this species is a good target for treatment.

[c] Provided sufficient drug levels can be achieved.

[d] Note the MICs exhibited by this species are similar to those exhibited by *A. fumigatus* but clinical data are insufficient to establish a breakpoint.

lations of *in vitro* results with *in vivo* response have been reported for mould infections. Clinical failures in the treatment of aspergillosis and hyalohyphomycosis have been correlated with itraconazole MICs of >8 μg/ml and amphotericin B MICs of >2 μg/ml (145–147). There are also failures and breakthrough infections with moulds reported in patients on caspofungin therapy (6, 148). Moreover, there are experiences with treating infections with other groups of fungi, such as *Scedosporium*, where the finding of greater *in vitro* susceptibility to voriconazole than to amphotericin B translates into better clinical outcomes in patients treated with this drug (149).

Amphotericin B MICs determined by either reference method are ≤1.0 μg/ml for most mould species, including most mucoraceous moulds (150, 151). High amphotericin B MICs (>2 μg/ml) have been reported for *Purpureocillium lilacinum*, most *Scedosporium* and *Pseudallescheria* spp., and some isolates of *Alternaria* spp., *Aspergillus* spp. (especially *A. terreus* and *A. flavus*), *Fusarium* spp., *Talaromyces* (formerly *Penicillium*) *marneffei*, *Phialophora* spp., and *S. schenckii* (53, 151, 152), and infections with these species are often refractory to treatment (153).

The EUCAST breakpoints for amphotericin B with *A. fumigatus* and *A. niger* are ≤1.0 μg/ml (susceptible) and >2.0 μg/ml (resistant) (Table 7). *A. terreus* has not been assigned breakpoints as it is not considered a good target for amphotericin B, and for *A. flavus* and *A. nidulans*, insufficient clinical data are available for breakpoint selection.

MICs of itraconazole are also usually ≤1.0 for *A. fumigatus* and ≤2.0 μg/ml for most other moulds; the exceptions are its high MICs for *Aspergillus ustus/calidoustus* (some isolates), *Fusarium solani*, *F. oxysporum*, *P. lilacinum*, *S. schenckii*, *S. prolificans*, *Trichoderma longibrachiatum*, and many mucoraceous moulds (152, 154). EUCAST has established itraconazole breakpoints for *A. flavus*, *A. fumigatus*, *A. nidulans*, and *A. terreus* (S, ≤1 mg/liter; R, >2 mg/liter) (Table 7). Azole resistance in *A. fumigatus* isolates has been associated with target gene mutations (*Cyp51A* gene, efflux pumps and upregulation of the target gene) (see chapter 130). Such alterations may confer resistance to itraconazole only, to itraconazole and posaconazole, or may induce pan-azole resistance. Typically, the itraconazole MICs are elevated to a much greater extent than those of posaconazole and voriconazole, so itraconazole may serve as an initial azole-resistance screening compound in *Aspergillus*.

Voriconazole and posaconazole display an extended spectrum of antimould activity and, particularly for posaconazole, good *in vitro* activity against the mucoraceous moulds has been reported (150, 151, 155, 156). Publications have agreed on epidemiological breakpoints for *A. fumigatus* tested by the CLSI and EUCAST methods (157–159). The consensus epidemiological cutoff results were ≤1.0 μg/ml for itraconazole, ravuconazole, and voriconazole, and ≤0.25 μg/ml for posaconazole. The EUCAST breakpoints reflect these accepted ECVs: voriconazole breakpoints are ≤1.0 μg/ml (susceptible) and >2.0 μg/ml (resistant) for *A. fumigatus*, *A. flavus*, *A. nidulans*, and *A. terreus*, while posaconazole breakpoints are ≤0.125 μg/ml (susceptible) and >0.25 μg/ml (resistant) for *A. fumigatus* and *A. terreus* (Table 7). The breakpoint for posaconazole is conservative and one step below the ECOFF/ECV due to the variable bioavailability of the current oral solution and the pharmcokinetic/pharmacodynamic data suggesting the wild-type MIC is only just covered. It is likely that the breakpoint may be elevated if future formulations of posaconazole provide better exposure in the clinical setting.

Publications have cited resistance to itraconazole and some cross-resistance to voriconazole in *A. fumigatus* from certain chronic clinical conditions necessitating long-term use of these agents (10, 160). More recently, there have been reports from the Netherlands and other countries of primary azole resistance in 6 to 12.8% of recent clinical *A. fumigatus* isolates (13, 18–20). This has been linked to the significant environmental use of azoles for plant protection, and a correlation of frequency of such resistant isolates in the environment (most of which harbor a unique TR_{34}/L98H/L98H alteration) with the proportional global use of antifungal pesticides has been established (21). Resistance frequencies therefore vary. A recent publication of more than 700 clinical isolates of *Aspergillus* spp. showed that only 2.2% had itraconazole MICs of ≥4 μg/ml (resistant), although a further 9.6% had an MIC of 2.0 μg/ml (intermediate) (161). Cross-resistance to posaconazole was encountered in 53.5% of 43 isolates with elevated itraconazole MICs, whereas only 7% of the isolates appeared cross-resistant to voriconazole, which corresponds to the degree of similarity in the molecular structures of the drugs (161).

MICs/MECs of the echinocandins are usually <1.0 μg/ml for most *Aspergillus* spp. (144, 162). However, a study that tested 81 isolates of *Aspergillus flavus* by EUCAST methodology found them to be uniformly resistant to caspofungin, anidulafungin, and micafungin, with MIC_{50} results at >16.0 μg/ml (162). In contrast, a study employing CLSI methodology reported that 95% of 432 isolates of *A. flavus* yielded a caspofungin MIC of ≤0.25 μg/ml (144). Higher MICs/MECs are often reported for other mould species for which the echinocandins usually demonstrate low to moderate activity (53, 162). However, MICs of <1.0 μg/ml have been reported for some isolates of dimorphic fungi, *Acremonium* spp., *Phialophora* spp., and *S. apiospermum* (162, 163).

For the echinocandins, *Aspergillus* species have been characterized as susceptible or nonsusceptible based on their wild-type distributions and epidemiological cutoff values, and based on large numbers of isolates in a multicenter collaboration, species-specific caspofungin ECVs were proposed, ranging from 0.25 to 1.0 μg/ml (144). However, unacceptable variation in the MIC range for caspofungin against *Aspergillus* is found if various publications are compared over laboratories and time, as has been reported for caspofungin testing of *Candida*. Until this variability has been reduced to an acceptable level, it will not be meaningful to adopt ECOFFs/ECVs or attempt to establish breakpoints (164–168). As there are clinical data to suggest that *A. fumigatus* isolates may acquire echinocandin resistance and that infections due to such isolates may fail therapy, it is of utmost importance to optimize and validate echinocandin testing of *Aspergillus* (6).

Broth Microdilution Method for Dermatophytes

Susceptibility testing of dermatophytes has lagged behind that of other moulds, but the M38-A2 broth microdilution method has been successfully adapted, with minor modifications, to the testing of dermatophytes (169). These modifications include the use of oatmeal agar for inoculum preparation when testing *Trichophyton rubrum* in order to induce conidium formation, and 4 to 5 days of incubation at 35°C for MIC determination (80% growth inhibition endpoints). Two isolates of *Trichophyton* spp. have been validated as reference strains (Table 3).

Colorimetric Methods

The YeastOne method has also been evaluated for moulds, and it appears to be a suitable method producing results that are comparable to reference methods (170–172). The measurement of metabolic activity by reading the Alamar

Blue color change (optical density) produced when tetrazolium salts (yellow) are cleaved to their formazan derivative (purple), using a microtiter plate spectrophotometer, has also been evaluated for moulds (173, 174). Again, further evaluations, including interlaboratory studies, are needed with more isolates and species, and, particularly important, such studies should whenever possible include wild-type as well as resistant isolates for as many species as possible.

Agar-Based Alternative Approaches for Filamentous Fungi

As for yeasts, agar-based methods have been applied to susceptibility testing of moulds, including agar dilution, disk diffusion, and Etest methods and semisolid agar (175).

Agar Dilution Methods

Agar dilution methods involve the preparation of 10× double dilutions of the agent, which are incorporated into molten agar. Drug-containing plates are inoculated with suspensions of the organism being tested. In one study, results with an itraconazole-resistant isolate tested by agar incorporation and broth microdilution correlated well with outcome in a mouse model of infection (145). Since standard methods are not available, the size of the inoculum varies among the different studies. Such methods are particularly useful for the high throughput required for environmental surveillance and for initial and early screening for azole resistance of clinical cultures, as testing can be done directly from the primary plate before a pure culture is available. Plates with four wells containing different concentrations of three antifungal agents in RPMI-1640 with 2% glucose agar (itraconazole 4 mg/liter, posaconazole 0.5 mg/liter, and voriconazole 1 mg/liter) and a control well have been developed for this purpose (Balis Laboratorium VOF, Boven Leeuwen, Netherlands) (41). Surveillance plates are inoculated with 50 µl of a test suspension and examined after 24, 48, and 72 h of incubation at 37°C.The use of such screening plates may reduce the number of isolates that need further reference testing by 90% and at the same time provide an early indication of azole susceptibility patterns (Fig. 1).

Disk Diffusion Method

Disk diffusion methodology has been evaluated for amphotericin B, anidulafungin, caspofungin, micafungin, itraconazole, posaconazole, and voriconazole against a wide range of opportunistic pathogenic moulds (176–180). The CLSI Subcommittee has published an approved method for testing caspofungin, amphotericin B, and the triazoles against nondermatophyte filamentous fungi (M51-A1) (32). It is similar to that for yeasts (M44-A2) (31) but employs Mueller-Hinton agar not supplemented with methylene blue or increased dextrose, as in a collaborative multicenter study these conditions were found to be unsuitable for many moulds (177). The inoculum concentration is prepared as for CLSI M38-A3, and plates are inoculated in the same way as for yeast disk diffusion methodology. After incubation at 35°C for 16 to 24 h for mucoraceous moulds, 24 h for *Aspergillus* spp., and 48 h for other moulds, there should be a confluent lawn of growth surrounding a circular inhibition zone, which is measured to the nearest whole millimeter (177). Good levels of overall categorical agreement were found as compared to CLSI M38 results when testing large numbers of isolates from many mould species. However, there were reservations about using amphotericin B disks except with mucoraceous moulds, and the itraconazole disks should not currently be used to test mucoraceous moulds but are suitable for other genera (177). Three strains have been selected as QC isolates (*A. fumigatus* ATCC MYA-3626, *P. variotii* ATCC MYA-3630, and *C. krusei* ATCC 6258) for which zone diameter ranges have been established for amphotericin B, itraconazole, posaconazole, and voriconazole (181).

Neo-Sensitabs Diffusion Method for Moulds

This commercial agar diffusion test (Rosco, Denmark) is available in Europe for antifungal susceptibility testing of yeast, although early studies proved disappointing for some drug-organism combinations when testing moulds (178). A more recent study compared susceptibility results obtained with Neo-Sensitabs with those obtained by Etest, although there was no comparison with a reference method. A group of 100 isolates including *Aspergillus* spp., *Fusarium* spp., *Scedosporium* spp., mucoraceous and other moulds were tested with amphotericin B, caspofungin, itraconazole, posaconazole, and voriconazole. When the recently published epidemiological cutoff points for moulds were applied (144, 159), high levels of categorical agreement were reported between these two tests with all drugs except amphotericin B (182). However, few resistant strains, and none with molecular evidence of resistance mutations, were included.

Etest and Other Gradient Strips

Numerous studies have assessed the utility of Etest for testing mould pathogens (179, 182–186). Since the trailing effect is not a major problem for azole testing against most moulds, Etest inhibition ellipses are usually sharp and MICs are easily interpreted. In contrast with the echinocandins, there may be quite heavy background growth within the inhibition zone, but much heavier growth within the inhibition ellipse may indicate resistance (6). Overall, comparisons of Etest and M38-A methods have demonstrated better agreement when testing the triazoles (>90%) than amphotericin B (>80%). Amphotericin B Etest MICs for *A. flavus*, *S. apiospermum*, and *S. prolificans* are usually higher than reference values, especially after 48 h of incubation (184). In a case study, an isolate of *A. fumigatus* was found to be multiresistant to azoles with corresponding genetic mutation and also appeared resistant to caspofungin by Etest, with an MIC of >32 mg/liter, although this resistance had not been detected by broth microdilution testing (6). On molecular testing, the isolate was shown to have upregulation in the expression of the *FKS* gene and displayed reduced susceptibility to caspofungin in an animal model of infection. The reliability of the Etest method and the clinical relevance of its MICs for moulds should be addressed. Although Etest strips for amphotericin B, anidulafungin, ketoconazole, posaconazole, voriconazole, and caspofungin are commercially available, the U.S. FDA has not to date approved any antifungal strip for clinical use in susceptibility testing of moulds.

Molecular Tests

The application of molecular methods to the detection of resistance in isolates of filamentous fungi has proved to be easier in mould isolates than in yeast as resistance mechanisms appear to be fewer. A multiplex PCR assay to detect the three most frequent *A. fumigatus cyp51A* gene mutations leading to triazole resistance and cross-resistance has been developed (187). Such a test could be beneficial particularly in the azole-naive setting, as it included the most common environmental resistance mechanism also seen in isolates from invasive infections (TR_{34}/L98H). However, in the setting of chronic aspergillosis, a huge variety of CYP51A mutations have been associated with mono-, multi-, or pan-azole resistance, and up to 40% of the resistant isolates are found to have other underlying mechanisms apart from target gene mutations, thereby rendering the negative predictive value of absence of mutations unacceptably low (21, 188). Furthermore, two new alterations have been described recently in azole-resistant isolates found in either azole-naive patients or in the environment, suggesting rapid molecular detection of azole resistance in *A. fumigatus* in the routine setting may be a complex and difficult task (189–191). The molecular mechanisms behind echinocandin resistance in *Aspergillus* are less well understood. *FKS1* gene mutations have been induced in laboratory strains and shown to confer resistance, but have so far not been detected in clinical isolates. Moreover, additional mechanisms not involving the *FKS* target gene have been found in isolates exposed to cell wall digestion and subsequently cultured in the presence of caspofungin (192). Gene expression profiling by Northern blotting and real-time PCR has revealed overexpression of the *FKS1* gene leading to reduced susceptibility in an isolate of *A. fumigatus* from a patient who had failed caspofungin therapy (6).

Fungicidal Activity

A CLSI study has demonstrated that laboratories can reliably perform MFC testing (50) and time-kill curves. In contrast to what is observed with yeasts, the azoles appear to have a certain degree of fungicidal activity, as mentioned above, for a variety of common and rare opportunistic mould pathogens. MFCs of 0.2 to 4 μg/ml have been reported with the triazoles for various mould species (50). However, standardization of this procedure is needed to reliably assess the potential value of the MFC endpoint in patient management. These standardization efforts should include the correlation of *in vitro* results with the clearing of target organs of the infecting organism in animal models and the further clinical relevance of MFC data. Recent studies incorporating an indicator of metabolic activity [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] in microdilution broth-based formats have examined fungicidal activity of amphotericin B and voriconazole and have revealed a concentration-dependent sigmoid pattern of fungicidal effects (193). Such developments will need careful evaluation and standardization.

SUMMARY AND CONCLUSIONS

A great deal of progress has been achieved in the field of antifungal susceptibility testing with both yeast and filamentous fungi since testing began in earnest in the early 1980s. Standardized broth macrodilution and microdilution methods are available for testing moulds and yeasts, as are standardized disk diffusion methods for systemically active antifungal drugs. Progress is also being made in establishing the relationship between test results and patient responses to therapy in varied clinical settings and with many of the currently available antifungal agents. Breakpoints are available for common yeast species with most systemically active drugs, and there are some breakpoints for *A. fumigatus*. Moreover, some commercial methods have been approved for the antifungal susceptibility testing of *Candida* spp., but care should be taken in selecting appropriate breakpoints based on the ECV for these methods, which may vary from those produced by the standardized reference tests. Often, identifying the yeast or mould pathogen to the species level is sufficient to direct initial therapy (see chapter 129), but given the increasing problem of resistance to azole antifungals in *A. fumigatus* in some countries, the use of drug-containing screening agars is to be encouraged for primary isolates to provide an early indication of resistance while isolates are being purified for reference testing. The strong move towards consensus in the standardized methodology employed and the principles by which breakpoints are selected in the United States and Europe has meant that internationally agreed ECV breakpoints can be applied for some drug-fungus combinations. This has helped to improve surveillance of resistance patterns worldwide and will help in the further development of clinically relevant breakpoints.

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Taxonomy and Classification of Human Parasitic Protozoa and Helminths

FRANCIS E. G. COX

132

The term *parasite* is traditionally applied to several disparate groups of eukaryotic organisms ranging from single-celled protozoa to birds and even mammals; however, it is mostly used with reference to protozoa and helminth worms (including nematodes, cestodes, trematodes, and acanthocephalans). Medical parasitology is concerned with the 200 or so species of helminth worms and about 80 species of protozoans that infect humans. Many of them are rare and accidental parasites, but more than 100 species are commonly found in humans. Some of them are responsible for a disproportionate number of important diseases, particularly in the warmest parts of the world, and they invariably receive the most attention. This chapter is concerned with the broad overall classification of the parasitic protozoa and helminth worms commonly encountered in humans. More detailed information can be found in chapters 136 to 147 of this volume.

Taxonomy is the study of the classification of living organisms, and classification is the science of establishing and defining systematic groups of organisms in a hierarchical manner that reflects evolutionary past and present relationships between the groups. The classification of higher organisms has in the past been largely determined using morphological criteria, structural similarities, and the fossil record; however, there is now increasing reliance on molecular techniques to establish evidence of relatedness. The basic unit in classification is the species, a grouping in which, among higher organisms, all members are morphologically similar and able to interbreed successfully. The universally used system for the classification of eukaryotic organisms is the Linnaean system, in which species are given two names, the generic name and the specific name; thus, humans are *Homo sapiens*. All organisms are placed in specific taxonomic groups, or taxa. Species are grouped into families and families into orders, orders into classes, classes into phyla, and phyla into one of six kingdoms (see chapter 113 and below), each taxonomic group, or taxon, subsuming the characteristics of the taxon above it. Taxa are frequently subdivided (e.g., suborders and subclasses, infraorders, superfamilies), resulting in a plethora of layers often impenetrable even to experts in taxonomy. This chapter errs on the side of simplicity rather than complexity.

Morphological characteristics have long been the gold standards of systems of classification and remain so for the identification of parasites. In recent years, however, other methods have been increasingly relied on for estimating evolutionary distances, the basis of rational hierarchical classifications. Some of the methods used include molecular analyses, particularly DNA and RNA sequences, distance matrices as measures of genetic distances, maximum likelihoods of the probability of particular relationships, maximum parsimony based on estimates of the smallest number of evolutionary events necessary to account for observable data, and Bayesian probability theory based on an assumption of prior probabilities. There is at present no consensus as to which methods are likely to produce scientifically sound and acceptable classifications of parasites, so all current systems must be regarded as interim working schemes.

The Linnaean system of classification of eukaryotes is logical and scientifically sound; however, it does not necessarily meet the needs of those interested not in the finer points of taxonomy but in having a system that is comprehensible and usable (in the words of John Corliss, a "user-friendly" system) (1). This subject is discussed in some detail in a parasitological (and equally mycological) context by Tibayrenc (2), who identifies some 24 concepts of species and goes on to say that "biological researchers need more pragmatic approaches that can be understood by nonspecialists. Decision makers need precise answers for cost-effective and efficient control measures against transmissible diseases." This is what underlies the adoption of the systems of classification described in this chapter, which are intended to serve as frameworks within which researchers and others, particularly those concerned with the diseases caused by parasites and not the parasites themselves, can communicate with one another.

CLASSIFICATION OF THE PROTOZOA

The single-celled protozoa are, and always have been, difficult to classify and have always constituted an enigmatic group. From the mid-1970s until fairly recently, the consensus among scientists was that there were five kingdoms of living organisms, Prokaryota (bacteria), Animalia (animals), Plantae (plants), Fungi (fungi), and Protista (an unnatural assemblage of single-celled eukaryotic organisms with affinities to animals [Protozoa], plants [Protophyta],

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and Fungi) (3). The affinities of these single-celled organisms have long been the subjects of controversy and frequent reappraisal on a somewhat arbitrary basis. Three things have made it possible to put the classification of the protozoa on a sound scientific basis: a new understanding of symbiogenesis, advances in molecular and biochemical techniques, and the use of multigene trees. Symbiogenesis is the phenomenon whereby eukaryotic cells have acquired prokaryotic cell organelles such as mitochondria, chloroplasts, and flagella, resulting in permanent associations between the organelles and the host cell (4). Studies of these associations have provided new insights into the evolution of various protozoan groups. Our understanding of symbiogenesis (coupled with the use of molecular and biochemical techniques and the construction of multigene trees) has now made it possible to arrange single-celled organisms into groups based on evolutionary distances and has enabled taxonomists to organize all living organisms within realistic and evolutionarily sound overall schemes—but not necessarily in a hierarchical way. The most important aspect of this classification is that its major elements are compatible with more traditional classifications. Essentially, there are now six kingdoms: the five classical groups, Bacteria, Animalia, Fungi, Plantae, and Chromista (sister to the Plantae, sometimes referred to as the Stramenopila), plus Protozoa (5–7). With the gradual removal of a number of former protozoa to the Chromista (now a massive kingdom with 10 phyla) and also to the plant and fungal kingdoms, the diversity in the former Protista has become very much reduced to what was formerly known as the Protozoa, and thus the use of the word Protista becomes redundant.

The classification used in this chapter, based on that developed by Cavalier-Smith (5–9), places the Protozoa as the basal eukaryotic kingdom and reestablishes the validity of the kingdom Protozoa; it thus brings the protozoa and helminths under the same rules, whereby all species are named according to the guidelines set out in the International Code of Zoological Nomenclature (http://www.iczn.org). From a practical point of view, this confers stability in the naming of species and ensures priority for the names originally given while permitting corrections or amendments when necessary. It must be remembered, however, that the International Code is not a panacea for all taxonomic problems. This revised classification has necessitated the redistribution of some taxa, including some parasites of humans. In this chapter, some of the generic and specific names are different from those in the 10th edition of this *Manual*, but all such changes can be justified on grounds of new discoveries and interpretations.

There are estimated to be more than 200,000 named species of single-celled eukaryotic organisms (10), of which only about 10,000 (some 5%) are parasitic; thus, any system of classification must primarily satisfy zoologists and protozoologists working with free-living protozoa. Traditionally, the phylum Protozoa embraced four great groups of single-celled organisms recognized on the basis of their mode of locomotion: Rhizopoda or Sarcodina (amoebae, moving by pseudopodia), Mastigophora (flagellates, moving by flagella), Ciliophora or Ciliata (ciliates, moving by cilia), and Sporozoa (sporozoans, spore-forming protozoans without any obvious means of locomotion). Rapid developments in our understanding of the protozoa during the 1960s and 1970s necessitated a new classification. In 1980, the Society of Protozoologists published a classification (11) that recognized seven phyla: Sarcomastigophora (amoebae and flagellates), Apicomplexa (essentially equivalent to the Sporozoa), Ciliophora (ciliates), Microspora (now classified with the phylum Fungi), Myxozoa (now classified in the phylum Animalia), Ascetospora, and Labyrinthomorpha (groups of little interest to parasitologists). However, by the beginning of the 1990s, a number of protozoologists (12, 13) had reverted to what was essentially the traditional four "groups": the flagellated protozoa, the amoeboid protozoa, the ciliated protozoa, and the sporozoans (including the microsporidians). This approach had the merit of simplicity and maintained clear links with the traditional classification, but it was merely a classification of convenience and did not stand up to rigorous analysis at either the evolutionary or molecular level. Further attempts to resolve this problem resulted in an "interim user-friendly" classification (1) that served its purpose well until more rational and natural classifications emerged.

Over the past decade or so, developments in molecular biology have given us a clearer understanding of phylogenetic relationships involving particular groups of single-celled eukaryotic organisms, but unfortunately this has once again resulted in a plethora of classifications. In 2005, a quarter of a century after the publication of the Society of Protozoologists' classification, another attempt was made by the then International Society of Protozoologists to produce an "updated and comprehensive classification" of the single-celled eukaryotes or protists (14), which has since been superseded (15). The authors of these classifications abandoned the traditional higher taxa in favor of a series of groups and ranks but succeeded merely in producing a classification so complex that it is unlikely to be of use to any scientists except specialist protozoologists working with free-living organisms. It now seems unlikely that protozoologists, particularly those working with free-living organisms, will ever come up with a scheme of classification acceptable to everyone. These apparent vacillations have confused parasitologists, most of whom are concerned with fewer than 0.05% of all named species that affect humans, who simply require a stable framework within which to classify parasites—particularly those of medical and veterinary importance.

The classification in Table 1, based on that by Cavalier-Smith (5–9), embraces traditional and novel elements, meets the requirements of parasitologists, and bridges the gap between those working with free-living protozoa and those working with parasitic protozoa. There are several major and important differences between the traditional (1980) classification (11) and the one outlined below. There are two important changes from the schemes of classification found in most medical and veterinary textbooks. First, the microsporidians, some of which infect humans, mainly immunocompromised individuals, have traditionally been classified with the Protozoa *sensu stricto* but are now classified with the Fungi (16). Second, the taxonomic position of *Blastocystis* has always been enigmatic, and this genus has been shuffled between the Fungi and Protozoa but is now placed in the kingdom Chromista (8, 17).

With the removal of these groups, the Protozoa becomes a kingdom containing 11 to 13 phyla, depending on which particular classification is used, of which six contain parasites that infect humans. The phylum Amoebozoa, containing the amoebae, is equivalent to the old Rhizopoda (Sarcodina); the flagellates belonging to the former Mastigophora are now distributed among three phyla, Metamonada, Percolozoa, and Euglenozoa. Two phyla remain unchanged, the Ciliophora, containing the ciliates, and the Apicomplexa (Sporozoa). The status of the phylum Apicomplexa requires some explanation. Sporozoa is the traditional name applied to a large group of sexually repro-

TABLE 1 Outline classification of the parasitic protozoa (kingdom Protozoa) found in humans

Phylum	Class	Order	Important species	Other species
Group 1: The flagellates				
Metamonada (flagellates)	Trepomonadea (intestinal flagellates)	Diplomonadida	*Giardia duodenalis*[a]	*Enteromonas hominis*
	Retortamonadea (intestinal flagellates)	Retortamonadida		*Chilomastix mesnili*, *Retortamonas intestinalis*
	Trichomonadea (intestinal and related flagellates)	Trichomonadida	*Dientamoeba fragilis*, *Trichomonas vaginalis*	*Trichomonas tenax*, *Pentatrichomonas hominis*
Percolozoa (flagellates and amoebae)	Heterolobosea (free-living and opportunistic flagellates and flagellated amoebae)	Schizopyrenida	*Naegleria fowleri*	
Euglenozoa (flagellates)	Kinetoplastidea (blood- and tissue-inhabiting flagellates)	Trypanosomatida	*Leishmania donovani*, *L. infantum*, *L. major*, *L. tropica*, *L. braziliensis*, *L. mexicana* *Trypanosoma cruzi*, *T. brucei gambiense*[b], *T. brucei rhodesiense*[b]	*Leishmania aethiopica*, *L. archibaldi*, *L. amazonensis*, *L. colombiensis*, *L. garnhami*, *L. guyanensis*, *L. killicki*, *L. lainsoni*, *L. naiffi*, *L. panamensis*, *L. peruviana*, *L. pifanoi*, *L. shawi*, *Leishmania* sp. (Thailand), *Trypanosoma rangeli*
Group 2: The amoebae				
Amoebozoa (amoebae)	Amoebaea (amoebae, free-living and opportunistic parasites)	Acanthopodida	*Acanthamoeba castellanii*, *Balamuthia mandrillaris*	*Acanthamoeba astronyxis*, *A. culbertsoni*, *A.hatchetti A. heylyi*, *A. polyphaga*, *A. rhydodes*, *Acanthamoeba* spp.
	Archamoebea (intestinal amoebae)	Euamoebida	*Entamoeba histolytica*, *E. coli*, *E. dispar*, *E. hartmanni*	*Entamoeba gingivalis*, *E. moshkovskii*, *E. chattoni*, *E. polecki*, *Endolimax nana*, *Iodamoeba buetschlii*
Group 3: The sporozoans				
Apicomplexa (sporozoans and dinoflagellates)	Coccidea (sporozoan parasites)	Eimeriida	*Cryptosporidium parvum*, *Toxoplasma gondii*, *Cyclospora cayetanensis*	*Cryptosporidium baileyi*, *C. canis*, *C. felis*, *C. hominis*, *C. meleagridis*, *Sarcocystis hominis*, *S. lindemanni*, *S. suihominis* *Cystoisospora* (*Isospora*) *belli*, *C. natalensis*
		Piroplasmida	*Babesia microti*	*Babesia duncani*, *B. divergens*, *B. gibsoni*, *Babesia* spp.[c]
		Haemosporida	*Plasmodium knowlesi*, *P. falciparum*, *P. malariae*, *P. ovale*, *P. vivax*	
Group 4: The ciliates				
Ciliophora (ciliates)	Litostomatea (free-living and parasitic ciliates)	Trichostomatia	*Balantidium coli*	
Kingdom Chromista				
Bigyra	Blastocystea		*Blastocystis hominis*	

[a] Also known as *Giardia intestinalis* or *Giardia lamblia*. Molecular and epidemiological evidence suggests that there is more than one assemblage of *Giardia* spp. that infect humans and, possibly, more than one species.

[b] *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* are subspecies of *T. brucei* but are often referred to as species *T. gambiense* and *T. rhodesiense*.

[c] Molecular phylogenetic studies suggest that humans harbor a small number of *Babesia* spp. that cannot be identified as *B. microti*, *B. divergens*, *B. gibsoni*, or *B. duncani*.

ducing, spore-forming protozoans with recognizably similar morphology at the electron microscope level and comparable life cycles. In the 1970s, a new parasite genus, *Perkinsus*, containing parasites of marine molluscs, which was quite different from any of the other sporozoans, was allocated to this group. The phylum Sporozoa was downgraded to a class and the name of the phylum was changed to Apicomplexa in order to incorporate two classes, Sporozoea and Perkinsea (11). Not all parasitologists liked this change, but the word Apicomplexa has gradually crept into the literature and is now widely used. It is now clear that *Perkinsus* spp. do not belong with the sporozoans and should be classified close to, or with, the dinoflagellates (18). Since its original description, the taxon Apicomplexa has also acquired a number of nonparasitic flagellated species belonging to the order Colpodellida; therefore, Apicomplexa now includes both the traditional sporozoans and a number of nonparasitic species. Unfortunately, Apicomplexa has now become largely synonymous with Sporozoa, and the two names are often used interchangeably. Nevertheless, the compact group of species of most interest to parasitologists, the sporozoans *sensu stricto*, are members of the class Coccidea in the subphylum Sporozoa, a taxon included in Table 1. Therefore, when considering these important parasitic protozoans, reference should be made to the subphylum Sporozoa and sporozoans rather than to the phylum Apicomplexa and apicomplexans. More information about the parasitic protozoa that affect humans can be found in chapters 136 to 142 in this volume.

CLASSIFICATION OF THE HELMINTH WORMS

Although usually considered together by parasitologists, helminth worms actually constitute three entirely separate phyla, Nematoda, Platyhelminthes, and Acanthocephala. The nematodes and acanthocephalans have affinities close to the arthropods, whereas the affinities of the platyhelminths lie with the annelids and molluscs. For these reasons, these three groups are considered separately.

CLASSIFICATION OF THE NEMATODES

Estimates of the numbers of named nematodes range from 15,000 to 20,000, and some experts believe that there may be more than a million. Nematodes occupy every conceivable habitat, but fewer than 10% of named species are parasites of vertebrates and, of these, very few are parasitic in humans. The classification of nematodes faces the problems encountered when classifying the protozoa; the interests of most scientists concerned with free-living forms contrast with the interests of the minority interested in parasites—particularly those of medical or veterinary importance. It is unlikely, therefore, that there will ever be a system of classification satisfactory to both kinds of interest and, as for the protozoa, the views of the majority will always tend to dominate.

Nematodes are typically bilaterally symmetrical, round, and tubular in shape, with an outer cuticle, a hydrostatic body cavity, the pseudocoelum (a gut that runs from mouth to anus), and a well-developed reproductive system with separate sexes. Respiratory and circulatory systems are absent. The typical life cycle consists of an egg, four larval stages, and male and female adults. The classification of the nematodes has been traditionally based on the morphological characteristics of the adult male and, to a lesser extent female, worms—particularly the nature of the cuticle; the shape of the mouth and lips, the length of the esophagus, cephalic papillae, vulval appendages; and the nature of the male bursa. All these features are easily observable and can be measured, but, as indicators of evolutionary relationships, they are not really satisfactory. Since 1974, there have been at least 10 different major attempts to devise systems of classification that more closely reflect evolutionary relationships, and recent classifications have tended to place more weight on evidence obtained using molecular techniques than on morphology. Early classifications divided the nematodes into two classes, the Phasmida and Aphasmida, based on the presence or absence of paired granular secretory organs, the caudal phasmids. These major groups were subsequently changed to Adenophorea and Secernentea, the latter group containing most of the nematodes of medical importance (19). The most widely used classification of the nematodes is that developed over a number of years by the Centre for Agricultural Bioscience International, formerly the Commonwealth Agricultural Bureau Institute, and brought together in the *Keys to the Nematode Parasites of Vertebrates* (20). The editors of these volumes agree that this classification is somewhat dated but, regardless of whatever new classifications emerge, the keys are, and will remain, the most useful source of information for those involved in the day-to-day tasks of identifying nematodes of human, veterinary, and agricultural importance. This is the classification used in this chapter and shown in Table 2.

A radically different classification of the nematodes developed by De Ley and Blaxter is gaining some acceptance among those working with free-living (the majority of) nematodes (21). This classification, based largely on the use of morphological data and small subunit rDNA sequences, embeds the Secernentea within the Adenophorea, thus undermining the traditional two-class classification. This has necessitated many other changes, so this classification is very different from the traditional one. This classification is still in the process of being developed, so the version adopted in this chapter is essentially the traditional one with some minor modifications. As such, it is compatible with the classifications used in most parasitological textbooks. More information about the nematodes that affect humans can be found in chapters 143, 144, and 147 in this volume.

CLASSIFICATION OF THE PLATYHELMINTHES: TREMATODES

The phylum Platyhelminthes is usually divided into three classes, the flatworms or flukes, Monogenea and Digenea, and the tapeworms, Cestodaria. There are no monogenean parasites that affect humans, and the tapeworms are considered in the next section, so this section is restricted to the Digenea. There are about 18,000 species of digeneans belonging to over 140 families, by far the largest group of metazoan parasites. All are dorsally flattened and leaflike, with a well-developed alimentary system with a blind gut and an oral sucker surrounding the mouth and also a ventral sucker, both of which are used for attachment to the host. The classification of digenetic trematodes is reasonably straightforward, because all species are parasites; thus, it is not necessary to take into account the requirements of scientists concerned with free-living organisms. The classification of the digeneans has, therefore, remained fairly stable, as can be seen from an examination of Yamaguti's monumental *Synopsis of Digenetic Trematodes of Vertebrates* (22) and the Centre for Agricultural Bioscience International's three-volume *Keys to the Trematoda* (23–25).There is also a very good classification by Olson and colleagues (26) based partly on DNA sequences. These authors believed that it was necessary to reassign some species between the Echinostomida, a polyphyletic group, and the Plagiorchiida, both of which contained nonnatural groupings. These changes have had little impact on the classification of parasites of medical interest but have been incorporated into Table 3 by bringing closely related groups nearer together, but otherwise the classification remains very much a traditional one. More information about the trematodes that affect humans can be found in chapters 146 and 147 of this volume.

CLASSIFICATION OF THE PLATYHELMINTHES: CESTODES

The classification of the cestodes, or tapeworms, presents very few problems, largely because they have distinctive morphological characteristics. There are more than 1,000 species characterized by the possession of a distinct anterior

TABLE 2 Outline classification of the parasitic nematodes (kingdom Animalia, phylum Nemathelminthes [Nematoda, roundworms]) found in humans

Order	Superfamily	Family	Important species	Other species
Class 1: Adenophorea (Asphasmidea)				
Enoplida	Trichinelloidea (Trichuroidea)	Trichinelliidae (Trichuriidae)	*Trichinella spiralis*, *Trichuris trichiura*	*Trichinella britovi*, *T. murrelli*, *T. nativa*, *T. nelsoni*, *T. papuae*, *T. pseudospiralis*, *T. zimbabwensis*, *Trichuris suis*, *T. vulpis*, *Capillaria* (*Paracapillaria*) *philippensis*, *Capillaria* spp.
Class 2: Secernentea (Phasmidea)				
Rhabditida	Rhabditoidea	Strongyloididae	*Strongyloides fuelleborni*, *S. stercoralis*	*Strongyloides*, *Procynornis*
	Spiruroidea	Gongylonematidae		*Gongylonema pulchrum*
Strongylida	Ancylostomatoidea	Ancylostomatidae	*Ancylostoma duodenale*, *Necator americanus*	*Ancylostoma brazilienese*, *A. caninum*, *A. ceylanicum*
	Metastrongyloidea	Metastrongylidae		*Metastrongylus elongatus*
		Angiostrongylidae		*Parastrongylus cantonensis*, *P. costaricensis*
	Strongyloidea	Chabertiidae		*Oesophagostomum bifurcum*, *Oesophagostomum* spp., *Ternidens deminutus*
		Sygamidae		*Mammomonogamus laryngeus*
	Thelazioidea	Thelaziidae		*Thelazia californiensis*, *Thelazia callipaeda*
	Trichostrongyloidea	Trichostrongylidae		*Trichostrongylus axei*, *T. brevis*, *T. capricola*, *T. colubriformis*, *T. orientalis*, *T. probolurus*, *T. skrjabini*, *T. vitrinus*, *Trichostrongylus* spp., *Haemonchus contortus*
		Dioctophymatidae		*Dioctophyme renale*
Oxyuroida	Oxyuroidea	Oxyuridae	*Enterobius vermicularis*	*E. gregorii*
	Physalopteroidea	Physalopteridae		*Physaloptera caucasica*
Ascarida	Ascaridoidea	Ascarididae	*Ascaris lumbrioides*	*Ascaris suum*, *Bayliascaris procyonis*, *Lagochilascaris minor*, *Parascaris equorum*, *Toxocara canis*, *T. cati*
		Anisakidae		*Aisakis physeteris*, *A. simplex*, *Phocanema decipiens*
Spirurida	Dracunculoidea	Dracunculidae	*Dracunculus medinensis*	
	Gnathostomatoidea	Gnathostomatidae	*Gnathostoma spinigerum*	*G. binucleatum*, *Gnathostoma* spp.
	Filarioidea	Onchocercidae	*Brugia malayi*, *B. timori*, *Loaloa*, *Mansonella perstans*, *Wuchereria bancrofti*, *Onchocerca volvulus*	*Brugia beaveri*, *B. guyanensis*, *Dipetalonema* spp., *Dirofilaria immitis*, *D. repens*, *D. striata*, *D. tenuis*, *D. ursi*, *Mansonella ozzardi*, *M. streptocerca*, *M. semiclarum*, *M. rodhaini*

TABLE 3 Outline classification of the trematode parasites (kingdom Animalia, phylum Platyhelminthes, class Trematoda, subclass Digenea [flukes]) found in humans

Order	Superfamily	Family	Important species	Other species
Diplostomida	Diplostomoidea	Diplostomidae		*Alaria alata*, *A. americana*, *Diplostomum spathaceum*, *Neodiplostomum seoulensise*
	Schistosomatoidea	Schistosomatidae	*Schistosoma haematobium*, *S. japonicum*, *S. mansoni*, *S. mekongi*, *S. intercalatum*	*Schistosoma bovis*, *S. malayensis*, *S. mattheei*, *Schistosoma* spp., *Austrobilharzia terrigalensis*, *Bilharziella polonica*, *Giganobilharzia sturniae*, *G. huttoni*, *Heterobilharzia americana*, *Microbilharzia variglandis*, *Orientobilharzia turkestanicum*, *Trichobilharzia* spp.
		Clinostomidae		*Clinostomum complanatum*
Plagiorchiida	Gymnophalloidea	Gymnophallidae		*Gymnophalloides seoi*
	Echinostomatoidea	Echinostomatidae		*Acanthoparyphium kurogamo*, *A. tyosenens*, *Artyfechinostomum*, *malayanum*, *Echinostomum echinatum*, *E. hortense*, *E. ilocanum*, *E. revolutum*, *Echinostoma* spp., *Echinoparyphium recurvatum*, *Echinochasmus* spp., *Hypoderaeum conoideum*
		Fasciolidae	*Fasciola hepatica*, *Fasciolopsis buski*	*Fasciola gigantica*
	Opisthorchioidae	Heterophyidae		*Apophallus donicus*, *Heterophyes heterophyes*, *Heterophyes* spp., *Haplorchis* spp., *Metagonimus yokogawai*, *M. takahashi*, *M. miyatai*, *Centrocestus cuspidatus*, *C. formosanus*, *Stellantchasmus falcatus*, *Stichodora* spp., *Cryptocotyle lingua*
		Opisthorchidae	*Clonorchis sinensis*, *Opisthorchis felineus*, *O. viverrini*	*Opisthorchis guayaquilensis*, *Metorchis* spp., *Prosthodendrium molenkampi*
	Paramphistomoidea	Zygocotylidae	*Gastrodiscoides hominis*	*Watsonius watsoni*
	Plagiorchioidea	Lecithodendriidae		*Phaneropsolus bonnei*
		Paragonimidae	*Paragonimus westermani*	*Paragonimus africanus*, *P. heterotremus*, *P. mexicanus*, *P. miyazakii*, *P. ohirai*, *P. pulmonalis*, *P. skrjabini*, *P. uterobilateralis*, *Paragonimus* spp.
		Plagiorchiidae		*Plagiorchis* spp.
		Troglotrematidae		*Nanophyetus salmincola*
		Achillurbainiidae		*Achillurbainia* spp.
	Dicrocoelioidea	Dicrocoeliidae	*Dicrocoelium dendriticum*	*Eurytrema pancreaticum*

TABLE 4 Outline classification of the cestode parasites (kingdom Animalia, phylum Platyhelminthes, class Cestoda [tapeworms]) found in humans

Order	Family	Important species	Other species
Pseudophyllidea	Diphyllobothriidae	*Diphyllobothrium latum*	*D. dalliae*, *D. klebanovskii*, *D. pacificum*, *Diphylobothrium* spp., *Diplogonoporus grandis*, *Diplogonoporus* spp., *Ligula intestinalis*, *Schistocephalus solidus*, *Spirometra mansoni*, *Spirometra* spp.
Cyclophyllidea	Anoplocephalidae		*Bertia* spp., *Bertiella mucronata*, *B. studeri*, *Inermicapsifer cubensis*, *I. madagascariensis*, *Mathevotaenia symmetrica*
	Davaineidae		*Buginetta alouattae*, *Raillietina celebensis*, *R. demerariensis*
	Dipylidiidae		*Dipylidium caninum*
	Hymenolepididae	*Hymenolepis nana*	*Hymenolepis diminuta*
	Mesocestoidae		*Mesocestoides variabilis*, *M. lineatus*
	Taeniidae	*Taenia saginata*, *T. solium*, *Echinococcus granulosus*, *E. multilocularis*	*Taenia taeniaeformis*, *T. multiceps*, *Echinococcus vogeli*, *E. ologarthrus*, *Multiceps* spp.

scolex and a body that consists of "segments" called proglottids. There are 14 orders in the class Cestoda, of which two contain species that infect humans: the Pseudophyllidea, which attach by means of grooves on the sides of the scolex, and the Cyclophyllidea, which attach by means of four cup-like suckers assisted in some cases by hooks. The classification adopted here (Table 4) is based on the one given by Khali (27) and widely used in textbooks. More information about the cestodes that affect humans can be found in chapter 145 of this volume.

CLASSIFICATION OF THE ACANTHOCEPHALAN WORMS

The Acanthocephala, or thorny-headed worms, contains about 1,100 species, all intestinal parasites mainly of fish. Two species are occasional parasites of humans, *Macracanthorhynchus hirudinaceus* and *Moniliformis moniliformis*. Neither is an important pathogen. An outline classification of the acanthocephalan worms that affect humans is shown in Table 5.

The aim of this chapter is to present an outline classification of the parasites that affect humans in order to provide a framework within which particular genera or species can be placed. There are several lists of parasites available in the literature, the most authoritative and comprehensive, but now somewhat dated, is Ashford and Crewe (28); there is also an excellent list of foodborne intestinal parasites by Fried and colleagues (29). There is also a lot of information on various websites, but much of this must be treated with caution. The most useful accounts of the more important parasites, particularly those that occur in the tropics and subtropics, are the 22nd edition of *Manson's Tropical Diseases* (30) and the 10th edition of *Topley and Wilson's Microbiology and Microbial Infections* (12).

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TABLE 5 Classification of the acanthocephalan parasites (phylum Acanthognatha [thorny- or spiny-headed worms]) found in humans

Class	Order	Family	Important species	Other species
Archiacanthocephala (Archaeacanthocephala)	Moniliformida	Moniliformidae	None	*Moniliformis*
	Oligacanthorhynchida	Oligacanthorhynchidae	None	*Macracanthorhynchus hirudinaceus*, *M. ingens*

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Specimen Collection, Transport, and Processing: Parasitology

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Routine diagnostic parasitology generally includes laboratory procedures designed to detect organisms within clinical specimens by using morphological criteria rather than culture, biochemical tools, and/or physical growth characteristics (Table 1). Parasite identification is frequently based on brightfield microscopic analysis of concentrated and/or stained preparations. Small organisms often require high magnification such as with oil immersion (×1,000). Electron microscopy for species characterization of organisms has been replaced in recent years mainly by molecular methods (PCR and sequence analyses of selected genes). Furthermore, new commercial test kits designed especially for detection of fecal antigens (e.g., coproantigens of *Giardia* spp., *Cryptosporidium* spp., and *Entamoeba histolytica* [Table 2] or circulating *Plasmodium* antigens in blood) have expanded the methodological repertoire. In addition to methods for direct parasite detection (morphology, antigens, and DNA), methods for indirect detection of parasite infections demonstrating specific antibodies directed to a variety of native or recombinant parasite antigens have been developed and made commercially available (Table 3). Diagnostic techniques are available to detect a large range of protozoan and helminth species in different clinical specimens. An important precondition for reliable diagnostic results is the proper collection, processing, and examination of clinical specimens. Based on the biology of the parasites and the procedural features of the tests, multiple specimens must often be submitted and examined before the suspected organism(s) is found and its identity is confirmed or a suspected infection can be excluded.

SPECIMEN COLLECTION AND TRANSPORT

Various collection methods are available for specimens suspected of containing parasites or parasitic elements (Tables 4, 5, 6, and 7) (1–7). When collection methods are selected, the decision should be based on a thorough understanding of the value and limitations of each. The final laboratory results are based on parasite recovery and identification and depend on the initial handling of the specimen. Unless the appropriate specimens are properly collected and processed, these infections may not be detected. Therefore, specimen rejection criteria have become much more important for all diagnostic microbiology procedures. Diagnostic laboratory results based on improperly collected specimens may require inappropriate expenditures of time and supplies and mislead the physician. As a part of any continuous quality improvement program for the laboratory, the generation of test results must begin with stringent criteria for specimen acceptance or rejection. In addition, diagnostic laboratories should provide clear information on preanalytical requirements to the physician.

All fresh specimens should be handled carefully, since each specimen represents a potential source of infectious material. Safety precautions should include proper labeling of fixatives; specific areas designated for specimen handling (biological safety cabinets may be necessary under certain circumstances, such as parasite cultures); proper containers for centrifugation; acceptable discard policies; appropriate policies of no eating, drinking, or smoking in work areas; and, if applicable, correct techniques for organism culture and/or animal inoculation. Precautions must be followed when applicable, particularly when blood and other body fluids are being handled (8, 9).

Collection of Fresh Stool

Collection of stool for parasite detection should always be performed before barium is used for radiological examination. Stool specimens containing the opaque, chalky sulfate suspension of barium are unacceptable for examination, and intestinal protozoa may be undetectable for 5 to 10 days after barium is given to the patient. Certain substances and medications also interfere with the detection of intestinal protozoa, including mineral oil, bismuth, antibiotics (metronidazole and tetracyclines), antimalarial agents, and nonabsorbable antidiarrheal preparations. After administration of any of these compounds, parasites may not be recovered for a week to several weeks. Therefore, specimen collection should be delayed for 5 to 10 days or at least 2 weeks after barium or antibiotics, respectively, are administered (1, 2, 10, 11). Some laboratories add the following comment to their negative reports: "Certain antibiotics such as metronidazole or tetracycline may interfere with the recovery of intestinal parasites, particularly the protozoa."

Fecal specimens should be collected in clean, wide-mouthed containers; often, a waxed cardboard or plastic container with a tight-fitting lid is selected for this purpose. The specimens should not be contaminated with water or urine because water may contain free-living organisms that can be mistaken for human parasites, and urine may destroy

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TABLE 1 Body sites and possible parasites recovered[a]

Site	Parasites
Blood	
Red blood cells	*Plasmodium* spp., *Babesia* spp.
Leukocytes	*Leishmania* spp., *Toxoplasma gondii*
Whole blood/plasma	*Trypanosoma* spp., microfilariae
Bone marrow	*Leishmania* spp., *Trypanosoma cruzi*, *Plasmodium* spp.
Central nervous system	*Taenia solium* (cysticerci), *Echinococcus* spp., *Naegleria fowleri*, *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Toxoplasma gondii*, microsporidia[b], *Trypanosoma* spp.
Cutaneous ulcers	*Leishmania* spp., *Acanthamoeba* spp.
Intestinal tract	*Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba coli*, *Entamoeba hartmanni*, *Endolimax nana*, *Iodamoeba bütschlii*, *Blastocystis hominis*, *Giardia duodenalis* (*G. lamblia*, *G. intestinalis*), *Chilomastix mesnili*, *Dientamoeba fragilis*, *Pentatrichomonas hominis*, *Balantidium coli*, *Cryptosporidium* spp., *Cyclospora cayetanensis*, *Cystoisospora belli*, *Enterocytozoon bieneusi*[b], *Encephalitozoon* sppb[c], *Sarcocystis* spp., *Ascaris lumbricoides*, *Enterobius vermicularis*, hookworm, *Strongyloides stercoralis*, *Trichuris trichiura*, *Hymenolepis nana*, *Hymenolepis diminuta*, *Taenia saginata*, *Taenia solium*, *Diphyllobothrium latum*, *Clonorchis sinensis* (*Opisthorchis*), *Paragonimus* spp., *Schistosoma* spp., *Fasciolopsis buski*, *Fasciola hepatica*, *Metagonimus yokogawai*, *Heterophyes heterophyes*
Liver, spleen	*Echinococcus* spp., *Entamoeba histolytica*, *Leishmania* spp., microsporidia[b], *Capillaria hepatica*, *Clonorchis sinensis* (*Opisthorchis*), *Fasciola hepatica*
Lungs	*Cryptosporidium* spp.[c], *Echinococcus* spp., *Paragonimus* spp., *Toxoplasma gondii*, helminth larvae
Muscle	*Trichinella* spp., *Taenia solium* (cysticerci), *Onchocerca volvulus* (nodules), *Trypanosoma cruzi*, microsporidia[b], *Sarcocystis* spp.
Skin	*Leishmania* spp., *Onchocerca volvulus*, microfilariae, *Sarcoptes scabei*, *Dermatobia hominis*, *Tunga penetrans*
Urogenital system	*Trichomonas vaginalis*, *Schistosoma* spp., microsporidia[b], microfilariae
Eyes	*Acanthamoeba* spp., *Toxoplasma gondii*, *Loa loa*, *microsporidia*[b], *Thalezia* spp., *Taenia solium*(cysticercosis)

[a] Parasites include trophozoites, cysts, oocysts, spores, adults, larvae, eggs, and amastigote and trypomastigote stages. This table does not include every possible parasite that could be found in a particular body site. However, the most likely organisms are listed.

[b] Traditionally classified with the protozoa, microsporidia are now known to be more closely related to the fungi.

[c] Disseminated in severely immunosuppressed individuals.

motile organisms. Stool specimen containers should be placed in plastic bags when transported to the laboratory for testing. If postal delivery services are used, any diagnostic specimens must be packed according to national or international rules (e.g., labeling with UN code 3373; three-container approach). Specimens should be identified with the patient's name and identification number, physician's name, and the date and time the specimen was collected. The specimen must also be accompanied by a request form indicating which laboratory procedures should be performed. The presumptive diagnosis or relevant travel history information is helpful and should accompany the test request. In some situations, it may be necessary to contact the physician for additional patient history.

In the past, it has been recommended that a normal examination for stool parasites before therapy include three specimens. Three specimens, collected as outlined above, have also been recommended for posttherapy examinations. However, a patient who has received treatment for a protozoan infection should be checked 3 to 4 weeks after therapy, and those treated for *Taenia* infections should be checked 5 to 6 weeks after therapy. In many cases, the posttherapy specimens are not collected, often as a cost containment measure; if the patient becomes symptomatic again, additional specimens can be submitted (3, 4).

Although some recommend collection of only one or two specimens, there are differences of opinion regarding this approach. It has also been suggested that three specimens be pooled and examined as a single specimen; again, this approach is somewhat controversial. However, physicians should be aware that the probability of detecting clinically relevant parasites in a single stool specimen may be as low as 50 to 60% but is >95% if three samples are examined (4).

If a series of three specimens is collected, they should be submitted on separate days. If possible, the specimens should be submitted every other day; otherwise, the series of three specimens should be submitted within no more than 10 days. If a series of six specimens is requested, the specimens should also be collected on separate days or within no more than 14 days. Many organisms, particularly the intestinal protozoa, do not appear in the stool in consistent numbers on a daily basis, and the series of three specimens is considered the minimum for an adequate examination. Multiple specimens from the same patient should not be submitted on the same day. One possible exception would be a patient who has severe, watery diarrhea, in whom any organisms present might be missed because of a tremendous dilution factor related to fluid loss. These specimens should be accepted only after consultation with the physician. It is also not recommended that the three specimens be submitted one each day for 3 consecutive days; however, use of this collection time frame would not be cause enough to reject the specimens.

To evaluate patients who are at risk for giardiasis, the negative predictive value of some of the immunoassays on a single stool specimen is not sufficiently high to exclude the possibility of a *Giardia* infection. In cases in which the

TABLE 2 Commercially available kits for immunodetection of parasitic organisms or antigens in stool samples[a]

Organism and kit name	Manufacturer and/or distributor	Type of test[b]	Comment(s)[c]
Cryptosporidium spp.			Detects *C. hominis*, different *C. parvum* genotypes, and other species, depending on intensity of the infection
ProSpecT *Cryptosporidium* Microplate Assay	Remel/Thermo Scientific	EIA	Can be used with unpreserved, frozen, or formalin-preserved stool. Stool in Cary-Blair transport medium is also acceptable; http://www.remel.com
Xpect *Cryptosporidium* Kit	Remel/Thermo Scientific	IC	Can be used with unpreserved, frozen, or preserved stool
PARA-TECT *Cryptosporidium*	Medical Chemical Corp.	EIA	Can be used with unpreserved, frozen, SAF-, formalin-, or TOTAL-FIX®-preserved stool; http://www.med-chem.com
Crypto-CELISA	Cellabs	EIA	Can be used with unpreserved, frozen, SAF- or formalin-preserved stool; http://www.cellabs.com.au
Crypto Cel	Cellabs	DFA	Can be used with unpreserved, frozen, or formalin-preserved stool
Cryptosporidium II Test	TechLab/Alere	EIA	Can be used with unpreserved, frozen, SAF- or formalin-preserved stool. Transport media such as Cary-Blair or C&S are also acceptable; http://www.alere.com
Entamoeba histolytica			Differentiates between *E. histolytica* and *E. dispar*
Entamoeba histolytica II Test	TechLab/Alere	EIA	Requires unpreserved or frozen stool
Entamoeba CELISA Path	Cellabs	EIA	Requires unpreserved or frozen stool. Transport media such as Cary-Blair or C&S are acceptable
Entamoeba histolytica/E. dispar group			Does *not* differentiate *E. histolytica* from *E. dispar*
ProSpectT *Entamoeba histolytica*	Remel/Thermo Scientific	EIA	Requires unpreserved or frozen stool. Cary-Blair transport medium is acceptable
Giardia spp.			
ProSpecT *Giardia* ProSpecT *Giardia* EZ	Remel/Thermo Scientific	EIA	Can be used with unpreserved, frozen, SAF-, or formalin-preserved stool
PARA-TECT *Giardia*	Medical Chemical	EIA	Can be used with unpreserved, frozen, SAF-, formalin-, or TOTAL-FIX®-preserved stool
Giardia-CELISA	Cellabs	EIA	Can be used with unpreserved, frozen, SAF-, or formalin-preserved stool
Giardia-Cel	Cellabs	DFA	Can be used with unpreserved, frozen, SAF-, or formalin-preserved stool
Xpect *Giardia*	Remel/Thermo Scientific	Cartridge IC	Can be used with unpreserved, frozen, or formalin-preserved stool
Giardia II	TechLab/ Alere	EIA	Can be used with unpreserved, frozen, or formalin-preserved stool
Combination tests: *Cryptosporidium* and *Giardia*			
ProSpecT *Giardia/Cryptosporidium*	Remel/Thermo Scientific	EIA	Can be used with unpreserved, frozen, or formalin-preserved stool
MERIFLUOR *Cryptosporidium/Giardia*	Meridian Bioscience	DFA	Can be used with unpreserved, frozen, SAF-, or formalin-preserved stool; http://www.meridianbioscience.com
PARA-TECT *Cryptosporidium/Giardia* DFA	Medical Chemical	DFA	Can be used with unpreserved, frozen, SAF-, formalin-, and selected single vial-preserved stool
Crypto/Giardia-Cel	Cellabs	DFA	Can be used with fresh, frozen, or formalin-preserved stool
ImmunoCard STAT! *Cryptosporidium/Giardia*	Meridian Bioscience	Cartridge IC	Can be used with fresh, frozen, or formalin-preserved stool
Xpect Giardia/Cryptosporidium	Remel/Thermo Scientific	IC	Can be used with fresh, frozen, or formalin-preserved stool
Giardia/Cryptosporidium Chek	TechLab/Alere	EIA	Can be used with fresh, frozen, or formalin-preserved stool
Giardia/Cryptosporidium Quick Chek	TechLab/Alere	IC	Can be used with fresh, frozen, or formalin-preserved stool
Triage Parasite Panel	Biosite Diagnostics, Inc.	IC	Requires unpreserved or frozen stool; combination test with *Giardia* and *E. histolytica/E. dispar* group; does not differentiate between *E. histolytica* and *E. dispar*; http://www.alere.com/ww/en.html

[a] This is not a complete listing of all available products but reflects readily available information.
[b] EIA, enzyme immunoassay; DFA, direct fluorescent antibody; IC, immunochromatography.
[c] URLs are given only the first time the company name appears in the table.

TABLE 3 Commercially available kits or antigens for immunodetection of specific serum antibodies[a]

Disease (organism)	Manufacturer and/or distributor[b]	Type of test[c]	Comment(s)
Protozoa			
Toxoplasmosis (*Toxoplasma gondii*)			See chapter 138 for serologic tests.
Amebiasis (*Entamoeba histolytica*)	Bordier Affinity	EIA	Serology is considered the best diagnostic tool for extraintestinal amebiasis. Early extraintestinal infections may be missed (follow-ups recommended); infections with *E. dispar* do not induce detectable antibodies.
	NovaTec	EIA	
	Scimedx Corp.	EIA	
Chagas' disease (*Trypanosoma cruzi*)	Hemagen Diagnostics	EIA	
	InBios	EIA, IC	
		EIA	
	NovaTec	EIA	
	Operon	EIA	
	Oxford Biosystems	EIA	
	Scimedx Corp.		
Leishmaniasis	Bordier Affinity Products	EIA	
	NovaTec	EIA	
	Scimedx Corp.	EIA	
Helminths			
Ascariasis	NovaTec	EIA	
	Oxford Biosystems	EIA	
	Scimedx Corp.	EIA	
Cysticercosis (*Taenia solium*)	NovaTec	EIA	Cross-reactions in cases of other helminthic infections (especially echinococcosis) may occur.
	Scimedx Corp.	EIA	
Schistosomiasis (*Schistosoma* spp.)	Bordier Affinity Products	EIA	
	NovaTec	EIA	
	Oxford Biosystems	EIA	
	Scimedx Corp.	EIA	
Cystic echinococcosis (*Echinococcus granulosus*)	Bordier Affinity Products	EIA	Not species-specific. Cases of alveolar echinococcosis cross-react. False-positive reactions may occur for patients with other helminth infections.
	Launch Diagnostics	EIA	
	Oxford Biosystems	EIA	
Alveolar echinococcosis (*E. multilocularis*)	Bordier Affinity Products	EIA	Screening test for alveolar echinococcosis; cases of cystic echinococcosis may cross-react.
Strongyloidiasis (*Strongyloides stercoralis*)	Bordier Affinity Products	EIA	Cross-reactions in patients with other helminth infections may occur.
	InBios	EIA	
	Scimedx Corp.	EIA	
Toxocariasis (*Toxocara canis*)	Bordier Affinity Products	EIA	
	NovaTec	EIA	
	Scimedx Corp.	EIA	
Trichinellosis (*Trichinella spiralis*)	Oxford Biosystems	EIA	Numerous cross-reactions with other helminth infections may occur.
	Scimedx Corp.	EIA	
Filariasis	Bordier Affinity Products	EIA	
	NovaTec	EIA	

[a] This is not a complete listing of all available products but reflects readily available information.

[b] Bordier Affinity Products (http://www.bordier.ch); Hemagen (http://www.hemagen.com); InBios (http://www.inbios.com); Launch Diagnostics Limited (http://launchdiagnostics.com); NovaTec (http://www.novatec-id.com); Operon (http://operon.es); Oxford Biosystems and Cadama (http://oxfordbiosystems.com); Scimedx Corp. (http://scimedx.com).

[c] Abbreviations: EIA: enzyme immunoassay; IC: rapid immunochromatography (some are dipstick, cartridge, or other rapid test formats).

clinical suspicion for *Giardia* infection is moderate or high and the first assay yields a negative result, testing of a second specimen is recommended (12).

Fresh specimens are mandatory for the recovery of motile trophozoites (amebae, flagellates, or ciliates). The protozoan trophozoite stage is normally found in cases of diarrhea; the intestinal tract contents move through the system too rapidly for cyst formation to occur. Once the stool specimen is passed from the body, trophozoites do not encyst but may disintegrate if not examined or preserved within a short time after passage. However, most helminth eggs and larvae, coccidian oocysts, and microsporidial spores survive for extended periods. Liquid specimens should be examined within 30 min of passage, not 30 min from the time they reach the laboratory. If this general time recommendation of 30 min is not possible, the specimen should be placed in one of the available fixatives (2). Soft (semiformed) specimens may have a mixture of protozoan trophozoites and cysts and should be examined within 1 h of passage; again, if this time frame is not possible, preservatives should be used. Immediate examination of formed specimens is not as critical; in fact, if the specimen is examined any time within 24 h after passage, the protozoan cysts should still be intact.

TABLE 4 Specimen preparation and procedures, recommended stain(s) and relevant parasites, and additional information

Body site	Procedures[a] and specimens	Recommended methods and relevant parasites[a]	Additional information
Blood	Microscopy[b]: thin and thick blood films. Fresh blood (preferred) or EDTA-blood (fill EDTA tube completely with blood and then mix)	Giemsa stain (all blood parasites); hematoxylin-based stain (sheathed microfilariae) Malaria: thick and thin blood films are definitely recommended and should be prepared within 30 to 60 min of blood collection via venipuncture (other tests may be used complementarily)	Most drawings and descriptions of blood parasites are based on Giemsa-stained blood films. Although Wright's stain (or Wright-Giemsa combination stain) works, stippling in malaria may not be visible, and the organisms' colors may not match the descriptions. However, with other stains (those listed previously, in addition to some of the "quick" blood stains), the organisms should be detectable on the blood films.
	Concentration methods: EDTA-blood Antigen detection: EDTA-blood for malaria, serum or plasma for circulating antigens (hemolyzed blood can interact in some tests)	Buffy coat, fresh blood films for detection of moving microfilariae or trypanosomes QBC, a screening method for blood parasites (hematocrit tube contains acridine orange), has been used for malaria, *Babesia*, trypanosomes, and microfilariae. It is usually impossible to identify malaria organisms to the species level; requires high levels of training. Commercial immunoassay test kits for malaria and some microfilariae. Sensitivity is not higher than for thick films for *Plasmodium* spp., much more sensitive for *Leishmania* spp. (peripheral blood is used from immunodeficient patients only).	The use of blood collected with anticoagulant (rather than fresh) has direct relevance to the morphology of malaria organisms seen in peripheral blood films. If the blood smears are prepared after more than 1 h, stippling may not be visible, even if the correct pH buffers are used. Also, if blood is kept at room temperature (with the stopper removed), the male microgametocyte may exflagellate and fertilize the female macrogametocyte, and development continues within the tube of blood (as it would in the mosquito host). The ookinete may actually resemble *Plasmodium falciparum* gametocytes.
	PCR[c]: EDTA-blood, ethanol fixed or unfixed thin and thick blood films, coagulated blood, possibly with hemolyzed or frozen blood samples	Sequencing of PCR product is often used for species or genotype identification.	PCR: so far, no commercial tests are available; high laboratory standards are needed (may work with frozen, coagulated, or hemolyzed blood samples).
	Specific antibody detection: serum or plasma, anticoagulated or coagulated blood (hemolyzed blood can cause problems in some tests)	Most commonly used are EIA (many test kits commercially available), EITB (commercially available for some parasites), and IFA.	Many laboratories use in-house tests; only a few fully defined antigens are available; sensitivities and specificities of the tests should be documented by the laboratory.
Bone marrow	Biopsy samples or aspirates Microscopy: thin and thick films with aspirate collected in EDTA Cultures: sterile material in EDTA or culture medium PCR: aspirate in EDTA	Giemsa stain (all blood parasites) Culture for *Leishmania* spp. (or *Trypanosoma cruzi*) PCR for blood parasites, including *Leishmania* and *Toxoplasma* spp. and other rare parasites	*Leishmania* spp. amastigotes are recovered in cells of the reticuloendothelial system; if films are not prepared directly after sample collection, infected cells may disintegrate. Sensitivity of microscopy is low; use only in combination with other methods.
Central nervous system	Microscopy: spinal fluid and CSF (wet examination, stained smears), brain biopsy (touch or squash preparations, stained)	Stains: Giemsa (trypanosomes, *Toxoplasma* spp.); Giemsa, trichrome, or calcofluor (amebae [*Naegleria* spp.-PAM, *Acanthamoeba* spp. or *Balamuthia* spp.-GAE]); Giemsa, acid-fast, PAS, modified trichrome, silver methenamine (microsporidia) (tissue Gram stains also recommended for microsporidia in routine histologic preparations); H&E, routine histology (larval cestodes, *Taenia solium* [cysticerci], *Echinococcus* spp.)	If CSF is received (with no suspect organism suggested), Giemsa is the best choice; however, modified trichrome or calcofluor is also recommended as a second stain (amebic cysts, microsporidia). If brain biopsy material is received (particularly from an immunocompromised patient), PCR is recommended for diagnosis and identification to the species or genotype level.

(Continued on next page)

TABLE 4 Specimen preparation and procedures, recommended stain(s) and relevant parasites, and additional information

Body site	Procedures[a] and specimens	Recommended methods and relevant parasites[a]	Additional information
	Culture: sterile aspirate or biopsy material (in physiological saline)	Free-living amebae (exception: *Balamuthia* spp. do not grow in the routine agar/bacterial overlay method). *Toxoplasma* spp. can be cultured in tissue culture media.	A small amount of the sample should always be stored frozen for PCR analyses in case the results of the other methods are inconclusive.
	PCR: aspirate or biopsy material, native, frozen, or fixed in ethanol	Protozoa and helminths; species and genotype characterization	
Cutaneous ulcers	Microscopy: aspirate, biopsy material (smears, touch or squash preparations, histologic sections)	Giemsa (*Leishmania* spp.); H&E, routine histology (*Acanthamoeba* spp., *Entamoeba histolytica*)	Most likely causative parasites would be *Leishmania* spp., which stain with Giemsa. PAS could be used to differentiate *Histoplasma capsulatum* from *Leishmania* spp. in tissue. Sensitivity of microscopy may be low.
	Cultures (less common)	*Leishmania* spp., free-living amebae (often bacterial contaminations)	
	PCR: aspirate, biopsy material, native, frozen, or fixed in ethanol	*Leishmania* spp.(species identification), free-living amebae	In immunocompromised patients, skin ulcers have been documented with amebae as causative agents. Cultures of material from cutaneous ulcers may be contaminated with bacteria; PCR would be the method of choice.
Eye	Microscopy: biopsy material (smears, touch or squash preparations), scrapings, contact lens, sediment of lens solution Culture: native material (see above) in PBS supplemented with antibiotics if possible to avoid bacterial growth PCR: native material in physiological NaCl or PBS, ethanol, or frozen	Calcofluor (*Acanthamoeba* spp. cyst only); Giemsa (amebic trophozoites, cysts); modified trichrome (preferred) or silver methenamine stain, PAS, acid-fast (microsporidial spores); H&E, routine histology (cysticerci, *Loa loa*, *Toxoplasma* spp.) Cultures: free-living amebae and *Toxoplasma* spp. Free-living amebae, *Toxoplasma*, microsporidial species and genotype identification	Some free-living amebae (most commonly *Acanthamoeba*) have been implicated as a cause of keratitis. Although calcofluor stains the cyst walls, it does not stain the trophozoites. Therefore, in suspected cases of amebic keratitis, both stains should be used. H&E (routine histology) can be used to detect and confirm cysticercosis. The adult worm of *Loa loa*, when removed from the eye, can be stained with a hematoxylin-based stain (Delafield's) or can be stained and examined by routine histology. Microsporidial confirmation to the species or genotype level is done by PCR and sequence analyses; however, the spores can be found by routine light microscopy with modified trichrome and/or calcofluor stain. Sensitivity of microscopic methods may be low.
Intestinal tract	Stool and other intestinal material Microscopy: stool, sigmoidoscopy material, duodenal contents (all fresh or preserved (see Table 4), direct wet smear, concentration methods Anal impression smear Adult worms or tapeworm segments (proglottids)	Concentration methods: formalin-ethyl acetate sedimentation of formalin- or SAF-fixed stool samples (most protozoa); flotation or combined sedimentation flotation methods (helminth ova); agar or Baermann concentration (larvae of *Strongyloides* spp.; unpreserved stool required) Direct wet smear (direct examination of unpreserved fresh material is also used); (motile protozoan trophozoites; helminth eggs and protozoan cysts may also be detected) Stains: trichrome or iron hematoxylin (intestinal protozoa); modified trichrome (microsporidia); modified acid-fast (*Cryptosporidium* spp., *Cyclospora* spp., *Cystoisospora* spp.)	Stool fixation with formalin or formalin-containing fixatives preserves trophozoite morphology, allows prolonged storage (room temperature) and long transportation, and prevents hatching of *Schistosoma* spp. eggs but makes *Strongyloides* larval concentration impossible and impedes further PCR analyses Taeniid eggs cannot be identified to the species level. Microsporidia: confirmation to the species or genotype level requires PCR; however, modified trichrome and/or calcofluor stain can be used to confirm the presence of spores.

(Continued on next page)

TABLE 4 Specimen preparation and procedures, recommended stain(s) and relevant parasites, and additional information

Body site	Procedures[a] and specimens	Recommended methods and relevant parasites[a]	Additional information
		Adhesive cellulose tape, no stain (*Enterobius vermicularis*)	
		Carmine stains (rarely used for adult worms or cestode segments). Proglottids can usually be identified to the genus level (*Taenia* spp., *Diphyllobothrium* spp., *Hymenolepis* spp.) without using tissue stains	Four to six consecutive negative tapes are required to rule out infection with pinworm (*Enterobius vermicularis*). Worm segments can be stained with special stains. However, after dehydration through alcohols and xylenes (or xylene substitutes), the sexual organs and the branched uterine structure are visible, allowing identification of the proglottid to the species level.
	Antigen detection: fresh native or frozen material; suitability of fixation is test dependent PCR: native material, fresh, frozen or ethanol-fixed	Commercial immunoassays (Table 2), e.g., EIA, FA, cartridge formats (*Entamoeba histolytica*, the *Entamoeba histolytica*/*E. dispar* group, *Giardia* spp., and *Cryptosporidium* spp.). In-house tests for *Taenia solium* and *Taenia saginata*.	Coproantigens can be detected in the prepatent period and independently from egg excretion. Due to potential inhibition after DNA extraction from stool samples, concentration or isolation methods may be required before DNA extraction. However, new DNA isolation kits facilitate isolation of high-quality DNA from stool. Sequence analyses may be required for species or genotype identification.
	Biopsy material Microscopy: fixed for histology or touch or squash preparations for staining PCR: see above	No commercial tests available. Primers for genus or species identification of most helminths and protozoa are published. H&E, routine histology (*Entamoeba histolytica*, *Cryptosporidium* spp., *Cyclospora* spp., *Cystoisospora* spp., *Giardia* spp., microsporidia); less common findings include *Schistosoma* spp., hookworm, or *Trichuris* spp.	Special stains may be helpful for the identification of microsporidia (tissue Gram stains, silver stains, PAS, and Giemsa) or coccidia (modified acid-fast stain).
Liver and spleen	Biopsy samples or aspirates Microscopy: unfixed material in physiological NaCl; fixed for histology Culture: sterile preparation of native material	Examination of wet smears for *Entamoeba histolytica* (trophozoites), protoscolices of *Echinococcus* spp. or eggs of *Capillaria hepatica*. Giemsa stain (*Leishmania* spp., other protozoa and microsporidia); H&E (routine histology)	Definite risks are associated with punctures (aspirates and/or biopsy) of spleen or liver lesions (*Echinococcus* spp). Always keep a small amount of material frozen for PCR.
	Animal inoculation: sterile preparation of native material PCR: native material, frozen or ethanol fixed	For *Leishmania* (not common) Intraperitoneal inoculation of *E. multilocularis* cyst material for viability test after long-term chemotherapy Species or genotype identification (e.g., *Echinococcus* spp.)	
Respiratory tract	Sputum, induced sputum, nasal and sinus discharge, bronchoalveolar lavage fluid, transbronchial aspirate, tracheobronchial aspirate, brush biopsy sample, open-lung biopsy sample Microscopy: unfixed material, treated for smear preparation PCR: unfixed native material, frozen or fixed in ethanol	Helminth larvae (*Ascaris* spp., *Strongyloides* spp.), eggs (*Paragonimus* spp., *Capillaria* spp.), or hooklets (*Echinococcus* spp.) can be recovered in unstained respiratory specimens. Stains: Giemsa for many protozoa, including *Toxoplasma* spp., tachyzoites, modified acid-fast stains (*Cryptosporidium* spp.); modified trichrome (microsporidia) Routine histology (H&E; silver methenamine stain, PAS, acid-fast, tissue Gram stains for helminths, protozoa, and microsporidia)	Immunoassay reagents (FA) are available for diagnosis of pulmonary cryptosporidiosis. Routine histologic procedures allow identification of any of the helminths or helminth eggs in the lung. Disseminated toxoplasmosis and microsporidiosis are well documented, with organisms being found in many different respiratory specimens.

(Continued on next page)

TABLE 4 Specimen preparation and procedures, recommended stain(s) and relevant parasites, and additional information

Body site	Procedures[a] and specimens	Recommended methods and relevant parasites[a]	Additional information
Muscle	Biopsy material Microscopy: unfixed, touch and squash preparations or fixed for histology and EM PCR: unfixed or native, frozen or ethanol fixed	Larvae of *Trichinella* spp. can be identified unstained (species identification with single larvae by PCR). H&E, routine histology (*Trichinella* spp., cysticerci); silver methenamine stain, PAS, acid-fast, tissue Gram stains, EM (rare microsporidia). Microsporidial identification to the species level requires subsequent sequencing.	If *Trypanosoma cruzi* organisms are present in the striated muscle, they could be identified in routine histology preparations. Modified trichrome and/or calcofluor stain can be used to confirm the presence of microsporidial spores. Larvae of *Trichinella* spp. may be detected in heavy infections only. Biopsies are not recommended as standard procedures.
Skin	Aspirates, skin snips, scrapings, biopsy samples	See Cutaneous ulcer (above)	Any of the parasites can be identified by routine histology procedures, but the sensitivities of these methods may be low.
	Microscopy: wet examination, stained smear (or fixed for histology or EM) PCR: unfixed native, frozen or fixed in ethanol	Wet preparations (microfilariae), Giemsa-stained smears or H&E, routine histology (*Onchocerca volvulus*, *Dipetalonema streptocerca*, *Dirofilaria repens*, other larvae causing cutaneous larva migrans [zoonotic *Strongyloides* spp., hookworms], *Leishmania* spp., *Acanthamoeba* spp., *Entamoeba histolytica*, microsporidia, and arthropods [*Sarcoptes scabiei* and other mites]) Primers for most parasite species available	
Amniotic fluid	PCR (and/or culture): native material Animal inoculation (*Toxoplasma*)	PCR based on the detection of highly repetitive gene sequences is the method of choice.	Only applicable to confirm suspected prenatal *Toxoplasma* spp. infections
Urogenital system	Vaginal discharge, saline swab, transport swab (no charcoal), air-dried smear for FA, urethral discharge, prostatic secretions, urine (single unpreserved, 24-h unpreserved, or early-morning specimen). Microscopy: wet smears, smears of urine sediment, stained smears Cultivation: vaginal or urethral discharge or swab preparations Molecular: native material, frozen or fixed in ethanol	Giemsa, immunoassay reagents (*Trichomonas vaginalis*); Delafield's hematoxylin (microfilariae); modified trichrome (microsporidia); H&E, routine histology, PAS, acid-fast, tissue Gram stains (microsporidia). Direct examination of urine sediment for *Schistosoma haematobium* eggs or microfilariae Identification and propagation of *T. vaginalis* (commercial plastic envelope culture systems available); moving trophozoites can be detected microscopically (or in Giemsa-stained smears)	Although *T. vaginalis* is probably the most common parasite identified, there are others to consider, the most recently implicated organisms being in the microsporidial group. Microfilariae could also be recovered and stained. Fixation of urine with formalin prevents hatching of *Schistosoma* eggs. Material must be put into culture medium immediately after collection; do not cool or freeze.

[a] CSF, cerebrospinal fluid; EIA, enzyme immunoassay; EITB, enzyme-linked immunoelectrotransfer blot (Western blot); EM, electron microscopy; FA, fluorescent antibody; GAE, granulomatous amebic encephalitis; GI, gastrointestinal; H&E, hematoxylin and eosin; IFA, indirect immunofluorescence assay; PAM, primary amebic encephalitis; PAS, periodic acid-Schiff stain; PBS, phosphate-buffered saline; QBC, quantitative buffy coat.

[b] Many parasites or parasite stages may be detected in standard histologic sections of tissue material. However, species identification is difficult, and additional examinations may be required. Usually, these techniques are not considered first-line methods. Additional methods like EM are carried out only by specialized laboratories and are not available for standard diagnostic purposes. EM examination for species identification has largely been replaced by PCR.

[c] Material/specimens suitable for PCR: native (unfixed), in saline, PBS (ethanol), or frozen; avoid formalin.

Preservation of Stool

If there are delays from the time of specimen passage until examination in the laboratory, the use of stool fixative should be considered. To preserve protozoan morphology and prevent the continued development of some helminth eggs and larvae, the stool specimens can be placed in fixative either immediately after passage (by the patient, using a collection kit) or once the specimen is received by the laboratory. Several fixatives are available; however, regardless of the fixative selected, adequate mixing of the specimen and preservative is mandatory. Specimens preserved in stool fixatives should be stored at room temperature. It is also important to use the correct ratio of stool and fixative to ensure proper fixation. If commercial vials are used, they

TABLE 5 Fecal specimens for parasites: options for collection and processing

Options	Pros	Cons
Although physicians can order stools for parasitology when deemed appropriate, many laboratories include in their protocols the rejection of stools from inpatients who have been in-house for >3 days.	Patients may become symptomatic with diarrhea after they have been inpatients for a few days; symptoms are usually not attributed to parasitic infections but are generally due to other causes.	There is always a chance that the problem is related to a nosocomial parasitic infection (rare), but *Cryptosporidium* spp., *Giardia* spp., and microsporidia may be possible considerations.
Examination of a single stool (O&P examination)	If parasites are diagnosed in the first sample or if the patient becomes asymptomatic after collection of the first stool, subsequent specimens may not be necessary. However, with some intestinal parasitic infections, patients may alternate with constipation and diarrhea.	Diagnosis from examination of a single stool specimen depends on the parasite load in the specimen. Of organisms present, 40 to 60% are found with only a single stool exam; two O&P examinations are acceptable, but three specimens are more sensitive; any patient remaining symptomatic would require additional testing. In a series of three stool specimens, frequently not all three specimens are positive and/or may be positive for different organisms (may be a cost-effective approach).
Examination of a second stool specimen only after the first one is negative and the patient is still symptomatic	With additional examinations, yield of protozoa increases (*Entamoeba histolytica*, 22.7%; *Giardia* spp., 11.3%; and *Dientamoeba fragilis*, 31.1%).	Assumes that the second (or third) stool specimen is collected within the recommended 10-day time frame for a series of stools; protozoa are shed periodically. May be inconvenient for the patient, and the correct diagnosis might be delayed.
Examination of a single stool and a *Giardia* immunoassay	If the examinations are negative and the patient's symptoms subside, further testing is likely not required.	Patients may exhibit symptoms (off and on), so it may be difficult to rule out parasitic infections with only a single stool and immunoassay. If the patient remains symptomatic, even if the *Giardia* immunoassay is negative, other protozoa may be missed (*Cryptosporidium* spp., *E. histolytica*/*E. dispar*, and *D. fragilis*).
Pooling of three specimens for examination; one concentration and one permanent stain are performed. The laboratory pools the specimens.	Three specimens are collected over 7–10 days, which may save time and expense.	Procedure not recommended. Decreases strongly the sensitivity of the procedure; organisms present in low numbers may be missed due to the dilution factor.
Permanent stained smears are performed, one from each of the three specimens; subsequently, three specimens are pooled for a single concentration on the pooled specimen.	Three specimens are collected over 7–10 days; would maximize recovery of protozoa in areas of the United States where these organisms are most common.	Might miss light helminth infection (eggs and larvae) due to the pooling procedure.
Three stool specimens are collected, but samples of stool from all three are put into a single vial (patient is given a single vial only).	Pooling of the specimens would require only a single vial.	Absolutely not recommended. Lack of sensitivity; proper mixing of specimen and fixative complicates patient collection and depends on patient compliance.
Immunoassays are performed only for selected patients[a] (children <5 yr old, children from day care centers, patients with immunodeficiencies, and patients from diarrheal outbreaks) for intestinal protozoa.	Would be more cost-effective than performing immunoassay procedures on all specimens	The competence and the information needed to group patients are often not available in the laboratory. Ordering guidelines for clients are highly recommended (see Table 7).

[a] It is difficult to recognize an early outbreak situation in which screening of all specimens for *Giardia* spp., *Cryptosporidium* spp., or both may be relevant. If it appears that an outbreak is in the early stages, performing the immunoassays on request can be changed to screening all stools.

are marked with a "fill-to" line for the addition of stool to the container.

When selecting an appropriate fixative, keep in mind that a permanent stained smear is mandatory for a complete examination for parasites (1, 2, 9, 10, 13; chapter 134). Make sure that the fixative you are using is compatible with the kit or the method you have selected. It is also important to remember that disposal regulations for compounds containing mercury are becoming stricter; each laboratory must check applicable regulations to help determine fixative options.

Formalin

Formalin is an all-purpose fixative appropriate for helminth eggs and larvae and for protozoan cysts. Two concentrations are commonly used: 5%, which is recommended for preservation of protozoan cysts, and 10%, which is recommended for helminth eggs and larvae. Although 5% is often recommended for all-purpose use, most commercial manufacturers

TABLE 6 Approaches to stool parasitology: test ordering[a]

Patient and/or situation	Test ordered	Follow-up test ordered
Patients with diarrhea and HIV or other cause of immune deficiency Potential waterborne outbreak from (municipal) water supply	*Cryptosporidium* or *Giardia/Cryptosporidium* immunoassay[b,c] (see Table 2)	If immunoassays are negative and symptoms continue, special tests for microsporidia (modified trichrome stain) and coccidia (modified acid-fast stain) and O&P examination should be performed.
Patient with diarrhea (nursery school, day care center, camper or backpacker) Patient with diarrhea and potential waterborne outbreak (resort setting) Patient from area in the United States where *Giardia* is the most common organism found	*Giardia* or *Giardia/Cryptosporidium* immunoassay[b,c]	If immunoassays are negative and symptoms continue, special tests for microsporidia and coccidia and O&P examination should be performed.
Patient with diarrhea and relevant travel history Patient with diarrhea who is a past or present resident of a developing country Patient in area of the United States where parasites other than *Giardia* spp. are found	O&P examination, the *Entamoeba histolytica/E. dispar* group immunoassay, confirmation for *E. histolytica*, *Cryptosporidium* or *Giardia/Cryptosporidium* immunoassay[b,c], test for *Strongyloides stercoralis* (even in the absence of eosinophilia)	If examinations are negative and symptoms continue, special tests for coccidia and microsporidia should be performed.
Patient with unexplained eosinophilia (may be low or high); may or may not have diarrhea; may be immunocompromised, often due to receipt of steroids; patient may present with hyperinfection and severe diarrhea (symptoms may also include pneumonia and/or episodes of sepsis and/or meningitis).	O&P examination with emphasis on helminths (unfixed stool, for sedimentation/flotation techniques); it is very important to make sure that an infection with *S. stercoralis* has been ruled out—particularly if the patient is immunosuppressed or may become immunosuppressed from therapy, etc. (unfixed stool, Baermann concentration or agar plate culture).	If the O&P examinations are negative, agar plate cultures for *S. stercoralis* are recommended, particularly if the history is suggestive for this infection. The serological detection of specific antibody is an additional diagnostic option.
Patient with diarrhea (suspected foodborne outbreak)	Test for *Cyclospora cayetanensis* (modified acid-fast stain)	If test is negative and symptoms continue, special procedures for microsporidia and other coccidia and O&P examination should be performed.

[a] Modified from reference 2.

[b] Depending on the particular immunoassay kit used, various single or multiple organisms may be included. Selection of a particular kit depends on many variables such as clinical relevance, cost, ease of performance, training, personnel availability, number of test orders, training of physician clients, sensitivity, specificity, equipment, and time to result. Very few laboratories handle this type of testing in exactly the same way. Many options are clinically relevant and acceptable for patient care.

[c] Two stool specimens should be tested using an immunoassay in order to rule out an infection with *Giardia* spp.; fecal immunoassays may also be negative in cases with a low parasite load.

provide 10%, which is more likely to kill all helminth eggs. To help maintain organism morphology, the formalin can be buffered with sodium phosphate buffers (i.e., neutral formalin). Selection of specific formalin formulations is at the user's discretion. Aqueous formalin permits the examination of the specimen as a wet mount only, a technique much less accurate than a stained smear for the identification of intestinal protozoa.

Protozoan cysts (not trophozoites), coccidian oocysts, helminth eggs, and larvae are well preserved for long periods in 10% aqueous formalin. Formalin heated to 60°C can be used for specimens containing helminth eggs, since in cold formalin, some thick-shelled eggs may continue to develop, become infective, and remain viable for long periods. Several grams of fecal material should be thoroughly mixed in 5% or 10% formalin (ratio, 1:10).

Formaldehyde vapor concentrations must be monitored and maintained at concentrations below the 8-hour time-weighted average. In the United States, these limits are 0.75 ppm and the 15-minute short-term exposure limit (i.e., 2.0 ppm) (1). However, these limits may vary from country to country. Generally, the amount of formaldehyde used in microbiology is quite small; laboratory monitoring values are usually well below the required maximum concentrations. Initial monitoring must be repeated any time there is a change in production, equipment, process, personnel, or control measures that may result in new or additional exposure to formaldehyde. Stool fixatives that contain formaldehyde indicate that fact on the label. A number of single-vial fixative collection systems are now available; although they may not contain formaldehyde, the actual formulas are proprietary.

Sodium Acetate-Acetic Acid-Formalin (SAF)

Both the concentration and the permanent stained smear can be performed with specimens in SAF. It is a liquid fixative, much like the 10% formalin described previously. The sediment is used to prepare the permanent smear, and it is frequently recommended that the stool material be placed on an albumin-coated slide to improve adherence to the glass (14, 15).

TABLE 7 Fecal fixatives: pros and cons

Stool preservative	Pros	Cons
Formalin	Good overall fixative for stool concentration. Easy to prepare; long shelf life. Concentrated sediment can be used with different stains[a] but not with all immunoassays (see Table 5).	Does not preserve trophozoites well. Does not adequately preserve organism morphology for a good permanent stained smear; not optimal for all immunoassays; not appropriate for molecular diagnosis (PCR).
SAF	Can be used for concentration and permanent stained smears. Contains no mercury compounds. Easy to prepare; long shelf life. Concentrated sediment can be used with most of the new immunoassay methods and special stains.	Poor adhesive properties; albumin-coated slides recommended. Protozoan morphology is better if iron hematoxylin stain is used for permanent stained smears (trichrome is not quite as good). May be a bit more difficult to use; however, this is really not a limiting factor. Not appropriate for molecular diagnosis (PCR).
Schaudinn's fixative (mercury base)	Fixation of slides prepared from fresh fecal specimens or samples from the intestinal mucosal surfaces. Provides excellent fixation of protozoan trophozoites and cysts.	Not generally recommended for concentration procedures. Contains mercuric chloride; creates disposal problems. Poor adhesive qualities with liquid or mucoid specimens.
PVA (mercury base)	Can prepare permanent stained smears and perform concentration techniques (less common). Provides excellent fixation of protozoan trophozoites and cysts. Specimens can be shipped to the laboratory for subsequent examination; organism morphology excellent after processing. Suitable for PCR analysis. This formulation is considered the gold standard against which all other fixatives are evaluated (organism morphology after permanent staining).	*Trichuris trichiura* eggs and *Giardia* spp. cysts are not concentrated as easily as from formalin-based fixative. *Strongyloides stercoralis* larval morphology is poor (better from formalin-based preservation). *Cystoisospora belli* oocysts may not be visible from PVA-fixed material (better from formalin-based preservation). Contains mercury compounds and may pose a disposal problem (chemical waste). May turn white and gelatinous when it begins to dehydrate or when refrigerated. Difficult to prepare in the laboratory. Specimens containing PVA cannot be used with the immunoassay methods.
Modified PVA (copper or zinc base)	Can prepare permanent stained smears and perform concentration techniques (less common). Many workers prefer the zinc substitutes over those prepared with copper sulfate. Does not contain mercury compounds.	Overall protozoan morphology of trophozoites and cysts is poor when they are fixed in modified PVA, compared with organisms fixed in mercuric chloride-based fixatives. Zinc-based fixatives appear to be some of the better alternatives. Staining characteristics of protozoa are not consistent; some are good, and some are poor. Organism identification may be difficult, particularly with small protozoan cysts (*Endolimax nana*).
Single-vial systems (with or without PVA)	Can prepare permanent stained smears and perform concentration techniques. Can perform immunoassays. May not contain formalin or mercury compounds. Unless organism numbers are rare, acceptable organism recovery and identification are possible; additional training may be required to recognize the organisms because the overall morphology is not comparable to that seen with mercury-based fixatives. Concentration, permanent stains, some immunoassays, and some molecular testing can be performed.	Overall protozoan morphology of trophozoites and cysts is not as good as that of organisms fixed with mercuric chloride-based fixative; similar to modified PVA options. Staining characteristics of protozoa are not consistent; some are good, and some are poor. Identification of *Endolimax nana* cysts may be difficult. Not all immunoassays can be performed from stool specimens in these fixatives. However, in spite of the cons, single-vial systems are becoming more widely used for concentrations, permanent stained smears, and fecal immunoassays.

[a] Modified acid-fast and modified trichrome stains.

SAF is considered to be a "softer" fixative than mercuric chloride. The organism morphology is not quite as sharp after staining as that of organisms originally fixed in solutions containing mercuric chloride. The pairing of SAF-fixed material with iron hematoxylin staining provides better organism morphology than does staining of SAF-fixed material with trichrome (personal observation). Although SAF has a long shelf life and is easy to prepare, the smear preparation technique may be a bit more difficult for less experienced personnel who are not familiar with fecal specimen techniques. Laboratories that have considered using only a single preservative have selected this option. Helminth eggs and larvae, protozoan trophozoites and cysts, coccidian oocysts, and microsporidian spores can be recovered using this method.

Schaudinn's Fluid

Schaudinn's fluid containing mercuric chloride can be used with fresh stool specimens or samples from the intestinal mucosal surface. Many laboratories that receive specimens from in-house patients (with no delay in delivery times) often select this approach. Permanent stained smears are then prepared from fixed material. A concentration technique using Schaudinn's fluid-preserved material is also available but is not widely used (1, 10, 11).

Polyvinyl Alcohol (PVA)

PVA is a plastic resin that is normally incorporated into Schaudinn's fixative. The PVA powder serves as an adhesive for the stool material (i.e., when the stool-PVA mixture is spread onto the glass slide, it adheres because of the PVA component). Fixation is still accomplished by Schaudinn's fluid itself. Perhaps the greatest advantage in the use of PVA is that a permanent stained smear can be prepared. Like SAF, PVA fixative solution is recommended as a means of preserving cysts and trophozoites for later examination and permits specimens to be shipped (by regular mail service) from any location in the world to a laboratory for subsequent examination. PVA is particularly useful for liquid specimens and should be used in the ratio of 3 parts PVA to 1 part fecal specimen (16).

Modified Polyvinyl Alcohol

Although fixatives that do not contain mercury compounds have been developed, some of the substitute compounds have not provided the quality of fixation necessary for good protozoan morphology on the permanent stained smear. Copper sulfate has been tried but does not provide results equal to those seen with mercuric chloride (17). Zinc sulfate has proven to be an acceptable mercury substitute and is used with trichrome stain. Substitutes containing zinc have become widely available; each manufacturer has a proprietary formula for the fixative (18, 19).

Single-Vial Collection Systems

Several manufacturers now have available single-vial stool collection systems, similar to SAF or modified PVA methods. From the single vial, both the concentration and permanent stained smear can be prepared. It is also possible to perform immunoassays from some of these vials. Ask the manufacturer about all diagnostic capabilities (concentrate, permanent stained smear, immunoassay procedures, and molecular analyses) and for specific information indicating that there are no formula components that would interfere with any of the methods. Like the zinc substitutes, these formulas are proprietary (19). New formulations continue to be developed, and some of them appear to adhere to the slide without the use of albumin or PVA. These formulations can be used to perform concentrations and some of the immunoassays and to prepare permanent stained smears. One commercially available single-vial fixative of stool specimens is mercury-, formalin- and PVA-free, preserves morphology, and alleviates many disposal and monitoring problems encountered by laboratories.

Collection of Blood

Depending on the life cycle, a number of parasites may be recovered in a blood specimen, either whole blood, buffy coat preparations, or various types of concentrations (2, 10, 11). These parasites include *Plasmodium* spp., *Babesia* spp., *Trypanosoma* spp., *Leishmania* spp., *Toxoplasma* spp., and microfilariae. Although some organisms may be motile in fresh, whole blood, species identification is normally accomplished from the examination of permanent stained blood thin and/or thick films. Blood films can be prepared from fresh, whole blood collected with no anticoagulants, anticoagulated blood, or sediment from the various concentration procedures.

Unless it is certain that well-prepared slides will be available, it is necessary to request a tube of fresh blood (EDTA anticoagulant is preferred) and prepare the smears. The tube should be filled with blood to provide the proper blood/anticoagulant ratio. For detection of stippling, the smears should be prepared within 1 h after the specimen is drawn. After that time, stippling may not be visible on stained films; however, the overall organism morphology will still be excellent. Most laboratories routinely use commercially available blood collection tubes; preparation of EDTA collection tubes in-house is neither necessary nor cost-effective.

The time the specimen was drawn should be clearly indicated on the tube of blood and also on the result report. The physician will then be able to correlate the results with any fever pattern (most likely seen in a semi-immune patient with past exposure to malaria with antibodies) or other symptoms that the patient may have. In immunologically naive patients or travelers with no previous exposure to malaria, there may be no periodicity at all; symptoms will mimic many other infections or medical problems. There should also be comments on the test result report sent back to the physician stating that one negative specimen does not rule out the possibility of a parasitic infection (1, 20).

Collection of Specimens from Other Body Sites

Although clinical specimens for examination can be obtained from many other body sites, these specimens and appropriate diagnostic methods are not as commonly performed as those used for the routine stool specimen or for blood specimens (21, 22). Most specimens from other body sites (Table 1) are submitted as fresh specimens for further testing.

DIRECT DETECTION BY ROUTINE METHODS

Intestinal Tract Specimens

The most common specimen submitted to the diagnostic laboratory is the stool specimen, and the most commonly performed procedure in parasitology is the ova and parasite (O&P) examination, which is composed of three separate protocols: the direct wet mount, the concentration, and the permanent stained smear. The direct wet mount requires fresh stool, is designed to allow the detection of motile protozoan trophozoites, and is examined microscopically at low and high dry magnifications (×100, entire 22- by 22-mm coverslip [larvae, larger helminth eggs]; ×400, one-third to one-half of a 22- by 22-mm coverslip [protozoan cysts and/or trophozoites, smaller helminth eggs]). If the specimens are received in the laboratory in stool collection fixatives, the direct wet preparation may be eliminated from the routine O&P examination.

The second part of the O&P examination is the concentration, which is designed to facilitate the recovery of protozoan cysts, coccidian oocysts, microsporidial spores, and helminth eggs and larvae. Both flotation (zinc sulfate, zinc chloride, and others) and sedimentation methods are available, the most common procedure being the formalin-ethyl acetate sedimentation method (formerly called the formalin-ether method). The concentrated specimen is examined as a wet preparation, with or without iodine (personal preference), using low and high dry magnifications (×100 and ×400) as indicated for the direct wet smear examination.

It is important to remember that large, heavy helminth eggs (unfertilized *Ascaris* eggs) or eggs that are operculated (trematode and some cestode eggs) do not float optimally when flotation fluids with densities of <1.35 are used; both the surface film and sediment must be examined by this method. It is also important to note that the flotation media with high densities may change the morphological characteristics of some parasites.

The third part of the O&P examination is the permanent stained smear, which is designed to facilitate the identification of intestinal protozoa. Several staining methods are available, the two most common being the Wheatley modification of the Gomori's tissue trichrome and the iron hematoxylin stains. This part of the O&P examination is critical for the confirmation of suspicious objects seen in the wet examination and identification of protozoa that might not have been seen in the wet preparation. The permanent stained smears are examined using oil immersion objectives (×600 for screening and ×1,000 for final review of ≥300 oil immersion fields).

Other specimens from the intestinal tract such as duodenal aspirates or drainage, mucus from the Entero-Test capsule technique, and sigmoidoscopy material can also be examined as wet preparations and as permanent stained smears after processing with either trichrome or iron hematoxylin staining. Adult and pediatric Entero-Test capsules are currently available from Nutri-Link Ltd., Newton Abbot, United Kingdom (www.nutri-linkltd.co.uk). Although not all laboratories examine these types of specimens, they are included to give some idea of the possibilities for diagnostic testing.

Amniotic Fluid

Methods for the diagnosis of congenital *Toxoplasma* infection are summarized in chapter 138. Amniotic fluid collected under sterile conditions allows both mouse or tissue culture inoculation and molecular diagnosis by PCR. PCR is the recommended test of choice for the detection of *Toxoplasma* in amniotic fluid.

Urogenital Tract Specimens

The identification of *Trichomonas vaginalis* is usually based on the microscopic examination of wet preparations of vaginal and urethral discharges and prostatic secretions or urine sediment. Multiple specimens may have to be examined before the organisms are detected. These specimens are diluted with a drop of saline and examined under low power (×100) and reduced illumination for the presence of motile organisms; as the jerky motility begins to diminish, it may be possible to observe the undulating membrane, particularly under high dry power (×400). Culture systems (InPouch TV; BioMed Diagnostics, San Jose, CA) that allow direct inoculation, transport, culture, and microscopic examination are available commercially (1, 23). Rapid tests that allow detection of *T. vaginalis* antigens and molecular testing kits (e.g., DNA probes, nucleic acid amplification methods) are also commercially available.

Stained smears such as Papanicolaou- or Giemsa-stained smears can be used, but they are usually not necessary for the identification of this organism and can be difficult to interpret. If a dry smear is received by the laboratory, it can be fixed with absolute methanol and stained with Giemsa stain; this approach is not optimal, but the smear can be examined and may confirm a positive infection. The number of false-positive and false-negative results reported on the basis of stained smears strongly suggests the value of confirmation by observation of motile organisms from the direct mount, from appropriate culture media, or from direct detection by more sensitive antigen detection or molecular methods (Table 3) (24).

Examination of urinary sediment may be indicated in certain filarial infections. Administration of the drug diethylcarbamazine (Hetrazan) has been reported to enhance the recovery of microfilariae from urine. The triple-concentration technique is recommended for the recovery of microfilariae (1). The membrane filtration technique can also be used with urine for the recovery of microfilariae (1). *Schistosoma haematobium* eggs can be concentrated by centrifugation of urine specimens; a membrane filter technique for the egg recovery has also been useful (1). Fresh samples or fixed samples in formalin should be used to prevent hatching of eggs.

Microsporidial spores of *Encephalitozoon intestinalis* can also be recovered from urine sediment. This organism primarily infects the intestinal tract but can also disseminate to the kidneys in immunocompromised individuals.

Sputum

Although not one of the more common specimens, expectorated sputum may be submitted for examination for parasites. Organisms in sputum that may be detected and may cause pneumonia, pneumonitis, or Loeffler's syndrome include the migrating larval stages of *Ascaris lumbricoides*, *Strongyloides stercoralis*, and hookworm; the eggs of *Paragonimus* spp.; *Echinococcus granulosus* hooklets; and *Entamoeba histolytica*, *Entamoeba gingivalis*, *Trichomonas tenax*, *Cryptosporidium* spp., and possibly, the microsporidia. In a *Paragonimus* infection, the sputum may be viscous and tinged with brownish flecks, which are clusters of eggs ("iron filings"), and may be streaked with blood. Sputum is usually examined as a wet mount (saline or iodine), using low and high dry power (×100 and ×400). The specimen is not concentrated before preparation of the wet mount. If the sputum is thick, an equal amount of 3% sodium hydroxide (NaOH) (or undiluted chlorine bleach) can be added; the specimen is thoroughly mixed and then centrifuged at 500 × *g* for 5 min. NaOH should not be used if one is looking for *Entamoeba* spp. or *T. tenax*. After centrifugation, the supernatant fluid is discarded, and the sediment can be examined as a wet mount with saline or iodine. If examination has to be delayed for any reason, the sputum should be fixed in 5 or 10% formalin to preserve helminth eggs or larvae or in PVA fixative to be stained later for protozoa.

Aspirates

The examination of aspirated material for the diagnosis of parasitic infections may be extremely valuable, particularly when routine testing methods have failed to demonstrate the organisms. These specimens should be transported to the laboratory immediately after collection. Aspirates include liquid specimens collected from a variety of sites where organisms might be found. The aspirates most commonly processed in the parasitology laboratory include fine-needle and duodenal aspirates. Fluid specimens collected by bronchoscopy include bronchoalveolar lavage fluid and bronchial washings (25).

Fine-needle aspirates may be submitted for slide preparation, culture, and/or molecular analyses. Aspirates of cysts and abscesses for amebae may require concentration by centrifugation, digestion, microscopic examination for motile organisms in direct preparations, and cultures and microscopic evaluation of stained preparations. Antigen detection and PCR are other possibilities, depending on individual laboratory testing options.

Bone marrow aspirates for *Leishmania* spp. amastigotes, *Trypanosoma cruzi* amastigotes, or *Plasmodium* spp. require Giemsa staining or the use of other stains for blood and tissues. Examination of these specimens may confirm an infection that has been missed by examination of routine blood films. In certain situations, culture, immunoassays for antigen detection, or PCR also provide more sensitive results (26).

Biopsy Specimens

Biopsy specimens are recommended for the microscopic detection of tissue parasites (Table 5). The following procedures may be used for this purpose in addition to standard histologic preparations: impression smears and teased and squash preparations of biopsy tissue from skin, muscle, cornea, intestine, liver, lung, and brain. Tissue to be examined by permanent sections or electron microscopy should be fixed as specified by the laboratories that will process the tissue. In certain cases, a biopsy may be the only means of confirming a suspected parasitic infection. Specimens that are going to be examined as fresh material rather than as tissue sections should be kept moist in saline and submitted to the laboratory immediately.

Detection of parasites in tissue depends in part on specimen collection and on having sufficient material to perform the recommended diagnostic procedures. Biopsy specimens are usually quite small and may not be representative of the diseased tissue. Multiple tissue samples often improve diagnostic results. To optimize the yield from any tissue specimen, all areas should be examined by as many procedures as possible. Tissues are obtained by invasive procedures, many of which are very expensive and lengthy; consequently, these specimens deserve the most comprehensive procedures possible.

Tissue submitted in a sterile container in sterile saline or on a sterile sponge dampened with saline may be used for cultures or molecular analyses of protozoa after mounts for direct examination or impression smears for staining have been prepared. Bacteriological transport media should be avoided. If cultures for parasites are to be made, sterile slides should be used for smear and mount preparation.

Blood

Depending on the life cycle, a number of parasites may be recovered in a blood specimen, either whole blood, buffy coat preparations, or various types of concentrations (1, 25). Although some organisms may be motile in fresh whole blood, species identification is normally accomplished from the examination of permanent stained blood films, both thick and thin films. Blood films can be prepared from fresh whole blood collected with no anticoagulants, anticoagulated blood, or sediment from the various concentration procedures. The recommended stain of choice is Giemsa stain; however, the parasites can also be seen on blood films stained with Wright's stain. Delafield's hematoxylin stain is often used to stain the microfilarial sheath; in some cases, Giemsa stain does not provide sufficient stain quality to allow differentiation of the microfilariae.

Thin Blood Films

In any examination of thin blood films for parasitic organisms, the initial screen should be carried out with the low-power objective (×100) of a microscope. Microfilariae may be missed if the entire thin film is not examined. Microfilariae are rarely present in large numbers, and frequently only a few organisms are present in each thin film preparation. Microfilariae are commonly found at the edges of the thin film or at the feathered end of the film because they are carried to these sites during spreading of the blood. The feathered end of the film, where the erythrocytes (RBCs) are drawn out into one single, distinctive layer of cells, should be examined for the presence of malaria parasites and trypanosomes. In these areas, the morphology and size of the infected RBCs are most clearly seen.

Depending on the training and experience of the microscopist, examination of the thin film usually takes 15 to 20 min (≥300 oil immersion fields) for the thin film at a magnification of ×1,000. Although some people use a 50× or 60× oil immersion objective to screen stained blood films, there is some concern that small parasites such as *Plasmodium* spp., *Babesia* spp., or *Leishmania* spp. may be missed at this lower total magnification (×500 or ×600) compared with the ×1,000 total magnification obtained using the more traditional 100× oil immersion objective. Because people tend to scan blood films at different rates, it is important to examine a minimum number of fields. If something suspicious has been seen in the thick film, the number of fields examined on the thin film is often considerably greater than 300. The request for blood film examination should always be considered a STAT procedure, with all reports (negative as well as positive) being relayed by telephone to the physician as soon as possible. If positive, notification of appropriate governmental public health agencies (local, state, and federal) should be done within a reasonable time frame in accordance with guidelines and laws.

Both malaria and *Babesia* infections have been missed with automated differential instruments, and therapy was delayed. Although these instruments are not designed to detect intracellular blood parasites, the inability of the automated systems to discriminate between uninfected RBCs and those infected with parasites may pose serious diagnostic problems (27).

Thick Blood Films

In the preparation of a thick blood film, the highest concentration of blood cells is in the center of the film. The examination should be performed at low magnification to detect microfilariae more readily. Examination of a thick film usually requires 5 to 10 min (approximately 100 oil immersion fields). The search for malarial organisms and trypanosomes is best done under oil immersion (×1,000). Intact RBCs are frequently seen at the very periphery of the thick film; such cells, if infected, may prove useful in malaria diagnosis, since they may demonstrate the characteristic morphology necessary to identify the organisms to the species level.

Blood Stains

For accurate identification of blood parasites, a laboratory should develop proficiency in the use of at least one good staining method. It is better to select one method that will provide reproducible results than to use several on a hit-or-miss basis. Blood films should be stained as soon as possible, since prolonged storage may result in stain retention. Delay in staining positive malarial smears may result in failure to demonstrate typical staining characteristics for individual species.

The most common stains are of two types. Wright's stain has the fixative in combination with the staining solution, so that both fixation and staining occur at the same time; therefore, the thick film must be laked before staining. In Giemsa stain, the fixative and stain are separate; therefore, the thin film must be fixed with absolute methanol before staining.

Buffy Coat Films

Trypanosomes, occasionally *Histoplasma capsulatum* (a fungus that manifests as small oval yeast cells resembling those of *Leishmania* amastigote stages), and, in immunocompromised patients, potentially *Leishmania* spp. (*L. infantum*, *L. chagasi*, and *L. donovani*) are detected within the large mononuclear cells in the buffy coat (a layer of leukocytes resulting from centrifugation of whole anticoagulated blood). The amastigote form of *Leishmania* spp. possesses a kinetoplast, a nucleus that stains dark red-purple, and the cytoplasm stains light blue. *H. capsulatum* appears as a large dot of nuclear material (dark red-purple) surrounded by a clear halo area. Trypanosomes in the peripheral blood also concentrate with the buffy coat cells (1).

Screening Methods

Microhematocrit centrifugation with use of the QBC malaria test, a glass capillary tube, and closely fitting plastic insert (QBC malaria blood tubes; Becton Dickinson, Tropical Disease Diagnostics, Sparks, MD) has been used for the detection of blood parasites. Tubes precoated with acridine orange provide a stain that induces fluorescence in the parasites. At the end of centrifugation of 50 to 60 µl of capillary or venous blood (5 min in a QBC centrifuge, 14,387 × *g*), parasites or RBCs containing parasites are concentrated into a 1- to 2-mm region near the top of the RBC column and are held close to the wall of the tube by the plastic float, making them readily visible by fluorescence microscopy. This method automatically prepares a concentrated smear, which represents the distance between the float and the walls of the tube. Once the tube is placed into the plastic holder (Para-viewer) and immersion oil is applied to the top of the hematocrit tube (no coverslip is necessary), the tube is examined with a 40× to 60× oil immersion objective (which must have a working distance of 0.3 mm or greater) (1). Cost of the equipment and technical expertise may limit use of this test methodology.

Antigen and DNA Detection for Blood Parasites

Several antigen detection tests are available. Many among them are designed for rapid and individual diagnoses. Most of the kits are not available in the United States but have proven to be very useful in other countries. Rapid tests are available to diagnose specifically *Plasmodium falciparum* or, on a genus level, *Plasmodium* spp. infections. These tests are simple to perform and can be applied during the more time-consuming microscopic identification of the thin and thick blood smears. However, one has to be aware that false-positive and false-negative reactions do occur (28–31). The general recommendation is to use these tests only in addition to the microscopic examination of thick and thin blood smears. Various PCR-based methods have been described in the scientific literature. Although the methods are more sensitive than standard microscopic examinations of blood films, they are not routinely used for malaria diagnosis.

Knott Concentration

The Knott concentration procedure is used primarily to detect the presence of microfilariae in the blood, especially when a light infection is suspected. Aqueous formalin is added to anticoagulated blood and centrifuged at 300 × *g* to concentrate microfilaria. The sediment is examined microscopically (×100 and ×400). A thick prep can be made with the remainder of the sediment. Microfilariae will be nonmotile in the wet smear; however, they will exhibit diagnostic morphologic characteristics when stained. The disadvantage of the procedure is that the microfilariae are killed by the formalin and are therefore not seen as motile organisms.

Membrane Filtration Technique

The membrane filtration technique using Nuclepore filters (25-mm Nuclepore filter [5-µm porosity]) has proved highly efficient in demonstrating filarial infections when microfilaremias are of low density. It has also been successfully used in field surveys (1).

Culture Methods

Very few clinical laboratories offer specific culture techniques for parasites. The methods for *in vitro* culture are often complex, while quality control is difficult and not really feasible for the routine diagnostic laboratory. In certain institutions, some techniques may be available, particularly where consultative services are provided and for research purposes.

Few parasites can be routinely cultured, and the only procedures that are in general use are for *Entamoeba histolytica*, *Naegleria fowleri*, *Acanthamoeba* spp., *Trichomonas vaginalis*, *Toxoplasma gondii*, *Trypanosoma cruzi*, *Encephalitozoon* spp., and the leishmanias. These procedures are usually available only after consultation with the laboratory and on special request. Commercial tests are available only for *T. vaginalis* (InPouch TV; BioMed Diagnostics, San Jose, CA).

Animal Inoculation and Xenodiagnosis

Most routine clinical laboratories do not have the animal care facilities necessary to provide animal inoculation capabilities for the diagnosis of parasitic infections. Host specificity for many animal parasite species is well known and limits the types of animals available for these procedures. In certain suspect infections, animal inoculation may be requested and can be very helpful in making the diagnosis, although animal inoculation certainly does not take the place of other, more routine procedures. Mouse inoculation with amniotic fluid has been used in the past for diagnosis of congenital toxoplasmosis; this method, however, has mostly been replaced by PCR. Intraperitoneal inoculation of *Echinococcus multilocularis* metacestode material from surgical resection or from biopsy samples from mice or gerbils is still the most reliable procedure available for viability testing after long-term chemotherapy.

Xenodiagnosis is a technique that uses the arthropod host as an indicator of infection. Uninfected reduviid bugs are allowed to feed on the blood of a patient who is suspected of having Chagas' disease (*T. cruzi* infection). After 30 to 60 days, feces from the bugs are examined over a 3-month time frame for the presence of developmental stages of the parasite, which are found in the hindgut of the vector. This type of procedure is used primarily in South America for fieldwork, and the appropriate bugs are raised in various laboratories specifically for this purpose.

Antigen Detection

The detection of parasite-specific antigen is indicative of current infection. Immunoassays are generally simple to perform. Some formats allow the processing of large numbers of tests at one time, thereby reducing overall costs; others are specially designed for rapid individual diagnoses. A major disadvantage of antigen detection is that in most cases the method can detect only a single pathogen at one time.

Therefore, additional parasitological examinations must be performed to detect other parasitic pathogens. The current commercially available immunoassays for the detection of intestinal protozoa have excellent sensitivity and specificity compared with routine microscopy (32–35). Specific ordering approaches using both immunoassays and routine O&P examinations are listed in Table 6. Rapid tests for the diagnosis of malaria should be used only in parallel to the examination of thick and thin blood smears.

Parasite DNA Detection

Molecular testing is becoming more readily available for most diagnostic laboratories. Assays for *Trichomonas vaginalis*, *Entamoeba histolytica*, *Giardia* spp., and *Cryptosporidium* spp. are commercially available and FDA-approved. Intestinal gastrointestinal panels are available that can test simultaneously for multiple gastrointestinal pathogens, including bacterial, viral, and parasitic pathogens. As with antigen detection, only select pathogens can be detected, and other pathogens may be missed.

Nucleic acid-based diagnostic tests (particularly PCR, with its inherent potential for highly efficient and specific amplification of DNA) have been developed for almost all species of parasites. However, only a few are routinely used in diagnostic settings. The main reason for this minor role of diagnostic PCR in parasitology is the fact that many parasite stages can be adequately diagnosed using established, more traditional techniques (microscopy, detection of antigens and antibodies, or *in vitro* cultivation) that are generally less expensive and technically less demanding than PCR. Therefore, diagnosis by PCR is of great value in cases in which these techniques are insufficient, that is, in cases in which (i) the immune response is not informative (e.g., acute infections, short-term follow-up after therapy, congenital infections); (ii) high sensitivity is needed because of low parasite levels (e.g., cutaneous leishmaniasis); or (iii) morphologically indistinguishable organisms need to be identified (e.g., *Entamoeba histolytica*/*Entamoeba dispar* and eggs of taeniid tapeworms).

Diagnostic PCR may become more widespread when simple, fully standardized (commercial) test kits are available and costs are reduced through the implementation of pre- and post-PCR automated techniques. Furthermore, the possibilities to not only detect and identify but also quantify organisms and determine their genotypes by analyzing the diagnostic PCR product extend the diagnostic power of PCR. Indeed, PCR coupled with genetic characterization is already widely used in parasitology to address questions such as parasite host range and host specificity, ways of transmission, and molecular epidemiology. Such genotyping applications should increase in the future with increasing knowledge of the relationship between genetic variation in parasites and features such as virulence or drug resistance. An important limitation of PCR-based diagnosis is the fact that sensitivity dramatically decreases with material stored for more than 1 day in formalin due to the fragmentation (fragment length of a few hundred base pairs) of the DNA. However, by selecting primers that produce PCR products as short as possible, which is recommended for real-time PCR, sensitivity might be reasonably high. Therefore, it seems to be the best choice to avoid formalin fixation if PCR analyses have to be considered. Such tests, however, are not yet widely available in parasitology.

APPENDIX

Parasite Images

Parasites image library and parasitological resources

University of Delaware

http://www.udel.edu/mls/dlehman/medt372/index.html (accessed 1 January 2015)

Centers for Disease Control and Prevention

http://www.dpd.cdc.gov/DPDx/HTML/Image_Library.htm (accessed 1 January 2015)

Oregon Public Health Laboratories Parasite Image Library

http://public.health.oregon.gov/LaboratoryServices/ImageLibrary/Pages/parlib.aspx (accessed 1 January 2015)

American Society for Parasitologists

http://amsocparasit.org/links-resources/parasite-Images (accessed 1 January 2015)

Parasitology Information

Centers for Disease Control and Prevention

http://www.dpd.cdc.gov/DPDx (accessed 1 January 2015)

World Health Organization

http://www.who.int (accessed 1 January 2015)

Medical Chemical Corporation

http://www.med-chem.com (accessed 1 January 2015)

NCBI National Library of Medicine (PubMed)

http://www.ncbi.nim.nih.gov/entrez/query.fcgi (accessed 1 January 2015)

Additional Parasitology Websites

Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand

http://www.med.cmu.ac.th/dept/parasite/Default.htm (accessed 1 January 2015)

United States Department of Agriculture (Agriculture and Research Service)

http://www.ars.usda.gov/is/np/systematics/animalpar.htm (accessed 5 July 2013)

Oklahoma State University

http://www.cvm.okstate.edu/instruction/kocan/vpar5333/vpar5333.Htm (accessed 5 July 2013)

University of California, Davis, Department of Nematology

http://ucdnema.ucdavis.edu/imagemap/nemmap/ENT156HTML/E156charac (accessed 5 July 2013)

University of Edinburgh

http://www.ed.ac.uk/cpb/websites/htm (accessed 5 July 2013)

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Reagents, Stains, and Media: Parasitology

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The evaluation of clinical specimens for ova and parasites in the clinical laboratory can involve the use of direct macroscopic examination of the specimen and microscopic examination of fresh and preserved specimens, as well as culture for some parasitic organisms. These examinations necessitate the use of a variety of stains, reagents, and media, the most common of which are discussed in this chapter.

Because many parasitic organisms cannot be cultured, microscopic examination is the mainstay of diagnostic parasitology. Examination after proficient staining of fresh and unconcentrated specimens, as well as preserved and/or concentrated specimens with permanent stained preparations, most often provides a rapid and accurate diagnosis. A variety of reagents and stains are available for these purposes, and each laboratory must decide which ones to use to best serve its patient population. In addition, because most specimens are submitted in fixatives and preservatives, the reader should note the specific interference of polyvinyl alcohol (PVA) and mercury reagents with immunoassays that are commonly used for parasitic diagnosis. As immunoassays and nucleic acid amplification assays become more commonly used for the detection of parasites, reagents needed to perform these test must be considered.

Caution should be taken when using reagents in the parasitology laboratory. Many routinely used compounds can be dangerous if not handled appropriately. For example, formalin and formaldehyde solutions can cause severe skin irritation and, if swallowed, can cause violent vomiting and diarrhea; mercury compounds are local irritants and systemic poisons that can be absorbed through the skin; phenol is a skin irritant, and exposure to large amounts can affect the central nervous system; and xylene can cause serious skin irritation, with extended exposure causing gastrointestinal, neurologic, and tissue damage (1). It is also important to remember that reagents that contain formalin and/or mercury require special disposal. Proper disposal should be made using state and federal regulatory guidelines.

REAGENTS

All of the following reagents are preservatives and/or fixatives. Table 1 lists several types of preservatives along with their content, specific permanent stained smears that can be performed, and immunoassays for which they can be used, as well as additional comments concerning the fixative. Today, most clinical laboratories purchase premixed, packaged vials of preservatives and fixatives/adhesives that can be either inoculated with fecal sample sent to the laboratory or sent home with the patient for home collection. A list of commercially available transport vials can be found in Table 2.

Formalin Preparations

Formalin

Formalin has been used in parasitology as an all-purpose preservative for concentration procedures. However, formalin should not be used to prepare smears for permanent stains, because the reagent does not adequately preserve organism morphology (2). It is easy to prepare and has a long shelf life. It is most routinely used as a preservative for stool and duodenal aspirate specimens. Formalin works well to preserve the morphologies of helminth ova and larvae and those of protozoan cysts, oocysts, and spores, although it does not preserve protozoan trophozoites well. Although both 5% and 10% solutions of formalin are currently used (5% for the best preservation of protozoan stages and 10% for ova and larvae), the 10% formulation is most widely used in clinical parasitology today. A formalin vial is often paired with a vial containing a fixative such as PVA or Schaudinn's to ensure that a permanent stained smear can be prepared and read. Formalin (10%) is prepared as indicated below:

Formaldehyde (37% to 40% HCHO solution)	100 ml
Saline (0.85% NaCl) OR distilled water	900 ml

Buffered formalin

Formalin buffered with sodium and potassium phosphates can be used to help maintain the morphology of parasites for long-term storage and is used for concentration procedures. To make buffered formalin, mix the following dry ingredients and store in a tightly closed container. Add 0.8 g of this mixture to 1 liter of 10% (or 5%) formalin.

Sodium phosphate, dibasic (Na_2HPO_4)...............	6.1 g
Potassium phosphate, monobasic (KH_2PO_4)........	0.15 g

Merthiolate-iodine-formalin (MIF)

Protozoa and helminth ova and larvae can be distinguished on direct wet mount after stool and duodenal aspirate speci-

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TABLE 1 Preservatives used in diagnostic parasitology (intestinal tract specimens)

Preservative	Preservative can be used for:		
	Concentrated examination	Permanent stained smear	Immunoassays
5% or 10% formalin	Yes	No	Yes (EIA, FA, rapid)
5% or 10% buffered formalin	Yes	No	Yes (EIA, FA, rapid)
MIF	Yes	Polychrome IV stain[e]	No No published data for immunoassay systems
SAF	Yes	Iron hematoxylin, trichrome (not as good)	Yes (EIA, FA, rapid)
Hg-PVA[a]	Yes	Trichrome or iron hematoxylin	No PVA plastic powder interferes with immunoassays
Cu-PVA[b]	Yes	Trichrome or iron hematoxylin	No PVA plastic powder interferes with immunoassays
Zn-PVA[c]	Yes	Trichrome or iron hematoxylin	Some, but not all PVA plastic powder interferes with immunoassays
Single-vial systems[d]	Yes	Trichrome or iron hematoxylin	Some, but not all If no PVA or mercury, may be compatible with fecal immunoassays
Schaudinn's (without PVA)[a]	No	Trichrome or iron hematoxylin	No Mercury interferes with immunoassays

[a] PVA (plastic powder used as "glue" to attach stool onto the glass slide/no fixation properties per se) and Schaudinn's fixative (mercuric chloride base) are still considered to be the gold standard against which all other fixatives are evaluated for organism morphology after permanent staining. Additional fixatives prepared with nonmercuric chloride-based compounds are being developed and tested.

[b] This modification uses a copper sulfate base rather than mercuric chloride.

[c] This modification uses a zinc base rather than mercuric chloride and apparently works well with both trichrome and iron hematoxylin stains.

[d] These modifications use a combination of ingredients (including zinc) but are prepared from proprietary formulas. The aim is to provide a fixative that can be used for the fecal concentration and permanent stained smear. Acceptability for use with immunoassays for *Giardia duodenalis* and *Cryptosporidium* species varies, so verify with the manufacturer's package insert for proper use. Testing for *Entamoeba histolytica* and/or the *E. histolytica*/*E. dispar* group still requires fresh or frozen specimens.

[e] This stain can be used in place of trichrome for staining fecal smears preserved with MIF, PVA, or SAF methods. It is available commercially.

[f] Immunoassays for *Giardia duodenalis* and *Cryptosporidium* spp.

mens are fixed in MIF. MIF allows not only fixation but also staining of the organisms, although organism morphology is not as good as when other permanent staining compounds are used. MIF can be used to perform concentration procedures. This method requires that two stock solutions listed below be combined into a fresh working solution immediately before use. In addition, after mixing specimens with MIF, the mixture must be left undisturbed for 24 h before preparation of smears from the bottom two layers of the three layers that will form. Parasitological examination of specimens placed in MIF can be performed for several weeks after preservation, making it very useful for field surveys. MIF is also readily available commercially.

Solution I (store in brown bottle)

Distilled water	50 ml
Formaldehyde	5 ml
Thimerosal (tincture of merthiolate, 1:1,000)	40 ml
Glycerol	1 ml

Solution II (Lugol's solution) (good for several weeks in a tightly stoppered brown bottle)

Distilled water	100 ml
Potassium iodide crystals (KI)	10 g
Iodine crystals (add after KI dissolves)	5 g

TABLE 2 Commercially available collection/transport vials for the recovery of parasites[a]

Company	Preservative(s)	Fixative(s)	Single-vial system
Alpha-Tec Systems (http://www.alphatecsystems.com, accessed 1/2/15)	10% formalin	SAF Zn-PVA	Proto-fix CLR
Medical Chemical Corporation (http://www.med-chem.com/, accessed 1/2/15)	10% formalin MIF	Cu-PVA LV-PVA Zn-PVA SAF	Total-Fix universal fixative (no mercury, formalin, or PVA)
Meridian Bioscience (http://www.meridianbioscience.com/, accessed 1/2/15)	10% formalin	LV-PVA SAF Zn-PVA	EcoFix universal fixative (no mercury or formalin; contains PVA)
Remel (http://www.remel.com, accessed 1/2/15)	5% formalin 10% formalin	PVA SAF Zn-PVA	
Scientific Device Laboratory (http://www.scientificdevice.com, accessed 1/2/15)		PVA SAF	Parasafe

[a] SAF, sodium acetate formalin; LV-PVA, low-velocity polyvinyl alcohol; Zn-PVA, zinc polyvinyl alcohol; MIF, merthiolate-iodine-formaldehyde; Cu-PVA, copper polyvinyl alcohol. Website access dates are in month/day/year format.

Combine 9.4 ml of solution I with 0.6 ml of solution II just before use.

PVA-Containing Preservatives and Fixatives

PVA acts as an adhesive for stool material, allowing the stool to adhere to glass slides. Several modifications are commercially available. The accompanying compound with the PVA, specifically, mercuric chloride, zinc sulfate, or cupric sulfate, acts as the preservative and allows fixation of protozoan cysts and trophozoites for use with trichrome or iron hematoxylin stains for permanent smears. All PVA-containing preservatives interfere with immunoassays. PVA is not an appropriate reagent to use for stool specimen concentration and should be paired with a reagent vial (formalin, MIF, SAF) that can be used for that purpose.

Hg-PVA

The Hg-PVA fixative uses mercuric chloride as the preservative. Protozoan morphology is best preserved with PVA-incorporating mercury compounds. However, due to the toxic nature of mercury compounds and the difficulty in preparation, most laboratories no longer prepare Hg-PVA.

Zn-PVA

The Zn-PVA fixative uses zinc sulfate in place of mercury as a preservative and fixative for protozoans. Specimens treated with Zn-PVA may also be stained with trichrome or iron hematoxylin stains for permanent smears.

Cu-PVA

The Cu-PVA fixative uses cupric sulfate in place of mercury as a preservative and fixative for protozoans. Specimens treated with Cu-PVA may also be stained with trichrome or iron hematoxylin stains for permanent smears.

Studies have shown that zinc sulfate provides a satisfactory, but not equal, substitute for mercury in the permanent staining procedures and that copper sulfate does not preserve morphology equal to that seen with mercuric chloride (3, 4).

Schaudinn's fixative/solution

Schaudinn's solution is a fixative made of mercuric chloride, distilled water, and 95% ethyl alcohol that gives excellent morphological preservation of protozoan organisms. It is used primarily in the preparation of permanent stained smears for parasitological examination from fresh, nonpreserved specimens. Specimens fixed in Schaudinn's solution can be used with either trichrome or iron hematoxylin stains. As with Hg-PVA, due to the toxic nature of mercuric compounds and the difficulty in preparation of this fixative, Schaudinn's solution is not routinely made in the clinical microbiology laboratory. In addition, Schaudinn's fixative is becoming increasingly more difficult to purchase commercially. Many laboratories are opting to use a mercury-free or low-level mercury single vial that can be used to prepare both a concentration and preparation for permanent stained smears. See "Nonmercury or low-level mercury, nonformalin fixatives: single-vial systems" below.

Sodium acetate-acetic acid-formalin (SAF)

SAF is very similar to formalin in that it is a liquid fixative and contains no mercury. However, unlike formalin, SAF can be used for both concentration techniques and permanent stained smears. The sediment from the concentration procedure is used for both the wet preparation and the permanent stain. Albumin-coated slides allow better adhesion of the concentrated material to the slide and are recommended for use with SAF. SAF is an acceptable substitute for PVA or Schaudinn's solution for permanent smears stained with either trichrome or iron hematoxylin. SAF is available commercially and has a long shelf life, but it can also readily be made in the laboratory by mixing the reagents listed below:

Sodium acetate	1.5 g
Glacial acetic acid	2.0 ml
Formaldehyde (37% to 40% HCHO solution)	4.0 ml
Distilled water	92.0 ml

Nonmercury or low-level mercury, nonformalin fixatives: single-vial systems

Several nonmercury, nonformalin proprietary fixatives are commercially available for both concentration of stool specimens and the preparation of permanently stained smears (Medical Chemical Corporation, Torrance, CA; Meridian Bioscience, Cincinnati, OH). There is also a low-level mercury, nonformalin proprietary fixative available that can be used as above (Alpha-Tec, Vancouver, WA). The beauty of these systems is that only one vial is needed to prepare both a concentration and a permanent stained smear and that the vials can be discarded along with other biohazardous waste in the laboratory. Acceptability for use in fecal immunoassays varies (check with manufacturers for specific uses). Special stains are also available for use with some of these fixatives.

STAINS

Table 3 lists the stains that are most commonly used to detect and aid in the identification of parasitic organisms. The description and procedures for stains used in parasitol-

TABLE 3 Stains used for parasitic identification

Organism	Stain(s) used for detection
Cryptosporidium spp., *Cystoisospora belli*, *Cyclospora cayetanensis*	Modified acid-fast stain
Cystoisospora and *Cyclospora* oocysts, *Sarcocystis* sporocysts	Autofluorescence with no stain using fluorescent microscopy
Cyclospora	Modified safranin stain
Naegleria spp., *Acanthamoeba* spp., *Balamuthia* spp.	Calcofluor white stain using fluorescent microscopy, trichrome stain
Acanthamoeba	Modified Field's stain, trichrome stain
Blood parasites (agents of malaria, microfilariae, and *Leishmania*, *Babesia*, and *Trypanosoma* spp.)	Giemsa or Wright's stain (rapid blood stains are also acceptable)
Microfilariae (specifically for sheaths and nuclei)	Delafield's hematoxylin stain; Giemsa stain also acceptable except for *Wuchereria bancrofti*
Parasitic helminth eggs/larvae and protozoan cysts	Iodine
Intestinal protozoan parasites	Iron hematoxylin stain, trichrome stain

TABLE 4 Commercially available stains for the detection of parasites

Company (website, access date [mo/day/yr])	Stains
Alpha-Tec Systems (http://www.alphatecsystems.com, accessed 1/2/15)	Giemsa stain and buffer Cryptosporidium stain set
Medical Chemical Corporation (http://www.med-chem.com, accessed 1/2/15)	Trichrome stain Iron hematoxylin stain Giemsa stain and buffer Wheatley's modified Gomori trichrome
Meridian Bioscience (http://www.meridianbioscience.com, accessed 1/2/15)	EcoStain Trichrome stain
Remel (http://www.remel.com, accessed 1/2/15)	Trichrome stain Trichrome quick stain
Scientific Device Laboratory (http://www.scientificdevice.com, accessed 1/2/15)	StainQuick trichrome Trichrome stain Cryptosporidium Kinyoun stain Modified trichrome stain

ogy are listed below. A list of commercially available parasitology stains can be found in Table 4.

Iodine (Lugol's or D'Antoni's)

A solution of iodine can be used when preparing direct or concentrated wet mounts for parasitological examination. These nonspecific dyes allow the differentiation of parasitic cysts from leukocytes, the former of which retain the iodine and appear light brown. Iodine can easily overstain eggs; thus, a wet prep without iodine should be used to detect eggs. Iodine stains can be purchased commercially for use in routine parasitology. These solutions should be stored in dark containers in a dark environment. D'Antoni's iodine preparation has the advantage of use without further dilution, whereas Lugol's iodine must first be diluted into a working solution (1:5 dilution in distilled water) before use. Working solutions of both iodine preparations fade with time and should be discarded and replaced when their dark tea color lightens.

Basic procedure

Place 1 drop of iodine on a slide; add to this a small amount of fecal specimen and mix until homogeneous. The iodine stains fecal material immediately, and the timing of this step is not important. Place a coverslip on top of the suspension and view under ×100 magnification. Examine any suspicious material under ×400 magnification.

Acid-Fast Stains (Modified)

Cryptosporidium, *Cystoisospora belli*, and *Cyclospora cayetanensis* are coccidian parasites that can cause diarrheal disease in humans. These organisms are more easily detected when a modified acid-fast stain is used. The modification to the acid-fast stains is the use of decolorizing agents that are less harsh than those used for staining mycobacteria. The carbol fuchsin and counterstain (methylene blue) reagents used for mycobacteria can be used for staining coccidian parasites; however, the decoloring agents are not interchangeable. In the modified acid-fast staining procedures, a 1% solution of sulfuric acid (1 ml of sulfuric acid in 99 ml of water) is used, as opposed to the 3% solution used in the acid-fast bacillus Kinyoun staining procedure, and a 5% sulfuric acid solution (5 ml of concentrated sulfuric acid in 95 ml of distilled water) is used, as opposed to the 3% HCl solution in 95% ethanol that is used in the acid-fast bacillus Ziehl-Neelsen staining procedure.

Modified Kinyoun stain (cold method)

Basic procedure (modified Kinyoun stain)

Specimen (1 or 2 drops) is applied to a slide, allowed to air dry, and fixed with absolute methanol for 1 min. Carbol fuchsin is applied to the slide for 5 min, and then the slide is rinsed with 50% ethanol, followed by a water rinse. Sulfuric acid (1%) decolorizer is added for 2 min, and the slide is rinsed again with water. Methylene blue is added for 1 min, and then the slide is rinsed again with water. The slide is then air dried and examined at ×100 to ×1,000 magnification.

Modified Ziehl-Neelsen stain (hot method)

Specimen (1 or 2 drops) is applied to a slide and allowed to air dry, then dry on a heating block (70°C) for 5 min. Place slide on rack and flood with carbol fuchsin. With an alcohol lamp or Bunsen burner, gently heat the slide until steam appears. Allow to stain for 5 min and then rinse. Decolorize with 5% sulfuric acid for 30 seconds and rinse. Counterstain with methylene blue for 1 minute and rinse. The slide is then air dried and examined at ×100 to ×1,000 magnification.

Blood Film Stains

Giemsa and Wright's stains

Examination of blood films for parasites includes the use of two common stains, the Giemsa stain and Wright's stain, both derivatives of the original Romanowsky stain. These stains are very similar, differing primarily in that no fixative is included in the Giemsa stain and the blood film must be fixed with absolute methanol before staining. Erythrocytic stippling, seen in some malaria infections, can be seen using only the Giemsa stain (5). Although stock solutions of these stains can be prepared in the laboratory, the procedure is very cumbersome and involves grinding of powdered stain with methanol and/or glycerol with a mortar and pestle, days to weeks of storage with shaking, and removal of supernatant or filtering before use. In addition, it is recommended that the Giemsa stain be prepared fresh each day of use by diluting the stain stock solution with phosphate-buffered water, pH 7 to 7.2 (6). Alternatively, Giemsa, Wright's, and Wright-Giemsa stains are readily available from commercial suppliers in liquid form and may need only dilution in a buffer solution. Blood films may be stained manually, but many laboratories rely on automated hematologic instruments for staining of thin (not thick) blood films, with acceptable results. These stains allow the detection of blood parasites, including the agent of malaria, microfilariae, and *Leishmania*, *Babesia*, and *Trypanosoma* species. Although for many years, Giemsa stain has been the stain of choice, the parasites can also be seen on blood films stained with Wright's stain, a Wright-Giemsa combination stain, or one of the more rapid stains such as Diff-Quik (American Scientific Products, McGaw Park, IL), Wright's Dip Stat Stain (Medical Chemical Corp.), or Field's stain. It is more appropriate to use a stain with which you are familiar rather than Giemsa, which is somewhat more complicated to use. Polymorphonuclear leukocytes can serve as the quality control organism for any of the blood stains. Any parasites present will stain like the polymorphonuclear leukocytes, regardless of the stain used.

Basic procedure

Cover the surface of a thin blood film with stain for 1 to 3 min. Add an equal volume of buffered water to the slide and mix by gently blowing on the surface of the slide. Wait 4 to 8 min and flood the slide with buffered water. Coplin jars with stain can also be used. Allow the slide to dry and examine it under ×1,000 magnification. Thick films must be laked in distilled water or treated with saponin before performance of the staining procedure described previously (6, 7). Satisfactorily stained smears show the following characteristics for the Giemsa and Wright's stains: erythrocytes, pale red and light tan, reddish, or buff, respectively; nuclei of leukocytes, purple with pale purple cytoplasm and bright blue with contrasting light cytoplasm, respectively; eosinophilic granules, bright purple-red and bright red, respectively; and neutrophilic granules, deep pink-purple and pink or light purple, respectively.

Hematoxylin Stains

Delafield's hematoxylin stain

Delafield's hematoxylin stain is used for thin and concentrated blood films for the detection of microfilariae and may show greater detail of the nuclei and sheaths than that shown with Giemsa and Wright's stains. Although the stain is not procedurally difficult to prepare, it does involve an aging process of 1 week followed by 1 month before it can be used. Delafield's hematoxylin stain is not readily available commercially and is used only in special circumstances. Preparation of the stain involves dissolving 180 g of aluminum ammonium sulfate in 1 liter of distilled water, heating until dissolved, and cooling (ammonium alum). Hematoxylin crystals (4 g) are dissolved in 25 ml of 95% ethyl alcohol, and the solution is then added to 400 ml of the ammonium alum. The solution is then covered with a cotton plug and exposed to sunlight and air for 1 week, after which it is filtered. To this solution, 100 ml each of glycerol and 95% ethyl alcohol is added. This solution is placed in sunlight for at least 1 month (8).

Basic procedure

Allow the specimen on a slide to air dry. Lake blood films with distilled water for 15 min and fix in absolute methanol for 5 min, followed by air drying. Stain for 10 to 15 min and rinse in water. Air dry, add a coverslip with mounting fluid, and examine the slide under ×1,000 magnification.

Iron hematoxylin stain

Iron hematoxylin stains are used for the detection, identification, and enumeration of intestinal protozoan parasites. There are many derivations of the iron hematoxylin stain, all of which can be used with fresh fecal specimens, fixed specimens containing PVA, or specimens preserved in Schaudinn's solution or SAF to make permanent stained smears. Solutions of hematoxylin-ethanol and ferrous ammonium sulfate-hydrochloric acid are combined in equal parts to make a working iron hematoxylin stain, and both solutions are available commercially.

Basic procedure

Place prepared slides in 70% ethanol for 5 min. Place the slide in 70% ethanol with iodine for 5 min if mercury-based fixatives are being used and then again in 70% ethanol for 5 min more (these last two steps are not necessary for non-mercury-based fixatives). Wash the slide in running tap water for 10 min, followed by placement in iron hematoxylin working solution for 5 min. After this staining step, wash again in running tap water for 10 min and then place the slide in the following reagents for 5 min each: 70% ethanol, 95% ethanol, 100% ethanol (twice), and xylene (or a substitute) (twice). Add Permount, and add a coverslip. Examine under ×1,000 magnification.

Trichrome stain (Wheatley trichrome stain)

The Wheatley trichrome stain, a modification of the Gomori tissue stain, is used for the detection, identification, and enumeration of intestinal protozoan parasites (9). This stain uses chromotrope 2R and light green SF stains to visually distinguish internal elements of protozoan parasitic cysts and trophozoites. Trichrome staining is usually performed on fixed fecal specimens containing PVA or Schaudinn's solution-preserved specimens. MIF- or SAF-preserved specimens may also be stained with the trichrome stain, as well as specimens preserved with single-vial systems. In addition, some proprietary stains are also available that may work better with these and other fixatives to make permanent stained preparations. Trichrome stain can be easily prepared in the laboratory or purchased from a commercial supplier.

Basic procedure

Place a prepared slide in 70% ethanol for 5 min. For mercury-based fixatives only, place the slide in 70% ethanol with iodine for 1 minute (fresh specimens) or for as long as 10 min (PVA-fixed air-dried specimens). Then place again in 70% ethanol for 5 min (twice). Place in trichrome stain for 10 min, followed by a 1- to 3-s rinse in 90% ethanol with acetic acid. Dip the slide several times in 100% ethanol, and then place it in 100% ethanol for 3 min (twice), followed by xylene for 5 to 10 min (twice). Add Permount, and add a coverslip. Dry overnight or for 1 h at 37°C. Examine under ×1,000 magnification. If 95% alcohol is substituted for 100% ethanol and xylene substitutes are used, it is important to increase the dehydration times for both the alcohol and xylene substitute by at least 5 to 10 min.

Acid-fast trichrome stain

The acid-fast trichrome staining technique allows the detection of acid-fast coccidia (*Cryptosporidium* spp., *C. belli*, and *C. cayetanensis*) (10). Smears prepared from fresh or preserved fecal material can be used in this staining procedure.

Basic procedure

Place a prepared, air-dried slide in absolute methanol for 5 to 10 min and then again allow it to air dry. Place in carbol fuchsin solution for 10 min before rinsing in tap water. Decolorize with 0.5% acid-alcohol and rinse in tap water. Place in trichrome stain for 30 min at 37°C. Rinse in acid-alcohol for 10 s and then dip the slide several times in 95% alcohol for

TABLE 5 Media used for cultivation of parasites

Medium	Organism(s)	Comment(s)
Acanthamoeba monoxenic culture	*Acanthamoeba* and *Naegleria* species	Acanthamoeba medium plus nonnutrient agar overlaid with *Escherichia coli* or *Enterobacter aerogenes* Used for cerebrospinal fluid, tissue, or soil samples
Balamuth's aqueous egg yolk infusion medium	Intestinal amebae	Cannot grow *Balamuthia* *Balamuthia* grows only in cell culture lines
Boeck and Drbohlav's Locke-egg-serum (LES) medium	Intestinal amebae	Inspissated egg base slant with serum and rice powder
Buffered charcoal-yeast extract (BCYE) agar	*Acanthamoeba* species	Some manufacturers' media support growth better than others. Some of these media support growth of trophozoites and/or cysts.
Cysteine-peptone-liver-maltose (CPLM) medium	*Trichomonas vaginalis*	Methylene blue dye is added to aid visualization of the organisms.
Defined medium (14, 17) for pathogenic *Naegleria* species	*Naegleria fowleri*	Defined medium (14, 17)
DGM-21A medium	*Acanthamoeba* species	Defined medium
Diamond's Trypticase-yeast extract-maltose (TYM) complete medium, Diamond's complete medium modified by Klass	*Trichomonas vaginalis*	TYM contains no antibiotics; a nonselective medium Klass modification contains penicillin G, streptomycin sulfate, and amphotericin B to inhibit bacterial overgrowth.
Evans' modified Tobie's medium	*Leishmania* species and *Trypanosoma cruzi*	Uses beef extract, defibrinated horse blood, and phenol red as a pH indicator
InPouch TV (Biomed Diagnostics, Inc.)	*Trichomonas vaginalis*	Commercially available
Lash's casein hydrolysate-serum medium	*Trichomonas vaginalis*	Contains beef blood serum, which is absent from other media for *T. vaginalis*
LYI-S-2 medium	*Entamoeba histolytica*	Similar to TYI-S-33
M-11 medium	*Acanthamoeba culbertsoni*	Defined medium containing 11 amino acids
Modified Columbia agar	*Trichomonas vaginalis*	Solid agar medium; *T. vaginalis* colonies change pH and appearance of the agar plates.
Nelson's medium	*Naegleria fowleri*	Contains fetal calf serum, which is necessary for growth of *N. fowleri*
NIH medium	*Leishmania* and *Trypanosoma* species	Similar to Evans' modified Tobie's medium NaCl base agar medium
Novy-MacNeal-Nicolle (NNN) medium	*Leishmania* and *Trypanosoma* species	
NNN medium with Offutt's modifications	*Leishmania* species	Contains blood in base medium, differentiating it from NNN medium
4 N (NNNN) medium	*Leishmania* and *Trypanosoma* species	Uses a sugar base Uses NIH medium overlay
Nonnutrient agar with live or dead bacteria	*Acanthamoeba* species	Some manufacturers' media support growth better than others.
Proteose peptone-yeast extract-glucose (PPYG) medium	*Acanthamoeba* species	Basic medium to support growth of *Acanthamoeba* species
Peptone-yeast extract-glucose (PYG) medium	*Acanthamoeba* species	Similar to PPYG medium
SCGYEM medium (17)	*Naegleria fowleri*	Undefined medium
Schneider's *Drosophila* medium with 30% fetal calf serum	*Leishmania* and *Trypanosoma* species	Liquid medium; less costly than blood-based agars
Trichomonas culture system	*Trichomonas vaginalis*	Commercially available
Trypticase soy agar with 5% sheep, rabbit, or horse blood	*Acanthamoeba* species	Some manufacturers' media support growth better than others.
TYI-S-33 medium	*Entamoeba histolytica*	Contains no antibiotics; a nonselective medium
TYSGM-9 medium	*Entamoeba histolytica*	Contains penicillin G and streptomycin sulfate to inhibit bacterial overgrowth
U.S. Army Medical Research Unit (USAMRU)	*Leishmania* species	Particularly useful in isolation of *Leishmania brasiliensis* complex
Yeager's LIT (liver infusion tryptose) medium	*Trypanosoma cruzi*	Hemin and antibiotics are added to isolate *T. cruzi* from triatoma gut specimens.
YI-S medium	*Entamoeba histolytica*	Similar to TYI-S-33 medium

10 s. Place the slide in 95% alcohol for 30 s and then allow it to air dry. Examine at ×1,000 magnification.

Other Stains

Modified Field's stain

Modified Field's stain facilitates the identification of *Acanthamoeba* species. This stain was evaluated and shown to give very good contrast compared with other stains such as Wright's, Giemsa, Ziehl-Neelsen, and trichrome stains. For information on preparation and use of modified Field's stain, refer to the article by Pirehma and colleagues (11).

Modified safranin stain

Modified safranin stain allows the detection of *Cyclospora* oocysts in formalin-fixed specimens and fecal concentrates. The stain most commonly used in the past for these organisms was the modified acid-fast stain; however, tremendous variations in staining properties can be seen with this stain. The modified safranin stain reportedly uniformly stains oocysts of *Cyclospora*. It has also been shown to be fast, reliable, and easy to perform (12).

Basic procedure

Place a prepared, thin smear of stool on a 60°C slide warmer until dried. Cover smear with a 1% safranin solution and heat in a microwave oven at full power (650 W) for 30 to 60 seconds. Rinse the smear with tap water for 30 s, counterstain with 1% aqueous methylene blue for 1 min, rinse with tap water, and air dry. Examine at ×1,000 magnification.

Calcofluor white stain

Calcofluor white, one of a number of optical brighteners, binds to cellulose and chitin and fluoresces best when exposed to long-wavelength UV light. These properties allow its use in detecting fungi, including *Pneumocystis jirovecii* and free-living amebae like *Naegleria*, *Acanthamoeba*, and *Balamuthia* species, as well as the larvae of *Dirofilaria* species (its cuticle contains chitin). Calcofluor white is available through several commercial suppliers and is also easily made in the laboratory by following the manufacturer's recommendations.

Basic procedure

Place the specimen on a slide and allow it to air dry. Fix the slide in methanol for 1 to 2 min, rinse with distilled water, and allow it to air dry. Add 1 or 2 drops of 10% KOH, place 1 or 2 drops of calcofluor white solution on the specimen for 3 min, and view the slide with a fluorescent microscope and barrier filter (300 to 412 nm) at ×100 to ×400 magnification.

MEDIA

Cultures can be performed for only a few parasitic organisms, including *Acanthamoeba* species, *Naegleria fowleri*, *Plasmodium* species, intestinal amebae (like *Entamoeba histolytica*), *Trichomonas vaginalis*, *Leishmania* species, and *Trypanosoma* species (6). However, most clinical diagnostic microbiology laboratories do not provide parasite cultures. Cultures of these organisms are done only at large reference laboratories or research facilities. The exception is *Trichomonas vaginalis*, for which commercial products have been adapted for the routine laboratory. Many of the media listed in Table 5 are not commercially available but are listed for reference. Additional information regarding these media is available in the literature (1, 2, 11, 17–21).

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General Approaches for Detection and Identification of Parasites

LYNNE S. GARCIA, GRAEME P. PALTRIDGE, AND ROBYN Y. SHIMIZU

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This chapter discusses various approaches and diagnostic methods currently used for the diagnosis of parasitic infections. Assuming that clinical specimens have been properly collected and processed according to specific specimen rejection and acceptance criteria, the examination of prepared wet mounts, concentrated specimens, permanent stained smears, thick and thin blood films, and various culture materials can provide critical information leading to organism identification and confirmation of the suspected cause of clinical disease (1–6). With the exception of a few fecal immunoassay kits, the majority of this diagnostic work depends on the knowledge and microscopy skills of the microbiologist. The field of diagnostic parasitology has taken on greater importance during the past few years for a number of reasons. Expanded world travel has increased the potential levels of exposure to a number of infectious agents, as well as expanding epidemiologic boundaries and organism changes in pathogenicity. It is important to be aware of those organisms commonly found within certain areas of the world and the makeup of the patient population being serviced at your institution, particularly if immunocompromised patients are frequently seen as a part of your routine patient population. It is also important for the physician and microbiologist to recognize and understand the efficacy of any diagnostic method for parasite recovery and identification. Specific information on specimen collection and processing can be found in chapter 133. In order to become proficient in diagnostic medical parasitology, there is no substitute for performing extensive benchwork and the required microscopy associated with this type of testing.

STOOL SPECIMENS

For review, see the list of options for the collection of fecal specimens in chapter 133, Table 3, and Table 6. Algorithms for the processing of stool specimens are presented in Fig. 1, 2, and 3. The procedures that normally comprise the ova and parasite (O&P) examination are provided below and include the direct wet mount in saline, the concentration, and the permanent stained smear (1–4, 6–14).

Direct Wet Mount in Saline

The purpose of a direct wet mount is to confirm the possibility of infection with certain protozoa and helminths, to assess the worm burden of the patient, and to look for organism motility (Table 1) (1, 10, 13). Any fresh stool specimens that have not been refrigerated and that have been delivered to the laboratory within specified time frames are acceptable for testing; however, it is much more important to examine liquid or soft stools, rather than formed stools. Liquid and soft stools are much more likely to contain motile protozoan trophozoites than cysts, which do not demonstrate motility. Low-power examination (magnification, ×100) of the entire coverslip preparation (22 mm by 22 mm) and high dry power examination (magnification, ×400) of at least one third to one half of the coverslip area are recommended before the preparation is considered negative. Often, results from the direct smear examination should be considered presumptive; however, some organisms (*Giardia duodenalis* [*G. lamblia*, *G. intestinalis*] cysts and trophozoites, *Entamoeba coli* cysts, *Iodameba bütschlii* cysts, helminth eggs and larvae, and *Cystoisospora belli* oocysts) can be definitively identified. Reports of results obtained by this method should be considered preliminary, with the final report available after the results of the concentration wet mount and permanent stained smear are available.

If iodine is added to the preparation for increased contrast, the organisms will be killed and motility will be lost. Specimens that arrive in the laboratory in stool preservatives do not require a direct smear examination; proceed to the concentration and permanent stained smear. Examination of the wet mount using iodine is not required, but is the decision of each user performing this type of microscopy.

Concentration Wet Mount

The purpose of the concentration method is to separate parasites from fecal debris and to concentrate any parasites present through either sedimentation or flotation (1, 6, 10, 13). The concentration is specifically designed to allow recovery of protozoan cysts, coccidian oocysts, microsporidian spores (now classified with the fungi), and helminth eggs and larvae (Table 2). Any stool specimen that is fresh or preserved is acceptable for testing. Wet mounts prepared from concentrated stool are examined in the same manner as that used for the direct wet mount method. The addition of too much iodine may obscure helminth eggs (the eggs may resemble debris); the use of iodine is an individual decision. Often, results from the concentration examination should be considered presumptive; however, some organisms

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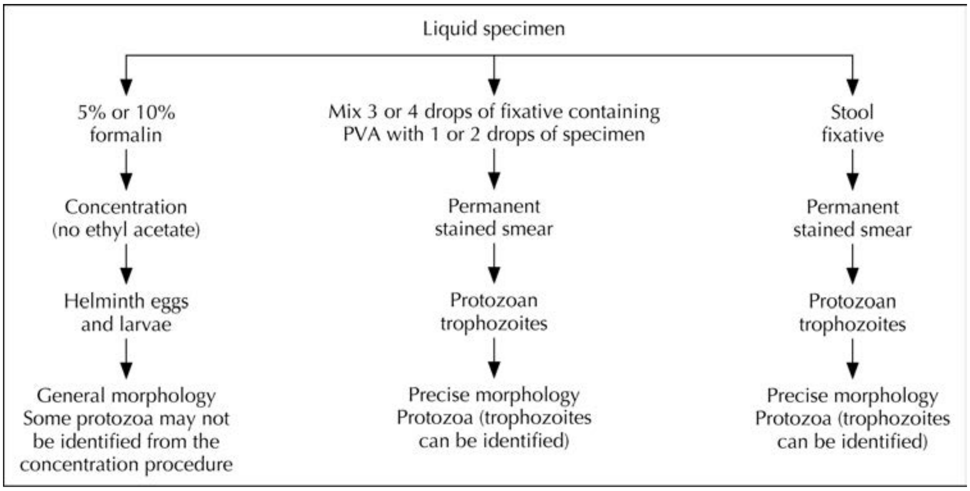
Liquid specimen
5% or 10% formalin
Concentration (no ethyl acetate)
Helminth eggs and larvae
General morphology Some protozoa may not be identified from the concentration procedure
Mix 3 or 4 drops of fixative containing PVA with 1 or 2 drops of specimen
Permanent stained smear
Protozoan trophozoites
Precise morphology Protozoa (trophozoites can be identified)
Stool fixative
Permanent stained smear
Protozoan trophozoites
Precise morphology Protozoa (trophozoites can be identified)

FIGURE 1 Processing liquid stool for O&P examination. Either PVA or Schaudinn's fixative can be used for the preparation of the permanent stained smear. Organism motility is seen when saline is used; iodine kills the organisms, so motility will no longer be visible. The use of ethyl acetate may remove the entire specimen and pull it into the layer of debris that will be discarded (liquid specimen normally contains mucus); centrifuge at 500 × *g* for 10 min (normal centrifugation time), but do not use ethyl acetate in the procedure. In general, laboratories have switched to nonmercury substitutes; the original Schaudinn's fixative contains mercuric chloride. However, in some instances the term "Schaudinn's fixative" is still used to describe not the original fixative but a formulation that is prepared with a copper or zinc base or other proprietary compounds. When fixatives are selected, it is important to know the contents in order to comply with disposal regulations. Reprinted from reference 9. doi:10.1128/9781555817381.ch135.f1

(*G. intestinalis* [*G. lamblia*, *G. duodenalis*] cysts, *E. coli* cysts, *I. bütschlii* cysts, helminth eggs and larvae, and *I. belli* oocysts) can be definitively identified. As with the direct wet mount, results obtained by the concentration wet mount should be considered preliminary, with the definitive report available after the results of the permanent stained smear are available.

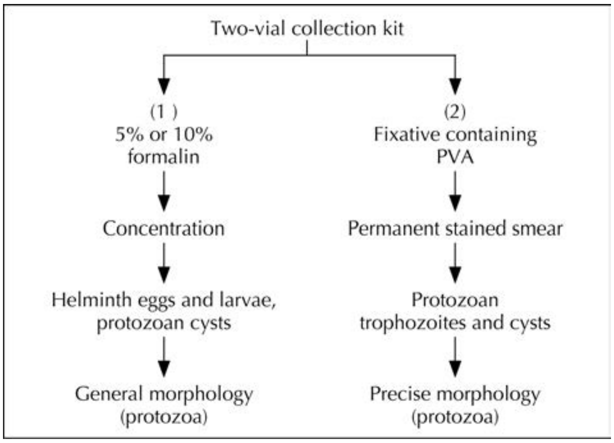
Two-vial collection kit
(1) 5% or 10% formalin
Concentration
Helminth eggs and larvae, protozoan cysts
General morphology (protozoa)
(2) Fixative containing PVA
Permanent stained smear
Protozoan trophozoites and cysts
Precise morphology (protozoa)

FIGURE 2 Processing preserved stool for O&P examination (two-vial collection kit). The formalin can be buffered or nonbuffered, depending on the laboratory protocol in use. Fixative prepared with mercuric chloride provides the best organism preservation. Alternatives are available, including zinc-based PVA, copper sulfate-based PVA, SAF, and the one of the single vial fixatives that requires no adhesive (PVA or albumin). Reprinted from reference 9.
doi:10.1128/9781555817381.ch135.f2

The formalin-ethyl acetate sedimentation concentration procedure is the most commonly used procedure, and the recommended centrifugation speed and time are 500 × *g* and 10 min, respectively. In this procedure, the use of ether has been replaced with ethyl acetate. However, it is important to remember that ethyl acetate should not be used for liquid specimens or those containing a great deal of mucus. The ethyl acetate may pull the liquid/mucus specimen contents into the debris layer, which will be discarded. Although the recovery of parasites from a liquid specimen or one containing a lot of mucus may not be successful, this simple centrifugation approach is still recommended. The standard zinc sulfate flotation procedure does not detect operculated or heavy eggs; when using this method, both the surface film and sediment should be examined before a negative result is reported.

Permanent Stained Smears

Trichrome, Iron-Hematoxylin, or Iron-Hematoxylin/Carbol Fuchsin

The permanent stained smear provides contrasting colors for both the background debris and the parasites present (Table 3, Table 4, and Table 5) (1, 6, 10, 13). Permanent stained stool smears are designed to allow examination and recognition of detailed organism morphology under oil immersion magnification (magnification, ×1,000). This method is primarily designed to allow the recovery and identification of the more common intestinal protozoan trophozoites and cysts, excluding the coccidia (unless the iron-hematoxylin/carbol fuchsin method is used) and microsporidia. Oil immersion examination of a minimum of 300× oil immersion fields is recommended; additional fields may be required if suspect organisms have been seen in the wet mounts. The use of a specific time for slide examination

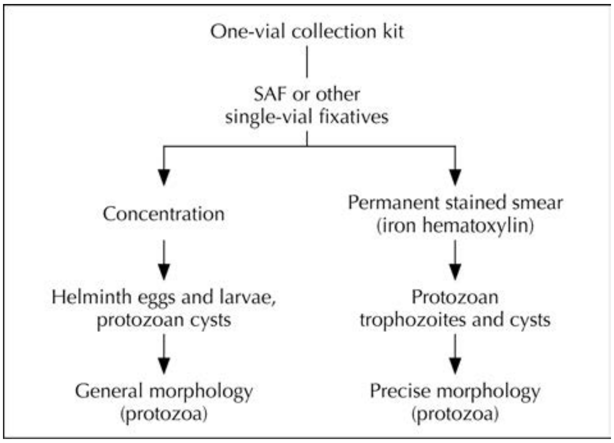
One-vial collection kit
SAF or other single-vial fixatives
Concentration
Permanent stained smear (iron hematoxylin)
Helminth eggs and larvae, protozoan cysts
Protozoan trophozoites and cysts
General morphology (protozoa)
Precise morphology (protozoa)

FIGURE 3 Processing preserved stool for O&P examination (one-vial collection kit). There are single-vial collection systems for which the formulas are proprietary; however, many contain zinc sulfate as one of the key ingredients. With the exception of SAF, compatibility of these fixatives with immunoassay reagents is not always possible, particularly if the fixative contains PVA. Other good options include Unifix, Total-Fix (single-vial fixative with no mercury, formalin, or PVA) (Medical Chemical Corp.), or Z-PVA (Medical Chemical Corp.) with trichrome stain, as well as Ecofix with Ecostain (Meridian Biosciences, Cincinnati, OH). If the iron hematoxylin method containing the carbol fuchsin step is used, the coccidian oocysts will stain pink (*Cryptosporidium* spp., *Cyclospora cayetanensis*, or *Cystoisospora belli*). Also, when using SAF, some recommend the use of an adhesive such as egg albumin to "glue" the fecal material onto the slide prior to staining. It is highly recommended that special stains be performed for the detection and identification of the coccidia (modified acid-fast stains) and the microsporidia (modified trichrome stains) from concentrated sediment to enhance organism recovery. Coccidian oocysts of *Cystoisospora belli* can easily be detected in the concentration sediment wet mount; however, unless a very heavy infection is present, *Cryptosporidium* spp. oocysts may not be seen without special modified acid-fast stains. The small size of the microsporidian spores prevents identification without the use of special modified trichrome stains and microscopic examination with a 100× oil immersion objective. Reprinted from reference 9.
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is not recommended, since each microbiologist screens the slides at a different rate. Although review of 300 fields seems like a time-consuming procedure, it takes less time than one would assume. This is particularly true for a trained microscopist in microbiology. Based on their expertise, patient population, and percentage of positive specimens, different laboratories may approach the examination of permanent stained smears by using different guidelines. Some laboratories may use a 60× oil immersion objective for screening purposes (magnification, ×600); however, it is important to examine a sufficient number of fields at a total magnification of ×1,000 before reporting the specimen as negative (no parasites seen).

Modified Acid-Fast Staining

The modified acid-fast staining method is used to provide contrasting colors for the background debris and the parasites present and to allow examination and recognition of the acid-fast characteristic of the organisms under high dry magnification (magnification, ×400) (1, 6, 15). Organisms that can be identified with this stain are the coccidia *Cryptosporidium* spp., *Cyclospora cayetanensis*, and *C. belli*. It is important to remember that *C. cayetanensis* stains much more acid-fast variable than *Cryptosporidium* spp. However, occasionally one can see acid-fast variability with *Cryptosporidium*, particularly if the decolorizer is too strong (16). A 1% acid destain is sufficient to provide excellent results for all of the coccidia. Although some microsporidian spores are acid-fast positive, their small size makes recognition very difficult; modified trichrome stains are recommended for the detection of microsporidian spores. Oil immersion examination of a minimum of 300× oil immersion fields is recommended.

Both hot and cold modified Ziehl-Neelsen and Kinyoun acid-fast staining methods are excellent for staining coccidian oocysts. Limitations of the procedure are generally related to specimen handling, including proper collection, centrifugation speed and time, and the percentage acid used for the destain step.

Modified Trichrome

Modified trichrome stains were primarily designed to allow recovery and identification of microsporidial spores from centrifuged stool specimens; internal morphology (horizontal and diagonal stripes of the polar tubule) may be seen in some spores under oil immersion (magnification, ×1,000). Any stool specimen that is submitted fresh or preserved in formalin, sodium acetate-acetic acid-formalin (SAF), or the single vial fixative is acceptable. Polyvinyl alcohol (PVA)-fixed specimens are unacceptable Oil immersion examination of a minimum of 300× oil immersion fields is recommended. The identification of microsporidial spores may be possible; however, their small size makes recognition difficult, particularly in infections with few organisms in the clinical specimen. Identification to the species level requires electron microscopy or molecular methods such as PCR.

Immunoassay Methods

Immunoassay reagents are available commercially for several of the protozoan parasites, including *G. intestinalis* (*G. Lamblia*, *G. duodenalis*), *Cryptosporidium* spp., the *Entamoeba*

TABLE 1 Diagnostic characteristics of organisms in wet mounts

Specimen	Protozoa	Helminths
Stool, other specimens from gastrointestinal tract, urogenital system	Size, shape, stage (trophozoite, precyst, cyst, oocyst), motility (fresh specimens only), refractility, cytoplasm inclusions (chromatoidal bars, glycogen vacuoles, axonemes, axostyles, median bodies, sporozoites)	Egg, larvae, or adult; size; internal structure: Egg: embryonated, opercular shoulders, abopercular thickenings or projections, hooklets, polar filaments, spines Larvae: head and tail morphology, digestive tract Adult: nematode, cestode, or trematode

TABLE 2 Identification of helminth eggs[a]

1. Eggs small (~25–40 μm)	
A. Operculate, generally oval, shoulders (egg <35 μm); *Clonorchis*, operculated shoulders, bile stained, may be an abopercular knob, miracidium present, but difficult to see	*Clonorchis* (*Opisthorchis*) spp. (Chinese liver fluke) or intestinal flukes: (*Heterophyes* or *Metagonimus yokogawai*)
B. Thick, radially striated shell (six-hooked oncosphere, individual eggs resemble those of *Taenia* spp.; eggs passed in egg packets containing 6–10 eggs; each egg is 2540 μm)	*Dipylidium caninum* (dog, cat tapeworm); note: egg packets could measure >150 μm
C. Thick, radially striated shell (six-hooked oncosphere may not be visible in every egg from formalinized fecal specimens) (eggs cannot be identified to species level without special stains) (each egg is 30–47 μm)	*Taenia* spp. (*T. saginata*, beef tapeworm; *T. solium*, pork tapeworm)
D. Thin eggshell, clear space between developing shell and embryo, spherical or subspherical, containing a six-hooked oncosphere; polar filaments (filamentous strands) present between thin egg shell and embryo (each egg is 31–43 μm)	*Hymenolepis nana* (dwarf tapeworm)
2. Eggs medium (~40–100 μm)	
A. Egg barrel-shaped, with clear polar plugs (each egg is 50–54 by 20–23 μm)	*Trichuris trichiura* (whipworm)
B. Egg flattened on one side, may contain larva (each egg is 70–85 by 60–80 μm)	*Enterobius vermicularis* (pinworm)
C. Egg with thick, tuberculated (bumpy) capsule (in decorticate eggs, capsule may be missing) (each egg is 45–75 by 35–50 μm)	*Ascaris lumbricoides* (large roundworm), fertilized eggs
D. Egg bluntly rounded at ends, thin shell (contains developing embryo at the 8–16 ball stage of development) (each egg is 56–75 by 36–40 μm)	Hookworm
E. Operculate, operculum break in shell sometimes hard to see, smooth transition from shell to operculum; small "bump" may be seen at abopercular end (each egg is 58–75 by 40–50 μm)	*Diphyllobothrium* spp, (broad fish tapeworm)
F. Thin eggshell, clear space between developing shell and embryo, spherical or subspherical, containing a six-hooked oncosphere; *no* polar filaments (filamentous strands) present between thin eggshell and embryo (each egg is 70–85 by 60–80 μm)	*Hymenolepis diminuta* (rat tapeworm)
3. Eggs large (~100–180 μm)	
A. Egg with opercular shoulders into which the operculum fits (looks like teapot lid and flange into which lid fits), abopercular end somewhat thickened—not always visible (each egg is 80120 by 48–60 μm); egg has been described as "urn-shaped."	*Paragonimus* spp. (lung fluke)
B. Egg tapered at one or both ends; long thin shell containing developing embryo (each egg is 73–95 by 40–50 μm)	*Trichostrongylus* spp.
C. Egg with thick, tuberculated (bumpy) capsule (in decorticate eggs, capsule may be missing) (each egg is 85–95 by 43–47 μm)	*Ascaris lumbricoides* (large roundworm); unfertilized eggs
D. Egg spined, ciliated miracidium larva may be seen, lateral spine very short (each egg is 70–100 by 55–65 μm)	*Schistosoma japonicum* (blood fluke, stool) *Schistosoma mekongi* (rounder and smaller than *S. japonicum*); measure 50–65 by 30–55 μm
E. Egg spined, ciliated miracidium larva may be seen, spine terminal (each egg is 112–170 by 40–70 μm)	*Schistosoma haematobium* (blood fluke, urine)
F. Egg spined, ciliated miracidium larva may be seen, spine terminal (each egg is 140–240 by 50–85 μm)	*Schistosoma intercalatum* (blood fluke, stool)
G. Egg spined, ciliated miracidium larva may be seen, large lateral spine (each egg is 114–180 by 45–70 μm)	*Schistosoma mansoni* (blood fluke, stool)
H. Egg >85 μm, operculum break in shell sometimes hard to see; smooth transition from shell to operculum; egg passed in undeveloped stage (each egg is 130–140 by 80–85 μm)	*Fasciolopsis buski* (giant intestinal fluke) or *Fasciola hepatica* (sheep liver fluke) or *Echinostoma* spp.

[a] This table does not include every possible helminth that could be found as a human parasite; however, the most likely helminth infections are included.

histolytica-Entamoeba dispar group, and *Entamoeba histolytica* (Table 6). These methods (enzyme immunoassay [EIA], fluorescent-antibody assay [FA], and immunochromatographic assay [cartridge]) are designed to detect the antigens of select organisms; a negative result does not rule out the possibility that these organisms/antigen are present in low numbers or that other intestinal parasites are etiologic agents causing disease, including *Dientamoeba fragilis*, the microsporidia, and helminth parasites (1, 17–19). Immunoassay reagents are currently under development and trial for *D. fragilis* and *Blastocystis* spp. Fecal immunoassays for the microsporidia are available; however, none are currently FDA-approved for use within the United States.

Molecular Methods

In January 2013, the FDA approved the first test that can simultaneously detect 11 common viral, bacterial, and parasitic (*Cryptosporidium*, *Giardia*) causes of infectious gastroenteritis from a single patient sample (xTAG Gastrointestinal Pathogen Panel; Luminex, Inc., Austin, TX)

TABLE 3 Diagnostic characteristics of organisms in permanent stained smears

Specimen	Protozoa	Helminths
Stool, other specimens from gastrointestinal tract, urogenital system	Size, shape, stage (trophozoite, precyst, cyst, oocyst, spore) Nuclear arrangement, cytoplasm inclusions (chromatoidal bars, vacuoles, axonemes, axostyles, median bodies, sporozoites, polar tubules) *Balantidium coli* trophozoites and cysts may not be visible due to excess stain retention.	Egg, larvae, and/or adults may not be identified because of excess stain retention or distortion

There are also several molecular tests that are in clinical trials for the detection of select gastrointestinal parasites. These tests are molecular gastrointestinal panels and target the most commonly occurring bacterial, viral, and parasitic stool pathogens. Although there are laboratory-developed tests for most parasites, these are not commercially available or available only in specialized testing centers. When such tests are used, there should be attention given to the use of internal amplification controls to detect inhibition, since common specimens, such as blood and stool, contain PCR inhibitors. Thorough validation is required before these are implemented for clinical testing.

ADDITIONAL TECHNIQUES FOR STOOL EXAMINATION

Although the routine O&P examination consisting of the direct wet mount, the concentration, and the permanent stained smear is an excellent procedure recommended for the detection of most intestinal parasites, several other diagnostic techniques are available for the recovery and identification of specific parasitic organisms (1, 6, 10). Most laboratories do not routinely offer all of these techniques, but many can be performed relatively simply and inexpensively. Occasionally, it is necessary to examine stool specimens for the presence of scolices and proglottids of cestodes and adult nematodes and trematodes to confirm the diagnosis and/or for species identification (Table 7). A method for the recovery of these stages is also described in this chapter.

Culture of Larval-Stage Nematodes

Nematode infections giving rise to larval stages that hatch in soil or in tissues may be diagnosed by using fecal culture methods to concentrate the larvae (1, 6, 10, 15). *Strongyloides stercoralis* larvae are the most common larvae found in stool specimens. Depending on the fecal transit time through the intestine and the patient's condition, rhabditiform and filariform larvae may be present. Caution must be exercised when handling larval cultures because infective filariform larvae may be present. If there is a delay in the preservation of the stool specimen, then embryonated ova as well as larvae of hookworm may be present. Culture of feces for larvae is useful for (i) revealing their presence when they are too scanty to be detected by concentration methods; (ii) distinguishing whether the infection is due to

TABLE 4 Key to identification of intestinal amebae (permanent stained smear)

1.	Trophozoites present	2
	Cysts present	7
2.	Trophozoites measure >12 µm	3
	Trophozoites measure <12 µm	4
3.	Karyosome central, compact; peripheral nuclear chromatin evenly arranged; "clean" cytoplasm	*Entamoeba histolytica*[a]
	Karyosome eccentric, spread out; peripheral nuclear chromatin unevenly arranged; "dirty" cytoplasm	*Entamoeba coli*
4.	Peripheral nuclear chromatin	5
	Other than above	6
5.	Karyosome central, compact (nucleus looks like a target); peripheral nuclear chromatin evenly arranged; "clean" cytoplasm	*Entamoeba hartmanni*
	Karyosome large, blot-like; extensive nuclear variation (nuclear variants may include those with peripheral chromatin)	*Endolimax nana*
6.	No peripheral chromatin, karyosome large, junky cytoplasm	*Iodameba bütschlii*
	No peripheral chromatin, karyosome variable, clean cytoplasm	*Endolimax nana*
7.	Cysts measure >10 µm (including any shrinkage "halo")	8
	Cysts measure <10 µm (including any shrinkage "halo")	10
8.	Single *Entamoeba*-like nucleus with large inclusion mass	*Entamoeba polecki*[b]
	Multiple nuclei	9
9.	Four *Entamoeba*-like nuclei, chromatoidal bars have smooth, rounded ends	*Entamoeba histolytica*[a]
	Five or more *Entamoeba*-like nuclei, chromatoidal bars have sharp, pointed ends (chromatoidal bars often not present)	*Entamoeba coli*
10.	Single nucleus (may be "basket" nucleus), large glycogen vacuole	*Iodameba bütschlii*[c]
	Multiple nuclei	11
11.	Four *Entamoeba*-like nuclei, chromatoidal bars have smooth, rounded ends (nuclei may also number only two)	*Entamoeba hartmanni*
	Four karyosomes, no peripheral chromatin, round to oval shape	*Endolimax nana*

[a] *Entamoeba histolytica* refers to the *Entamoeba histolytica/Entamoeba dispar* group/complex. *E. histolytica* (pathogenic) can be determined by finding red blood cells in the cytoplasm of the trophozoites. Otherwise, on the basis of morphological grounds *E. histolytica* (pathogen) and *E. dispar* (nonpathogen) cannot be differentiated. A report should read: *Entamoeba histolytica/E. dispar* group (or complex).

[b] It is very difficult to differentiate *Entamoeba polecki* trophozoites from those of the *E. histolytica/E. dispar* group, *E. coli*, or *E moshkovskii*.

[c] Although some *I. bütschlii* cysts are larger than 10 µm, the majority of cysts measure 9 to 10 µm and the typical glycogen vacuole ensures the proper identification.

TABLE 5 Key to identification of intestinal flagellates

1.	Trophozoites present	2
	Cysts present	7
2.	Pear shaped	3
	Shape other	6
3.	Two nuclei, sucking disk present	*Giardia duodenalis* (*G. lamblia*, *G. intestinalis*)
	One nucleus present	4
4.	Costa length of body	*Pentatrichomonas hominis*
	No costa	5
5.	Cytostome present, >10 μm	*Chilomastix mesnili*
	Cytostome present, <10 μm	*Retortamonas intestinalis* or *Enteromonas hominis*
6.	Ameba shaped, one or two fragmented nuclei	*Dientamoeba fragilis*
	Oval, one nucleus	*Enteromonas hominis*
7.	Oval or round cyst	8
	Lemon-shaped cyst	9
8.	Four nuclei, median bodies, axoneme, >10 μm	*Giardia duodenalis* (*G. lamblia*, *G. intestinalis*)
	Two nuclei, no fibrils, <10 μm	*Enteromonas hominis*
9.	One nucleus, (shepherd's) crook fibril	*Chilomastix mesnili*
	One nucleus, bird's beak fibril	*Retortamonas intestinalis*

S. stercoralis or hookworm on the basis of rhabditiform larval morphology by allowing hookworm egg hatching to occur, releasing first-stage larvae; and (iii) allowing the development of larvae into the filariform stage for further differentiation.

Fecal culture methods are especially helpful for the detection of light infections with hookworm, *S. stercoralis*, and *Trichostrongylus* spp. and for the specific identification of parasites. Also, such techniques are useful for obtaining a large number of infective-stage larvae for research purposes. One enhanced recovery method, the agar plate culture for *Strongyloides*, and several culture techniques are described in this chapter. Since these procedures are less common, brief descriptions are included.

Agar Plate Culture for *S. stercoralis*

Agar plate cultures are recommended for the recovery of *S. stercoralis* larvae and tend to be more sensitive than some of the other diagnostic methods (1, 6, 10). Approximately 2 g of fresh stool (about 1 in. in diameter) is placed in the center of the agar plate, and the plate is sealed to prevent accidental infections and held for 2 days at room temperature. As the larvae crawl over the agar, they carry bacteria with them, thus creating visible tracks over the agar. The plates are examined under the microscope for confirmation of the presence of larvae, the surface of the agar is then washed with 10% formalin, and final confirmation of larval identification is made via wet examination of the sediment from the formalin washings. Although specific agar formulations are often provided, any agar plate where the bacterial colonies are easily seen can be used for this purpose. However, it is recommended that the plates be drier rather than very moist, to avoid bacterial swarming that could obscure the tracking on the agar surface.

Baermann Technique

Another method of examining a stool specimen suspected of having small numbers of *Strongyloides* larvae is the use of a modified Baermann apparatus. The Baermann technique uses a funnel apparatus and relies on the principle that active larvae migrate from a fresh fecal specimen that has been placed on a wire mesh with several layers of gauze, which are in contact with tap water (1). Larvae migrate through the gauze into the water and settle to the bottom of the funnel, where they can be collected and examined. The main difference between this method and the Harada-Mori and petri dish methods is the greater amount of fresh stool used, possibly providing a better chance of larval recovery in a light infection. Besides being used for patient fecal specimens, this technique can be used to examine soil specimens for the presence of larvae. However, the agar plate method is considered to be a more sensitive method for the recovery of *S. stercoralis* larvae.

Harada-Mori Filter Paper Strip Culture

To detect light infections with hookworm, *S. stercoralis*, and *Trichostrongylus* spp., as well as to facilitate specific identification, the Harada-Mori filter paper strip culture technique is very useful (1, 7, 10). The technique requires filter paper (strip cut to fit into a 15-ml test tube) to which fresh fecal material (about the size of a raisin to a small grape) is added to the middle of the filter paper and a test tube into which the filter paper is inserted. Moisture is provided by adding water to the tube (within a half-inch of the stool, but not covering the stool), which continuously soaks the filter paper by capillary action. Incubation under suitable conditions favors hatching of ova and/or development of larvae. Fecal specimens to be cultured should not

TABLE 6 Commercially available immunoassays for detection of intestinal parasites

Fresh stool		Preserved in 5–10% formalin, SAF, or other single-vial system[a]	
No concentration	Concentration	No concentration	Concentration
Enzyme immunoassay (EIA)	DFA[b]	EIA	DFA[b]
Cryptosporidium spp.	*Cryptosporidium* spp.	*Cryptosporidium* spp.	*Cryptosporidium* spp.
G. lamblia[a]	*G. lamblia*	*G. lamblia*	*G. lamblia*
E. histolytica		Immunochromatographic assay (cartridge format)	
E. histolytica/*E. dispar* group		*Cryptosporidium* spp.	
Immunochromatographic assay (cartridge format)		*G. lamblia*	
Cryptosporidium spp.			
G. lamblia			
E. histolytica/*E. dispar* group			

[a] Diagnostic kits retain the *Giardia lamblia* designation.

[b] DFA, direct fluorescent-antibody assay.

TABLE 7 Additional helminth recovery and identification techniques (other than O&P examination)

Organism	Specimen	Procedure
Nematodes		
S. stercoralis	Fresh stool, not refrigerated (all organisms can be recovered by any of the procedures indicated in the column to the right)	Harada-Mori filter paper strip
Hookworm		Filter paper/slant culture
Trichostrongylus spp.		Charcoal culture
		Baermann test
		Agar plate culture (primarily for *S. stercoralis*)
Hookworm	Fresh stool, refrigeration acceptable	Direct smear (Beaver)[a]
Ascaris lumbricoides		Dilution egg count (Stoll)[a]
Trichuris trichiura		Either method acceptable for estimation of worm burden[a]
Enterobius vermicularis	Scotch tape preparations, paddles, anal swab, other collection devices	Direct microscopic examination
Trematodes		
Schistosoma spp.	Fresh stool, not refrigerated	Egg hatching test
	Fresh urine (24-h and single collection)	Egg viability test
Cestodes		
Tapeworms	Proglottids (gravid in alcohol)	India ink injection
	Stool in 5% to 10% formalin	Scolex search

[a] Although these two methods have been used in the past, currently the World Health Organization recommendation is the Kato-Katz thick fecal smear method with the McMaster egg count method being an excellent alternative for monitoring large scale eradication programs. In the majority of diagnostic laboratories, none of these procedures are routinely performed; generally their use is limited to relevant geographic areas and/or studies (24).

be refrigerated, since some parasites are susceptible to cold and may fail to develop after refrigeration. Also, caution must be exercised in handling the filter paper strip itself, since infective *Strongyloides* larvae may migrate upward as well as downward on the paper strip.

Filter Paper/Slant Culture Technique (Petri Dish)

An alternative technique for culturing *Strongyloides* larvae is a filter paper/slant culture on a microscope slide placed in a glass or plastic petri dish (1). As with the techniques described above, sufficient moisture is provided by continuous soaking of filter paper in water. Fresh stool material is placed on filter paper, which is cut to fit the dimensions of a standard (1 by 3 in.) microscope slide. The filter paper is then placed on a slanted glass slide in a glass or plastic petri dish containing water. This technique allows direct examination of the culture system with a dissecting microscope to look for nematode larvae and free-living stages of *S. stercoralis* in the fecal mass or the surrounding water without having to sample the preparation.

Egg Studies and Scolex Search

Estimation of Worm Burdens

The only human parasites for which it is reasonably possible to correlate egg production with adult worm burdens are *Ascaris lumbricoides*, *Trichuris trichiura*, and the hookworms (*Necator americanus* and *Ancylostoma duodenale*). The specific instances in which information on approximate worm burdens is useful are when one is determining the intensity of infection, deciding on possible chemotherapy, and evaluating the efficacies of the drugs administered. With current therapy, the need for the monitoring of therapy through egg counts is no longer as relevant. Remember that egg counts are estimates; you will obtain count variations regardless of how carefully you follow the procedure. If two or more fecal specimens are being compared, it is best to have the same individual perform the technique with both samples and to do multiple counts. A number of methods have been described (1, 4, 7, 10, 13, 20).

Hatching of Schistosome Eggs

When schistosome eggs are recovered from either urine or stool, they should be carefully examined to determine viability. The presence of living miracidia within the eggs indicates an active infection that may require therapy. The viability of the miracidia can be determined in two ways: (i) the cilia of the flame cells (primitive excretory cells) may be seen on a wet smear by using high dry power and are usually actively moving, and (ii) the miracidia may be released from the eggs by the use of a hatching procedure (1). The eggs usually hatch within several hours when placed in 10 vol of dechlorinated or spring water (hatching may begin soon after contact with the water). The eggs that are recovered in the urine (24-h specimen collected with no preservatives) are easily obtained from the sediment and can be examined under the microscope to determine viability. A sidearm flask has been recommended, but an Erlenmeyer flask is an acceptable substitute.

Because adult worms occasionally reside in veins other than their normal site, both urine and stool specimens must be collected. Specimens should be collected without preservatives and should not be refrigerated prior to processing. Hatching does not occur until the saline is removed and nonchlorinated water is added. If a stool concentration is performed, use saline throughout the procedure to prevent premature hatching. Make sure that the light is not too close to the side arm or top layer of water in the Erlenmeyer flask. Excess heat kills the miracidia. The lamp light mimics the sun shining on a water source, and the hatched larvae tend to swim toward the light. The absence of live miracidia does not rule out the presence of schistosome eggs. Nonviable eggs or eggs that failed to hatch are not detected by this method. Microscopic examination of direct or concentrated specimens should be used to demonstrate the presence or absence of eggs. Egg viability can be determined by placing some stool or urine sediment (the same material used for the hatching flask) on a microscope slide. Low-power magnification (×100) can be used to locate the eggs. Individual eggs can be examined with high dry magnification (×400); moving cilia on the flame cells (primitive excretory system) confirm egg viability.

Search for Tapeworm Scolices

Since therapy for the elimination of tapeworms is usually very effective, a search for the tapeworm scolex is rarely requested and is no longer clinically relevant. However, stool specimens may have to be examined for the presence of scolices and gravid proglottids of cestodes for proper species identification. This procedure requires mixing a small amount of feces with water and straining the mixture through a series of wire screens (graduated from coarse to fine mesh) to look for scolices and proglottids. Remember to use standard precautions and to wear gloves when performing this procedure. The appearance of scolices after therapy is an indication of successful treatment. If the scolex has not been passed, it may still be attached to the mucosa; the parasite is capable of producing more segments from the neck region of the scolex, and the infection continues.

EXAMINATION OF OTHER SPECIMENS FROM THE INTESTINAL TRACT

Examination for Pinworm

A roundworm parasite that has a worldwide distribution and that is commonly found in children is *Enterobius vermicularis*, known as pinworm or seatworm. The adult female worm migrates out of the anus, usually at night, and deposits her eggs on the perianal area. The adult female (8 to 13 mm long) may occasionally be found on the surface of a stool specimen or on the perianal skin. Since the eggs are usually deposited around the anus, they are not commonly found in feces and must be detected by other diagnostic techniques. Diagnosis of pinworm infection is usually based on the recovery of typical eggs, which are described as thick-shelled, football-shaped eggs with one slightly flattened side. Often each egg contains a fully developed embryo and becomes infective within a few hours after being deposited. Commercial collection devices are available and can be used for specimen collection, similar to the approach with cellulose tape indicated below (1). Many clinicians may treat patients on the basis of clinical symptoms without confirmation of the suspected diagnosis of pinworm infection.

Cellulose Tape Preparations

The most widely used procedure for the diagnosis of pinworm infection is the cellulose tape (adhesive cellophane tape) method (1, 8, 11, 12). Specimens should be obtained in the morning, before the patient bathes or goes to the bathroom. The tape is applied to the anal folds and is then placed sticky side down on a microscope slide for examination. At least four to six consecutive negative slides should be observed before the patient is considered free of infection.

Anal Swabs

The anal swab technique (1) is also available for the detection of pinworm infections; however, most laboratories use the cellulose tape method because it eliminates the necessity of preparing and storing swabs. At least four consecutive negative preparations should be observed before the patient is considered negative; some recommend six consecutive negative preparations.

A paraffin-coated swab should be gently rubbed over the perianal surface and into the folds. Place the swab into the anal opening about 1/4 in. and then replace the swab in the tube. The tube containing the swab is half filled with xylene substitute and is allowed to stand for a few minutes. After centrifugation, the sediment is examined microscopically for the presence of eggs.

Sigmoidoscopy Material

Material obtained from sigmoidoscopy can be helpful in the diagnosis of amebiasis that has not been detected by routine fecal examinations, and the procedure is recommended for this purpose. However, usually a series of at least three routine stool examinations for parasites should be performed for each patient before a sigmoidoscopy examination is done (1).

Material from the mucosal surface should be aspirated or scraped and must not be obtained with cotton-tipped swabs. At least six representative areas of the mucosa should be sampled and examined (six samples, six slides). Usually, the amount of material is limited and should be processed immediately to ensure the best examination possible (Table 8). Three methods of examination can be performed. All three are acceptable; however, depending on the availability of trained personnel, the availability of proper fixation fluids, or the amount of specimen obtained, one or two procedures are recommended. If the amount of material limits the examination to one procedure, the use of one of the fecal fixatives is highly recommended for the subsequent preparation of permanent stains.

Although the fecal immunoassays are rarely performed on sigmoidoscopy specimens, they can be used on fecal specimens for the detection of the *Entamoeba histolytica*/*E. dispar* group or confirmation of the pathogen *Entamoeba histolytica*. However, this testing requires fresh or frozen stool; preserved stool is not acceptable.

Physicians performing sigmoidoscopy procedures may not realize the importance of selecting the proper fixative for material to be examined for parasites when using the concentration and permanent stained smear methods. It is recommended that a parasitology specimen tray (containing Schaudinn's fixative, a liquid fixative containing PVA, 5 or 10% formalin, or one of the single vial fixatives) be provided or that a trained technologist be available at the time of sigmoidoscopy to prepare the slides.

Direct Saline Mount

If there is no lag time after collection and a microscope is available in the immediate vicinity, some of the material should be examined as a direct saline mount for the presence of motile trophozoites (1). A drop of material is mixed with a drop of 0.85% sodium chloride and is examined under low light intensity for the characteristic movement of amebae. It may take time for the organisms to become acclimated to this type of preparation; thus, motility may not be obvious for several minutes. There will be epithelial cells, macrophages, and possibly polymorphonuclear leukocytes (PMNs) and erythrocytes, which will require a careful examination to reveal amebae.

Since specific identification of protozoan organisms can be difficult when only the direct saline mount is used, this technique should be used only when there is sufficient material left to prepare permanent stained smears.

Permanent Stained Smear

Most of the material obtained at sigmoidoscopy can be smeared (gently) onto a slide and immediately immersed in SAF or one of the single vial fixatives (1). These slides can then be stained with trichrome or iron hematoxylin stain and examined for specific cell morphology, either protozoan or otherwise. The procedure and staining times are identical to those for routine fecal smears.

If the material is bloody, contains a lot of mucus, or is a "wet" specimen, a few (no more than 2 or 3) drops of fixative containing PVA can be mixed with 1 or 2 drops

TABLE 8 Recovery of parasites from other intestinal tract specimens

Source	Organism	Procedure
Sigmoidoscopy specimens		
Unpreserved	Ameba trophozoites (motility)	Direct wet mount, immunoassay tests[a]
Air-dried smears	Coccidia	Modified acid-fast stain
	Microsporidia	Modified trichrome, optical brighteners, immunoassay tests[b]
Preserved 5–10% formalin or SAF or one of single vial fixatives	Helminth eggs and larvae (rare), ameba and flagellate cysts[c] and trophozoites (SAF and one of single vial fixatives)	Concentration wet mount, immunoassay tests
	Coccidia	Modified acid-fast smear, immunoassay tests
	Microsporidia	Modified trichrome, optical brighteners, immunoassay tests[b]
Polyvinyl alcohol (PVA) fixative	Helminth eggs and larvae (rare), ameba and flagellate cysts[c]	Wet mount
	Ameba and flagellate cysts[c] and trophozoites	Permanent stained smear
Schaudinn's fixative	Ameba and flagellate cysts[c] and trophozoites	Permanent stained smear
Duodenal specimens[d]		
Unpreserved Entero-Test capsule	Helminth eggs and larvae, trophozoites (motility) of *Giardia*	Direct wet mount
Preserved 5–10% formalin or SAF or one of the single vial fixatives	Helminth eggs and larvae, *Giardia* trophozoites	Concentration wet mount, immunoassay tests (depending on validation documentation) Permanent stained smear
Entero-Test capsule	Coccidia	Modified acid-fast smear, immunoassay tests
	Microsporidia	Modified trichrome, optical brighteners, immunoassay tests[b]
Fecal fixatives containing PVA (adhesive)	Flagellate trophozoites	Permanent stained smear
Anal impression smear	Pinworm adult and eggs	No stain, cellulose tape preparation and other collection devices
Adult worm or segments	Helminth adult worms or proglottids	Carmine stain (rarely used), India ink
Tissue biopsy specimen	Helminth eggs, larvae, and adults; protozoan cysts; trophozoites; oocysts; sporozoites; and spores	Touch preparations, squash preparations, permanent stains, histology

[a] Immunoassay tests for the *Entamoeba histolytica/E. dispar* group or *Entamoeba histolytica* require fresh or frozen stool; preserved stool specimens are not acceptable for testing. Immunoassays for *Cryptosporidium* spp. and *Giardia lamblia* (name designation on commercial kits) are approved for use on stool; immunoassay use for other intestinal tract specimens (sigmoidoscopy) may or may not be appropriate, depending on specimen source, consistency, volume, and appropriate validation documentation.

[b] Some genus-specific immunoassay reagents for the microsporidia are available commercially but are not FDA approved.

[c] Although cysts may be present in stool, sigmoidoscopy specimens are often obtained from patients with severe diarrhea or dysentery. In these cases, the cyst forms are usually absent; trophozoites would be the most likely stage seen, particularly in the case of *Entamoeba histolytica*.

[d] Duodenal specimens are often submitted as aspirates; in such cases the volume may be sufficient to perform concentrations. However, if small amounts of duodenal mucus and/or biopsy material are obtained, squash preparations preserved with Schaudinn's fixative are preferred. This approach may require the use of slides precoated with albumin to facilitate adhesion. Mucus obtained from the Entero-Test capsule string may be treated as a fresh specimen; the string can also be immediately placed in preservative after retrieval, and the mucus can be processed as a permanent stained smear from a number of fixative options (with and without PVA).

of material directly on the slide, which is allowed to air dry (a 37°C incubator can be used) for at least 2 h before staining. If time permits, the smears should be allowed to dry overnight; they can be routinely stained with trichrome stain and examined as a permanent mount.

Material from sigmoidoscopy can be placed in small amounts of SAF or one of the single vial fixatives. After fixation for 30 min, the specimen can be centrifuged at 500 × *g* for 10 min, and smears from the small amount of sediment can be prepared for permanent staining. The most relevant organism for consideration when sigmoidoscopy is performed is *E. histolytica*, the morphology of which is normally seen from the permanent stained smear. However, if fresh stool or aspirated material is obtained, then both the permanent stained smear and an antigen detection reagent kit for the detection of the *Entamoeba histolytica/E. dispar* group or confirmation of the pathogen *Entamoeba histolytica* can be used. If enough material is present for only a single procedure, then the permanent stained smear is recommended, particularly if the iron hematoxylin stain (incorporating the carbol fuchsin step) is used (1). This method would enhance the detection of the coccidia, which are modified acid-fast positive.

Duodenal Contents

Duodenal Drainage

In infections with *G. intestinalis* (*G. lamblia*, *G. duodenalis*) or *S. stercoralis*, routine stool examinations may not reveal the organisms. Duodenal drainage material can be submitted for examination (Table 8).

A fresh, unpreserved specimen should be submitted to the laboratory; the amount may vary from <0.5 ml to several milliliters of fluid. The specimen may be centrifuged (at 500 × *g* for 10 min) and should be examined immediately as a wet mount for motile organisms (iodine may be added later to facilitate identification of any organisms present). If the specimen cannot be completely examined within 2 h after it is taken, any remaining material should be preserved in one of the fecal fixatives for permanent staining.

If the duodenal fluid contains mucus, this is where the organisms tend to be found. Therefore, centrifugation of the specimen is important, and the sedimented mucus should be examined. *Giardia* trophozoites may be caught in mucus strands, and the movement of the flagella on the trophozoites may be the only subtle motility seen for these flagellates. *Strongyloides* larvae are usually very motile. Immunoassay methods for *Cryptosporidium* spp. and *G. intestinalis* (*G. lamblia*, *G. duodenalis*) can also be used with fresh or formalinized material; however, duodenal fluid is not included in many of the package inserts as an acceptable specimen.

If the amount of duodenal material submitted is very small, rather than using any of the specimen for a wet smear examination, permanent stains can be prepared. This approach provides a more permanent record, and the potential problems with unstained organisms, very minimal motility, and a lower-power examination can be avoided by using oil immersion examination of the stained specimen at ×1,000 magnification.

Duodenal Capsule Technique (Entero-Test)

A method of sampling duodenal contents that eliminates the need for intestinal intubation has been devised and consists of the use of a length of nylon yarn coiled inside a gelatin capsule (1, 6, 7, 10). The yarn protrudes through one end of the capsule, and this end of the line is taped to the side of the patient's face. The capsule is then swallowed, the gelatin dissolves in the stomach, and the weighted string is carried by peristalsis into the duodenum. The weight is released and passes out in the stool when the line is retrieved after a period of 4 h. Bile-stained mucus clinging to the yarn is then scraped off with gloved fingers and is collected in a small petri dish. Usually 4 or 5 drops of material are obtained. Adult and pediatric capsules are currently available from Nutri-Link Ltd, United Kingdom (http://www.nutri-linkltd.co.uk/).

The specimen should be examined immediately as a wet mount for motile organisms. Organism motility is similar to that described above for duodenal drainage. If the specimen cannot be completely examined within an hour after the yarn has been removed, the material should be preserved in SAF or one of the single vial fixatives or fixative containing PVA. Mucus smears should be prepared.

The pH of the terminal end of the yarn should be checked to ensure adequate passage into the duodenum (a very low pH means that it never left the stomach). Also, since the bile duct drains into the intestine at this point, the terminal end of the yarn should be a yellow-green color.

UROGENITAL SPECIMENS

Several parasites may be recovered and identified from urogenital specimens. Although the most common pathogens are probably *Trichomonas vaginalis* and *Schistosoma haematobium*, other organisms, such as the microsporidia, are becoming much more important (Table 9). Also, the membrane filtration for the recovery of microfilariae can be performed from urine.

Direct Wet Mount

The identification of *T. vaginalis* is usually based on the examination of a wet preparation of vaginal and urethral discharges and prostatic secretions or urine sediment and may require the testing of multiple specimens to confirm the diagnosis. These specimens are diluted with a drop of saline and are examined under low power and reduced illumination for the presence of actively motile organisms. As the jerky motility begins to diminish, it may be possible to observe the undulating membrane, particularly under high dry power (magnification, ×400).

While the membrane filtration technique can be used for the recovery of microfilariae, examination of urinary sediment may be indicated in certain filarial infections. The occurrence of microfilariae in urine has been reported with increasing frequency in *Onchocerca volvulus* infections in Africa.

TABLE 9 Detection of urogenital parasites

Organism	Procedure
Trichomonas vaginalis	Wet mount (motility)
	Culture
	Giemsa stain (if dry smear submitted—prefix in absolute methanol and allow smear to dry prior to staining)
	Direct fluorescent antibody
	DNA probe
	Latex agglutination
	Enzyme-linked immunoassay
	Rapid immunochromatographic assay (dipstick)
Schistosoma haematobium	Wet mount (urine sediment)
	Membrane filtration
	Tissue section (Squash prep and/or histology section)
Microfilariae	Knott concentration
	Membrane filtration (Nuclepore filter)
	Giemsa stain
	Delafield's hematoxylin stain
Microsporidia	Modified trichrome stain[a]
	Optical brighteners (calcofluor white)[a]
	Immunoassay tests[a,b]
	Routine histology
	Electron microscopy or molecular methods for identification to the genus, species levels

[a] Staining procedures and fluorescent antibody tests for organism detection performed on centrifuged sediment (500 × *g* for 10 min).

[b] Immunoassays are available commercially but are not yet FDA approved for use within the U.S.

Urine is collected in a bottle, this volume is recorded, and thimerosal (1 ml/100 ml of urine) is added. The specimen is placed in a funnel fitted with tubing and a clamp; this preparation is allowed to settle overnight. On the following day, 10 to 20 ml of urine is withdrawn and centrifuged. The supernatant is discarded, and the sediment is resuspended in 0.85% NaCl. This preparation is again centrifuged, and 0.5 to 1.0 ml of the sediment is examined under the microscope for the presence of nonmotile microfilariae. The membrane filtration technique can also be used with urine for the recovery of *Schistosoma haematobium* eggs (1). This approach uses a 25-mm Nuclepore filter (5-μm porosity) (1, 6, 10).

The use of stained smears is usually not necessary for the identification of *T. vaginalis*. Many times the number of false-positive and false-negative results reported on the basis of stained smears strongly suggests the value of confirmation by observation of motile organisms from the direct mount, from appropriate culture media (1, 6, 10, 20), or from direct detection with immunoassay reagents (1).

Stained smears may be prepared from material obtained from the membrane filtration techniques used for the recovery of microfilariae; Delafield's hematoxylin or Giemsa stain may be used. It is important to remember that Giemsa stain may not adequately stain the sheath, and correct identification of the organisms may require staining with a hematoxylin-based stain.

Some microsporidial infections can also be diagnosed from the examination of urine sediment that has been stained by one of the modified trichrome methods or by using optical brightening agents such as calcofluor white (1). Multiple methods are recommended for confirmation of the diagnosis. It is recommended that any patient suspected of microsporidiosis submit both stool and urine for examination.

Culture

Specimens from women for culture (for *T. vaginalis*) may consist of vaginal exudate collected from the posterior fornix on cotton-tipped applicator sticks or genital secretions collected on polyester sponges. Specimens from men can include semen, urethral samples collected with a polyester sponge, or urine. Urine samples collected from the patient should be the specimen first voided in the morning. It is critical that clinical specimens be inoculated into culture medium as soon as possible after collection (1, 12, 20). Although collection swabs can be used, there are often problems with specimens drying prior to culture; immediate processing is mandatory for maximum organism recovery. Another approach would be to use the plastic envelope methods (*Trichomonas* Culture System [Empyrean Diagnostics, Mountain View, CA] or InPouch TV [BioMed Diagnostics, San Jose, CA]), which are simplified techniques for transport and culture (4). The following control strain should be available when using these cultures for clinical specimens: *T. vaginalis* ATCC 30001. Many media for the isolation of *T. vaginalis* are available, and some of these can be purchased commercially and have relatively long shelf lives, particularly the plastic envelope methods.

If no trophozoites are seen after 4 days of incubation, then discard the tubes and report the culture as negative. Results for patient specimens should not be reported as positive unless control cultures are positive. Since culture may take as long as 3 to 4 days and the clinical specimens may contain nonviable organisms, it is recommended that microscopic examination of wet smears be performed as well (possible dead organisms may be present, although they will be difficult to see).

Antigen Detection (*Trichomonas vaginalis*)

The culture method is considered to be very sensitive for the diagnosis of trichomoniasis; however, due to the time and effort involved, some laboratories have decided to use some of the new immunoassay detection kits (1). The Osom Trichomonas Rapid Test (Genzyme Diagnostics, Cambridge, MA) is an immunochromatographic method for antigen detection using the dipstick format. Results are available within 10 min, and according to the manufacturer, there is a 95% agreement with the reference standard (culture and wet mount). The Affirm VPIII DNA probe technology (Becton, Dickinson and Co, Franklin Lakes, NJ) offers a dependable, rapid means for the early identification of three organisms causing vaginitis: *Candida* species, *Gardnerella vaginalis*, and *Trichomonas vaginalis*. Also, the APTIMA *Trichomonas vaginalis* Assay (GenProbe, San Diego, CA) is available.

SPECIMENS FROM OTHER BODY SITES

When routine testing methods have failed to demonstrate the organisms, the examination of aspirated material for the diagnosis of parasitic infections may be extremely valuable (Table 10). Specimens should be transported to the laboratory immediately after collection. Aspirates include liquid specimens collected from a variety of sites as well as fine-needle aspirates and duodenal aspirates. Fine-needle aspirates are often collected by the cytopathology staff who process the specimens, or they may be collected and sent to the laboratory directly for slide preparation and/or culture. Fluid specimens collected by bronchoscopy include bronchoalveolar lavage and bronchial washing fluids.

Procedural details for processing sigmoidoscopic aspirates and scrapings for the recovery of *E. histolytica* and techniques for preparation of duodenal aspirate material have been presented earlier in this chapter.

Biopsy specimens are recommended for use in the diagnosis of parasitic infections in tissues (Table 10). In addition to standard histologic preparations, the following can be used: impression smears and teased and squash preparations of biopsy tissue from skin, muscle, cornea, intestine, liver, lung, and brain. Tissue to be examined as permanent sections or by electron microscopy should be fixed as specified by the laboratories that will process the tissue, and in certain cases, testing of a biopsy specimen may be the only means of confirming a suspected parasitic problem. Specimens that are going to be examined as fresh material rather than as tissue sections should be kept moist in saline and submitted to the laboratory immediately.

Detection of parasites in tissue depends on specimen collection and the retrieval of sufficient material for examination. Biopsy specimens are usually quite small and may not be representative of the diseased tissue. Multiple tissue samples often improve diagnostic results. To optimize the yield from any tissue specimen, examine all areas and use as many procedures as possible. Tissues are obtained from invasive procedures, many of which are very expensive and lengthy: consequently, these specimens deserve the most comprehensive procedures possible.

Tissue submitted in a sterile container on a sterile sponge dampened with saline may be used for cultures of protozoa after mounts for direct examination or impression smears for staining have been prepared. If cultures for parasites will be made, use sterile slides for smear and mount preparation or inoculate cultures prior to smear preparation.

Bone Marrow

Bone marrow aspirates to be evaluated for *Leishmania* amastigotes, *Trypanosoma cruzi* amastigotes (African trypanosomiasis organisms are not relevant), *Plasmodium* spp., or *Toxoplasma gondii* (bone marrow is a less common site for this organism) require Giemsa staining. However, if stained with a hematoxylin/Giemsa combination or other blood stains, the organisms will be visible as well. If specimens are to be processed for culture, it is important to maintain sterility of the specimen prior to inoculation of media for parasitology cultures. After inoculation of appropriate media, the remaining specimen can be processed for smear preparation and staining.

Brain

Generally, when cerebrospinal fluid or brain aspirates or biopsy specimens are received, the most likely parasites would include the free-living amebae *Acanthameba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, or *Sappinia* spp. The use of non-nutrient agar (with bacterial overlays) cultures is recommended for *Acanthameba* spp. and *N. fowleri;* quality control cultures with known positive organisms are recommended as a basis for acceptable interpretation of patient culture results. *Sappinia* spp. will also grow using the non-nutrient agar with bacterial overlay culture system. If free-living ameba cultures are ordered for central nervous system specimens, the agar plates should be incubated at 37°C (room air, no CO_2) (1, 6, 10). Unfortunately, cultures for *B. mandrillaris* are difficult to perform; consultation with the Centers for Disease Control and

TABLE 10 Specimen, possible parasite recovered, and appropriate tests (other than intestinal tract)[a]

Body site	Specimen	Possible parasites	Tests
Bone marrow	Aspirate	*Leishmania* spp., *Trypanosoma cruzi*, *Plasmodium* spp., *Toxoplasma gondii*	Giemsa, culture (if relevant)
Brain	Tissue biopsy specimen, cerebrospinal fluid	*Naegleria* spp., *Acanthameba* spp., *Sappinia* spp.	Giemsa, trichrome, culture
		Balamuthia mandrillaris, *Entamoeba histolytica*	Giemsa, trichrome
		Toxoplasma gondii	Giemsa, immunospecific reagent, culture, PCR
		Microsporidia (*Encephalitozoon* spp., *Trachipleistophora anthropophthera*)	Modified trichrome, acid-fast stain, Giemsa, optical brightening agent (calcofluor white), histology[b] (methenamine silver, PAS,[c] tissue Gram stains), electron microscopy
		Taenia solium (cysticerci), *Echinococcus* spp.	Routine histology[b]
Eye	Cornea, conjunctiva, contact lens, lens solutions[d]	Microsporidia (*Encephalitozoon* spp., *Trachipleistophora* spp., *Anncaliia* sp., *Nosema* spp., *Microsporidium* spp.)	Acid-fast stain, Giemsa, modified trichrome, methenamine silver, optical brightening agent calcofluor white), histology[b] (methenamine silver, PAS,[c] tissue Gram stains), electron microscopy
		Acanthameba spp.	Giemsa, trichrome, culture, calcofluor white (cysts only)
		Toxoplasma gondii	Giemsa, immunospecific reagent, culture
	Larval or adult worms	*Loa loa*, *Dipetalonema*, *Thalezia*, *Dirofilaria immitis*, *Gnathostoma* spp., *Toxocara canis*, *Taenia solium* (cysticerci), *Echinococcus* spp.	Direct examination, routine histology[b]
	Fly larvae, adult lice	Myiasis, lice infestation	Direct examination
Kidney, bladder	Biopsy specimens	Microsporidia (*Encephalitozoon* spp., *Enterocytozoon bieneusi*)	Modified trichrome, acid-fast stain, Giemsa, optical brightening agent (calcofluor white), histology[b] (methenamine silver, PAS,[c] tissue Gram stains), electron microscopy
		Schistosoma haematobium	Direct examination
	Adult worm, eggs	*Dioctyphyma renale*	Direct examination
Liver, spleen	Aspirates, biopsy specimens	*Echinococcus* spp.	Wet mount, routine histology[b]
		Clonorchis sp., *Opisthorchis* spp. *Capillaria hepatica*, *Toxocara canis*, *T. cati*	Routine histology[b]
		Toxoplasma gondii, *Leishmania donovani*	Giemsa, culture
		Cryptosporidium spp.	Modified acid-fast stain, immunospecific reagents
		Microsporidia (*Encephalitozoon* spp., *Enterocytozoon bieneusi*)	Modified trichrome, acid-fast stain, Giemsa, optical brightening agent (calcofluor white), histology[b] (methenamine silver, PAS,[c] tissue Gram stains), electron microscopy
		Entamoeba histolytica[e]	Wet mount, trichrome
Lymph node, lymphatics	Aspirates, biopsy specimens	*Toxoplasma gondii*, *Trypanosoma cruzi*, *Trypanosoma brucei rhodesiense*, *T. brucei gambiense*	Direct examination, routine histology,[b] Giemsa, culture
		Microsporidia	Modified trichrome, acid-fast stain, Giemsa, optical brightening agent (calcofluor white), histology[b] (methenamine silver, PAS,[c] tissue Gram stains), electron microscopy
		Wuchereria bancrofti, *Brugia malayi*, *Brugia* spp.	Thick blood films, concentration, membrane filtration

(Continued on next page)

TABLE 10 Specimen, possible parasite recovered, and appropriate tests (other than intestinal tract)[a]

Body site	Specimen	Possible parasites	Tests
Lung	Sputum (expectorated or induced), bronchoalveolar lavage fluid, transbronchial aspirates, brush biopsy specimens, open lung biopsy specimens	*Ascaris lumbricoides*, *Strongyloides stercoralis*, hookworm, *Paragonimus* spp., *Echinococcus granulosus*	Wet mount, routine histology[b]
		Microsporidia (*Encephalitozoon* spp., *Enterocytozoon bieneusi*)	Modified trichrome, acid-fast stain, Giemsa, optical brightening agent (calcofluor white), histology[b] (methenamine silver, PAS,[c] tissue Gram stains), electron microscopy
		Toxoplasma gondii	Giemsa, immunospecific reagent, culture
		Cryptosporidium spp.	Modified acid-fast stain, immunospecific reagent
	Saliva	*Entamoeba gingivalis*, *Trichomonas tenax*	Trichrome
Muscle	Biopsy specimen	*Trichinella* spp.	Wet examination, squash preparation, routine histology[b]
		Microsporidia (*Pleistophora* spp., *Nosema* spp., *Trachipleistophora hominis*)	Modified trichrome, acid-fast stain, Giemsa, optical brightening agent (calcofluor white), histology[b] (methenamine silver, PAS,[c] tissue Gram stains), electron microscopy
		Sarcocystis spp., *Baylisascaris procyonis*, *Ancylostoma* spp., *Taenia solium* (cysticerci), *Multiceps* (coenurus), *Echinococcus* spp., *Spirometra* (spargana), *Onchocerca volvulus* (nodules), *Gnathostoma* spp., *Trypanosoma cruzi*	Routine histology[b]
Nasopharynx, sinus cavities	Scraping, biopsy specimens, aspirates	Microsporidia (*Encephalitozoon* spp., *Enterocytozoon bieneusi*, *Trachipleistophora hominis*)	Modified trichrome, acid-fast stain, Giemsa, optical brightening agent (calcofluor white), histology[b] (methenamine silver, PAS,[c] tissue Gram stains), electron microscopy
		Acanthameba spp.	Giemsa, trichrome, culture, calcofluor white (cysts only)
		Naegleria spp.	Giemsa, trichrome, culture
		Leishmania spp.	Giemsa, culture
Rectal tissue	Scraping, aspirate, biopsy specimens	*Schistosoma mansoni*, *S. japonicum*	Direct examination
Skin	Skin snips	*Onchocerca volvulus*, *Mansonella streptocerca*	Giemsa, routine histology[b]
	Scraping, aspirates, biopsy specimen	*Leishmania* spp.	Giemsa, culture, routine histology[b]
		Acanthameba spp.	Giemsa, trichrome, culture, calcofluor (cysts only)
		Entamoeba histolytica, *Schistosoma* spp.	Routine histology[b]

[a] This table does not include every possible parasite that could be found in a particular body site. Parasite stages include trophozoites, cysts, oocysts, spores, adults, larvae, eggs, hooklets, amastigotes, and trypomastigotes. Although PCR methods have been used in the research setting for most of the organisms listed in the table, reagents are generally not commercially available.

[b] Routine histology can be used for the detection and identification of many parasites. In some cases, it may be the only means of diagnosis.

[c] PAS, periodic acid-Schiff stain.

[d] Although eye specimens are much preferred, free-living amebae have been cultured from patient contact lenses and lens solutions; we would not reject these specimens. An exception would be *unopened* commercial lens care solutions; these solutions would be rejected.

[e] The examination of abscess aspirates for the presence of *Entamoeba histolytica* trophozoites is an uncommon procedure and not always reliable in diagnosing extraintestinal amebiasis; serologic tests would be preferred.

Prevention (CDC) is recommended if this infection is suspected. The remaining specimen can then be processed for smear preparation and staining. Although *T. gondii* would also be seen in stained smears, one could also use immunospecific reagents, cell line culture, or PCR. Spinal fluid should not be diluted before examination. Impression smears from tissues should be prepared and stained with a blood stain. The material is pressed between two slides, with the smear resulting when the slides are pulled apart (one across the other). The smears are allowed to air dry and are then processed like a thin blood film (fixed in absolute methanol and stained with one of the blood stains).

Patients with primary amebic meningoencephalitis are rare, but the examination of spinal fluid may reveal the amebae, usually *Naegleria fowleri*. Unspun sedimented spinal fluid should be placed on a slide, under a coverslip, and observed for motile amebae; smears can also be stained with trichrome, Wright's, or one of the blood stains. Spinal fluid, exudate, or tissue fragments can be examined by light microscopy or phase-contrast microscopy. Care must be taken not to confuse leukocytes with actual organisms and vice versa. The spinal fluid may be clear or appear cloudy or purulent (with or without erythrocytes), with a cell count of from a few hundred to more than 20,000 leukocytes (primarily neutrophils) per ml. Failure to find bacteria in this type of spinal fluid should alert one to the possibility of primary amebic meningoencephalitis; however, false-positive bacterial Gram stains have been reported due to the excess debris. Isolation of these organisms from tissues can be done by using special media. When spinal fluid is placed in a counting chamber, organisms that settle to the bottom of the chamber tend to round up and look very much like leukocytes. For this reason, it is better to examine the spinal fluid on a slide directly under a coverslip, not in a counting chamber.

Possible infection with microsporidia (the most likely organism would be *Encephalitozoon* spp.; less common would be *Trachipleistophora antropophtera*) should also be considered. Specific methods would include modified trichrome, acid-fast, and blood stains; a nonspecific optical brightening agent (calcofluor white); routine histology (methenamine silver, periodic acid-Schiff [PAS], and tissue Gram stains); and electron microscopy. Electron microscopy or immunospecific reagents would be required to identify the microsporidia to the genus and species levels.

Helminth parasite stages such as *Taenia solium* cysticerci and hydatid cysts of *Echinococcus* spp. would generally be identified through examination of routine histologic slides; however, confirmation of hydatid disease could also be made from the hooklets seen in the hydatid cyst fluid contents.

Eyes

Eye specimens could include those from the cornea, conjunctiva, contact lens, or contact lens solutions. Although eye specimens are preferred, *Acanthameba* spp. have been cultured from patient contact lenses and lens solution. These specimens would be acceptable; however, due to risk management issues, the laboratory should not accept unopened commercial lens care solutions. These solutions could be referred to laboratories or agencies, such as the U.S. Food and Drug Administration (FDA), that handle testing and approval of commercial products. Also, the presence of *Acanthameba* in a lens solution does not automatically equate to *Acanthameba* keratitis; it is only suggestive at best. A corneal scraping is recommended. After appropriate media have been inoculated, smears from acceptable specimens should be prepared and stained using a blood stain or trichrome stains. Although *T. gondii* could be seen in stained smears, one could also use an immunospecific reagent or PCR for confirmation. Most individuals who are infected with *T. gondii* do not develop ocular disease; however, two populations are at risk, the immunocompromised patient with HIV and neonates who have been exposed transplacentally to the mother's acute infection.

Also, microsporidia are highly suspect from this body site, and specific methods would include modified trichrome, acid-fast, and blood stains; a nonspecific optical brightening agent (calcofluor white); routine histology (methenamine silver, PAS, and tissue Gram stains); and electron microscopy. Electron microscopy or immunospecific reagents would be required to identify the microsporidia to the genus and species levels, which could include *Encephalitozoon* spp., *Trachipleistophora* spp., *Nosema* spp., *Vittaforma* spp., *Anncaliia* spp., and *Microsporidium* spp. These eye infections are frequently seen in immunocompetent individuals with no underlying disease. It is also important to recognize the possible confusion between microsporidial spores and bacteria on a Gram-stained smear.

Ocular diseases caused by nematodes include onchocerciasis, loiasis, dirofilariasis, gnathostomiasis, theileriasis, and toxocariasis. Diagnosis of onchocerciasis can be accomplished by confirming the presence of microfilariae in a corneal biopsy or from the microscopic examination of multiple skin snips. The diagnosis of loiasis can be confirmed from the detection of circulating microfilariae of *Loa loa* in the blood or direct eye examination for the presence of the adult worm. Ocular disease can occur with the migration of *Dirofilaria immitis* larvae through periorbital or palpebral tissue; however, laboratory diagnosis of this infection is rare. Diagnosis of infections with *Gnathostoma* spp. and *Toxocara canis* is difficult but should be considered in patients with marked eosinophilia and elevated IgE. In the case of visceral and ocular larva migrans, serology for toxocariasis may be very helpful.

Eye disease can also be caused by cestodes, including *T. solium* (cysticercosis) and *Echinococcus* spp. (hydatid disease). These infections would generally be identified through direct ophthalmoscopic demonstration of the larval *T. solium*, computed tomography scans for hydatid cysts, or examination of routine histologic slides.

Ocular myiasis and infestations with lice are also possible; laboratory personnel may be asked to identify various fly larvae (*Dermatobia*, *Gasterophilus*, *Oestrus*, *Cordylobia*, *Chrysomia*, *Wohlfahrtia*, *Cochliomyia*, and *Hypoderma*) and/or lice (*Pediculus humanus corporis* or *P. humanus capitis* [documented, but rare] or *Pthirus* [*Pthirus*] *pubis* [more common] or *Demodex* spp. [blepharitis]).

Kidneys and Bladder

The kidneys serve as the primary site for the adult worm *Dioctyphyma renale*. These worms generally live in the pelvis of the right kidney or in body cavities. Although these infections have been isolated from dogs in many areas of the world, they tend to be uncommon in humans. Infections can be confirmed at autopsy, by the migration of worms from the urethra, by discharge of worms from the skin over an abscessed kidney, or by recovery of eggs in the urine. Possible infection with microsporidia (the most likely organism would be *Encephalitozoon* spp.; less common would be *Enterocytozoon bieneusi*) should also be considered. Specific methods would include modified trichrome, acid-fast, and blood stains; a nonspecific optical brightening agent (calcofluor white); routine histology (methenamine silver, PAS, and tissue Gram stains); and electron microscopy. Electron microscopy or immunospecific reagents would be required to identify the microsporidia to the genus and species levels.

Mucosa from the bladder wall may reveal eggs of *Schistosoma haematobium* (tissue squash preparation) when they are not being recovered in the urine. The eggs in the bladder wall should be checked for viability by either a hatching technique or microscopic observation of the functioning flame cells within the miracidium larva; the hatching test would be most appropriate for a urine specimen (1).

Liver and Spleen

Although the liver and spleen can serve as sites for a number of organisms as the secondary site, specific organisms that need to be considered for these organs as primary sites include *Leishmania donovani*, *Toxoplasma gondii*, *Echinococcus* spp., *Toxocara canis*, *Toxocara cati*, *Capillaria hepatica*, *Clonorchis* sp., *Opisthorchis* spp., *Cryptosporidium* spp., *Entamoeba histolytica*, *Encephalitozoon* spp., and *Encephalitozoon intestinalis* (liver). Typical methods for diagnosis would include wet mounts, routine histology, blood stains, culture, modified acid-fast stains, immunospecific reagents, and trichrome stains (routine and modified).

Examination of aspirates from lung or liver abscesses may reveal trophozoites of *E. histolytica*; however, demonstration of the organisms may be difficult (1). Liver aspirate material should be taken from the margin of the abscess rather than the necrotic center. The organisms are often trapped in the viscous pus or debris and do not exhibit typical motility. A minimum of two separate portions of exudate should be removed (more than two are recommended). The first portion of the aspirate, usually yellowish white, rarely contains organisms. The last portion of the aspirated abscess material is reddish and is more likely to contain amebae. The best material to be examined is that obtained from the actual wall of the abscess. The Amebiasis Research Unit, Durban, South Africa, has recommended the use of proteolytic enzymes to free the organisms from the aspirate material.

After the addition of the enzyme streptodornase to the thick pus (10 U/ml of pus), the mixture is incubated at 37°C for 30 min and shaken repeatedly. After centrifugation (500 × *g* for 5 min), the sediment may be examined microscopically as wet mounts or may be used to inoculate culture media. Some of the aspirate can be mixed directly with PVA on a slide and examined as a permanent stained smear (1). In a suspected case of extraintestinal amebiasis, many laboratories prefer to use a serologic diagnostic approach.

Aspiration of cyst material for the diagnosis of hydatid disease is a dangerous procedure and is usually performed only when open surgical techniques are used for cyst removal. Aspirated fluid usually contains hydatid sand (intact and degenerating scolices, hooklets, and calcareous corpuscles). Some older cysts contain material that resembles curded cottage cheese, and the hooklets may be very difficult to see. Some of this material can be diluted with saline or 10% KOH or concentrated HCL; usually, scolices or daughter cysts will have disintegrated. However, the diagnosis can be made from seeing the hooklets under high dry power (magnification, ×400) or using special stains. The absence of scolices or hooklets does not rule out the possibility of hydatid disease, since some cysts are sterile and contain no scolices and/or daughter cysts. Histologic examination of the cyst wall should be able to confirm the diagnosis.

Lungs

Expectorated Sputum and Induced Sputum

Although it is not one of the more common specimens, expectorated sputum may be submitted for examination for parasites (Table 10). Organisms in sputum that may be detected and that may cause pneumonia, pneumonitis, or Loeffler's syndrome include the migrating larval stages of *Ascaris lumbricoides*, *S. stercoralis*, and hookworm; the eggs of *Paragonimus* spp.; *Echinococcus granulosus* hooklets; the protozoa *E. histolytica* and *Cryptosporidium* spp.; and possibly the microsporidia (1). In a *Paragonimus* infection, the sputum may be viscous and tinged with brownish flecks ("iron filings"), which are clusters of eggs, and may be streaked with blood. Although *Entamoeba gingivalis* and *Trichomonas tenax* may be found in sputum, they are generally indicators of poor oral hygiene and/or periodontal disease, not pulmonary disease.

A sputum specimen should be collected properly so that the laboratory receives a "deep sputum" sample from the lower respiratory tract for examination rather than a specimen that is primarily saliva from the mouth. If the sputum is not induced, then the patient should receive specific instructions regarding collection.

Care should be taken not to confuse *E. gingivalis*, which may be found in the mouth and saliva, with *E. histolytica*, which could result in an incorrect suspicion of pulmonary abscess. *E. gingivalis* usually contains ingested PMNs, while *E. histolytica* may contain ingested erythrocytes but not PMNs. *T. tenax* would also be found in saliva from the mouth and thus would be an incidental finding and normally not an indication of pulmonary problems.

Direct Wet Mount

Sputum is usually examined as a wet mount (saline or iodine), using low and high dry power (magnifications, ×100 and ×400, respectively). The specimen is not concentrated before preparation of the wet mount. If the sputum is thick, an equal amount of 3% sodium hydroxide (or undiluted chlorine bleach) can be added; the specimen is thoroughly mixed and then centrifuged. However, NaOH destroys any protozoan trophozoites that might be present. After centrifugation, the supernatant fluid is discarded, and the sediment can be examined as a wet mount with saline or iodine. If examination must be delayed for any reason, the sputum should be fixed in 5 or 10% formalin to preserve helminth eggs or larvae or in one of the fecal fixatives to be stained later for protozoa. Another option is the use of a dithiothreitol-based product such as Sputasol (Oxoid Code #SR0233A, Remel, Lenexa, KS). Sputasol is formulated to break down mucus and liberate organisms without killing them.

Permanent Stained Smears

If *Cryptosporidium* spp. are suspected (which is rare), then acid-fast or immunoassay techniques normally used for stool specimens can be used (1). Trichrome or iron hematoxylin stains of material may aid in differentiating *E. histolytica* from *E. gingivalis*, and Giemsa stain may better define larvae and juvenile worms.

Bronchoscopy Aspirates

Fluid specimens collected by bronchoscopy may be lavage or washing fluids, with bronchoalveolar lavage fluids preferred. Specimens are usually concentrated by centrifugation prior to microscopic examination of stained preparations. Organisms that may be detected in such specimens are *Paragonimus* spp., *S. stercoralis*, *T. gondii*, *Cryptosporidium* spp., and the microsporidia.

If *T. gondii* is suspected, one of the blood stains, immunospecific reagents, and/or culture (tissue culture) can be used to confirm the diagnosis. A number of cell lines have been used (human foreskin fibroblast is one example), and most routine cell lines work well for the growth and isolation of this organism.

Lymph Nodes and Lymphatics

Material from lymph nodes and lymphatics may confirm parasitic infections (toxoplasmosis, Chagas' disease, trypanosomiasis, microsporidiosis, or filariasis) and should be processed as follows. Fluid material can be examined under low power (magnification, ×100) and high dry power (magnification, ×400) as a wet mount (diluted with saline) for the presence of motile organisms.

Material obtained from lymph nodes should be processed for tissue sectioning and as impression smears that should be processed as thin blood films and stained with one of the blood stains. Appropriate culture media can also be inoculated, again making sure that the specimen has been collected under sterile conditions.

If microsporidia are suspected, modified trichrome stains can be used; calcofluor white and immunoassay methods (currently under development) are also excellent options (1). If *T. gondii* is suspected, Giemsa stain, immunospecific reagents, and/or culture (tissue culture) can be used to confirm the diagnosis.

Specific filarial infections generally are caused by *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia* spp. In most cases, the microfilariae can be recovered and identified through examination of thick blood films, specific concentration sediment, and/or membrane filtration methods (1).

Muscle

Muscle is considered the primary site for the following organisms: *Sarcocystis* spp., microsporidia (*Pleistophora* spp., *Trachipleistophora hominis*, or *Nosema* spp.), *Gnathostoma* spp., *Trichinella* spp. larvae, and cestode larval forms (coenurus [*Taenia* spp.], cysticercosis [*T. solium*], and sparganum). As a secondary site, *T. cruzi* amastigotes can also be found in muscle; *Baylisascaris procyonis* and *Ancylostoma* spp. are also possibilities, as are hydatid cysts (*Echinococcus* spp.). In most cases, biopsy specimens processed by routine histologic methods provide the most appropriate specimens for examination and confirmation of the causative agent.

The presumptive diagnosis of trichinosis is often based on patient history: ingestion of raw or rare pork, walrus meat, bear meat, or horse meat; diarrhea followed by edema and muscle pain; and the presence of eosinophilia. Generally, the suspected food is not available for examination. The diagnosis may be confirmed by finding larval *Trichinella* spp. in a muscle biopsy specimen. The encapsulated larvae can be seen in fresh muscle if small pieces are pressed between two slides and examined under the microscope (1). Larvae are usually most abundant in the diaphragm, masseter muscle, or tongue and may be recovered from these muscles at necropsy. Routine histologic sections can also be prepared.

Human infection with any of the larval cestodes may present diagnostic problems, and frequently, the larvae are referred for identification after surgical removal (1). In addition to *E. granulosus* (hydatid disease) and the larval stage of *Taenia solium* (cysticercosis), other larval cestodes occasionally cause human disease. The larval stage of tapeworms of the genus *Multiceps*, a parasite of dogs and wild canids, is called a coenurus and may cause human coenurosis. The coenurus resembles a cysticercus but is larger and has multiple scolices developing from the germinal membrane surrounding the fluid-filled bladder. These larvae occur in extraintestinal locations, including the eye, central nervous system, and muscle.

Human sparganosis is caused by the larval stages of tapeworms of the genus *Spirometra*, which are parasites of various canine and feline hosts; these tapeworms are closely related to the genus *Diphyllobothrium*. Sparganum larvae are elongated, ribbon-like larvae without a bladder and with a slightly expanded anterior end lacking suckers. These larvae are usually found in superficial tissues or nodules, although they may cause ocular sparganosis, a more serious disease.

Finding prominent calcareous corpuscles in the tapeworm tissue frequently supports the diagnosis of larval cestodes; specific identification usually depends on referral to specialists.

Nasopharynx and Sinus Cavities

Organisms that might be found in these body sites include the microsporidia, *Acanthameba* spp., and *Naegleria*. Specimens submitted for examination could include scrapings, aspirates, and/or biopsy specimens. A number of the special stains would include modified trichrome, acid-fast, and tissue Gram stains. Giemsa, calcofluor white, and regular trichrome stains would also be appropriate. If *Naegleria* or *Acanthameba* spp. are suspected, culture is highly recommended. In certain cases of mucocutaneous leishmaniasis, *Leishmania* spp. could also be found from these body sites; one of the blood stains would be recommended for confirmation in both aspirates and biopsy specimens; cultures would also be an option (1).

Rectal Tissue

Often when a patient has an old, chronic infection or a light infection with *Schistosoma mansoni* or *Schistosoma japonicum*, the eggs may not be found in the stool and an examination of the rectal mucosa may reveal the presence of eggs. The fresh tissue should be compressed between two microscope slides and examined under the low power of the microscope (low-intensity light) (1). Critical examination of these eggs should be made to determine whether living miracidia are still found within the egg. Treatment may depend on the viability of the eggs; for this reason, the condition of the eggs should be reported to the physician.

Skin

The use of skin snips is the method of choice for the diagnosis of human filarial infections with *O. volvulus* and *Mansonella streptocerca* (1, 7). Microfilariae of both species occur chiefly in the skin, although *O. volvulus* microfilariae may rarely be found in the blood and occasionally in the urine. Skin snip specimens should be thick enough to include the outer part of the dermal papillae. With a surgical blade, a small slice may be cut from a skin fold held between the thumb and forefinger, or a slice may be taken from a small "cone" of skin pulled up with a needle. Significant bleeding should not occur, and there should be just a slight oozing of fluid. Corneal-scleral punches (either Holth or Walser type) have been found to be successful in taking skin snips of uniform size and depth and an average weight of 0.8 mg (range, 0.4 mg to 1.2 mg); this procedure is easy to perform and is painless. In African onchocerciasis, it is preferable to take skin snips from the buttock region (above the iliac crest); in Central American onchocerciasis, the preferred skin snip sites are from the shoulders (over the scapula).

Skin snips are placed immediately in a drop of normal saline or distilled water and are covered so that they will not dry; teasing of the specimen with dissecting needles is not necessary but may facilitate release of the microfilariae. Microfilariae tend to emerge more rapidly in saline; however, in either solution, the microfilariae usually emerge within 30 min to 1 h and can be examined with low-intensity light and the 10× objective of the microscope. To see definitive morphological details of the microfilariae, allow the snip preparation to dry, fix it in absolute methyl alcohol, and stain it with Giemsa or one of the other blood stains.

Skin biopsy specimens used for the diagnosis of cutaneous amebiasis (*Entamoeba histolytica* or *Acanthameba* spp.) and cutaneous leishmaniasis should be processed for tissue sectioning and subsequently stained by the hematoxylin and eosin technique (21).

Although cutaneous disease is an unusual presentation for schistosomiasis, it does occur with skin lesions as the only manifestation. Based on routine histologic examination of skin biopsies, eggs can be found in the cellular infiltrate from within the lesion. When evaluating patients with unusual skin lesions, a complete history may reveal travel to an area where schistosomiasis is endemic.

Material containing intracellular *Leishmania* organisms can be aspirated from below the ulcer bed through the uninvolved skin, not from the surface of the ulcer. The surface of the ulcer must be thoroughly cleaned before specimens are taken; any contamination of the material with bacteria or fungi may prevent recovery of the organism from culture. Aspirated material is placed on a slide and stained with Giemsa or one of the blood stains.

Some prefer to perform a punch biopsy through the active margin of the lesion (after cleaning the lesion); good results have also been seen with the use of dermal scrapings from the bottoms of the ulcers. When microscopic examinations of dermal scrapings of both the ulcer bottom and active margins are combined, the sensitivity of diagnosis may increase to 94% (21). Aspirate culture has been shown to be the most sensitive method for the diagnosis of patients with chronic ulcers. However, any successful culture depends on the prevention of contamination with bacteria and/or fungi; sampling of the ulcer must be done correctly in order to prevent false-negative culture results.

BLOOD

Depending on the life cycle, a number of parasites may be recovered in a blood specimen, either whole blood or buffy coat preparations, or following concentration by various types of procedures. These parasites include *Plasmodium*, *Babesia*, and *Trypanosoma* species, *Leishmania donovani*, and microfilariae. Although some organisms may be motile in fresh, whole blood, species identification is usually accomplished from the examination of permanent stained thick and thin blood films. Blood films can be prepared from fresh, whole blood collected with no anticoagulants, anticoagulated blood (EDTA is recommended; heparin is acceptable, but organism morphology is not as good; other anticoagulants are not recommended), or sediment from the various concentration procedures. Although for many years Giemsa stain has been the stain of choice, the parasites can also be seen on blood films stained with Wright's stain, a Wright/Giemsa combination stain, or one of the more rapid stains such as Diff-Quik (American Scientific Products, McGaw Park, IL), Wright's Dip Stat stain (Medical Chemical Corp., Torrance, CA), or Field's stain. It is more appropriate for personnel to use a stain with which they are familiar rather than having to use Giemsa stain, which is somewhat more complicated to use. PMNs serve as the quality control cell for any of the blood stains. Any parasites present stain like the PMN nuclear and cytoplasmic material, regardless of the stain used. Delafield's hematoxylin stain is often used to stain the microfilarial sheath; in some cases, Giemsa stain does not provide sufficient stain quality to allow differentiation of the microfilariae (Table 11). When handling blood, as well as other clinical specimens, standard precautions should be observed (1, 4, 5, 22).

Preparation of Thick and Thin Blood Films

Microfilariae and trypanosomes can be detected in fresh blood by their characteristic shape and motility; however, specific identification of the organisms requires a permanent stain. Two types of blood films are recommended. Thick films allow a larger amount of blood to be examined, which increases the possibility of detecting light infections (1, 5, 12). However, only experienced workers can usually make species identification with a thick film, particularly in the case of malaria, and the morphological characteristics of blood parasites are best seen in thin films.

The accurate examination of thick and thin blood films and identification of parasites depend on the use of absolutely clean, grease-free slides for preparation of all blood films. Old (unscratched) slides should be cleaned first in detergent and then with 70% ethyl alcohol; new slides should additionally be cleaned with alcohol (use gauze, not cotton) and allowed to dry before use.

Thick and thin blood films should be handled on a STAT basis (ordering, collection, processing, examination, reporting). Blood films should be prepared when the patient is admitted or seen in the emergency room or clinic; typical fever patterns are frequently absent, and the patient may not be suspected of having malaria. If malaria remains a possible diagnosis, after the first set of negative smears, samples should be taken at intervals of 6 to 8 h for at least 3 successive days. Often, after a day or two, other etiologic agents may be suspected and no additional blood specimens will be received. Another option is to collect blood immediately on admission; if the initial blood films are negative, collect daily specimens for 2 additional days (ideally between paroxysms if present; however, there is often no periodicity seen). Using either collection option, quality patient care depends on the fact that both the physician and laboratory staff know that one negative set of blood films does not eliminate *Plasmodium* spp. as possible etiologic agents.

After a finger stick, the blood should flow freely; blood that has to be "milked" from the finger is diluted with tissue fluids, which decrease the number of parasites per field. An alternative approach to the fingerstick is collection of fresh blood containing anticoagulant (preferably EDTA) for the preparation of blood films. Ideally, the smears should be prepared within 1 h after the specimen is drawn. After that time, stippling may not be visible on stained films and other morphologic changes will occur the longer the blood stands; however, the overall organism morphology may still be acceptable within 1 to 2 h.

The time that the specimen was drawn should be clearly indicated on the tube of blood and on the result report. The physician will then be able to correlate the results with any symptoms that the patient may have. There should also be some indication on the slip that is sent back to the physician that one negative specimen does not rule out the possibility of a parasitic infection.

Thick Blood Films

To prepare the thick film, place 2 or 3 small drops of capillary blood directly from the fingerstick (no anticoagulant) on an alcohol-cleaned slide. With the corner of another slide and using a circular motion, mix the drops and spread them over an area 2 cm in diameter. Continue stirring for 30 s to prevent the formation of fibrin strands that may obscure the parasites after staining. If blood containing an anticoagulant is used, 1 or 2 drops may be spread over an area about 2 cm in diameter; it is not necessary to continue stirring for 30 s, since there will be no formation of fibrin strands. If too much blood is used or any grease remains on the slide, the blood may flake off during staining. Allow the film to air dry (room temperature) in a dust-free area. Never apply heat to a thick film, since heat fixes the blood, causing the erythrocytes to remain intact during staining; the result is stain retention and an inability to identify the parasites. However, the thick films can be placed in a 37°C incubator for 10 to 15 min to dry; this seems to work quite well. Remember: do not make the films too thick; one should be able to see newsprint through the wet film prior to drying. After the thick films are thoroughly dry, they can be laked to remove the hemoglobin. To lake the films, place them in buffer solution before staining for 10 min or directly into a dilute, buffered aqueous Giemsa stain. If thick films are to be stained at a later time, they should be laked before storage (1). Although not as commonly used, other methods for the preparation of combination thick/thin blood films are available (1, 6).

TABLE 11 Techniques for the recovery and identification of blood parasites (EDTA or heparin)[a] (25)

Organism	Procedure	Stain
Malaria parasites	Thick and thin films	Giemsa, Wright's, Field's, rapid stains
	QBC[b]	Stain not relevant (centrifugation, acridine orange, microscopy)
	ParaSight F[c]	Stain not relevant (HRP-2 immunochromatographic assay)
	Binax Now Malaria[d]	Stain not relevant (HRP-2 and aldolase immunochromatographic assay)
	PATH IC Falciparum malaria IC[e]	Stain not relevant (HRP-2 immunochromatographic assay)
	Malaria Ag-CELISA[f]	Stain not relevant (HRP-2 immunochromatographic assay)
	Rapimal Dipstick[f]	Stain not relevant (HRP-2 immunochromatographic assay)
	Rapimal Cassette[f]	Stain not relevant (HRP-2 immunochromatographic assay)
	OptiMAL[g]	Stain not relevant (pLDH immunochromatographic assay)
Babesia spp.	Thick and thin films	Giemsa, Wright's, Field's, rapid stains
Microfilariae	Thick and thin films, Knott concentration, membrane filtration, gradient centrifugation	Giemsa, Wright's, or Delafield's hematoxylin
	QBC[b]	Stain not relevant
	Binax Now ICT Filariasis[d]	Rapid dipstick format
	Filariasis Ag-CELISA[f]	Enzyme immunoassay (EIA)
	TropBio[h]	Rapid dipstick format
Trypanosomes	Thick and thin films, buffy coat smears, triple centrifugation, culture	Giemsa, Wright's, Field's, rapid stains
	QBC[b]	Stain not relevant
Leishmaniae	Thick and thin films, buffy coat smears, culture	Giemsa, Wright's, Field's, rapid stains

[a] Molecular techniques are still experimental and are not always available; it is always important to verify FDA approval within the United States (contact the manufacturer). For those working outside of the United States, there are many other commercially available immunoassays for malaria detection; however, numerous publications are available for those specifically listed in the table.

[b] QBC-Blood Parasite Detection Method (Becton Dickinson Tropical Disease Diagnostics, Sparks, MD).

[c] ParaSight F-Rapid Test for *P. falciparum* malaria (Becton Dickinson Tropical Disease Diagnostics, Sparks, MD). This test is not licensed for diagnostic use in the United States. This test is a dipstick format.

[d] Binax Now Malaria (all four *Plasmodium* species) (Alere, Inc., Waltham, MA), Binax Now ICT Filariasis (*Wuchereria bancrofti*) (Binax, Inc., Portland, ME). These tests are in a dipstick format, and positive controls are available for both tests.

[e] PATH IC Falciparum malaria IC test (PATH, Seattle, WA). This test is in a dipstick format.

[f] Filariasis Ag-CELISA (*Wuchereria bancrofti*), Malaria Ag-CELISA (*P. falciparum*). Rapimal Dipstick (*P. falciparum*), Rapimal Cassette (*P. falciparum*) (Cellabs, Sydney, New South Wales, Australia).

[g] OptiMAL (differentiates between *P. falciparum* and *P. vivax*) (Flow, Inc., Portland, OR). This test is in a dipstick format.

[h] TropBio (James Cook University, Townsville, Queensland, Australia).

Thin Blood Films

The thin blood film is routinely used for specific parasite identification, although the number of organisms per field is much reduced compared with the number in the thick film. The thin film is prepared exactly as one used for a differential count, and a well-prepared film is thick at one end and thin at the other (one layer of evenly distributed erythrocytes with no cell overlap). The thin, feathered end should be at least 2 cm long, and the film should occupy the central area of the slide, with free margins on both sides. Holes in the film indicate the presence of grease on the slide. After the film has air-dried (do not apply heat), it may be stained. The necessity for fixation before staining depends on the stain selected.

Staining Blood Films

For accurate identification of blood parasites, a laboratory should develop proficiency in the use of at least one good staining method (1, 8, 6, 10). Since prolonged storage may result in stain retention, blood films should be stained on the same day or within a few days of collection. If thick blood films are not prepared or received, it is possible to stain one of the thin blood films as a thick film and examine the thick portion of the thin film. During staining, the red blood cells are laked, thus leaving the white blood cells, platelets, and any parasites present.

Wright's stain has the fixative in combination with the staining solution, so that both fixation and staining occur at the same time; therefore, the thick film must be laked before staining. In aqueous Giemsa stain, the fixative and stain are separate; thus, the thin film must be fixed with absolute methanol before staining.

When slides are removed from either type of staining solution, they should be dried in a vertical position. After being air-dried, they may be examined under oil immersion by placing the oil directly on the uncovered blood film. If slides are going to be stored for a considerable length of time for teaching or legal purposes, they should be protected with a coverglass by being mounted in a medium such as Permount. Blood films that have been stained with any of the Romanowsky stains and that have been mounted with Permount or other resinous mounting media are susceptible to fading of the basophilic elements and generalized loss of stain intensity. An antioxidant, such as 1% (by vol) 2,6-di-*t*-butyl-*p*-cresol (butylated hydroxytoluene; Sigma-Aldrich), can be added to the mounting medium. Without the addition of this antioxidant, mounted stained blood films eventually become pink; stained films protected with this compound generally remain unchanged in color for many years.

Giemsa Stain

Each new lot number of Giemsa stain should be tested for optimal staining times before being used on patient specimens. If the blood cells appear to be adequately stained, the timing and stain dilution should be appropriate to demonstrate the presence of malaria and other parasites. The use of prepared liquid stain or stain prepared from the powder depends on personal preference; there is apparently little difference between the two preparations.

The commercial liquid stain or the stock solution prepared from powder should be diluted approximately the same amount to prepare the working stain solution (1, 5). Stock Giemsa liquid stain is diluted 1:10 with buffer for both thick and thin blood films, with dilutions ranging from 1:10 up to 1:50. Staining times usually match the dilution factor (e.g., 1:20 for 20 min or 1:50 for 50 min). Some people prefer to use the longer method with more dilute stain for both thick and thin films. The phosphate buffer used to dilute the stock stain should be neutral or slightly alkaline (pH 7.0 to 7.2). Phosphate buffer solution may be used to obtain the right pH. In some laboratories, the pH of tap water may be satisfactory and may be used for the entire staining procedure and the final rinse. Some workers recommend the use of pH 6.8 to emphasize Schüffner's dots.

Giemsa stain colors the blood components as follows: erythrocytes, pale red; nuclei of leukocytes, purple with pale purple cytoplasm; eosinophilic granules, bright purple-red; and neutrophilic granules, deep pink-purple. In malaria parasites, the cytoplasm stains blue and the nuclear material stains red to purple-red. Schüffner's dots and other inclusions in the erythrocytes stain red. The nuclear and cytoplasmic staining characteristics of the other blood parasites such as *Babesia* spp., trypanosomes, and leishmaniae are like those of the malaria parasites. While the sheath of microfilariae may not always stain with Giemsa, the nuclei within the microfilaria itself stain blue to purple.

Wright's Stain

Wright's stain is available in liquid form and a powder form, which must be dissolved in anhydrous, acetone-free methyl alcohol before use. Since Wright's stain contains alcohol, the thin blood films do not require fixation before staining. Thick films stained with Wright's stain are usually inferior to those stained with Giemsa solution. Great care should also be taken to avoid excess stain precipitate on the slide during the final rinse. Before staining, thick films must be laked in distilled water (to rupture and remove erythrocytes) and air-dried. The staining procedure is the same as that for thin films, but the staining time is usually somewhat longer and must be determined for each batch of stain. Wright's stain colors blood components as follows: erythrocytes, light tan, reddish, or buff; nuclei of leukocytes, bright blue with contrasting light cytoplasm; eosinophilic granules, bright red; and neutrophilic granules, pink or light purple.

In malaria parasites, the cytoplasm stains pale blue and the nuclear material stains red. Schüffner's dots and other inclusions in the erythrocytes usually do not stain or stain very pale with Wright's stain. Nuclear and cytoplasmic staining characteristics of the other blood parasites such as *Babesia* spp., trypanosomes, and leishmaniae are like those seen in the malaria parasites. While the sheath of microfilariae may not always stain with Wright's stain, the nuclei within the microfilaria itself stain pale to dark blue.

Other Stains for Blood

Although Giemsa and Wright's stains are excellent options, the parasites can also be seen on blood films stained with a Wright/Giemsa combination stain or one of the more rapid stains such as Diff-Quik (American Scientific Products), Wright's Dip Stat stain (Medical Chemical Corp.), or Field's stain. PMNs serve as the quality control organism for any of the blood stains. Any parasites present stain like the PMNs, regardless of the stain used. Remember, when using any of the blood stains, that color variations are common.

Proper Examination of Thin and Thick Blood Films

In cases where malaria parasites have not been indicated as the suspect organism, the initial screen of the thin blood film should be carried out with the low-power objective of a microscope because microfilariae may be missed if the entire thin film is not examined. Microfilariae are rarely present in large numbers, and frequently, only a few organisms occur in each thin film preparation. Microfilariae are commonly found at the edges of the thin film or at the feathered end of the film because they are carried to these sites during the process of spreading the blood. This approach to thin film examination is particularly important in cases where a suspect organism has not been indicated. The feathered end of the film where the erythrocytes are drawn out into one single, distinctive layer of cells should be examined for the presence of malaria parasites and trypanosomes. In these areas, the morphology and size of the infected erythrocytes are most clearly seen.

In the case of a suspected malaria diagnosis, the request for blood film examination should always be considered a STAT procedure, with all reports (negative as well as positive) being reported by telephone to the physician as soon as possible (1, 5). Examination of the thin film should include viewing of 200× to 300× oil immersion fields at a magnification of ×1,000. Although some people use a 50× or 60× oil immersion objective to screen stained blood films, there is some concern that small parasites such as *Plasmodium* spp., *Babesia* spp., or *Leishmania donovani* may be missed at this smaller total magnification (×500 or ×600), although they are usually detected at the total magnification of ×1,000 obtained with the more traditional 100× oil immersion objective. Because people tend to scan blood films at different rates, it is important to examine a minimum number of fields, regardless of the time that it takes to perform this procedure. If something suspicious has been seen in the thick film, often the number of fields examined on the thin film may be considerably greater than 200 to 300.

Diagnostic problems with the use of automated differential instruments have been reported (1). Both malaria and *Babesia* infections can be missed with these instruments, and therapy is therefore delayed. Because these instruments are not designed to detect intracellular blood parasites, any reliance on the automated systems for discrimination between uninfected erythrocytes and those infected with parasites may pose serious diagnostic problems.

In the preparation of a thick blood film, the greatest concentration of blood cells is in the center of the film. A search for parasitic organisms on the entire thick film should be carried out initially at low magnification (10× objective) to detect microfilariae more readily. Examination of a thick film usually requires 3 to 5 min (approximately 100 oil immersion fields). The search for malarial organisms and trypanosomes is best done under oil immersion (total magnification, ×1,000). Close examination of the very periphery of the thick film may reveal intact erythrocytes; such cells, if infected, may prove useful in malaria diagnosis, since the characteristic morphology necessary to identify the organisms to the species level is more easily seen.

Immunochromatographic Tests for Malaria

Immunochromatography relies on the migration of liquid across the surface of a nitrocellulose membrane. Using monoclonal antibodies prepared against a malaria antigen target that has been incorporated onto the strip of nitrocellulose, these tests are based on the capture of parasite antigen from peripheral blood. Currently, the malaria antigens used for these rapid diagnostic tests are histidine-rich protein 2 (HRP-2), parasite lactate dehydrogenase (pLDH), and *Plasmodium* aldolase (Table 11). These dipsticks offer the possibility of more rapid, nonmicroscopic methods for malaria diagnosis. The tests are easy to perform and interpret; however, there are a number of questions that remain concerning the relevant uses for this type of testing, especially considering the fact that the Binax Now Malaria Test is now FDA approved (Alere, Inc, Waltham, MA). A positive-control specimen is also available.

Sensitivity remains a problem, particularly for nonimmune populations. Parasite densities of >100 parasites/μl (0.002% parasitemia) should be detected and are reasonable targets to expect from dipsticks for *Plasmodium falciparum* diagnosis. However, this level of sensitivity is at the lower end of the capability of most devices using capture methods for HRP-2 or pLDH. This level of sensitivity is probably as good as clinical laboratory staff in nonspecialized laboratories with limited exposure to malaria cases could expect to provide using microscopy diagnosis. One of the potential benefits would be for inexperienced evening staff for whom the dipstick identification of a life-threatening parasitemia with *P. falciparum* could prevent a missed infection. However, a negative test could not be accepted and would require confirmation by microscopic examination of both thick and thin blood films for the detection of parasitemia below the present threshold of detection by these rapid tests. Also, there are rare false positives for patients with certain rheumatologic disorders; this is noted in the package insert and has been confirmed by users in this patient population. The U.S. military has conducted FDA-approved trials because of possible use of the devices in the field.

Concentration Procedures

Buffy Coat Films

L. donovani, trypanosomes, and *Histoplasma capsulatum* (a fungus with intracellular elements resembling those of *L. donovani*) may occasionally be detected in the peripheral blood. The parasite or fungus is found in the large mononuclear cells that are found in the buffy coat (a layer of leukocytes resulting from centrifugation of whole citrated blood). The nuclear material stains dark red-purple, and the cytoplasm is light blue (*L. donovani*). *H. capsulatum* appears as a dot of nuclear material (dark red-purple) surrounded by a clear halo area. Trypanosomes in the peripheral blood also concentrate with the buffy coat cells.

Use alcohol-cleaned slides for preparation of the blood films. A microhematocrit tube can also be used; the tube is carefully scored and snapped at the buffy coat interface, and the leukocytes are prepared as a thin film. The tube can also be examined prior to removal of the buffy coat under low and high dry powers of the microscope. If trypanosomes are present, the motility may be observed in the buffy coat. Microfilaria motility would also be visible.

QBC Microhematocrit Centrifugation Method

Microhematocrit centrifugation with use of the QBC malaria tube, a glass capillary tube and closely fitting plastic insert (QBC malaria blood tubes; Becton Dickinson Tropical Disease Diagnostics, Sparks, MD), has been used for the detection of blood parasites (1). At the end of centrifugation of 50 to 60 μl of capillary or venous blood (5 min in a QBC centrifuge, 14,387 × *g*), parasites or erythrocytes containing parasites are concentrated into a small, 1- to 2-mm region near the top of the erythrocyte column and are held close to the wall of the tube by the plastic float, thereby making them readily visible by microscopy. Tubes precoated with acridine orange provide a stain that induces fluorescence in the parasites. This method automatically prepares a concentrated smear that represents the distance between the float and the walls of the tube. Once the tube is placed into the plastic holder (Paraviewer) and immersion oil is applied onto the top of the hematocrit tube (no coverslip is necessary), the tube is examined with a 40× to 60× oil immersion objective (it must have a working distance of 0.3 mm or greater).

Although a malaria infection could be detected by this method (which is much more sensitive than the thick or the thin blood smear), appropriate thick and thin blood films need to be examined to accurately identify the species of the organism causing the infection.

Knott Concentration

The Knott concentration procedure is used primarily to detect the presence of microfilariae in the blood, especially when a light infection is suspected (1, 12). The disadvantage of the procedure is that the microfilariae are killed by the formalin and are therefore not seen as motile organisms. An alternative blood concentration is the citrate-saponin method, where microfilaria can be seen as actively motile organisms—until killed for staining with dilute acetic acid—and where they remain straightened out, which aids in detecting identification landmarks (23).

Membrane Filtration Technique

The membrane filtration technique is highly efficient in demonstrating filarial infections when microfilaremias are of low density. This method is unsatisfactory for the isolation of *Mansonella perstans* microfilariae because of their small size. A 3-μm-pore-size filter could be used for recovery of this organism. Other filters with similar pore sizes are not as satisfactory as the Nuclepore filter (1).

Delafield's Hematoxylin

Some of the material that is obtained from the concentration procedures can be allowed to dry as thick and thin films and then stained with Delafield's hematoxylin, which demonstrates greater nuclear detail as well as the microfilarial sheath, if present. In addition, fresh thick films of blood containing microfilariae can be stained by this hematoxylin technique (1).

Triple-Centrifugation Method for Trypanosomes

The triple-centrifugation procedure may be valuable in demonstrating the presence of trypanosomes in the peripheral blood when the parasitemia is light (1). After repeated centrifugation of the supernatant, the sediment is examined as a wet preparation or is stained as a thin blood film.

SUMMARY

This chapter covers various approaches and diagnostic methods currently in use for the diagnosis of parasitic infections. If clinical specimens have been properly collected and processed according to specific specimen rejection and acceptance criteria, the examination of prepared wet mounts, concentrated specimens, permanent stained smears, blood films, and various culture materials provides detailed information leading to parasite identification and confirmation of the suspected etiologic agent (1, 2, 4, 9, 12, 13, 20). Although other tests, such as immunoassay diagnostic kits, continue to become available commercially, the majority of medical parasitology diagnostic work depends on the knowledge and microscopy skills of the microbiologist.

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PARASITES

Plasmodium and *Babesia**

BOBBI S. PRITT

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Plasmodium and *Babesia* are intraerythrocytic protozoan parasites that cause malaria and babesiosis, respectively. Both organisms are transmitted through the bite of an infected arthropod and cause significant human morbidity and mortality worldwide. Although they differ in their epidemiology and life cycle, there is overlap between both clinical and diagnostic features of these two parasites, and for these reasons, they are discussed together in this chapter.

TAXONOMY

Plasmodium and *Babesia* are genera in the Haemosporida (aka Haemosporidia, Haemospororina, or Haemospororida) and Piroplasmida orders, respectively. They both belong to the phylum Apicomplexa, a large complex group of eukaryotic single-celled parasites characterized by a specialized apical complex of rhoptries, polar rings, micronemes, subpellicular microtubules, conoid, and an apicoplast (1). The apicoplast is a plastid organelle thought to have been acquired by an ancient secondary endosymbiosis of a red alga and its chloroplast. While the apicoplast resembles a chloroplast, it is not involved in photosynthesis and instead is thought to be involved in the synthesis of heme, lipids, and isoprenoids (1).

PLASMODIUM

Description of the Agent

Members of the *Plasmodium* genus infect a wide range of birds, mammals, reptiles, and amphibians worldwide using blood-feeding dipteran vectors (2). Despite there being at least 200 named *Plasmodium* species, only five regularly infect humans: *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi* (3). These species designations were originally based on morphologic characteristics, with *P. ovale* being one of the last to be described due to its morphologic similarities to *P. vivax* (4). Recently, multilocus genetic analysis identified the presence of polymorphisms in *P. ovale*, leading to the description of classic and variant strains (5). It is now widely accepted that *P. ovale* actually comprises two distinct, closely related species that coexist in the same geographic regions without interbreeding (6). In order to avoid confusion with a new species designation, the names *Plasmodium ovale curtisi* and *P. ovale wallikeri* have been proposed for the classic and variant strains, respectively (5, 6).

P. knowlesi has only recently been recognized as a significant cause of zoonotic malaria in parts of Southeast Asia (7). This species predominantly infects macaque monkeys (genus *Macaca*) but has been reported as the cause of malaria in 27.7% of cases in Malaysian Borneo hospitals in one study (8), and it has been identified in patients from northwestern Singapore (9), southern Thailand (10), central Vietnam (11), and the Philippines (12). Individual cases have also been reported from western travelers to Southeast Asia, including individuals from Finland, Sweden, Austria, Spain, Great Britain, and the United States (7).

Epidemiology and Transmission

Malaria is arguably one of the most important infectious diseases in the world, with 3.4 billion people at risk of disease (3). According to the World Health Organization (WHO), recent worldwide malaria control efforts have produced a 29% reduction in malaria mortality globally between 2000 and 2012; however, there were still an estimated 207 million cases and 627,000 deaths in 2012 (3). Approximately 90% of deaths occur in sub-Saharan Africa, where a close link between malaria mortality and poverty is observed. Seventy-seven percent of deaths occur in children younger than 5 years of age.

Malaria is found primarily in the tropics and subtropics today (Table 1). However, it was once widespread throughout many temperate regions, including Europe and North America (13). The *Plasmodium* parasite is transmitted primarily through the bite of an infected *Anopheles* mosquito (Fig. 1) and competent mosquito vectors are found throughout the world. A list of countries with endemic malaria is available through the Centers for Disease Control and Prevention (CDC) Yellow Book (14). Today, cases of malaria in the United States and Europe are almost exclusively imported from individuals traveling from countries with ongoing malaria transmission, although occasional autochthonous cases have been reported (15–18). In the United States, the majority of imported malaria is detected in individuals who travel to endemic countries to visit friends and relatives (VFRs) (19). These individuals often do not

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*This chapter contains information presented by William O. Rogers in chapter 133 of the 10th edition of this *Manual.*

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TABLE 1 Characteristics of *Plasmodium* infections[a]

Diagnostic criterion	*P. falciparum*	*P. malariae*	*P. vivax*	*P. ovale*	*P. knowlesi*
Incubation period (days)	8–11	18–40	10–17	10–17	9–12[b]
Fever cycle	36–48 hours, "malignant tertian or subtertian"	72 hours, "benign quartan"	44–48 hours, "benign tertian"	48 hours, "tertian"	24 hours, "quotidian"
Stage of erythrocyte infected	All stages	Old cells	Young cells	Young cells	All stages
Degree of possible parasitemia	High	Low; <2%	Low; <2%	Low; <2%	High
Sequestration of infected erythrocytes	Yes	No	No	No	Possibly[b]
Usual degree of disease severity	Moderate to severe, life-threatening	Mild to moderate	Moderate to severe, occasionally life-threatening	Mild	Moderate to severe, life-threatening
Degree of anemia	Severe	Mild to moderate	Moderate to severe	Mild	Severe
Involvement of the central nervous system	Common	Rare	Occasional	Rare	Common
Nephrotic syndrome	Rare	Common	Occasional	Rare	Rare
Degree of host inflammatory response	High	Low	Very high	Low	High
Relapses	No	No, but recrudescences ≤50 years later	Yes	Yes	Not likely[b]
Area of endemicity	Large range; tropics and subtropics, especially Africa and Asia	Narrow range; tropics	Large range; tropics, subtropics, and temperate regions; relatively absent from West Africa	Tropics; sub-Saharan Africa and Southeast Asia	Narrow range; Southeast Asia
Proportion in area of endemicity	80–90%	0.5–3%	50–80%	5–8%	1–60%

[a] Adapted from references 7, 21, and 31.

[b] Information is lacking due to the limited number of reported human cases.

consider themselves at risk for malaria and may not seek travel advice from their physician or take malaria prophylaxis. In 2011, 1,925 cases of malaria were reported in the United States by the CDC (19), with 70% of cases being reported in VFRs.

Less common means of malaria transmission are via blood transfusion and across the placenta to the developing fetus. In 2011, the CDC detected one transfusion-related case of malaria and two congenitally acquired cases (19), making these extremely rare means of transmission in the United States. In most countries where malaria is nonendemic, the blood supply is partially protected through use of screening questionnaires that defer individuals from donating blood if they have recently traveled to malaria-endemic countries or have had malaria recently. In the United States, the American Association of Blood Banks (AABB) recommends that individuals traveling to areas of endemicity be deferred from donating blood products for 1 year after their return, while previously infected patients and individuals living in malaria-endemic regions for 5 years or more should be deferred for 3 years after successful treatment (20). In some European countries and Australia, antigen detection methods are used to screen individuals with possible exposure to malaria, thus shortening the potential deferral time; in comparison, there are no assays that are approved or cleared by the U.S. Food Drug Administration (FDA) in the United States for malaria donor blood screening (20).

Clinical Significance

Patients are asymptomatic for the initial 7- to 30-day incubation period during which parasites replicate in the liver (21). It is only with subsequent infection and destruction of host erythrocytes that parasite antigens such as glucose phosphate isomerase are released into the blood and stimulate cytokine production and resultant fever. The classic malarial fever paroxysm begins with rigors and chills, followed by an abrupt onset of fever which lasts for 1 to 2 hours. The paroxysm resolves with profuse sweating and a return to normal temperature. With time, infected cells may begin to rupture in synchrony, producing the classic (but infrequently observed) "tertian" or "quartan" fever cycles (Table 1). Fever is commonly preceded or accompanied by severe headache, malaise, and myalgia (21).

Infection can range from mild or asymptomatic disease, usually in individuals with pre-existing immunity, to severe, life-threatening disease. Severe malaria is defined by WHO criteria (22) and includes features such as unarousable coma, generalized convulsions, respiratory distress, hypoglycemia, circulatory collapse, renal failure, and hyperparasitemia (greater than 100,000 parasites/μl of blood). Young children,

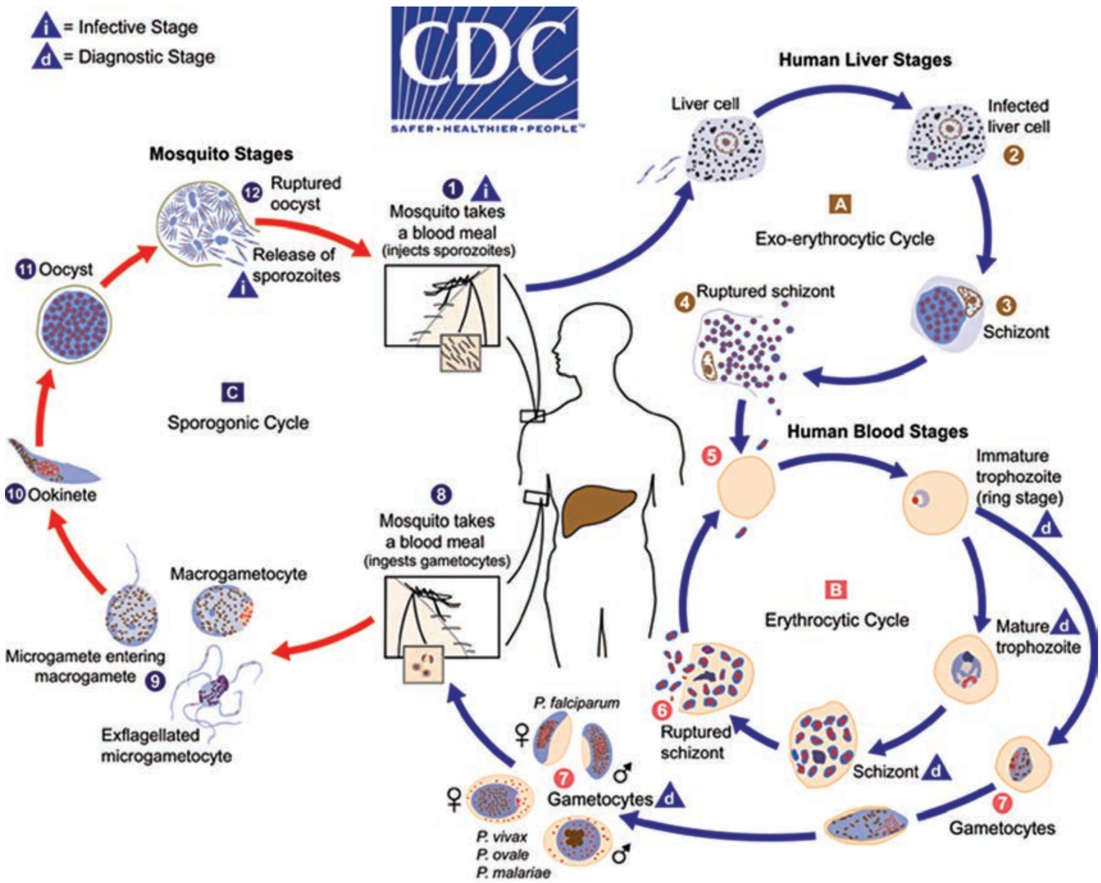
i = Infective Stage
d = Diagnostic Stage
CDC
SAFER·HEALTHIER·PEOPLE
Human Liver Stages
Liver cell
Infected liver cell
2
A
Exo-erythrocytic Cycle
3 Schizont
4 Ruptured schizont
Mosquito Stages
12 Ruptured oocyst
Release of sporozoites
1 Mosquito takes a blood meal (injects sporozoites)
11 Oocyst
C
Sporogonic Cycle
10 Ookinete
8 Mosquito takes a blood meal (ingests gametocytes)
Macrogametocyte
Microgamete entering macrogamete 9
Exflagellated microgametocyte
Human Blood Stages
5
Immature trophozoite (ring stage)
B
Erythrocytic Cycle
Mature trophozoite
6 Ruptured schizont
Schizont
7 Gametocytes
P. falciparum
♀
♂
7 Gametocytes
P. vivax
P. ovale
P. malariae

FIGURE 1 The *Plasmodium* parasite is transmitted to humans when an infected female *Anopheles* mosquito takes a blood meal and inoculates sporozoites into the bloodstream (1). Sporozoites infect hepatocytes (2) and undergo asexual reproduction to form schizonts (3) in a process called exoerythrocytic schizogony (A). After 5 to 15 days, liver schizonts rupture to release hundreds of thousands of merozoites into the blood (4) which then infect erythrocytes (5). *P. vivax* and *P. ovale* have a dormant hypnozoite stage, which may remain in the liver and cause relapsing disease weeks to months later, while other human *Plasmodium* species do not have this stage. In erythrocytes, merozoites undergo asexual reproduction to form trophozoites and then schizonts in a process called erythrocytic schizogony (B). Schizonts rupture to release merozoites, which then infect other erythrocytes (6). This process is repeated every 1 to 3 days, resulting in the production of thousands to millions of infected erythrocytes in several days. Some merozoite-infected erythrocytes do not undergo asexual reproduction but instead develop into male microgametocytes and female macrogametocytes (7). When ingested by a female *Anopheles* mosquito during a blood meal (8), microgametocytes exflagellate to release microgametes, which penetrate macrogametes and form zygotes (9) via sexual reproduction in the sporogonic cycle (C). Zygotes become motile and elongated to form ookinetes (10) which invade the mosquito's midgut wall and develop into oocysts (11). Over a period of 8 to 15 days, oocysts grow, rupture, and release thousands of sporozoites (12), which travel to the mosquito's salivary glands to be inoculated into a new human host during the mosquito's next blood meal (1). Life cycle courtesy of the CDC DPDx (http://www.cdc.gov/dpdx/).
doi:10.1128/9781555817381.ch136.f1

nonimmune individuals, and pregnant women and their fetuses are at greatest risk of severe disease (3).

P. falciparum infection has the highest morbidity and mortality of the four human *Plasmodium* species, being responsible for nearly all cases of severe malaria and malaria deaths worldwide (21). The high morbidity and mortality of *P. falciparum* infection is attributed in part to the parasite's ability to infect and destroy all stages of erythrocytes, leading to high levels of parasitemia and resultant severe anemia, splenomegaly, and jaundice. In contrast, the other *Plasmodium* species preferentially infect older erythrocytes (*P. malariae*) or young erythrocytes (*P. vivax* and *P. ovale*) and cause a lesser degree of total erythrocyte destruction (21).

P. falciparum virulence is also attributed to a phenomenon called cytoadherence, accomplished through insertion of parasite-derived adhesion molecules from the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family onto the surface of infected erythrocytes during later stages of parasite development. The PfEMP1 molecules allow for clumping of uninfected erythrocytes around infected erythrocytes (rosetting) and receptor-mediated cytoadherence of infected erythrocytes to endothelial cells (sequestration)

(23). Accumulation of infected erythrocytes within the microvasculature is associated with metabolic acidosis, hypoxia, and release of detrimental inflammatory cytokines, particularly in the lung, kidney, and brain. During pregnancy, a special form of cytoadhesion occurs between infected erythrocytes and syncytiotrophoblasts in the placenta (23). Symptoms resulting from the high *P. falciparum* parasitemia and sequestration in the microvasculature include acute renal failure, respiratory distress, abortion, intrauterine growth retardation, mental status changes, coma, and death. Other *Plasmodium* species do not sequester and generally cause less severe disease (21).

Additional species-associated manifestations include splenic rupture (*P. vivax*), nephrotic syndrome (*P. malariae* in children), hypoglycemia (*P. falciparum* in children), extensive erythrocyte destruction with hemoglobinuria and kidney failure ("blackwater fever," *P. falciparum*), recrudescent disease (*P. malariae*), and relapses due to reactivation of hypnozoites in the liver (*P. ovale* and *P. vivax*) (21). Fatal cases have also been reported in infections with *P. knowlesi*. Given that *Plasmodium* infection can cause a diverse spectrum of symptoms, including gastrointestinal and respiratory manifestations, it is essential that malaria be considered in the differential diagnosis for anyone with fever and recent exposure to malaria-endemic areas.

Collection, Transport, and Storage of Specimens

Since *P. falciparum* infection can cause rapidly progressive, fatal disease, blood collection and testing for malaria should always be performed on a STAT basis (24, 25). Preparation of thick and thin blood films is considered the gold standard for diagnosis of malaria. If a laboratory does not have the expertise to perform examination of thin and thick blood films, then consideration should be given to use of rapid antigen tests for preliminary screening (see "Diagnosis" below) or rapid transport to a neighboring laboratory that can receive and test the blood shortly after it is drawn. Testing options for malaria should be available 7 days a week on a 24-hour basis (25).

Blood is ideally collected via finger prick with immediate (bedside) preparation of thick and thin smears. However, it is more common in nonendemic settings to collect blood by venipuncture for rapid transport to the laboratory (25). EDTA is the preferred anticoagulant, as the use of other anticoagulants such as heparin may cause significant parasite distortion (25). Prolonged storage in EDTA may also cause parasite distortion, parasite maturation into sexual life cycle stages, and loss of diagnostic features such as stippling; therefore, blood films should be prepared within 1 hour of collection (25, 26). A single set of blood films may be insufficient to detect *Plasmodium* parasites. The Clinical and Laboratory Standards Institute (CLSI) recommends preparation of two thin and two thick blood films on initial evaluation of the patient, with preparation of repeat films every 6 to 8 hours if necessary for up to 3 days before excluding malaria from the clinical differential diagnosis (25).

Additional EDTA whole blood may be refrigerated for supplemental testing such as PCR if necessary. Unfortunately, parasite morphology rapidly degrades in stored blood and is not usually adequate for later identification or for species identification by morphologic methods. Chapters 133 and 135 of this *Manual* and the CLSI guidelines for diagnosis of blood-borne parasites (25) provide detailed directions for collecting and preparing blood films. Requests for blood films should be accompanied by important patient information such as clinical signs and symptoms, travel history, and receipt of malaria chemoprophylaxis or therapeutic antimalarial agents, which might suppress parasitemia or alter parasite morphology.

Direct Examination

Microscopy

Giemsa-Stained Blood Films

Microscopic examination of Giemsa-stained thick and thin blood films is the traditional method for malaria diagnosis. Accurate interpretation of this time-honored method relies on the availability of trained and experienced microscopists, high quality reagents, and well-maintained light microscopes. The thick film contains 1 to 2 drops of blood that have been lysed (laked) on the slide by placement into a hypotonic solution (25). This releases intracellular parasites and allows for examination of 20 to 30 layers of blood. The thick blood film is approximately 10 to 20 times more sensitive than the thin film, with a reported detection threshold of 10 to 50 parasites/µl of blood, or approximately 0.0002 to 0.001% parasitemia, assuming a total erythrocyte count of 5 × 10^6/µl of blood (27). Given this high sensitivity, the thick film is ideal for screening and parasite detection. Under field conditions, the estimated sensitivity may be somewhat lower (100 to 500 parasites/µl of blood) (27, 28).

Given the greater sensitivity of the thick film compared to the thin film, efforts should be made to quickly prepare and examine the thick film so that it can be used as the primary screening method. A common mistake is to use too much blood on the slide, thus requiring longer times for the film to dry. A well-made thick film should be approximately 1.5 to 2.0 cm in diameter and of a thickness through which newsprint can barely be read (25). Drying of the thick film can be enhanced by placing the slides in a laminar flow hood or under the gentle breeze from a fan. Exposure to heat is not recommended since it may cause fixation of the blood and interfere with the laking process (25). Gently scratching grooves into the carrier slide with the edge of another glass slide while spreading the blood to the appropriate diameter will facilitate adhesion to the slide and obviate the need for extended drying times without negatively affecting the microscopic morphology (29); films may be safely stained as soon as the film is visibly dry.

Thin films are made in the same manner as hematology blood films. A single drop of blood is spread on the slide in such a manner as to produce a feathered edge. The films are then fixed in methanol prior to staining.

Staining of thick and thin films is best performed with Giemsa at a pH of 7.0 to 7.2 to highlight potential stippling and other features of the parasites (25) (see chapter 134 for further information on malaria stains). Wright-Giemsa and the rapid Field stain may also be used. After staining, a well-prepared thick film should have a relatively clean background of lysed erythrocytes, leukocyte nuclei, and platelets, while thin films will demonstrate pale blue-gray to light pink erythrocytes and a well-formed feathered edge. Parasites will have pale to deep blue cytoplasm and pink-red chromatin (25).

Both thick and thin slides should first be screened at low power using the 10× objective for identification of microfilariae, followed by examination under oil immersion with the 100× objective (25). Examination of 200 to 300 microscopic fields on thick and thin films using the 100× objective should be performed before reporting a specimen as negative (25). Considerable practice is required to accurately differentiate parasites from platelets, stain debris, and

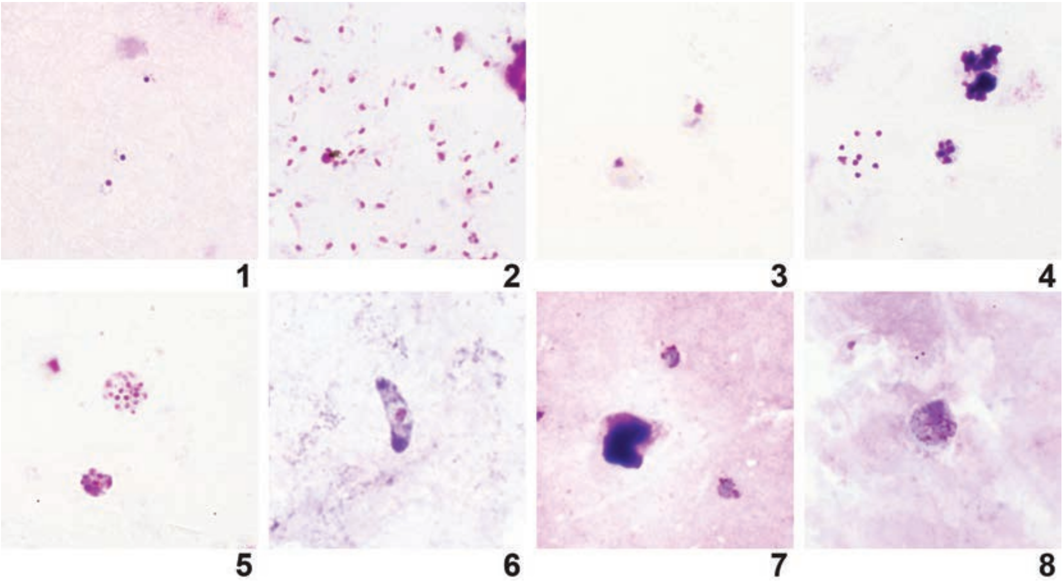

FIGURE 2 *Plasmodium* morphology on thick blood films encompasses the entire spectrum of forms including trophozoites, schizonts, and gametocytes. Shown are *P. falciparum* early trophozoites in a moderate (1) and heavy (2) infection; *P. vivax* early trophozoites (3); *P. malariae* (4) and *P. vivax* (5) schizonts; and *P. falciparum* (6), *P. malariae* (7), and *P. vivax* (8) gametocytes. Neutrophils are shown for size comparison in panels 4 and 7. (Giemsa, ×1,000.) doi:10.1128/9781555817381.ch136.f2

leukocyte granules on the thick film (Fig. 2), but the additional training effort is rewarded by the increased sensitivity that this method provides. The species of the infecting organism can be determined using the thick film, but this is best accomplished using the thin film. Thin films provide ideal erythrocyte and parasite morphology for species determination since they are fixed in methanol prior to staining, thus maintaining the structure of the erythrocytes and intraerythrocytic parasites (25). The area of the thin film that provides optimal parasite morphology is where the erythrocytes have minimal overlap and maintain their central pallor (Fig. 3), parasites outside of this region may be considerably distorted. Species determination is made using a number of morphologic features including the size and shape of the infected erythrocyte, presence of intracytoplasmic inclusions (e.g., stippling), stages of the parasite present in peripheral blood, and specific characteristics of each parasite stages (Table 2 and Fig. 4 through 8). Atlases

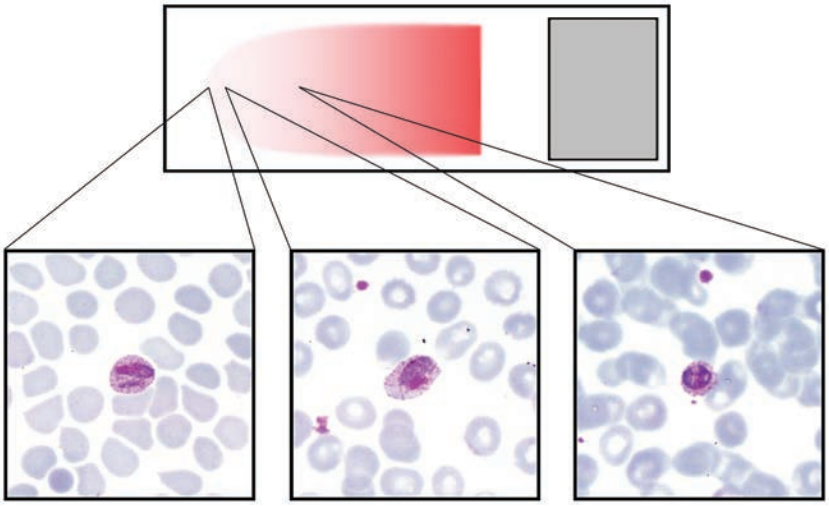

FIGURE 3 The ideal location for microscopic examination of a thin film is the region of the feathered edge where the erythrocytes have minimal overlap and maintain their central pallor (middle). In this location, the erythrocyte infected with *P. ovale* is clearly enlarged and ovoid. When examining similar cells in regions of the film that are too thin (left) or thick (right), the morphology is distorted and may be misleading. doi:10.1128/9781555817381.ch136.f3

TABLE 2 Comparative morphology of *Plasmodium* spp. in Giemsa-stained thin films[a]

Diagnostic criterion	*P. falciparum*	*P. malariae*[b]	*P. vivax*	*P. ovale*	*P. knowlesi*
Size and shape of infected erythrocytes	Normal size and shape	Normal or slightly smaller size, normal shape	Normal or enlarged size, may appear molded against neighboring erythrocytes	Normal or enlarged size, frequently oval, may be fimbriated	Normal size and shape
Cytoplasmic inclusions	Occasional Maurer's clefts; larger (comma-shaped) and less numerous than Schüffner's dots	Ziemann's dots rarely seen; requires deliberate over-staining	Schüffner's dots/ stippling; may not be present in early trophozoites	Dark Schüffner's/ James' dots/ stippling; may not be present in early trophozoites	Irregular stippling in late trophozoites and schizonts
Parasite stages in peripheral blood	Early trophozoites and gametocytes	All stages	All stages	All stages	All stages
Multiply infected erythrocytes	Common	Rare	Occasional	Occasional	Common
Early trophozoite characteristics	Delicate rings, <1/3 diameter of the erythrocyte, frequently with double chromatin dots ("headphone" form); often at edge of erythrocyte ("appliqué/accolé form")	Rings ≤1/3 diameter of the erythrocyte; chromatin dot may appear unattached in center of ring ("bird's eye" form)	Rings ≥1/3 diameter of the erythrocyte; larger chromatin dot than *P. falciparum*	Rings ≥1/3 diameter of the erythrocyte; similar to *P. vivax*	Rings ≤1/3 diameter of the erythrocyte; double chromatin dots, rare appliqué forms; resembles *P. falciparum* early trophozoites
Mature trophozoites	Not typically seen in peripheral blood, compact thick rings	Compact cytoplasm, round, oval, basket or band-shaped, dark brown pigment	Amoeboid trophozoites, fine golden-brown pigment	More compact and less amoeboid than *P. vivax*, dark brown pigment	Slightly amoeboid; band forms common; scattered clumps of golden-brown pigment; resembles *P. malariae* mature trophozoites
Schizont characteristics	Not typically seen in peripheral blood, 8–24 merozoites	6–12 merozoites, often radially arranged around central pigment ("rosette" or "daisy head" schizont)	12–24 merozoites	6–14 merozoites	10–16 merozoites
Gametocyte characteristics	Crescent- or banana-shaped; distorting the shape of the erythrocyte	Round to oval; filling most of the erythrocyte	Round to oval; filling most of the erythrocyte	Round to oval; filling most of the erythrocyte	Round to oval; filling most of the erythrocyte

[a] Adapted from references 7, 21, and 31.

[b] Identification of *P. malariae* in patients with recent travel to Southeast Asia should raise the possibility of *P. knowlesi* infection, given the morphologic similarities of these two parasites. In this situation, severe clinical disease and a high parasite burden are consistent with *P. knowlesi* infection.

and other resources are widely available for differentiating the various *Plasmodium* species (24, 30–33).

The size of the infected erythrocytes is a particularly important feature for determining the infecting *Plasmodium* species, since enlarged infected erythrocytes are characteristic of *P. ovale* and *P. vivax* infection, particularly in the later parasite stages, while small to normal-sized infected erythrocytes are characteristic of *P. malariae* and *P. falciparum* infection. Thus, the size of the infected cell can quickly allow the microscopist to halve the number of species in the differential diagnosis. The presence of Schüffner's stippling supports the diagnosis of *P. ovale* and *P. vivax* infection. Care must be taken, however, not to confuse the finer Schüffner's dots with the larger, coarser Maurer's clefts that may occur in *P. falciparum* infections. Stippling and Maurer's clefts are most commonly seen when smears are made shortly after blood collection and the pH of the Giemsa stain is 7.0 to 7.2.

The life cycle stages present are also very useful for identification of species. Infection with *P. falciparum* usually consists solely of early trophozoites ("rings") and, less commonly, gametocytes; intermediate stages (maturing trophozoites and schizonts) are only rarely seen since they are sequestered in the microvascular beds. When intermediate stages of *P. falciparum* are present, they may indicate severe, overwhelming infection. In contrast to *P. falciparum*, a spectrum of forms including late-stage trophozoites and schizonts are commonly seen with other species.

When examining peripheral blood films, it is important to remember that mixed malaria infections are not uncommon

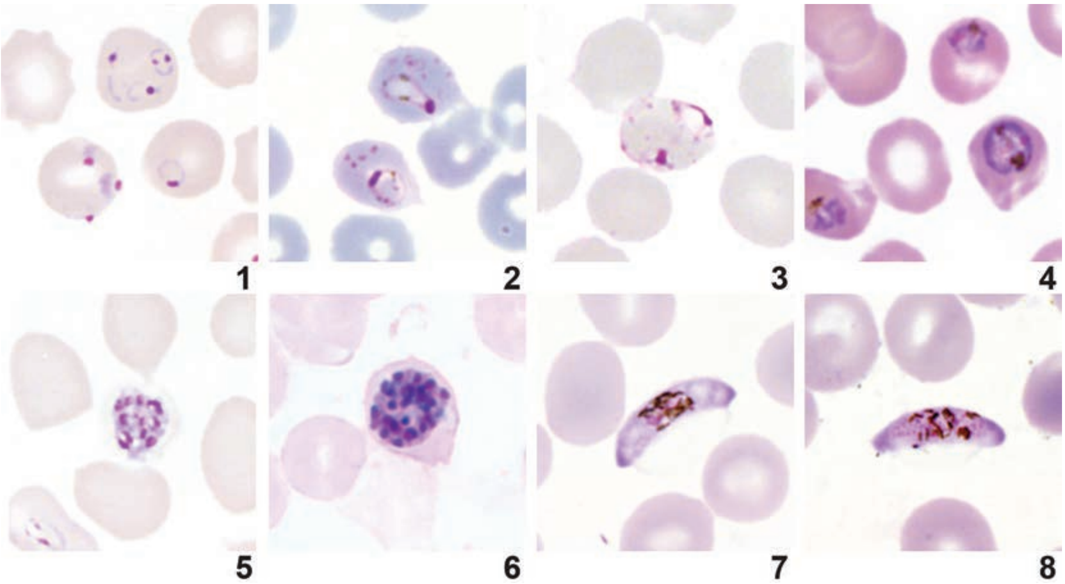

FIGURE 4 *P. falciparum*, successive developmental stages in Giemsa-stained thin blood films: early stage trophozoites/rings with a "headphone" form (1), rings with Maurer's clefts (2), rings with appliqué forms and Maurer's clefts (3), maturing trophozoites (4), early stage schizont (5), mature schizont (6) (courtesy of the CDC DPDx), macrogametocyte (7), and microgametocyte (8). Rings (1, 2, 3) and, less commonly, gametocytes (7, 8) are found in peripheral blood, while other stages (4–6) are typically sequestered in the microvasculature.
doi:10.1128/9781555817381.ch136.f4

and that *P. falciparum* may be found in conjunction with less virulent species in the same patient. It is also important to remember that other microorganisms may be seen on peripheral blood films such as *Babesia* intraerythrocytic parasites (see *Babesia* section below) and extracellular microfilariae (chapter 144), trypanosomes (chapter 137), and *Borrelia* spirochetes (chapter 59). Intraleukocytic *Ehrlichia*/*Anaplasma* morulae may also be seen on the peripheral blood film, although this is not the preferred method for detection of these bacteria (chapter 65).

When *Plasmodium* species are identified, it is important to quantify the degree of parasitemia to help guide initial ther-

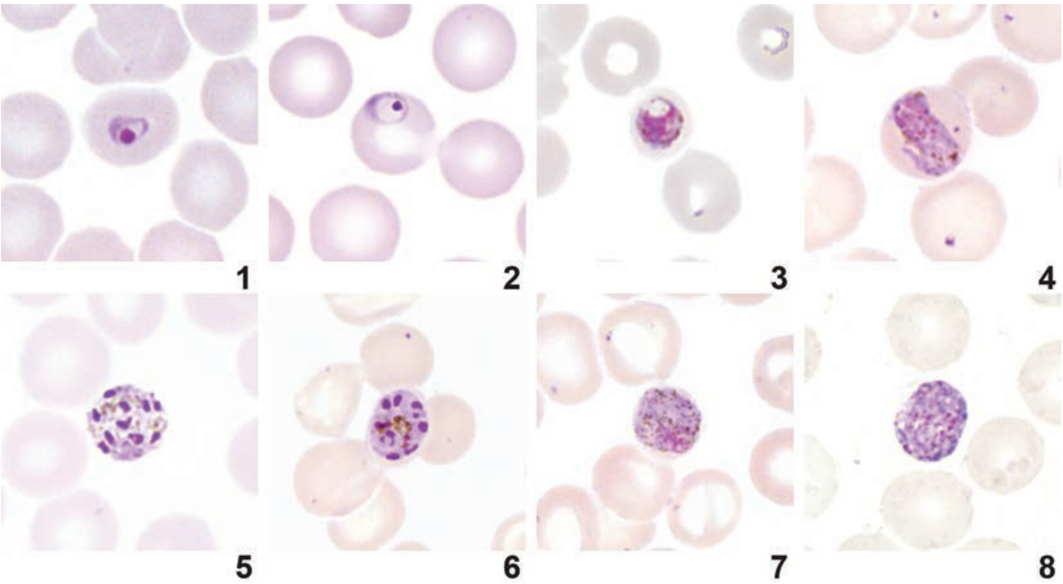

FIGURE 5 *P. malariae*, successive developmental stages in Giemsa-stained thin blood films: early stage trophozoite/ring (1), "bird's eye" ring form (2), mature trophozoites with "basket" form (3), mature trophozoite with "band" form (4), mature schizont (5), mature schizont showing rosette form with central pigment (6), macrogametocyte (7), and microgametocyte (8).
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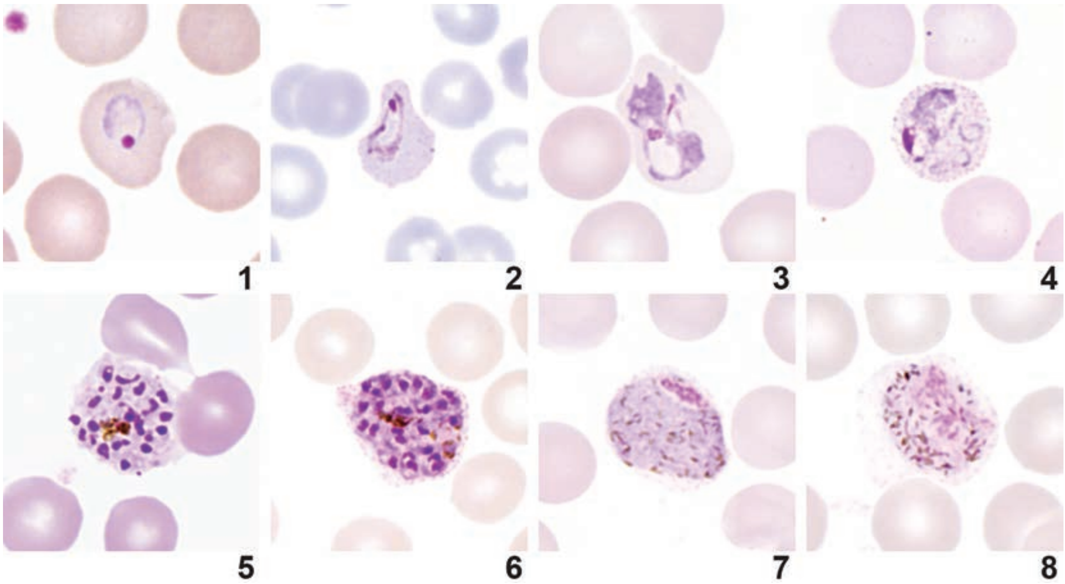

FIGURE 6 *P. vivax*, successive developmental stages in Giemsa-stained thin blood films: early stage trophozoite/ring, (1), maturing ring (2), mature amoeboid trophozoites (3, 4), mature schizonts (5, 6), macrogametocyte (7), microgametocyte (8). Note the Schüffner's dots (2, 4, 6, 8) and frequent molding of infected cells to neighboring erythrocytes. The infected erythrocytes are slightly larger than noninfected cells. doi:10.1128/9781555817381.ch136.f6

apy, predict patient prognosis, and monitor response to treatment. This may be done using either the thin or thick film, and formulas for this purpose are widely available (24, 25, 31, 32, 34). When using the thick film for quantitation, it is common practice to express the number of parasites in relation to a standard number of leukocytes per microliter of blood (8,000/μl), although it is preferable to substitute the patient's actual leukocyte count when known. Results are reported as the number of parasites per microliter of blood. When using the thin film, results are reported as the percentage of infected erythrocytes in the fields examined (number of infected cells counted/total number of erythrocytes counted × 100). When calculating the degree of parasitemia with either the thick or thin film, it is important to not include parasite sexual stages

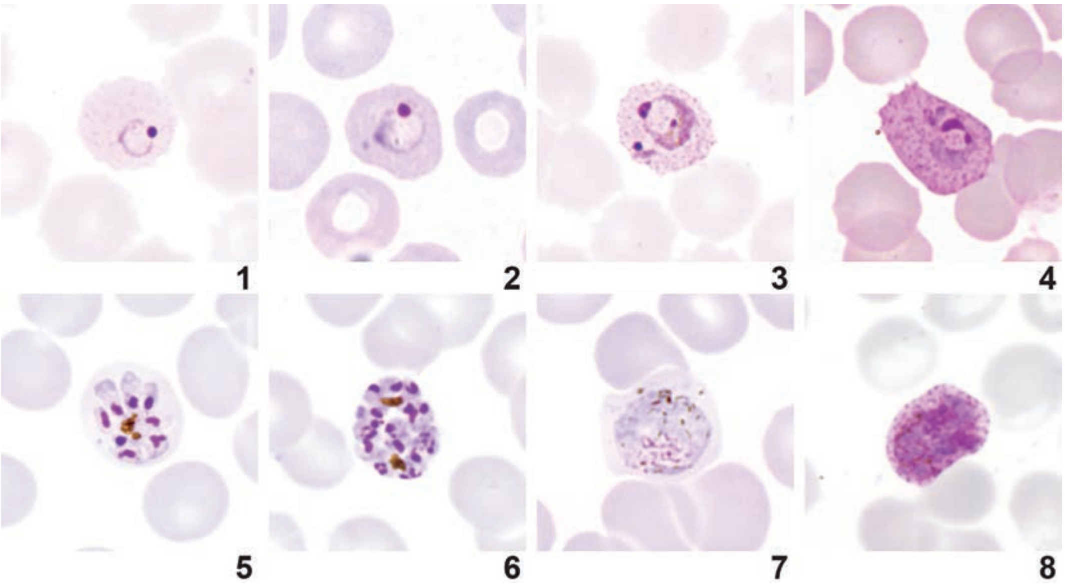

FIGURE 7 *P. ovale*, successive developmental stages in Giemsa-stained thin blood films: early stage trophozoite/ring (1, 2), developing trophozoite (3), mature trophozoite with "comet cell" morphology (4); early stage schizont (5), mature schizont (6), macrogametocyte (7), microgametocyte (8). Note the Schüffner's dots (3, 4, 8) and oval/elongated shape of the infected cells (4, 6, 8). The infected erythrocytes are slightly larger than noninfected cells. doi:10.1128/9781555817381.ch136.f7

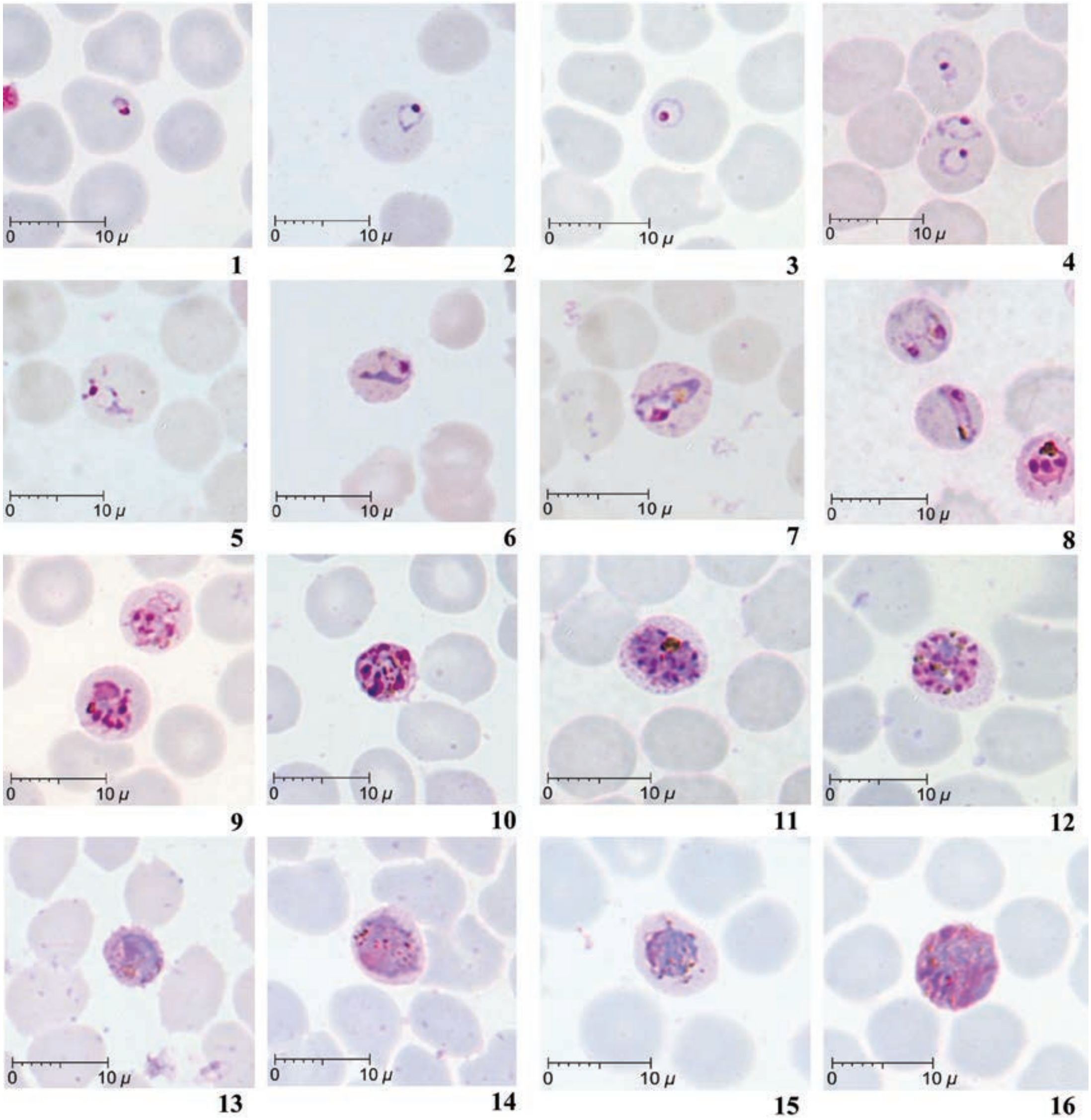

FIGURE 8 *P. knowlesi*, successive developmental stages in Giemsa-stained thin blood films: early stage trophozoites/rings (1 to 4), developing trophozoite (5 to 7), mature trophozoite with "band" form (8), early stage schizont (9 and 10), mature schizont (11 and 12), immature gametocyte (13), mature microgametocyte (14 and 16), mature macrogametocyte (15). (Images reproduced from figures in reference 33 with permission.) doi:10.1128/9781555817381.ch136.f8

(gametocytes) in the total count since they are not infectious to humans and are not killed by most antimalarial drugs. With the thin film, it is also important to count consecutive fields, even if they do not include parasites, and not to count extracellular parasites. Multiply infected erythrocytes are only counted as a single infected cell. Regardless of the method used for calculating the degree of parasitemia, it is important to use the same method for initial and post-treatment specimens (28). Given the possibility of inter-observer variability, it may be prudent to limit the number of individuals performing the assessment on sequential specimens from the same patient.

Other Microscopy Methods

Other microscopic methods are less commonly used for the identification of malaria parasites in whole blood, including stains for nucleic acid and hemozoin (Table 3). Of these, the most commonly used is acridine orange (AO), a DNA-binding fluorescent dye that is excited at 490 nm, producing a yellow or apple-green fluorescence (28). Use of this method requires a fluorescence microscope or light microscope with appropriate adaptor. Identification of infected cells is relatively straightforward since mature erythrocytes lack DNA and do not produce a fluorescent signal, while parasites contain DNA and fluoresce brightly. However, leukocyte nuclei and Howell-Jolly bodies within erythrocytes will also fluoresce, occasionally making identification of low parasite levels challenging. A number of studies found AO staining to have a similar sensitivity and specificity to traditional Giemsa-stained thick films, with the ability to reliably detect <100 parasites/μl or 0.002% parasitemia (28). This method may also allow for more rapid screening than with traditional Giemsa-stained films (27, 28, 35). Another technique that uses

TABLE 3 Comparison of malaria diagnostic direct methods[a]

Method	Test	Advantages	Disadvantages	Comments	Reference(s)
Giemsa stain	Thick film	Screens large volume of blood; high sensitivity	Subjective; species differentiation is not easily accomplished	Gold standard for detection; requires experienced and skilled microscopists	21, 25, 29, 31, 32, 34
	Thin film	Preserves parasite morphology; good for species determination	Subjective; less sensitive than the thick film for screening	Gold standard for species determination; requires experienced and skilled microscopists	
Fluorescent DNA/RNA stains	AO or BCP-stained films	May decrease screening time	Non-specific stain; may be hard to interpret	Requires fluorescence microscope	35, 101
	QBC	High sensitivity for *P. falciparum*	Species determination and calculation of percent parasitemia is difficult; less sensitive for non-*P. falciparum* species	Requires fluorescence microscope and centrifuge	102–104
	Flow cytometry	Automated	Variable sensitivity		105
Hemozoin detection	Dark-field microscopy	No stain used. Abundant pigment in macrophages suggests poor prognosis	Poor sensitivity		106, 107
	Histology/FFPE tissues	Allows for correlation with morphologic features	Requires tissue biopsy; hemozoin may be confused with formalin or anthracotic pigment		108
Antigen detection	Kits for detection of HRP-2, aldolase, and parasite LDH	Rapid, ease of use and interpretation; some kits appropriate for field use	Relatively expensive compared to blood films, variable sensitivity for low percent parasitemia and non-*P. falciparum* infections	Performance characteristics vary widely by kit	36
Nucleic acid detection	DNA/RNA hybridization	Non-amplification technique; does not require the same degree of contamination controls as PCR	Poor sensitivity, expensive, requires sophisticated equipment and facilities for high complexity testing	First nucleic acid detection method; not widely used clinically	109, 110
	PCR	High sensitivity and specificity, equal to or exceeding thick film; some assay designs give excellent detection of mixed infections	Expensive, requires sophisticated equipment and facilities for high complexity testing; potential for amplicon contamination; not usually performed rapidly	Multiple laboratory-developed methods; no FDA-approved tests; clinical availability limited to reference and public health laboratories	41, 44–46, 111
	Loop-mediated isothermal amplification (LAMP)	Sensitivity and specificity comparable to PCR, some tests suitable for field use	Expensive (but less than PCR), no commercial options at this time	Not widely used clinically	112

[a] AO, acridine orange; BCP, benzothiocarboxypurine; FFPE, formalin-fixed, paraffin-embedded; HRP-2, histidine-rich protein 2; LDH, lactate dehydrogenase; QBC, quantitative buffy coat; WBC, white blood cell.

AO staining is the quantitative buffy coat or QBC (QBC Diagnostics, Becton Dickinson, Port Matilda, PA). In this method, whole blood is stained with AO and spun in a micro-hematocrit tube. The buffy coat layer in the microcentrifuge tube is then directly examined using a fluorescence microscope with a specialized long-focal-length objective (28).

Rarely, malaria pigment (hemozoin) is detected via dark-field microscopy or in histologic tissue sections. In tissue sections, the pigment is concentrated within infected sequestered erythrocytes and macrophages in the capillaries of infected organs such as the lung, kidney, liver, brain, and placenta (Fig. 9).

Antigen Detection

Within the past decade, there has been a marked expansion of commercially available immunochromatographic tests for rapid detection of *Plasmodium* antigens (28, 36, 37). These tests, commonly referred to as rapid diagnostic tests (RDTs), are rapid and relatively easy to use, and many

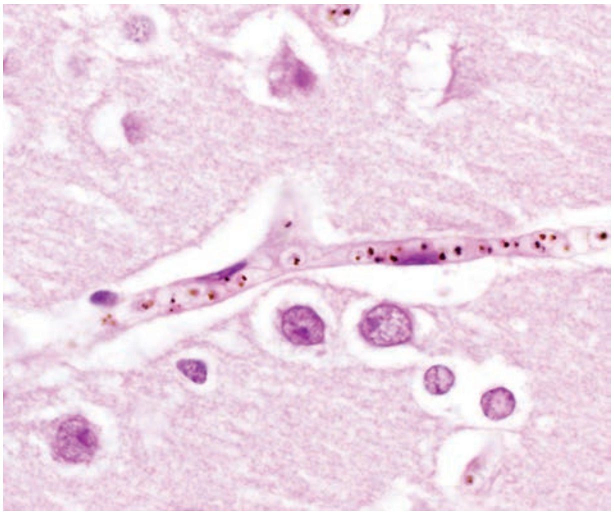

FIGURE 9 Cerebral malaria due to *P. falciparum* on tissue section (hematoxylin and eosin, ×400). The capillary in the center of the image contains multiple infected erythrocytes, seen primarily by their brown-black hemozoin pigment.
doi:10.1128/9781555817381.ch136.f9

are stable in field conditions (36). They now form an integral part of the WHO's strategy for combating malaria worldwide (36).

The antigens detected by the commercially available RDTs are parasite lactate dehydrogenase (pLDH), histidine-rich protein 2 (HRP-2), and *Plasmodium* aldolase (31). Some tests specifically detect *P. falciparum* and/or *P. vivax*, while others offer only pan-*Plasmodium* results. There are no specific tests for *P. ovale*, *P. malariae*, or *P. knowlesi* (37). At this time, the only FDA-approved RDT is the BinaxNow Malaria Test (Alere, Inc., Waltham, MA), which targets *P. falciparum* HRP-2 antigen and a pan-malarial antigen.

In general, malaria RDTs perform almost as well as microscopy for detection of *P. falciparum* at moderate or high parasite levels, but suffer from lower sensitivity for detection of non-*P. falciparum* infections and low levels of parasitemia for all species (28, 37). While parasite levels of 100 parasites/µl (0.002% parasitemia) are commonly seen in the clinical setting, many RDTs cannot reliably detect parasites at this level, even when the infecting species is *P. falciparum* (28, 37). The WHO, the Foundation for Innovative New Diagnostics (FIND), and the Special Programme for Research and Training in Tropical Diseases (TDR) launched a massive study in 2006, comparing commercially RDTs for their ability to detect *P. falciparum* and *P. vivax* at high (2,000 to 5,000 parasites/µl) and low (200 parasites/µl) concentrations (36). The publication of their most recent results, containing data on over 120 RDTs, demonstrates that RDT performance varies widely by kit, manufacturer, and even lot number (36). In these studies, the FDA-approved BinaxNow Malaria Test had 100% sensitivity for detection of *P. falciparum* at high levels but sensitivities of 91.1, 85, and 10% for detection of *P. falciparum* at low levels, *P. vivax* (high levels), and *P. vivax* (low levels), respectively (36). As with many immunochromatographic assays, false positives may be observed in the presence of autoantibodies such as rheumatoid factor. Antigen may be detected for days after successful treatment; therefore, RDTs should not be used to monitor outcomes of treatment (37).

Given the limitations of RDTs, it is widely recommended that all results be confirmed with standard thick and thin blood films (28, 31). In nonendemic settings such as the United States and Europe, laboratories that may benefit from RDT use include those that lack the expertise to interpret traditional blood films or desire a more rapid diagnostic method for general or point-of-care testing (38–40). Rapid diagnostic tests may be particularly beneficial in STAT laboratories and during the evening shift, where expertise for sensitive detection of blood parasites using thick films is lacking; in these settings, the ability to immediately detect cases with high *P. falciparum* parasitemia may be life-saving.

Nucleic Acid Detection

Most nucleic acid detection methods use PCR for nucleic acid amplification and detection of *Plasmodium* parasite DNA, with the 18S small subunit rRNA gene being the most common target. Numerous assays have been described, including conventional (12, 41, 42) and real-time PCR (43–47) formats for *Plasmodium* detection, species differentiation (including *Plasmodium ovale curtisi* and *P. ovale wallikeri*), and identification of parasite resistance markers to antimalarial drugs (see "Antimicrobial Susceptibilities" below). Most PCR assays are laboratory developed, although some commercial tests exist; unfortunately, none of these has been approved or cleared by the FDA.

Although there is tremendous variability between published assays, PCR-based tests are generally recognized as having improved sensitivity over the traditional thick film, with detection limits below 10 parasites/µl of blood. A quantitated *P. falciparum* standard is available through the WHO (48) which can be used to determine the analytical sensitivity of new and existing molecular assays.

Tests for detection of *Plasmodium* nucleic acid are primarily limited to specialized reference or public health laboratories and are not generally suited for rapid diagnosis in the clinical setting. However, they may be useful for detection of low parasitemia, confirmation of suspected infection, differentiation of *Plasmodium* from *Babesia* parasites, *Plasmodium* species determination, and sensitive detection of mixed infections (31).

Despite the potential advantages of PCR assays, implementation in endemic resource-limited settings poses many challenges due to the associated expense and need for highly specialized equipment and facilities. Fortunately, isothermal amplification techniques, such as nucleic acid sequence-based amplification (NASBA) and loop-mediated isothermal amplification (LAMP), offer simpler and less expensive tests for detection of *Plasmodium* DNA and RNA, respectively (49, 50). One recently described innovative electricity-free system utilizes a calcium oxide-based heat-generating reaction within an improvised insulated container for malaria LAMP testing (51). Other methods of *Plasmodium* nucleic acid amplification that may be suitable for resource-limited settings include nucleic acid lateral flow immunoassay (NALFIA) (52) and PCR-based enzyme-linked immunosorbent assay (PCR-ELISA) (53). Further studies are needed to determine the performance characteristics of these alternative assay designs in a variety of clinical settings.

When positive results are obtained with malaria nucleic acid tests, thick or thin blood films should be examined so that the percent parasitemia can be determined. Quantitative PCR may one day replace the subjective manual parasite counts that suffer from lack of inter-observer reproducibility. Unfortunately, quantitative PCR results do not correlate well with the conventionally obtained percent parasitemia. This is because conventional counts do not include gametocytes and extracellular forms, and multiply infected cells are only counted once; in contract, PCR will detect nucleic acid from each parasite in the specimen.

Serologic Tests

Serologic testing plays little role in diagnosis of acute malaria given that antibodies are frequently absent at the time of patient presentation. Also, a positive test indicates previous exposure but does not differentiate between acute and past infection. For these reasons, serology has its greatest utility in epidemiologic studies, blood donor screening (54), and occasionally for evaluating relapsing, recrudescent, or untreated malaria in nonimmune patients. Indirect fluorescent antibody (IFA) tests using antigens prepared from the four human *Plasmodium* species have been previously described (55, 56) and are available at specialized testing facilities. These tests are time-consuming and subjective but are highly sensitive and specific.

Other Diagnostic Laboratory Methods

Other nonconventional techniques for laboratory diagnosis include laser-desorption mass spectrometry (57) for detection of hemozoin in whole blood, and recently, detection of hemozoin-generated vapor nanobubbles across intact skin (58). These techniques hold promise for rapid and affordable malaria testing in the future.

Antimicrobial Susceptibilities

Parasite resistance to antimalarial medications is a serious problem worldwide (59). Chloroquine was a mainstay of treatment and prophylaxis for many years, but due to widespread *P. falciparum* resistance, it has been supplanted with other drugs such as atovaquone-proguanil (Malarone), doxycycline, and sulfadoxine-pyrimethamine (59–61). Chloroquine-susceptible *P. falciparum* is now only found in Mexico, the Caribbean, and parts of the Middle East and Central America (59). *P. falciparum* resistance to mefloquine and sulfadoxine-pyrimethamine, as well as *P. vivax* tolerance to chloroquine and primaquine, has also been detected in some endemic regions (59, 62). Only rare instances of quinine resistance have been reported, allowing this drug to remain a therapy for severe malaria (63, 64). Artemisinin compounds (e.g., artesunate, artemether) have been recognized as superior drugs for both malaria treatment and are now recommended by the WHO as first-line therapy, in combination with other antimalarial agents, for both uncomplicated and complicated disease (65). Unfortunately, decreased *in vivo* susceptibility to artesunate has been detected in *P. falciparum* strains along the Thai-Cambodian border, a historic site for emerging resistance to antimalarial agents (66). Containment interventions are urgently needed to prevent global spread of these partially resistant *P. falciparum* artemisinin-resistant strains.

Detection of antimalaria resistance may be performed using *in vitro* susceptibility testing (67), although this method is not available clinically. A more practical approach is through molecular detection of single-nucleotide polymorphisms that have been associated with antimalarial resistance, such as *dhps/dhft* (sulfadoxine-pyrimethamine), *Pfcrt* (chloroquine), *pfmdr* (chloroquine, mefloquine, and quinine), and *pfATPase6/pfmdr* (artemisinin) (62), using PCR (47) and DNA microarray formats (64, 68).

Evaluation, Interpretation, and Reporting of Results

Detection of malaria parasites by any diagnostic method is considered a critical result and must be reported to the clinical team immediately. The final report for blood smear evaluation should include the *Plasmodium* species and calculated degree of parasitemia. It is important to convey when a mixed infection or late stage forms of *P. falciparum* are suspected, since these findings may have treatment implications. Similarly, parasite load above 100,000 parasites/µl (approximately 2% parasitemia) in nonimmune individuals is indicative of severe disease requiring urgent therapy (69), while parasite levels above 10% suggest a role for exchange transfusion (70). It may be useful to relay to the clinical team that a single set of negative blood films does not exclude the diagnosis of malaria and that serial smears should be performed if clinically indicated (25).

Asexual forms of the parasites may be seen in repeat blood films for 2 to 3 days following appropriate treatment. If the level of parasitemia does not fall by 75% in the first 48 hours of treatment or parasites are still present after day 3, then parasite resistance or lack of patient compliance with treatment should be considered. Gametocytes may circulate for 2 weeks or more, since they are not killed by most antimalarial drugs, and therefore should be excluded from the parasite count or reported separately.

If expertise for *Plasmodium* species determination or differentiation between *Plasmodium* and *Babesia* parasites is not available in the primary laboratory, it is important to inform the clinician that *Plasmodium* or *Babesia* parasites are identified and that blood and/or films are being sent to a reference or public health laboratory for further analysis. It is important to mention in the preliminary report that the potentially deadly *P. falciparum* cannot be excluded.

BABESIA

Description of the Agent

Members of the genus *Babesia* infect a wide range of wild and domestic animals worldwide using primarily ixodid tick vectors (71, 72). Despite there being more than100 named *Babesia* species, only a few are known to regularly infect humans (72). *Babesia microti* is responsible for the vast majority of human cases in the U.S. Infection with *B. duncani* and *B. duncani*-like organisms have also been reported in Washington, Oregon, and California (73–75), and rare cases of *B. divergens*-like organisms have been detected in Kentucky, Missouri, and Washington state (72, 76). In Europe, *B. divergens* causes the majority of cases, with fewer cases attributable to *B. venatorum* and *B. microti* (77–80). Elsewhere a number of different *Babesia* species have been detected in humans, including *B. microti*-like organisms in Taiwan (81) and Japan (82), a new *Babesia* sp., the KO-1 strain, in South Korea (83), and uncharacterized species in Mexico, Brazil, Columbia, Egypt, Mozambique, South Africa, China, and India (72). Rare unverified human cases have also been attributed to *B. canis* and *B. bovis* (84).

Traditionally *Babesia* species were classified into small and large forms based on size, with *B. microti* and *B. divergens* being considered small form species (trophozoites <2.5 µm) (77). More recently, genetic analysis of the 18S rRNA gene has been used to clarify *Babesia* phylogeny and divide the piroplasms into four to five clades (77, 85). This analysis shows that *B. microti* and *B. divergens* are not closely related and they are now assigned to different clades (85).

Epidemiology and Transmission

In contrast to malaria, babesiosis is a disease of the temperate regions, including parts of North America and Europe. In the United States, classic hot spots for babesiosis due to *B. microti* include the northeastern coast (e.g., Martha's Vineyard, Nantucket, and Long Island), upper Midwest states, and areas of the Atlantic and South-Central states, while a small number of cases due to other *Babesia* species have been described in Missouri, Kentucky, Washington, Oregon, and California (72). National U.S. surveillance was begun in 18 states where babesiosis is endemic and New York City on 1 January 2011 (86), with 1,124 probable and confirmed cases reported in the first year. The majority of cases were reported by just seven states; in order of increasing numbers of cases, these were Minnesota, Rhode Island, Connecticut, Wisconsin, New Jersey, Massachusetts, and New York. The majority of patients were ≥60 years of age (range 1 to 98 years) and most did not recall a tick bite (86). Given that most infections with *Babesia* are asymptomatic, the actual number of infections is probably much greater than reported.

Significantly fewer cases of babesiosis have been reported in Europe. To date, 30 or more cases of babesiosis due to *B. divergens* have been reported, primarily from patients in France, Great Britain, and Ireland, while only sporadic cases of babesiosis due to *B. venatorum* and *B. microti* infection have been documented (72, 77). *B. divergens* is primarily a bovine babesiosis that has a significant economic impact on the livestock industry (87).

Most cases of babesiosis are acquired through the bite of an infected ixodid tick (Fig. 10). In nature, disease is maintained between invertebrate and vertebrate hosts. Humans readily become infected but are considered dead-end hosts. *Ixodes scapularis* (the blacklegged or deer tick) and *Ixodes ricinus* (the castor bean tick or sheep tick) are the most common vectors in the U.S. and Europe, respectively. The primary vertebrate host in the life cycle of *B. microti* in the U.S. is the white-footed mouse, *Peromyscus leucopus*, and other rodents and shrews may serve as the host for *B. microti* cases in Europe. The primary hosts for *B. divergens* and *B. venatorum* in Europe are cattle and deer, respectively. Following a tick bite, *Babesia* sporozoites enter the blood and directly infect circulating erythrocytes to form trophozoites resembling malarial parasites. There is no liver stage as seen in malaria and thus no latent exoerythrocytic forms. Trophozoites divide asexually via budding, rather than schizogony as in malaria, to form similar-appearing merozoites and gametocytes (84). Lysis of infected erythrocytes releases merozoites into the blood, where they infect new erythrocytes. As with malaria, sexual reproduction with fertilization of the gametes occurs in the gut of the arthropod host. Fertilized gametes migrate across the intestinal wall and travel to the salivary glands to develop into sporoblasts.

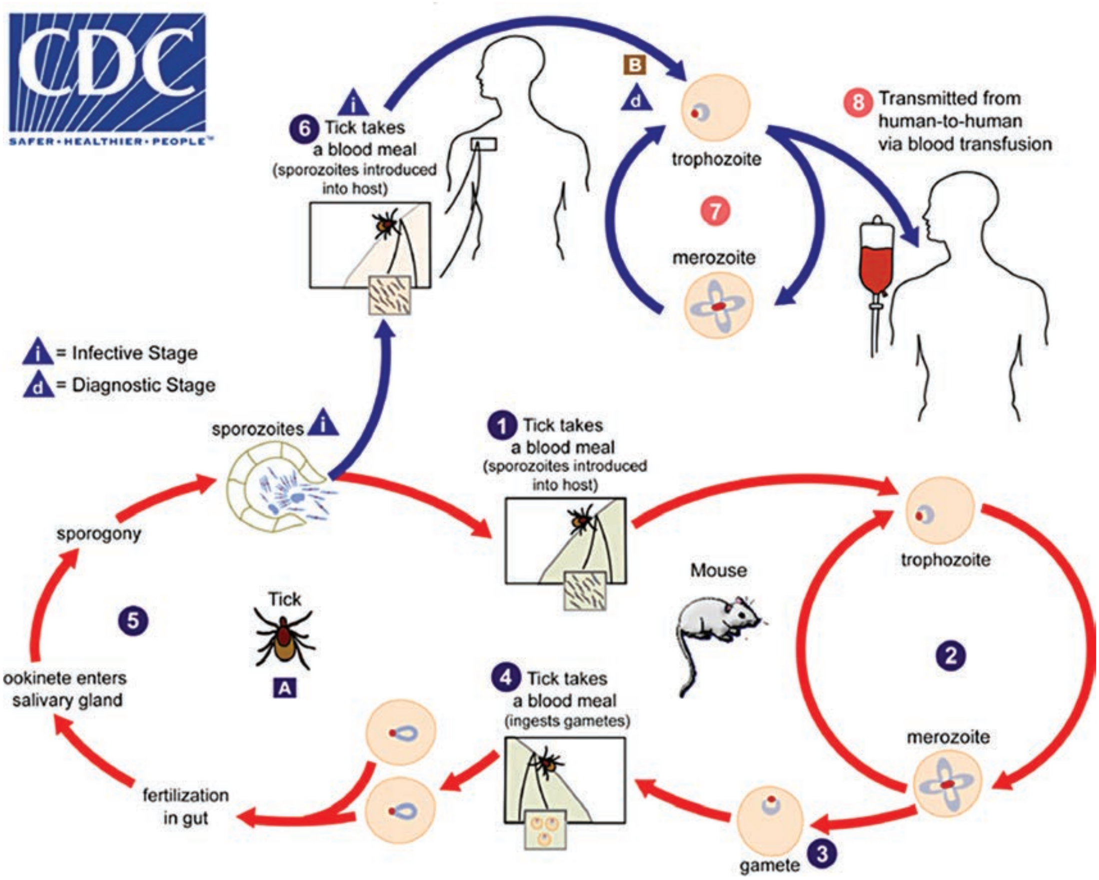
CDC
SAFER·HEALTHIER·PEOPLE
6 Tick takes a blood meal (sporozoites introduced into host)
i
B
d
trophozoite
7
merozoite
8 Transmitted from human-to-human via blood transfusion
i = Infective Stage
d = Diagnostic Stage
sporozoites i
1 Tick takes a blood meal (sporozoites introduced into host)
sporogony
5
Tick
A
Mouse
trophozoite
2
merozoite
ookinete enters salivary gland
4 Tick takes a blood meal (ingests gametes)
fertilization in gut
gamete 3

FIGURE 10 *Babesia microti* life cycle. The normal zoonotic life cycle involves a rodent (primarily *Peromyscus leucopus*, the white-footed mouse) and an *Ixodes* tick. The rodent becomes infected when an infected tick introduces *B. microti* sporozoites when taking a blood meal (1). Sporozoites enter the rodent erythrocytes and most undergo asexual reproduction (2), while some differentiate into female and male gametes (3). Ticks become infected when ingesting *Babesia* gametes in the blood of an infected rodent (4). Within the tick (A), the gametes undergo sexual reproduction to produce sporozoites (5). As with rodents, humans become infected through the bite of an infected *Ixodes* tick (6). Sporozoites enter human erythrocytes (B) and undergo asexual replication (7). Less commonly, humans are infected via blood transfusion (8). doi:10.1128/9781555817381.ch136.f10

The final step in maturation occurs when the tick takes a blood meal; sporozoites develop from the sporoblasts and are injected into the host during the final hours of feeding. The seasonality of babesiosis corresponds with the activity of the tick vectors, with most cases occurring in the spring, summer, and fall (86).

Less commonly, human babesiosis is acquired through receipt of infected blood products (88) or by vertical transmission across the placenta (89). Blood transfusion is recognized as an increasingly important source of infection (86, 88, 90), with 10 cases identified in the United States in 2011 (86). The largest review of transfusion-related babesiosis in the United States identified 159 cases during 1979–2009, with 156 cases due to *B. microti* and 3 due to *B. duncani* (90). Significantly fewer cases of transfusion-transmitted babesiosis have been reported in Europe (88). As expected, most implicated blood donations were collected during times of the year that ticks are active; however, a number of cases occurred from blood donated in the winter. In the latter cases, the original infections were presumably acquired by donors during the tick-biting season and remained dormant in the blood for months without causing symptoms. Most transfusion-transmitted cases have been associated with the receipt of whole blood, but cases associated with whole blood-derived platelets have also been reported (88, 90). The number of transfusion-related cases appears to be on the rise in the United States, and there is a need for a viable and cost-effective screening method for blood products. At this time, there is no FDA-approved test for this purpose and prevention is performed using only a donor questionnaire and indefinite donation deferral of patients with a history of babesiosis (88).

Clinical Significance

Symptoms of babesiosis due to *B. microti* and *B. duncani*/*B. duncani*-like parasites can range from asymptomatic or mild to life-threatening. When present, symptoms may include fever and a nonspecific influenza-like illness. Severe disease may behave like malaria, with high fever, chills, night sweats, myalgia, hemolytic anemia, hematuria, hepatosplenomegaly, and jaundice. Life-threatening complications include disseminated intravascular coagulation, acute renal or respiratory failure, congestive heart failure, and coma. Asplenia, immunosuppression, and advanced age are risk factors for severe disease (77).

Most patients with *B. microti* infection have a functioning spleen and symptoms tend to be mild. In contrast, patients infected with *B. divergens* in Europe are generally asplenic or immunocompromised and present with severe disease. Most infections with *B. divergens*-like agents in the United States have similar characteristics. At this point, only a small number of infections with *B. venatorum* have been reported, but preliminary evidence suggests that disease is relatively mild (77).

Collection, Transport, and Storage of Specimens

Collection and transport of whole blood specimens for babesiosis testing are performed in the same manner as for malaria. It is important to remember that *Ixodes scapularis*, the main vector of *Babesia microti*, is also a vector for other tick-borne pathogens, such as *Anaplasma phagocytophilum* and *Borrelia burgdorferi*, the agents of anaplasmosis and Lyme disease, respectively. Consideration should be given to concurrent testing for these agents.

Requests for blood films should be accompanied by important patient information such as clinical signs and symptoms, travel history, immune status, and presence or absence of a functioning spleen.

Direct Examination

Microscopy

As with malaria, the traditional method for diagnosis of babesiosis is microscopic examination of Giemsa-stained thick and thin blood films. Further details regarding the preparation and use of blood films are provided in the previous section on malaria.

Babesia spp. parasites may present a diagnostic challenge on blood films due to the many morphologic similarities they share with *Plasmodium* species (specifically *P. falciparum*) on thick and thin films (Fig. 11 and 12). However, a number of diagnostic features can be used to differentiate between *Babesia* and *Plasmodium* in most instances (Table 4). In general, *Babesia* parasites demonstrate greater pleomorphism in size and shape than *P. falciparum* parasites, with ovoid, elliptical, pear, racket, and spindle shapes commonly seen. Extracellular forms are also common. Rarely, classic tetrads ("Maltese crosses") are seen. It is important to note that only ring-type forms are seen in babesiosis. The presence of stippling, hemozoin pigment, schizonts, or plasmodial-type gametocytes excludes the diagnosis of babesiosis. It is not possible to differentiate the various human *Babesia* species by morphology; this requires molecular methods.

When the morphology is insufficient for differentiation of *Babesia* and *Plasmodium* parasites, it is helpful to obtain a complete travel history, examine multiple blood samples for parasites, or perform an alternative detection method (e.g., PCR).

Determining the degree of parasitemia may be useful for patient management. Parasitemia levels usually vary between 1 and 10% but may reach up to 80% (72, 77). Monitoring the parasite load in conjunction with treatment may be especially important in immunocompromised and asplenic patients who are at risk for prolonged or relapsing disease (91).

Nucleic Acid Detection

Nucleic acid amplification assays such as PCR may be useful in instances of low parasitemia and when parasite morphol-

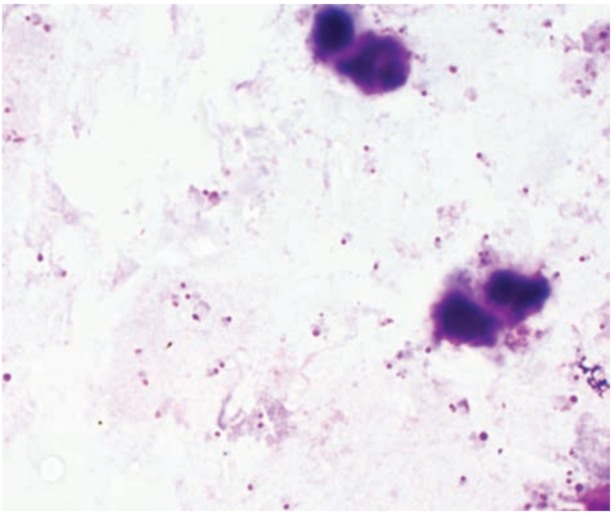

FIGURE 11 *Babesia* morphology on thick blood film consists of ring forms resembling trophozoites of *P. falciparum*. Some pleomorphism is apparent. doi:10.1128/9781555817381.ch136.f11

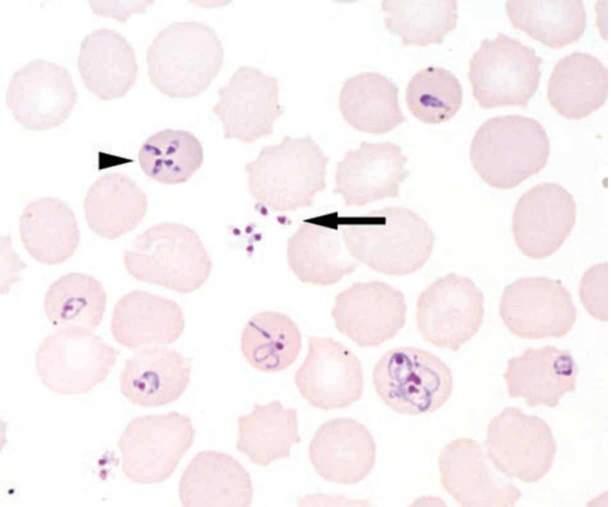

FIGURE 12 *Babesia* morphology on thin blood film. Note the presence of small intraerythrocytic ring forms resembling early trophozoites of *P. falciparum*, as well as thicker and markedly pleomorphic ring forms. Characteristic extracellular forms (arrow) and a tetrad form (arrow head) are also seen.
doi:10.1128/9781555817381.ch136.f12

ogy is inconclusive. Assays have been described for *B. microti* (92–95) and *B. divergens* (95). A real-time PCR assay for detection of *B. microti* reports a detection limit of approximately 0.0001% parasitemia, which is superior to the detection limit of thick films (0.0002 to 0.001% parasitemia). Sequencing of 18S rDNA has also been used for molecular characterization of *Babesia* species (78, 95). There are no FDA-approved *Babesia* PCR assays at this time.

Isolation Procedures

In rare instances, it may be necessary to inoculate the blood from a patient with suspected babesiosis into a laboratory animal such as a golden hamster (*B. microti*) (94, 96) or gerbil (*B. divergens*) (78, 87) to confirm the presence of a low-level infection or determine the viability of an organism detected only by DNA testing. This is rarely performed in the clinical laboratory but may be available following consultation at the CDC or at other specialized centers.

Serologic Tests

Serologic testing plays little role in the diagnosis of acute babesiosis, but may be useful for epidemiologic studies and in cases of chronic disease. Antibodies to *B. microti* typically appear 2 weeks after the onset of illness and may be detectable for several years after infection (71, 72). The indirect fluorescence immunoassay (IFA) (97, 98) is the recommended method for detection of serum antibodies against *B. microti* and has a relatively high sensitivity (88 to 96%) and specificity (90 to 100%) (71, 97). Premade slides for *B. microti* IFA testing are commercially available in the United States and Europe. Interpretation of IFA results are subjective and the cutoff titer for defining a positive result varies by laboratory; titers of 1:128 to 1:256 may be associated with a higher specificity (71). Low antibody titers or negative results may be seen when serum is obtained early in disease or when patients are immunosuppressed or asplenic, while false positives may occur in patients with connective tissue disorders (e.g., rheumatoid arthritis). *B. microti* IFA serology does not demonstrate significant cross-reactivity with antibodies to *B. duncani*, *B. divergens*, or *B. venatorum* (97), so specific assays must be used for these organisms (99, 100).

Evaluation, Interpretation, and Reporting of Results

Detection of *Babesia* parasites by any diagnostic method should be considered a clinically significant result and should be promptly reported to the clinical team. The final blood film report should ideally include the calculated degree of parasitemia. When the blood film examination is negative, the report may include a statement that a single set of negative blood films does not exclude the diagnosis of babesiosis and that serial smears should be performed if clinically indicated.

If expertise for differentiation of *Babesia* and *Plasmodium* parasites is not available in the primary laboratory, it is important to inform the clinician that *Plasmodium* or *Babesia* parasites are identified and blood and/or films are being sent to a reference or public health laboratory for further analysis.

TABLE 4 Comparative morphology of *Babesia* spp. and *P. falciparum*

Diagnostic features	*P. falciparum*	*Babesia* spp.
Size of infected RBC	Normal	Normal
Parasite stages in peripheral blood	Ring forms, less commonly crescent-shaped gametocytes; may see high parasitemia; other stages only rarely seen	Ring forms only; heavy infections common
Parasite characteristics	Delicate rings, <1/3 diameter of the RBC; may have headphone and appliqué forms; multiply infected RBCs common	Delicate rings, 1/3 to 1/6 diameter of the RBC; pleomorphic with ovoid, spindled, and amoeboid forms; multiply infected RBCs common; single or multiple chromatin dots; rarely tetrad forms
Red blood cell inclusions	Occasionally Maurer's clefts	None
Extracellular forms	Rare	Common
Pigment	Brown-black hemozoin	None
Fever cycle	36–48 hours	No periodicity
Area of endemicity	Tropical and subtropical regions	Temperate regions

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Leishmania and *Trypanosoma*

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Leishmania spp. and *Trypanosoma* spp. are protozoa belonging to the family Trypanosomatidae. Leishmaniasis is principally a zoonosis, and the organisms are obligate intracellular parasites transmitted to humans by bites from infected female sand flies. For *Leishmania* in the Old World, there is only one subgenus, *Leishmania*; however, in the New World, the genus has been split into subgenera (*Leishmania* and *Viannia*) according to the development of the organism in the digestive tract (peripylarian or suprapylarian) of the sand fly (1). Depending on the geographic area, many different species can infect humans, producing a variety of diseases (cutaneous, diffuse cutaneous, mucocutaneous, and visceral diseases) (Table 1). The spectrum of clinical presentation is dependent on the host's cell-mediated immune response (2).

Trypanosoma spp. are hemoflagellate protozoa that live in the blood and tissue of the human host. American trypanosomiasis (Chagas' disease) is produced by *Trypanosoma cruzi*, which belongs to the subgenus *Schizotrypanum* and is confined to the American continent. *Trypanosoma rangeli* belongs to the subgenus *Schizotrypanum*, produces an asymptomatic infection, and is also present only on the American continent. African trypanosomiasis (sleeping sickness) is caused by *Trypanosoma brucei gambiense* and *T. brucei rhodesiense* species belonging to the subgenus *Trypanozoon* and is confined to the central belt of Africa. African trypanosomes and *T. rangeli* are transmitted directly into the bite wound by salivary secretions from the insect vector, whereas *T. cruzi* is transmitted through contamination of the bite wound with the feces from the reduviid bug (Table 2). The first documented case of human trypanosomiasis caused by *Trypanosoma evansi* was detected in India (3).

LEISHMANIA SPP.

Recent estimates suggest that there are approximately 350 million people at risk of acquiring leishmaniasis, with 112 million currently infected. More than 400,000 new cases are reported annually (4). New species of *Leishmania*, particularly in the New World, are being detected frequently. The taxonomy of leishmaniasis is controversial and in a state of dynamic flux. Species differentiation is currently based on molecular techniques rather than geographical distribution and clinical presentation (5–7).

Life Cycle and Morphology

The parasite has two distinct phases in its life cycle, amastigote and promastigote (Fig. 1). The amastigote stage (Leishman-Donovan body) is found in reticuloendothelial cells of the mammalian host. The amastigote form is small, is round or oval, measures 3 to 5 μm, and contains a large nucleus and small kinetoplast (Fig. 1 and 2). This stage undergoes multiplication within the reticuloendothelial cells of the host.

Upon ingestion during a blood meal by the insect vector (sand fly), the amastigote transforms into the promastigote stage (Fig. 3). Promastigotes multiply in the gut of the insect, transform to metacyclic promastigotes, and migrate to the hypostome of the sand fly, where they are released when the next blood meal is taken. The complete life cycle in the sand fly is 4 to 18 days. Upon inoculation into the bite site, the promastigote changes to the amastigote form after being engulfed by tissue macrophages. This form change helps to defeat the host's immune response. Changes in the parasite's surface molecules play an important role in macrophage attachment and evading the host's immune response, including manipulating the macrophage's signaling pathways (2, 8, 9).

The life cycles of *Leishmania* organisms are similar for cutaneous, mucocutaneous, and visceral leishmaniasis, except that infected reticuloendothelial cells can be found throughout the body in visceral leishmaniasis.

Epidemiology and Transmission

All adult female sand flies transmitting leishmaniasis belong to the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. There are >30 species of sand flies that can transmit leishmaniasis. The disease is considered primarily a zoonosis, with natural reservoirs, including rodents, opossums, anteaters, sloths, and dogs. In certain areas of the world where the disease is endemic, the infection can be transmitted by a human-vector-human cycle. The infection may also be transmitted by direct contact with an infected lesion or mechanically through bites by stable or dog flies (*Stomoxys calcitrans*). If the blood meal of the fly is interrupted and it restarts its meal on another host, it can regurgitate part of the last meal with salivary juices into the bite site, thereby infecting that host. Greater than 90% of cutaneous leishmaniasis cases occur in Afghanistan, Algeria, Brazil, Iran, Iraq, Peru, Saudi Arabia, and Syria. There has been an increase in the number of cases among military personnel deployed in Afghanistan, Iraq, and Kuwait (27). Autochthonous human infections have been described in Texas and Oklahoma (10–12). Most of the diagnosed cases of mucocutaneous leishmaniasis are from Bolivia, Brazil, and Peru.

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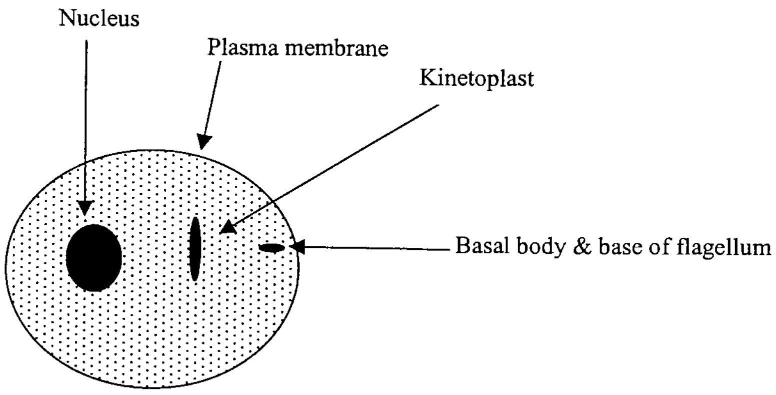
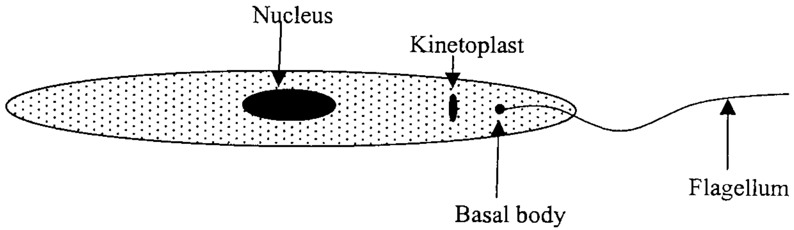


Amastigote



Promastigote

FIGURE 1 Life cycle stages of *Leishmania* spp. doi:10.1128/9781555817381.ch137.f1

Visceral leishmaniasis may exist as an endemic, epidemic, and sporadic disease. The disease is a zoonosis except in India and Brazil, where kala-azar is an anthroponosis (13). Natural reservoirs are wild Canidae and various rodents for *Leishmania donovani*; dogs, other Canidae, and rats for *Leishmania infantum*; and Canidae and cats for *L. infantum* (*Leishmania chagasi*) in the Americas. Individuals with post-kala-azar dermal leishmaniasis may be very important reservoirs for maintaining the infection during interendemic periods. More than 90% of the cases of visceral leishmaniasis are found in Bangladesh, Brazil, India, Nepal, and Sudan.

Clinical Significance

Depending on the species involved, infection with *Leishmania* spp. can result in cutaneous, diffuse cutaneous, mucocutaneous, or visceral disease (Table 1) (14). A large number of disease variations have been described, which makes classical disease categories confusing (5). In areas of endemicity, *Leishmania* coinfection with human immunodeficiency virus (HIV)-positive patients is common. If coinfected patients remain severely immunocompromised, approximately one-quarter will die within the first month of being diagnosed with leishmaniasis. The leishmanial infection will manifest itself like an opportunistic infection, and parasites will be detected in atypical sites (15). The use of highly active antiretroviral therapy has significantly improved the prognosis of patients infected with HIV and visceral leishmaniasis (16).

The first sign of cutaneous disease is the appearance of a firm, painless papule at or near the insect bite site. The incubation period may be as short as 2 weeks (*Leishmania major*) or as long as several months to 3 years (*Leishmania tropica* and *Leishmania aethiopica*). Papules may be intensely pruritic and will grow to 2 cm or more in diameter. Lesions may progress from a simple papule or erythematous macule to a nodule and ulcerate within days to weeks. In simple cutaneous leishmaniasis, the infection remains localized at the insect bite site, where a definite self-limiting granulomatous response develops. Lesions have been mistaken for basal cell carcinoma, tropical pyoderma, sporotrichosis, or cutaneous mycobacterial infections.

Mucocutaneous leishmaniasis is produced most often by the *Leishmania braziliensis* complex. The primary lesions are similar to those found in other infections of cutaneous leishmaniasis. Untreated primary lesions may develop into the mucocutaneous form in up to 80% of cases. Metastatic spread to the nasal or oral mucosa may occur in the presence of the active primary lesion or many years later after the primary lesion has healed. Mucosal lesions do not heal spontaneously, and secondary bacterial infections are frequent and may be fatal. A small number of mucocutaneous leishmaniasis cases have been reported with *L. donovani* and *L. aethiopica*. Differential diagnoses have included lymphoma, midline granuloma, Wegener's granulomatosis, paracoccidioidomycosis, histoplasmosis, cutaneous mycobacterial infection, syphilis, and leprosy.

Clinical features of the visceral disease vary from asymptomatic, self-resolving infections to frank visceral leishmaniasis. The incubation period may be as short as 10 days and as long as 2 years; usually it is within 2 to 4 months. Common symptoms include fever, anorexia, malaise, weight loss, and, frequently, diarrhea. Visceral leishmaniasis is an important

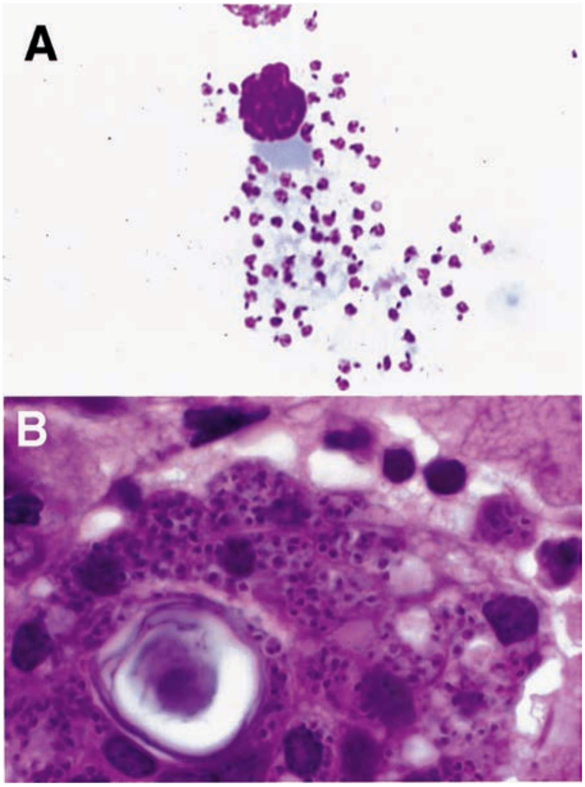


FIGURE 2 (A) *Leishmania donovani* amastigotes from a splenic press preparation (Giemsa stain); (B) *Leishmania donovani* in liver (hematoxylin and eosin stain). doi:10.1128/9781555817381.ch137.f2

opportunistic infection in individuals infected with HIV. Concomitant HIV infection dramatically increases the risk of developing active visceral leishmaniasis in asymptomatic individuals and accelerates the progression to AIDS. Individuals coinfected with HIV may not elicit common symptoms seen in immunocompetent individuals, whereas the leukopenia will be unusually severe (15). Common clinical signs include nontender hepatomegaly and splenomegaly, lymphadenopathy, and occasionally acute abdominal pain; darkening of facial, hand, foot, and abdominal skin (kala-azar) is often seen in light-skinned persons in India. Anemia, cachexia, and marked enlargement of liver and spleen are noted as the disease progresses. Death may ensue after a few weeks or after 2 to 3 years in chronic cases. The majority of infected individuals are asymptomatic or have very few or minor symptoms, which resolve without therapy. There has been a significant increase in leishmaniasis in organ transplant recipients since 1990. Most of the reported cases in organ transplant recipients have been visceral leishmaniasis, with a much smaller number of mucocutaneous leishmaniasis cases and, rarely, cutaneous leishmaniasis cases (17, 18). Differential diagnoses in the acute stage include amebic liver abscess, Chagas' disease, malaria, typhoid, typhus, and schistosomiasis. Subacute or chronic disease has been confused with malnutrition, bacteremia, brucellosis, histoplasmosis, leukemia, lymphoma, malaria, mononucleosis, and schistosomiasis.

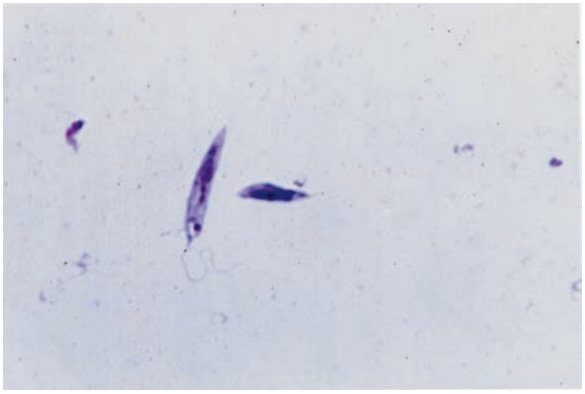


FIGURE 3 *Leishmania donovani* promastigotes (Giemsa stain). doi:10.1128/9781555817381.ch137.f3

Postdermal leishmaniasis or post-kala-azar dermal leishmaniasis (PKDL) is a condition seen in India and the Sudan in some patients unsuccessfully treated for visceral leishmaniasis. PKDL usually occurs 6 months or later after completion of therapy. This syndrome is rarely seen in Latin America but has been reported to occur in patients coinfected with HIV. The macular or hypopigmented dermal lesions are associated with few parasites, whereas erythematous and nodular lesions are associated with abundant parasites. This condition must be differentiated from leprosy, syphilis, and yaws.

Diagnosis

In areas where the disease is endemic, the diagnosis may be made on clinical grounds. Prolonged fever, progressive weight loss, anemia, leukopenia, hypergammaglobulinemia, and pronounced hepatomegaly and splenomegaly are highly suggestive of visceral leishmaniasis. The development of one or more chronic skin lesions with a history of exposure in an area of endemicity is suggestive of cutaneous leishmaniasis. In many areas of the world where the disease is endemic, laboratory testing (microscopy, culture, PCR, antigen tests, and serology) is almost impossible to obtain. Definitive diagnosis depends on detecting either the amastigotes in clinical specimens or the promastigotes in culture. Whenever leishmaniasis is suspected, multiple specimens should be taken and all of the diagnostic techniques should be employed if possible. When infected cells are in low numbers in specimens, one method may be successful in detecting the infection, while others may be negative.

Collection of Specimens

All cutaneous lesions should be thoroughly cleaned with 70% alcohol, and extraneous debris (the eschar and exudates) should be removed. After debridement, with precautions being taken to prevent bleeding, the base of the ulcer can be scraped with a scalpel blade to obtain an exudate for slide preparation, culture, or PCR. Specimens can be collected from the margin of the lesion by aspiration, scraping, or punch biopsy or by making a slit with a scalpel blade. Material scraped from the wall of the slit should be smeared onto a number of slides. PCR-based methods for the diagnosis of leishmaniasis have used a variety of specimens, including urine. PCR has been shown to be more sensitive than direct microscopy, histology, and culture, but availability is limited mainly to large hospitals or clinics. The PCR-based methods have not been standardized, and multicentered studies to validate these tests have not been done (19–22).

TABLE 1 Features of human leishmanial infections

Species	Disease type(s)[a]	Recommended specimen(s)	Geographic distribution
L. (*L.*) *donovani*	VL	Bone marrow or spleen biopsy specimen	Africa, Asia
	CL, MCL, DL	Skin or mucosal biopsy specimen	
L. (*L.*) *infantum*[b]	VL	Bone marrow or spleen biopsy specimen	Africa, Europe, Mediterranean area, Southeast Asia, Central and South America
L. (*L.*) *killicki*	CL	Skin biopsy specimen	Algeria, Tunisia
L. (*L.*) *tropica*	CL	Skin biopsy specimen	Afghanistan, India, Turkey, former USSR
L. (*L.*) *major*	CL	Skin biopsy specimen	Afghanistan, Africa, Middle East, former USSR
L. (*L.*) *aethiopica*	CL, DCL, MCL	Skin or mucosal biopsy specimen	Ethiopia, Kenya, former USSR, Yemen
L. (*L.*) *mexicana*	CL, DCL, MCL	Skin or mucosal biopsy specimen	Belize, Guatemala, Mexico, Texas
L. (*V.*) *braziliense*	CL, MCL	Skin or mucosal biopsy specimen	Central and South America
L. (*V.*) *peruviana*	CL	Skin biopsy specimen	Colombia, Costa Rica, Panama
L. (*V.*) *garnhami*	CL	Skin biopsy specimen	Venezuela
L. (*V.*) *colombiense*	CL	Skin biopsy specimen	Colombia, Panama
L. (*V.*) *panamensis*	CL	Skin biopsy specimen	Colombia, Costa Rica, Panama
L. (*V.*) *guyanensis*	CL, MCL	Skin or mucosal biopsy specimen	Brazil, Colombia, French Guiana, Peru
L. (*V.*) *venezuelensis*	CL	Skin biopsy specimen	Venezuela
L. (*V.*) *lainsoni*	CL	Skin biopsy specimen	Brazil
L. (*V.*) *shawii*	CL	Skin biopsy specimen	Brazil
L. (*L.*) *amazonensis*	CL, DCL	Skin or mucosal biopsy specimen	Brazil, Venezuela
L. (*V.*) *naffi*	CL	Skin biopsy specimen	Brazil, Caribbean Islands
L. (*L.*) *pifanoi*	CL, DCL	Skin or mucosal biopsy specimen	Brazil, Venezuela

[a] CL, cutaneous leishmaniasis; DCL, diffuse cutaneous leishmaniasis; DL, diffuse leishmaniasis; MCL, mucocutaneous leishmaniasis; VL, visceral leishmaniasis.
[b] *L.* (*L.*) *infantum* in the Old World is *L.* (*L.*) *chagasi* in the New World.

Laboratorians may want to contact their state public health laboratory or the Centers for Disease Control and Prevention (CDC) for diagnostic information and help in specimen selection and available tests. The CDC has the website address http://www.cdc.gov/ and posts diagnostic guidelines, including "Practical Guide for Laboratory Diagnosis of Leishmaniasis" (http://www.cdc.gov/parasites/leishmaniasis/resources/pdf/cdc_diagnosis_guide_leishmaniasis.pdf).

The core of tissue from a punch biopsy specimen can be used to make imprints or touch preparations on a slide. A tissue core should also be submitted for histological examination. Recognition of amastigotes in tissues is more difficult than in smears or imprints because the organisms tend to be crowded within the cells, to appear small, and to be cut at various angles. Fine-needle aspiration can also be performed by using a sterile syringe containing sterile preservative-free buffered saline (0.1 ml) and a 26-gauge needle. The needle is inserted under the outer border of the lesion, the needle is rotated several times, and tissue fluid is aspirated into the needle. Tissue obtained by splenic puncture yields the highest rate of positive specimens; however, this procedure carries significant risk to patients, particularly those with coagulation disorders. Other specimens for the detection of visceral leishmaniasis include lymph node aspirates, liver biopsy specimens, sternal aspirates, iliac crest bone marrow specimens, and buffy coat preparations of venous blood. Amastigotes with reticuloendothelial cells have been detected in bronchoalveolar lavage fluid, pleural effusions, and biopsy specimens collected from the gastrointestinal tracts and oropharynxes of HIV-positive patients. Indi-

TABLE 2 Characteristics of trypanosomiasis

Characteristic	*T. brucei rhodesiense*	*T. brucei gambiense*	*T. cruzi*	*T. rangeli*
Vector	Tsetse fly (*Glossina*)	Tsetse fly (*Glossina*)	Reduviid bug (*Panstrongylus*, *Rhodnius*, *Triatoma*)	Reduviid bug (*Rhodnius*)
Primary reservoir	Animals	Humans	Animals	Animals
Illness	Acute, <9 mo	Chronic, months to years	Acute, chronic	Asymptomatic
Epidemiology[a]	Anthropozoonosis	Anthroponosis	Anthropozoonosis	Anthropozoonosis
Diagnostic stage	Trypomastigote	Trypomastigote	Trypomastigote, amastigote	Trypomastigote
Recommended specimen(s)	Blood, CSF, chancre, and lymph node aspirate	Blood, CSF, chancre, and lymph node aspirate	Blood, chagoma, and lymph node aspirate	Blood

[a] Anthropozoonosis, transmission involving a human-animal-human cycle; anthroponosis, transmission involving a human-human cycle.

viduals with post-kala-azar dermal leishmaniasis have large numbers of parasites in the skin, particularly those with erythematous and nodular lesions (23).

Direct Examination

Microscopic Detection

Amastigote stages are found within macrophages or close to disrupted cells (Fig. 2). This stage can be recognized by its shape, size, staining characteristics, and, especially, the presence of an intracytoplasmic kinetoplast. The cytoplasm will stain light blue, and the nucleus and kinetoplast will stain red or purple with Giemsa stain. Amastigotes can be differentiated from intracellular fungal organisms because they will not stain positive with periodic acid-Schiff, mucicarmine, or silver stain. Intracellular fungal organisms do not have a kinetoplast that can be seen in amastigote stages.

PCR Detection

Molecular techniques (none are U.S. Food and Drug Administration [FDA] approved) for the detection of leishmanial DNA or RNA have been used for diagnosis, prognosis, and species identification. These methods are considered more sensitive than slide examination or culture, particularly for the detection of mucocutaneous leishmaniasis, for which organisms are difficult to culture (4, 24–39). Generally, organisms in mucocutaneous lesions are scant and difficult to detect microscopically. Because infections caused by species of the *Leishmania* subgenus *Viannia* are considered more aggressive and are more likely to result in treatment failure, molecular techniques to identify the organism to the species and strain levels can be very important for therapy (40–42). Multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST) have been used to identify *Leishmania* species and strains, but this depends on having culturable isolates, and in some cases, these methods were not discriminative enough. To improve on isolate identification, various rRNA and DNA targets, such as the miniexon or spliced leader, rRNA internal transcribed spacer (ITS), 7SL RNA gene, heat shock protein 70 gene, and cytochrome *b* gene, have been used (43–47). Multiple targets are necessary due to gene polymorphism within the target sequences.

Culture

If material is to be cultured, it must be collected aseptically. Tissues should be minced prior to culture. Culture media successfully employed to recover organisms include Novy, MacNeal, and Nicolle's medium (NNN) and Schneider's *Drosophila* medium supplemented with 30% fetal bovine serum (48). Cultures, incubated at 25°C, should be examined twice weekly for the first 2 weeks and once a week thereafter for up to 4 weeks before the culture is declared negative. Promastigote stages can be detected microscopically in wet mounts and then stained with Giemsa stain to observe their morphology.

Animal Inoculation and Culture

Animals such as the golden hamster can be inoculated with patient material. Animals are inoculated intranasally for cutaneous and mucocutaneous leishmaniasis and intraperitoneally for visceral leishmaniasis. It may take 2 to 3 months before an animal becomes positive. A combination of tissue smears, culture, and animal inoculation may be needed to optimize the laboratory diagnosis of the infection. PCR testing, if available, can be used as a supplemental diagnostic procedure (34, 49).

Skin Testing

The leishmanin (Montenegro) test (not available in Canada or the United States), a delayed-type hypersensitivity reaction, is useful for epidemiological surveys of a population to identify groups at risk of infection. This test may be available from clinics and institutions in areas of endemicity. The heavier the parasite burden and the longer the length of time of infection, the more likely one will have a positive skin test. Although skin test antigens are commercially available outside the United States, there is a lack of product standardization and stability. Positive reactions are usually seen in cutaneous and mucocutaneous leishmaniasis; however, patients with active visceral and diffuse cutaneous leishmaniasis exhibit negative reactions. Post-kala-azar patients may also exhibit a negative reaction. This test is of no value for the diagnosis of visceral leishmaniasis.

Serologic Tests

Serologic tests (none are FDA approved) are available for research or epidemiologic purposes; however, they are not very useful for the diagnosis of mucocutaneous and visceral leishmaniasis. In kala-azar, there is a large increase in gamma globulins, both immunoglobulin G (IgG) and IgM. This is the basis for the aldehyde or formol-gel test, which has been used as a screening test in areas of endemicity (50). The addition of 1 drop of formalin to 1 ml of serum promotes the precipitation of immunoglobulins. A number of serologies, including indirect fluorescent-antibody assay (IFA), enzyme-linked immunoassay (ELISA), and immunoblot tests have been developed for diagnostic purposes; however, they are not widely available except in areas of endemicity (51–53). An ELISA, dipstick test, and rapid immunochromatographic strip using *L. infantum*/*L. chagasi* recombinant k39 antigen has good sensitivity and specificity in diagnosing visceral leishmaniasis in immunocompetent people (54–57). Visceral leishmaniasis patients coinfected with HIV may have no detectable antileishmania antibodies (14, 58, 59). Serologic testing is available at some referral laboratories and the CDC. The detection of urinary antigens has been used for the diagnosis of visceral leishmaniasis (60).

Treatment and Prevention

Lesions in simple cutaneous leishmaniasis generally heal spontaneously. Treatment options have included cryotherapy, heat, photodynamic therapy, surgical excision of lesions, and chemotherapy (61). Chemotherapeutics include azoles (fluconazole, ketoconazole, posaconazole), amphotericin B, miltefosine, paromomycin, pentavalent antimonials, and pentamidine. Treatment is advocated to reduce scarring in cosmetic areas and to prevent dissemination and/or relapse of the infection. Although the optimal treatment for cutaneous leishmaniasis is unknown, standard therapy consists of injections of antimonial compounds. Intralesional antimonial therapy may be given to patients with a limited number of cutaneous lesions (≤3) whereas intramuscular or intravenous therapy should be given for more-disseminated infections. Topical therapy should not be advocated for mucocutaneous infections. Response to therapy varies depending on the species of *Leishmania* and the type of disease (62, 63); therefore, it is important to identify the species of *Leishmania* causing the infection (39, 64, 65). The risk of relapse is quite high within the first 6 to 12 months posttherapy (66, 67). Patients clinically cured of *L.* (*V.*) *braziliensis* infection, which is noted for its chronicity, latency, and metastasis, with mucosal membrane involvement, have been found to be PCR positive up to 11 years posttherapy (68).

Visceral leishmaniasis can be fatal if not treated, and individuals coinfected with HIV must be treated aggressively. In addition, the individual may have to be treated for life to present relapse of visceral leishmaniasis. Drugs most commonly used to treat visceral leishmaniasis include amphotericin B, miltefosine, paromomycin, and pentavalent antimonials (sodium stibogluconate, meglumine antimonite). Liposomal amphotericin B has the highest therapeutic efficacy, whereas resistance to antimonial drugs is common in India and Nepal. Miltefosine is an effective oral agent, although failure rates as high as 30% have been reported.

To ensure that treatment has been effective, follow-up smears and cultures should be done 1 to 2 weeks posttherapy. PCR testing has been used to monitor the progress of therapy (68, 69).

In areas where leishmaniasis is endemic, vaccination is still a major goal for eliminating leishmaniasis. Inoculating the serous exudate from naturally acquired lesions of cutaneous leishmaniasis into an inconspicuous area of the body of a nonimmune person has been effective; however, vaccines against other forms of leishmaniasis have not worked. Vaccination with exudates from individuals with mucocutaneous leishmaniasis should never be tried due to the extensive pathology associated with this infection. Other possible prevention methods include spraying dwellings with insecticides, applying insect repellents to the skin, and using fine-mesh bed netting. Reservoir control has been unsuccessful in most areas, although in areas where canines may be a reservoir host, pyrethroid-impregnated collars are being used to prevent infections. Individuals with lesions should be warned to protect the lesion from insect bites, and patients should be educated about the possibility of autoinoculation or infection.

AMERICAN TRYPANOSOMIASIS

Trypanosoma cruzi

American trypanosomiasis (Chagas' disease) is a zoonosis caused by *Trypanosoma cruzi*. There are 100 million persons at risk of infection in 18 Latin American countries, and 16 to 18 million persons are actually infected (15). Patients can present with either acute or chronic disease. Chagas' disease was considered a disease of rural areas; however, it is now ubiquitous due to social-pattern changes as a result of migration from rural to urban areas. A very serious problem is disease acquisition through blood transfusion and organ transplantation (20, 70). A large number of patients with positive serology can remain asymptomatic (71).

Life Cycle and Morphology

Trypomastigotes (Fig. 4 and 5) are ingested by the reduviid bug (triatomids, kissing bugs, or conenose bugs) as it obtains a blood meal. The trypomastigotes transform into epimastigotes (Fig. 4 and 6) that multiply in the posterior portion of the midgut. After 8 to 10 days, metacyclic trypomastigotes develop from the epimastigotes. These metacyclic trypomastigotes passed in the feces of the reduviid bug are the infectious stage for humans.

Humans contract Chagas' disease when the reduviid bug defecates while taking a blood meal and metacyclic trypomastigotes in the feces are rubbed or scratched into the bite wound or onto mucosal surfaces. In humans, *T. cruzi* can be found in two forms, as amastigotes and trypomastigotes (Fig. 4, 6, and 7). The trypomastigote form is present in the blood and infects the host cells. The amastigote form multiplies within the cell, eventually destroying the cell, and both amastigotes and trypomastigotes are released into the blood.

The trypomastigote is spindle shaped and approximately 20 μm long; it characteristically assumes a C or U shape in stained blood films (Fig. 6). Trypomastigotes occur in the blood in two forms, a long, slender form and a short, stubby one. The short, stubby form is thought to be the infectious form for the vector. The nucleus is situated in the center of the body, with a large, oval kinetoplast located at the posterior end. A flagellum arises from the basal body and extends along the outer edge of an undulating membrane until it reaches the anterior end of the body, where it projects as a free flagellum. When the trypomastigotes are stained with Giemsa stain, the cytoplasm stains blue and the nucleus, kinetoplast, and flagellum stain red or violet. The amastigote (2 to 6 μm in diameter) is indistinguishable from those found in leishmanial infections. It contains a large nucleus and a rod-shaped kinetoplast that stains red or violet with Giemsa stain, and the cytoplasm stains blue (Fig. 2 and 7).

Epidemiology and Transmission

Chagas' disease is a zoonosis occurring throughout the American continent, including Central and South America, California, Louisiana, and Texas (72). It involves reduviid bugs living in close association with reservoirs (dogs, cats, armadillos, opossums, raccoons, and rodents). Human infections occur mainly in rural areas in which poor sanitary and socioeconomic conditions and poor housing provide excellent breeding places for reduviid bugs. Chagas' disease is found in 18 countries in Central and South America. There have been 6 autochthonous cases identified in the United States (72). The disease distribution has been broken into two ecological zones: the southern cone, where the reduviid vector lives inside the human home, and the northern South America, Central America, and Mexico zone, where the reduviid lives inside and outside the home. Strains of *T. cruzi* have large differences in infectivity of potential vectors, antigenicity, histotropism, pathogenicity, and response to therapy (73–76). Based on molecular epidemiology, *T. cruzi* has been broken into 6 genotypes or lineages (77, 78). Most human infections in the southern cone are due to *T. cruzi* II, whereas in the northern cone, *T. cruzi* I predominates (79, 80). Transmission by blood or blood product transfusion is a serious concern in areas of endemicity, and serologic screening of blood donors can be used, whereas in areas where the disease is not endemic, questionnaires may be used to defer prospective donors from areas of endemicity (81, 82).

Clinical Significance

In addition to contracting *T. cruzi* infections through the insect's bite wound or exposed mucous membranes, one can be infected by blood transfusion, placental transfer, organ transplant, and accidental ingestion of parasitized reduviid bugs or their feces in food or drink (81–85). Most congenital infections are asymptomatic. A localized inflammatory reaction may ensue at the infection site with the development of a chagoma (erythematous subcutaneous nodule) or Romaña's sign (edema of the eyelids and conjunctivitis).

The incubation period following exposure is usually 1 to 2 weeks. Most patients have nonspecific symptoms or no symptoms at all. Acute systemic signs occur around the second to third week of infection and are characterized by high fevers, hepatosplenomegaly, myalgia, erythematous rash, acute myocarditis, lymphadenopathy, and subcutaneous edema of face, legs, and feet. The acute phase of Chagas' disease in immunosuppressed patients is manifested as acute myocarditis or acute encephalitis, with high mortality rates. Most acute cases resolve over a period of 2 to 3 months

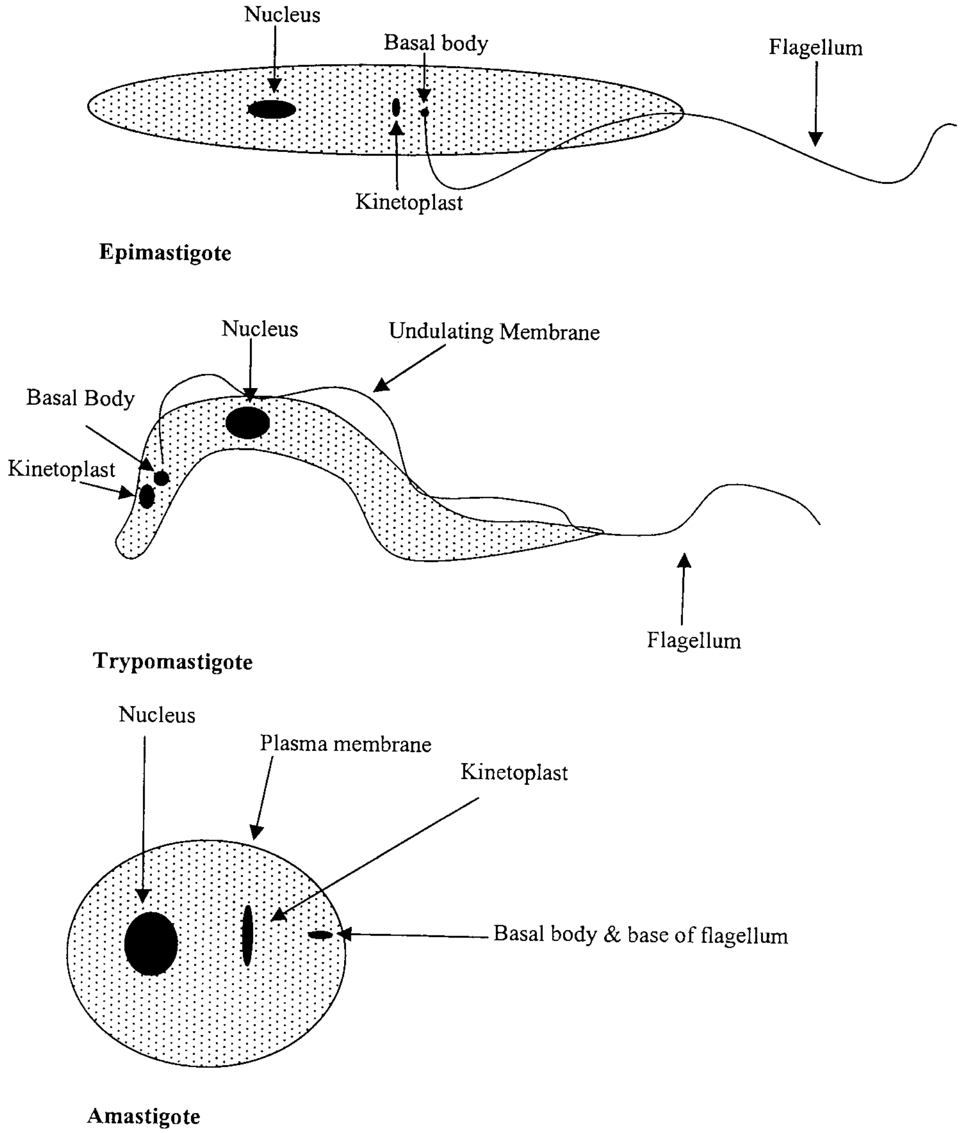


FIGURE 4 Life cycle stages of trypanosomes. doi:10.1128/9781555817381.ch137.f4

into an asymptomatic chronic stage (indeterminate phase or clinical latency period). Approximately 70% of the individuals with chronic Chagas' disease remain asymptomatic (indeterminate phase); however, they are still capable of transmitting the infection. The remaining 30% of these individuals with chronic Chagas' disease develop myocarditis or symptoms associated with denervation of the digestive tract. Chronic Chagas' disease may develop years or decades after undetected infection or after the diagnosis of acute disease. The most frequent clinical sign of chronic Chagas' disease is cardiomyopathy manifested by cardiomegaly and conduction changes. Some patients are more likely to develop megaesophagus or megacolon. Gastrointestinal Chagas' disease is rare outside of Argentina, Bolivia, Brazil, Chile, and Paraguay (southern-cone countries). The "mega" condition has been associated with the destruction of ganglion cells, resulting in dysmotility and causing dysphagia, aspiration, and regurgitation in patients with megaesophagus and severe constipation in patients with megacolon. In chronic Chagas' disease, autoimmunity may also be responsible for tissue destruction in addition to the tissue destruction caused by the parasite. Reactivation of Chagas' disease in HIV-positive patients usually leads to very high parasitemia and can occur in other immunosuppressed patients (86). Central nervous system (CNS) involvement is seldom observed, but in HIV-coinfected individuals, CNS involvement is frequently noted and acute fatal meningoencephalitis and granulomatous encephalitis have been described for these patients. Congenital transmission from mother to fetus can occur in both the acute and chronic phases of the disease. Congenital infections can cause abortion, prematurity, neurological sequelae, and mental deficiency (71). Infants of seropositive mothers should be monitored for up to a year after birth to rule out infection. Transmission of the infection during transplantation of solid organs and other tissues from seropositive donors has become a significant problem (70). Although transplantation of any organ or tissue from a seropositive donor should be regarded as infectious, the risk of transmission of the infection is dependent on other factors. Some recipients do not develop infections;

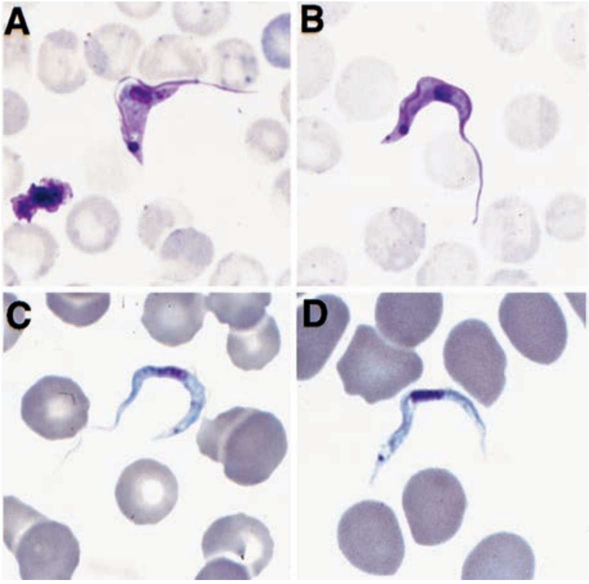


FIGURE 5 (A and B) *Trypanosoma cruzi* trypomastigotes; (C and D) *Trypanosoma brucei gambiense* trypomastigotes. doi:10.1128/9781555817381.ch137.f5

however, all should be serially monitored for signs of infection.

Diagnosis

Health care personnel working with specimens from patients suspected of having Chagas' disease should follow the blood-borne-pathogen guidelines using universal precautions. Trypomastigotes are highly infectious.

Collection of Specimens

A definitive diagnosis depends on demonstration of trypomastigotes in the blood, amastigote stages in tissues, or positive PCR and serologic tests (Table 3). Aspirates from chagomas and enlarged lymph nodes can be examined for amastigotes and trypomastigotes. Histological examination of biopsy specimens may also be done. Trypomastigotes may easily be detected in the blood in acute disease; however, in chronic disease, this stage is rare or absent, except during febrile episodes. Trypomastigotes appear in the blood about 10 days after infection and persist through the acute phase.

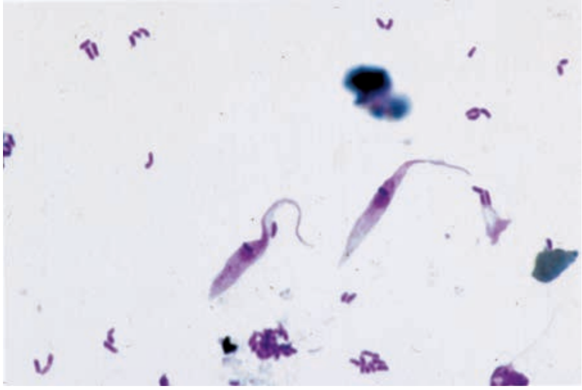


FIGURE 6 *Trypanosoma cruzi* epimastigotes (Giemsa stain). doi:10.1128/9781555817381.ch137.f6

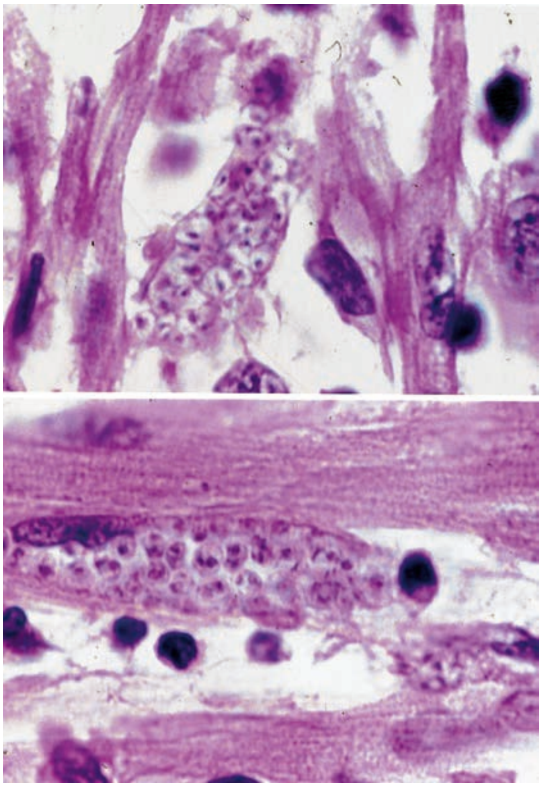


FIGURE 7 *Trypanosoma cruzi* amastigotes in heart muscle (hematoxylin and eosin stain). doi:10.1128/9781555817381.ch137.f7

Laboratorians may want to contact their state public health laboratory or the CDC for diagnostic information and help in specimen selection and available tests.

Direct Examination

Microscopic Detection

Trypomastigotes may be detected in wet mounts of blood or by using thin and thick blood films or the buffy coat concentration technique (87). The stain of choice is Giemsa for both amastigote and trypomastigote stages. Amastigotes

TABLE 3 Diagnostic methods to detect Chagas' infections

Method[a]	Use during indicated infection stage		
	Acute	Indeterminate	Chronic
Direct			
Direct microscopy	+	–	–
Thick and thin blood films	+	–	–
PCR	+	+	+
Blood culture	+	–	–
Xenodiagnosis	+	+	+
Indirect			
IFA (IgM and IgG)	+	+	+
EIA	–	+	+
IHA	–	+	+
RIPA/WB	–	+	+

[a] IFA, indirect fluorescent-antibody assay; EIA, enzyme immunoassay; IHA, indirect hemagglutinin assay; RIPA, radioimmunoprecipitation assay; WB, Western blotting.

can be differentiated from fungal organisms because they will not stain positive with periodic acid-Schiff, mucicarmine, or silver stain. In areas where kala-azar occurs, amastigote stages look similar, and infections of *L. donovani* and *T. cruzi* must be differentiated by PCR, immunoassay, culture (epimastigote for *T. cruzi* versus promastigote for *L. donovani*), serologic tests, animal inoculation, or xenodiagnosis techniques (88). Patient history, including geographic and/or travel history, and confirmation of organisms in striated muscle rather than reticuloendothelial tissues are very strong evidence for *T. cruzi* rather than *L. donovani* as the causative agent.

PCR Detection

Although not routinely available except in specialized centers, PCR (not FDA approved) has been used to detect as few as one trypomastigote in 20 ml of blood and has been useful in treatment follow-up (20, 88–90). To improve on detection of positive patients, real-time PCR using multiple gene targets has been advocated (91, 92). Multiple targets are needed due to polymorphism within the gene targets. The PCR-based methods have not been standardized, and multicenter studies to validate these tests have not been done. There have been few studies where various PCR methods used for diagnostic purposes have been compared.

Culture and Animal Inoculation

Aspirates, blood, and tissues can also be cultured. The medium of choice is NNN (48). Cultures, incubated at 25°C, should be examined for epimastigote stages twice weekly during the first 2 weeks and once per week thereafter for up to 4 weeks before they are considered negative. If available, laboratory animals (rats or mice) can be inoculated and the blood can be observed for trypomastigotes.

Xenodiagnosis

Although xenodiagnosis is used less frequently for clinical diagnosis in areas of endemicity, trypanosome-free reduviid bugs are allowed to feed on individuals suspected of having Chagas' disease. The feces, hemolymph, hindgut, and salivary glands can be examined microscopically for flagellated forms over a period of 3 months, or PCR methods can be used to detect infected bugs and provide a rapid diagnosis (93). Xenodiagnosis is positive in less than 50% of the seropositive patients. Some patients may develop a severe anaphylactic reaction to the reduviid bug's salivary secretion.

Serologic Tests

Serologic tests using blood and saliva for the diagnosis of Chagas' disease include complement fixation (Guerreiro-Machado test), chemiluminescence, IFA, indirect hemagglutination, and ELISA (94, 95). Most of these tests use an epimastigote antigen, and cross-reactions have been noted for patients infected with *Trypanosoma rangeli*, *Leishmania* spp., *Toxoplasma gondii*, and hepatitis (4, 96). The use of synthetic peptides and recombinant proteins has improved the sensitivity and specificity of the serodiagnostic techniques; however, their reliability for diagnosis is questionable (81, 97–102). Several diagnostic enzyme immunoassay (EIA) methods using parasite lysate and recombinant antigens have been approved by the FDA for screening blood donors. The sensitivity and specificity of these serologic tests for screening blood donors have improved so that single-assay screening may be justified rather than the two-assay screening method previously recommended. The FDA and AABB (American Association of Blood Banks) require donated blood be screened for Chagas' antibodies, and it is recommended that UNOS (United Network Organ Sharing) test tissue donors for the presence of Chagas' antibodies. Alternative diagnostic methods to confirm serology-positive tests are recommended. Follow-up blood specimens should be reexamined 1 to 2 months after therapy by the techniques described above. A difficult problem using serology has been determining whether a patient has been reinfected or if treatment has been unsuccessful. Changes in *T. cruzi*-specific T cell and antibody responses were determined to be an excellent marker for therapeutic response. Serologic testing is available at referral laboratories and the CDC.

Treatment and Prevention

Nifurtimox (Lampit) and benznidazole (Radamil) reduce the severity of acute Chagas' disease. Other drugs, namely, allopurinol, fluconazole, itraconazole, and ketoconazole, have been used to treat a limited number of patients. Treatment of individuals with chronic Chagas' disease slowed the development of cardiomyopathy and decreased mortality. Response to therapy can be monitored by measuring the reduction in antibody response or the reduction in parasite load by PCR (76, 90, 103). Surgery has been successfully used to treat cases of chagasic heart disease, megaesophagus, and megacolon.

Until recently, control of Chagas' disease has been mainly through the use of insecticides to eliminate the reduviid vector. Construction of reduviid-proof dwellings and health education are essential for effective control programs. Bed nets are also effective in preventing infections. Serologic screening of blood products for transfusion from areas in which the disease is endemic is highly recommended (95, 103, 104). An alternative approach to serologic testing for blood donors is the use of questionnaires to defer prospective donors from areas of endemicity.

Trypanosoma rangeli

T. rangeli infects humans and other vertebrates in both Central and South America and is often found in areas where *T. cruzi* is also present (105, 106). Human infections are asymptomatic, and trypomastigotes have been noted to persist in the blood for longer than a year. *T. rangeli* and *T. cruzi* can use the same triatomid vector to transmit infections. *T. rangeli* infections can be transmitted by inoculation of triatomid saliva during feeding or by the vector's feces (107). In some areas, *T. rangeli* infections are five to six times more frequent than infections with *T. cruzi*. Trypomastigotes can be detected from the blood of infected patients by using thin and thick blood smears and buffy coat concentration techniques. The parasites can be stained with Giemsa or Wright stain. Microscopically, the trypomastigote cannot be differentiated from African trypanosomes, which do not occur in the Americas. *T. rangeli* trypomastigotes can be differentiated from *T. cruzi* trypomastigotes based on the smaller size of the kinetoplast. PCR methods have been used to detect infections in humans and vectors (107, 108). Infections can also be detected by xenodiagnosis. In addition, blood can be cultured (Tobie's medium or NNN) (48) or injected into laboratory animals (mice) and examined for epimastigotes and trypomastigotes, respectively. Although there are no serologic tests to detect *T. rangeli* infections, serologic cross-reactions have been noted to occur with tests for *T. cruzi* (109). To differentiate between the two infections, a clinical history should be obtained and acute- and convalescent-phase sera should be tested by serology or different test methods, namely, PCR and microscopic analysis of blood. There are no treatment recommendations for *T. rangeli* infections.

AFRICAN TRYPANOSOMIASIS

African trypanosomiasis is limited to the tsetse fly belt of Central Africa, where there are over 60 million people at risk for African trypanosomiasis (110). Fewer than 10,000 cases are reported per annum. The West African (Gambian) form of sleeping sickness, noted for its chronicity and responsible for 99% of the sleeping sickness cases, is caused by *T. brucei gambiense*, whereas the East African (Rhodesian) form, noted for its acute morbidity and mortality within months of infection, is caused by *T. brucei rhodesiense* (111). *T. brucei gambiense* infections can last for months to years, with slow CNS involvement. In some areas of endemicity, civil strife has disrupted both the health care infrastructure and vector control, which has led to a resurgence of this disease.

Life Cycle and Morphology

T. brucei rhodesiense and *T. brucei gambiense* are closely related and morphologically indistinguishable. In the past, differentiation was based on clinical signs and geographic area; however, differentiation can now be accomplished using isoenzyme characteristics and DNA and RNA methods.

The trypomastigote forms (Fig. 4 and 6) in the blood range from long, slender-bodied organisms with a long flagellum to short, fat, stumpy forms without a free flagellum (14 to 33 µm long and 1.5 to 3.5 µm wide). The short, stumpy forms are the infective stage for the tsetse fly.

Using Giemsa or Wright stain, the granular cytoplasm stains pale blue and contains dark-blue granules and possibly vacuoles. The centrally located nucleus stains reddish. The kinetoplast is located at the organism's posterior end and stains reddish; the remaining intracytoplasmic flagellum (axoneme) may not be visible. The flagellum arises from the kinetoplast, as does the undulating membrane. The flagellum runs along the edge of the undulating membrane until the undulating membrane merges with the trypanosome body at the organism's anterior end. At this point, the flagellum becomes free to extend beyond the body. Trypanosomal forms are ingested by the tsetse fly when a blood meal is taken and transform to epimastigotes (Fig. 4). The organisms multiply in the gut of the fly, and after approximately 2 weeks, the organisms migrate back to the salivary glands. Human are infected when metacyclic forms from the salivary glands are introduced into the bite site as the blood meal is taken by the tsetse fly. The trypomastigote has the ability to change the surface coat of the outer membrane, helping the organism evade the host's humoral immune response (112).

Epidemiology and Transmission

The development cycle in the tsetse fly varies from 12 to 30 days and averages 20 days. Fewer than 10% of tsetse flies become infective after obtaining blood from infected patients. Both female and male tsetse flies can transmit the infection. Infections can also occur through placental transfer from mother to fetus and by needle sticks.

Although there is no evidence of animal-to-human transmission of *T. brucei gambiense*, trypanosomal strains isolated from hartebeest, kob, chickens, dogs, cows, and domestic pigs in West Africa are identical to those isolated from humans in the same area. Evidence suggests that transmission may be entirely interhuman. The tsetse fly vectors of Rhodesian trypanosomiasis are game feeders (including cattle) that may transmit the disease from human to human or from animal to human.

There is molecular evidence of multistrain introduction of the infection with parasites of the *T. brucei* complex that can have epidemiological implications as to virulence, pathogenicity, and response to therapy (113). This phenomenon has not received much attention.

Clinical Significance

After a bite by an infected tsetse fly, a local inflammatory reaction that resolves spontaneously within 1 to 2 weeks can be detected at the bite site. The bite site chancre can be painful, presenting as an erythematous indurated nodule that may ulcerate. The trypomastigotes gain entrance to the bloodstream, causing a symptom-free low-grade parasitemia that may continue for many months. The infection may self-cure during this period without development of symptoms or lymph node invasion. Chancres may be confused with insect bites and bacterial skin infections, with resolution occurring within a few weeks.

The clinical course and disease progression are more acute with *T. brucei rhodesiense* than with *T. brucei gambiense* infections. Diagnostic symptoms include irregular fever, lymph node enlargement (particularly those of the posterior triangle of the neck, known as Winterbottom's sign, which is prominent in *T. brucei gambiense* infections), delayed sensation to pain (Kerandel's sign), and erythematous skin rashes. In addition to lymph node involvement, the spleen and liver become enlarged. Stage I of African trypanosomiasis is when the trypomastigotes multiply in the subcutaneous tissues, blood, and lymph (hemolymphatic phase). Stage II occurs when the trypomastigotes cross the blood-brain barrier to initiate infection of the central nervous system (neurologic phase). With Gambian trypanosomiasis, the blood lymphatic stage (stage I) may last for years before the sleeping sickness syndrome occurs (CNS involvement, meningoencephalitis stage, stage II). When symptoms occur in a patient infected with Gambian trypanosomiasis, the patient is already in the advanced stages of disease, with CNS involvement.

Laboratory findings include anemia, granulocytopenia, increased sedimentation rate, and marked increases in serum IgM. The sustained high IgM levels are a result of the parasite producing variable antigen types to evade the patient's defense system (112). In an immunocompetent host, the lack of elevated serum IgM rules out African trypanosomiasis. A diagnostic differential may include brucellosis, HIV infection, leishmaniasis, malaria, relapsing fever, tuberculosis, and typhoid fever.

Upon trypomastigote invasion of the CNS, the sleeping sickness stage of the infection is initiated (stage II). Gambian trypanosomiasis is characterized by steady, progressive meningoencephalitis, behavioral changes, apathy, confusion, coordination loss, and somnolence. *T. brucei rhodesiense* produces a more rapid, fulminating disease, and death may occur before there is extensive CNS involvement. In the terminal phase of the disease, the patient becomes emaciated, leading to profound coma and death, usually from secondary infections. Cerebrospinal fluid (CSF) findings include increased protein and IgM levels, lymphocytosis, and morular cells of Mott. Morular (mulberry) cells are altered plasma cells whose cytoplasm is filled with proteinaceous droplets. Morular cells are not seen in all patients; however, they are characteristic of African trypanosomiasis. World Health Organization criteria for CNS involvement, besides findings of trypomastigotes in the CSF, include a white blood cell count of greater than 5 cells and increased protein levels in the CNS fluid. The diagnostic differential may include cryptococcosis, HIV, meningitis, Parkinson's disease, psychiatric disorders, viral encephalitis, and space-occupying lesions.

Diagnosis

Because most of the infections occur in rural areas, sophisticated diagnostic techniques are not readily available and diagnosis is dependent on simple direct detection methods. Definitive diagnosis depends upon demonstration of trypomastigotes in blood, lymph node aspirate, sternum bone marrow, chancre fluid, and CSF. Trypomastigotes can be more readily detected in body fluids in infections due to *T. brucei rhodesiense* than in those due to *T. brucei gambiense* because of substantially higher parasitemias. Due to periodicity, parasite numbers in the blood vary, and a number of techniques must be used to detect the trypomastigotes. Laboratorians may want to contact their state public health laboratory or the CDC for diagnostic information and help with specimen selection and available tests.

Collection of Specimens

Trypomastigotes are highly infectious, and health care personnel must be cautious and adhere to blood-borne-pathogen guidelines using universal precautions when handling blood, CSF, or aspirates. Microscopic specimens should be examined as rapidly as possible due to potential lysis of trypomastigotes. Serial exams of fluids may be necessary to detect trypomastigotes especially with Gambian trypanosomiasis. Blood can be collected from either finger stick or venipuncture. Venous blood should be collected in a tube containing EDTA. Multiple blood exams should be performed before trypanosomiasis is ruled out. Parasites are found in high numbers in the blood during the febrile period and in low numbers in the afebrile periods. If CSF is examined, a volume greater than 1 ml, preferably 5 ml or more, should be collected. In cases in which trypomastigotes are in undetectable numbers in the blood, they may be seen in aspirates of inflamed lymph nodes; however, attempts to demonstrate them in tissue are not practical. Blood and CSF specimens should be examined every 6 months during therapy to evaluate the clinical response and up to 2 years after therapy.

Direct Examination

Microscopic Detection

In addition to taking thin and thick blood films, determining the buffy coat concentration is recommended to detect the parasites. Parasites can be detected (i) on thick blood smears when numbers are greater than 2,000/ml, (ii) by determining the hematocrit concentration in a capillary tube or by quantifying buffy coats (QBC) when numbers are greater than 100/ml, and (iii) by anion-exchange chromatography when numbers are greater than 4/ml (114). Unfortunately, anion exchange is not easily adapted to clinical laboratories or field studies (115). In suspected and confirmed cases of trypanosomiasis, a lumbar puncture is mandatory to rule out CNS involvement (stage II). CSF examination must be conducted by using centrifuged sediments (116). The CSF should be examined immediately because the trypomastigotes begin to autolyze within 10 min. Detection of trypomastigotes in the CSF allows immediate classification of stage II illness (CNS involvement).

PCR Detection

Referral laboratories have used molecular methods (PCR, not FDA approved) to detect infections and differentiate species, but these methods are not routinely used in the field (21, 48, 115, 117). The PCR-based methods have not been standardized, and multicenter institutional studies to validate these tests have not been done. There have been few studies where the various PCR methods used for diagnostic purposes have been compared.

Culture and Animal Inoculation

Small laboratory animals (rats and guinea pigs) have been used to detect infections. *T. brucei rhodesiense* is more adaptable to cultivation (Tobie's medium [48]) and animal infection than *T. brucei gambiense*; however, cultivation is not practical for most diagnostic laboratories.

Serologic Tests

Serologic techniques (not FDA approved) that have been used for epidemiologic screening include IFA, ELISA, indirect hemagglutination assay, the card agglutination trypanosomiasis test (CATT), and LATEX/*T. b. gambiense*. Serologic tests are normally used for screening, with the definitive diagnosis of infection dependent on microscopic observation of trypomastigotes. The CATT is effective in screening the population for suspected cases of *T. brucei gambiense* but not *T. brucei rhodesiense*. Major serodiagnostic problems with the CATT include false-positive results due to malarial infections and the fact that many in the population have elevated antibody levels due to exposure to animal trypanosomes that are noninfectious to humans. The CATT does not differentiate between current and past infections (115, 118, 119). Both the CATT and LATEX/*T. b. gambiense* have good negative predictive values. Markedly elevated serum and CSF IgM concentrations are of diagnostic value. CSF antibody titers should be interpreted with caution because of the lack of reference values and the possibility of CSF containing serum due to a traumatic tap. Intrathecal production of immunoglobulins can be found in a number of neuroinflammatory diseases. LATEX/IgM has been developed for field use to measure CSF concentrations of IgM (115).

Antibodies to galactocerebrosides and neurofilaments as well as elevated concentrations of interleukin-10 and CSF protein levels may be markers for CNS involvement; all of these have been used to monitor CNS infections (115, 120). ELISA has been used to detect antigen in serum and CSF. These biomarker tests (antigen detection) are not widely used due to the limited sensitivity of the test when there are limited numbers of trypomastigotes in the blood or CSF (121, 122). This method can also be used for clinical staging of the disease to determine whether there was CNS infection and as a follow-up to therapy.

Treatment and Prevention

All patients determined to have active infections should be treated. The drugs used and the course of treatment are dependent on the trypanosomal species and the clinical staging of the disease (123). Suramin (Bayer 205; Naphuride or Antrypol) is the drug of choice for treating the early blood or lymphatic stage of *T. brucei rhodesiense* infections, whereas pentamidine isethionate (Lomidine) is the drug of choice for treating the early stages of *T. brucei gambiense* infections (111, 124, 125). Melarsoprol (mel B or Arsobal) is the drug of choice when CNS involvement is suspected with *T. brucei rhodesiense* infections (111, 125). This drug may be given with a corticosteroid to reduce possible encephalopathy.

Difluoromethyornithine (DFMO; eflornithine or Ornidyl) is a cytostatic drug effective against the acute and late stages of *T. brucei gambiense* infections (123). It can be used alone or with oral nifurtimox. The effectiveness of therapy can be judged microscopically by the absence of trypomastigotes in the blood, lymph fluid, or CSF and by a decrease in CSF white blood cells (126). CSF antibodies (IgM) decrease, as do levels of interleukin-10, after successful therapy (127). Any individual treated for African trypanosomiasis should be monitored every 6 months for 2 years after completion of therapy (120).

Population-screening programs have been used to control *T. brucei gambiense* infections. The use of vector control measures has met with limited success (75). The most effective control measures include an integrated approach to reduce the human reservoir of infection and the use of insecticide and fly traps. Use of *N*,*N*-diethyl-meta-toluamide (DEET) and permethrin-impregnated clothing or another insect repellent has not been proved to be particularly effective against tsetse flies, but it prevents other insect bites. Tsetse flies are attracted to clothing with bright and dark colors. Persons visiting areas in which the infection is endemic should wear protective clothing (long-sleeved shirts and long trousers). Vaccines are not available.

OTHER TRYPANOSOMES INFECTING HUMANS

Trypanosoma congolense

Only one case of human infection with this parasite has been reported and confirmed by DNA identification (128). The patient had a mixed infection with *T. brucei* and was successfully treated with pentamidine.

Trypanosoma evansi

The first human case of *T. evansi* was diagnosed in India (3). This organism is normally considered a parasite of animals (buffalo, camels, cattle, horses, and rats) and has a very wide geographic distribution (Africa, Asia, and Central and South America). The infection is transmitted mechanically by blood-sucking insects, such as stable flies or horseflies. In animals, the incubation period is 5 to 60 days, and the severity of the disease varies from no symptoms to weakness, weight loss, anemia, abortions, and death. In the above-mentioned human case, the patient complained of transient fevers and sensory disorders. Fever peaks were noted every 7 to 10 days, and large numbers of parasites were detected in the blood at the time of fevers. No parasites were observed in the CSF. The patient was treated with suramin. Normally, human serum has natural trypanolytic activity, but this patient was determined to have a mutation in apolipoprotein L-1 (APOL1), which has trypanolytic activity (129). Laboratory diagnosis is usually done by examination of blood and lymph node aspirates or biopsy specimens. *T. evansi* can be cultured in mice and rats (130).

T. evansi cannot be differentiated from *T. brucei gambiense*, *T. brucei rhodesiense*, or *T. rangeli* microscopically.

Trypanosoma lewisi

Human cases of *T. lewisi* infections have been described in India and Thailand in pediatric patients (130–132). Trypomastigotes were detected in the blood of these patients. In these cases, the patients fully recovered from the infection with no therapy. Symptoms included prolonged fever, thrombocytopenia hepatosplenomegaly, and elevated liver enzymes.

The kinetoplast is subterminal to the posterior end of the trypomastigote, and the nucleus is found at the anterior end, terminating where the flagellum is free of the trypomastigote body. *T. lewisi* is a natural infection of wild rats and is considered nonpathogenic. The intermediate host is the flea, in whose gut the parasite multiplies and gives rise to epimastigotes that are found in the rectum and feces. The infection is passed to susceptible rats by ingestion of fleas or their feces. Human infections are thought to be transmitted in a similar fashion.

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Toxoplasma

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Toxoplasmosis is caused by infection with the parasite *Toxoplasma gondii*. It is one of the most common parasitic infections in humans and is most typically asymptomatic. However, in select clinical situations it can cause severe and disabling disease, making accurate and timely diagnosis vital.

TAXONOMY

Toxoplasma gondii is included in the phylum Apicomplexa, class Coccidea, subclass Coccidiasina, order Eimeriida, suborder Eimeriorina, family Sarcocystidae (*Sarcocystis*), subfamily Toxoplasmatinae, and genus *Toxoplasma*.

LIFE CYCLE

Toxoplasma gondii is a protozoan parasite that infects most species of warm-blooded animals, including humans. Members of the cat family Felidae are the only known definitive hosts for the sexual stages of *T. gondii* and thus are the main reservoirs of infection. The three stages of this obligate intracellular parasite are (i) tachyzoites (trophozoites), which rapidly proliferate and destroy infected cells during acute infection; (ii) bradyzoites, which slowly multiply in tissue cysts; and (iii) sporozoites in oocysts (Fig. 1). Tachyzoites and bradyzoites occur in body tissues; oocysts are excreted in cat feces (Fig. 2). Cats become infected with *T. gondii* by carnivorism or by ingestion of oocysts. Cats that are allowed to roam outside are much more likely to become infected than domestic cats that are confined indoors. After tissue cysts or oocysts are ingested by the cat, viable organisms are released and invade epithelial cells of the small intestine, where they undergo an asexual cycle followed by a sexual cycle and then form oocysts, which are then excreted. The unsporulated (i.e., noninfective) oocyst takes 1 to 5 days after excretion to become sporulated (infective). Although cats shed oocysts for only 1 to 2 weeks, large numbers may be shed, often exceeding 100,000 per g of feces. Oocysts can survive in the environment for several months to more than a year and are remarkably resistant to disinfectants, freezing, and drying but are killed by heating to 70°C for 10 min (1).

EPIDEMIOLOGY AND TRANSMISSION

Serologic prevalence data indicate that toxoplasmosis is one of the most common infections of humans throughout the world (2). Because *T. gondii* organisms are rarely detected in humans with toxoplasmosis, serologic examination is used to indicate the presence of the infection by detecting *Toxoplasma*-specific antibodies. The prevalence of positive serologic titers increases with age. In many areas of the world, infection is more common in warm climates and at lower altitudes than in cold climates and mountainous regions. This distribution is probably related to conditions favoring the sporulation and survival of oocysts. Variations in prevalences of infection between geographic areas and between population groups within the same locale are also probably due to differences in exposure. A high prevalence of infection in France (50 to 85%) has been related to a preference for eating raw or undercooked meat. However, a high prevalence in Central America has been related to the frequency of stray cats in a climate favoring the survival of oocysts. In U.S. military recruits in 1962, seroprevalence rates of up to 30% were found in people living along the sea coast, with rates of less than 1% in the Rocky Mountains and the desert Southwest. More-recent data comparing antibody prevalences in U.S. military recruits in 1962 and 1989 indicated a one-third decrease in seropositivity (3). The overall seroprevalence in the United States as determined with specimens collected by the Third National Health and Nutritional Assessment Survey (NHANES III) between 1988 and 1994 among persons 12 or more years of age was found to be 22.5%, with seroprevalence among women of childbearing age (15 to 44 years) of 15% (4). More recently, in analysis of NHANES data, *T. gondii* seroprevalence was shown to have declined in U.S.-born persons 12 to 49 years old, from 14.1% in 1988 to 1994 to 9.0% in 1999 to 2004 (5).

Human infection may be acquired in several ways: (i) ingestion of undercooked contaminated meat containing *T. gondii* cysts; (ii) ingestion of oocysts from hands, food, soil, or water contaminated with cat feces; (iii) organ transplantation or blood transfusion; (iv) transplacental transmission; and (v) accidental inoculation of tachyzoites. The two major routes of transmission of *Toxoplasma* to humans are oral and congenital. In humans, ingesting either the tissue cyst or the oocyst results in the rupture of the cyst wall (6), which releases organisms that invade the intestinal epithelium, disseminate throughout the body, and multiply intracellularly. The host cell dies and releases tachyzoites, which invade adjacent cells and continue the process. The tachyzoites are pressured by the host's immune response to transform into bradyzoites and form tissue cysts, most commonly in skeletal muscle, myocar-

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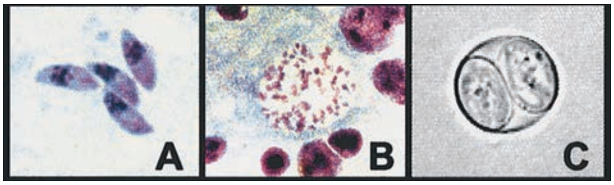

FIGURE 1 Three life stages of *T. gondii*. (A) Tachyzoites (Giemsa stain); (B) cyst with bradyzoites in brain tissue (Giemsa stain); (C) sporulated oocysts, unstained. (Photographs courtesy of J. P. Dubey, U.S. Department of Agriculture, Beltsville, MD). doi:10.1128/9781555817381.ch138.f1

dium, and brain; these cysts may remain throughout the life of the host. Recrudescence of clinical disease may occur if the host becomes immunosuppressed and the cysts rupture, releasing the parasites.

Recently, researchers have begun to appreciate that differences in transmission, reactivation, and disease severity may also be explained by the different genotypes of *T. gondii* that occur in different parts of the world (7).

Prevention

Risk factors for *T. gondii* infection identified in epidemiologic studies include eating raw or undercooked pork, mutton, lamb, beef, ground meat products, oysters, clams, mussels, and wild-game meat, kitten ownership, cleaning a cat litter box, contact with soil (gardening and yard work), and eating raw or unwashed vegetables or fruits (1, 4, 8–10). Recommendations for prevention of toxoplasmosis for all persons, including pregnant women, were originally discussed at a conference at the Centers for Disease Control and Prevention and published (1), and now have been updated (see the Division of Parasitic Diseases [DPDM] Web page http://www.cdc.gov/parasites/toxoplasmosis/prevent.html). These recommendations included the following: (i) food should be cooked to safe temperatures (beef, lamb, and veal roasts and steaks to at least 145°F, with a 3-min rest; pork, ground meat, and wild game to 160°F; and poultry to at least 165°F, with a 3-min rest); (ii) fruits and vegetables should be peeled or washed thoroughly before being eaten; (iii) cutting boards, dishes, counters, utensils, and hands should always be washed with hot soapy water after they have come into contact with raw meat, poultry, seafood, or unwashed fruits or vegetables; (iv) individuals should wear gloves when gardening and during any contact with soil or sand, because cat waste might be in soil or sand, and wash hands afterwards; and (v) pregnant women should avoid changing cat litter, if possible. Pregnant women who must change cat litter should use gloves and then wash their hands thoroughly. The litter box should

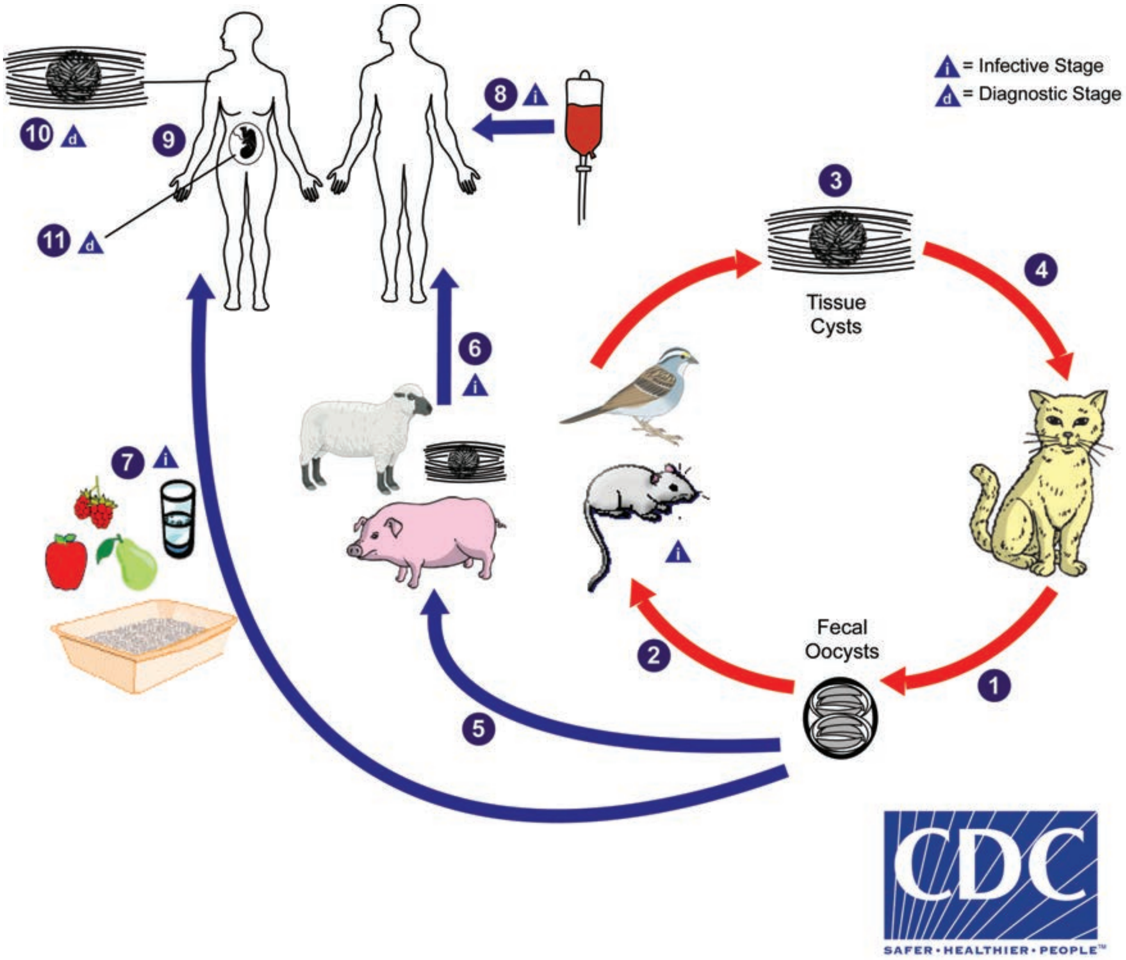

FIGURE 2 Life cycle of *Toxoplasma gondii*. doi:10.1128/9781555817381.ch138.f2

be changed daily because *T. gondii* oocysts require more than 1 day to become infectious. Pregnant women should be encouraged to keep their cats inside and not adopt or handle stray cats. Cats should be fed only canned or dried commercial food or well-cooked table food, not raw or undercooked meats. Several outbreaks have been reported in association with drinking untreated water contaminated by oocysts. Freezing meats for several days at subzero temperatures (<0°F) greatly reduces the risk of infection.

CLINICAL SIGNIFICANCE

Toxoplasmosis can be categorized into four groups: (i) acquired by an immunocompetent patient, (ii) acquired or reactivated by an immunodeficient patient, (iii) congenital, and (iv) ocular. Methods of diagnosis and their interpretations may differ for each clinical category.

Toxoplasma infection acquired by immunocompetent individuals is generally an asymptomatic infection. However, 10 to 20% of patients with acute infection may develop cervical lymphadenopathy and/or a flu-like illness. The clinical course is benign and self-limited; symptoms usually resolve within weeks to months.

Immunodeficient patients often have central nervous system (CNS) disease but may have myocarditis or pneumonitis. In patients with AIDS, toxoplasmic encephalitis is the most common cause of intracerebral mass lesions and is thought to be due to reactivation of chronic infection. Toxoplasmosis in patients being treated with immunosuppressive drugs may be due to either newly acquired or reactivated latent infection (11, 12).

Congenital toxoplasmosis results from an acute primary infection acquired by the mother during pregnancy. The incidence and severity of congenital toxoplasmosis vary with the trimester during which infection was acquired. Because treatment of the mother may reduce the severity of manifestations in the infant due to congenital infection, prompt and accurate diagnosis is extremely important. Many infants with subclinical infection at birth will subsequently develop signs or symptoms of congenital toxoplasmosis; however, prompt treatment may help prevent subsequent symptoms.

Ocular toxoplasmosis, an important cause of chorioretinitis in the United States, may be the result of congenital or acquired infection (13). Acquired infection is now thought to be more common than congenital infection. Congenitally infected patients can be asymptomatic until the second or third decade of life, when lesions develop in the eye, presumably due to cyst rupture and subsequent release of tachyzoites and bradyzoites. Chorioretinitis is characteristically bilateral in patients with congenital infection but is often unilateral in individuals with acute acquired *T. gondii* infection.

Recent data have suggested an association between *T. gondii* infection and various neurologic or psychiatric syndromes, including schizophrenia, Alzheimer disease, and even suicide (14–16). These findings are intriguing but require further study to validate.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Serum, plasma, cerebrospinal fluid (CSF), ocular fluid, and amniotic fluid may be tested for antibodies and/or parasite DNA (2).

Collection for Determination of Parasite DNA

Blood samples should be collected with an anticoagulant; CSF, ocular fluid, and amniotic fluids do not need an anticoagulant. All samples should be shipped and stored at 4°C prior to testing.

Collection for Antibody Determination

Blood specimens to be tested for the presence of antibodies should be allowed to clot and be centrifuged, and the serum should be removed and shipped to a reference laboratory. Hemolysis does not seem to interfere with the antibody reaction in most tests. Serum and CSF specimens may be stored for several days at 4°C or frozen for longer storage. Specimens may be shipped at ambient temperature unless they will be in transit for more than 1 week or will be subjected to temperatures above 30°C. To avoid evaporation of small volumes, ocular fluids should be stored and shipped frozen. CSF and ocular fluid should be tested in parallel with a serum sample drawn on the same date. Long-term storage should take place at −20°C or below.

If the determination of immune status is the reason for testing, a single serum specimen is satisfactory; acute- and convalescent-phase specimens are not necessary. In situations in which determining the time of infection is important, specimens drawn at least 3 weeks apart may or may not be useful. In most cases, detection of an increasing immunoglobulin G (IgG) or IgM titer is not possible because the titers have already reached a plateau by the time the initial sample is drawn. If two specimens are to be compared, they should be tested in parallel. Results from tests done at different times, in different laboratories, or with different procedures should not be compared quantitatively, only qualitatively as positive or negative.

For tests other than serology, contact a reference laboratory for instructions before collecting specimens to ensure proper collection and handling.

DIRECT EXAMINATION

Microscopy

Only very rarely can the diagnosis of toxoplasmosis be documented by the observation of parasites in patient specimens (17, 18). Secretions, excretions, body fluids, and tissues are potential specimens for direct observation of parasites but are generally unrewarding. Fluid specimens, such as heparinized blood or CSF, should be centrifuged, and the sediment should be smeared on a microscope slide. The slides should be air dried, fixed in methanol, and stained with Giemsa stain for microscopic examination. Tachyzoites may be observed as free organisms or within host cells, such as leukocytes. Well-preserved tachyzoites are crescent shaped and stain well, but degenerating organisms may be oval and stain poorly. Tissue imprints stained with Giemsa stain may reveal *T. gondii* cysts.

Antigen Detection

Immunologic techniques have been used to identify parasites in tissue sections or tissue cultures; fluorescein isothiocyanate- or peroxidase-labeled antisera may be useful in detecting tachyzoites in tissue sections. Enzyme immunoassay (EIA) antigen detection techniques lack sensitivity for human samples and are not recommended.

Nucleic Acid Detection Techniques

PCR technology for *Toxoplasma* has been used to detect congenital infections, toxoplasmic encephalitis in AIDS patients, and ocular disease, with various degrees of success (19–26). The most important use of PCR is for prenatal diagnosis of congenital toxoplasmosis using amniotic fluid. When maternal serological results indicate potential infection during pregnancy, PCR of amniotic fluid has been shown to be more sensitive for the confirmation of fetal infection than the conventional methods of inoculation of mice and tissue culture cells and fetal blood testing for IgM (27, 28). PCR technology for *Toxoplasma* is offered at the Toxoplasma Serology Laboratory, Palo Alto, CA, and by a few commercial laboratories. Commercial kits are not yet available. The most common PCR targets include the 18S ribosomal DNA, B1, and AF146257 genes. The requestor should be aware that the levels of reliability of PCR tests may vary widely (19, 29–32). PCR testing by low-density microarrays has been used successfully in the evaluation of granulomatous lymphadenitis (33). Recently, loop-mediated isothermal amplification has been used to detect *Toxoplasma* DNA in blood (34).

Cell-Mediated Immune Responses

T-cell response has been studied in newborns with congenital toxoplasmosis, and a recent study has suggested that measurement of interferon gamma production by T cells in response to stimulation by specific *Toxoplasma* antigens may add to the diagnosis of congenital infection (35). The interferon gamma release assay has been used successfully to detect cell-mediated responses in congenitally infected infants (36).

ISOLATION PROCEDURES

Parasites can be isolated with limited success by inoculating patient tissue or body fluids into either mice or tissue culture cells (37). Fresh tissue samples are ground in saline with a mortar and pestle and inoculated intraperitoneally into mice or directly into tissue culture flasks. The mice should be monitored for 4 to 6 weeks; if the organism is virulent for mice, the parasites can often be demonstrated in the peritoneal fluid after 5 to 10 days. However, if the organism is relatively avirulent for mice, as is usually the case, the mice may not be killed by the infection. If they survive for 6 weeks, serum samples should be obtained for serologic testing. If antibodies are present, the mouse brains should be examined for the presence of *T. gondii* cysts. If cysts are not observed, the murine host may not have been the ideal host. Inoculate additional mice with brain homogenate from the initially inoculated mice and observe and recheck after 6 weeks. *T. gondii* grows in a variety of tissue culture cells. A cytopathic effect may be detected on direct examination after 24 to 96 h in culture. Giemsa staining may reveal parasite structure, but parasitized cells may be difficult to detect. Immunofluorescence allows more-sensitive detection of the organisms. The following procedure has been used with some success for parasite isolation from amniotic fluids (2, 38). Centrifuge a 10-ml sample of amniotic fluid at 1,000 × *g*. Resuspend the sediment in 8 ml of minimum essential medium. Inoculate 1 ml into coverslip cultures of the human embryonic fibroblast cell line MRC5 in 24-well plates. Incubate the cultures for 96 h with one change of medium at 24 h; fix the cultures with cold acetone. Examine the coverslips by indirect immunofluorescence for the presence of *T. gondii*. The use of tissue culture cells for isolation permits a more rapid diagnosis than mouse inoculation; both methods can be useful for diagnosing congenital toxoplasmosis.

SEROLOGIC TESTS

Serologic testing for *T. gondii*-specific antibodies is the most commonly used method for diagnosis of toxoplasmosis. Many tests for the detection of antibodies to *Toxoplasma* have been used since Sabin and Feldman developed the methylene blue dye test (DT) (2, 32, 39). Commercial kits for agglutination tests, indirect fluorescent-antibody (IFA) tests, and EIAs are available worldwide. Because of difficulties in obtaining specimens from patients with clinically documented toxoplasmosis, commercial kit sensitivity and specificity may not be based on documented case specimens but rather on a comparison of results obtained with another kit. Consequently, the true sensitivity and specificity of a kit are generally not known or determined. The rates stated by the manufacturer or published in articles may vary depending upon the samples chosen for testing. Sensitivity and specificity rates determined in prospective studies, when random samples are tested as received for *Toxoplasma* testing, will usually differ from those determined in retrospective studies, when the samples have been chosen as potential problem samples to increase the probability of detecting false-positive or false-negative reactions.

When laboratory personnel decide to initiate *Toxoplasma*-specific antibody testing or when they decide to switch to a different antibody detection kit, the user must carefully review the manufacturer's package insert and published literature for information on the sensitivity and specificity rates. The user should perform an in-laboratory comparison of kits by using positive and negative samples confirmed by a toxoplasmosis reference laboratory. Tables 1 and 2 list commercial kits available in the United States and references to published evaluations. However, the test kit industry is in a great deal of flux; company and kit names may change.

In the United States, initial testing for the presence of IgG antibodies in most laboratories is usually performed with an EIA or IFA commercial kit. Results may be stated in international units (based on the WHO international standard reference serum for *Toxoplasma* [32, 40] distributed by Public Health Wales, Singleton Hospital, Swansea, Wales, United Kingdom) as an index (specific to each kit), as an optical density value (specific to each kit), or as a geometric mean titer. Numerical results are not comparable from kit to kit; comparison may be made only qualitatively as negative (nonreactive or not infected) or positive (reactive or infected). Although elevated *Toxoplasma*-specific IgG levels have been suggested as an indicator of recent infection, high levels may last for many years after primary infection and should not be relied upon for this purpose.

To more definitely distinguish acute and chronic infections, detection of *Toxoplasma*-specific IgM antibodies has been used. The most important use of IgM test results is that a negative reaction essentially excludes recent infection. A guide to the general interpretation of *Toxoplasma* IgG and IgM serology results is presented in Table 3. IFA test IgM titers generally increase within 1 week of the onset of symptoms and revert to negative within 6 to 9 months of infection. False-positive reactions caused by rheumatoid factor and false-negative reactions caused by blockage by *Toxoplasma*-specific IgG may occur in IFA IgM tests and indirect EIAs for IgM when whole serum samples are tested. To decrease the effects of these interfering factors, specimens should be treated to obtain only the IgM fraction for testing.

The IgM capture EIA eliminates potential interference by IgG and other isotypes by binding only IgM antibodies; unbound antibodies are removed by washing the plate, which eliminates the need for serum fractionation. The most important advantage of the IgM capture EIA compared to those of the IFA test is the increased detection of congenital infections: the IgM enzyme-linked immunosorbent assay

TABLE 1 *Toxoplasma* IgG kits available commercially in the United States

Type of test and company[a]	Kit	Reference(s)
IFA		
GenBio	ImmunoFA *Toxoplasma* IgG	
Hemagen	Virgo Toxo IgG	
Inverness	*Toxoplasma* IgG	
Meridian	*Toxoplasma* IgG	
EIA		
Abbott Laboratories	IMx Toxo IgG	100–105
	AxSYM Toxo IgG	106–108
Bayer Diagnostics	Immuno 1	105
	Advia Centaur	109
Beckman Coulter	Access Toxo G	100
bioMérieux Vitek	Vidas Toxo IgG	101, 103, 107, 108, 110
Bio-Rad	Platelia Toxo G	103
Biotecx	OptiCoat Toxo IgG ELISA	
BioTest Diagnostics	Toxo IgG	
Diagnostic Products Corp.	Immulite *Toxoplasma* IgG	106, 110, 111
Diamedix	*Toxoplasma* IgG	
DiaSorin	*Toxoplasma* IgG	
	Liaison	63, 112
GenBio	ImmunoDOT Torch	
Hemagen	*Toxoplasma* IgG	
Inverness	Toxo IgG II	
Roche Diagnostics	Elecsys Toxo IgG	
Trinity Biotech	Captia *Toxoplasma gondii* IgG	
Latex		
Biokit	Toxogen	

[a] Abbott Laboratories, Diagnostics Division, North Chicago, IL 60064; Bayer Diagnostics, 511 Benedict Ave., Tarrytown, NY 10591; Beckman Coulter, 4300 N. Harbor Blvd., Fullerton, CA 92834; Biokit USA, 113 Hartwell Avenue, Lexington, MA 02173; bioMérieux, 595 Anglum Dr., Hazelwood, MO 63042; Bio-Rad, 4000 Alfred Nobel Dr., Hercules, CA 94547; Biotecx Laboratories, 6023 S. Loop East, Houston, TX 77033; BioTest Diagnostics Corp., 66 Ford Rd., Suite 131, Denville, NJ 07834; Diagnostic Products Corp., 5700 W. 96th St., Los Angeles, CA 90045; Diamedix Corp., 2140 N. Miami Ave., Miami, FL 33127; DiaSorin, P.O. Box 285, Stillwater, MN 55082; GenBio, 15222 Avenue of Science, San Diego, CA 92128; Hemagen Diagnostics, 34–40 Bear Hill Rd., Waltham, MA 02154; Inverness Medical Professional Diagnostics, 2 Research Way, Princeton, NJ 08540; Meridian Bioscience, 3471 River Hills Dr., Cincinnati, OH 45244; Roche Diagnostics Corp., 9115 Hague Road, Indianapolis, IN 46250.

(ELISA) was positive for 73% of serum samples from newborn infants with proven congenital toxoplasmosis, whereas only 25% of the same serum samples were found positive by an IFA IgM test (41). Although the capture EIA system is more efficient at detecting acute infections, some persons may have undetectable or low-level IgM antibodies, and some persons may have detectable IgM antibodies beyond 2 years postinfection (2, 42). Therefore, determining the relative time of infection is not possible with this system alone. Many commercial companies market an EIA kit for IgM; some use the indirect EIA format with a serum pretreatment step, while others use the IgM capture format. False-positive IgM reactions due to unknown factors may be a problem with commercially available kits (43).

The *Toxoplasma* IgG avidity test is an additional tool to help discriminate between past and recently acquired infections (2, 24, 32, 35, 44–47). The test is based on the observation that during acute infection, IgG antibodies bind antigen weakly or have low avidity, while during chronic infection, antibodies bind antigen more strongly (high avidity). Depending on the method used, a high-avidity result indicates infection acquired more than 3 to 5 months before. However, a low-avidity result does not indicate a recently acquired infection because low-avidity antibodies may be detectable for a year postinfection. The Vidas Toxo Avidity assay by bioMérieux is the only kit approved by the Food and Drug Administration (FDA). Multiple commercial kits are available from companies outside the United States: Ani Labsystems (48), bioMérieux (23, 49), and DiaSorin (50).

Other tests may be of assistance in determining current infection. Assays for *Toxoplasma*-specific IgA antibodies should always be performed in addition to IgM assays for newborns suspected of having congenital infection (51–53). Results of IgA testing in adults have been less consistent (54). The presence of *Toxoplasma*-specific IgE antibodies may also contribute to the determination of acute infections, although reports of the utility of IgE antibody detection have been mixed (55, 56). Immunoblot assays may be useful in determining congenital infections (57, 58) and ocular infections (59). These and other assays (30, 32, 60) are available in the United States at the Toxoplasma Serology Laboratory, Palo Alto Medical Foundation, Palo Alto, CA (http://www.pamf.org/serology/; phone, 650-853-4828) and at many of the Toxoplasma Reference Laboratories in Europe (for example, the National Reference Centre for Toxoplasmosis, Maison Blanche Hospital, University Reims Champagne, Ardenne, France).

A guideline for the clinical use and interpretation of serologic tests for *Toxoplasma gondii* was published (61) by the Clinical and Laboratory Standards Institute (formerly called the National Committee for Clinical Laboratory

TABLE 2 *Toxoplasma* IgM kits available commercially in the United States

Type of test and company[a]	Kit	Reference(s)
IFA		
GenBio	ImmunoFA *Toxoplasma* IgM	
Hemagen	Virgo Toxo IgM	
Inverness	*Toxoplasma gondii* IgM	
Meridian	*Toxoplasma* IgM	
EIA		
Abbott Laboratories	IMx Toxo IgM	103, 104, 113, 114
	AxSYM Toxo IgM	100, 107
Bayer Diagnostics	Immuno 1	
	Advia Centaur	109
Beckman Coulter	Access Toxo M	100, 113, 115, 116
bioMérieux Vitek	Vidas Toxo IgM	100, 103, 107, 117
Bio-Rad	Platelia Toxo IgM	103, 113, 118, 119
Biotecx	OptiCoat Toxo IgM	
BioTest Diagnostics	Toxo IgM	110, 111
Diagnostic Products Corp.	Immulite	
Diamedix	*Toxoplasma* IgM	112
DiaSorin	*Toxoplasma* IgM	
	Liaison	
Hemagen	*Toxoplasma* IgM	
Trinity Biotech	Captia *Toxoplasma gondii* IgM	
Inverness	Toxo IgM	

[a] Abbott Laboratories, Diagnostics Division, North Chicago, IL 60064; Bayer Diagnostics, 511 Benedict Ave., Tarrytown, NY 10591; Beckman Coulter, 4300 N. Harbor Blvd., Fullerton, CA 92834; Biokit USA, 113 Hartwell Ave., Lexington, MA 02173; bioMérieux, 595 Anglum Dr., Hazelwood, MO 63042; Bio-Rad, 4000 Alfred Nobel Dr., Hercules, CA 94547; Biotecx Laboratories, 6023 S. Loop East, Houston, TX 77033; BioTest Diagnostics Corp., 66 Ford Rd., Suite 131, Denville, NJ 07834; Diagnostic Products Corp., 5700 W. 96th St., Los Angeles, CA 90045; Diamedix Corp., 2140 N. Miami Ave., Miami, FL 33127; DiaSorin, P.O. Box 285, Stillwater, MN 55082; GenBio, 15222 Avenue of Science, San Diego, CA 92128; Hemagen Diagnostics, 34–40 Bear Hill Rd., Waltham, MA 02154; Inverness Medical Professional Diagnostics, 2 Research Way, Princeton, NJ 08540; Meridian Bioscience, 3471 River Hills Dr., Cincinnati, OH 45244.

Standards or NCCLS) and is available for purchase on their website at www.clsi.org.

CLINICAL USE OF IMMUNODIAGNOSTIC TESTS

There are four groups of patients for whom diagnosis of toxoplasmosis is critical: pregnant women with infection during gestation, congenitally infected newborns, patients with chorioretinitis, and immunocompromised individuals.

Determination of Immune Status

An algorithm for serological testing for immune status and acute acquired infection is shown in Fig. 3. Three times at which baseline information about an individual's immune status would be useful include the following: (i) before con-

TABLE 3 Guide to general interpretation of *Toxoplasma* serology results obtained with IgG and IgM commercial assays

IgG result	IgM result	Report/interpretation for humans (except infants)
Negative	Negative	No serological evidence of infection with *Toxoplasma*
Negative	Equivocal	Possible early acute infection or false-positive IgM reaction. Obtain a new specimen for IgG and IgM testing. If results for the second specimen remain the same, the patient is probably not infected with *Toxoplasma*.
Negative	Positive	Possible acute infection or false-positive IgM result. Obtain a new specimen for IgG and IgM testing. If results for the second specimen remain the same, the IgM reaction is probably a false positive.
Equivocal	Negative	Indeterminate. Obtain a new specimen for testing or retest this specimen for IgG in a different assay.
Equivocal	Equivocal	Indeterminate. Obtain a new specimen for both IgG and IgM testing.
Equivocal	Positive	Possible acute infection with *Toxoplasma*. Obtain a new specimen for IgG and IgM testing. If results for the second specimen remain the same or if the IgG test becomes positive, both specimens should be sent to a reference laboratory with experience in the diagnosis of toxoplasmosis for further testing.
Positive	Negative	Infected with *Toxoplasma* for more than 1 yr
Positive	Equivocal	Infected with *Toxoplasma* for probably more than 1 yr or false-positive IgM reaction. Obtain a new specimen for IgM testing. If results with the second specimen remain the same, both specimens should be sent to a reference laboratory with experience in the diagnosis of toxoplasmosis for further testing.
Positive	Positive	Possible recent (within the last 12 mo) infection, or false-positive IgM reaction. Send the specimen to a reference laboratory with experience in the diagnosis of toxoplasmosis for further testing.

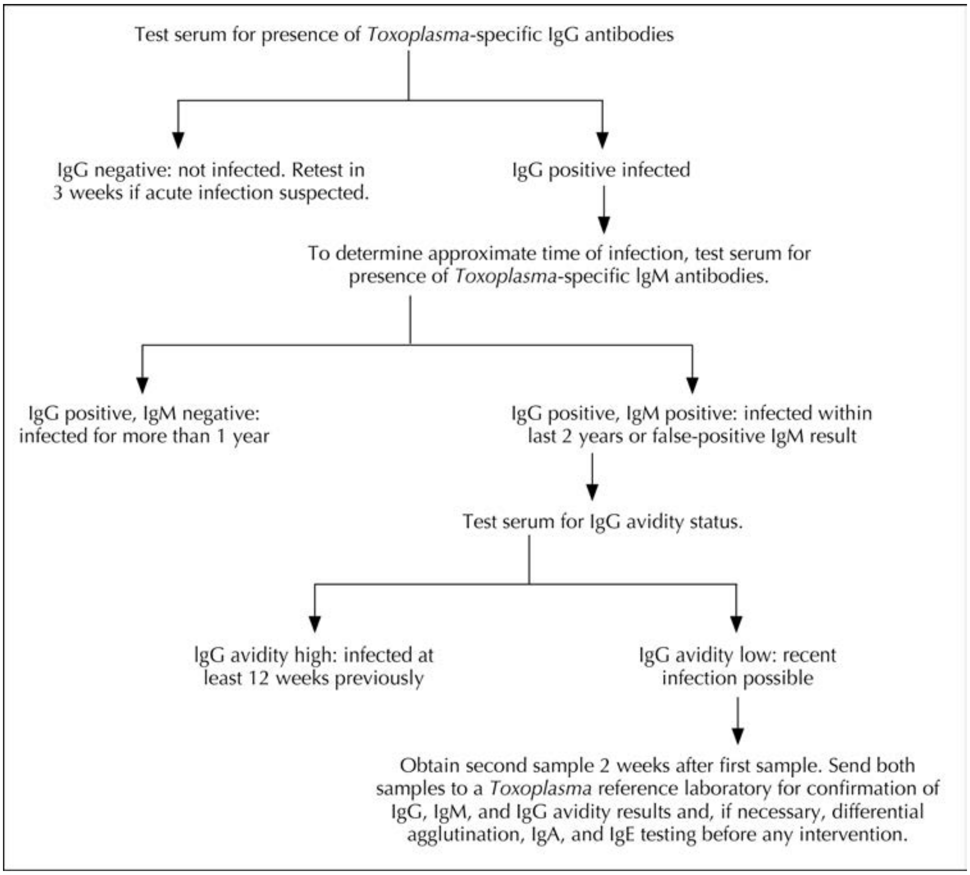
Test serum for presence of *Toxoplasma*-specific IgG antibodies
IgG negative: not infected. Retest in 3 weeks if acute infection suspected.
IgG positive infected
To determine approximate time of infection, test serum for presence of *Toxoplasma*-specific IgM antibodies.
IgG positive, IgM negative: infected for more than 1 year
IgG positive, IgM positive: infected within last 2 years or false-positive IgM result
Test serum for IgG avidity status.
IgG avidity high: infected at least 12 weeks previously
IgG avidity low: recent infection possible
Obtain second sample 2 weeks after first sample. Send both samples to a *Toxoplasma* reference laboratory for confirmation of IgG, IgM, and IgG avidity results and, if necessary, differential agglutination, IgA, and IgE testing before any intervention.

FIGURE 3 Algorithm for the serodiagnosis of toxoplasmosis in people greater than 1 year of age. doi:10.1128/9781555817381.ch138.f3

ception; (ii) before receiving immunosuppressive therapy; and (iii) after the initial determination of positive HIV-1 status. Screening one serum specimen with a sensitive test for IgG antibodies, such as the DT, the IFA test, or EIA, is sufficient. A negative test result indicates that the patient has not been infected. A positive result of any degree indicates infection with *T. gondii* at some undetermined time.

Diagnosis of Acute Acquired Infections

If an acute acquired infection is suspected, the patient's serum specimen should be tested for the presence of *Toxoplasma*-specific antibodies (Fig. 3). A negative result in the DT, IgG IFA test, or IgG EIA essentially excludes a diagnosis of acute *Toxoplasma* infection in an immunocompetent person. Very rarely, early after infection, a person may present with a positive IgM and a negative IgG result, which will soon turn positive. Demonstration of seroconversion from a negative titer to a positive titer or a >4-fold increase in titer confirms the diagnosis of recent infection when specimens drawn several weeks apart are tested in parallel with the same test. However, such situations are rare because specimens are usually drawn after titers have peaked, too late to observe titer changes after initial infection. The presence of typical lymphadenopathy suggestive of acute toxoplasmosis, the presence of a high DT or IgG IFA titer (≥300 IU/ml or ≥1:1,000), and the presence of specific IgM are indicative of acute infection (49, 62, 63). If the patient has clinical illness compatible with toxoplasmosis, but the IgG titer is low, a follow-up test 3 weeks later should show an increase in the antibody titer if the illness is due to acute toxoplasmosis and the host is not severely immunosuppressed.

Results of an EIA for IgM and an IgG avidity assay can provide additional evidence for or against acute infection when IgG antibodies are present (2, 32, 64). A negative IgM test essentially rules out infection in the previous 6 months. A positive IgM titer combined with a positive IgG titer may be suggestive of acute infection (due to persistent IgM antibodies) or may be a false-positive reaction.

Diagnosis during Pregnancy

Congenital toxoplasmosis occurs when a woman passes the infection to her fetus after acquiring a primary infection during pregnancy or, more rarely, when a pregnant woman is immunocompromised and a previously acquired infection is reactivated. Congenital toxoplasmosis can also occur, as more recently described, when secondary infection with a different genotype occurs (7, 39, 65). The rate of transmission of infection to the fetus ranges from 11% in the first trimester to 90% in the late third trimester, with an overall transmission rate of approximately 30 to 50%. In France and Austria, the prevention, diagnosis, and treatment of congenital toxoplasmosis begin with mandatory serologic testing of all women before or soon after conception. The cost-effectiveness of adopting this approach for all pregnant women in the United States is controversial, although modeling suggests that it may be cost-effective; this approach does serve as a model for managing individual pregnant patients (30, 66, 67).

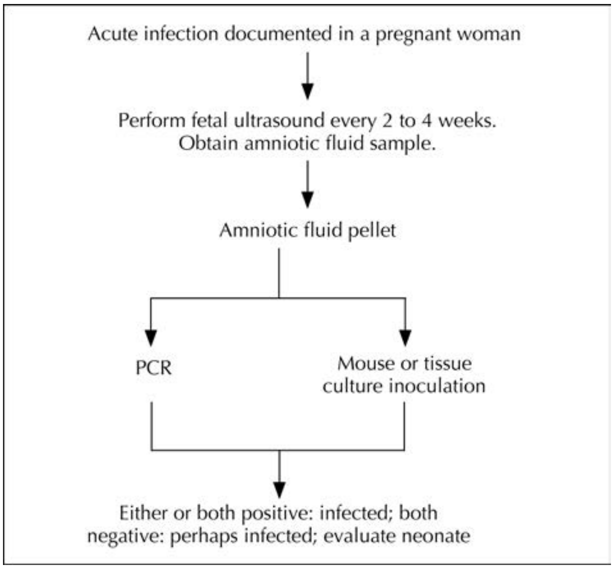
Acute infection documented in a pregnant woman
Perform fetal ultrasound every 2 to 4 weeks. Obtain amniotic fluid sample.
Amniotic fluid pellet
PCR
Mouse or tissue culture inoculation
Either or both positive: infected; both negative: perhaps infected; evaluate neonate

FIGURE 4 Algorithm for the diagnosis of antenatal congenital toxoplasmosis. doi:10.1128/9781555817381.ch138.f4

Immunocompetent women who have IgG antibody before conception are generally considered immune and so at very little risk for transmission of infection to the fetus. As noted above, newer research suggests that infection with a second genotype may occasionally lead to congenital transmission. Women who are seronegative are considered at risk for infection and in France are tested monthly during pregnancy for IgG antibody. If a woman is first tested after conception and has *Toxoplasma*-specific IgG antibodies, IgM and IgG avidity testing should be done to determine if acute infection has occurred during pregnancy (2, 39) (Fig. 3). A high-avidity result in the first 12 weeks of pregnancy essentially rules out an infection acquired during gestation. A low IgG avidity result cannot be used as an indicator of recent infection, because some individuals have persistent low IgG avidity for many months after infection. Immunodiagnosis of acute infection in a pregnant woman should be confirmed by a toxoplasmosis reference laboratory prior to intervention (30, 31, 35, 39).

When the diagnosis of acute toxoplasmosis has been made in a pregnant woman, she can be treated and the fetus can be tested for evidence of infection. The strategy used by Daffos et al. (68) involved initiating treatment with spiramycin once acute maternal infection was indicated and then obtaining amniotic fluid and fetal blood samples between 20 and 26 weeks of gestation for testing (Fig. 4). Amniotic fluid PCR (at 18 weeks of gestation or later) is the test of choice to establish the intrauterine diagnosis of congenital toxoplasmosis (44, 69–72). In addition, fetal ultrasound examinations were performed every 2 to 4 weeks until delivery to search for several nonspecific signs of infection: cerebral or hepatic calcifications, hydrocephalus, hepatomegaly, or ascites.

If collected, fetal blood should be tested for *Toxoplasma*-specific IgG, IgM, and IgA antibodies. Clotted blood should be inoculated into mice or tissue culture cells to demonstrate parasitemia. Nonspecific markers of infection should be evaluated; these include leukocytes, eosinophils, platelets, total IgM, gamma-glutamyltransferase, and lactate dehydrogenase (69). Most infected fetuses have one or more abnormal nonspecific tests, most commonly an elevated total IgM level or an elevated gamma-glutamyltransferase level (58, 67, 69, 73). Demonstrating *Toxoplasma*-specific IgM or IgA antibodies in fetal serum or isolating the parasite from fetal leukocytes is a definitive diagnosis of fetal infection.

Diagnosis in Newborns

Diagnosis of *Toxoplasma* infection in newborns is made through a combination of serologic testing, parasite isolation, and nonspecific findings (58, 69, 74–76). An attempt should be made to isolate *T. gondii* from the placenta, amniotic fluid, and cord blood if the diagnosis has not already been established (Fig. 5). *T. gondii* has been isolated from 95% of the placentas of congenitally infected newborns when the mother has not been treated and from approximately 81% when the mother has been treated. However, *T. gondii* can be isolated from the placentas of uninfected newborns as well. The child's serum should be tested for total IgG and IgM antibody levels and *Toxoplasma*-specific IgG, IgM, and IgA antibodies. CSF should be analyzed for cells, glucose, protein, total IgG antibody, and *Toxoplasma*-specific IgG and IgM antibodies and directly examined for *T. gondii* tachyzoites. A child suspected of having congenital toxoplasmosis should have a thorough general, neurologic, and ophthalmologic examination and a computed tomographic scan of the head (magnetic resonance imaging does not demonstrate calcifications). Because the diagnosis can take several months to confirm, clinicians may have to treat patients based upon early signs, symptoms, and serology while awaiting definitive confirmation. Although the complexity of diagnosing congenital infection necessitates the use of multiple costly laboratory tests, the benefit of early diagnosis and treatment and the cost of unnecessary treatment justify establishing the correct diagnosis.

Persistent or increasing IgG antibody levels in the infant compared with the mother as measured by the DT or IFA test and/or a positive result for *Toxoplasma*-specific IgM or IgA are diagnostic of congenital infection. Demonstration by IgG and IgM Western blotting of serum antibodies in the newborn that are directed against unique *Toxoplasma* epitopes not found in the mother's serum is also evidence of congenital infection (31, 32, 39, 46, 77).

Placental leak can occasionally lead to false-positive IgM or IgA measurements in newborns. Positive tests for these antibodies usually must be confirmed by repeat testing for IgM at 2 to 4 days of life and repeat testing for IgA at 10 days of life. Passively transferred maternal IgG has a half-life of approximately 1 month. Maternal antibodies can be detected for several months and have been reported at up

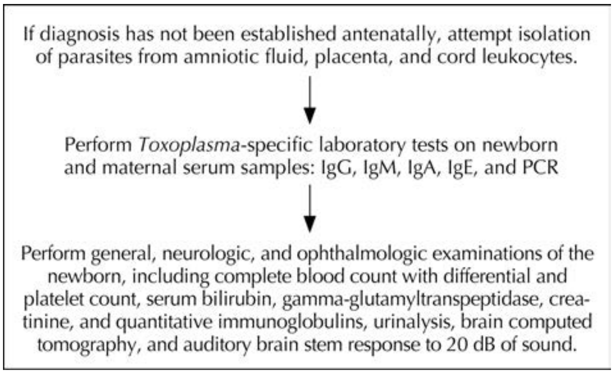
If diagnosis has not been established antenatally, attempt isolation of parasites from amniotic fluid, placenta, and cord leukocytes.
Perform *Toxoplasma*-specific laboratory tests on newborn and maternal serum samples: IgG, IgM, IgA, IgE, and PCR
Perform general, neurologic, and ophthalmologic examinations of the newborn, including complete blood count with differential and platelet count, serum bilirubin, gamma-glutamyltranspeptidase, creatinine, and quantitative immunoglobulins, urinalysis, brain computed tomography, and auditory brain stem response to 20 dB of sound.

FIGURE 5 Algorithm for the diagnosis of neonatal toxoplasmosis. doi:10.1128/9781555817381.ch138.f5

to 1 year of age. Untreated congenitally infected newborns will begin to produce *Toxoplasma*-specific IgG antibody within approximately 3 months. Treatment of an infected child may delay antibody production until 9 months of age and on rare occasion may prevent production altogether. The persistence of a positive IgG result at 12 months of life in the child confirms infection. Demonstration of a decrease in antibody load (*Toxoplasma*-specific IgG antibody divided by total IgG) can be helpful in differentiating maternal antibody from fetal antibody.

Although rarely performed, demonstration of IgM antibody or local *Toxoplasma*-specific IgG antibody production in CSF not contaminated with peripheral blood can help confirm the diagnosis of congenital toxoplasmosis. The calculation is made by dividing the *Toxoplasma*-specific antibody titer in the body fluid by the *Toxoplasma*-specific antibody titer in the serum and multiplying the result by the concentration of gamma globulin in serum divided by the concentration of gamma globulin in the body fluid. A result of 4 or greater corresponds to significant antibody production.

A long-term prospective study is under way in the United States to define optimal therapeutic regimens for the treatment of congenital toxoplasmosis (8, 78). Clinicians should contact Rima McLeod, University of Chicago Hospitals, Chicago, IL (phone, 773-834-4152) regarding the treatment of infected children.

Diagnosis of Ocular Infection

Toxoplasma chorioretinitis results from both acute infection and congenital infection (79, 80). In addition to demonstration of IgG antibody to *Toxoplasma* in the serum of a person with compatible eye lesions, demonstration of the local production of antibody and detection of parasite DNA in aqueous humor have been used to document active ocular toxoplasmosis (2, 20, 23, 81). When the formula described in the section above is used to calculate results obtained in eye fluids and results obtained in serum, a value of 8 or greater suggests acute ocular toxoplasmosis. If the serum DT titer is greater than 1:1,000, it is usually not possible to calculate local antibody production.

Diagnosis in Immunocompromised Hosts

A wide variety of immunosuppressed hosts, including patients with hematologic and solid-tumor malignancies, transplant recipients, and those with autoimmune disorders, have been described as having severe, often fatal, toxoplasmosis. The disease is most often related to reactivation of latent infection and commonly involves the CNS, although a wide spectrum of clinical manifestations has been reported. Diagnosis can be very difficult for these patients, as IgM antibody is usually not detectable, and the presence of IgG antibody only confirms chronic infection. In the absence of serologic evidence of acute infection, diagnosis can be confirmed by demonstration of the organism histologically or cytologically as replicating within tissue or by isolation or identification of its nucleic acids in a site such as amniotic fluid, CSF, bronchoalveolar fluid, or placenta, in which the encysted organism would not be present as part of a latent infection.

Persons undergoing organ or bone marrow transplantation can benefit from pretransplant testing for *Toxoplasma*-specific IgG antibodies to determine immune status because they are either at risk for acute acquired infection if they are seronegative before transplantation or at risk for reactivation if they are seropositive before transplantation (2, 82). Those with acute acquired infection will usually develop detectable *Toxoplasma*-specific IgG and IgM antibodies, while those with reactivation will not have a detectable *Toxoplasma*-specific IgM response. Serial measurement of *Toxoplasma* DNA in peripheral blood by PCR has been advocated by some as a supplement to serology for monitoring for development of toxoplasmosis in bone marrow transplant patients (21, 22). Seronegative transplant recipients of hearts from seropositive donors can develop toxoplasmic myocarditis that mimics organ rejection.

Toxoplasmic encephalitis is the most frequent CNS opportunistic infection of AIDS patients and is uniformly fatal if untreated. Among people who died with AIDS from 1992 through 1997 in the United States, 7.2% developed toxoplasmic encephalitis during the course of their AIDS (11, 83). It is recommended that all HIV-infected persons be tested for *Toxoplasma*-specific IgG antibodies soon after the diagnosis of HIV infection to detect latent infection (11, 83). If *Toxoplasma*-seropositive, adult/adolescent patients who have a $CD4^+$ T-lymphocyte count of <100/µl should be administered treatment as described in the treatment section. Most AIDS patients with toxoplasmic encephalitis have demonstrable IgG antibodies to *T. gondii*. However, approximately 3% of AIDS patients with toxoplasmic encephalitis do not have *Toxoplasma*-specific antibody in their serum. Local production of *Toxoplasma*-specific IgG antibody in CSF has been demonstrated for persons with AIDS and with toxoplasmic encephalitis (31, 32). When the formula described above for toxoplasmosis in the newborn is used, a result of greater than 1 corresponds to significant antibody production.

TREATMENT

In general, physicians treat four types of patients with *T. gondii* infection: (i) pregnant women with acute infection to prevent fetal infection (30, 67, 68, 76, 84, 85); (ii) congenitally infected infants (74, 78, 86–88); (iii) immunosuppressed persons, usually with reactivated disease (11, 21, 22, 31, 83, 89–91); and (iv) patients with acute and recurrent ocular disease (79, 80, 92–95). Drugs are also prescribed for preventive or suppressive treatment of HIV-infected persons (11, 83). The currently recommended drugs work primarily against the actively dividing tachyzoite form of *T. gondii* and do not eradicate encysted organisms (bradyzoites).

The most common drug combination used to treat congenital toxoplasmosis consists of pyrimethamine and a sulfonamide (sulfadiazine is recommended in the United States) plus folinic acid in the form of leucovorin calcium to protect the bone marrow from the toxic effects of pyrimethamine. Pyrimethamine inhibits dihydrofolate reductase, which is important in the synthesis of folic acid and produces a reversible depression of the bone marrow. Sulfonamides inhibit synthesis of dihydrofolic acid, also important in the synthesis of folic acid. The two drugs work synergistically against *T. gondii*. Due to toxicity in early pregnancy, pyrimethamine and sulfadiazine therapy is generally recommended for use in pregnant women at ≥18 weeks of gestation (39, 60). After the 18th week, pyrimethamine and sulfadiazine may be given if fetal infection is confirmed by amniocentesis or cordocentesis. Spiramycin (available through the FDA [phone, 301-796-0563 or 301-796-1400; if no response, then 301-796-3763]) is recommended for pregnant women with acute toxoplasmosis when fetal infection has not been confirmed in an attempt to prevent the transmission of *T. gondii* from the mother to the fetus (39, 60). Randomized prospective studies of treatment during acute infection in pregnant women have not been performed. Some researchers have questioned, or been unable to demonstrate, the effectiveness of treatment during pregnancy in preventing congenital infection (76, 96) or sequelae in infants (96). One hypothesis for the lack of effectiveness is that *T. gondii* tachyzoites transform into bradyzoites within days of infection, probably coinciding with the serological and cell-mediated responses (96). Therefore, by the time an infection is detected in a pregnant woman, tachyzoites already may have been transmitted to the fetus, inflicted damage, and converted to encysted bradyzoites that do not respond to therapy. Nevertheless, a multicenter observational study found that the treatment of acute *T. gondii* infection in pregnancy was associated with a reduction of sequelae in infants but not a reduction in maternal-fetal transmission (68). Pyrimethamine and sulfadiazine (plus leucovorin) are the drugs generally used to treat infants with congenital toxoplasmosis and have led to improved outcomes compared with those of historic controls (39, 74, 78).

In immunosuppressed persons with toxoplasmosis, a regimen of pyrimethamine and sulfadiazine plus leucovorin is the preferred treatment (11, 31, 60, 83). Clindamycin is a second alternative for use in combination with pyrimethamine and leucovorin in those who cannot tolerate sulfonamides (11, 60, 83, 91, 97). Leucovorin prevents the hematologic toxicities associated with pyrimethamine therapy. Atovaquone in combination with either pyrimethamine or sulfadiazine has sufficient activity to be considered for treatment in some less severely affected adult patients (60, 98). The role of other drugs in the treatment of systemic toxoplasmosis has not been defined by controlled trials. In general, alternative drugs, such as azithromycin, clarithromycin, and dapsone, should be used in combination with another drug, preferably pyrimethamine (31).

Because relapse often occurs after toxoplasmosis in HIV-infected patients, maintenance therapy (secondary prophylaxis) with pyrimethamine plus sulfadiazine (first choice) or pyrimethamine plus clindamycin (alternative) is recommended (10, 82). Secondary prophylaxis for toxoplasmosis may be discontinued in patients with a sustained increase in CD4$^+$ counts (e.g., 6 months) to >200 cells/µl in response to highly active antiretroviral therapy if the patient has completed the initial therapy and has no symptoms or signs attributable to toxoplasmosis. For prophylaxis to prevent an initial episode of *T. gondii* in *Toxoplasma*-seropositive persons with a CD4$^+$ T-lymphocyte count of less than 100 cells/µl, trimethoprim-sulfamethoxazole is recommended as the first choice, with alternatives consisting of dapsone plus pyrimethamine, or atovaquone with or without pyrimethamine. Leucovorin is given with all regimens, including pyrimethamine (11, 83).

Pyrimethamine and sulfadiazine are often used for persons with ocular disease (80). Clindamycin, in combination with other antiparasitic medications, is also frequently prescribed for ocular disease (93). A variety of newer agents have been tried in the treatment of ocular toxoplasmosis, including atovaquone (99), rifabutin, trovafloxacin, azithromycin, and clarithromycin (100). In addition to prescribing antiparasitic drugs, physicians may add corticosteroids to reduce ocular inflammation. However, the optimal treatment for ocular toxoplasmosis remains to be defined by controlled trials.

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Pathogenic and Opportunistic Free-Living Amebae

GOVINDA S. VISVESVARA

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Small, free-living amebae belonging to the genera *Naegleria*, *Acanthamoeba*, and *Balamuthia* have been identified as agents of central nervous system (CNS) infections of humans and other animals (1–37). Only one species of *Naegleria* (*N. fowleri*), several species of *Acanthamoeba* (e.g., *A. castellanii*, *A. culbertsoni*, *A. hatchetti*, *A. healyi*, *A. polyphaga*, *A. rhysodes*, *A. astronyxis*, *A. lenticulata*, and *A. divionensis*), and the only known species of *Balamuthia*, *Balamuthia mandrillaris*, are known to cause disease. *Acanthamoeba* spp. also cause infection of the human cornea, *Acanthamoeba* keratitis (38–44). Further, both *Acanthamoeba* spp. and *B. mandrillaris* have been identified as agents of cutaneous infections in humans (7, 12, 17, 19, 22, 33, 34, 45). *Sappinia diploidea*, a free-living ameba normally found in soil contaminated with the feces of elk and buffalo, was identified in an excised brain lesion from a 38-year-old immunocompetent man who developed a bifrontal headache, blurry vision, and loss of consciousness following a sinus infection (46). This ameba was recently identified as *Sappinia pedata* based on a real-time PCR assay (47). Additionally, *Paravahlkampfia francinae*, a new species of the free-living ameba genus *Paravahlkampfia*, was recently isolated from the cerebrospinal fluid (CSF) of a patient with a headache, sore throat, and vomiting, symptoms typical of primary amebic meningoencephalitis (PAM) caused by *Naegleria fowleri*. The patient recovered within a few days, indicating that some of the previously reported patients with PAM who survived may have been infected with *P. francinae* (48). These findings clearly indicate that there are probably other small amebae that may also cause encephalitis in humans.

The concept that these small, free-living amebae may occur as human pathogens was proposed by Culbertson and colleagues, who isolated *Acanthamoeba* sp. strain A-1 (now designated *A. culbertsoni*) from tissue culture medium thought to contain an unknown virus (49). They also demonstrated the presence of amebae in brain lesions of mice and monkeys that died within a week after intracerebral inoculation with *A. culbertsoni*. Culbertson et al. hypothesized that similar infections might exist in nature in humans. In 1965, Fowler and Carter were the first to describe a fatal infection due to free-living amebae in the brain of an Australian patient (50). The infection is now believed to have been due to *N. fowleri*, as reported in reference 50.

TAXONOMY

Until recently, the classical taxonomic classification was based largely on morphologic, ecologic, and physiologic criteria. According to this system, *Acanthamoeba* and *Balamuthia*, along with a heterogeneous group of amebae that include both free-living (e.g., *Hartmannella*, *Vahlkampfia*, and *Vannella*) and parasitic (e.g., *Entamoeba histolytica*) amebae, were classified under the phylum Protozoa, subphylum Sarcodina, superclass Rhizopodea, class Lobosea, and order Amoebida. *Naegleria* was classified under the class Heterolobosea, order Schizopyrenida, and family Vahlkampfiidae. *Sappinia* was classified under the class Amoebozoa: Lobosea, order Euamoebida, and family Thecamoebidae. Recent information based on modern morphological approaches, biochemical pathways, and molecular phylogenetics has led to the abandonment of the older hierarchical systems consisting of the traditional "kingdom," "phylum," "class," "subclass," "superorder," and "order" and replacement with a new classification system. According to this new schema, the eukaryotes have been classified into six clusters or "supergroups," namely, Amoebozoa, Opisthokonta, Rhizaria, Archaeplastida, Chromalveolata, and Excavata. *Acanthamoeba* and *Balamuthia* are included under the supergroup Amoebozoa: Acanthamoebidae; *Sappinia* is included under the supergroup Amoebozoa: Flabellinea: Thecamoebidae; and *Naegleria fowleri* is included under the supergroup Excavata: Heterolobosea: Vahlkampfiidae (51). The classification of amebae presented here is supported by the International Society of Protistologists. However, other classification schemes may also be used.

DESCRIPTION OF THE AGENTS

Acanthamoeba spp.

Acanthamoeba has two stages in its life cycle, a feeding and multiplying stage, the trophozoite and the cyst (a dormant, resistant stage). The trophozoite measures 15 to 45 µm in length and produces from the surface of its body fine, tapering, hyaline projections called acanthopodia (see Fig. 5). The trophozoite is uninucleate, although binucleate forms are occasionally seen. The nucleus is characterized by a centrally located, large, dense nucleolus. It feeds on *Escherichia coli* or other Gram-negative bacteria and divides by binary fission. During cell division, the nucleus divides by conventional mitosis, in which the nucleolus and the nuclear membrane disappear. It has no flagellate stage but produces a double-walled cyst (10 to 25 µm) (see Fig. 6) with a wrinkled outer wall (the ectocyst) and a stellate, polygonal, or even round inner wall (the endocyst) (52).

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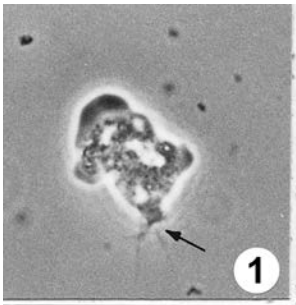
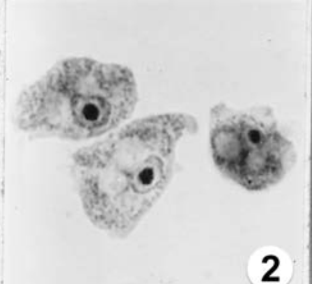
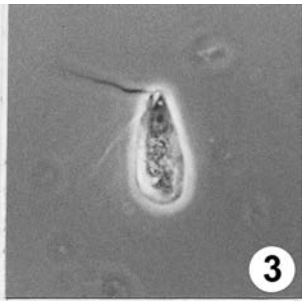
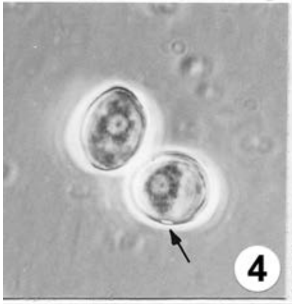
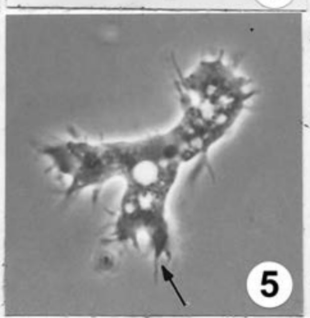
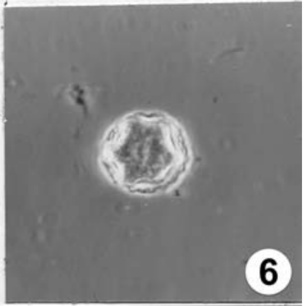


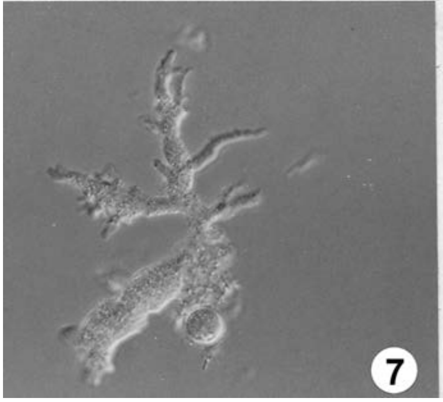


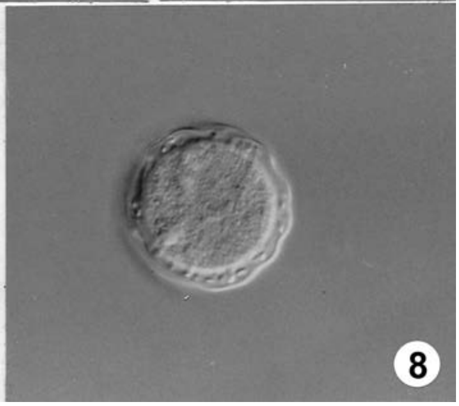












FIGURES 1 through 4 *N. fowleri*. (1) Trophozoite, phase contrast (note the uroid and filaments at the arrow); (2) trophozoite, trichrome stain; (3) biflagellate, phase contrast; (4) smooth-walled cyst, phase contrast (note the pore at the arrow). All magnifications, approximately ×835. doi:10.1128/9781555817381.ch139.f1-4

FIGURES 5 and 6 *A. castellanii*. (5) Trophozoite, phase contrast (note the acanthopodia at the arrow); (6) double-walled cyst, phase contrast. Both magnifications, approximately ×835. doi:10.1128/9781555817381.ch139.f5&6

FIGURES 7 and 8 *B. mandrillaris*. (7) Trophozoite, phase contrast; (8) cyst, phase contrast. Both magnifications, approximately ×1,140. doi:10.1128/9781555817381.ch139.f7&8

Cysts are resistant to many physical and chemical environmental pressures, including desiccation (53).

The genus *Acanthamoeba* contains as many as 24 species in three groups, with groupings based largely on morphologic characteristics. In group I are included those species that have large trophozoites and cysts that measure more than 18 µm. Group II is the largest group, and its members are the most widespread; the endocysts are usually stellate, polygonal, triangular, and oval and measure less than 18 µm. Group III includes those species with cysts that measure less than 18 µm, and the endocysts are either round or oval. Because such morphological characters may change with environmental pressures and culture conditions, efforts were made to utilize nonmorphologic characters for use in taxonomic classification. Therefore, the 18S rRNA gene was considered a good target for the classification and diagnosis of *Acanthamoeba* infection since it has multiple copies and is evolutionarily stable. In the case of *Acanthamoeba*, a substantial sequence variation was seen not only between the species but also within the same species, identified based on morphological features. *Acanthamoeba* is therefore classified into 18 genotypes known as T1 to T18 (54–56).

Naegleria

N. fowleri has three stages, a trophozoite, a flagellate, and a cyst, in its life cycle. The trophozoite is a small, slug-like ameba measuring 10 to 25 µm long that exhibits an eruptive locomotion by producing smooth hemispherical bulges. The posterior end, termed the uroid, appears to be sticky and often has several trailing filaments. It is uninucleate and is characterized by a centrally located, large, dense nucleolus. It feeds on *Escherichia coli* or other Gram-negative bacteria and divides by binary fission. During cell division, the nucleus divides by promitosis, in which the nucleolus elongates and divides into two polar bodies, and the nuclear membrane persists. During its life cycle, this ameba produces a transient pear-shaped biflagellate stage, resulting from altered environmental conditions, and smooth-walled cysts (Fig. 1 to 4). The flagellates do not have cytostomes (mouths). Cysts are usually spherical and measure 7 to 15 µm, and the cyst wall may have one or more pores plugged with a mucoid material (52).

Balamuthia

Balamuthia trophozoites are in general irregular in shape; a few, however, may be slug-like. They are uninucleate, but binucleate forms may occasionally be seen. The nucleus has a large centrally located nucleolus. The pattern of nuclear division in *Balamuthia* is termed metamitosis, in which the nuclear membrane breaks down and the nucleolus eventually disappears (32, 33). Occasionally, the nucleus of *Balamuthia*, especially in tissue sections, may have more than one nucleolus. Unlike *Acanthamoeba* and *Naegleria fowleri*, *Balamuthia* is not known to feed on bacteria. In the laboratory, it feeds on mammalian tissue culture cells, such as monkey kidney cells, human lung fibroblasts, or human brain microvascular endothelial cells. Actively feeding amebae may be 12 to 60 µm long, with a mean length of 30 µm (Fig. 7). The trophic forms while feeding on tissue culture cells produce broad pseudopodia without any clearly discernible movement. However, when tissue culture cells are destroyed, the trophozoites resort to a spider-like walking movement by producing finger-like determinate pseudopodia (33). Like *Acanthamoeba*, *Balamuthia* does not have a flagellate stage. Cysts are generally uninucleate and spherical and measure 6 to 30 µm in diameter (Fig. 8). Under a light microscope, each cyst appears to have an irregular and slightly wavy outer wall and a round inner wall. A layer of refractile granules immediately below the inner cyst wall is often seen in mature cysts. Under an electron microscope, the cyst wall can be seen to consist of three walls: a thin, irregular outer ectocyst; a thick, electron-dense inner endocyst; and a middle amorphous layer, the mesocyst (32, 33).

EPIDEMIOLOGY

Infection with *N. fowleri* was first described in 1965 in Australia and has now been identified virtually all over the world. In the United States, PAM has been reported from 1931 to 2012 and is known to affect primarily young males who frequently dive and jump into warm freshwater in the summer, when the temperature of the water in lakes and ponds, especially in the southern tier of the United States, is expected to be high. Recent occurrences of PAM in northern states such as Minnesota (13) and Kansas (37) indicate that the geographic range of *N. fowleri* is changing. It has been postulated that higher temperatures due to climate change might be responsible for increases in ambient water temperatures and, therefore, for increases in the chances of contracting *N. fowleri* infections in areas that had been free of this infection (6, 13). Poorly treated municipal water systems have been implicated in the acquisition of PAM (24, 37). However, the fact that two recent cases of PAM in Louisiana were associated with disinfected municipal water underscores the changing epidemiology of PAM (37). It is, however, believed that the risk for infection might be reduced if appropriate measures, such as holding the nose shut by using nose clips and/ or not stirring up sediments while swimming or doing water sports in freshwater lakes and other bodies of water, are taken (36).

Both *Acanthamoeba* and *Balamuthia* infections have been identified from many parts of the world. However, they are not associated with a particular time of the year. *Acanthamoeba* infections are found mostly in people with immunosuppression either because of other underlying infections or because of transplantation (2, 7, 9, 12, 17–19, 21, 22, 25, 30), whereas infections with *Balamuthia* are found in both immunocompromised and immunocompetent individuals (8, 11, 20, 22, 26, 27, 29, 33, 34, 45). Recently, *B. mandrillaris* has been identified as an agent of encephalitis in solid organ transplant recipients (4, 5).

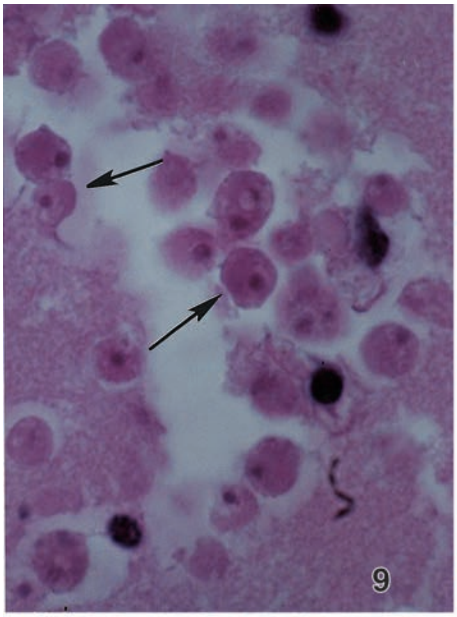
N. fowleri and *Acanthamoeba* spp. are commonly found in soil, freshwater, sewage, and sludge and even on dust in the air. Several species of *Acanthamoeba* have also been isolated from brackish water and seawater and from ear discharges, pulmonary secretions, nasopharyngeal mucosa samples, maxillary sinus samples, mandibular autografts, and stool samples (19, 22, 27, 34). These amebae normally feed on bacteria and multiply in their environmental niche as free-living organisms. *Acanthamoeba* spp. have also been known to harbor *Legionella* spp., *Mycobacterium avium*, and other bacterial pathogens, such as *Listeria monocytogenes*, *Burkholderia pseudomallei*, *Vibrio cholerae*, and *Escherichia coli* serotype O157, which signifies a potential expansion of the public health importance of these organisms (57, 58). Additionally, pure cultures of *A. polyphaga* have been used to isolate *Legionella pneumophila* (59), *Legionella anisa* (60), and, recently, *Mycobacterium massiliense* (61) from human clinical specimens, such as sputa, liver, and lung abscess specimens, and even from human feces. Obligate intracellular pathogens, such as *Chlamydia*, *Chlamydophila*, and *Chlamydia*-like bacteria, have been found in ~5% of *Acanthamoeba* isolates, and *Chlamydophila pneumophila*, a respiratory pathogen, can survive and grow within *Acanthamoeba* (57). Whether endosymbiont-bearing *Acanthamoeba* strains serve as reservoirs for these bacteria, some of which are potential pathogens for humans, is unknown.

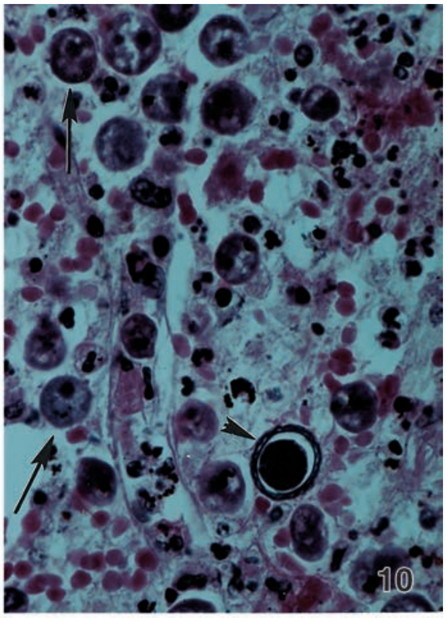
Although *B. mandrillaris* has been isolated from soil and dust (62–64) and its DNA found in the environment (65), not much is known about the environmental niche of *B. mandrillaris* and its feeding habits. It is, however, believed that its habitat is similar to those of *Acanthamoeba* and *Naegleria* and that it feeds on small amebae and possibly flagellates (66).

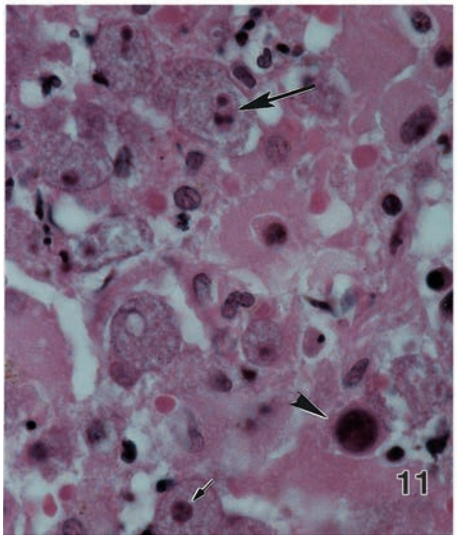
CLINICAL SIGNIFICANCE

Naegleria Meningoencephalitis

In 1966, Butt (3) described the first case of CNS infection caused by *N. fowleri* in the United States and coined the term "primary amebic meningoencephalitis" (PAM). PAM is an acute fulminating disease with an abrupt onset that occurs generally in previously healthy children and young adults who had contact with freshwater an average of 5 days before the onset of symptoms. It is characterized by a severe headache, spiking fever, a stiff neck, photophobia, and a coma, leading to death within an average of 5.3 days (range, 1 to 12 days) after the onset of symptoms (3, 6, 19, 22, 24, 27, 28, 34, 36, 37, 50). The portal of entry of the *N. fowleri* amebae is the nasal passages. When people swim in lakes and other bodies of freshwater that harbor these amebae, the amebae may enter the nostrils of the swimmers, make their way into the olfactory lobes via the cribriform plate, and cause acute hemorrhagic necrosis, leading to destruction of the olfactory bulbs and the cerebral cortex. Only a few patients have survived. Upon autopsy, large numbers of amebic trophozoites, many with ingested erythrocytes and brain tissue, are usually seen interspersed with brain tissue







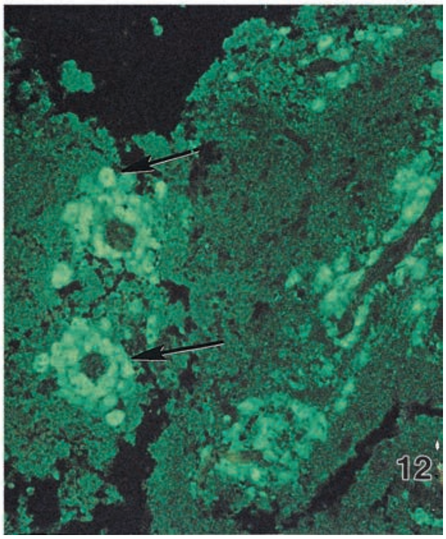


FIGURE 9 Large numbers of *N. fowleri* trophozoites (arrows) in a section of CNS tissue, showing extensive necrosis and destruction of brain tissue. Magnification, approximately ×564. doi:10.1128/9781555817381.ch139.f9

FIGURE 10 *A. culbertsoni* trophozoites (arrows) and a cyst (arrowhead) around a blood vessel in a section of CNS tissue from a GAE patient. Magnification, approximately ×489. doi:10.1128/9781555817381.ch139.f10

FIGURE 11 *B. mandrillaris* trophozoites and a cyst (arrowhead) in a brain section from a GAE patient. Note the double (small arrow) and triple (large arrow) nucleolar elements within the nuclei of the trophozoites. Magnification, approximately ×413. doi:10.1128/9781555817381.ch139.f11

FIGURE 12 Immunofluorescence localization of *B. mandrillaris* in a brain section from a GAE patient. Note the fluorescent amebae (arrows) around blood vessels. Magnification, approximately ×188. doi:10.1128/9781555817381.ch139.f12

(Fig. 9). It is believed that *N. fowleri* directly ingests brain tissue by producing food cups, or amebostomes, as well as by exerting contact-dependent cytolysis, possibly mediated by a multicomponent system consisting of a heat-stable hemolytic protein, a heat-labile cytolysin, and/or phospholipase enzymes (67). Cysts of *N. fowleri* are not usually seen in brain tissue. It was believed until recently that *N. fowleri* infects only humans. In March 1997, however, a report of the first case of PAM in a South American tapir was published, indicating that PAM can occur in animals other than humans (16). Additionally, a report of PAM in Holstein cattle, associated with drinking of surface waters, indicates that this disease is probably more common than is currently appreciated (31).

Acanthamoeba Encephalitis

Several species of *Acanthamoeba* (*A. culbertsoni*, *A. castellanii*, *A. polyphaga*, *A. astronyxis*, *A. healyi*, and *A. divio-*

nensis) have been known to cause a chronic granulomatous amebic encephalitis (GAE), primarily in immunosuppressed (either because of iatrogenic suppression or because of transplantation, or because of human immunodeficiency virus infection or AIDS), chronically ill, or otherwise debilitated persons with no previous history of exposure to recreational freshwater (1, 2, 7, 9, 12, 17–19, 21, 22, 25, 27, 30, 34). There is one report, however, of an immunocompetent person with GAE (35). GAE has an insidious onset and is usually chronic, lasting for more than a week and sometimes even for months (17, 22, 27, 34). It is characterized by a headache, confusion, dizziness, drowsiness, seizures, and sometimes hemiparesis. Cerebral hemispheres are usually the most heavily affected CNS tissue. They are often edematous, with extensive hemorrhagic necrosis involving the temporal, parietal, and occipital lobes. Amebic trophozoites and cysts are usually scattered throughout the tissue. Many blood vessels are thrombotic and exhibit fibrinoid necrosis; they are also surrounded by polymorphonuclear leukocytes, amebic trophozoites, and cysts (Fig. 10). Multinucleated giant cells forming granulomas may be seen in immunocompetent patients. Some patients, especially those with human immunodeficiency virus infection or AIDS, develop chronic ulcerative skin lesions, abscesses, or erythematous nodules (17). It is believed that the route of invasion of and penetration into the CNS is hematogenous, probably from a primary focus in the lower respiratory tract or the skin (17, 19, 22, 27, 34). *Acanthamoeba* spp. also cause infections of the CNS of animals other than humans. Such infections have been recorded in gorillas, monkeys, dogs, ovines, bovines, horses, and kangaroos (10, 19, 22, 23, 27, 34).

Balamuthia (Leptomyxid) Encephalitis

It was believed that all cases of GAE were caused by *Acanthamoeba* spp. Although a number of these cases were confirmed by serologic techniques as being caused by *Acanthamoeba* spp., the causative organisms in a few cases could not be definitively identified. Since cysts were found in the brain tissues of the patients, it was believed that the infections were caused by some other species of *Acanthamoeba* that did not cross-react with the anti-*Acanthamoeba* sera used in the serologic test (19, 22, 27, 32, 34). *B. mandrillaris* (leptomyxid ameba) was definitively identified by an indirect immunofluorescence test as the causal agent in these and many other cases (4, 5, 8, 10, 11, 19, 22, 23, 26, 29, 33–35). The rabbit anti-*B. mandrillaris* serum used in the indirect immunofluorescence test was made by using culture-derived *Balamuthia* organisms that had been isolated from the CNS tissue of a baboon (32). It is now known that a number of primates, including gorillas, gibbons, baboons, orangutans, and monkeys, as well as dogs, sheep, and horses, have died of CNS infections caused by *B. mandrillaris* (10, 19, 20, 22, 23, 27, 32–34). According to one report, Hispanic Americans have a high incidence of *B. mandrillaris* infection. It is not clearly known whether this is due to environmental factors, genetic predisposition, limited access to medical care, or other socioeconomic factors and pressures (68).

The pathology and pathogenesis of *B. mandrillaris*-induced GAE are similar to those of *Acanthamoeba*-induced GAE. Both trophozoites and cysts are found in CNS tissue (Fig. 11), and their sizes overlap those of *Acanthamoeba* trophozoites and cysts (19, 20, 22, 27, 32, 34, 69). Hence, it is difficult to differentiate *Balamuthia* from *Acanthamoeba* spp. in tissue sections under a light microscope. In some cases, *Balamuthia* trophozoites in tissue sections appear to have more than one nucleolus in the nucleus (Fig. 11). In such cases, it may be possible to distinguish *Balamuthia* amebae from *Acanthamoeba* organisms on the basis of nuclear morphology, since *Acanthamoeba* trophozoites have only one nucleolus. In most cases, electron microscopy, immunohistochemical techniques, or both are necessary to identify *Balamuthia* organisms. Ultrastructurally, the cysts are characterized by three layers in the cyst wall: an outer wrinkled ectocyst, a middle structureless mesocyst, and an inner thin endocyst (32–34). *Balamuthia* amebae are antigenically distinct from *Acanthamoeba* organisms; they can easily be distinguished by immunofluorescence or other immunochemical assays (Fig. 12) (8, 10, 11, 19, 20, 22, 23, 26, 27, 29, 32–34, 69).

Acanthamoeba Keratitis

Acanthamoeba spp. also cause a painful vision-threatening disease of the human cornea, *Acanthamoeba* keratitis. If the infection is not treated promptly, it may lead to ulceration of the cornea, loss of visual acuity, and eventually blindness and enucleation (17, 19, 21, 22, 27, 38–44). *Acanthamoeba* keratitis is characterized by severe ocular pain, a 360° or partial paracentral stromal ring infiltrate, recurrent corneal epithelial breakdown, and a corneal lesion refractory to the commonly used ophthalmic antibacterial medications. *Acanthamoeba* keratitis in the early stages is frequently misdiagnosed as herpes simplex virus keratitis because of the irregular epithelial lesions, stromal infiltrative keratitis, and edema that are commonly seen in herpes simplex virus keratitis (38–44). A nonhealing corneal ulcer is often the first clue that *Acanthamoeba* keratitis may be the problem.

The first case of *Acanthamoeba* keratitis in the United States was reported in 1973 in a south Texas rancher with a history of trauma to his right eye (40). Both the trophozoite and cyst stages of *A. polyphaga* were demonstrated to be present in corneal sections and were repeatedly cultured from corneal scrapings and biopsy specimens. Between 1973 and July 1986, 208 cases were diagnosed and reported to the Centers for Disease Control (CDC) (43). The numbers of cases increased gradually between 1973 and 1984, and a dramatic increase began in 1985. An in-depth epidemiologic and case-control study (43) revealed that a major risk factor was the use of contact lenses, predominantly daily-wear or extended-wear soft lenses, and that patients with *Acanthamoeba* keratitis were significantly more likely than controls to use homemade saline solution instead of commercially prepared saline (78 and 30%, respectively), to disinfect their lenses less frequently than recommended by the lens manufacturers (72 and 32%), and to wear their lenses while swimming (63 and 30%). Based on a case-control study conducted by the CDC to investigate a recent increase in the *Acanthamoeba* keratitis cases during 2004 to 2007, it was revealed that a national increase in the number of such cases was associated with the use of Advanced Medical Optics Complete MoisturePlus multipurpose contact lens solution (44). Further, another study revealed that most contact lens solutions marketed in the United States do not have sufficient disinfection activity against *Acanthamoeba* spp. (70).

COLLECTION, HANDLING, AND STORAGE OF SPECIMENS

For isolation of *N. fowleri*, CSF and brain tissue (especially surrounding the nasal olfactory bulbs) should be obtained aseptically. For *Acanthamoeba* and *Balamuthia*, CSF, brain, lungs, and ulcerated skin tissues, and for *Acanthamoeba* in the case of *Acanthamoeba* keratitis, corneal scrapings or corneal biopsy tissue should be obtained aseptically. The specimens should be kept at room temperature (24 to 28°C) and should never be frozen. Personnel handling the specimens must take appropriate precautions, such as wearing surgical masks and gloves and working in a biological safety cabinet. Remaining tissues must be preserved in 10% neutral buffered Formalin so that they can be examined histologically for amebae (69).

CLINICAL AND LABORATORY DIAGNOSIS

Methods of Examination

Direct Examination

Since no distinctive clinical features differentiate PAM from pyogenic or bacterial meningitis, direct examination of the sample as a wet mount preparation is of paramount importance in the diagnosis of PAM and other diseases caused by these amebae. In PAM, the CSF is usually pleocytotic, with a preponderance of polymorphonuclear leukocytes and no bacteria. The CSF pressure may be elevated. The CSF glucose level may be normal or slightly reduced, but the CSF protein level is increased, ranging from 1 to 10 mg/ml. Microscopic detection of amebic organisms in the CSF is the only means of diagnosing PAM. CSF should be examined *in situ* microscopically for the presence of *N. fowleri* amebae with directional movement. Since the amebae tend to attach to the surface of the container, the container should be shaken gently; then, a small drop of fluid should be placed on a clean microscope slide and covered with a no. 1 coverslip. The CSF may have to be centrifuged at 500 × *g* for 5 min to concentrate the amebae. After the specimen has been centrifuged, most of the supernatant is carefully aspirated and the sediment is gently suspended in the remaining fluid. A drop of this suspension is prepared as described above for microscopic observation. Giemsa or trichrome staining should be performed on CSF smears to visualize the nuclear morphology of the amebae. The slide preparation should be examined under a compound microscope with 10× and 40× lens objectives. Phase-contrast optics is preferable. If regular bright-field illumination is used, the slide should be examined under diminished light. The slide may be warmed to 35°C (to promote amebic movement), and amebae, especially *N. fowleri*, if present, can easily be detected by their active directional movements. Rarely, flagellates with two flagella may be seen. Currently, this is the most sensitive method available for the diagnosis of PAM.

Permanently Stained Preparations

A small drop of the sedimented CSF or other sample is placed in the middle of a slide, which is allowed to stand in a moist chamber for 5 to 10 min at 37°C. This will allow any amebae to attach to the surface of the slide. Several drops of warm (37°C) Schaudinn's fixative are dropped directly onto the sample, which is allowed to stand for 1 min. The slide is then transferred for 1 h to a Coplin jar containing the fixative. It may be stained with Wheatley's trichrome or Heidenhain's iron hematoxylin stain. Corneal scrapings smeared onto microscope slides may be fixed with methanol and stained with Hemacolor stain (Harleco, a division of EM Industries, Inc.) (27, 34). CSF can also be processed via cytospinning whenever possible and stained with Wright-Giemsa stain. Amebae, if present, can be distinguished based on their nuclear morphology (13), and the result should be reported to the clinician so that the patient can be treated with appropriate medication. This is especially important in the case of PAM caused by *N. fowleri*. Gram staining is not useful in the detection of amebae. Further, a false-positive Gram stain may lead to inaccurate diagnosis and hence to inappropriate therapy, which may result in patient death. A recent report suggests the possibility of extra CNS dissemination of *N. fowleri*, and therefore, the organism might pose a risk of transmission of PAM via organ transplantation (15). A few reports describing the transplantation of solid organs from donors infected with *B. mandrillaris* (4, 5, 15) underscores the importance of a correct and timely diagnosis.

Antigen Detection

Pathogenic *N. fowleri* is morphologically indistinguishable from nonpathogenic *Naegleria* at the trophic stage. Differences between these amebae, however, have been demonstrated antigenically by the gel diffusion, immunoelectrophoretic, and immunofluorescence techniques, as well as by their isoenzyme patterns, and these techniques have been utilized to identify the amebae isolated in culture (22, 27, 34). Similarly, antigenic differences have also been shown among various species of *Acanthamoeba* (17, 19, 22, 27, 34). Additionally, *N. fowleri*, *Acanthamoeba* spp., and *B. mandrillaris* have been identified in tissue sections by histochemical methods (2–13, 15, 16, 21–23, 25–27, 29, 30, 32–35, 69).

Nucleic Acid Detection in Clinical Materials

Acanthamoeba genotypes have been identified in corneal tissue, tear fluid, and brain and lung tissue, as well as in the environment. A number of studies have analyzed the mitochondrial DNA and 18S rRNA gene to understand the inter- and intraspecies diversity and phylogeny of *Acanthamoeba* spp. (17, 22, 27, 34). However, only a few studies have used this technique to identify *Acanthamoeba* keratitis or *Acanthamoeba* GAE by using patient specimens (1, 2, 7, 17, 22, 27, 29, 33, 54, 55, 71). This is probably because of the lack of reagents from commercial sources.

For the detection of *Acanthamoeba*, Booton et al. (54) used nuclear single-subunit ribosomal DNA (rDNA) sequences and the genus-specific primers JDP1 (5′-GGCCCAGATCGTTTACCGTGAA-3′) and JDP2 (5′-TCTCACAAGCTGCTAGGGGAGTCA-3′), which amplify a region of the single-subunit rDNA that permits genotypic identification of an *Acanthamoeba* isolate following sequence analysis.

In the case of *Balamuthia*, all isolates analyzed so far seem to be largely similar on the basis of their 18S rRNA gene sequences, whereas mitochondrial 16S rRNA gene sequences show variability of about 1.8%, in contrast to those of *Acanthamoeba* isolates (72). A PCR assay for the detection of *B. mandrillaris* has been described (72). A PCR assay to detect *B. mandrillaris* in Formalin-fixed archival tissue specimens has also been described (73).

In the case of *N. fowleri*, however, different isolates show similar nuclear 18S rRNA sequences, but variation in the internal transcribed spacer (ITS) sequences has been used to identify six different genotypes (I, II, III, IV, V, VI) (74), although only three genotypes (genotypes I, II, and III) circulate in North America (75). Sequencing of the 5.8S rRNA gene and ITS1 and ITS2 of *N. fowleri* not only can differentiate *N. fowleri* from other *Naegleria* spp. and *Paravahlkampfia* spp. but also can be used in the genotypic analysis of *N. fowleri* strains (6, 13, 22, 24, 27, 34, 37, 48, 71, 74, 75). The recently unraveled genome of *N. fowleri* (76) offers great promise not only in elucidating the pathogenic mechanisms but also in the development of better drug targeting and thus help in the survival of patients infected with this deadly pathogen.

A real-time multiplex PCR test that simultaneously identifies *Acanthamoeba*, *Balamuthia*, and *N. fowleri* in CSF specimens has been developed at the CDC (71). In this assay, PCR primers and TaqMan probes targeting three regions of the 18S rRNA gene are used. The real-time multiplex PCR assay is a fast, sensitive, and robust assay that detects all three pathogenic free-living amebae, *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*, simultaneously in a single specimen and has many advantages over conventional PCR. This test is being used at the CDC to identify *Acanthamoeba*, *Balamuthia*, and *N. fowleri* in patient specimens with great success. Based on its limited sensitivity and specificity, this assay can identify a single ameba in a specimen. Unfortunately, these molecularly based tests are not routinely available in clinical laboratories because of the lack of commercially available reagents.

ISOLATION

The recommended procedure for isolating free-living pathogenic amebae from biological specimens is as follows.

Materials

1. Page's ameba saline (52). Physiological saline or phosphate-buffered saline solutions that are normally available in clinical laboratories are not suitable, as the sodium chloride concentrations in these solutions will prevent the growth of amebae, especially *N. fowleri*.

2. Petri dishes containing 1.5% Difco agar made with Page's ameba saline (nonnutrient agar plate) (52). These plates can be stored at 4°C for up to 3 months. Chocolate agar with blood, Trypticase soy agar, and Lowenstein-Jensen agar have been used sometimes. These are not suitable because bacteria that coat the plates or bacteria from the clinical sample may overgrow and either prevent the growth of amebae, especially when they are in small numbers, or obscure their presence.

3. Eighteen- to 24-h-old cultures of *E. coli* or *Enterobacter aerogenes*.

Preparation of Agar Plates

1. Remove the plates from the refrigerator and place them in a 37°C incubator for 30 min.

2. Add 0.5 ml of ameba saline to a slant culture of *E. coli* or *Enterobacter aerogenes*. Gently scrape the surface of the slant with a sterile bacteriologic loop (do not break the agar surface). Using a sterile Pasteur pipette, gently and uniformly suspend the bacteria. Add 2 or 3 drops of this suspension to the middle of a warmed (37°C) agar plate, and spread the bacteria over the surface of the agar with a bacteriologic loop. The plate is then ready for inoculation.

Inoculation of Plates with Specimens

1. For CSF samples, centrifuge the CSF at 500 × *g* for 5 to 8 min. With a sterile serologic pipette, carefully transfer all but 0.5 ml of the supernatant to a sterile tube, and store the tube at 4°C for possible future use. Mix the sediment with the remaining fluid. With a sterile Pasteur pipette, place 2 or 3 drops in the center of the agar plate precoated with bacteria, and incubate in room air at 37°C.

2. For tissue samples, gently grind a small piece of the tissue in a small amount of ameba saline. With a sterile Pasteur pipette, place 2 or 3 drops of the mixture in the center of the agar plate. Incubate the plate in room air at 37°C for CNS and lung tissues and at 30°C for tissues from other sites (e.g., skin and cornea).

3. Handle water and soil samples in the same manner as CSF and tissue specimens, respectively.

4. Control cultures are recommended for comparative purposes, although care should be exercised to prevent cross-contamination of patient cultures.

Examination of Plates

1. Using the low-power (10×) lens objective of a microscope, observe the plates daily for 7 days for amebae.

2. If you see amebae anywhere, circle that area with a wax pencil. With a fine spatula, cut a small piece of agar from the circled area and place it face down on the surface of a fresh agar plate precoated with bacteria; incubate as described above. Both *N. fowleri* and *Acanthamoeba* spp. can easily be cultivated in this way and, with periodic transfers, maintained indefinitely. When the plate is examined under a microscope, the amebae will look like small blotches, and if they are observed carefully, their movement can be discerned. After 2 to 3 days of incubation, the amebae will start to encyst. If a plate is examined after 4 to 5 days of incubation, trophozoites as well as cysts will be visible. *B. mandrillaris*, however, will not grow on agar plates seeded with bacteria. While *B. mandrillaris* can be grown on monkey kidney or lung fibroblast cell lines, on human brain microvascular endothelial cells, and axenically in a complex medium (32, 33, 77–80), such techniques are not routinely available.

Identification and Culture

Identification of living organisms to the genus level is based on characteristic patterns of locomotion, morphologic features of the trophozoite and cyst forms, and results of enflagellation experiments. Immunofluorescence or immunoperoxidase tests using monoclonal or polyclonal antibodies (available at the Centers for Disease Control and Prevention) will be helpful in differentiating *Acanthamoeba* spp. from *B. mandrillaris* in fixed tissue (4, 7–12, 19–23, 25, 27, 29, 32–35, 69).

Enflagellation Experiment

1. Mix 1 drop of the sedimented CSF containing amebae with about 1 ml of sterile distilled water in a sterile tube, or with a bacteriologic loop scrape the surface of a plate that is positive for amebae, transferring a loopful of scraping to a sterile tube that contains approximately 1 ml of distilled water.

2. Gently shake the tube and transfer a drop of this suspension to the center of a coverslip whose edges have been coated thinly with petroleum jelly. Place a microscope slide over the coverslip and invert the slide. Seal the edges of the coverslip with Vaspar. Place the slide in a moist chamber and incubate as before for 2 to 3 h. In addition, incubate the tube as described above.

3. Periodically examine the tube and the slide preparation microscopically for free-swimming flagellates. *N. fowleri* has a flagellate stage; *Acanthamoeba* spp. and *B. mandrillaris* do not. If the sample contains *N. fowleri*, about 30 to 50% of the amebae will have undergone transformation into pear-shaped biflagellate organisms (Fig. 3).

Other Culture Methods

Axenic Culture

Acanthamoeba spp. can easily be cultivated axenically, without the addition of serum or host tissue, in many different types of nutrient media, e.g., proteose peptone-yeast extract-glucose medium, Trypticase soy broth medium, and chemically defined medium (78). *N. fowleri*, however, requires media containing fetal calf serum or brain extract, e.g., Nelson's medium. A chemically defined medium has only recently been developed for *N. fowleri* (78). *B. mandrillaris* cannot be cultivated on agar plates with bacteria. It can, however, be cultivated on mammalian cell lines or a complex axenic medium (78).

Axenic cultures of *Acanthamoeba* spp. and *N. fowleri* can be established as follows. An actively growing 24- to 36-h-old ameba culture is scraped from the surface of the plate, suspended in 50 ml of ameba saline, and centrifuged at 500 × *g* for 5 min. The supernatant is aspirated, and the sediment is inoculated into proteose peptone-yeast extract-glucose medium or Nelson's medium, depending on the ameba isolate, and incubated at 37°C. Gentamicin, to a final concentration of 50 µg/ml, is added aseptically to the medium before the amebae are inoculated. Three subcultures into the antibiotic-containing medium at weekly intervals are usually sufficient to eliminate the associated bacteria (*E. coli* or *Enterobacter aerogenes*).

Cell Culture

Acanthamoeba spp., *B. mandrillaris*, and *N. fowleri* can also be inoculated onto many types of mammalian cell cultures. Shell vial cultures normally used in the isolation of viruses are suitable for the isolation of the amebae provided that antifungal agents (amphotericin B) are not included in the antibiotic mix. The amebae grow vigorously in these cell cultures and produce cytopathic effects somewhat similar to those caused by viruses (78–80). Because of such cytopathic effects, *Acanthamoeba* organisms were mistaken for transformed cell types presumed to contain viruses and were erroneously termed lipovirus and Ryan virus (17, 22, 27, 34).

Animal Inoculation

Two-week-old Swiss Webster mice weighing 12 to 15 g each can be infected with these amebae. The mice are anesthetized with ether, and a drop of ameba suspension is instilled into their nostrils. Mice infected with *N. fowleri* die within 5 to 7 days after developing characteristic signs, such as ruffled fur, aimless wandering, partial paralysis, and finally coma and death. Mice infected with *Acanthamoeba* spp. and *B. mandrillaris* may die of acute disease within 5 to 7 days or may die of chronic disease after several weeks. In all cases, the presence of amebae in the mouse brain can be demonstrated either by culture or by histologic examination.

Serology

The serologic techniques discussed here have been developed as research tools and are not routinely available to clinical laboratories. Antibodies (detected by complement fixation, indirect fluorescent-antibody assay [IFA], precipitin, etc.) to *Acanthamoeba* spp. have been shown to be present in the sera of patients with GAE, upper respiratory tract distress, optic neuritis, macular disease, and keratitis (1, 17, 19, 22, 27, 30, 34, 37, 39, 40, 81).

An antibody response to *N. fowleri*, however, has not yet been defined. Most of the patients with *N. fowleri* PAM die very shortly after infection (5 to 10 days), before they have time to produce detectable levels of antibody. In one case, however, in which the patient survived PAM, an antibody response was detected by 10 days after hospitalization, ultimately reaching a titer of 1:4,096 (22, 27, 28, 34, 67).

Since the recognition of *Balamuthia* GAE is relatively new, not much information is available on the serologic responses of patients infected with *Balamuthia*. According to a recent report, four patients with confirmed *Balamuthia* GAE infection had high titers of antibody to *Balamuthia*, whereas six serum specimens from patients with encephalitis of unknown causes (10% of the sera) had titers of 64 and above and none of the control sera had titers of 64 or above (26). It is therefore possible that patients with *Balamuthia* GAE may be diagnosed premortem by using this serologic test (4, 22, 26, 27, 34). According to Schuster et al. (81), IFA can be successfully used to screen GAE due to *Acanthamoeba* and *Balamuthia* in patients whose clinical presentation, laboratory results, and neuroimaging findings are suggestive of amoebic encephalitis, thus enabling an earlier diagnosis and earlier start of antimicrobial therapy. Recently, Kucerova et al. showed by SDS-PAGE and Western blot assay that several isolates irrespective of their isolation from humans or animals or from different geographic locales are basically similar, underscoring the similarity of the isolates (82).

TREATMENT

There is no single drug that is effective against systemic acanthamebiasis. A number of antimicrobials have shown efficacy against amebae *in vitro*, but there is no assurance that these same drugs will be effective clinically. An important consideration in corneal infections is using drugs that are not only amebicidal but also cysticidal. As long as cysts remain viable, the infection can recur. Jones et al. (40) found that paromomycin, clotrimazole, and hydroxystilbamidine isethionate were active against *A. polyphaga in vitro*. In a recent study, azithromycin and several phenothiazine compounds protected rat glioma cells from destruction by *Acanthamoeba* (27). Culbertson et al. and others found sulfadiazine to be active against experimental *Acanthamoeba* infections in mice (17, 27, 49). *In vitro* studies indicate that chlorhexidine gluconate and polyhexamethyl biguanide have excellent amebicidal and cysticidal properties, and they have been used topically in the treatment of *Acanthamoeba* keratitis with success. Several patients with *Acanthamoeba* keratitis have been successfully treated with different drug combinations administered over a long period (22, 27, 38–41). For example, in one study, treatment with 0.1% propamidine isethionate (Brolene) eye drops and 0.15% dibromopropamidine ointment together with topical neomycin sulfate was successful in the management of *Acanthamoeba* keratitis (39). Although a few patients with *Acanthamoeba* sp. GAE have survived, most have died in spite of treatment with several drug combinations. The prognoses of patients without CNS infection but with disseminated cutaneous ulcers due to *Acanthamoeba* spp. are good. For example, a patient with Down's syndrome and an immunoglobulin A deficiency who also had undergone cadaveric renal transplantation developed a biopsy specimen-confirmed *Acanthamoeba* skin ulcer. The patient was successfully cured of the infection after prolonged therapy (more than 8 months) with a regimen that included topical as well as systemic administration of a combination of drugs, including topical application of chlorhexidine gluconate solution followed by 2% ketoconazole cream. He also received pentamidine isethionate intravenously for 1 month and thereafter was given oral itraconazole therapy for 8 months; this regimen resulted in complete healing of the cutaneous ulcers

(30). Recently, several patients with *Acanthamoeba* CNS infections have also been cured with a combination of pharmaceuticals that included amikacin, voriconazole, sulfa drugs, and miltefosine (1, 22, 34, 35).

Pathogenic *N. fowleri* is exquisitely susceptible to amphotericin B *in vitro*, and the minimum amebicidal concentrations were determined to be 0.02 to 0.078 μg/ml for three different clinical isolates of *N. fowleri* (22, 27, 28, 34, 50). Although many patients with PAM have been treated with amphotericin B, only two patients have survived after receiving intrathecal and intravenous injections of amphotericin B alone or in combination with miconazole (22, 27, 28, 34).

In vitro studies indicate that *B. mandrillaris* is susceptible *in vitro* to pentamidine isothiocyanate and that patients with *B. mandrillaris* infection may benefit from treatment with this drug (4, 5, 45). Although most patients with *B. mandrillaris* GAE have died of this disease, several patients have survived after treatment initially with pentamidine isethionate and subsequently with a combination of sulfadiazine, clarithromycin, and fluconazole.

The anticancer and antiparasitic drug miltefosine and the antifungal drug voriconazole were tested *in vitro* against *Balamuthia mandrillaris*, *Acanthamoeba* spp., and *Naegleria fowleri*. *Balamuthia* organisms exposed to <40 μM concentrations of miltefosine survived, while concentrations of ≥40 μM were amebicidal. *Acanthamoeba* spp. recovered from exposure to 40 μM but not 80 μM miltefosine. The inhibitory and amebicidal concentrations for *N. fowleri* were 40 and 55 μM, respectively. Voriconazole had little or no inhibitory effect on *Balamuthia* at concentrations up to 40 μg/ml but had a strong inhibitory effect upon *Acanthamoeba* spp. and *N. fowleri* at all drug concentrations through 40 μg/ml. The ability of miltefosine and voriconazole to penetrate into brain tissue and CSF and their low toxicity make them attractive possibilities in the treatment of the amebic encephalitides. In combination with other antimicrobials, these two drugs may form the basis of an optimal therapy for treatment of *Acanthamoeba*, *Balamuthia*, and *Naegleria* infections (79). For example, miltefosine in conjunction with other pharmaceuticals has been used in the successful treatment of *Acanthamoeba* (1), *Balamuthia* (4, 15, 83), and *N. fowleri* (84).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Most clinical laboratories rely on the agar plate technique for the isolation and identification of small, free-living, and pathogenic *N. fowleri* and *Acanthamoeba* spp. An agar plate inoculated with patient CSF should be microscopically examined every day for the presence of amebae. If amebae are seen, an area with amebae should be scraped and inoculated into ~1 ml of distilled water and incubated at 37°C and examined every 10 min for 1 h. If flagellates are seen, the clinician should be informed that the CSF is positive for *Naegleria* (possibly *N. fowleri*) amebae. If no flagellates are seen, even after 2 h, then the amebae should be examined under high magnification (×400) for the presence of fine thorn-like processes (acanthopodia). The clinician should then be informed of the presence of amebae, probably *Acanthamoeba*. Wet mount preparations should always be confirmed with permanently stained smears (trichrome, hematoxylin, Giemsa-Wright). If other techniques, like PCR or IFA, are available, then the identity of the amebae should be confirmed. Since many clinical laboratories may not have the necessary expertise and/or equipment, they usually send the specimens to an outside laboratory like the CDC for identification and interpretation. Also, antimicrobial testing is currently not available in most clinical laboratories. Further, it is well known, at least for these free-living amebae, that what works *in vitro* may not always work *in vivo*. For example, fluconazole has no activity *in vitro* against *Balamuthia* but is one of the drugs of choice for treatment. Several patients have survived after receiving fluconazole given along with other drugs; this approach may represent synergistic activities.

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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Intestinal and Urogenital Amebae, Flagellates, and Ciliates

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Entamoeba histolytica and *Giardia duodenalis* infections are two of the most common protozoal infections seen worldwide and are of serious concern on a global scale due to their prevalence and the pathogenicity of their causative agents. Most of the other protozoa described in this chapter are nonpathogenic organisms. Nevertheless, detection and differentiation of nonpathogens from true pathogens in clinical specimens is useful in that their presence indicates exposure to fecal contamination and examination of additional specimens may reveal pathogenic protozoa. The pathogenic and nonpathogenic organisms are categorized as indicated in Table 1; however, reports of disease in patients infected with those organisms considered nonpathogenic are found in the literature.

Microscopic examination of stool specimens continues to be one of the main tools used in the laboratory diagnosis of intestinal amebic, flagellate, and ciliate infections. The goal of microscopy is to identify pathogenic protozoa, differentiate between these and nonpathogenic species, and properly discriminate among various artifacts that may be present. Antigen detection methods such as enzyme immunoassays (EIAs), immunochromatographic assays, and direct fluorescent-antibody (DFA) assays are available for the detection of pathogens such as *E. histolytica*/*Entamoeba dispar* group, *E. histolytica*, and *G. duodenalis*. Culture for the intestinal ameba is generally not feasible, readily available, or clinically relevant except in certain limited situations but is useful still in the diagnosis of *Trichomonas vaginalis*. Nucleic acid-based techniques have been developed, and reports in the literature for select pathogens are increasing. One of the most important developments related to the molecular detection of parasites is the advent of panels for detection of multiple agents of gastrointestinal disease, including combinations of bacterial, viral, and parasitic agents (1). More data from clinical evaluations, particularly related to detection of intestinal parasites, is needed to assess the performance of these systems, but on the whole they appear more sensitive than conventional methods. Multiplex molecular testing is not yet widely available for intestinal parasites, but as more kits are approved by the U.S. Food and Drug Administration (FDA), they will be a viable alternative for many laboratory settings. These assays will be particularly useful to diagnose diseases like amebiasis, for which microscopy does not allow differentiation of pathogenic versus nonpathogenic *Entamoeba* species. See the sections below and refer to chapters 6, 7, and 135 for additional information.

AMEBAE

Taxonomy

The amebae that parasitize the intestinal tracts of humans belong to three genera: *Entamoeba*, *Endolimax*, and *Iodamoeba*. They all belong to the phylum *Amoebozoa*, class *Archamoebea*, and order *Euamoebida*. These organisms move by means of cytoplasmic protrusions called pseudopodia (see chapter 132 of this *Manual*). While *Dientamoeba fragilis* was once classified as an ameba, it is now grouped with the flagellates; however, it is still identified microscopically on the basis of morphologic comparison to amebae.

Description of the Agents

Of the 11 species of intestinal amebae, *E. dispar*, *Entamoeba hartmanni*, *Entamoeba coli*, *Entamoeba polecki*, *Entamoeba gingivalis*, *Endolimax nana*, and *Iodamoeba bütschlii* are nonpathogenic for humans. *Blastocystis hominis* is also considered pathogenic, although there is still some debate.Little is known about the pathogenicity of *Entamoeba moshkovskii* and *Entamoeba bangladeshi*. *E. histolytica* is pathogenic for humans, causing invasive intestinal and extraintestinal amebiasis.

Epidemiology, Transmission, and Prevention

All amebae have a common and relatively simple life cycle. The cyst is the infectious form and is acquired by ingestion of contaminated material such as water and food or by direct fecal-oral transmission. Once the cyst reaches the intestinal tract, excystation occurs, releasing trophozoites. Encystment occurs in the colon, presumably when conditions become unfavorable for the trophozoites. Cysts are passed in the feces and remain viable in the environment for days to weeks in water and soil if protected from desiccation. Improvements in sanitary conditions are necessary to prevent infections in areas where the organisms are endemic. Research to develop a vaccine against *E. histolytica* is ongoing, but none is currently available.

Collection, Transport, and Storage of Specimens

For detection of the amebae, laboratories predominantly receive stool specimens for examination. Both fresh and

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TABLE 1 Intestinal and urogenital amebae, flagellates, and ciliates of humans

Parasite	Pathogenic	Nonpathogenic
Amebae	*Entamoeba histolytica*[a]	*Entamoeba dispar* *Entamoeba moshkovskii*[b] *Entamoeba bangladeshi*[c]
	Blastocystis hominis	*Entamoeba hartmanni* *Entamoeba coli* *Entamoeba polecki* *Entamoeba gingivalis*[d] *Endolimax nana* *Iodamoeba bütschlii*
Flagellates	*Giardia duodenalis* *Trichomonas vaginalis* *Dientamoeba fragilis*	*Chilomastix mesnili* *Pentatrichomonas (Trichomonas) hominis* *Trichomonas tenax*[d] *Enteromonas hominis* *Retortamonas intestinalis*
Ciliates	*Balantidium coli*	

[a] A distinction between *E. histolytica*, *E. dispar*, *E. moshkovskii*, and *E. bangladeshi* cannot be made on the basis of morphology unless ingested RBCs are seen in the cytoplasm of the trophozoite, indicative of *E. histolytica*.

[b] *E. moshkovskii* may be found in human stool specimens more predominantly in certain areas of endemicity. A free-living ameba, it is nonpathogenic and morphologically identical to *E. histolytica/E. dispar* (7, 30).

[c] *E.bangladeshi* has been recently identified from human stool specimens of both symptomatic and asymptomatic individuals. It is morphologically identical to *E. histolytica/E. dispar* (13).

[d] *E. gingivalis* and *T. tenax* are found in the oral cavity and related specimens.

preserved specimens are useful for the diagnosis of infection, depending on the methodology employed and the circumstances of the laboratory. If fresh specimens are received for the detection of organism motility, they must be examined quickly; wet mounts for the detection of motility cannot be performed on preserved specimens. Other sample types, such as aspirates and tissue samples, may be received and are appropriate for testing depending on the organism suspected. For a more detailed description of collection, refer to chapter 133 of this *Manual*.

Direct Examination

Microscopy

All diagnostic stages of the amebae (trophozoite and cyst) can be detected in fecal specimens. The key morphologic features of amebae must be used to differentiate among the various species and to distinguish between somatic cells and other material. Trophozoites must be distinguished from epithelial cells and macrophages. Cysts must be distinguished from polymorphonuclear cells. Also, yeast, pollen, molds, food particles, and other debris present in feces may cause confusion (Table 2; Fig. 1 and 2).

Morphologic examination of fecal specimens can be accomplished with fresh wet mount preparations, wet mounts of concentrated material, and permanent-stained smears. Each of these three types of preparations may be useful for visualizing certain key characteristics. Stained and unstained wet mounts of concentrated material can also be useful for identification, particularly for certain cysts such as those of *E. coli* and *I. bütschlii*. Iodine provides color and contrast, both of which may aid in the identification of organisms in wet preparations. However, morphologic examination with permanent-stained smears by oil immersion microscopy (magnification, ×1,000) is the most useful procedure (2).

Trophozoite motility is visible only in saline wet mounts of fresh feces and is often difficult to detect. The arrangement, size, and pattern of nuclear chromatin help differentiate species within the genus *Entamoeba* from other intestinal amebae. The size and position of the nuclear karyosome are also important morphologic features. A ring of nuclear chromatin surrounding the karyosome, resembling a bull's-eye, is characteristic of *Entamoeba*. *Endolimax*, *Iodamoeba*, and the flagellate *Dientamoeba* lack peripheral chromatin. The cytoplasm of the trophozoites may contain granules and ingested material such as red blood cells (RBCs), bacteria, yeasts, and molds. It is important to note that it is very difficult to differentiate trophozoites of *I. bütschlii* from *E. nana* trophozoites. This is true even on the permanent-stained smear (3). The characteristics of cysts are less variable than those of trophozoites. To aid in differentiation among the genera, the cytoplasm should be examined for the presence of chromatoidal bodies and vacuoles, particularly the large glycogen vacuole seen in *I. bütschlii*.

Evaluation, Interpretation, and Reporting of Results

It is important to remember that identification may not be possible on the basis of one morphologic feature or the characteristics of a single organism in the preparation. Nuclear and cytoplasmic features can vary within species and may overlap between species, making identification challenging. Mixed infections are not uncommon and can be missed in a cursory examination. A complete, overall assessment of the slide is necessary for correct identification. It is important to use an accurate micrometer to measure life cycle stages. Size is reliable only for the differentiation of *E. histolytica/E. dispar* from *E. hartmanni*. Also, on permanent-stained smears, shrinkage may occur, affecting the apparent size of the organism. Results of microscopy should clearly indicate the full taxonomic name of the organisms detected along with the forms of the organisms seen (trophozoites versus cysts). Quantitation of the amebae on the final report is not appropriate.

E. histolytica

Description of the Agent

The development of axenic culture methods was a key step in confirming the existence of two species among organisms that had been identified as *E. histolytica* based solely on microscopic findings. Using organisms obtained by such

TABLE 2 Key features of trophozoites and cysts of common intestinal amebae and *Blastocystis hominis*[a]

Organism	Trophozoites	Cysts
E. histolytica/E. dispar	Size[b]: 12–60 µm; invasive forms, >20 µm Motility: Progressive, directional, rapid Nucleus[c]: 1; peripheral chromatin evenly distributed; karyosome small, compact, centrally located; may resemble *E. coli* Cytoplasm: Finely granular, like "ground glass"; may contain bacteria Note: RBCs in cytoplasm diagnostic for *E. histolytica* infection	Size: 10–20 µm; spherical, centrally located Cytoplasm: Chromatoidal bodies may be present; elongate with blunt rounded edges; may be round or oval
E. hartmanni	Size: 5–12 µm Motility: Nonprogressive Nucleus: 1; peripheral chromatin like *E. histolytica/E. dispar*, may appear as solid ring; karyosome small, compact, centrally located or eccentric Cytoplasm: Finely granular, bacteria, no RBCs Note: Accurate measurement essential for differentiation from *E. histolytica/E. dispar*	Size: 5–10 µm; spherical Nucleus: Mature cyst, 4; immature cyst, 1 or 2 (very common); peripheral chromatin fine, evenly distributed, may be difficult to see; karyosome small, compact, centrally located Cytoplasm: Chromatoidal bodies usually present, like in *E. histolytica/E. dispar*
E. coli	Size: 15–50 µm Motility: Sluggish, nondirectional Nucleus: 1; peripheral chromatin clumped and uneven, may be solid ring; karyosome large, not compact, diffuse, eccentric Cytoplasm: Granular, usually vacuolated; contains bacteria, yeast, no RBCs Note: Can resemble *E. histolytica/E. dispar*; coinfection seen; stained smear essential	Size: 10–35 µm; spherical, rarely oval or triangular Nucleus: Mature cyst, 8; occasionally ≥16; immature cyst, ≥2; peripheral chromatin coarsely granular, unevenly arranged; may resemble *E. histolytica/E. dispar*; karyosome small, usually eccentric but may be central Cytoplasm: Chromatoidal bodies less frequent than in *E. histolytica/E. dispar*; splintered, with rough, pointed ends Note: May be distorted on permanent-stained smear due to poor penetration of fixative
E. nana	Size: 6–12 µm Motility: Sluggish, nonprogressive Nucleus: 1; no peripheral chromatin; karyosome large, "blot like" Cytoplasm: Granular, vacuolated; may contain bacteria Note: May be tremendous nuclear variation; can mimic *E. hartmanni* and *D. fragilis*	Size: 5–10 µm; oval, may be round Nucleus: Mature cyst, 4; immature cyst, 2; no peripheral chromatin; karyosome smaller than those in trophozoites but larger than those in *Entamoeba* spp. Cytoplasm: Chromatoidal bodies rare; small granules occasionally seen
I. bütschlii	Size: 8–20 µm Motility: Sluggish, nonprogressive Nucleus: 1; no peripheral chromatin; karyosome large, may have "basket nucleus" Cytoplasm: Coarsely granular, may be highly vacuolated; bacteria, yeast, and debris may be seen Note: Stained smear essential; nucleus may appear to have a halo with chromatin granules fanning around karyosome	Size: 5–20 µm; oval to round Nucleus: Mature cyst, 1; no peripheral chromatin; karyosome large, usually eccentric Cytoplasm: No chromatoidal bodies; small granules occasionally present Note: Glycogen present; large, compact, well-defined mass; cysts may collapse owing to large glycogen vacuole space
B. hominis[d]	Very difficult to identify; rarely seen	Size: 2–200 µm; generally round Description: Usually characterized by a large, central body (looks like a large vacuole) surrounded by small, multiple nuclei; central body area can stain various colors (trichrome) or remain clear

[a] Adapted from reference 3.

[b] Size ranges are based on wet preparations (with permanent stains, organisms usually measure 1 to 2 µm less).

[c] Nuclear and cytoplasmic descriptions are based on permanent-stained smears.

[d] Description of central body form.

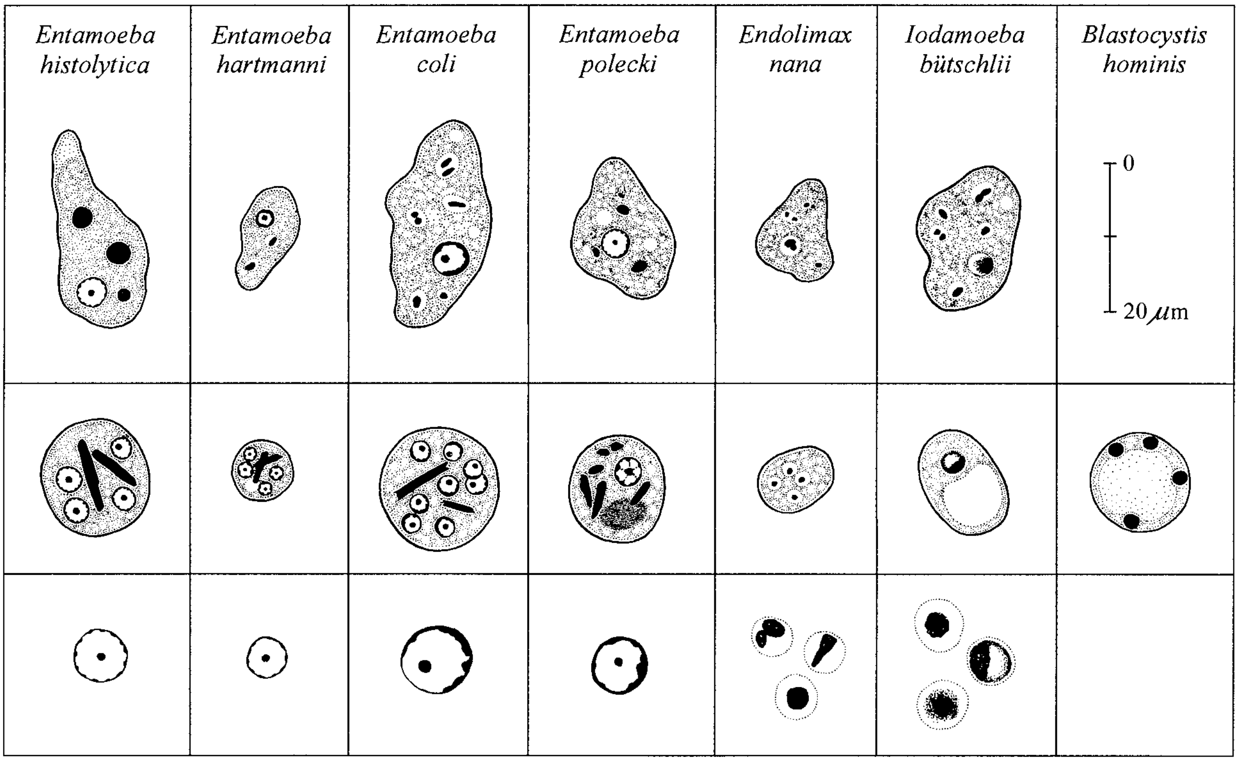

FIGURE 1 Intestinal amebae of humans. (Top row) Trophozoites. *E. histolytica* is shown with ingested RBCs. This is the only microscopic finding that allows differentiation of the pathogenic species *E. histolytica* from the nonpathogenic species *E. dispar*. An ameboid form of *B. hominis* is rarely seen and is difficult to identify. (Middle row) Cysts. For *B. hominis* the central body form is depicted. (Bottom row) Trophozoite nuclei, shown in relative proportion. doi:10.1128/9781555817381.ch140.f1

cultures, Sargeaunt and Williams (4) performed isoenzyme analysis of several glycolytic enzymes and identified electrophoretic banding patterns, or zymodemes. Two groups were identified on the basis of these patterns: pathogenic zymodemes (invasive isolates) and nonpathogenic zymodemes (noninvasive isolates). The zymodeme patterns represent stable genetic differences and do not interconvert (5). Additional genetic, biochemical, and immunologic evidence has supported the existence of two distinct species. Diamond and Clark (6) redescribed the two species as *E. histolytica* Schaudinn 1903, which is the invasive human pathogen, and *E. dispar* Brumpt 1925, which is noninvasive and does not cause disease.

A third species of *Entamoeba*, *E. moshkovskii*, has been recognized and is generally considered a free-living ameba that may be detected in human stool specimens. It is morphologically indistinguishable from *E. histolytica*/*E. dispar*. The epidemiology of *E. moshkovskii* is not well understood. The prevalence varies depending on the population studied from relatively rare up to nearly 50% of the identified *Entamoeba* species found in stool (7), and coinfections with *E. histolytica* and *E. dispar* are not uncommon (8). It has been reported as an agent associated with gastrointestinal disease in the absence of other known causes (9–11), and newly documented acquisition of *E. moshkovskii* in children has been associated with diarrhea (12). Most recently, Royer et al. (13) reported a fourth species, *E. bangladeshi* nov. sp., Bangladesh, which is also morphologically indistinguishable from *E. histolytica*/*E. dispar*. It was identified in stool specimens from symptomatic and asymptomatic children in Bangladesh; specimens were positive for *E. histolytica*/*E. dispar* by microscopy but negative by PCR targeting *E. histolytica*, *E. dispar*, and *E. moshkovskii*. More research is needed to understand the role of both *E. moshkovskii* and *E. bangladeshi* in human disease and the importance of laboratory diagnosis (7, 13, 14).

Epidemiology, Transmission, and Prevention

E. histolytica can be found worldwide but is more prevalent in tropical and subtropical regions. In areas where the organism is endemic, up to 50% of people may be infected. In temperate climates with poor sanitation, infection rates can approach those seen in tropical regions. Humans are the primary reservoir; infection occurs by ingestion of cysts from material contaminated with feces such as water and food. Sexual transmission also occurs.

Asymptomatic *E. histolytica* infection is equally distributed between the genders, while invasive amebiasis affects men predominantly. In the United States, groups with a higher incidence of amebiasis include immigrants from South and Central America and Southeast Asia. Also, residents of the southern United States and institutionalized individuals are more likely than others to be infected. In one study, short-term travelers to areas where *E. histolytica* and *E. dispar* are endemic were found to be at higher risk of infection with the pathogenic species, *E. histolytica*, than

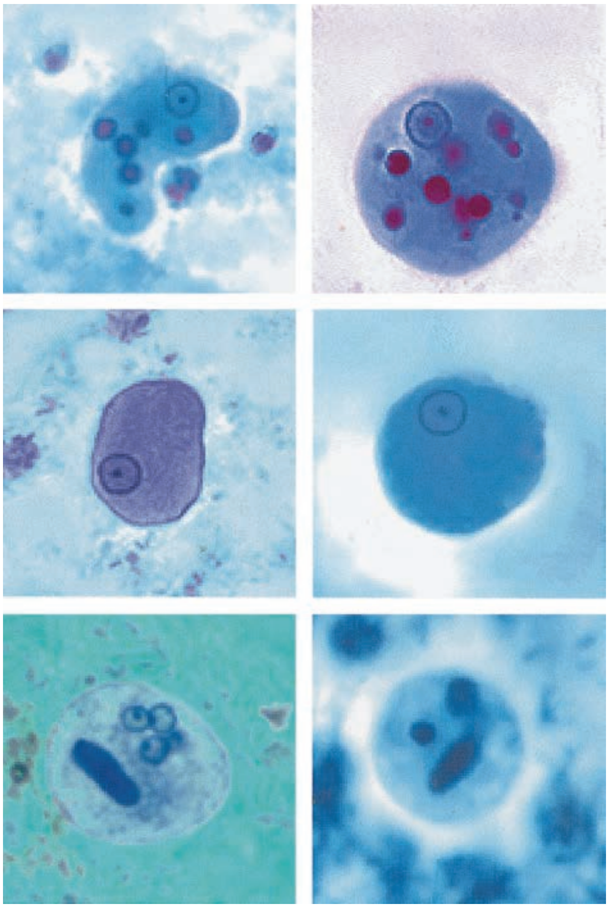

FIGURE 2 (Top row) *Entamoeba histolytica* trophozoites. Note the ingested RBCs in the cytoplasm. (Middle row) *E. histolytica/Entamoeba dispar* trophozoites. Note the absence of RBCs in the cytoplasm. (Bottom row) *E. histolytica/E. dispar* cysts. These cysts cannot be identified to the species level on the basis of morphology. Organisms are stained with Wheatley's trichrome stain. Courtesy of L. Garcia.
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were residents, who were more likely to harbor the nonpathogenic species, *E. dispar* (15). In homosexual males, the infection is often transmitted by sexual behavior, with up to 30% found to be infected with *E. histolytica/E. dispar* in certain studies (16). These infections are most often asymptomatic. Among HIV-infected patients in the United States, the incidence of diagnosed *E. histolytica* disease is low (13.5 cases per 10,000 person-years) (17). HIV-infected individuals in non-Western countries, such as Taiwan and Korea, do have a higher risk of invasive amebiasis, in contrast to findings from the United States (18, 19). These differing rates of invasive disease may be attributed to the higher endemicity of *E. histolytica* in the Asia-Pacific regions (20).

Clinical Significance

Among the estimated 500 million people infected each year with *E. histolytica*, there are ~50 million cases of colitis and liver abscess and ~100,000 deaths (21). The discrepancy between the number of people infected with *E. histolytica* and the morbidity and mortality rates is explained by the existence of two morphologically similar yet distinct species: one capable of producing disease (*E. histolytica*) and the other not (*E. dispar*). *E. dispar* appears to be at least 10 times more common than *E. histolytica* (14). A more precise determination of prevalence is possible using newer molecular methods to detect and differentiate among the species of *Entamoeba* and may change our understanding of the epidemiology of these infections (22).

Infection with *E. histolytica/E. dispar* can result in different clinical presentations: asymptomatic infection, symptomatic infection without tissue invasion, and symptomatic infection with tissue invasion. The majority of infections with *E. histolytica/E. dispar* are asymptomatic. Individuals with such infections will have a negative or weak serologic response and will primarily pass cysts in their stools. Zymodeme analysis shows that most asymptomatic individuals are infected with the noninvasive species *E. dispar* (21, 23). However, it appears that infection with both *E. histolytica* and *E. dispar* can be asymptomatic, with cyst stages being passed in the stool (24). Asymptomatic *E. histolytica* infection may be due to the existence of genetically distinct invasive and noninvasive strains of *E. histolytica* (25).

Intestinal disease results from the penetration of the amebic trophozoites into the intestinal tissues. Approximately 10% of infected individuals have clinical symptoms, presenting as dysentery, colitis, or, rarely, ameboma. The incubation period varies from a few days to several months. Various molecules such as adhesins, amebapores, and proteases have been associated with lysis of the colonic mucosa in intestinal amebiasis (26), and evidence supports the role of caspase-3-dependent apoptotic death as a major mechanism of host cell destruction (27). The 260-kDa galactose- or *N*-acetylgalactosamine-specific lectin of *E. histolytica* is an important virulence factor, mediating the attachment of the ameba to the intestinal epithelium and contact-dependent cytolysis (28). Symptoms of amebic dysentery include diarrhea with cramping, lower abdominal pain, low-grade fever, and the presence of blood and mucus in stool. The ulcers produced by intestinal invasion by trophozoites start as superficial localized lesions that deepen into the classic flask-shaped ulcers of amebic colitis. The ulcers are separated by segments of normal tissue but can coalesce. Amebae can be found at the advancing edges of the ulcer but usually not in the necrotic areas. Abdominal perforation and peritonitis are rare but serious complications. A more chronic presentation occurs with amebic colitis. It is characterized by intermittent diarrhea over a long period and can be misdiagnosed as ulcerative colitis or irritable bowel syndrome. Ameboma, a localized tumor-like lesion, results from chronic ulceration and may be mistaken for malignancy. Histologically, it consists of granulomatous tissue.

Extraintestinal disease occurs with the hematogenous spread of the organism. It can occur with or without previous symptomatic intestinal infection. The liver is the most common site of extraintestinal disease, followed by the lungs, pericardium, brain, and other organs including skin. Symptoms can be acute or gradual and may include low-grade fever, right-upper-quadrant pain, and weight loss. Up to 5% of individuals with intestinal symptoms develop liver abscess. However, up to 50% of individuals with liver abscess have no history of gastrointestinal disease.

Direct Examination

The laboratory diagnosis of amebiasis can be made by the examination of feces, material obtained from sigmoidoscopy, tissue biopsy specimens, and abscess aspirates. Serologic testing is also useful for the diagnosis of extraintestinal amebiasis. The choice of methods used by each laboratory is

TABLE 3 Sensitivity and specificity of diagnostic tests for amebiasis[a]

Test and specimen type	Colitis		Liver abscess, sensitivity (%)
	Sensitivity (%)	Specificity (%)	
Microscopy			
Stool	>60	10–50	<10
Abscess fluid	NA[b]	NA	<25
Culture with isoenzyme analysis	Lower than antigen or PCR tests	Gold standard	<25
Stool antigen detection (ELISA)[c]	>95	>95	Usually negative
Serum antigen detection (ELISA)	65 (early)	>90	~75 (late); ~100 (first 3 days)
Abscess antigen detection (ELISA)	NA	NA	~100 (before treatment)
Saliva antigen detection	Not done	Not done	70
PCR, stool	70–>95[d]	>90	Not done
Serum antibody detection (ELISA)	>90	>85	70–80 (acute stage); >90 (convalescent stage)

[a] Adapted from reference 30 with permission.
[b] NA, not available.
[c] ELISA, enzyme-linked immunosorbent assay.
[d] Sensitivities of PCR assays are dependent on comparator methods and PCR techniques, with real-time PCR demonstrating increased sensitivity over conventional PCR.

dependent on the available resources, funding, and clinical need. A summary of the laboratory techniques and their performance characteristics is presented in Table 3.

Microscopy

As discussed above, the most important part of the standard microscopic examination of stool and other specimens is the permanent-stained smear. Direct wet preparations and concentration procedures may also be useful (Fig. 1 and Table 2; see also chapter 135). Detection of trophozoites and cysts does not, however, allow differentiation of the pathogenic species, *E. histolytica*, from the nonpathogenic species, *E. dispar* (Fig. 1 and 2). The presence of ingested RBCs in the cytoplasm of the trophozoites is commonly regarded as diagnostic of *E. histolytica* infection. However, the majority of patient samples do not contain trophozoites with ingested RBCs (14). In addition to concerns about sensitivity, Haque et al. (29) found that 16% of *E. dispar* isolates had ingested RBCs; thus, this distinction between the two species is not absolute and may affect specificity (30, 31). In tissue specimens, only the trophozoite is found, and its presence is considered diagnostic of invasive *E. histolytica* disease.

Antigen Detection

For a more definitive differentiation of *E. histolytica* and *E. dispar*, methods other than microscopy are necessary. Zymodeme analysis can accomplish this differentiation, but it requires culture of the organisms from the specimen and is too expensive and complex for routine laboratory use. Antigen-based methods for the detection of *E. histolytica*/*E. dispar* are commercially available in the United States, including one that detects *E. histolytica*/*E. dispar*, *G. duodenalis*, and *Cryptosporidium parvum* in an immunochromatographic cartridge format (32). The antigen assays offer a more sensitive method for detection than microscopy and, depending on the kit used, allow specific detection of *E. histolytica* (14). A listing of commercially available parasite antigen detection kits is given in chapter 133 and has been recently reviewed (14).

The antigen detection assays are designed for use with fresh, fresh-frozen, or unfixed human fecal specimens. As mentioned previously, depending on the kit used, the group *E. histolytica*/*E. dispar* or the individual species can be detected in the feces. Of the available kits, only TechLab (Blacksburg, VA) offers an *E. histolytica*-specific EIA kit, *E. histolytica* II, which detects the Gal- or GalNAc-binding lectin specific to the pathogenic species (29, 33). A rapid point-of-care test that is specific for *E. histolytica* adherence lectin (*E. histolytica* Quik Chek; TechLab) has been reported and would be useful particularly in the developing world; this test is not yet available commercially either in or outside the United States (34, 35). All the antigen detection methods are relatively simple and are more sensitive and specific than microscopy (Table 3) (29, 32, 36). In comparison to amplified methods such as PCR, antigen detection using stool specimens may be less sensitive and specific for detection of *E. histolytica*/*E. dispar*, depending on the population studied (37, 38). To date, none of the available products can be used with preserved stool (i.e., formalin), as the fixative appears to denature the antigens. Although some reports of the use of preserved stool have appeared (39), more work is needed to identify additional antigens that withstand fixation before it would be practical to offer antigen tests in laboratories that receive only preserved stool specimens. Antigen assays have been used to test a number of other sample types such as serum, pus, and saliva (40, 41); the detection of antigen in serum may prove to be a sensitive means of diagnosing amebic liver abscess and intestinal disease (30, 41). Because <10% of patients with amebic liver abscesses have concurrent intestinal disease with amebae detectable in the stool, methods such as routine ova and parasite examination are not useful. Microscopic examination or culture of pus from liver abscesses likewise lacks sensitivity. In a study by Haque et al. (41), serum antigen detection by using the TechLab *E. histolytica* II kit was a sensitive method for diagnosis with samples collected prior to treatment with metronidazole. Serum antigenemia appears to clear after treatment, suggesting possible utility to monitor therapy; however, this use is still experimental (30, 41).

Nucleic Acid Detection Techniques

Nucleic acid amplification techniques, such as PCR, have been developed for the detection and differentiation of *E. histolytica* and *E. dispar*. The most common genomic targets include the rRNA- or species-specific episomal repeats. Conventional PCR has been applied to specimens such as stool, liver, or brain aspirates and cerebrospinal fluid and can detect both trophozoite and cyst DNA (42–46). More recently, real-time PCR has been used for the detection of *E. histolytica* and *E. dispar* (37, 47). Haque et al. (48) recently reported on the use *E. histolytica* real-time PCR on blood, saliva, and urine samples for detection of amebic liver abscess and amebic colitis, finding that a combination of testing both urine and saliva increased diagnostic sensitivity, with 97 and 89% of amebic liver abscess and colitis cases detected, respectively; blood was less sensitive, being positive in 49% of amebic liver abscess cases and 36% of colitis cases examined. Several researchers have reported methods for multiplex molecular detection of and differentiation among the *Entamoeba* species, including *E. histolytica*, *E. dispar*, and *E. moshkovskii* (14, 42, 49, 50). Use of such a multiplex assay would permit a more accurate diagnosis than microscopy and allow targeted therapy for only true *E. histolytica* infections. As mentioned previously, simultaneous detection from stool of multiple intestinal parasites or multiple organisms, including bacteria and viruses, is of interest as there is significant overlap in the clinical presentations. Reports include detection of various combinations, including *E. histolytica*, *G. duodenalis*, and *C. parvum* using real-time PCR (51) and *E. histolytica*, *G. duodenalis*, *Cryptosporidium* spp., and *D. fragilis* using tandem multiplex real-time PCR (52). At the time of writing, there is one FDA-cleared multiplex molecular test, xTAG Gastrointestinal Panel (Luminex Molecular Diagnostics, Austin, TX), for testing on fresh or frozen stool samples, which detects 11 agents of gastroenteritis, including the parasites *E. histolytica*, *G. duodenalis*, and *Cryptosporidium* spp. It is a bead-based assay utilizing nucleic acid extraction followed by endpoint PCR amplification and bead-based detection. Initial reports demonstrate the utility of the test, but the numbers of *E. histolytica* infections detected is low (1, 53). As more data are generated and other FDA-cleared multiplex molecular tests are made available, this approach will have a significant impact on many aspects of laboratory diagnosis of amebiasis.

PCR on stool specimens has proven to be more sensitive and specific than microscopy and at least as sensitive as antigen detection, depending on the study (Table 3) (30, 37, 54). For routine use in clinical laboratories, a PCR method would ideally involve a relatively simple sample preparation procedure and allow the use of preserved material. Different extraction techniques, some of which are relatively simple, and the use of fresh and preserved material have been reported (55–57). Stool can present challenges due to the presence of PCR inhibitors (58), so the inclusion of an amplification control is useful (51).

Serologic Tests

Serologic testing is a valuable aid, in conjunction with antigen detection or PCR, for the diagnosis of symptomatic, invasive disease. Multiple serologic methods have been used, including indirect hemagglutination, complement fixation, latex agglutination, and EIA (14, 30). These methods are most useful in populations where *E. histolytica* is not endemic. Of patients with biopsy-proven intestinal amebiasis, 85% have serum antibodies. For patients with extraintestinal disease, serologic tests have a sensitivity approaching 99%. For asymptomatic intestinal diseases, serology is generally not useful unless the patient has invasive infection. Persons infected with *E. dispar* do not produce detectable antibodies. After cure of invasive amebiasis, serum antibodies may persist for up to 10 years; this can complicate diagnosis in areas where infection is endemic (59).

EIAs for the detection of IgM and IgG are widely used, and most are based on the detection of antilectin antibodies, which appear over 1 week after symptoms of *E. histolytica* infection (30). Indirect hemagglutination is useful and highly specific but may lack sensitivity compared to EIAs (30). Several investigators have reported the utility of IgA antibody testing and a link to partial immunity in individuals with detectable antilectin IgA (41, 60, 61). There are several commercially available serologic kits for diagnosis of amebiasis, some of which are available in the United States. For a review of serologic testing, see the article by Fotedar et al. (14) and also refer to chapter 7 in this *Manual*.

Treatment

On the basis of the 1997 World Health Organization conference, treatment is not recommended for *E. dispar* infections; if *E. histolytica*/*E. dispar* is detected (no differentiation of species) in symptomatic patients, the physician must evaluate the total clinical presentation to decide whether treatment is indicated. The detection of *E. histolytica* requires treatment of the patient regardless of the symptoms. The use of diagnostics that are species specific (i.e., antigen detection or PCR) allows for targeted chemotherapy.

The drugs used for the treatment of amebiasis are of two classes: luminal amebicides for cysts (paromomycin, iodoquinol, and diloxanide furoate) and tissue amebicides for trophozoites (metronidazole, tinidazole, and dehydroemetine) (62). Invasive disease should be treated with a tissue amebicide followed by a luminal amebicide. Tissue amebicides are not appropriate for treatment of asymptomatic infections (cysts). No high-level resistance to amebicides has been detected to date. Follow-up stool examination is always necessary because of potential treatment failures. Chemoprophylaxis is never appropriate because it may lead to drug resistance and limit the utility of drugs such as metronidazole (21, 63).

Evaluation, Interpretation, and Reporting of Results

Laboratory reporting of *Entamoeba* infection must account for the ability of a particular methodology to detect and differentiate pathogenic and nonpathogenic species. This is based on the report of a World Health Organization panel of experts that made recommendations concerning the reporting and treatment of amebiasis (21). If a microscopic diagnosis is made on the basis of the detection of trophozoites and/or cysts and no method is used to differentiate the two species, the report should indicate "*E. histolytica*/*E. dispar* detected." Laboratory reports must indicate whether trophozoites and/or cysts are present, due to differences in therapy (3).

With the increasing awareness of other *Entamoeba* species, reporting as *E. histolytica*/*E. dispar* complex (*E. histolytica*, *E. dispar*, *E. moshkovskii*, and *E. bangladeshi*) might be considered, but care must be taken to convey the proper interpretation of these results, reflecting the lack of differentiation of the pathogenic and nonpathogenic species. Laboratories may also choose to further test stools with *E. histolytica*/*E. dispar* detected with an *E. histolytica*-specific test such as antigen or PCR. This reflexive testing could allow more specific and appropriate treatment and be a cost-effective testing algorithm.

Antigen detection methods may or may not allow species differentiation, and the laboratory report should accurately reflect these facts. Reporting of PCR results that are specific for *E. histolytica* or other *Entamoeba* species such as *E. dispar* should state that nucleic acid was detected or not detected. Due to the ability of amplified methods to detect nonviable organisms, the use of PCR for determination of treatment failure should be interpreted cautiously. PCR does not allow for the determination of the parasite forms present, in contrast to the TechLab *E. histolytica* II.

Blastocytis hominis

Taxonomy

Since its first description in 1912, the taxonomic classification of *B. hominis* has changed and is still somewhat unclear (64, 65). Current classification of *Blastocystis* is as follows: kingdom *Chromista*, phylum *Bigyra*, class *Blastocystea*, and order *Blastocystida* (see chapter 132). Molecular studies indicate that *Blastocystis* is closely related to *Proteromonas lacertae* (66–68). Though *P. lacertae* is a flagellate, interestingly, *B. hominis* does not possess a flagellum and is nonmotile. Genetic, biochemical, and immunologic analyses have revealed that great diversity exists within the species (69–71). Recent studies have shown that *Blastocystis* spp. in humans and animals can be divided into 12 or more species (71).

Description of the Agent

The life cycle of *B. hominis* includes vacuolar and fecal cyst forms, granular forms, and ameboid forms. *B. hominis* is capable of pseudopod extension and retraction. The exact nature of the life cycle of this organism and the infective form has yet to be confirmed experimentally. The cyst form can be varied depending on whether the cyst is observed in fresh stool or in culture (68). *B. hominis* produces pseudopods and reproduces by binary fission or sporulation. It is strictly anaerobic and is capable of ingesting bacteria and other debris. The membrane-bound central body occupies up to 90% of the cell and may function in reproduction. Both thin- and thick-walled cysts have been observed. The classic form is usually seen in human stool specimens; it characteristically contains a large central body or vacuole. The more amebic form is more difficult to see in human specimens and has been seen in diarrheal stool specimens (3).

Epidemiology, Transmission, and Prevention

B. hominis is a common intestinal parasite of humans and animals, with a worldwide distribution (68). Depending on the geographic location, it may be detected in 1 to 40% of fecal specimens. Human-to-human, animal-to-human, and waterborne modes of transmission have been proposed (68, 71–73). The organism is polymorphic, consisting of four major forms. The thick-walled cysts are thought to be responsible for external transmission via the fecal-oral route; the thin-walled cysts are thought to cause autoinfection (74). The cyst form is the most recently described form of the life cycle stages. The cysts can vary in shape but are mostly ovoid or spherical. The central vacuole form (also referred to as the central body form) is the most common form found in clinical stool samples. The large central vacuole can occupy the majority of the cellular volume. The ameboid form is rarely reported, and the granular form can be seen when culturing the parasite.

Clinical Significance

The role of *B. hominis* in human disease is still controversial, and studies supporting and refuting its pathogenicity have been published (75–77). Most recent epidemiological studies point to the fact that *B. hominis* is a pathogen. Since many genotypes appear to exist, there is strong evidence that there are pathogenic and nonpathogenic species (68). Regardless of the number of publications on each side of this debate, clinicians may decide to treat patients with *B. hominis* infection. When *B. hominis* is present in large numbers in the absence of other pathogens, it may be the cause of gastrointestinal disease. The most common symptoms cited include recurrent diarrhea without fever, vomiting, and abdominal pain. The symptoms may be more pronounced and prolonged in patients with underlying conditions such as HIV infection, neoplasia, and abnormal intestinal tract function (78, 79). Other studies suggest that symptomatic patients receiving treatment for *B. hominis* infection may improve due to the elimination of another, undetected pathogen (75). In a study of HIV-infected individuals, Albrecht et al. (80) concluded that, even in patients with severe underlying immunodeficiencies, *B. hominis* is not pathogenic and its detection does not justify treatment.

Direct Examination

Microscopy

Diagnosis of infection is made by detection of the organism, typically the central body form, by routine microscopic stool examination (Table 2; Fig. 1 and 3). The size of the central body form can vary tremendously, from 2 to 200 µm. Extensive size variation exists within and between isolates. The vacuolar form contains a large central vacuole that occupies ~90% of the cell (68, 81). Examination of permanent-stained smears is the procedure of choice. Exposure to water before fixation (for the concentration method) will lyse the trophozoites and central body forms, yielding false-negative results. Some type of quantitation (few, moderate, or many) should be included in the laboratory report. Direct wet mounts using iodine as a stain are not recommended, as trichrome staining is more sensitive (68).

Other Diagnostic Methods

Culture of the organism from stool is possible and has been reported to be more sensitive than microscopy but is not routinely available (82). A serologic response to *B. hominis* has been detected using techniques such as EIA and fluorescent-antibody testing (68, 83, 84). It is suggested that this antibody response supports the role of *B. hominis* as a human pathogen but is not useful for diagnosis and should be limited to

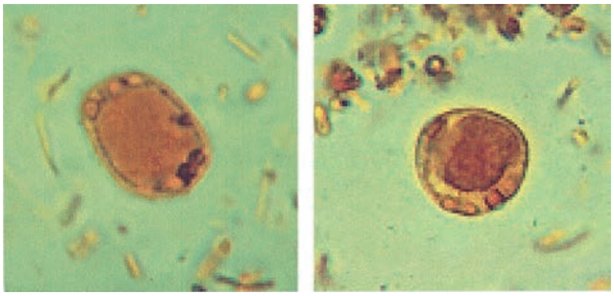

FIGURE 3 *Blastocystis hominis* on an iodine wet mount preparation. Note the peripheral nuclei and the central body form. Courtesy of L. Garcia. doi:10.1128/9781555817381.ch140.f3

epidemiological and serologic studies. In asymptomatic individuals, a serologic response may require exposure of up to 2 years before it is detectable (85). A review by Tan (68) describes in detail molecular approaches to the diagnosis and characterization of *B. hominis*. Molecular approaches to organism detection might be more sensitive based on some studies but are still not routinely available in the clinical laboratory setting (86–88).

Treatment

Until the role of *B. hominis* as an intestinal pathogen is clearly established, treatment decisions must be based on the overall clinical presentation. Current recommendations for treatment include the use of metronidazole, iodoquinol, or trimethoprim-sulfamethoxazole (62). Metronidazole appears to be the most appropriate choice at present. *In vitro* data on the susceptibility of *B. hominis* to various drugs are limited (77), but resistance to metronidazole has been reported (89). A failure to clear organisms from the stool has been demonstrated in patients treated with metronidazole and trimethoprim-sulfamethoxazole.

Evaluation, Interpretation, and Reporting of Results

For most laboratories, testing and reporting for *B. hominis* is limited to detection of the organism by microscopic means. There can be confusion in identifying *Blastocystis* correctly, since the organism can be confused with yeast, fat globules, and even *Cyclospora* spp. It is not necessary to state the form of the organism present (e.g., central body form). This is the sole intestinal parasite that should be routinely reported with quantitation to aid clinicians in determining its clinical significance. Additional commentary may be added to the laboratory report explaining the role of *B. hominis* in disease and the need to exclude other causes for the clinical condition.

NONPATHOGENIC AMEBAE

The other species of intestinal amebae are considered nonpathogenic; except for *E. polecki*, they have a worldwide distribution and are more prevalent in warmer climates. They must, however, be differentiated from the pathogenic species, *E. histolytica*. A permanently stained smear is often essential to accomplish this goal (Table 2; Fig. 1).

E. hartmanni is a separate species that is morphologically similar to *E. histolytica/E. dispar* (Fig. 4). Size is the key differentiating characteristic. *Entamoeba coli* trophozoites may be difficult to differentiate from *E. histolytica/E. dispar* trophozoites on wet preparations. The mature cyst of *Entamoeba coli* may be refractory to fixation, making it less visible in permanent-stained smears but still detectable by the wet mount method (Fig. 5). It has been reported as the most common ameba isolated from human stool specimens. *E. polecki* is associated with pigs, and in certain areas of the world, such as Papua New Guinea, it is the most common human intestinal parasite. The trophozoite shares characteristics with both *E. histolytica/E. dispar* and *Entamoeba coli*; the cyst normally has one nucleus. *E. gingivalis* was the first parasitic ameba of humans to be described. It is found in the soft tartar between teeth and can be recovered from sputum. In the trophozoite, the cytoplasm often contains ingested leukocytes. A cyst form has not been observed.

E. nana, like *E. hartmanni*, is one of the smaller intestinal amebae. It is seen in most populations as frequently as *Entamoeba coli*. There is a great deal of nuclear variation, and it can mimic *D. fragilis* and *E. hartmanni* (Fig. 6). The cysts of *E. nana* are usually oval, and both the trophozoites and the cysts are commonly present in fecal material. *I.*

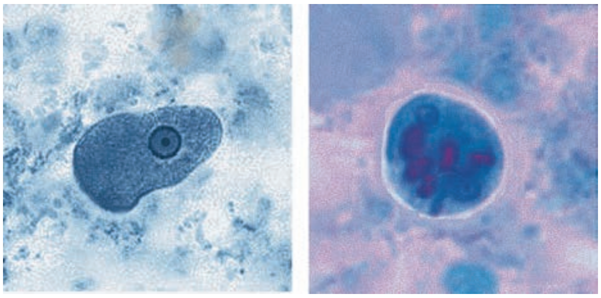

FIGURE 4 *Entamoeba hartmanni* trophozoite (left) and cyst (right). Note the "bull's-eye" nucleus in the trophozoite and the numerous chromatoidal bars within the cyst. Organisms are stained with Wheatley's trichrome stain. Courtesy of L. Garcia. doi:10.1128/9781555817381.ch140.f4

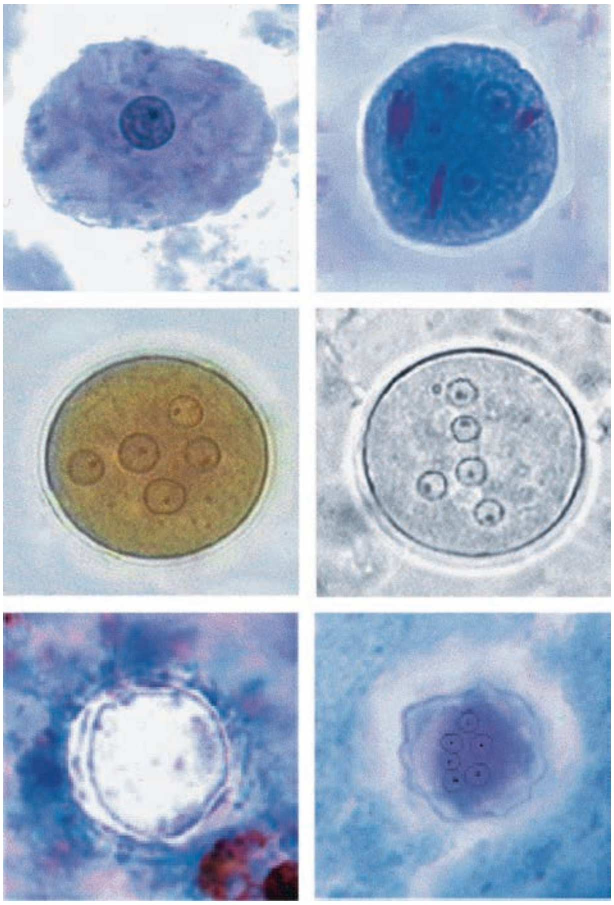

FIGURE 5 (Top row) *Entamoeba coli* trophozoite (left; trichrome) and cyst (right; trichrome). Note the large eccentric karyosome in the trophozoite and the chromatoidal bars with sharp pointed ends in the cyst. (Middle row) *Entamoeba coli* cyst in iodine (left) and in saline (right). The nuclei are visible; the presence of five or more nuclei is confirmatory for *Entamoeba coli*. (Bottom row) *Entamoeba coli* cyst (left; trichrome) and cyst (right; trichrome). Both cysts are very distorted and shrunken, although the nuclei can be seen in the cyst on the right. This type of shrinkage is typical of *Entamoeba coli* cysts on permanent-stained smears. Courtesy of L. Garcia. doi:10.1128/9781555817381.ch140.f5

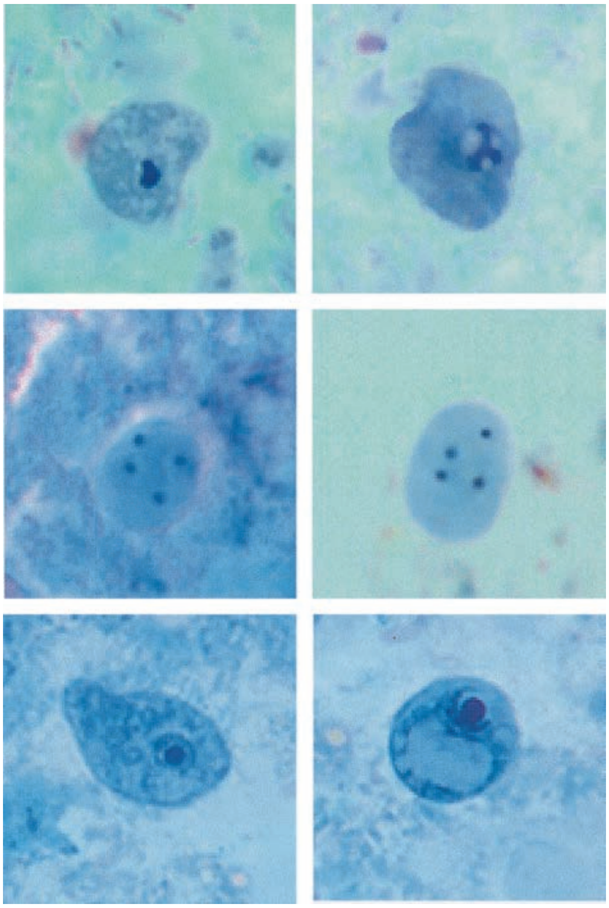

FIGURE 6 (Top row) *Endolimax nana* trophozoites. Note the typical nucleus on the left and the nuclear variation on the right. (Middle row) *E. nana* cysts. Note the typical four nuclei, none of which have peripheral chromatin. (Bottom row) *I. bütschlii* trophozoite (left) and cyst (right). Note the "basket nucleus" in the cyst, along with the large glycogen vacuole. Organisms are stained with Wheatley's trichrome stain. Courtesy of L. Garcia.
doi:10.1128/9781555817381.ch140.f6

bütschlii has the same distribution as other nonpathogenic amebae but is less common than *Entamoeba coli* and *E. nana*. The trophozoite of *I. bütschlii* may be similar to that of *E. nana*, and differentiation between them is difficult. The cyst is very characteristic; it is round to oval and may contain a large glycogen vacuole (Fig. 6).

Treatment is not recommended for infections with any of the nonpathogenic amebae. Methods of prevention of infection with all these amebae include improved personal hygiene and improved sanitary conditions.

FLAGELLATES

Taxonomy

There are six genera of flagellates that parasitize the intestinal tracts of humans; they belong to phylum *Metamonada*, class *Trichomonadea* (*Dientamoeba* and *Trichomona*), class *Trepomonadea* (*Giardia* and *Enteromonas*), and class *Retortamonadea* (*Chilomastix* and *Retortamonas*) (Fig. 7; see chapter 132).

Description of the Agents

Some flagellates are commensals that reside in the intestinal tract and are harmless to the individual. The flagellates that are pathogenic to humans include *G. duodenalis*, *D. fragilis*, and *T. vaginalis*. The nonpathogenic organisms are *Chilomastix mesnili*, *Enteromonas hominis*, *Retortamonas intestinalis*, and *Pentatrichomonas (Trichomonas) hominis*. As mentioned above, *D. fragilis* has been reclassified as a flagellate and appears to be closely related to the trichomonads.

Epidemiology, Transmission, and Prevention

Transmission of flagellates, with the exception of *T. vaginalis*, is initiated via the ingestion of contaminated food or water. Of the members of the six genera, all except *D. fragilis* and *Trichomonas* spp. are transmitted in a cyst form. To date, only a trophozoite form has been observed for *D. fragilis* and *Trichomonas* spp., though for *D. fragilis* the presence of a cyst form is being challenged. Once in the intestine, the organism excysts, releasing trophozoites that attach to the intestinal epithelium. Completion of the life cycle in humans culminates in the release of viable cysts into the environment via the feces. Infection with any of the flagellates indicates exposure to feces regardless of the pathogenicity of the organism; preventing this exposure is the key. Measures to improve sanitary conditions are necessary to prevent the spread of infection. As yet, there are no human flagellate vaccines available.

Collection, Transport, and Storage of Specimens

For the detection of flagellates and ciliates, laboratories predominantly receive stool specimens for microscopic examination. String test samples (Entero-Test; Nutri-Link Ltd., Newton Abbot, United Kingdom) can be submitted for *Giardia* as well, which is described in detail below. Fresh or preserved specimens can be submitted, but preserved stool is the specimen of choice for most routine clinical laboratories. If fresh specimens are submitted and observed for motility, they must be examined within a specific time period depending on the consistency of the stool. Stool specimens for immunoassay or DFA assay can be fixed in formalin, which can also be used for an ova and parasite exam if immunoassays are negative for *G. duodenalis*. Additional information on specimen collection and transport can be found in chapter 133 of this *Manual*.

Direct Examination

Microscopy

In the clinical laboratory, wet preparations and permanent-stained smears of fecal material are still the predominant specimens used to diagnose infections with flagellates. The flagellates have greater morphologic diversity relative to one another than do the amebae (described above), making determination of the genus easier (Table 4; Fig. 7). To aid in the identification of the trophozoite, key features to be noted are the shape, size, number, and position of flagella; the number of nuclei; and the presence of a spiral groove, cytostome, and characteristic features such as a sucking disk or undulating membrane. Typically, the size, shape, and number of nuclei are diagnostic characteristics used to identify cysts. Examination of permanent-stained smears is always recommended for diagnosis of infection because the wet mount may not be reflective of all organisms and stages present in the specimen.

Other Diagnostic Methods

Antigen-based tests for organisms such as *G. duodenalis* have increased in popularity over the years due to the ease of

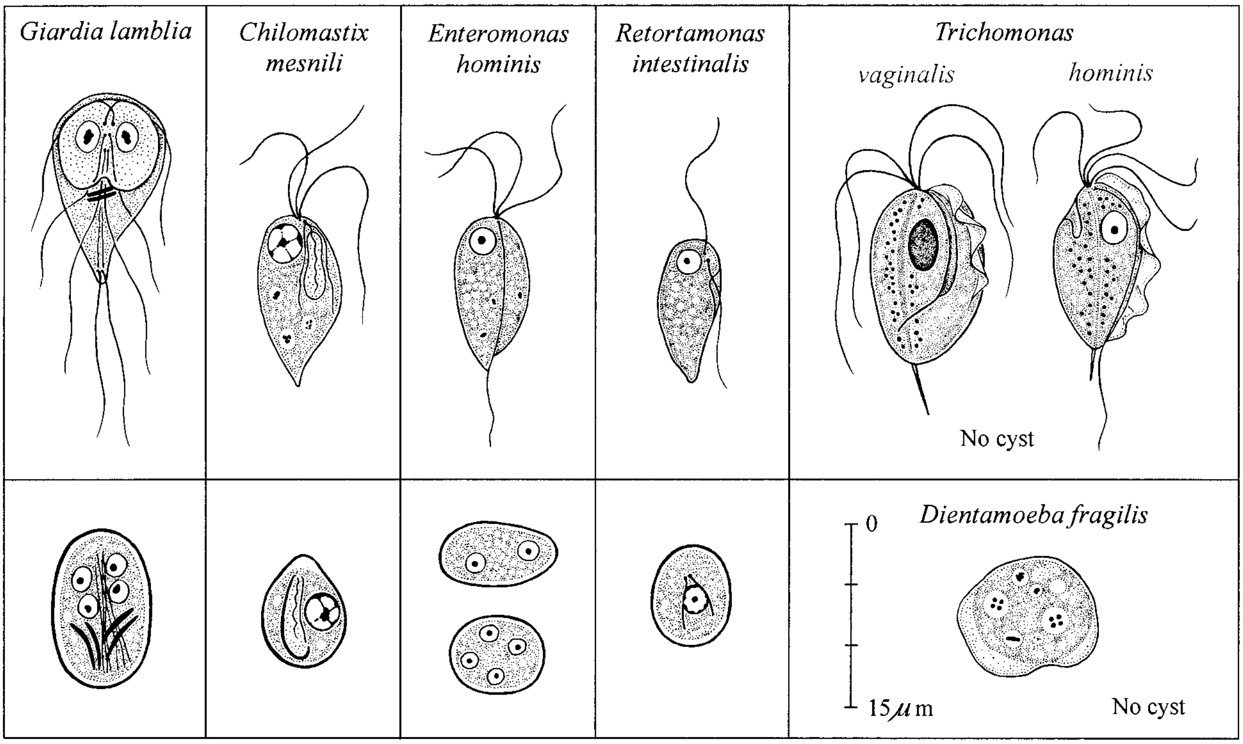

FIGURE 7 Intestinal and urogenital flagellates of humans. (Top row) Trophozoites. *T. vaginalis* is found in urogenital sites; all other flagellates are intestinal. (Bottom row) Cysts. *D. fragilis* trophozoite is shown; no cyst stage. doi:10.1128/9781555817381.ch140.f7

use and increased throughput compared to microscopy. In addition, nucleic acid amplification methods have been developed to detect *T. vaginalis* in clinical samples and *G. duodenalis* in water and clinical samples but are not yet routinely available in the clinical laboratory. Both antigen and amplification tests for the flagellates are discussed in more detail below. Serologic assays are available for the diagnosis of *G. duodenalis* infection but are not routinely used in the clinical setting.

Evaluation, Interpretation, and Reporting of Results

It is important that a representative portion of the slide, either a wet mount or a permanent smear, be scanned before a final opinion on a specimen is given. It is also important to use an accurate micrometer to measure life cycle stages. Reports of microscopy results should clearly indicate the full taxonomic names of the organisms detected along with the forms of the organisms seen (trophozoites versus cysts) (2). Quantitation of the number of organisms seen is not appropriate for the flagellates.

Giardia duodenalis

Taxonomy

G. duodenalis and the other *Giardia* spp. are classified as belonging to the phylum *Metamonada* (flagellates), subphylum *Conosa*, class *Trepomonadea* (intestinal flagellates), order *Diplomonadida* (see chapter 132 of this *Manual*).

Description of the Agent

G. duodenalis is an intestinal flagellate that infects both humans and animals and is the most common cause of intestinal parasitosis in humans worldwide. Because the literature refers to this organism as *Giardia lamblia*, *Giardia intestinalis*, and *G. duodenalis*, it is evident that there has been debate about the classification and nomenclature of this flagellate. There is an extensive review of taxonomy related to *Giardia* that explores in depth the taxonomy of this genus. Currently *G. duodenalis* is the accepted species name in regards to human infections. According to Feng and Xiao (90), there are six species: *G. agilis*, *G. ardeae*, *G. muris*, *G. microti*, *G. psittaci*, and *G. duodenalis*.

It is now accepted that there is considerable genetic diversity within *G. duodenalis*. Based on molecular biology, the genus *Giardia* is subdivided into major genotypes containing subgenotypes. The major genotypes of *G. duodenalis* that are associated with human infections are assemblages A and B. Assemblage A is associated with a mixture of both human and animal isolates, whereas assemblage B is typically associated with human isolates only. Most zoonotic animal-to-human infections occur with assemblage A (91).

Epidemiology, Transmission, and Prevention

Infection with *G. duodenalis* occurs through fecal-oral transmission or the ingestion of cysts in contaminated food or water, and an inoculum of only 10 to 100 cysts is sufficient for human infection (92). Individuals more commonly infected in developed countries are children in day care centers, hikers, and immunocompromised individuals. Among immunocompromised individuals, infections have been documented in people with AIDS and hypogammaglobulinemia and those affected by malnutrition. Prevalence rates for this pathogen range from 1 to 7% in industrialized countries and from 5 to 50% in developing countries. Of the intestinal flagellates, *G. duodenalis* is the flagellate most frequently isolated in the United States. In high-risk domestic settings,

TABLE 4 Key features of trophozoites and cysts of common intestinal and urogenital flagellates[a]

Organism	Trophozoites	Cysts
D. fragilis	Size[b]: 5–15 µm; shaped like amebae Motility: Nonprogressive; pseudopodia are angular Flagella: None Nucleus[c]: 1 (40%) or 2 (60%); no peripheral chromatin; karyosome clusters of 4–8 granules Note: Trophozoites not visible in unstained preparation; variation in size between trophozoites can exist on the same smear; cytoplasm is finely granular, and vacuoles may be present.	One report of cyst stage being present
G. duodenalis	Size: 10–20 µm long, 5–15 µm wide; pear shaped Motility: Falling leaf Flagella: 4 lateral, 2 ventral, 2 caudal Nucleus: 2; not visible in unstained preparation; small central karyosomes present Note: Sucking disk is prominent on ventral side of trophozoite; organism is spoon shaped from side view; 2 axonemes are present.	Size: 8–19 µm long, 7–10 µm wide; oval, ellipsoidal, or round Nucleus: 4 nuclei usually located on one end; not distinct in unstained preparation; no peripheral chromatin; karyosomes smaller than those in trophozoite Cytoplasm: Staining can cause shrinkage where cytoplasm pulls away from the cyst wall; 4 median bodies present. Note: Poorly defined longitudinal fibers may be present
C. mesnili	Size: 6–24 µm long, 4–8 µm wide; pear shaped Motility: Stiff, rotary Flagella: 3 anterior, 1 in cytostome Nucleus: 1; not visible in unstained preparation Cytoplasm: Prominent cytostome extends over 1/3 to 1/2 of body; spiral groove across ventral surface can be hard to see; vacuoles present	Size: 6–10 µm long, 4–6 µm wide; lemon shaped with anterior hyaline knob Nucleus: Same as trophozoite; difficult to see in unstained preparation; indistinct central karyosome present Cytoplasm: Curved fibril alongside cytostome known as "shepherd's crook"
P. hominis	Size: 5–15 µm long, 7–10 µm wide; pear shaped Motility: Jerky, rapid Flagella: 3–5 anterior, 1 posterior (extends beyond end of body) Nucleus: 1; not visible in unstained preparation Cytoplasm: Central longitudinal axostyle; undulating membrane runs the entire length of body Note: May have tremendous nuclear variation; can mimic *E. hartmanni* and *D. fragilis*	No cyst stage
T. vaginalis	Size: 7–23 µm long, 5–15 µm wide Motility: Jerky, rapid Flagella: 3–5 anterior, 1 posterior Nucleus: 1; not visible in unstained preparation Note: Undulating membrane extends 1/2 the length of body; no free posterior flagella	No cyst stage
E. hominis	Size: 4–10 µm long, 5–6 µm wide; oval Motility: Jerky Flagella: 3 anterior, 1 posterior Nucleus: 1; not visible in unstained preparation Note: One side of the body is flat; posterior flagellum extends free posteriorly or laterally.	Size: 4–10 µm long, 4–6 µm wide; elongate or oval in shape Nucleus: 1–4; not visible in stained preparation Note: Resemble *E. nana* cysts; fibrils or flagella usually not seen
R. intestinalis	Size: 4–9 µm long, 3–4 µm wide; pear shaped or oval Motility: Jerky Flagella: 1 anterior, 1 posterior Nucleus: 1; not visible in unstained preparation Note: Very difficult to identify; rarely seen; prominent cytostome which extends 1/2 the length of body	Size: 4–9 µm long, 5 µm wide; pear or lemon shaped Nucleus: 1; not visible in unstained preparation Note: Resemble *Chilomastix* cysts; bird beak fibril arrangement; shadow outline of cytostome with supporting fibrils extends above nucleus

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[a] Adapted from reference 3.

[b] Size ranges are based on wet preparations (with permanent stains, organisms usually measure 1 to 2 µm less).

[c] Nuclear and cytoplasmic descriptions are based on permanent-stained smears.

such as day care centers, prevalence rates can reach 90% (93). A *Giardia* vaccine is available for dogs and cats; this may affect the prevalence of infections in humans (94).

Infection occurs when viable cysts are ingested, excyst, and transform into trophozoites. After excystation, the trophozoite, which appears to have a propensity for the duodenum, attaches to the mucosal epithelium. Trophozoites attach to the epithelium via a sucking disk located on the ventral side of the parasite. During the course of infection, the parasites remain attached to the epithelium and do not invade the mucosa. In the intestine, trophozoites divide by binary fission to produce two identical daughter trophozoites. As they move down toward the large intestine, the trophozoites encyst, and infective cysts are excreted into the environment (3).

Clinical Significance

The majority of individuals infected with *G. duodenalis* are asymptomatic. Asymptomatic versus symptomatic infection may be due to the existence of two different strains of *Giardia* with different levels of virulence. Preliminary studies have grouped *G. duodenalis* into two groups, group A and group B. Group A appears to be more pathogenic and is associated with symptomatic infection. Isoenzyme and molecular studies show that group A and group B differ from one another and appear to be no more related than *E. histolytica* and *E. dispar*. The genes homologous between group A and group B *Giardia* isolates show an identity of ~81 to ~89% (95–97). In symptomatic individuals, acute *G. duodenalis* infections can mimic infections with other protozoal, viral, and bacterial pathogens. After an incubation period of ~12 to ~20 days, patients can experience nausea, chills, low-grade fever, epigastric pain, and a sudden onset of watery diarrhea. Diarrhea is often explosive and presents as foul smelling without the presence of blood, cellular exudate, or mucus. Individuals can develop subacute or chronic infections with symptoms such as recurrent diarrhea, abdominal discomfort and distention, belching, and heartburn. In patients with chronic cases of giardiasis, diarrhea can lead to dehydration, malabsorption, and impairment of pancreatic function (98).

Direct Examination

Microscopy

Diagnosis of *G. duodenalis* infection is often established by the microscopic examination of stool for the presence of cysts and/or trophozoites, although many laboratories have migrated to antigen-based assays. Stained smears are more helpful in identifying the trophozoite stage of the infection, although this stage can be identified in wet mount preparations (Table 4; Fig. 7 and 8). Examination of stool specimens may not be diagnostic because cyst forms can be trapped in mucus, making them difficult to detect on smears. Also, the excretion pattern of cysts can be cyclical. In these instances, other methods, such as the string test (Entero-Test), should be used to obtain clinical samples. The string test procedure consists of a patient swallowing a weighted capsule containing gelatin and a tightly wound string. After ~5 h, the string is removed from the patient and the adherent material is examined as a wet mount or permanent smear (3). Endoscopy can also be used to collect clinical specimens. Because this procedure is invasive, it is used only for diagnosis of disease in patients with perplexing clinical presentations (93).

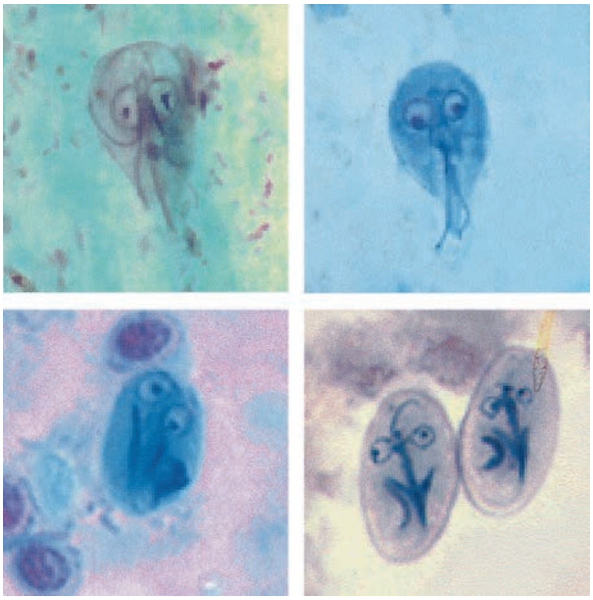

FIGURE 8 (Top row) *Giardia duodenalis* trophozoites. Note the typical nuclei, axonemes, and curved median bodies. (Bottom row) *G. duodenalis* cysts. The cysts on the right are stained with iron-hematoxylin, and those on the left are stained with Wheatley's trichrome stain. Courtesy of L. Garcia.
doi:10.1128/9781555817381.ch140.f8

Antigen Detection

Other assays that have gained wide acceptance for the detection of *G. duodenalis* in stool specimens are EIAs, DFA assays, and immunochromatographic assays. These methods have very high specificities (90 to 100%) and sensitivities that range from 63 to 100% (99–104). Although these methods have proven to be fairly sensitive and specific compared to the routine staining of fecal smears (trichrome staining), other important pathogens may be missed if a wet mount and permanent-stained smear are not examined. The DFA assay widely used is the Merifluor reagent assay (Meridian Bioscience, Inc., Cincinnati, OH), but it does require the availability of a fluorescent microscope. DFA assays have a sensitivity and specificity of >95%.

Immunoassays are available and are useful for testing large numbers of patient specimens, and some studies have shown the performance of the EIA to be excellent compared to that of the ova and parasite exam (100, 102, 105). Four available immunoassays are the ProSpecT *Giardia* EZ (Remel, Lenexa, KS), the ColorPAC *Giardia* assay (Becton Dickinson and Co., Sparks, MD) (101), the TechLab *Giardia* II assay, and the *Giardia/Cryptosporidium* Chek from TechLab. The Tri-Combo parasite screen (TechLab) is another immunoassay for *Giardia*, *Cryptosporidium*, and *E. histolytica* but is not yet approved by the FDA (106). These assays have very good sensitivity and specificity, often >90% (103, 106, 107).

It is very important to note that if a patient is immunocompromised, has traveled, or is suspected of coming into contact with an unusual pathogen, the ova and parasite examination should be performed. Some studies suggest that the use of an immunoassay does not necessarily eliminate the need for more than one stool specimen. Positive specimens from asymptomatic patients may be missed if only one stool specimen is tested using an immunoassay (108). Some immunoassays are affected by specimens contaminated with blood. It was documented in one study that blood can interfere with the performance of the ProSpecT assay and potentially cause false-positive results (101). Immunochromatographic detection methods have also been developed for use in the clinical laboratory (32, 105, 109, 110). The ImmunoCard STAT (Meridian Bioscience) has been developed to detect *G. duodenalis* and *C. parvum* antigens in stool specimens by using rapid solid-phase qualitative immunochromatography. It is recommended that patients negative by the ImmunoCard STAT who remain symptomatic be tested using a routine ova and parasite exam (109). The Triage Micro parasite panel (Alere, Waltham, MA) is a rapid immunoassay that can detect not only *G. duodenalis* antigens but also those of *E. histolytica/E. dispar* and *C. parvum* in stool specimens (111). Antigens in stool specimens are immobilized on a membrane containing specific antibodies to the respective intestinal parasites. The formation of color bars occurs on a specific area of the membrane depending on the parasite present in the stool. Laboratories should check with the manufacturer's package insert to determine what specimens, formalin-fixed or fresh, are compatible with each assay. The Quik Chek (TechLab) is also available for rapid diagnosis of *Giardia* and *Cryptosporidium* infections. Although there are several methods now available for the detection of *G. duodenalis*, parameters such as workload, skill levels of technologists, and the availability of necessary equipment should be considered in choosing a specific method, along with performance characteristics of the assay (112). Refer to chapter 133 for a complete list of current available assays for *Giardia* detection in the clinical laboratory.

Nucleic Acid Detection Techniques

Some investigators are now using molecular tools to diagnose *G. duodenalis* infection with clinical samples (51–53, 91, 113, 114). One group has developed an oligonucleotide microarray with excellent sensitivity and specificity for the detection of *G. duodenalis* and other intestinal parasites in stool specimens. The assay was also able to discriminate between *Giardia* assemblages A and B (115). A multiplex PCR assay to detect specific genotypes of *G. duodenalis* has also been developed that may potentially aid in epidemiologic investigations of outbreaks (116). It is important to note that molecular detection of *G. duodenalis* has not been widely implemented; to date there is one FDA-approved assay, as mentioned above (1, 53).

Because of the potential for contamination of municipal water supplies, routine monitoring of water for parasitic protozoa is recommended as a public health control measure. *G. duodenalis* cysts can remain viable for 2 to 3 months in cold water and are fairly resistant to killing by routine chlorine treatments. DFA assays are commonly used to detect *G. duodenalis* cysts in water samples, but PCR is also being used for this purpose (117). The advantage of PCR is the increased sensitivity compared to those of fluorescence assays or assays that use special stains. Research continues to enhance the ability of investigators to detect viable organisms, which is a better indication of poor water quality (118).

Treatment

For individuals diagnosed with giardiasis, the treatment of choice is metronidazole, tinidazole, or nitazoxanide (62). Alternatives include paromomycin, furazolidone, and quinacrine or other nitroimidazoles such as ornidazole and secnidazole (119). In most immunocompetent hosts, infection is self-limiting; however, treatment lessens the duration of symptoms and prevents transmission. Other drugs that have been used to treat giardiasis are albendazole, mebendazole, and bacitracin (120), and there are other therapeutics on the horizon. Albendazole, mebendazole, and paromomycin have lower activity against *Giardia* than do the nitroimidazoles (119). Resistance to metronidazole and other agents has been observed clinically and *in vitro* (63). *In vitro* resistance-testing assays are available but lack the standardization required for the clinical laboratory (121).

Evaluation, Interpretation, and Reporting of Results

As with any of the organisms mentioned in this chapter, it is important to evaluate more than one stool specimen for the presence of *G. duodenalis*. For *Giardia*, this could include up to five or six stool specimens to increase the chance of recovery. This is especially important for diagnosing *G. duodenalis* infection, since organisms can be passed in the stool intermittently on a more cyclical basis. During the staining procedure, the cysts can be shrunken or distorted, which may affect the ability of the clinical laboratory scientists to read the smear. There are several techniques available for the diagnosis of giardiasis in the clinical laboratory. Some methods might offer somewhat better sensitivity but need to fit into the overall workflow of the laboratory. EIAs offer somewhat enhanced sensitivity but cannot always replace the ova and parasite exam due to the prevalence of other pathogens in the patient population being tested. Adding assays, such as the EIA, might increase detection of *Giardia* but add to the bottom-line cost per testing if the physicians continue to order a routine ova and parasite exam as well.

Dientamoeba fragilis

Taxonomy

D. fragilis is currently grouped in the kingdom *Protozoa*, class *Trichomonadea*, and order *Trichomonadida* (see chapter 132).

Description of the Agent

Despite the lack of external flagella, this parasite is currently classified as a flagellate but has historically been grouped with the amebae. Electron microscopy and antigenic analysis have aided in the classification of this organism and have demonstrated that it is closely related to *Trichomonas* and *Histomonas* species. Phylogenetic analysis of small-subunit rRNA sequences has also confirmed the relationship between *D. fragilis* and *Histomonas* (122, 123).

Epidemiology, Transmission, and Prevention

D. fragilis is found worldwide and is known to cause a noninvasive diarrheal illness in humans. Colonization in humans is similar to that seen with other intestinal parasites; typically, the cecum and the proximal part of the colon are affected. The mode of transmission of *D. fragilis* is still uncertain; one hypothesis concerning the spread of *D. fragilis* is that transmission occurs within the eggs of *Enterobius vermicularis* or *Ascaris lumbricoides* (124). Several studies provide circumstantial evidence to support *E. vermicularis* as a vector, documenting a greater-than-expected coinfection rate with the two organisms (125). Recent animal studies suggest the possibility of a cyst form for *D. fragilis*, but this has not been definitively proven (126, 127).

Though *D. fragilis* has a worldwide distribution, prevalence rates for this organism vary quite substantially from 0% in Prague to 42% in Germany (125). There appears to be a higher prevalence in certain groups of individuals such as missionaries, Native Americans living in Arizona, and institutionalized individuals (128). In the United States, the prevalence is reported to be quite low. This may be due to underreporting attributable to difficulties associated with identifying the organism in clinical samples. In addition, Johnson and Clark (129) identified the existence of two genetically distinct types of *D. fragilis* with a resulting sequence divergence of ~2% (129, 130). The degree of divergence appears to be similar to that seen with *E. histolytica* and *E. dispar*. Since asymptomatic infections occur, this finding may lend support to the possibility that two distinct species exist, though there is no consensus on this matter (125, 129).

Clinical Significance

The frequency of symptomatic disease ranges from 15 to 25% in adults, and symptomatic disease is more common in children, in whom up to 90% of those infected have clinical signs (93, 131, 132). Symptoms include fatigue, insufficient weight gain, diarrhea (often intermittent), abdominal pain, anorexia, and nausea. Studies have linked *D. fragilis* infection to biliary infection (133), irritable bowel syndrome (26), allergic colitis (134), and diarrhea in HIV-infected patients (135). One case report describes a patient who presented in the emergency room with acute appendicitis that was attributed to *D. fragilis* infection (136). Some individuals, mainly children, also experience unexplained peripheral blood eosinophilia (3, 137). Diarrhea is seen predominantly during the first 1 to 2 weeks after the onset of disease. The number of *D. fragilis* organisms can vary greatly from day to day, which is similar to those of other intestinal protozoa. Abdominal pain can persist for 1 to 2 months (131). Although *D. fragilis* has been implicated in the above-mentioned clinical situations, the organism is isolated from patients with no apparent clinical symptoms. In addition, the lack of an animal model affects researchers' ability to detect specific pathological manifestations (125).

Direct Detection

Microscopy

Diagnosis of *D. fragilis* infection is similar to that of infections with other intestinal protozoa, and detection of the trophozoite in fresh or preserved stool is warranted to establish infection. Laboratories using direct microscopy may be at a disadvantage, since the trophozoite can degenerate if not placed in fixative. Nonfixed trophozoites can appear rounded and refractile and are more difficult to identify microscopically. Morphologically, the trophozoite of *D. fragilis* contains one or two nuclei (binucleate), with two nuclei being more common. Well-trained laboratory personnel can identify *D. fragilis* trophozoites in stool specimens, but because no cyst stage exists, diagnosis from the wet mount can be difficult. Use of a permanent-stained smear is the recommended procedure for detection (Table 4; Fig. 7 and 9).

Other Diagnostic Methods

Culture has been shown to be more sensitive than microscopy; however, it is not recommended for the routine clinical laboratory (125). Antigen and antibody techniques have been used as diagnostic tools for the detection of *D. fragilis* but are not commercially available (138, 139). *D. fragilis* DNA has been detected using PCR with fresh, unpreserved stool. Molecular techniques have the promise of being more sensitive than microscopy (140–142). Multiplex PCR assays are being developed for the detection of *D. fragilis* (52).

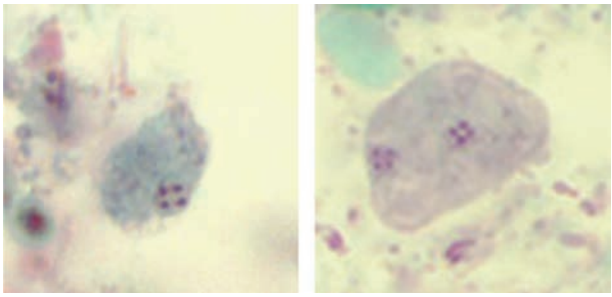

FIGURE 9 *Dientamoeba fragilis* trophozoites. The trophozoites can have one nucleus (left) or two nuclei (right). No cyst stage is known for this organism. Trophozoites are stained with Wheatley's trichrome stain. Courtesy of L. Garcia. doi:10.1128/9781555817381.ch140.f9

Treatment

The treatment of choice for symptomatic individuals is iodoquinol (diiodohydroxyquin) (62). Alternate choices include paromomycin, tetracycline, or metronidazole (62). Metronidazole, iodoquinol, and tetracycline have been used to treat children (3). Additional therapeutic options include diphetarsone, carbarsone, erythromycin, hydroxyquinoline, and secnidazole (125, 143). If *E. vermicularis* is detected concomitantly, the treatment regimen should also include mebendazole (131).

Evaluation, Interpretation, and Reporting of Results

The recovery of *D. fragilis* is greatly enhanced by the collection of at least three stool specimens (2, 93). Trophozoites have been recovered from soft and formed stools, indicating the need to evaluate all types of samples (131). Low incidence in a community setting for *D. fragilis* may be due to the inability of the laboratory to accurately identify the organism.

Trichomonas vaginalis

Taxonomy

T. vaginalis is a flagellate belonging to the kingdom *Protozoa*, class *Trichomonadea*, and order *Trichomonadida* (see chapter 132 of this *Manual*). Unlike the other members of the order, which inhabit the intestinal tract, *T. vaginalis* infects the urogenital tract.

Description of the Agent

The life cycle includes only the trophozoite stage; there is no cyst stage. The organism is similar in morphology to the other trichomonads and is characterized by a pear shape with a prominent axostyle and an undulating membrane that stops halfway down the side of the trophozoite (Table 4; Fig. 7 and 10). It is a facultative anaerobe that divides by binary fission, and it cannot survive long outside the host.

Epidemiology, Transmission, and Prevention

T. vaginalis is a pathogenic flagellate that infects the urogenital tracts of males and females. The infection is primarily a sexually transmitted infection and is thought to be the most common curable sexually transmitted infection among sexually active young women. Worldwide there are an estimated 250 million cases of *Trichmonas* infection each year, with an overall estimated prevalence of 4.5% (144). Prevalence is estimated to be higher in women (8%) than in

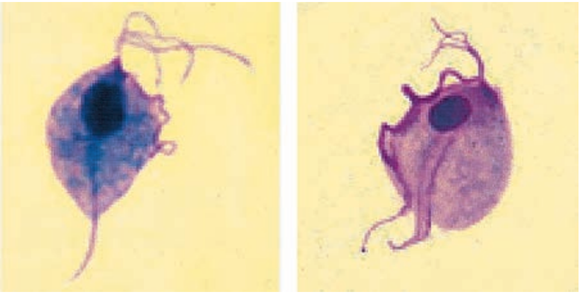

FIGURE 10 *Trichomonas vaginalis* trophozoites. Note the undulating membrane, which comes about halfway down the body. Although the membrane appears to come down farther on one of the trophozoites (right), this appearance occurs because of the photographic angle. Also, note the axostyle that penetrates the bottom of the organism. No cyst stage is known for this organism. Trophozoites are stained with Giemsa stain. Courtesy of L. Garcia. doi:10.1128/9781555817381.ch140.f10

men (1%) (144, 145). It is hypothesized that hormones in women may play a large role in infection and persistence of *Trichomonas* infections in women. The incidence of trichomoniasis differs depending on the population examined, varying from 5 to 60% in various studies. Factors such as lower socioeconomic status, multiple sex partners, and poor personal hygiene are linked to a higher incidence of infection. The prevalence of *Trichomonas* infections appears to be higher in older women, as shown by several studies (146–148). This is in contrast to other common sexually transmitted diseases, such as chlamydia, for which prevalence is typically higher in younger women. One study screened male patients to assess the prevalence in that population. The prevalence was ~6.6%, but the average age of *T. vaginalis*-infected men was 39.9 years, which was much higher than those infected with *Chlamydia trachomatis* or *Neisseria gonorrhoeae* (146). This parallels what is seen in women, as described above. Several studies have described the relationship between *T. vaginalis* and the possibility of lethal prostate cancer (149–151). Some researchers are exploring the use of a serodiagnostic test that would identify those at risk for prostate cancer related to seropositivity for *T. vaginalis* (152).

The estimated infection rates cited in the literature may indeed be too low because (i) trichomoniasis is not a reportable disease in the United States and other countries; (ii) the infection, particularly in men, can be asymptomatic; and (iii) laboratory tests used for diagnosis vary in their sensitivities. Despite these rates of infection and their serious medical consequences, trichomoniasis has not received adequate attention from public health and sexually transmitted disease prevention programs (153).

Clinical Significance

Infection in females can result in vaginitis, cervicitis, and urethritis (153). The vaginal discharge is classically described as copious, liquid, greenish, frothy, and foul smelling. The onset of symptoms, such as intense vaginal and vulvar pruritus and discharge, is often sudden and occurs during or after menstruation. The vaginal pH is usually elevated above the normal pH of 4.5, and dysuria occurs in 20% of women with *T. vaginalis* infection. Infection has also been associated with premature rupture of membranes, premature birth, and posthysterectomy cuff infections (154–156). In men, the most common symptomatic presentation is urethritis. Up to 50% of infected women are asymptomatic carriers. In men, the majority of infections are asymptomatic. Asymptomatic carriers serve as a reservoir for transmission and also remain at risk for developing disease. Trichomoniasis has been implicated as a cofactor in the transmission of HIV (157, 158). In one study, men with symptomatic *Trichomonas* urethritis were found to have increased HIV concentrations in seminal plasma compared to HIV-infected men without urethritis (159).

Neonates can acquire the organism during passage through the infected birth canal (153). Reports have also documented *T. vaginalis* as a cause of neonatal pneumonia (160). A recent report of bilateral conjunctivitis in a male without genital infection was reported (211). Multilocus sequence typing has been used to characterize different strains of *T. vaginalis*. Techniques such as this can be useful for population-based studies and perhaps strain selection for future vaccine development (161).

Direct Detection

Microscopy

The diagnosis of *T. vaginalis* infection is commonly based on the examination of wet preparations of vaginal and urethral discharges, prostatic secretions, and urine sediments. Permanent stains such as Papanicolaou and Giemsa stains can be used, but the organisms may be difficult to recognize (162). For wet preparations, vaginal specimens are routinely collected during a speculum examination, but studies suggest that self-collected or tampon-collected specimens may be used successfully (163, 164). Specimens should be mixed with a drop of physiologic saline and examined microscopically within 1 h under low power (magnification, ×100) with reduced illumination. Specimens should never be refrigerated. The presence of actively motile organisms with jerky motility is diagnostic. The movement of the undulating membrane may be seen as the motility of the trophozoite diminishes. Polymorphonuclear cells are often present. The sensitivity of the wet preparation test with vaginal specimens is between 50 and 70%, depending on the skill of the microscopist and other factors. The sensitivity of microscopy in males is low, and additional testing, such as culture of urethral swab, urine, and semen, is required for optimal sensitivity. Perhaps the most important factor affecting the sensitivity of wet mount testing is the time between collection and examination of the specimen. Viability of the organism is essential for the detection of motility on the wet mount and drops off precipitously with time. Amies gel agar transport medium can maintain the viability for culture of *T. vaginalis* on swabs held at room temperature for 24 ± 6 h before inoculation of the specimen into a culture pouch (165). Because the morphology of *P. hominis*, a nonpathogenic intestinal flagellate, is very similar to that of *T. vaginalis*, care must be taken to ensure that specimens are not contaminated with fecal material.

Culture

Culture has greater sensitivity (>80%) than the wet mount method and is considered the gold standard method for the detection of *T. vaginalis*. Specimens must be collected properly and inoculated immediately into the appropriate medium, such as modified Diamond's, Trichosel, or Hollander's medium. Due to cost and convenience, this approach is not routinely used. Culture systems (InPouchTV [BioMed Diagnostics, San Jose, CA] and the system of Empyrean Diagnostics, Inc. [Mountain View, CA]) that allow direct inoculation, transport, culture, and microscopic examination are commercially available (166, 167). In situations in which immediate transport of specimens is not feasible, the use of these transport/culture devices should be encouraged. Studies have also shown that a delayed inoculation protocol is as sensitive as immediate inoculation, allowing the results of microscopy to be used to determine whether further culture is necessary (168). Serologic testing is not useful for the diagnosis of trichomoniasis.

Antigen Detection

Several antigen detection methods have been developed for *T. vaginalis* and offer the advantage of being rapid and easy to perform. A latex agglutination test (TV Latex; Kalon Biological, Guildford, United Kingdom) has been shown to have excellent sensitivity (169) but is not available in the United States. An immunofluorescence assay (Light Diagnostics *T. vaginalis* DFA; Chemicon International, Temecula, CA) is available in the United States for testing directly from patient samples. An immunochromatographic capillary flow assay is available commercially for the qualitative detection of *T. vaginalis* antigens from vaginal swabs. The OSOM Trichomonas Rapid Test Kit (Sekisui Diagnostics, Tokyo, Japan) is a dipstick assay providing results in 10 min. In published studies, the OSOM test has demonstrated good sensitivity (67 to 94.7%) and specificity (98.8 to 100%) compared to various comparator assays, including wet mount, culture, and amplified testing (170–176). This assay would perhaps be more suitable for a clinic setting, where more-rapid results are desirable. A rapid point-of-care test is being developed for *T. vaginalis*. This assay features novel electrochemical endpoint detection and in initial studies appears to have high sensitivity and specificity (177).

Nucleic Acid Detection Techniques

The Affirm VPIII (Becton Dickinson) is a direct DNA probe test for the detection of organisms from vaginal swabs associated with vaginosis/vaginitis. It tests for the three most common syndromes associated with increased vaginal discharge: bacterial vaginosis (*Gardnerella vaginalis*), candidiasis (*Candida albicans*), and trichomoniasis (*T. vaginalis*). According to the manufacturer's package insert, the assay has a sensitivity and specificity of 90 and 98%, respectively, compared with wet mount and culture for *T. vaginalis* (package insert, Affirm VPIII, version no. 670160JAAG; Becton Dickinson). In a clinical evaluation of vaginal swab specimens from both symptomatic and asymptomatic females, the Affirm detected more *T. vaginalis*-positive samples than wet mount testing, although the difference was not statistically significant (178).

Nucleic acid-based amplification methods, such as PCR and transcription-mediated amplification (TMA), developed for the detection of *T. vaginalis* have been reported in the literature, though only one is currently FDA cleared. These amplification methods have demonstrated varying sensitivities depending on the genomic target, specimen type, and sex of the patient (172, 179–183). PCR has a sensitivity of 85 to 100% with vaginal swabs; its sensitivity with urine is lower, ranging from 60 to 80% (153, 184, 185). For men, PCR with urine and urethral swabs has been reported and appears to be more sensitive than conventional methods (186, 187). Several researchers have reported using TMA technology from Hologic/Gen-Probe (San Diego, CA) for detection of *T. vaginalis*, which is now FDA approved (148, 181, 182, 188, 189). Studies using the Trichomonas Aptima Combo 2 assay have shown the performance of the assay to be superior compared to other methodologies (147, 148, 189). The assay is approved for the detection of *T. vaginalis* infections from a wide variety of specimens such as clinician-collected vaginal specimens, endocervical swabs, ThinPrep liquid-based cytology samples, and urine samples (189). Samples can be automated for testing using either the Panther or the Tigris instrumentation. In addition to testing on adults, molecular testing is also being evaluated for the detection of *Trichomonas* in children (190). Some researchers have evaluated the Hologic/Gen-Probe assay for diagnosis of anorectal *T. vaginalis* using rectal swabs (146, 191). Another assay available outside the United States is the Seeplex STD 6 ACE Detection System (Seegene, Seoul, Korea), which detects *T. vaginalis* and other pathogens (192). This assay is a multiplex PCR assay. Detection employs six pairs of dual priming oligonucleotide primers targeted to unique genes of the specific pathogen. It is not clear if this assay will receive approval in the United States.

While the wet mount method provides a rapid result at a low cost, tests with the increased sensitivities, such as nucleic acid probes or amplification tests, may be indicated because of the impact of *T. vaginalis* infections on pregnancy and the link with HIV transmission (165, 187). The use of alternate specimen types such as urine makes amplified testing an important advancement for diagnosis of trichomoniasis. An algorithm to reflex specimens with negative wet mounts to culture or a more sensitive methodology may be a useful diagnostic approach (173). Because the true prevalence of *T. vaginalis* is unknown and the prevalence appears to be higher in older women, it is important for laboratories and clinicians to be aligned in regards to which patients should be tested (148).

Treatment

The recommended treatment for *T. vaginalis* infections is metronidazole or tinidazole (62, 193). For metronidazole, oral therapy is recommended over topical treatment. Tinidazole may be used as a first-line agent or for refractory cases previously treated with metronidazole (194). For treatment during pregnancy, metronidazole is the recommended therapy; however, it should be used cautiously, as data do not suggest that metronidazole treatment results in a reduction in perinatal morbidity (193, 195). All sexual partners of infected individuals should also receive treatment. Treatment failure with metronidazole is most often due to noncompliance or reinfection. True resistance to metronidazole has been documented and appears to be increasing (194, 196). While not routinely available, methods have been published for the *in vitro* determination of susceptibility. These methods have not been standardized, and the results can vary based on assay conditions (197, 198).

Evaluation, Interpretation, and Reporting of Results

A laboratory finding that is positive for *T. vaginalis* is considered diagnostic of trichomoniasis. As discussed above, both microscopy and culture are prone to lower sensitivities due to issues related to sampling and transport. Laboratories should have strict rejection criteria for *Trichomonas* culture and wet mount specimens that do not arrive within the specified time or transport conditions; such policies improve sensitivity, ensuring more-accurate results. In comparison to methods such as antigen detection and molecular methods, a negative result by these methods should be viewed cautiously and evaluated in conjunction with clinical symptoms.

With testing for *Trichomonas*, such as antigen detection and direct molecular probes or amplified tests, reported results should reflect the analyte that is detected. For example, a positive result for *T. vaginalis* by a direct DNA probe assay should state "*T. vaginalis* DNA detected." If testing is expanded to differing sample types, such as urine testing by amplified methods, the report should clearly state the specimen tested.

If a home-brew assay is used for the diagnosis of *Trichomonas*, it is required that the result be labeled to indicate its status as an in-house test in accordance with Clinical Laboratory Improvement Amendments regulations as follows: "This test was developed and its performance characteristics determined by [Laboratory Name]. It has not been cleared or approved by the U.S. Food and Drug Administration."

NONPATHOGENIC FLAGELLATES

C. mesnili is found worldwide and is generally considered nonpathogenic. Unlike *D. fragilis*, *C. mesnili* has both a trophozoite and a cyst stage. The organism is acquired through the ingestion of contaminated food or water and resides in the cecum and/or colon of the infected human or animal. The trophozoite is 6 to 24 µm long and contains a characteristic spiral groove that runs longitudinally along the body (Table 4; Fig. 7 and 11). Motility of the organism can sometimes be seen in fresh preparations, and the spiral groove may be exposed as the organism turns. Flagella are difficult to see in stained preparations. The trophozoite contains one nucleus, with a cytostome or oral groove in close proximity. The pear-shaped cyst retains the cytoplasmic organelles of the trophozoite, with a single nucleus and curved cytostomal fibril. Observing the organism in permanent-stained preparations makes identification more definitive.

P. hominis, formerly referred to as *Trichomonas hominis*, is a nonpathogenic flagellate that is similar to *D. fragilis* in that only the trophozoite stage has been observed. Although the organism is cosmopolitan in nature and is recovered from individuals with diarrhea, it is still considered nonpathogenic. The trophozoites typically inhabit the cecum. They are pyriform and contain an undulating membrane that runs the length of the parasite. The use of permanent smears is recommended for observation of these organisms in clinical specimens. The trophozoites may stain weakly, making them difficult to detect on stained smears (3).

Two additional nonpathogenic intestinal flagellates are *E. hominis* and *R. intestinalis*. Both *E. hominis* and *R. intestinalis* are found in warm or temperate climates, and infection is acquired through the ingestion of cysts. When clinical specimens are examined, it is important to note that cysts of *E. hominis* can resemble those of *E. nana*, although *E. nana* cysts containing two nuclei are rare. Because of the small sizes of *E. hominis* and *R. intestinalis*, it is difficult to detect these organisms even when permanent-stained smears are examined. This may lead to the underreporting of both organisms. *R. intestinalis* has been recovered from the pancreatic juice of a patient with small lesions of the pancreatic duct (199).

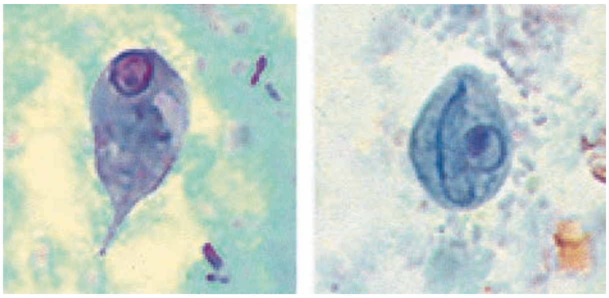

FIGURE 11 *Chilomastix mesnili* trophozoite (left) and cyst (right). Note the oral groove (feeding groove) at the right side of the trophozoite (clear area) and the curved fibril ("shepherd's crook") in the cyst. Also note the typical pear or lemon shape of the cyst. Organisms are stained with Wheatley's trichrome stain. Courtesy of L. Garcia.
doi:10.1128/9781555817381.ch140.f11

In general, treatment is not recommended for infections with the nonpathogenic flagellates. Improved personal hygiene and sanitary conditions are key methods for the prevention of infection.

CILIATES

Balantidium coli

Taxonomy

B. coli is a ciliate belonging to the phylum *Ciliophora*, class *Litostomatea*, order *Trichostomatia* (see chapter 132 of this *Manual*). Members of the phylum *Ciliophora* are protozoa possessing cilia in at least one stage of their life cycles. They also have two different types of nuclei, one macronucleus and one or more micronuclei. Over the last several years, molecular analysis has aided in the characterization of the genus *Balantidium* (200–202). Sequences of the genus are on file at GenBank based on the small-subunit rRNA. There is question as to whether the species isolated from humans, *B. coli*, and pigs, *B. suis*, are the same species (200).

Description of the Agent

This organism has both the trophozoite and cyst forms as part of its life cycle (Table 5; Fig. 12). The cyst form is the infective stage. After ingestion of the cysts and excystation, trophozoites secrete hyaluronidase, which aids in the invasion of the tissue. The trophozoite, which is oval and covered with cilia, is easily seen in wet mount preparations under

TABLE 5 Key features of the ciliate *B. coli*

Stage	Characteristics
Trophozoite	Shape and size: Ovoid with tapering anterior end; 50–100 µm long, 40–70 µm wide Motility: Rotary, boring; may be rapid Nuclei: 1 large kidney-bean-shaped macronucleus may not be distinct in unstained preparation; 1 small round micronucleus adjacent to macronucleus, difficult to see Cytoplasm: May be vacuolated; may contain ingested bacteria and debris; anterior cytostome Cilia: Body surface covered with longitudinal rows of cilia; longer near cytostome Note: May be confused with helminth eggs or debris on a permanent-stained smear; concentration or sedimentation examination recommended
Cyst	Shape and size: Spherical or oval; 50–70 µm in diam Nuclei: 1 large macronucleus, 1 micronucleus, difficult to see Cytoplasm: Vacuoles are visible in young cysts; in older cysts, internal structure appears granular Cilia: Difficult to see within the thick cyst wall

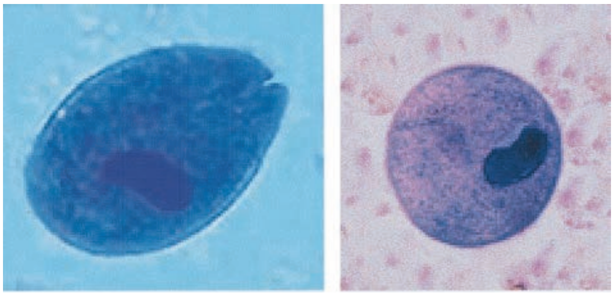

FIGURE 12 *Balantidium coli* trophozoite (left) and cyst (right). Note the single macronucleus visible in both the trophozoite and the cyst. The much smaller micronucleus is not visible. Organisms are stained with Wheatley's trichrome stain. Courtesy of L. Garcia. doi:10.1128/9781555817381.ch140.f12

low-power magnification. The cytoplasm contains both a macronucleus and a micronucleus, in addition to two contractile vacuoles. Motile trophozoites can be observed in fresh wet preparations, but the specimen must be observed soon after collection. The trophozoite is somewhat pear shaped and also contains vacuoles that may harbor debris such as cell fragments and ingested bacteria. Cyst formation takes place as the trophozoite moves down the large intestine.

Epidemiology, Transmission, and Prevention

B. coli exists in animal reservoirs such as pigs and chimpanzees, with pigs being the primary reservoir (200). The organism is the only pathogenic ciliate and the largest pathogenic protozoan known to infect humans. Transmission occurs by the fecal-oral route following ingestion of the cysts in contaminated food or water. Infection is more common in warmer climates and in areas where humans are in close contact with pigs. As with other intestinal protozoa, poor sanitary conditions lead to a higher incidence of infection. Prevalence of *Balantidium* varies by geographic location but overall is estimated to be between 0.02 and 1% (203). High-prevalence areas include areas of the Middle East, Papua New Guinea and West Irian, Latin America, and the Philippines (204, 205).

Clinical Significance

Infection with *B. coli* is most often asymptomatic; however, symptomatic infection can occur, resulting in bouts of dysentery similar to amebiasis (3, 206). Infection with *Balantidium* can be described in three ways: (i) asymptomatic host, carrying the disease; (ii) chronic infection, nonbloody diarrhea, including other symptoms such as cramping and abdominal pain; and (iii) fulminating disease consisting of mucoid and bloody stools (200). In addition, colitis caused by *B. coli* is often indistinguishable from that caused by *E. histolytica*. Symptoms typically include diarrhea, nausea, vomiting, headache, and anorexia. Fluid loss can be dramatic, as seen in some patients with cryptosporidiosis. The organism can invade the submucosa of the large bowel, and ulcerative abscesses and hemorrhagic lesions can occur. The shallow ulcers and submucosal lesions that result from invasion are prone to secondary infection by bacteria and can be problematic for the patient (207, 208). Death due to invasive *B. coli* infection has been reported (207). Infections associated with extraintestinal sites have been described (207–209). There have been several reports of *Balantidium* spreading from the intestine to the lung. Most of these cases have occurred in patients who are either elderly or immunocompromised. The disease presents as a pneumonia-like illness. In these documented cases, *Balantidium* has been recovered from specimens such as bronchial secretions and bronchial lavage specimens. It is hypothesized that extraintestinal colonization can occur between the lymphatic or circulatory system, by perforation through the colon, or through aspiration of fluid from the oral cavity (200). One case describes an individual with vertebral osteomyelitis and myelopathy, which is the first documented case of infection in the bone (210).

Direct Examination

Microscopy

Either ova and parasite examination of feces or histological examination of intestinal biopsy specimens establishes the diagnosis of *B. coli* infections. The diagnosis can be established only by demonstrating the presence of trophozoites in stool or tissue samples (209). It is very easy to identify these organisms in wet preparations and concentrated stool samples. Conversely, it can be challenging to identify *B. coli* from trichrome-stained permanent smears because the organisms are so large and have a tendency to overstain. This makes the organism less discernible and increases the chance of misidentification.

Treatment

The treatment of choice for *B. coli* infection is tetracycline, although it is considered an investigational drug when used in this context (62). Metronidazole and iodoquinol are therapeutic alternatives used in some cases (3). Nitazoxanide, which is a broad-spectrum antiparasitic drug, may be another alternative for treatment (200).

Evaluation, Interpretation, and Reporting of Results

The recovery of *B. coli* in humans is fairly uncommon despite its worldwide distribution. Pulmonary infections can occur, but the clinical laboratory scientist needs to make sure that this organism is not confused with motile ciliated epithelial cells that can be present in respiratory specimens. *Balantidium* spp. in wet mounts are very active parasites with uniform ciliation.

SUMMARY

Clinical laboratories are now given more choices for testing in diagnostic parasitology, with assays ranging from microscopy, culture, antigen detection, and nucleic acid amplification techniques for detection of the intestinal and urogenital amebae, flagellates, and ciliates. Molecular biology has the promise to deliver more sensitive and specific methods, with the availability of an FDA-cleared assay for detection of *T. vaginalis* from genital specimens and a multiplex assay for *E. histolytica*, *G. duodenalis*, and *Cryptosporidium* species detection from stool. There are other assays being designed and in clinical trials at the time of writing, and while these methods have not been fully adapted to the clinical diagnostic laboratory, their use in the coming years is certain to increase. In addition to these amplification tests, rapid point-of-care tests are available for organisms such as *T. vaginalis*. Results can now be available in real time for the clinician to manage patients directly in the exam setting. While these new test modalities are exciting, clinical laboratories are still faced with using microscopy for routine workup for stool specimens due to the lack of commercially available testing for all the relevant organisms. Some laboratories have switched to antigen-based methods, but many still rely on microscopy because antigen-based methods cannot detect all potential pathogens in a given stool specimen. Microscopy, as we know, cannot differentiate between pathogenic and nonpathogenic amebae and the different genotypes of *Giardia*. Together these exciting new areas will help increase the options that the clinical parasitology laboratory has for the diagnosis of intestinal parasitic infections.

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Cystoisospora, *Cyclospora*, and *Sarcocystis**

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Cystoisospora, *Cyclospora*, and *Sarcocystis* are intestinal coccidia of humans (Fig. 1 to 5). They have varied life cycles, epidemiologies, treatment requirements, and diagnostic methods. Oocysts of these coccidia are found in the feces of humans (Table 1), and diagnosis is based ultimately on demonstrating oocysts (*Cystoisospora* or *Cyclospora*) or sporocysts (*Sarcocystis*) in human stool samples.

TAXONOMY

Cystoisospora, *Cyclospora*, and *Sarcocystis* are in the phylum *Apicomplexa*, class *Coccidea*, order *Eimeriida*. *Cystoisospora belli*, *Sarcocystis hominis*, and *Sarcocystis suihominis* are in the family *Sarcocystidae*, while *Cyclospora cayetanensis* is in the family *Eimeriidae*. Many people are familiar with the old name for *Cystoisospora belli*, *Isospora belli*, but life cycle (1) and molecular studies indicate that the parasite is a member of the genus *Cystoisospora* (2), not *Isospora*.

DESCRIPTION OF THE AGENTS

Life Cycles

Cystoisospora belli

The life cycle is direct (monoxenous), but evidence exists that it can be facultatively heteroxenous (use two hosts). *C. belli* oocysts are passed in the feces unsporulated or partially sporulated (Fig. 1C and D and 2A and B). Oocysts generally complete sporulation within 72 h, although sporulation time varies between 24 h and >5 days, depending on temperature and humidity. Sporulated oocysts contain two sporocysts, each with four sporozoites, although *Caryospora*-like oocysts of *C. belli* (containing one sporocyst with eight sporozoites) have been reported and can comprise up to 5% of the sporulated oocysts in a sample (3). The prepatent period, the time it takes for unsporulated oocysts to appear in the feces after sporulated oocysts are ingested, is 9 to 17 days (4). The patent period, the time from when oocysts are first excreted in the feces until they can no longer be observed in the feces, is quite variable and depends on the immune status of the infected individual. Oocysts can usually be found for 30 to 50 days in immunocompetent patients, while immunosuppressed patients may continue to shed oocysts for 6 months or more (5). Recurrence of oocyst shedding is common. This prolonged oocyst shedding in immunosuppressed patients is presumably due to recycling of one or more schizogenous stages or activation of dormant extraintestinal monozoic tissue cysts (Fig. 4).

Developmental stages of *C. belli* have been reported for intestinal biopsy specimens of the duodenum, jejunum, and occasionally ileum, and oocysts can be aspirated directly from the duodenal contents. Intestinal development occurs predominantly in epithelial cells, although schizonts (meronts) are occasionally reported from the lamina propria or submucosa (6). At least two generations of schizonts, as well as macrogametocytes (female sexual stage), microgametocytes (male sexual stage), and unsporulated oocysts, have been observed.

C. belli sporozoites/merozoites are capable of traveling extraintestinally and becoming dormant as single-organism-containing tissue cysts (Fig. 4) in a variety of tissues, including lamina propria, mesenteric lymph nodes, liver, and spleen (7–9). These cysts are commonly termed monozoic tissue cysts. Monozoic tissue cysts in histological sections are thick walled and measure 12 to 22 by 8 to 10 µm, and each contains a single dormant sporozoite/merozoite of about 8 to 10 by 5 µm (7, 8). Presumably, the monozoic tissue cysts are capable of reactivating patent infections once immunity wanes. Monozoic tissue cysts can be present in the lamina propria in the absence of oocysts in stool samples (9).

The existence of these monozoic tissue cysts has led to speculation that a paratenic (transport) host may be involved in the life cycle of *C. belli* (7). Paratenic hosts are known to occur in the *Cystoisospora* species that infect cats and dogs, and it is probable that they occur in the life cycle of *C. belli*.

Cystoisospora natalensis

The life cycle is unknown but presumably monoxenous (direct). The oocysts are smaller and more spherical than those of *C. belli* (Table 1). Oocysts are passed in the stool unsporulated. At ambient temperature, oocysts can complete sporulation within 24 h (10). The prepatent and patent periods of *C. natalensis* are unknown. One individual passed unsporulated oocysts for at least 4 days (11). The validity of this species is questionable because oocysts of *C. natalensis* have been reported only for patients from South Africa and no reports of *C. natalensis* appear in the literature after 1955 (10, 11).

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*This chapter contains information presented by David S. Lindsay, Steve J. Upton, and Louis M. Weiss in chapter 138 of the 10th edition of this *Manual*.

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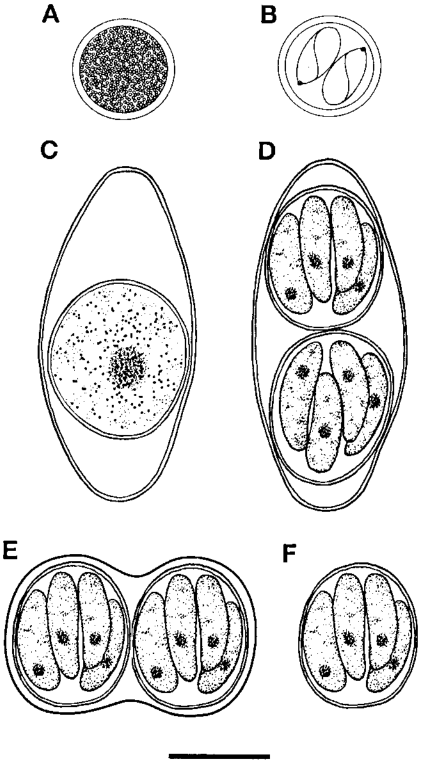

FIGURE 1 Line drawings of unsporulated and sporulated oocysts of *Cyclospora cayetanensis* (A and B) and *Cystoisospora belli* (C and D) and a sporulated oocyst (E) and sporocyst (F) of a *Sarcocystis* species from humans. Bar, 10 µm. doi:10.1128/9781555817381.ch141.f1

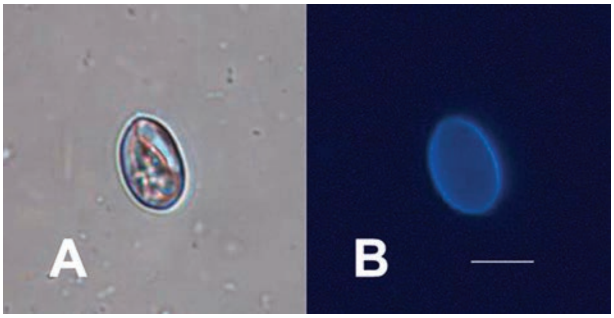

FIGURE 3 Sporocyst of a *Sarcocystis* species in a stool sample viewed using differential interference contrast microscopy (A) and autofluorescence using UV light (B). Bar, 10 µm. Courtesy of Alice E. Houk, Department of Biomedical Sciences and Pathobiology, Virginia Tech, Blacksburg, VA. doi:10.1128/9781555817381.ch141.f3

Cyclospora cayetanensis

The life cycle is monoxenous and involves only humans as hosts. Oocysts are passed in the stool unsporulated (Fig. 1A and 2C). At room temperature (23 to 25°C), small numbers of oocysts may sporulate within 10 to 12 days (3, 12) (Fig. 1B). However, many oocysts require 3 to 4 weeks for sporozoites to fully develop. Sporulated *C. cayetanensis* oocysts contain two sporocysts, each with two sporozoites. A structure termed a Stieda body is present in the end of each sporocyst. There are no Stieda bodies in the oocysts of *C. belli* or *Sarcocystis* species (Fig. 1). The precise prepatent period is not yet known. However, the onset of clinical signs following infection generally averages 7 to 8 days postinfection and lasts 2 to 3 weeks, but this may range from 1 to >100 days. The length of time that oocysts are shed in the feces is highly variable. Oocysts may be shed in the feces for anywhere from 7 days to several months. Relapse of diarrhea can occur in up to 25% of infected individuals (13). Indigenous infections are confined primarily to tropical, subtropical, or warm temperate regions of the world. Outbreaks occur in other areas of the world due to contaminated foodstuffs obtained from regions of endemicity (85).

Developmental stages of *C. cayetanensis* generally occur within epithelial cells of the lower duodenum and jejunum (14–16). There are two asexual generations followed by sexual stages and oocysts. Stages develop in a supranuclear location within enterocytes (16). An experimental attempt to infect seven healthy human volunteers by oral adminis-

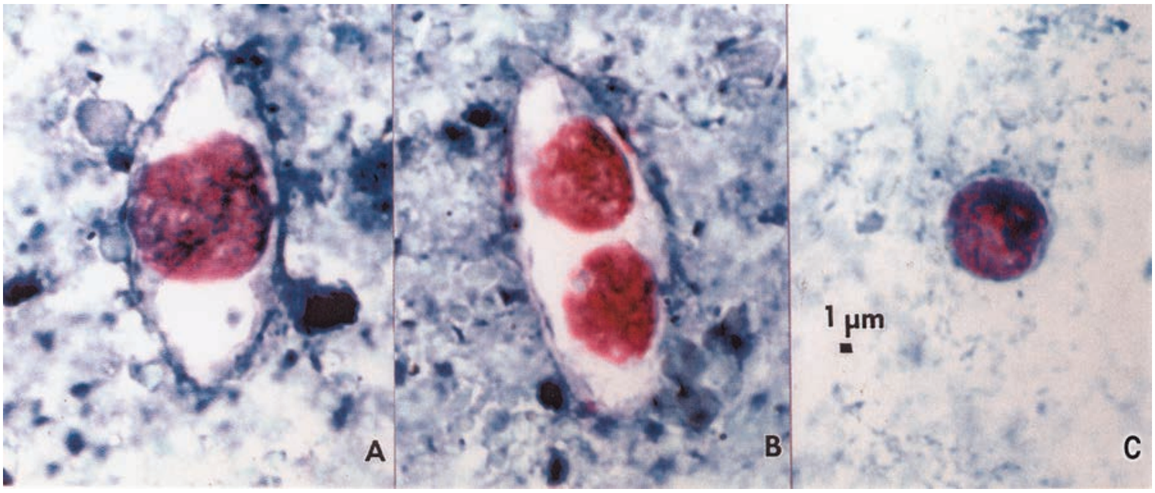

FIGURE 2 Modified Kinyoun's acid-fast-stained smears demonstrating a *Cystoisospora belli* oocyst with a sporont (A), a *C. belli* oocyst with two sporoblasts (B), and a *Cyclospora cayetanensis* oocyst with a sporont (C). Bar, 1µm. doi:10.1128/9781555817381.ch142.f2

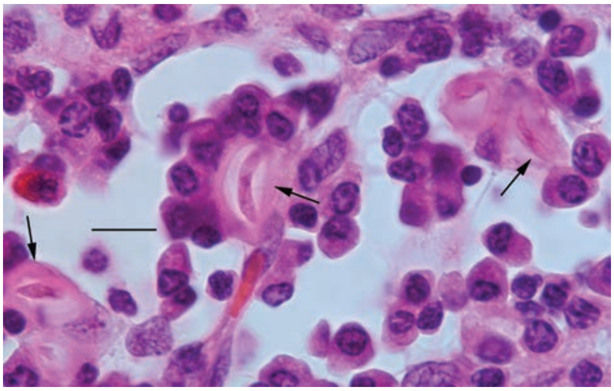

FIGURE 4 Hematoxylin and eosin-stained tissue section demonstrating several monozoic tissue cysts (arrows) of *Cystoisospora belli*. Note the thick wall that surrounds each single zoite. Bar, 10 μm. doi:10.1128/9781555817381.ch141.f4

tration of microscopically confirmed sporulated oocysts of *C. cayetanensis* was not successful, as none of the patients developed clinical signs or shed oocysts in their stools (17).

Sarcocystis hominis

The life cycle is heteroxenous. Humans are definitive hosts for *S. hominis*, and bovids are the intermediate hosts. Infection occurs when raw or undercooked meat containing sarcocysts is ingested. Known intermediate hosts include cattle (*Bos taurus*), American bison (*Bison bison*), water buffaloes (*Bubalus bubalis*), and wisents (European bison; *Bison bonasus*). These intermediate hosts harbor sarcocysts (muscle cysts) that are infective when ingested by humans. Infective bradyzoites (dormant merozoite-like stages) are present in the sarcocysts. The bradyzoites penetrate the human intestinal epithelium and develop as sexual stages (macrogametocytes and microgametocytes) in cells in the lamina propria of the intestine. Fertilization occurs, and the oocysts sporulate in the lamina propria. The oocysts are *Cystoisospora*-like and contain two sporocysts, each with four sporozoites. The oocyst wall often ruptures as the oocyst makes its way to the intestinal lumen. This results in the shedding of individual sporocysts in the feces (Fig. 3). Individual sporocysts contain four sporozoites. Both oocysts with two sporocysts and individual sporocysts can be seen in the feces of humans with intestinal *Sarcocystis* infection (Fig. 1E and F).

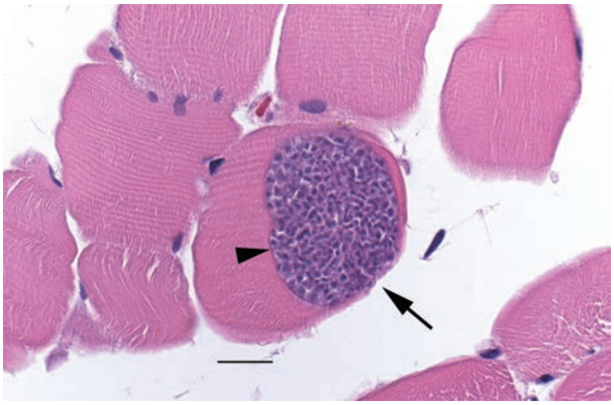

FIGURE 5 Sarcocyst (arrow) of a *Sarcocystis* species in a skeletal muscle biopsy specimen from a male Dutch patient obtained during an outbreak of muscular sarcocystosis (49) among visitors to Tioman Island off the east coast of Malaysia. A sarcocyst wall (arrowhead) surrounds hundreds of bradyzoites. Note the lack of inflammatory response. The patient's traveling partner was also confirmed to be positive by muscle biopsy. Bar, 10 μm. Courtesy of Douglas H. Esposito and Clifton Drew, National Center for Emerging and Zoonotic Infectious Diseases, CDC, Atlanta, GA. doi:10.1128/9781555817381.ch141.f5

Oocysts and sporocysts are fully sporulated when passed in the feces. For human volunteers, the prepatent period has been reported to be 8 to 39 days, and patent infections can last as long as 18 months. *S. hominis* occurs on all continents, anywhere cattle or buffaloes have access to human feces and humans ingest raw or undercooked beef.

Sarcocystis suihominis

The life cycle is similar to that described above for *S. hominis*, except that pigs are the intermediate hosts. The prepatent period is 9 to 10 days, and patency is in excess of 36 days. *S. suihominis* presumably occurs on all continents, anywhere swine have access to human feces and humans ingest raw or undercooked pork.

Human Muscular *Sarcocystis* Infection

Sarcocysts have been reported as incidental findings from tissue sections of both skeletal and cardiac muscle of nearly 100 humans worldwide. Humans become infected after ingesting *Sarcocystis* species sporocysts in contaminated food or water. One to two generations of precystic schizogony presumably occur in endothelial cells in capillaries throughout the body. The final generation of merozoites penetrates striated muscle cells and transforms into metrocytes. Metrocytes divide by endodyogeny to produce bradyzoites within the sarcocyst. Clinical signs probably arise from schizogony occurring in endothelial cells of capillaries and the host reaction to developing sarcocysts in muscles (i.e., eosinophilic myositis). There are at least seven distinct types of sarcocysts (18) present in human muscles. This suggests that as many as seven definitive hosts may be able to produce sporocysts infective for humans. Many of these sarcocysts appear similar to sarcocysts found in nonhuman primates.

EPIDEMIOLOGY, TRANSMISSION, AND PREVENTION

Cystoisospora belli

C. belli is found primarily in tropical, subtropical, and warm temperate regions, but reports of indigenous infections have been published from temperate areas as well. Most cases of infection in temperate areas involve foreign travel or homosexual contact. Transmission is via ingestion of sporulated oocysts and possibly the ingestion of raw or undercooked tissues from unknown paratenic hosts. An outbreak of *C. belli* infections involving ~90 patients was reported in the city of Antofagasta, Chile, in 1977 (19). It was associated with ingestion of vegetables contaminated with irrigation water from a sewage treatment plant (19). Improving sanitation and water quality in areas of endemicity will decrease transmission of *C. belli*.

Cyclospora cayetanensis

Cyclospora is endemic in Central and South America, the Caribbean, Mexico, Indonesia, Asia, Nepal, Africa, India, southern Europe, and the Middle East. In areas of endemicity, there is an increased risk of *Cyclospora* infection with contact with soil (20) and water (21). Three outbreaks of

TABLE 1 Structural data for *Cystoisospora*, *Cyclospora*, and *Sarcocystis* oocysts and sporocysts found in stool samples from humans

Species	Mean size (range) (μm)[a]	
	Oocysts	Sporocysts
Cystoisospora belli	32 × 14 (20–36 × 10–19)	14 × 10 (12–17 × 7–11)
Cystoisospora natalensis	Not given (25–30 × 21–24)	17 × 12 (not given)
Cyclospora cayetanensis	9 × 9 (8–10 × 8–10)	6 × 4 (6–7 × 3–4)
Sarcocystis hominis	19 × 15 (not given)	15 × 9 (13–17 × 8–11)
Sarcocystis suihominis	19 × 13 (19–20 × 12–15)	14 × 11 (12–14 × 10–11)

[a] Data have been rounded to the nearest micrometer.

waterborne *C. cayetanensis* infections were reported in a study of waterborne protozoal disease outbreaks from 2004 and 2010 (22). Infections in most temperate areas are correlated with the consumption of imported, contaminated fruits and vegetables, such as basil, raspberries, lettuce, mesclun, and snow peas. Two large outbreaks of cyclosporiasis occurred during the summer of 2013 (85). One was concentrated in Iowa (153 cases) and Nebraska (86 cases) and linked to a restaurant-associated salad mix that contained iceberg lettuce, romaine lettuce, red cabbage, and carrots (85). The second was associated with fresh cilantro (85) and was associated with a restaurant (22 of 30 patrons) in a large outbreak in Texas (278 cases).

Individuals in areas of endemicity should wear gloves when gardening to prevent exposure to oocysts of *C. cayetanensis*. Better washing of produce may help to remove *Cyclospora* oocysts, but many fruits are delicate. Most of the produce items implicated as transmitting *Cyclospora* are consumed raw, which does not lend itself to prevention by thermal means. Nonthermal treatments such as high hydrostatic pressure (23) have been shown to inactivate *Toxoplasma gondii* oocysts, and these methods may be effective in inactivating *Cyclospora* on produce.

Sarcocystis Species

Human intestinal *Sarcocystis* species are potentially present in any region in the world where cattle, buffaloes, and swine have access to human feces and the life cycle can be maintained. The cycle has not been detected in the United States. Cultural habits that include ingestion of raw meat or undercooked meat products help to maintain this life cycle in areas where *Sarcocystis* species are endemic. Cooking meat to an internal temperature of >67°C kills *T. gondii* tissue cysts in the meat (24), and this temperature should also kill tissue cysts of human-infective *Sarcocystis* species in meat products. Preventing cattle, buffaloes, and swine from consuming human feces will also break the cycle in areas of endemicity.

Most cases of human muscular *Sarcocystis* infection (Fig. 5) have been reported from the Far East, particularly Malaysia (25–27). One study of 100 consecutive autopsy cases from Malaysia found that 21% of tongue sections were positive for sarcocysts (27). This is probably an underrepresentation of the true prevalence because only a small amount of muscle can be examined histologically. Humans become infected by ingesting sporocysts in contaminated water (25) or food.

CLINICAL SIGNIFICANCE

Cystoisospora belli

C. belli can cause serious and sometimes fatal disease in immunocompetent individuals. Symptoms of *C. belli* infection include diarrhea, steatorrhea, headache, fever, malaise, abdominal pain, vomiting, dehydration, and weight loss (6, 12, 28, 29). Blood is not usually present in the feces. Eosinophilia is observed in many patients (30, 31). The disease is often chronic, with parasites present in the feces or biopsy specimens for several months to years. Recurrences are common and can occur as long as 10 years after successful treatment (32). Disease is more severe in infants and young children.

Clinical disease from *C. belli* infection is usually more severe in immunocompromised patients than in immunocompetent patients. *C. belli* infection produces diarrhea in AIDS patients that is often very fluid and secretory-like and leads to dehydration requiring hospitalization (30). Fever and weight loss are also common findings. Other opportunistic pathogens are also common copathogens in these patients. *C. belli* superinfection of the small bowel was seen in a patient who was immunosuppressed with systemic corticosteroids to aid in treatment of eosinophilic gastroenteritis (31). *C. belli* has been observed in both renal transplant (33) and liver transplant (34) patients. *C. belli*-induced intestinal lesions and responses to chemotherapy are usually similar to those observed in immunocompetent patients. *C. belli* has been observed in patients with concurrent Hodgkin's disease (6), non-Hodgkin's lymphoproliferative disease (35), human T-cell leukemia virus type 1-associated adult T-cell leukemia (36), and acute lymphoblastic leukemia and human T-cell leukemia virus type 1-associated T-cell lymphoma (37). These patients respond to specific anti-*C. belli* treatment. Extraintestinal cyst-like stages have been documented for AIDS patients and may play a role in relapse of infection (7). These usually contain a single merozoite-like stage (Fig. 4) and are called monozoic tissue cysts. Many thousands of these stages can be present (7).

Infections with *C. belli* in the gallbladder epithelium (38–40) and endometrial epithelium (41) have been reported, and oocysts have been observed in bile samples (42). Clinical signs in patients with parasites in these locations are not specific for coccidiosis, and parasites are located after tissue biopsy as part of a diagnostic workup. Infection of the biliary tract with *C. belli* was the first HIV-related opportunistic infection in one patient, and it may have represented an AIDS-defining infection in that case (40). The parasites probably reach these extraintestinal sites as merozoites from the gut or zoites from extraintestinal locations, and the epithelial cells of these tissues are permissive to parasite entrance and multiplication.

Cyclospora cayetanensis

Nonbloody watery diarrhea is the main clinical symptom of *C. cayetanensis* infection. Symptoms of nausea, fatigue, abdominal cramps, and fever were reported in >50% of clinical cases in one foodborne outbreak, with headache and vomiting occurring in 45 to 30% of these patients (13). Some individuals can be infected and show no clinical signs. In most immunocompetent patients, typical symptoms of cyclosporiasis include cycles of diarrhea with anorexia, malaise, nausea, and cramping and periods of apparent remission.

C. cayetanensis infection can be associated with biliary disease in both immunosuppressed patients and immunologically normal patients (43). Developmental stages of *C. cayetanensis* have been seen in the gallbladder epithelium of AIDS patients with acalculous cholecystitis (44). Oocysts can be observed in the bile of patients with active biliary disease.

Intestinal *Sarcocystis* Infections

Clinical *Sarcocystis* infections in humans can manifest primarily as intestinal disease if infected meat is ingested or as muscular disease if sporocysts are ingested (45). Intestinal disease occurs soon after consumption of infected meat (3 to 6 h) and is characterized by nausea, abdominal pain, and diarrhea. Intestinal disease can be more severe in individuals who have additional enteropathogens present in the gut. Intestinal *Sarcocystis* infection combined with invasion by Gram-positive bacteria has been associated with several cases of segmental enterocolitis in Thailand (46). Experimental studies with human volunteers have produced more-severe disease in those who have ingested pork containing *S. suihominis* than in those who have ingested beef containing *S. hominis* (45). Some individuals can be infected and show no clinical signs.

Muscular *Sarcocystis* Infections

Muscular *Sarcocystis* infections (Fig. 5) in humans are usually subclinical or associated with only mild clinical signs and are usually considered incidental findings (17, 47, 48). Clinical case reports are from Southeast Asia. Three outbreaks of acute muscular sarcocystosis have been reported from Malaysia (25, 26, 49). In the first reported outbreak, clinical signs associated with muscular *Sarcocystis* infection occurred in 7 of 15 members of a U.S. combat unit (25). The signs developed about 3 weeks after the troops returned from the jungle and were fever, myalgias, bronchospasm, fleeting pruritic rashes, transient lymphadenopathy, and subcutaneous nodules. Eosinophilia, elevated erythrocyte sedimentation rate, and elevated levels of muscle creatine kinase were present in these troops (25). The second (26) and third (49) outbreaks occurred in travelers returning from Tioman Island off the east coast of Malaysia. The second outbreak, during the summer of 2011, was in 32 patients, most of whom were from Germany (~50%), other European countries, North America, and Asia. Within days or weeks of returning home, most patients experienced fever and muscle pain (26). All had peripheral eosinophilia, and most had elevated serum creatinine phosphokinase levels (26). The third outbreak was reported in 65 patients returning from Tioman Island (26) and occurred during July and August of 2011 and 2012. The 65 patients originated from Germany (n = 25), France (n = 20), the Netherlands (n = 12), Switzerland (n = 3), Belgium (n = 2), Spain (n = 2), and Singapore (n = 1) (49). Most patients experienced fever and myalgia, while fewer had arthralgia, asthenia, headache, cough, and diarrhea (49).

Decreased immunity or cancer may make individuals more susceptible to muscular *Sarcocystis* infection. Sarcocysts have been reported in the heart of a patient with Hodgkin's disease (50) and the larynx of a patient with squamous cell carcinoma (51).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

The results obtained in the diagnostic laboratory are only as good as the material presented for testing. Choosing the appropriate sample and sample fixative is extremely important (52). Universal precautions should be followed when fresh stool samples are handled. If samples are to be sent to another laboratory for diagnosis, they should be fixed in an appropriate fixative. A 5% or 10% formalin solution is an appropriate fixative for stools suspected of containing intestinal coccidia. Formalin fixation does not interfere with some of the immunodetection methods currently employed to detect *Cryptosporidium* and *Giardia duodenalis*, which is a drawback of polyvinyl alcohol fixative. Oocyst structure lasts for several months when stools are stored at 4°C in formalin fixatives.

DIRECT EXAMINATION

Microscopy

Oocysts of *C. belli* and *C. cayetanensis* and sporocysts of *Sarcocystis* species are readily identified in fresh unstained wet mounts, based on their characteristic sizes and morphologies (Table 1; Fig. 1). This is especially true if oocysts and sporocysts are present in large numbers. Autofluorescence of oocysts of *C. belli* and *C. cayetanensis* and oocysts and sporocysts of *Sarcocystis* (Fig. 3) is an especially useful tool (53, 54) and has replaced many of the staining techniques previously used for these parasites in laboratories equipped with appropriate fluorescent microscopes.

Concentration techniques, such as formalin-ethyl acetate (rarely formalin-ether) sedimentation or sucrose centrifugal flotation, are helpful when few oocysts are present. Sucrose centrifugal flotation has been found to be superior to formalin-ether sedimentation for demonstrating oocysts of *C. cayetanensis* (55). The same should be true for oocysts of *C. belli* and sporocysts of *Sarcocystis* spp. Few laboratories employ sucrose concentration, and fortunately, direct wet smears can be very useful: their utility approaches that of sucrose centrifugal flotation when coupled with autofluorescence examination (55). Staining procedures may adversely affect the autofluorescence of oocysts and sporocysts.

Stained fecal smears have been widely used to demonstrate *C. cayetanensis* oocysts and, to a lesser extent, *C. belli* oocysts (53, 56). *C. belli* and *C. cayetanensis* oocysts stain red with the modified Kinyoun's acid-fast stain, and this method is widely used (Fig. 2). The main drawbacks are that staining can be variable and some oocysts do not stain (57). Oocysts usually do not stain with trichrome, chromotrope, or Gram-chromotrope stain (57). Some *C. cayetanensis* oocysts stain light blue with Giemsa stain (57). Variations on the safranin staining technique stain *C. cayetanensis* oocysts orange or pinkish orange, and heating and other treatments have been used to increase the staining frequency of oocysts. Flow cytometry has been used to detect *C. cayetanensis* oocysts in human stool samples (58). The results of flow cytometry examination were similar to those of microscopy, and preparation times for the two methods were similar (58).

A single negative stool specimen is not conclusive in the examination of stools for coccidial parasites; a total of three or more stool specimens collected on subsequent days need to be examined before coccidial infection can be ruled out. Liquid stool samples can be concentrated by centrifugation, and the pellet may be used for examination by use of wet mounts, concentration techniques, or stained smears.

Large numbers of oocysts may make diagnosis less challenging but do not always translate directly to the severity of clinical signs. Some individuals may excrete oocysts and be asymptomatic.

Cases of muscular *Sarcocystis* infection are diagnosed based on the detection of sarcocysts in muscle samples taken from biopsy specimens or postmortem samples.

Culture

In vitro culture of intestinal coccidial parasites is most often used as a tool to study developmental biology or to identify active chemotherapeutic agents. It presently has limited use in diagnosis of active human infection.

Only minimal development of *C. belli* occurs in human (ileocecal adenocarcinoma [HCT-8 cells] or epithelial carcinoma of the lung [A549]) or mammalian (African green monkey kidney [Vero] or Madin-Darby bovine kidney) cell cultures (59). No reports on development of *C. cayetanensis* in cell culture have been published. Bradyzoites of *S. suihominis* undergo sexual development and produce oocysts in cell culture (60). Vascular schizont stages of human *Sarcocystis* species have not been reported for *in vitro* systems, but continuous cultures of several mammalian and avian species have been reported.

Antigen Detection

The inability to produce stages in cell cultures and provide a source of diagnostic antigens has limited the usefulness of antigen detection for these coccidial parasites of humans.

Nucleic Acid Detection

There are no U.S. Food and Drug Administration-approved nucleic acid tests for the detection of infections with *C. belli*, *C. cayetanensis*, or *Sarcocystis* species. Several research laboratories have developed nucleic acid-based detection tests to demonstrate infection with these parasites in stool and tissue samples.

Detection of *C. belli* by PCR with primers based on internal transcribed spacer (ITS) region and small-subunit RNA sequences has been reported (2, 61, 62). Three different genotypes of *C. belli* can be identified with PCR and restriction fragment length polymorphism using MboII digestion (62) Coinfection of a single patient with two different genotypes has been observed (62). Molecular diagnosis of cystoisosporiasis employing extended-range PCR screening has been proven to detect *C. belli* in biopsy material from a patient who was fecal examination and histological examination negative on initial testing (63). Developmental stages were eventually observed in biopsy material and confirmed the findings (63). A real-time PCR using the internal transcribed spacer region 2 small-subunit RNA sequences has been developed to detect *C. belli* in stool samples (61).

Much attention has been placed on molecular methods to detect *C. cayetanensis* oocysts in stools, in water samples, and on produce because of the numerous outbreaks of *C. cayetanensis* infections (64). The 18S rRNA gene is presently the most frequently used target. Because *C. cayetanensis* is closely related to *Eimeria* species (65) from vertebrates, it is important that tests designed to detect *C. cayetanensis* in the water or on produce be examined for cross-reactivity to *Eimeria* spp. (66). *Cyclospora* species infecting mammals other than humans may also be present in water samples or on produce, and proofs of specificity are needed for these tests designed to look at environmental sources of *C. cayetanensis* and to detect *C. cayetanensis* oocysts on produce.

Quantitative PCR assays have been developed for *C. cayetanensis* oocysts in stool samples (67). This method detected DNA of the 18S ribosomal gene sequence from as little as 1 oocyst of *C. cayetanensis* per 5 ml of reaction volume. A multiplex PCR to detect *Cyclospora*, *Cystoisospora*, and microsporidia in stool samples has been developed (68).

S. hominis sporocysts in human stool can be detected using PCR-restriction fragment length polymorphism and sequencing of a partial 18S rRNA gene product. Several PCR methods have been developed to detect sarcocysts in the muscles of animals, and the U.S. Centers for Disease Control and Prevention (CDC) is presently attempting to develop a *Sarcocystis*-specific PCR to detect muscular *Sarcocystis* infection (26).

SEROLOGIC TESTS

The inability to obtain usable quantities of antigens from *C. belli*, *C. cayetanensis*, and *Sarcocystis* spp. has greatly limited the use of serologic diagnostic tests for these parasites in human stool samples. It is very difficult to obtain enough oocysts or sporocysts from feces to conduct serologic tests. The development of an enzyme-linked immunosorbent assay that measures IgG and IgM antibodies, using *C. cayetanensis* oocysts as antigen, has been reported (69). This study is questionable because the authors stated that they obtained their oocysts from experimentally infected guinea pigs, yet well-controlled studies indicate that humans are the only suitable host for *C. cayetanensis* (70). Attempts were made to develop an indirect fluorescent-antibody assay to detect IgG, IgA, and IgM specific for *C. cayetanensis*, using sectioned oocysts (71). None of the sera from four patients with positive stools reacted in the indirect fluorescent-antibody assay.

The CDC is presently attempting to develop a *Sarcocystis*-specific Western blot test to detect muscular *Sarcocystis* infection (49; D. H. Esposito and S. Handali, personal communication, April 2013). As of this writing, no test is available.

TREATMENT

Cystoisospora belli

The drug of choice for the treatment of *C. belli* is trimethoprim-sulfamethoxazole. A dose of trimethoprim (160 mg)-sulfamethoxazole (800 mg) two to four times a day for 10 to 14 days results in clearance of parasites, a decrease in diarrhea, and a decrease in abdominal pain within a mean of 2.5 days after treatment (72). Before the advent of combination antiretroviral therapy (cART), it was recommended that patients with HIV-1 infection and CD4$^+$ cell counts of <200 should receive secondary prophylaxis with trimethoprim (320 mg)-sulfamethoxazole (1,600 mg) once daily or three times a week to prevent relapse. In most cases, secondary prophylaxis is not needed once the CD4$^+$ count exceeds 200. However, a recent study described eight cases of chronic *C. belli* infection that persisted despite standard trimethoprim-sulfamethoxazole therapy, secondary prophylaxis, and good immunological and virological response to cART (73). Four patients died, two remained clinically ill, and two recovered (73).

For patients unable to tolerate sulfonamides due to allergy or intolerance, there is no standard treatment. Pyrimethamine at a dose of 50 to 75 mg/day is an effective alternative treatment for patients with sulfonamide allergies (74). Secondary prophylaxis using pyrimethamine at 25 mg/day can be used for patients not on cART (74). Pyrimethamine should be given with folinic acid (5 to 10 mg/day) to minimize bone marrow suppression. Another alternative agent is ciprofloxacin, a fluoroquinolone that inhibits topoisomerase. In a randomized study of 22 patients with cystoisosporiasis and HIV infection, all of the 10 patients who received trimethoprim-sulfamethoxazole had a cessation of diarrhea within 2 days, and 10 of 12 patients who received ciprofloxacin (500 mg twice daily) had a cessation of diarrhea within 4.5 days (75). All three patients (two with diarrhea and one without) who had persistent *C. belli* oocysts in their stools responded to trimethoprim-sulfamethoxazole treatment (75). In patients who responded to ciprofloxacin, continued prophylaxis with ciprofloxacin prevented recurrence of disease (75). Nitazoxanide has been used to treat *C. belli* infections (76, 77). Two patients who were given 500 mg of nitazoxanide twice daily for 3 days were oocyst negative after treatment (77). A patient treated with 500 mg of nitazoxanide twice daily for 7 days became oocyst negative by day 14 after treatment (76). Treatment failure was observed in a patient with biliary cystoisosporiasis and malabsorption when 2 g of nitazoxanide was given orally twice daily (78). Treatment failure was likely due to the lack of absorption of nitazoxanide and poor levels of the drug in the serum (78). Elevations in liver function tests and nausea are potential side effects of orally administered nitazoxanide.

In a study of eight AIDS patients with *C. belli* enteritis treated with diclazuril 200 mg/day for 7 days, treatment resulted in resolution of diarrhea in four of eight patients (79). Diclazuril 300 mg twice a day was reported to successfully treat *C. belli* in an AIDS patient who was hypersensitive to trimethoprim-sulfamethoxazole and pyrimethamine, and in this case, when the dose was decreased to 300 mg/day, the diarrhea recurred (80). Treatment with other antiprotozoal agents, such as metronidazole, tinidazole, quinacrine, and furazolidone, appears to be of little value for this infection.

Cyclospora cayetanensis

The drug of choice for the treatment of *C. cayetanensis* infection is trimethoprim (160 mg)-sulfamethoxazole (800 mg) given twice daily for 7 days (81, 82). Clearance of parasites, a decrease in diarrhea, and a decrease in abdominal pain occurred within a mean of 2.5 days after treatment. Patients on cART likely do not need secondary prophylaxis.

For patients unable to tolerate sulfonamides due to allergy or intolerance, there is no standard treatment. Nitazoxanide has been evaluated for activity against *C. cayetanensis*, and in these studies, its efficacy for the treatment of cyclosporiasis has been ~70% (83, 84). It should be appreciated, however, that only a few patients with cyclosporiasis were treated in any of these studies (83, 84). Another alternative agent is ciprofloxacin, a fluoroquinolone that inhibits topoisomerase. In a randomized study of 20 patients with cyclosporiasis, all of the 9 patients who received trimethoprim-sulfamethoxazole had a cessation of diarrhea within 3 days, and 10 of 11 who received ciprofloxacin (500 mg twice daily) had a cessation of diarrhea within 4 days; however, only 7 of 11 patients treated with ciprofloxacin cleared the organism from the stool (75). Anecdotal data suggest that the following drugs are ineffective: albendazole, azithromycin, pyrimethamine, nalidixic acid, norfloxacin, tinidazole, metronidazole, quinacrine, tetracycline, doxycycline, and diloxanime furoate (L. M. Weiss et al., unpublished data).

Sarcocystis hominis and *Sarcocystis suihominis*

There is no known treatment or prophylaxis for intestinal infection, myositis, vasculitis, or related lesions due to sarcocystosis in humans. It is clear that individuals who travel to Malaysia (25, 26, 49) should take precautions and not drink water or consume food potentially contaminated with sporocysts. Supportive therapy for patients with severe diarrhea is indicated. For six patients in Thailand with segmental necrotizing enteritis, treatment consisted of surgical resection of the affected areas of the intestine and antibiotics for the associated bacterial infection (46).

There is a case report of albendazole having efficacy in an outbreak of eosinophilic myositis due to *Sarcocystis* spp. (25). It is likely that steroids have a role in decreasing the inflammatory response in cases of myositis and vasculitis due to *Sarcocystis* spp. infection, but this has never been evaluated in a controlled trial.

Treatment Failure

There are no documented reports of drug-resistant strains of *C. belli*, *C. cayetanensis*, or *Sarcocystis* species. Development of resistance to trimethoprim-sulfamethoxazole has been suggested in one study of eight AIDS patients with *C. belli*, but other factors such as antigen-specific immune deficiency or generalized reduction in gut immunity could not be excluded (73). It appears that treatment failures are most likely to be related to poor drug absorption or distribution than to true drug resistance.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Both *C. belli* and *C. cayetanensis* are usually identified by stool examination and are rarely misidentified in human feces. Quantitation of the number of organisms found per high-power field is not required. Stool examinations that are reported as negative should indicate that at least three stool examinations are needed to detect organisms in 95% of infected individuals and that a single specimen may miss as many as 30% of infected patients. If acid-fast or similar stains are done on the stool examination and are positive, then this should be indicated in the report. Computer report notes can indicate that trimethoprim-sulfamethoxazole is the drug of choice for treatment of these parasites. PCR can be used for the identification of these organisms but is not commercially available and should be indicated as an experimental test. Serology is not currently used for diagnosis of these diseases in humans. Of the coccidia discussed here, only *C. cayetanensis* is reportable to state health departments and the CDC, as it has been associated with outbreaks and the contamination of human food sources.

S. hominis and *S. suihominis* can be identified by their characteristic morphology in stool specimens or in tissue biopsy specimens. They cannot be identified to the species level based on sporocyst structure and can only be identified as *Sarcocystis* spp. Due to the rarity of these infections, confirmation of the observed organisms should be obtained from experts in parasitology. Laboratory reports should indicate whether expert confirmation of the identity of these organisms has been obtained.

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Cryptosporidium

LIHUA XIAO AND VITALIANO CAMA

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Cryptosporidium spp. inhabit the brush borders of the gastrointestinal and respiratory epithelium of various vertebrates, causing enterocolitis, diarrhea, and cholangiopathy in humans (1). Immunocompetent children and adults with cryptosporidiosis usually have a short-term illness accompanied by watery diarrhea, nausea, vomiting, and weight loss. In immunocompromised persons, however, the infection can be protracted and life-threatening (2). In developing countries, cryptosporidiosis is one of the most important causes of moderate to severe diarrhea and diarrhea-associated death (3). *Cryptosporidium* spp. are well-recognized water- and foodborne pathogens in industrialized nations, having caused many outbreaks of human illness (4, 5).

TAXONOMY

Cryptosporidium spp. belong to the family *Cryptosporidiidae*, which is a member of the phylum *Sporozoa* (syn. *Apicomplexa*). The exact placement of *Cryptosporidiidae* in *Sporozoa* is uncertain. It was long considered a member of the class *Coccidea*, in the order of *Eimeriida*. Recent phylogenetic studies, however, indicate that *Cryptosporidium* spp. are more related to gregarines than to coccidia (6). Putative extracellular gregarine-like reproductive stages were described (7).

Recently, the taxonomy of *Cryptosporidium* has gone through revisions as the result of extensive molecular genetic studies and biologic characterizations of parasites from various animals (8, 9). There are >20 established *Cryptosporidium* species, including *C. hominis* in humans; *C. parvum* in humans and preweaned ruminants; *C. viatorum* in humans; *C. andersoni*, *C. bovis*, and *C. ryanae* in weaned calves and adult cattle; *C. xiaoi* and *C. ubiquitum* in sheep and goats; *C. suis* and *C. scrofarum* in pigs; *C. canis* in dogs; *C. felis* in cats; *C. cuniculus* in rabbits; *C. wrairi* in guinea pigs; *C. muris* and *C. tyzzeri* in rodents; *C. fayeri* and *C. macropodum* in marsupials; *C. meleagridis*, *C. baileyi*, and *C. galli* in birds; *C. varanii* and *C. serpentis* in reptiles; *C. fragile* in amphibians; and *C. molnari* and *C. scophthalmi* in fish. There are also >40 host-adapted *Cryptosporidium* genotypes that do not yet have species names, such as *Cryptosporidium* horse, skunk, and hedgehog genotypes (8–12). These species and genotypes biologically, morphologically, and phylogenetically belong to three groups: intestinal, gastric, and piscine species and genotypes (13). Most of these *Cryptosporidium* species and genotypes have not been found in humans.

DESCRIPTION OF THE AGENT

Cryptosporidium spp. are intracellular parasites that primarily infect epithelial cells of the intestine and biliary ducts. In severely immunosuppressed persons, the respiratory tract is sometimes involved. The involvement of the respiratory system in immunocompetent persons may be more common than previously believed, although its role in cryptosporidiosis transmission is not yet clear (14). The infection site varies according to species, but almost the entire development of *Cryptosporidium* spp. occurs between the two lipoprotein layers of the membrane of the epithelial cells.

Cryptosporidium infections in humans or other susceptible hosts start with the ingestion of viable oocysts (Fig. 1). Upon contact with gastric and duodenal fluid, four sporozoites are liberated from each excysted oocyst; invade the epithelial cells; develop to trophozoites surrounded by a parasitophorous vacuole; and undergo two or three generations of asexual multiplication and one generation of sexual reproduction, leading to the formation of new oocysts. The latter are sporulated *in situ*, excreted into the environment with feces, and can initiate infection in a new host upon ingestion without further development (Fig. 1). The time from ingestion of infective oocysts to the completion of endogenous development and excretion of new oocysts varies with species, hosts, and infection doses; it is usually between 4 and 10 days (15, 16). In addition to the classic coccidian developmental stages, a gregarine-like extracellular stage was described in *C. parvum*, which supposedly can go through multiplication via syzygy, a sexual reproduction process involving the end-to-end fusion of two or more parasites (7).

Currently, nearly 20 *Cryptosporidium* species and genotypes shave been reported in humans, including *C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. cuniculus*, *C. ubiquitum*, *C. viatorum*, *C. muris*, *C. suis*, *C. fayeri*, *C. andersoni*, *C. bovis*, *C. scrofarum*, *C. tyzzeri*, and *Cryptosporidium* horse, skunk, hedgehog, and chipmunk I genotypes. Humans are most frequently infected with *C. hominis* and *C. parvum*. The former almost exclusively infects humans and nonhuman primates and thus is considered an anthroponotic parasite, whereas the latter mostly infects humans and ruminants and thus is considered a zoonotic pathogen. Other species, such as *C. meleagridis*, *C. felis*, *C. canis*, *C. cuniculus*, *C. ubiquitum*, and *C. viatorum*, are less common. The remaining *Cryptosporidium* species and genotypes have been found only in a few human cases (17, 18). These *Cryptosporidium* spp. infect both immunocompetent and immunocom-

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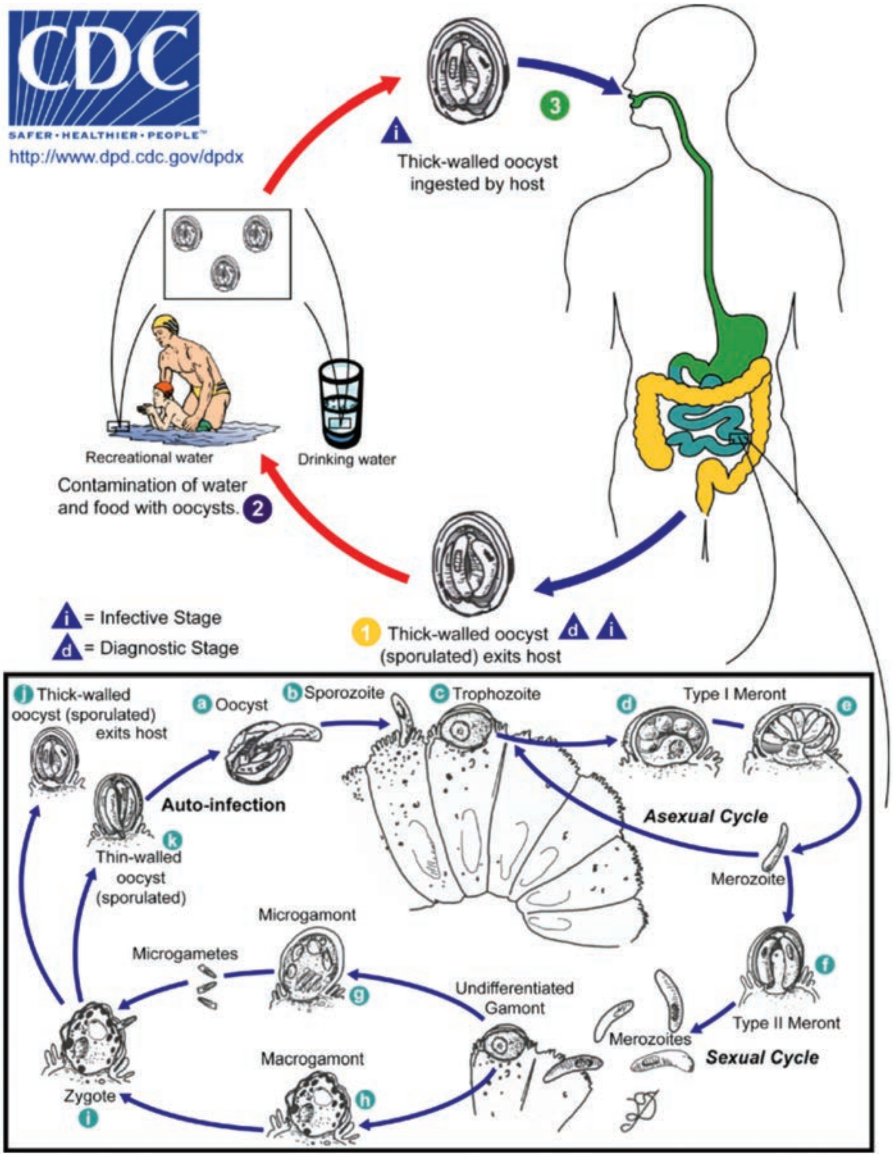
CDC
SAFER·HEALTHIER·PEOPLE™
http://www.dpd.cdc.gov/dpdx
i
Thick-walled oocyst
ingested by host
3
Recreational water
Drinking water
Contamination of water
and food with oocysts. 2
i = Infective Stage
d = Diagnostic Stage
1 Thick-walled oocyst d i
(sporulated) exits host
j Thick-walled
oocyst (sporulated)
exits host
a Oocyst
b Sporozoite
c Trophozoite
d
Type I Meront
e
Auto-infection
k
Thin-walled
oocyst
(sporulated)
Asexual Cycle
Merozoite
Microgamont
Microgametes
g
Undifferentiated
Gamont
f
Type II Meront
Merozoites
Sexual Cycle
Macrogamont
h
Zygote
i

FIGURE 1 Life cycle of *Cryptosporidium* spp. Sporulated oocysts, containing four sporozoites, are excreted by the infected host through feces and possibly other routes such as respiratory secretions (1). Transmission of *Cryptosporidium* spp. in humans occurs mainly through contact with infected persons (for *C. hominis* and *C. parvum*) or animals (for *C. parvum* mostly) and consumption of contaminated water and food (2). Following ingestion (and possibly inhalation) by a suitable host (3), excystation (a) occurs. The sporozoites are released and parasitize epithelial cells (b, c) of the gastrointestinal tract or other tissues such as the respiratory tract. In these cells, the parasites undergo asexual multiplication (schizogony or merogony) (d to f) and then sexual multiplication (gametogony), producing microgamonts (male) (g) and macrogamonts (female) (h). Upon fertilization of the macrogamonts by the microgametes (i), oocysts (j, k) develop that sporulate in the infected host. Two different types of oocysts are produced, the thick-walled oocyst (j), which is commonly excreted from the host; and the thin-walled oocyst (k), which is primarily involved in autoinfection. Oocysts are infective upon excretion, thus permitting direct and immediate fecal-oral transmission. Courtesy of DPDx (http://www.cdc.gov/dpdx/). doi:10.1128/9781555817381.ch142.f1

promised persons. The distribution of these species in humans varies among different geographic areas and socioeconomic conditions, with *C. canis* and *C. felis* mostly seen in humans in developing countries, *C. ubiquitum* mostly in industrialized nations, and *C. cuniculus* mostly in the United Kingdom. This is probably the result of differences in infection sources and transmission routes (17).

EPIDEMIOLOGY, TRANSMISSION, AND PREVENTION

Cryptosporidium spp. have a worldwide distribution, and their oocysts are ubiquitous in the environment. In the United States, the number of annual reported cases of cryptosporidiosis has increased more than 2-fold since 2004; there were 2,769 to 3,787 annual reported cases between

1999 and 2002, 3,505 to 8,269 between 2003 and 2005, 6,479 to 11,657 between 2006 and 2008, and 7,656 to 8,951 between 2009 and 2010 (19–22). Currently, it is estimated that there are ~750,000 cases of cryptosporidiosis in the United States each year, as 98.6% of cases remain undiagnosed or unreported (5). The estimated annual cost of hospitalization alone due to cryptosporidiosis in the United States is $45 million, with a per-case cost of $16,797 (23). Humans can acquire cryptosporidiosis through several transmission routes, such as direct contact with infected persons or animals and consumption of contaminated water (drinking or recreational) or food (1, 17). However, the relative role of each in the transmission of *Cryptosporidium* infection in humans is unclear.

Susceptible Populations

In developing countries, human *Cryptosporidium* infection occurs mostly in children younger than 2 years and is one of the top five causes of diarrhea in this age group (2, 3, 16). In industrialized nations, pediatric cryptosporidiosis occurs in children later than in developing countries, probably due to delayed exposures to contaminated environments as a result of better hygiene (19, 21, 22, 24, 25). Cryptosporidiosis is also common in elderly people in nursing homes, where person-to-person transmission probably plays a major role in the spread of *Cryptosporidium* infections (26). In the general population, a substantial number of adults are susceptible to *Cryptosporidium* infection, as sporadic infections occur in all age groups in the United States and the United Kingdom, and travel to developing countries and consumption of contaminated food or water can frequently lead to infection (27–30). Cryptosporidiosis is common in immunocompromised persons, including AIDS patients, persons with primary immunodeficiency, and cancer and transplant patients undergoing immunosuppressive therapy (1, 31–37). Hemodialysis patients with chronic renal failure and renal transplant patients commonly develop cryptosporidiosis (34, 38–41). In HIV-positive persons, the occurrence of cryptosporidiosis increases as the $CD4^+$ lymphocyte cell counts fall, especially below 200 cells/μl (32).

Anthroponotic versus Zoonotic Transmission

Studies in the United States and Europe have shown that cryptosporidiosis is more common among homosexual men than among persons in other HIV transmission categories (42), indicating that direct person-to-person or anthroponotic transmission of cryptosporidiosis is common. Contact with persons with diarrhea has been identified as a major risk factor for sporadic cryptosporidiosis in industrialized countries (28, 43–46). This is exemplified by the high prevalence of cryptosporidiosis in day care facilities and nursing homes and among mothers with young children in these countries.

Only a few case-control studies have assessed the role of zoonotic transmission in the acquisition of cryptosporidiosis in humans. In industrialized countries, contact with farm animals (especially cattle) is a major risk factor in sporadic cases of human cryptosporidiosis (28, 30, 43, 44, 47, 48). Contact with pigs, dogs, or cats was a risk factor for cryptosporidiosis in children in Guinea-Bissau and Indonesia in one study (49, 50). A weak association was observed between the occurrence of cryptosporidiosis in HIV-positive persons and contact with dogs in another study (51). In other studies, no increased risk in the acquisition of cryptosporidiosis was associated with contact with companion animals (52).

The distribution of *C. parvum* and *C. hominis* in humans is a reflection of the role of different transmission routes. Thus far, studies conducted in developing countries have shown a predominance of *C. hominis* in children or HIV-positive adults. This is also true for most areas in the United States, Canada, Australia, and Japan. In Europe and New Zealand, however, several studies have shown almost equal prevalence of *C. parvum* and *C. hominis* in both immunocompetent and immunocompromised persons (17). In contrast, children in the Middle East are mostly infected with *C. parvum* (53). The differences in the distribution of *Cryptosporidium* genotypes in humans are considered to be an indication of differences in infection sources (17, 48, 54, 55); the occurrence of *C. hominis* in humans is most likely due to anthroponotic transmission, whereas *C. parvum* in a population can be the result of both anthroponotic and zoonotic transmission. Thus, in most developing countries, it is possible that anthroponotic transmission of *Cryptosporidium* plays a major role in human cryptosporidiosis, whereas in Europe, New Zealand, and rural areas of the United States, both anthroponotic and zoonotic transmissions are important.

Recent subtyping studies based on sequence analyses of the 60-kDa glycoprotein (gp60) gene have shown that many *C. parvum* infections in humans are not results of zoonotic transmission (17). Among several *C. parvum* subtype families identified, IIa and IIc are the two most common families. The former has been identified in both humans and ruminants and thus can be a zoonotic pathogen, whereas the latter has been seen only in humans (13, 17, 56) and thus is an anthroponotic pathogen. In developing countries, most *C. parvum* infections in children and HIV-positive persons are caused by the subtype family IIc, with IIa largely absent, indicating that anthroponotic transmission of *C. parvum* is common in these areas (13, 17). In contrast, both IIa and IIc subtype families are seen in humans in developed countries. Even in the United Kingdom, where zoonotic transmission is known to play a significant role in the transmission of human cryptosporidiosis, anthroponotic transmission of *C. parvum* also occurs (57). Another *C. parvum* subtype family commonly found in sheep and goats, IId, is the dominant *C. parvum* subtype family in humans in Middle Eastern countries (53). Results of multilocus subtyping support the conclusions of gp60 subtyping studies (58–61).

Waterborne Transmission

Epidemiologic studies have frequently identified water as a major route of *Cryptosporidium* transmission in areas where the disease is endemic (62). In most tropical countries, *Cryptosporidium* infections in children usually peak during the rainy season; thus, waterborne transmission probably plays a role in the transmission of cryptosporidiosis in these areas (63–65). Seasonal variations in the incidence of human *Cryptosporidium* infection in industrialized nations have also been partially attributed to waterborne transmission (27, 28, 30, 66). In the United States, there is a late summer peak in sporadic cases of cryptosporidiosis (22, 28, 30), which is largely due to increased participation in recreational activities such as swimming and water sports (67). In a Canadian study, swimming in a lake or river was identified as a risk factor (45).

The role of drinking water in sporadic *Cryptosporidium* infection is not clear. In Mexican children living near the U.S. border, cryptosporidiosis is associated with consumption of municipal water instead of bottled water (68). In England, there is an association between the number of glasses of tap water drunk at home each day and the occurrence of sporadic cryptosporidiosis (43). In the United States, drinking untreated surface water was identified as a risk factor for the acquisition of *Cryptosporidium* in case-control studies (46, 69), and residents living in cities with surface-derived drinking water generally have higher antibody levels against *Cryptosporidium* in their blood than those living in cities with groundwater as drinking water, indicating that drinking water plays a role in the transmission of human cryptosporidiosis (70). Nevertheless, case-control studies conducted with both immunocompetent persons and AIDS patients in the United States and Canada have failed to show a direct linkage of *Cryptosporidium* infection to drinking water (71–73).

Numerous waterborne outbreaks of cryptosporidiosis have occurred in the United States, Canada, the United Kingdom, France, Australia, Japan, and other industrialized nations (4, 74). These include outbreaks associated with both drinking water and recreational water (swimming pools and water parks). After the massive cryptosporidiosis outbreak in Milwaukee, WI, in 1993, the water industry has adopted more-stringent treatments of source water. Currently, the number of drinking-water-associated outbreaks is in decline in the United States and the United Kingdom, and most outbreaks in the United States are associated with recreational water (30, 67). Even though five *Cryptosporidium* species are commonly found in humans, *C. parvum* and *C. hominis* are responsible for most cryptosporidiosis outbreaks, with *C. hominis* responsible for more outbreaks than *C. parvum* (17). This is even the case for the United Kingdom, where *C. parvum* and *C. hominis* are both common in the general population. Recently, there was one drinking-water-associated cryptosporidiosis outbreak caused by *C. cuniculus* (75). An outbreak of *C. meleagridis* also occurred in a high school dormitory in Japan, although the role of waterborne transmission in the occurrence of the outbreak was not clear (76).

Foodborne Transmission

Foodborne transmission is also important in cryptosporidiosis epidemiology. *Cryptosporidium* oocysts have been isolated often from fruits, vegetables, and shellfish (77–80). Direct contamination of food by fecal materials from animals or food handlers has been implicated in several foodborne outbreaks of cryptosporidiosis in industrialized nations. In most instances, human infections were usually due to consumption of contaminated fresh produce and unpasteurized apple cider or milk (81–87).

Very few case-control studies have examined the role of contaminated food as a risk factor in the acquisition of *Cryptosporidium* infection in areas where the disease is endemic. A pediatric study in Brazil failed to show any association between *Cryptosporidium* infection and diet or type of food hygiene (52). Case-control studies conducted in the United States, the United Kingdom, and Australia have actually shown a lower prevalence of *Cryptosporidium* infection in immunocompetent persons with frequent consumption of raw vegetables (28, 43, 46, 88), probably because of continuous exposure to *Cryptosporidium*-contaminated fresh produce. It is estimated that ~8% of *Cryptosporidium* infections in the United States are foodborne (5).

Prevention

As for any pathogens that are transmitted by the fecal-oral route, good hygiene is the key in preventing the acquisition of *Cryptosporidium* infection (89). Immunosuppressed persons especially should take necessary precautions in preventing the occurrence of cryptosporidiosis (90). This includes washing hands before preparing food and after using the bathroom, changing diapers, and contacting pets or soil (including gardening); avoiding drinking water from lakes and rivers, swallowing water in recreational activities, and drinking unpasteurized milk, milk products, and juices; and following safe-sex practices (avoiding oral-anal contact). During cryptosporidiosis outbreaks or when a community advisory to boil water is issued, individuals should boil water for 1 min to kill the parasite or use a tap water filter capable of removing particles <1 μm in diameter. Immunosuppressed persons also should avoid eating raw shellfish and should not eat uncooked vegetable salads and unpeeled fruits when traveling to developing countries (90).

CLINICAL SIGNIFICANCE

In developing countries, frequent symptoms of cryptosporidiosis in children include diarrhea, abdominal cramps, nausea, vomiting, headache, fatigue, and low-grade fever (2). The diarrhea can be voluminous and watery but usually resolves within 1 to 2 weeks without treatment. Not all infected children have diarrhea or other gastrointestinal symptoms, and the occurrence of diarrhea in children with cryptosporidiosis can be as low as 30% in community-based studies (91). Even subclinical cryptosporidiosis exerts a significant adverse effect on child growth, as infected children with no clinical symptoms experience growth faltering, both in weight and in height (1). Children can have multiple episodes of cryptosporidiosis, implying that the anti-*Cryptosporidium* immunity in children is short-lived or incomplete (92). Cryptosporidiosis has been associated with increased mortality in developing countries (3, 63, 93).

Unlike in developing countries, immunocompetent persons with sporadic cryptosporidiosis in industrialized nations usually have diarrhea (1, 29, 88). The median number of stools per day during the worst period of the infection is 7 to 9.5 (88). Other common symptoms include abdominal pain, nausea, vomiting, and low-grade fever (29, 88). The duration of illness has a mean or median of 9 to 21 days, with a median loss of 5 work or study days and hospitalization of 7 to 22% of patients (27, 29, 88). Patients infected with *C. hominis* are more likely to have joint pain, eye pain, recurrent headache, dizziness, and fatigue than those infected with *C. parvum* (94). There are significant differences among different *Cryptosporidium* species and *C. hominis* subtype families in clinical manifestations of pediatric cryptosporidiosis (92).

Cryptosporidiosis in immunocompromised persons, including AIDS patients, is frequently associated with chronic, life-threatening diarrhea (32). Sclerosing cholangitis and other biliary involvements are also common in AIDS patients with cryptosporidiosis (95). In severely immunocompromised persons, respiratory system involvement is common (96). Cryptosporidiosis in AIDS patients is associated with increased mortality and shortened survival (97, 98). Variations in the infection site (gastric infection, proximal small intestine infection, or ileocolonic infection versus panenteric infection) have been seen in AIDS patients with cryptosporidiosis. They may contribute to differences in clinical presentation and survival (99, 100). Likewise, different *Cryptosporidium* species and *C. hominis* subtype families are associated with different clinical manifestations in HIV-positive persons in developing countries (101).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Currently, most active *Cryptosporidium* infections are diagnosed by analysis of stool specimens. Stool specimens are usually collected fresh or in fixative solutions such as 10% buffered formalin and polyvinyl alcohol (PVA) (102). However, stool specimens fixed in formalin and mercury-based preservatives (such as LV-PVA) cannot be used for molecular diagnosis. For outbreak investigations, *Cryptosporidium* spp. present are frequently genotyped and subtyped by PCR methods, which require the use of fresh or frozen stool specimens or stools stored in Cary-Blair transport media or preserved in TotalFix (Medical Chemical Corporation, Torrance, CA), zinc PVA, or 2.5% potassium dichromate. It is recommended that whenever possible multiple specimens (three specimens passed at intervals of 2 to 3 days) from each patient should be examined if *Cryptosporidium* infection is suspected and the examination of the initial stool specimen is negative. This is because carriers with low oocyst shedding are common, and examination of individual specimens can lead to the detection of only 53% of infections (103).

Examination of intestinal or biliary biopsy is sometimes used in the diagnosis of cryptosporidiosis in AIDS patients (99). However, the sensitivity of the diagnosis depends on the location of tissues examined; the duodenum is usually infected with *Cryptosporidium* only in severe infection, and the terminal ileum and colon have significantly higher detection rates than the duodenum (103). Thus, upper endoscopic biopsies are much less sensitive than colonoscopy biopsies in diagnosing cryptosporidiosis.

DIRECT EXAMINATION

In clinical laboratories, *Cryptosporidium* spp. in stool specimens are commonly detected by microscopic examinations of oocysts or immunologic detection of antigens (104).

Microscopy

Stool specimens can be examined directly for *Cryptosporidium* oocysts by microscopy of direct wet-mount or stained fecal materials if the number of oocysts in specimens is high. *Cryptosporidium* oocysts in humans generally measure 4 to 6 μm. Occasionally, *C. muris* oocysts are also found, which are more elongated and measure 6 to 9 μm. Oocysts present are often concentrated using either traditional ethyl acetate or Weber-modified ethyl acetate concentration methods (104). Concentrated stool specimens can be examined by microscopy in several ways. *Cryptosporidium* oocysts can be detected by bright-field microscopy in direct wet mounts. This allows the observation of oocyst morphology and more accurate measurement of oocysts, which is frequently needed in biologic studies. Differential interference contrast can be used in microscopy, which produces better images and visualization of internal structures of oocysts. Most *Cryptosporidium* species look similar under microscopes and have similar morphometric measurements (8).

More often, *Cryptosporidium* oocysts in concentrated stool specimens are detected by microscopy after staining of the fecal smears. Many special stains have been used in the detection of *Cryptosporidium* oocysts, but modified acid-fast stains are the most commonly used (104), especially in developing countries, because of their low cost, ease of use, lack of need for special microscopes, and simultaneous detection of several other pathogens such as *Cystoisospora* (formerly *Isospora*) and *Cyclospora* (Fig. 2). Two stains widely used are the modified Ziehl-Neelsen acid-fast stain and modified Kinyoun's acid-fast stain (104). Oocysts are stained bright red to purple against a blue or green background (Fig. 2).

Direct immunofluorescence assays (DFAs) have been used increasingly in *Cryptosporidium* oocyst detection by microscopy, especially in industrialized nations. Compared to acid-fast staining, DFA has higher sensitivity and specificity (105). Many commercial DFA kits are marketed for the diagnosis of *Cryptosporidium*, most of which include reagents allowing simultaneous detection of *Giardia* cysts (Table 1). Oocysts appear apple green against a dark background in immunofluorescence microscopy (Fig. 3). Because of the high sensitivity and specificity, DFA has been used by some as the gold standard or reference test (105, 106). It has been shown that most antibodies in commercial DFA kits react with oocysts of almost all *Cryptosporidium* species, making identification to the species level impossible (107, 108).

Compared with DFA, the sensitivity of most microscopic methods is probably low. The detection limit for the combination of ethyl acetate concentration and DFA was shown to be 10,000 oocysts per gram of liquid stool and 50,000 oocysts per gram of formed stool (109, 110). A similar sensitivity was achieved with fecal specimens from dogs (111). The sensitivity of modified acid-fast staining was 10-fold lower than that of DFA (110), probably because acid-fast stains do not consistently stain all oocysts (112). The sensitivity of the DFA can be significantly improved by the incorporation of an oocyst isolation step using an immunomagnetic separation technique (113).

Antigen Detection

Cryptosporidium infection can also be diagnosed by the detection of *Cryptosporidium* antigens in stool specimens by immunoassays (104). Antigen capture-based enzyme immunoassays (EIAs) have been used in the diagnosis of cryptosporidiosis since 1990. In recent years, EIAs have gained popularity because they do not require experienced microscopists and can be used to screen a large number of samples (114). In clinical laboratories, several commercial EIA kits are commonly used (Table 1). High specificity (99 to 100%) has been generally reported for these EIA kits (105, 106, 115). Sensitivities, however, have been reported to range between 70% (105) and 94 to 100% (106, 116–118). Occasional false positivity of EIA kits is known to occur in the detection of *Cryptosporidium* (119, 120), and manufacturers' recalls of EIA kits have occurred because of high nonspecificity (121). If a patient is in the carrier state or undergoing self-cure, the number of oocysts may drop below the sensitivity levels of these kits (105). Most EIA kits have been evaluated only with human stool specimens presumably from patients infected with *C. hominis* or *C. parvum* (118). Their usefulness in the detection of *Cryptosporidium* spp. in animals may be compromised by the specificity of the antibodies.

In recent years, several lateral flow immunochromatographic assays have been marketed for rapid detection of *Cryptosporidium* in stool specimens (Table 1). In evaluation studies, these assays have been shown to have high specificities (>90%) and sensitivities (98 to 100%) (105, 122–127). However, sensitivities of 68 to 75% were shown in some studies for some assays (105, 128–130). High false-positive rates (positive predictive value, 56%) for several rapid assays have been reported recently in clinical diagnosis of cryptosporidiosis in the United States (131). This has prompted the Council of State and Territorial Epidemiologists to change the case definition of rapid assay-positive cases from confirmed cases to probable cases. It has also been shown recently that some rapid assay kits have low sensitivity (<35%) in detecting some *Cryptosporidium* species other than *C. hominis* and *C. parvum* (130).

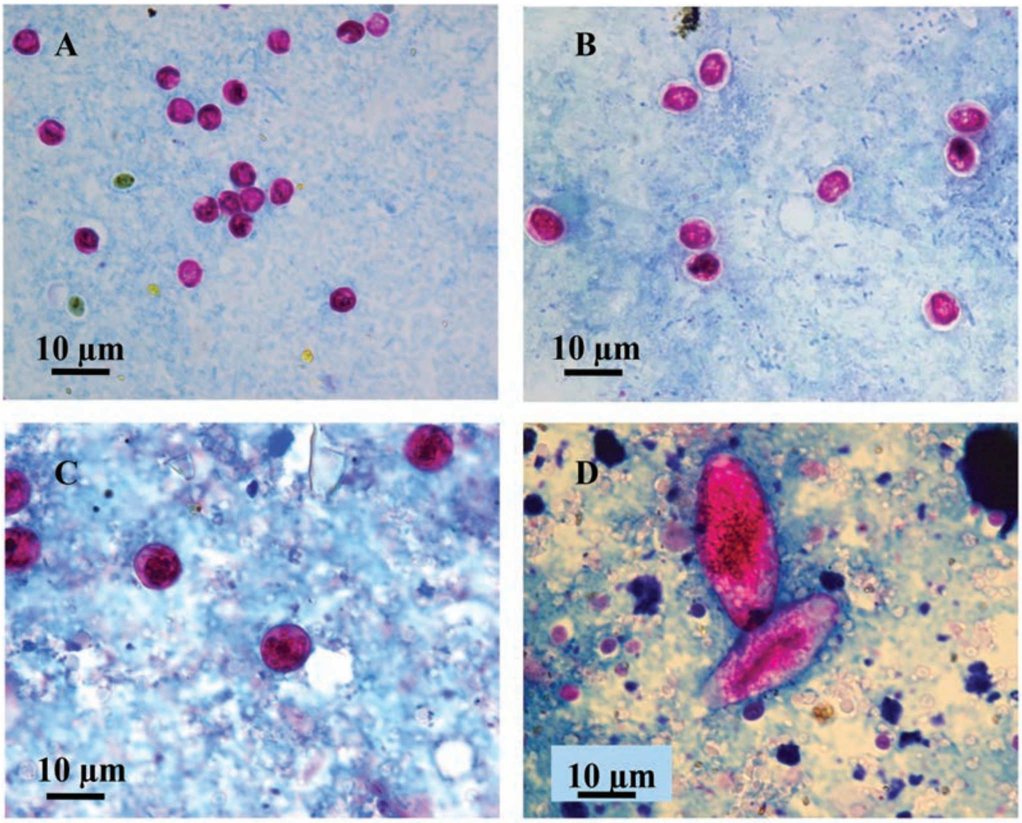

FIGURE 2 Oocysts of *Cryptosporidium hominis* (4 to 6 µm) (A), *Cryptosporidium muris* (6 to 8 µm) (B), *Cyclospora cayetanensis* (8 to 10 µm) (C), and *Cystoisospora belli* (20 to 30 µm by 10 to 20 µm) (D) stained by the modified Ziehl-Neelsen acid-fast stain.
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TYPING SYSTEMS

Molecular techniques, especially PCR and PCR-related methods, have been developed and used in the detection and differentiation of *Cryptosporidium* spp. for many years (132). A few of the PCR assays are commercially available (Table 1). Several genus-specific PCR-restriction fragment length polymorphism-based genotyping tools have been developed for the detection and differentiation of *Cryptosporidium* at the species level (133–137). Most of these techniques are based on the small-subunit rRNA gene. Other genotyping techniques are designed mostly for the differentiation of *C. parvum* and *C. hominis* and thus cannot detect and differentiate other *Cryptosporidium* spp. or genotypes (17). Their usefulness in the analysis of human stool specimens is compromised by their inability to detect *C. canis*, *C. felis*, *C. suis*, and *C. muris* (138).

Several subtyping tools have also been developed to characterize the diversity within *C. parvum* or *C. hominis* (17). One of the most commonly used techniques is sequence analysis of the gp60 gene (also known as gp40/15) (24, 56, 139, 140). Most of the genetic heterogeneity in this gene is present in the number of a trinucleotide repeat (TCA, TCG, or TCT), although extensive sequence differences are also present between families of subtypes. Multilocus mini- and microsatellite subtyping tools for *C. parvum* and *C. hominis* have also been developed (59, 141–144). The usefulness of subtyping tools has been demonstrated by the analysis of samples from foodborne and waterborne outbreaks of cryptosporidiosis (46, 60, 85, 87, 145–156).

ISOLATION PROCEDURES

The *in vitro* cultivation of *Cryptosporidium* spp. remains inefficient despite recent advances (157, 158). The low parasite yields and oocyst production have limited the usefulness of parasite culture in the isolation and diagnosis of *Cryptosporidium*. Nevertheless, *in vitro* cultivation of early *Cryptosporidium* developmental stages in several epithelial cell lines (HCT-8, Madin-Darby bovine kidney, Caco-2, etc.) has been used widely in research studies to assess potential drugs and oocyst disinfection methods and to characterize parasite development, differentiation, and biochemistry (157). The recent development of procedures for long-term maintenance of *C. parvum* and *C. hominis* in cell and cell-free culture, if verified, could promote the use of *in vitro* cultivation methods in routine diagnosis of *Cryptosporidium* infection (158, 159).

TREATMENT

Numerous pharmaceutical compounds have been screened for anti-*Cryptosporidium* activities *in vitro* or in laboratory animals (160–162). Some of those showing promise have been used in the experimental treatment of cryptosporidiosis in humans, but few have been shown to be effective in

TABLE 1 Some commercial diagnostic assays for the detection of *Cryptosporidium* oocysts, antigens, or DNA

Product	Manufacturer, country	Approval/ accreditation[a]	Intended use
Immunofluorescence assays			
Merifluor *Cryptosporidium/Giardia*	Meridian Biosciences, United States	FDA	Clinical
Aqua-Glo G/C kit	Waterborne Inc., United States	No	Environmental
Crypt-a-Glo FL, comprehensive kit	Waterborne Inc., United States	No	Veterinary
Crypto Cel[b]	Cellabs, Australia	No	Clinical/ environmental
Crypto/*Giardia* Cel[b]	Cellabs, Australia	No	Clinical/ environmental
Cryptosporidium parvum antibody (mouse monoclonal antibody)	Fitzgerald Industries International, Inc., United States	No	Research
Easy Stain	BTF, a bioMérieux company, Australia	ISO 34	Environmental
Enzyme immunoassays			
Cryptosporidium Ag, ELISA[b]	Cypress Diagnostics, Belgium	CE	Clinical
Ridascreen *Cryptosporidium*	R-Biopharm, Germany	CE	Clinical
Cryptosporidium II (direct antigen, visual or spectrophotometric)	TechLab, United States	FDA	Clinical
Cryptosporidium Quik Chek (membrane immunoassay)	TechLab, United States	FDA	Clinical
Giardia/Cryptosporidium Chek ELISA	TechLab, United States	FDA	Clinical
ProSpecT *Cryptosporidium* microplate	Thermo Fisher Scientific Remel, United States	FDA	Clinical
ProSpecT *Giardia/Cryptosporidium* microplate	Thermo Fisher Scientific Remel, United States	FDA	Clinical
IVD Research *Giardia*/Crypto Combo EIA	IVD Research Inc., United States	No	Research
Immunochromatography			
Bovine *Cryptosporidium* dipstick[b]	Cypress Diagnostics, Belgium	CE	Veterinary
Crypto/*Giardia* Duo-Strip[b]	Coris BioConcept, Belgium	CE	Clinical
Crypto-Strip C-1005 (Crypto Uni-Strip, Crypto-CIT)[b]	Coris BioConcept, Belgium	CE	Clinical
RIDA Quick *Cryptosporidium* (dipstick or cassette)[b]	R-Biopharm, Germany	CE	Clinical
RIDA Quick *Cryptosporidium/Giardia* Combi (dipstick or cassette)[b]	R-Biopharm, Germany	CE	Clinical
RIDA Quick *Cryptosporidium/Giardia/ Entamoeba* Combi (dipstick or cassette)[b]	R-Biopharm, Germany	CE	Clinical
Alere Triage Micro Parasite Panel	Alere, United States	FDA	Clinical (export only)
ColorPAC *Giardia/Cryptosporidium* rapid assay	Becton Dickinson, United States	FDA	Clinical
ImmunoCard STAT! *Cryptosporidium/Giardia* rapid assay	Meridian Bioscience, United States	CLIA	Clinical
IVD *Cryptosporidium* Antigen Detection Assay, Model CP-96	IVD Research Inc., United States	FDA	Clinical
Xpect *Cryptosporidium*	Thermo Fisher Scientific Remel, United States	FDA	Clinical
Xpect *Giardia/Cryptosporidium*	Thermo Fisher Scientific Remel, United States	FDA	Clinical
CoproStrip *Giardia/Cryptosporidium*[b]	Savyon Diagnostics Inc., Israel	No	Research
Crypto-*Giardia* Ag Rapid Test	Creative Diagnostics, United States	No	Research
Cryptosporidium Ag Rapid Test	Creative Diagnostics, United States	No	Research
Cryptosporidium Rapid Test	Creative Diagnostics, United States	No	Research
Crypto (card and blister formats)[b]	CerTest Biotec, Spain	CE	Clinical
Crypto-*Giardia* (card and blister)[b]	CerTest Biotec, Spain	CE	Clinical
Stick Crypto[b]	Operon, Spain	CE	Clinical
Stick Crypto-*Giardia*[b]	Operon, Spain	CE	Clinical
Molecular assays			
xTAG Gastrointestinal Pathogen Panel	Luminex Molecular Diagnostics, Inc., United States	FDA	Clinical
FilmArray System GI Panel	BioFire Diagnostics, United States	FDA/CE	Clinical
BD MAX Enteric Parasite Panel	Becton Dickinson, United States	CE	Clinical
KCRYP[b] (real-time PCR)	Ceeram S.A.S., France	CE	Food and environmental
KHCRYP[b] (real-time PCR)	Ceeram S.A.S., France	CE	Clinical
Cryptosporidium[b] (real-time PCR)	GeneSig (PrimerDesign Ltd.), United Kingdom	No	Research

[a] CE, *Conformité Européenne* or European Conformity (medical device that met standards set by the European Union and can be legally sold in those countries); FDA, approved by the U.S. Food and Drug Administration; ISO 34, produced in compliance with the International Standards Organization Guide 34; CLIA, laboratory protocols meet regulations of the Clinical Laboratory Improvement Amendment.

[b] Not available in the United States.

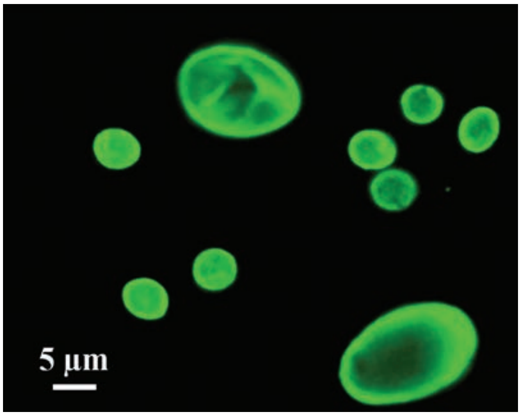

FIGURE 3 *Cryptosporidium hominis* oocysts (4 to 6 µm) and *Giardia duodenalis* cyst (11 to 14 µm by 7 to 10 µm) under immunofluorescence microscopy.
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controlled clinical trials (32, 163). Oral or intravenous rehydration and antimotility drugs are widely used whenever severe diarrhea is associated with cryptosporidiosis. Nitazoxanide is the only U.S. Food and Drug Administration-approved drug for the treatment of cryptosporidiosis in immunocompetent persons. Clinical trials have demonstrated that nitazoxanide can shorten clinical disease and reduce parasite loads (161, 164). This drug, however, is not effective in the treatment of *Cryptosporidium* infections in immunodeficient patients (163, 164). For this population, paromomycin and spiramycin have been used in the treatment of some patients, but their efficacy remains unproven (161–163).

In industrialized nations, the most effective treatment and prophylaxis for cryptosporidiosis in AIDS patients is the use of highly active antiretroviral therapy (HAART) (161, 162, 165). It is probably also an effective prevention for cryptosporidiosis in HIV-positive persons in developing countries (166). It is believed that the eradication and prevention of the infection are related to the replenishment of CD4$^+$ cells in treated persons and the antiparasitic activities of the protease inhibitors (such as indinavir, nelfinavir, and ritonavir) used in HAART (162, 165). Relapse of cryptosporidiosis is common in AIDS patients who have stopped HAART (167). In developing countries, protease inhibitors are generally not included in HAART regimens. Limited reports have shown that cryptosporidiosis is still common in HIV-positive patients receiving HAART in developing countries, although at lower frequencies than those generally reported in untreated HIV patients (166, 168, 169).

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Cryptosporidiosis is a notifiable disease in most states in the United States and in some other industrialized countries, although only ~1.4% of actual cases are diagnosed in the United States (5). Thus, the detection of the pathogen in stools or tissues should be reported to the local health department in addition to the physicians. Because most routine diagnostic tests cannot differentiate *Cryptosporidium* species, the detection of *Cryptosporidium* oocysts or antigens in stools or other specimens should be reported as *Cryptosporidium* positive without referring to the nature of species involved. From a public health point of view, the reporting of a significant number of cases above background levels in industrialized nations indicates the likely occurrence of outbreaks of cryptosporidiosis or false positivity of diagnostic kits (120, 121, 170, 171). In situations like this, it is crucial to have the test results verified with a confirmatory test such as DFA or PCR and to report them to the state or local public health department. The inclusion of both positive and negative controls in each test run and stringent adherence to recommended procedures will reduce the occurrence of test errors. In the United States, patients positive for *Cryptosporidium* by immunochromatographic assay-based rapid tests are considered by the Council of State and Territorial Epidemiologists as probable cryptosporidiosis cases. The diagnostic result requires confirmation by a second assay, such as DFA, EIA, or PCR.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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Nematodes*

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Nematodes are unsegmented cylindrical worms with a body cavity containing an alimentary canal and genital system; the sexes are separate. They lack circular muscles and move by flexing their bodies. There are four larval stages and the adult worm. In most instances, the third-stage larva is the infective stage.

Intestinal nematodes, including the soil-transmitted helminths, are the most common infections globally, with more than 1 billion people infected, especially in resource-poor settings where sanitation is inadequate. These parasites are a major cause of poor health, with the greatest morbidity in women and children, and are included in the list of neglected tropical diseases by the World Health Organization (WHO). Immigrants and refugees, travelers, and war veterans often unknowingly harbor helminths for years after leaving an area where helminths are endemic. Clinical features are usually directly proportional to the intensity of the worm infection. Global efforts to target soil-transmitted helminth infections in children with school-based chemotherapy have reduced their prevalence in many developing countries (http://www.who.int/intestinal_worms/en/). Strongyloidiasis may also benefit from a similar public health response in areas where it is highly endemic to overcome the epidemiological, diagnostic, and therapeutic challenges that *Strongyloides* poses (1).

Laboratory diagnosis is based primarily on microscopic examination of feces and differentiation of species based on morphology of eggs or larvae detected (Fig. 1 to 3). The sensitivity of this procedure depends on the stage of the infection and is affected by factors such as variability of the number of eggs or larvae excreted. Occasionally, adult worms may be processed by histology, and features of both the adult worm and the eggs may be used in identifying the parasite (Fig. 4).

Unless specified, antiparasitic susceptibility testing is not routinely performed in the clinical laboratory for these organisms. For more details about susceptibility testing for parasites, consult chapter 151.

TAXONOMY OF NEMATODES

There are a variety of morphological features which form the basis for taxonomy of nematodes. At the anterior end, mouthparts may possess structures for attachment or penetration. These may include spines, hooks, or cutting plates. The pharyngeal cavity may be short, hollowed out, or long and capillary. Esophageal shape also varies, but the esophagus usually ends in a muscular bulb. In the male, features of the tail may include caudal papillae and copulatory spicules (sclerotized copulatory aids). In the majority of cases, males carry more taxonomically useful information than females; the latter may often be unidentifiable to the species level in the absence of males. Studies strongly suggest that nematodes are actually related to the arthropods and priapulids in a newly recognized group, the Ecdysozoa (2, 3). All these nematodes belong to kingdom Animalia, subkingdom 3 Bilateria, and phylum Nemathelminthes (Nematoda) (see chapter 132).

ASCARIS LUMBRICOIDES (ROUNDWORM)

Taxonomy

A. lumbricoides belongs to the class Secernentea, order Ascarida, superfamily Ascaridoidea, and family Ascarididae.

Of the three genera of ascaridid nematodes regularly occurring as parasites of humans, only *Ascaris* is a true human parasite. The others (*Toxocara* and *Toxascaris*) are common parasites of cats and dogs and occasionally infect humans. Ascaridids are related to anisakids, with three distinct lips, but differ in that their life cycle is linked to terrestrial rather than aquatic conditions.

Ascaris suum was thought to be a separate species in pigs but has recently been found to be genetically similar to the human species *A. lumbricoides* (4).

Description

Eggs

Eggs of *A. lumbricoides* (Fig. 2A to C) are usually seen in two forms: unfertilized and fertilized. The egg shell of a fertilized egg (45 to 75 by 35 to 50 μm) consists of an inner lipid layer responsible for selective permeability, a chitin protein layer responsible for structural strength, and an outer vitelline layer. The inner layer contains a lipoprotein, ascaroside, which helps prevent formaldehyde, disinfectants, and other chemicals from contacting the embryo. The outer surface of the fertilized egg has an uneven deposit of mucopolysaccharide with adhesive properties. Eggs appear brown due to staining by bile. The unfertilized eggs have thinner walls and distorted mammillations. These eggs are usually more elongated in shape, measure 85 to 95 by 43 to 47 μm, and may have either a pronounced mammillated coat or little or no mammillated layer. The

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*This chapter contains information presented by Harsha Sheorey, Beverley-Ann Biggs, and Peter Traynor in chapter 141 of the 10th edition of this *Manual*.

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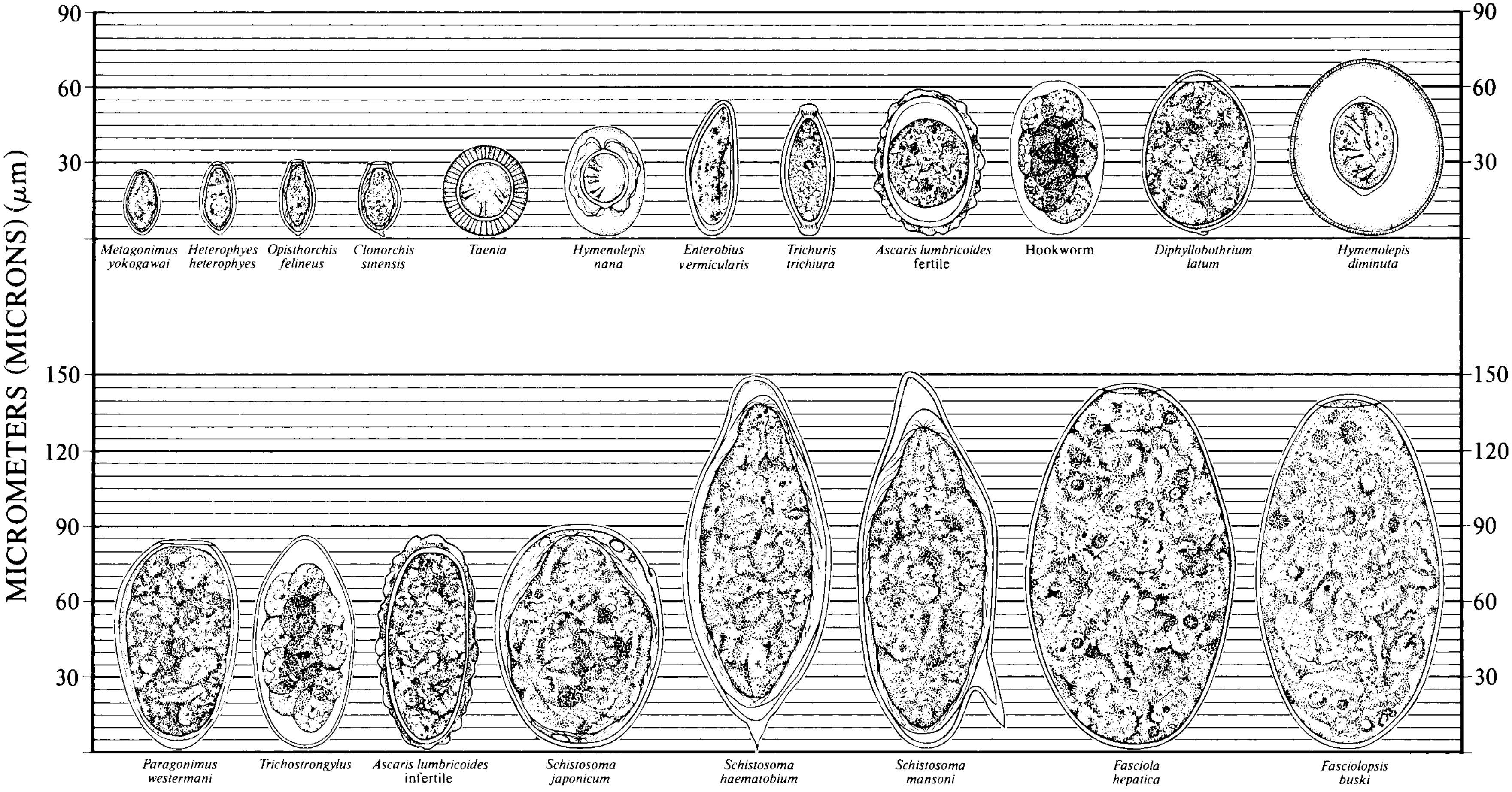
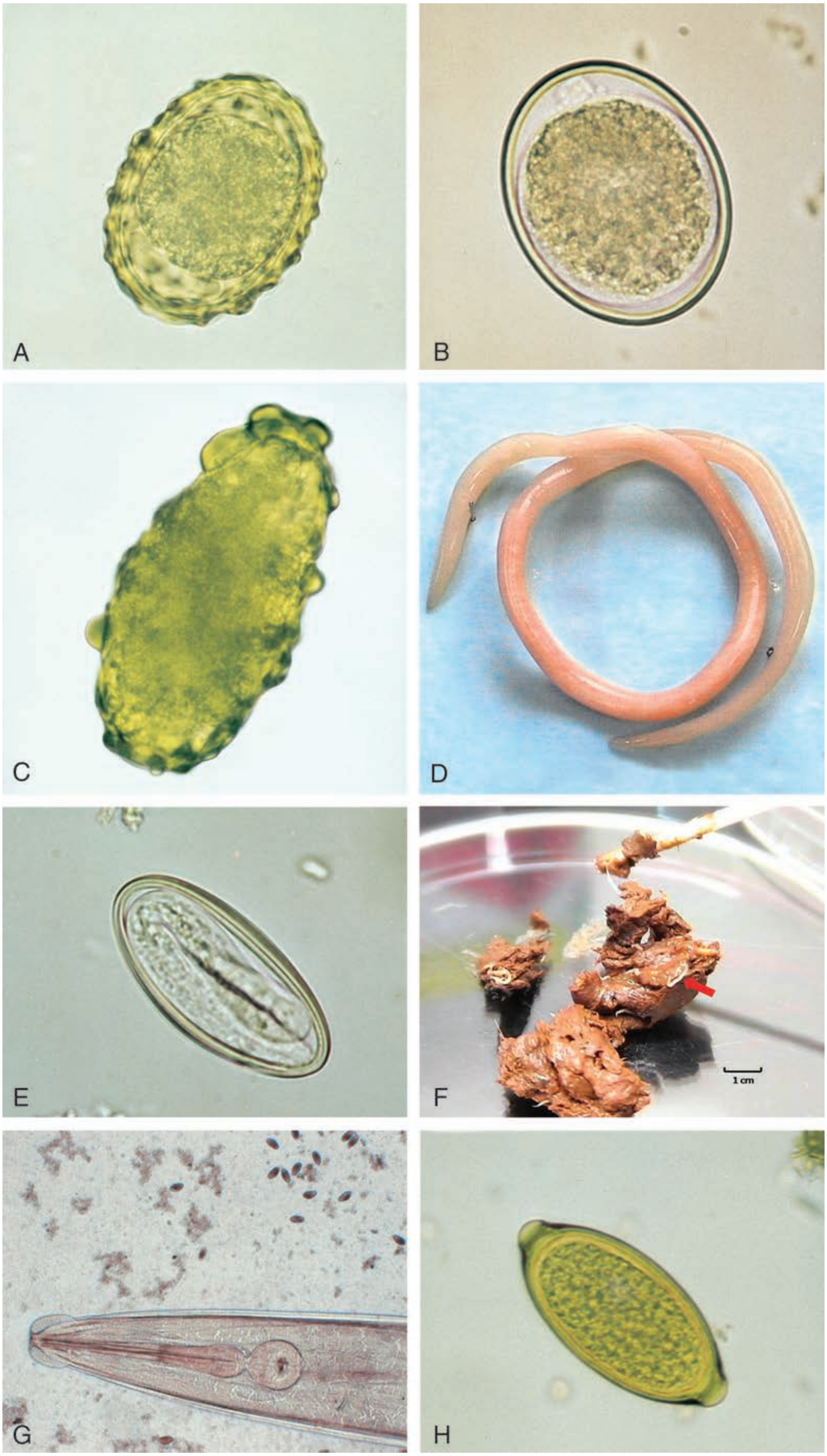

MICROMETERS (MICRONS) (μm)
90
60
30
150
120
90
60
30
Metagonimus yokogawai
Heterophyes heterophyes
Opisthorchis felineus
Clonorchis sinensis
Taenia
Hymenolepis nana
Enterobius vermicularis
Trichuris trichiura
Ascaris lumbricoides fertile
Hookworm
Diphyllobothrium latum
Hymenolepis diminuta
Paragonimus westermani
Trichostrongylus
Ascaris lumbricoides infertile
Schistosoma japonicum
Schistosoma haematobium
Schistosoma mansoni
Fasciola hepatica
Fasciolopsis buski

FIGURE 1 Relative sizes of helminth eggs. doi:10.1128/9781555817381.ch143.f1


A
B
C
D
E
F
1 cm
G
H

presence of only unfertilized eggs suggests that only female worms are present in the intestine. During the development of the eggs, the parent worm must supply the zygotes with sufficient nutrients to ensure progression to the infective second-stage larvae and subsequent survival.

Larvae

During an incubation period of 2 to 3 weeks in soil, first-stage larvae develop inside the eggs and molt to form the second-stage larvae. Following ingestion of eggs, the larvae hatch in the jejunum; second-stage larvae have a typical filariform appearance (measuring approximately 250 by 14 μm).They migrate across the intestinal wall and enter the circulation to reach the lungs, growing to approximately 560 by 28 μm. A further molt occurs in the lungs, and the larvae develop over 8 or 9 days to reach a size of 1.2 mm by 36 μm. They are carried to the throat and are swallowed. Growth is rapid after the fourth and final molt in the intestine.

Worms

Adult male and female *A. lumbricoides* worms (Fig. 2D) are colored light brown-pink when fresh, turning white with storage. Egg laying commences at 8 to 9 weeks, when females measure 15 to 20 cm, and they may reach 45 cm by 5 mm; males are generally smaller, at 15 to 31 cm by 3 mm. Males have a curved tail containing a cloaca, a pair of copulatory spicules, and caudal papillae. Adult worms live in the lumen of the small intestine and feed on digestion products.

Epidemiology and Prevention

A. lumbricoides infection is one of the most common human infections, with more than a billion people estimated to be infected worldwide. Although it has a worldwide distribution, it is most common in tropical and subtropical areas of the developed and underdeveloped worlds. It is particularly associated with crowding and poor sanitation. Contamination of soil by human excreta can result in a layer of eggs within silt that is easily disturbed, resulting in contamination of root and vegetable produce.

Preventive measures consist of health education about personal hygiene and sanitation, and drug therapy.

Transmission and Life Cycle

Eggs passed in feces (diagnostic stage) have an unsegmented ovum; this develops in the soil over 10 to 15 days to reach an infective stage larva (Table 1). Eggs may survive in this form for years under favorable conditions of heat and humidity. Infection follows the ingestion of embryonated eggs (infective stage) that hatch in the small intestine. Larvae penetrate the intestinal mucosa, and the venous circulation carries them to the lungs. From here they migrate to the trachea and are swallowed. They then complete their development into mature adult worms in the small intestine, with eggs produced after 60 to 70 days. A minority of individuals develop heavy infections and act as an important source of transmission.

The prepatent period (from egg ingestion to egg production) is approximately 2 months.

Clinical Significance

Initial infection is usually asymptomatic. Migration of larvae through the lungs may result in an eosinophilic pneumonitis with cough, fever, and dyspnea. This may occur up to 2 weeks after infection and lasts approximately 3 weeks. Bronchospasm may be prominent and is occasionally fatal.

The clinical features in established infections relate to the worm burden. Most infections are light and asymptomatic, but nonspecific abdominal symptoms may occur. There is risk of intestinal obstruction with increasing worm burden. Intestinal obstruction has a high mortality rate in some settings (5). Heavy infections are also associated with malnutrition and impaired growth in children.

Ectopic ascariasis occasionally occurs when adult worms attempt to escape the gut lumen. This may be a spontaneous event or occur in response to febrile illness, medication, or anesthesia. Worms may enter hepatobiliary and pancreatic ducts or the appendix and cause obstruction, resulting in biliary colic, cholecystitis, acute cholangitis, acute pancreatitis, appendicitis, or a hepatic abscess. It occurs more commonly in countries with high rates of infection where the parasite is endemic. (For more on the clinical significance of *A. lumbricoides*, see reference 6.)

Diagnosis

The primary diagnosis of infection is by demonstration of eggs in feces (7, 8). The fertilized eggs are round to oval, bile stained, mammillated, and thick walled. Both fertilized and unfertilized eggs may be found in the same specimen. Multiple specimens taken on separate days may be required. Concentration methods such as formalin-ethyl acetate sedimentation should be used for optimal yield. The flotation technique is unsuitable, as unfertilized eggs have a high density. Decorticated fertile and infertile eggs may be difficult to recognize (Fig. 2A to C). Motile larvae may sometimes be seen in expectorated sputum but rarely in feces. Occasionally, an adult worm (usually female) may be passed in feces or may spontaneously migrate out of the anus, mouth, or nares, particularly in children.

Identification is relatively straightforward because no other human parasite is as large as *A. lumbricoides*. However, it should be noted that earthworms of comparable size can occasionally be retrieved from toilets and submitted for identification. The distinguishing features of *Ascaris* adult worms are that they have tapering ends (Fig. 2D), a lateral white line along the entire length of the body, three lips at the anterior end, and a tough cuticle.

Treatment

Albendazole and mebendazole are effective (see chapter 149 and reference 9). These benzimidazoles should not be used in first trimester of pregnancy or in children <12 months of age. The WHO recommends use in second

FIGURE 2 Various eggs and adult worms of intestinal nematodes (magnification, ×850). (A) Fertile egg of *A. lumbricoides*. (B) Decorticated fertile egg of *A. lumbricoides*. (C) Infertile egg of *A. lumbricoides*. (D) *A. lumbricoides* adult worm with typical cylindrical body with tapering ends and thick cuticle. The *A. lumbricoides* adult worm is the largest of the human pathogenic nematodes, 15 to 35 cm in length. (E) Embryonated infective egg of *E. vermicularis*. (F) Adult worms in feces (arrow). (G) *E. vermicularis* adult female. Adult females are usually 8 to 13 by 0.3 to 0.5 mm. Shown is the anterior end of a female worm with the characteristic cervical alae. Typical eggs can also usually be seen in the field. (H) Egg of *T. trichiura*.
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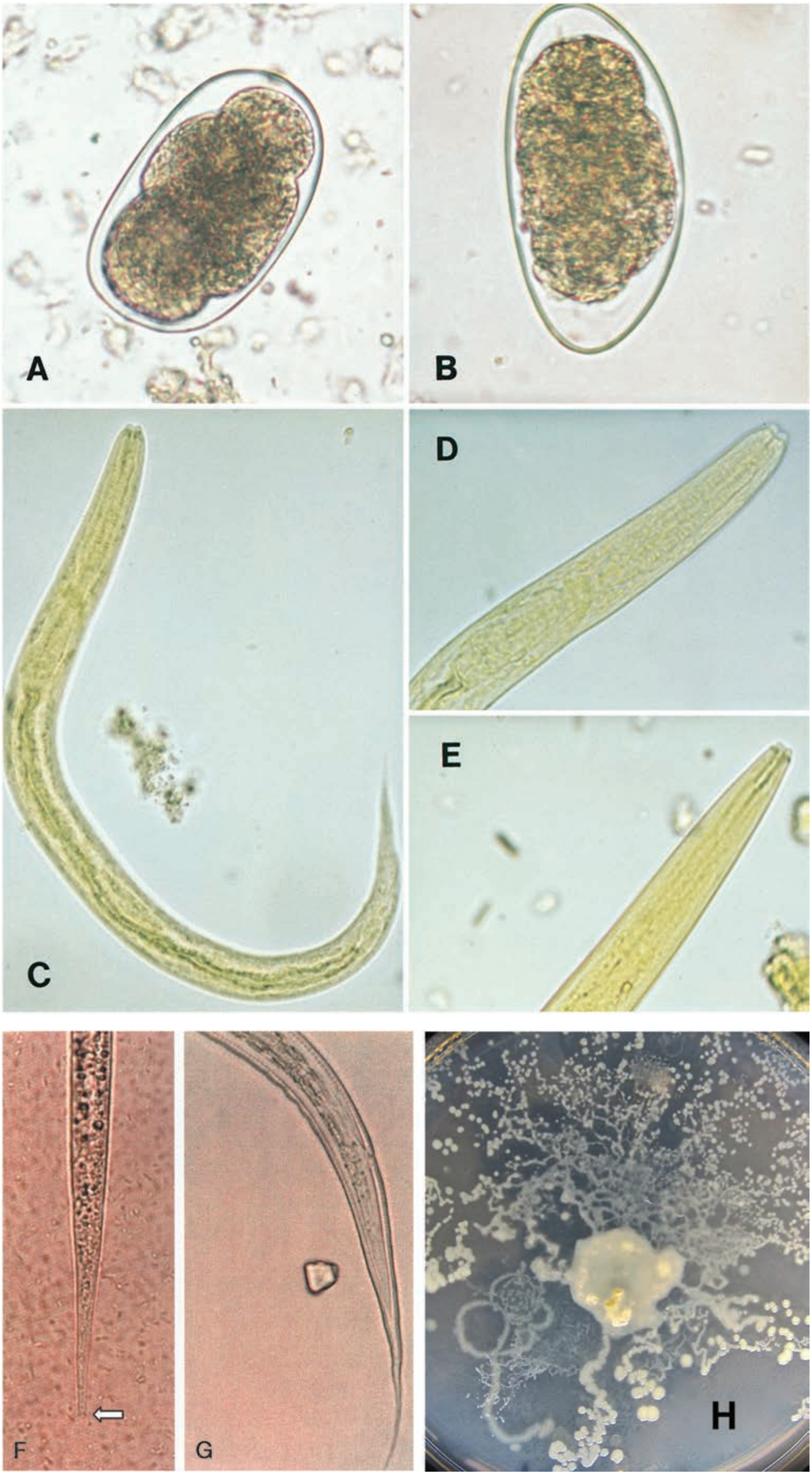
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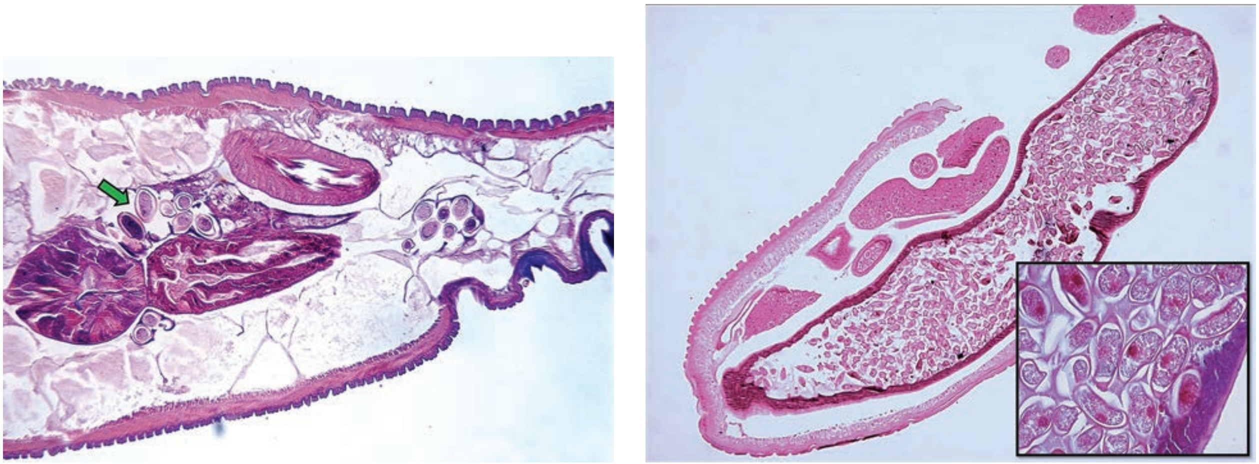

FIGURE 4 Sections of adult female worms taken at colonoscopy (hematoxylin-eosin stain). (A) *Enterobius vermicularis* female (×400) showing characteristic eggs (arrow); (B) *Trichuris trichiura* female (×100) showing characteristic eggs (inset, ×400). doi:10.1128/9781555817381.ch143.f4

and third trimesters of pregnancy and in children >12 months of age.

For a summary of *A. lumbricoides*, see Table 2.

ENTEROBIUS VERMICULARIS (PINWORM OR THREADWORM)

Taxonomy

E. vermicularis belongs to the class Secernentea, order Oxyurida, superfamily Oxyuroidea, and family Oxyuridae.

Only the genus *Enterobius* of the Oxyuridae regularly occurs in humans and other primates.

Description

Eggs

Pinworm eggs (Fig. 2E) are ovoid, 50 to 60 by 20 to 35 µm, and asymmetrically flattened on one side and appear colorless when recovered from the perianal skin. The outer layer of the egg shell is albuminous and sticky, enabling the egg to adhere readily. The egg contains an immature first-stage larva, but this develops rapidly to become infective.

Larvae

The larvae hatch in the small bowel and then migrate to the large bowl to complete molting and development.

Worms

Adult female worms (Fig. 2F and G) measure approximately 8 to 13 by 0.3 to 0.5 mm, with cervical alae or wing-like expansions at the mouth and a long, pointed tail (hence the name pinworm). Male worms are 2.5 by 0.2 mm but are rarely seen.

Enterobius gregorii was thought to be a sister species of *E. vermicularis*, and it has a slightly smaller spicule (sexual organ). However, its existence is controversial, and some experts think that *E. gregorii* is a younger or immature stage of *E. vermicularis*, as no differences have been found on molecular analysis (10).

Epidemiology and Prevention

E. vermicularis infection has a worldwide distribution and is more common in children than adults.

Preventive measures consist of health education about personal hygiene and sanitation, as well as drug therapy.

Transmission and Life Cycle

Adult worms inhabit the cecum, appendix, and ascending colon. The female migrates down the colon when mature and deposits her eggs on perianal and perineal skin (diagnostic stage). More than 10,000 eggs may be deposited, become infective within 6 h (Table 1), and remain viable for up to 5 days.

Transmission is either autoinfection, directly from the anal and perianal regions to the mouth, usually by fingernail contamination, or from exposure to a contaminated environment. When swallowed, the embryonated eggs (infective stage) hatch in the small bowel. Larvae migrate to the large bowel, where they mature into adult worms. Retroinfection, in which larvae migrate from the anal skin back to the rectum, is thought to be possible.

The prepatent period (from egg ingestion to egg production) is 3 to 4 weeks.

Clinical Significance

Children are most commonly infected. Many patients are asymptomatic or present with pruritus ani and perineal pruritus. The pruritus may be severe and is worse at night. Excoriation from scratching and secondary bacterial infection is often evident. With heavy worm burdens, poor con-

FIGURE 3 Eggs and larvae of intestinal nematodes (magnification, ×850). (A) Hookworm egg. (B) *Trichostrongylus* egg. (C) Rhabditiform, or first-stage, larva of *S. stercoralis*. (D) Anterior end of rhabditiform larva of *S. stercoralis* showing short buccal cavity. (E) Anterior end of rhabditiform hookworm larva showing long buccal cavity. (F) Tail end of filariform, or last-stage, larva of *S. stercoralis* with the notched tail (arrow). (G) Tail end of filariform, or last-stage, hookworm larva with tapering tail. (H) Agar plate culture showing tracks of bacterial colonies due to larval movement on the surface and inside the agar. The fecal specimen is placed in middle of the plate and sealed, as larvae are infectious. doi:10.1128/9781555817381.ch143.f3

TABLE 1 Usual times for completion of nematode life cycles under favorable conditions

Nematode(s)	Time for life cycle completion	
	Within host	In external environment
Ascaris lumbricoides	8 wk	10–15 days
Enterobius vermicularis	4–7 wk	6 h
Hookworms	4–7 wk	5–6 days
Strongyloides stercoralis	4 wk	3–4 days (direct)
Trichuris trichiura	10–12 wk	21 days

centration, enuresis, and emotional distress are features. Occasionally, the presence of worms in the appendix contributes to inflammation and true appendicitis (11). Reinfection (autoinfection or retroinfection) is also possible.

General symptoms, including abdominal pain, weight loss, and loss of appetite, may occur.

The presence of ectopic worms and eggs in several sites, including the female genitourinary tract, the epididymis, and the peritoneum, has been reported. They rarely cause serious complications. (For more on the clinical significance of *E. vermicularis*, see reference 6.)

Diagnosis

The primary diagnosis of infection is by demonstration of the presence of eggs on the skin in the perineal area by using the "sticky tape" method (eggs adhere to cellulose tape and can then be detected microscopically). Briefly, a strip of cellulose tape with the adhesive side outwards on a microscopy slide is pressed firmly against the right and left perianal folds. The tape is then spread back over the slide with the adhesive side down and examined directly under the microscope. Visibility of eggs can be improved by lifting the tape from the slide, adding a drop of xylene or xylene substitute, and pressing the tape back down on the slide. This helps clear the preparation and the eggs can be observed clearly (7, 8). If an opaque tape is submitted by mistake, a drop of immersion oil on the top of the tape will clear it enough for microscopy. Repeated preparations on 2 or 3 consecutive days may be required. Commercial collection kits (Evergreen Scientific, Los Angeles, CA, or Swube [Becton Dickinson], Sparks, MD) are available in some countries. Eggs are occasionally present in feces. Eggs are non-bile stained, ovoid, asymmetrical with a characteristic shape (concave on one side and flat on the other), smooth, and thick walled and may contain a partially or fully developed larva (Fig. 2E). Specimens should ideally be taken early in the morning before a bath or use of the toilet.

Adult female worms may be seen in feces, around the anal opening, or upon colonoscopy as white or cream-colored pin- or thread-like worms. Characteristic eggs may be seen within the distended female in histology section (Fig. 4A) or extruded from the genital pore by putting a little pressure on the worm (Fig. 2G).

Treatment

Albendazole and mebendazole are the drugs of choice. These drugs should be avoided in pregnancy. Pyrantel and piperazine are also effective and can be used in pregnancy (see chapter 149 and reference 9).

It is important to treat other family members and close contacts and to decontaminate the environment by washing bed linen and clothes. Advice should be given on adequate hygiene and hand washing.

For a summary of *E. vermicularis*, see Table 2.

HOOKWORMS

Taxonomy

Ancylostoma duodenale (Old World Hookworm)

A. duodenale belongs to the class Secernentea, order Strongylida, superfamily Ancylostomatoidea, and family Ancylostomatidae.

Necator americanus (New World Hookworms)

N. americanus belongs to the class Secernentea, order Strongylida, superfamily Ancylostomatoidea, and family Ancylostomatidae.

Ancylostoma and *Necator* are the two genera of Ancylostomatidae that infect humans. Other genera may occur as rare "accidental" parasites.

Description

Eggs

Eggs (Fig. 3A) of *A. duodenale* and *Necator americanus* are indistinguishable. They are oval, with a thin shell, and measure approximately 56 to 75 by 36 to 40 μm. They have a clear space between the developing embryo and the shell. When passed, eggs are only at the four-cell stage; development of larvae occurs in moist, shady, warm soil, where larvae hatch within 1 to 2 days.

Larvae

First-stage rhabditoid larvae (Fig. 3E and G) from the hatched eggs measure about 200 μm in length. They feed on organic debris and molt twice over a 5- to 8-day period. The infective, nonfeeding third-stage larvae measure from 500 to 700 μm, those of *Ancylostoma* being generally longer than those of *Necator*. These infective filariform larvae remain viable in the soil for several weeks.

Worms

For both species, male worms (5 to 11 mm) are shorter than females (7 to 13 by 0.4 to 0.5 mm), and *A. duodenale* is generally longer and more sturdily built than *N. americanus*. Adult worms are rarely seen, since they remain firmly attached to the intestinal mucosa, feeding on blood obtained by puncturing the capillary network in the intestinal mucosa. It has been estimated that a single *A. duodenale* worm can withdraw as much as 0.2 ml of blood per day, whereas *N. americanus* withdraws approximately 0.05 ml.

Epidemiology and Prevention

An estimated 740 million people are infected in poor regions of the tropics and subtropics, especially in Asia and sub-Saharan Africa (12, 13). In addition to drug therapy, preventive measures consist of health education about personal hygiene, the need to wear shoes, and avoidance of soil contamination.

TABLE 2 Summary of common nematodes

Parasite	Major clinical presentations of infection	Prepatent period	Laboratory findings	Treatment of choice
Ascaris lumbricoides	Symptoms relate to worm burden; most infections are light and asymptomatic; migratory phase, eosinophilic pneumonitis Symptoms of established infection include the following: • History of passing/vomiting worm • Mild gastrointestinal symptoms • Small-bowel obstruction in children with heavy worm burdens • Ectopic ascariasis involving appendix or hepatobiliary or pancreatic ducts • Malnutrition and growth retardation in children	2 mo	Demonstration of eggs in feces; identification of worm passed; eosinophilia low or absent	Albendazole or mebendazole
Enterobius vermicularis	Many infections are asymptomatic; pruritus ani occurs mainly at night; excoriation from scratching and secondary infection are common; general symptoms include weight loss and loss of appetite; occasionally ectopic, involving appendix or female genital tract	3–4 wk	Demonstration of eggs in "sticky tape" preparation; eosinophilia low or absent	Albendazole or mebendazole; prevent reinfection and treat contacts
Hookworms	Pruritic rash or "ground itch" on extremities at site of entry of larvae; migratory phase, cough and wheezing due to eosinophilic pneumonitis Symptoms of established infection include the following: • Iron deficiency anemia • Hypoproteinemia leading to chronic malnutrition and growth disorders in children • Enteropathy with gastrointestinal symptoms • Light infections may be asymptomatic	4–8 wk	Demonstration of eggs or larvae in feces; culture (agar plate method or Harada-Mori technique); eosinophilia, usually moderate	Albendazole or mebendazole; iron therapy if anemia is present
Strongyloides stercoralis	Chronic infection occurs due to autoinfection; recurrent, migratory, linear rash when larvae enter perianal skin, or "larva currens"; urticarial rashes also occur; enteropathy causing intermittent or chronic diarrhea, sometimes with malabsorption; pulmonary symptoms and hypereosinophilia may occur during autoinfection; Loeffler-like syndrome; Gram-negative bacterial septicemia or meningitis due to transfer of bowel flora by migrating larvae; hyperinfection syndrome (disseminated) in immunocompromised or debilitated individuals, leading to severe enteropathy and respiratory symptoms	2–4 wk	Demonstration of larvae in feces; culture (agar plate method or Harada-Mori technique); demonstration of antibodies in serum; eosinophilia, usually moderate	Ivermectin
Trichuris trichiura	Usually asymptomatic or mild gastrointestinal symptoms; epigastric pain, vomiting, distension, anorexia, and weight loss; *Trichuris* dysentery, sometimes with rectal prolapse, may occur in heavy infections; growth retardation in children due to chronic malnutrition and anemia	3 mo	Demonstration of eggs in feces; eosinophilia low or absent	Albendazole or mebendazole

Transmission and Life Cycle

Entry of third-stage or filariform larvae (infective stage) by direct penetration of the skin initiates human infection, followed by migration to the lungs within 10 days. *A. duodenale* can also infect if swallowed, but *N. americanus* cannot. *N. americanus* requires lung migration, whereas the ingestion of *A. duodenale* larvae can result in direct maturation to the adult stage in the intestine. Larvae leave the lungs after 3 to 5 weeks, pass through the trachea and pharynx, enter the gastrointestinal tract, and attach to the intestinal mucosa, where they mature into adults (Table 1). Attachment and the release of anticlotting factors result in blood loss, which is greater with *A. duodenale*. Hookworms may survive in the host for several years. Eggs are passed in the feces into the environment, where they hatch into rhabditiform larvae that mature into filariform larvae with potential to infect new hosts.

The prepatent period from larva penetration to egg production is 4 to 8 weeks.

Clinical Significance

The major clinical manifestation of hookworm infection is iron deficiency anemia due to intestinal blood loss and depletion of iron stores. The degree of iron deficiency induced by hookworms depends on the intensity and duration of infection, the species of hookworm, and the iron status of the host. Young children, adolescent girls, women of reproductive age, and pregnant women are most at risk for iron deficiency anemia because of inadequate dietary iron and increased demand for iron in childhood and as a result of fetal growth, or increased losses due to menstruation. Anemia during pregnancy is linked to maternal morbidity and mortality, impaired lactation, prematurity, and/or low-birth-weight infants.

Patients with light worm burdens are usually asymptomatic. Moderate and heavy worm burdens cause epigastric pain and tenderness, nausea, weight loss, and diarrhea. This may lead to protein-losing enteropathy and hypoalbuminemia. In those who develop iron deficiency anemia, common symptoms are lethargy and fatigue, exertional dyspnea, and palpitations. In children, chronic malnutrition and impaired growth and development may occur.

A pruritic rash is often present at the site of penetration of larvae ("ground itch"). This must be differentiated from cercarial dermatitis and creeping eruptions from other causes. In some patients, mild cough and wheezing occur in response to larval migration through the lungs (For more on the clinical significance of hookworms, see reference 6.)

Diagnosis

The primary diagnosis of infection is by detection of eggs in feces (7, 8). Multiple specimens taken on separate days may be required. For optimal yield, both direct and concentration methods such as formalin-ethyl acetate sedimentation should be used. Eggs are non-bile stained and usually have a 4- to 8-cell-stage embryo (Fig. 3A). Occasionally, a 16- to 32-cell-stage embryo or a developing larva may be seen, especially if there is delay in processing an unfixed fecal specimen. These larvae should be differentiated from *Strongyloides stercoralis* larvae by their morphological features (Fig. 3C to G). Hookworm eggs may be confused with *Trichostrongylus* eggs, which are generally larger or more elongated and in an advanced stage of cleavage (Fig. 3B).

Larvae are uncommon in adequately fixed fecal specimens, but motile larvae may be seen occasionally in expectorated sputum. Culture for the larval stage by the Harada-Mori technique or agar plate method is useful to detect light infections (see "*Strongyloides stercoralis*" below for details) (Fig. 3C to G).

Adult worms are very rarely passed in feces. Usually, the worm is firmly attached to the mucosa. A colonoscopic or gastroscopic specimen may be submitted for identification. The adult worm can be identified by the structure of the buccal capsule.

When hematological examination shows iron deficiency anemia, the diagnosis of hookworm infection should be considered for people who have been in areas where the infection is endemic.

Treatment

The two most practical and effective drugs for treating hookworm infections are albendazole and mebendazole. These benzimidazoles should not be used in the first trimester of pregnancy or in children <12 months of age. The WHO recommends use in second and third trimesters of pregnancy and in children >12 months of age. Pyrantel is also effective and can be used in pregnancy (see chapter 149 and reference 9).

Iron supplementation may be required for those who are deficient.

For a summary of hookworms, see Table 2.

STRONGYLOIDES STERCORALIS

Taxonomy

S. stercoralis belongs to the class Secernentea, order Rhabditida, superfamily Rhabditoidea, and family Strongyloididae.

Members of the family Strongyloididae exhibit an irregular alternation of generations, with a parasitic parthenogenetic female alternating with a free-living sexual generation. Only one genus, *Strongyloides*, occurs in humans.

Description, Transmission, and Life Cycle

Eggs

Eggs of the parasitic female are deposited within the mucosa of the small intestine and usually hatch before reaching the lumen. As a result, they are rarely excreted in the feces (*Strongyloides fuelleborni*, seen in Zimbabawe and Zambia, and a related species found in Papua New Guinea, are the exceptions; see below). Eggs of the free-living adult female are partially embryonated and oval and measure approximately 50 to 70 μm in length.

Larvae

First-stage, rhabditiform larvae (Fig. 3C, D, and F) measure approximately 180 to 380 by 14 to 20 μm and are characterized by a muscular esophagus comprising the anterior third of the body. A short buccal cavity (Fig. 3D) and a prominent genital primordium-located midbody (Fig. 3C) help to distinguish them from other nematodes such as hookworms (Fig. 3E). In the soil, first-stage larvae follow a direct or indirect course of development. In the direct cycle, larvae develop rapidly into infective third-stage, filariform larvae; in the indirect cycle, first-stage larvae develop into a free-living generation of adult male and female worms. Third-stage larvae are 500 to 600 by 16 μm, and their tails are notched. This feature separates them from hookworm larvae, which have long pointed tails (Fig. 3F and G).

Worms

Parasitic male forms do not occur. Parasitic females are small and thin, measuring approximately 2 to 3 mm in length and 30 to 50 µm in width. The anterior portion is thicker than the posterior and contains the esophagus.

The free-living adult female is approximately half the size of its parasitic counterpart, although it is nearly twice as thick (approximately 80 µm). While the reproductive systems are morphologically similar, the uterus in the free-living adult female contains significantly more eggs. The free-living adult male is slightly smaller than the female, approximately 50 µm in width.

Transmission occurs when filariform (infective-stage) larvae penetrate the skin or mucous membranes, enter the venous circulation, and are carried to the lungs. Larvae penetrate alveolar walls, migrate through the tracheobronchial tree, are swallowed, and finally reside in the mucosa of the upper small intestine. Here, they reach maturity and commence egg production; the eggs hatch immediately to release rhabditiform larvae, most of which are excreted in feces (diagnostic stage). In the soil, they undergo several molts to become infective filariform larvae (Table 1).

Some rhabditiform larvae develop into infective filariform larvae in the bowel lumen, penetrate the intestinal mucosa or perianal skin, and repeat the cycle of maturation within the same host. This process of autoinfection, uncommon among intestinal nematodes, results in chronic infections that may persist for 40 years or more.

The prepatent period (from larva penetration to egg production) is 2 to 4 weeks.

Epidemiology and Prevention

Strongyloides worms live in warm, moist soil and are widely distributed in the tropics and subtropics, a distribution similar to that of hookworms. There are an estimated 30 to 100 million patients infected with *S. stercoralis* worldwide. At-risk groups include those living in areas where the parasite is endemic and immigrants and refugees, indigenous peoples, and war veterans previously exposed in these regions.

Preventive measures include good personal hygiene and wearing shoes in areas where transmission occurs.

Clinical Significance

S. stercoralis infections are often chronic, lasting for several decades. Most patients have low worm burdens and are asymptomatic or have intermittent cutaneous and/or gastrointestinal symptoms. Intermittent or chronic diarrhea, abdominal pain and bloating, nausea, and anorexia are the main gastrointestinal symptoms.

Recurrent pruritic, serpiginous, erythematous rashes may occur due to larval migration in the skin ("larva currens"). These are seen most commonly on the buttocks, groin, and trunk; movement under the skin may continue for 1 to 2 days. Other, less distinctive urticarial and papular rashes also occur.

Although pulmonary symptoms are uncommon in uncomplicated strongyloidiasis, passage of larvae through the lungs may be associated with a cough, wheezing and dyspnea, and patchy infiltrates upon radiography (Loeffler-like syndrome).

Severe complicated strongyloidiasis may occur in immunocompromised or debilitated individuals and is due to accelerated autoinfection in the face of waning immunity. Hyperinfection syndrome is associated with the presence of many adult worms in the intestinal mucosa and penetration of the bowel wall by large numbers of filariform larvae. Large numbers of larvae in stool and sputum produce severe gastrointestinal and/or respiratory symptoms. These symptoms may be complicated by malabsorption or paralytic ileus. Gram-negative bacterial septicemia and meningitis may develop if enteric bacteria are spread into blood or cerebrospinal fluid during mucosal penetration by larvae. Larvae can occasionally disseminate to the central nervous system, peritoneum, liver, and kidneys. Disseminated strongyloidiasis may mimic many other diseases, including bacterial sepsis and tropical pulmonary eosinophilia. It should be considered a possibility for immunosuppressed patients who have been living in areas where strongyloidiasis is endemic. Patients on long-term corticosteroid therapy are most at risk for severe disease. Those with impaired cellular immunity due to other causes (e.g., use of immunosuppressive agents, hematological malignancies, and other chronic debilitating diseases) are also at risk.

Strongyloides species from animals can occasionally infect humans and produce persistent skin lesions similar to those produced by *S. stercoralis*. However, the life cycle is not completed and infection is not established (14). (For more on the clinical significance of *S. stercoralis*, see reference 6.)

Diagnosis

The laboratory diagnosis of strongyloidiasis can be difficult (7, 8). It is based on either demonstrating the presence of the larvae in feces or demonstrating antibodies to *S. stercoralis* in blood. Microscopy of feces may detect the first-stage (rhabditiform) larvae; eggs or adult worms are very rarely detected. The sensitivity of microscopy is generally low, as larvae are shed sporadically and numbers depend on the stage and severity of the infection. For this reason, multiple specimens collected over several days should be examined. Coproculture is an alternative method of detection of larvae in freshly passed stool specimen. Ideally, this should be attempted for all suspected cases, especially with patients from areas where the infection is endemic. It is important that the specimen be sent directly to the laboratory, without refrigeration or addition of preservative. Care should be exercised in the handling of these cultures, as the larvae present are filariform (infectious), capable of crossing intact skin. The various methods available for culture are as follows (for details, see reference 15):

1. Agar plate culture. A small amount of feces is placed on the center of an agar plate, and the plate is sealed and incubated at room temperature. If larvae are present, they travel away from the initial inoculum carrying bacteria from the feces. Colonies of these bacteria appear on the surface as tracks (or occasionally inside the agar) with a characteristic sinusoidal pattern (Fig. 3H). The plates are examined by microscopy for confirmation of the presence of larvae. This is the easiest culture method to perform and is now the recommended procedure (16).
2. Harada-Mori technique. The fecal specimen is smeared on the top of a strip of filter paper, dipping into 3 ml of water in a 15-ml sealed centrifuge tube. The tube is held at 25°C in the dark. After 7 to 10 days, the water can be tested by microscopy for the presence of larvae.
3. Petri dish methods. Either filter paper or watch glass is used to hold the specimen. The larvae migrate to fresh water and light.
4. Baermann technique. A modified funnel device allows sampling of the water used to moisten the fecal specimen placed on a wire mesh covered with gauze. This technique relies on the principle that larvae actively migrate out of the feces.

Occasionally, hookworm larvae may hatch in feces that have been left at warm temperatures for long periods before processing. In contrast to *S. stercoralis*, hookworm larvae have a long buccal cavity and inconspicuous genital primordium (Fig. 3C to E). The presence of larvae of *Strongyloides* in expectorated sputum or duodenal aspirate may also be demonstrated via an enteroscope (16). Filariform *Strongyloides* larvae may be seen in feces or sputum in cases of hyperinfection or in cases in which infection has been identified by the culture methods described above.

Although eosinophilia is common during acute infection, it does not reliably correlate with infection and may be intermittent in chronic infection. The concomitant use of steroids may significantly decrease eosinophilia in infected patients.

Demonstration of anti-*Strongyloides* antibodies in blood should be used as a screening test or as an adjunct for diagnosis. Used in conjunction with eosinophil count, it is very useful to monitor treatment. The decline in antibody levels is variable; it may first be observed after 6 months of effective treatment (17) and may be negative by 12 to 24 months (18). The sensitivity of the commercially available enzyme immunoassay (EIA) (*Strongyloides* IgG enzyme-linked immunosorbent assay [EIA-4208], DRG International, Inc., Springfield, NJ) using the *S. stercoralis* filariform antigens test is ~90% and is much higher than for the indirect hemagglutination test and the indirect fluorescent-antibody test. Although this test is approved in some countries (e.g., Therapeutic Goods Administration, Australia), it is not for sale in the United States (not FDA approved). Higher sensitivity (~95%) has been reported using the U.S. Centers for Disease Control and Prevention *Strongyloides* EIA for patients with proven infection (17). Sensitivity may be lower in the case of severely immunocompromised patients. The specificity of EIA is ~85%, as cross-reactions for patients with filarial and other nematode infections may occur. If antibodies are positive, efforts should be made to establish a parasitological diagnosis (microscopic and culture) and to exclude infection with other parasites that could result in cross-reacting antibodies. *Strongyloides* serology should be done for all candidates for immunosuppressive therapy (e.g., prior to organ transplantation or treatment for malignancies, etc.). Diagnosis by molecular techniques has been developed recently (19) but is not yet routinely available. A recent review of techniques for laboratory diagnosis of *Strongyloides* includes reference to molecular tests (20).

Treatment

Strongyloides stercoralis infection should always be treated because of the potential for developing severe complicated disease. Ivermectin is the drug of choice, but repeated cycles of treatment may be required for immunocompromised patients. Albendazole and mebendazole are less effective than ivermectin (thiabendazole, although effective, is no longer used due to frequent severe side effects) (16) (see chapter 149 and reference 9). These benzimidazoles should not be used in first trimester of pregnancy or in children <12 months of age. The WHO recommends use in second and third trimesters of pregnancy and in children >12 months of age.

Monitoring with stool microscopy, eosinophil counts, and serology is recommended until infection is eradicated.

For a summary of *S. stercoralis*, see Table 2.

STRONGYLOIDES FUELLEBORNI

Strongyloides fuelleborni is a nonhuman primate parasite found in monkeys in Central and Eastern Africa. A similar species is found in Papua New Guinea, but no animal vector has been identified. Most human infections are asymptomatic in adults but may cause a severe protein-losing enteropathy and abdominal distension ("swollen belly syndrome") in infants. In contrast to the case with *S. stercoralis*, ova of *S. fuelleborni* are found in feces in large numbers and resemble those of hookworms. The two *Strongyloides* species can be differentiated on the basis of adult worm morphology (14). Treatment is similar to that for *S. stercoralis*. If infection is untreated, the mortality rate is very high in infants.

TRICHURIS TRICHIURA (WHIPWORM)

Taxonomy

T. trichiura belongs to the order Enoplida, superfamily Trichinelloidea (Trichuroidea), and family Trichinelliidae (Trichuriidae).

Description

Eggs

Eggs of *T. trichiura* (Fig. 2H) are lemon shaped, with a mucous plug at both ends and an unsegmented nucleus. Eggs have a double shell, with the outer one bile stained; they measure 50 to 55 by 20 to 24 μm. Under favorable conditions in soil, the eggs become fully embryonated and infective in 2 to 4 weeks.

Larvae

Larvae emerge in the small intestine (the second-stage larvae measure about 260 by 15 μm in length). After a period of growth they pass into the cecum, where they embed in the mucosa.

Worms

Adult worms have a highly characteristic shape from which the name whipworm is derived. The long, thin anterior end lies in a burrow in the mucosa, and the thicker end, which contains the reproductive tract, extends into the intestinal lumen. Worms are whitish; the males (30 to 45 mm) are shorter than the females (35 to 50 mm) and have a coiled posterior end. Adult females produce up to 20,000 eggs/day and live for approximately 3 years.

Epidemiology and Prevention

Trichuris has a worldwide distribution and is often associated with *Ascaris* and hookworm infections in children in tropical and subtropical areas.

Preventive measures include health education about personal hygiene, avoidance of soil contamination, and drug therapy programs in areas where infection is endemic.

Transmission and Life Cycle

Transmission is direct via oral ingestion of embryonated eggs (infective stage) from contaminated soil. Following ingestion, larvae are released and pass into the large bowel, where they mature into adults in mucosal crypts. The eggs passed in feces contain an unsegmented ovum (diagnostic stage), and once the eggs are in warm, moist conditions in soil, they become infective 2 to 4 weeks after passage (Table 1).

The prepatent period (from egg ingestion to egg production) is 3 months.

Clinical Significance

The clinical features are related to the intensity of infection; light infections are asymptomatic or present with mild gastrointestinal symptoms.

Epigastric pain, vomiting, distension, anorexia, and weight loss may occur with heavier infections and *Trichuris* dysentery syndrome may be seen, in extreme cases complicated by rectal prolapse. Children with severe infections may develop growth retardation due to chronic malnutrition and anemia. (For more on the clinical significance of *T. trichiura*, see reference 6.)

Diagnosis

The primary diagnosis of infection is by detection of eggs in feces (7, 8). Multiple specimens collected on separate days may be required. Concentration methods such as formalin-ethyl acetate sedimentation should be used for optimal yield. The eggs are bile stained and double walled with mucoid plugs at both ends, giving them a characteristic tea tray appearance (Fig. 2H).

Adult worms are rarely passed in feces and are occasionally found upon colonoscopy (21). They have a coiled, long, thin anterior region and a thicker tail, giving them the appearance of a whip (hence the name whipworm). The characteristic eggs can be seen on histology section (Fig. 4B).

Treatment

Albendazole and mebendazole are the drugs of choice (see chapter 149 and reference 9). Failures are not uncommon, and multiple doses are required. These benzimidazoles should not be used in the first trimester of pregnancy or in children <12 months of age. The WHO recommends use in the second and third trimesters of pregnancy and in children >12 months of age.

For a summary of *T. trichiura*, see Table 2.

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

Specimens should be collected before antibiotics or antiparasitic drugs are given (for details, refer to chapter 133). It is important to transport and process feces specimens for parasitic examination as soon as possible. Clinicians and collection staff should be encouraged to either send fresh specimens to the laboratory without delay or use commercially available preservative kits. If delay is inevitable, specimens should be refrigerated or transported in commercially available vials or kits with a preservative such as polyvinyl alcohol or sodium acetate-acetic acid-formalin. If these are not available at the point of collection, preservatives should be added as soon as the specimen is received in the laboratory. Excellent directions for proper collection are available with these kits. Discussion on which preservative to use depends on various considerations such as whether permanent-stained smears or immunoassays are required, etc. (For details, see reference 15.) The use of two or three specimens collected over a period of 7 to 10 days is optimal. Refrigeration and the use of preservatives should be avoided if *Strongyloides* larval culture is required.

Specimens that are very small in volume or obviously dry should be rejected and a fresh specimen should be collected.

Worms retrieved at colonoscopy or submitted by the patient should be transported to the laboratory without delay. Worms can be preserved in 60% alcohol; avoid the use of formalin, as it causes contraction and hardening of tissues.

For sputum specimens, proper instructions should be given to patients, emphasizing requirements, i.e., avoidance of saliva and of use of a mouthwash before deep coughing (expectorating). Sputum and aspirates should be transported to the laboratory and processed immediately.

All specimens should be handled using standard precautions; e.g., latex gloves should be worn by anyone handling the specimens.

LABORATORY METHODS: DIRECT EXAMINATION

The specimen should be handled in a work cabinet (biological safety cabinet), and gloves and a gown should be worn. Examine the specimen and note its consistency. Look for any motile worms or segments of worms. Select and sample areas that look watery, purulent, or bloody. If feces are formed, sample from several areas of the specimen for the concentration technique.

Both the direct specimen and concentrate are examined as wet preparations diluted in saline and iodine. Scan the whole coverslip area at low-power magnification (×100). Most helminth eggs and larvae can be identified at this magnification, and diagnosis can be confirmed at high power (×400) on the basis of their shape, size, and characteristic features (Fig. 1 to 3). Smears should be prepared from a centrifuged deposit (500 × *g* for 10 min) for permanent staining, but this technique is not the preferred method for identification of nematode eggs or larvae; it is primarily to diagnose mixed infections with protozoa.

EVALUATION, INTERPRETATION, AND REPORTING OF RESULTS

Make note of the adequacy and quality of the specimen submitted. If it is inadequate, mention it on the report.

Any helminth eggs or larvae found in feces are significant and treatment is recommended, even if the patient is asymptomatic. There is no need to quantitate helminth parasites, as this does not necessarily correlate with clinical illness. However, in rare instances, this may be required for epidemiological studies or for clinical assessment of children.

The time of collection is also important, especially when *Enterobius* is suspected. If the specimen is inadequate, e.g., delayed, dry, or insufficient, a repeat specimen should be requested. A freshly collected specimen and/or use of preservative kits should be encouraged where possible.

Examples of interpretative comments used to ensure that adequate testing is performed are as follows.

1. Larvae of *S. stercoralis* not detected. A single negative result does not exclude the diagnosis of *Strongyloides*. Please send freshly collected feces to laboratory without delay.
2. Eggs of *E. vermicularis* not detected on the sticky tape preparation. A single negative result does not exclude the diagnosis of pinworm infection. Please send properly collected repeat specimen, if clinically indicated. Contact the laboratory for instructions if required.
3. No parasites detected. Excretion of parasite forms may be variable or intermittent. Repeat testing is recommended if clinically indicated. Contact the laboratory for instructions if required.

In a clinically significant case, if the first specimen is negative, ask for further specimens.

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Filarial Nematodes*

SOUMYA CHATTERJEE AND THOMAS B. NUTMAN

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Filarial worms are arthropod-transmitted nematodes or roundworms that dwell in the subcutaneous tissues and the lymphatics. Although eight filarial species commonly infect humans, four are responsible for most of the pathology associated with these infections. These are (i) *Wuchereria bancrofti*, (ii) *Brugia malayi*, (iii) *Onchocerca volvulus*, and (iv) *Loa loa*. In general, each of the parasites is transmitted by biting arthropods. The distribution and vectors of all the filarial parasites of humans are given in Table 1. Each goes through a complex life cycle that includes an infective larval stage carried by the insects and an adult worm stage that resides in humans, either in the lymph nodes or adjacent lymphatics or in the subcutaneous tissue.

All filariae share the unique characteristic of an adult female worm that produces microfilariae. These offspring either circulate in the blood or migrate through the skin. The microfilariae can then be ingested by the appropriate biting arthropod and develop into infective larvae that are capable of initiating the life cycle once more. Certain species (*W. bancrofti*, *Brugia* spp., and *L. loa*) circulate in the blood with a defined circadian rhythm or “periodicity,” which can be nocturnal (typically the lymphatic filariae) or diurnal (*L. loa*). Other species lack periodicity and are found in the peripheral blood at all hours of the day and night. When absent from the peripheral blood, the microfilariae of filarial parasites are found in the deeper visceral capillaries, particularly in the pulmonary capillaries. Because the adult worms are typically sequestered in the tissues, diagnosis of infection depends on finding microfilariae in either the blood or skin, depending on the species. Adult worms are long-lived (1), whereas the life spans of microfilariae range from 3 months to 3 years.

Microfilariae are relatively simple in their organization and structure (Fig. 1). They are vermiform and in stained preparations appear to be composed of a column of nuclei interrupted along its length by spaces and special cells that are the precursors of body organs or organelles. Some species of microfilariae are enveloped in a sheath, whereas others have no sheath (Table 1; Fig. 2).

All of the filariae are transmitted by species of blood-sucking arthropods such as mosquitoes, midges, black flies, and tabanid flies, in which the microfilariae develop to the infective larval stage (Table 1). Subsequent development of the infective larva to the gravid, adult stage in the vertebrate host requires several months and in some cases a year or more. Infection is generally not established unless exposure to infective larvae is intense and prolonged. Furthermore, the clinical manifestations of these diseases develop rather slowly.

Although these parasites are nonendemic in temperate or subtropical areas, they are often seen in individuals who have immigrated to, resided in, or traveled to tropical areas where filarial infection is endemic. There are significant differences in the clinical manifestations of filarial infections, or at least in the period over which these infections are acquired, between patients native to the areas where these infections are endemic areas and individuals who are travelers to or recent arrivals in these areas. Characteristically, the infection in previously unexposed individuals is more likely to be clinically symptomatic, compared to the clinically asymptomatic condition found in natives of the region where these infections are endemic (2, 3).

LYMPHATIC FILARIAL PARASITES

Taxonomy

Wuchereria bancrofti, *Brugia malayi*, and *Brugia timori* each belong to the phylum Nematoda, class Secernentea, subclass Spiruria, order Spirudida, superfamily Filarioidea, and family Filariidae.

Description of the Agents

There are three lymphatic-dwelling filarial parasites of humans—*Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. The adult worms usually reside in either the afferent lymphatic channels or the lymph nodes. These adult parasites may remain viable in the human host for decades. The morphologic appearance and other characteristics of the parasite can be found in Table 1 and Fig. 1 and 2.

Epidemiology and Transmission

Wuchereria bancrofti

W. bancrofti is the most common and widespread species of filaria infecting humans. It has an extensive distribution throughout tropical and subtropical areas of the world, including Asia and the Pacific Islands, Africa, areas of South America, and the Caribbean Basin. Humans are the only definitive host for this parasite and are therefore the natural reservoir for infection. There are both periodic

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TABLE 1 Filarial parasites of humans

Species	Distribution	Vector	Primary pathology	Location(s)	Periodicity	Size (μm)	Tail	Sheath	
								Presence	Staining properties
Wuchereria bancrofti	Tropics	Mosquito	Lymphatic, pulmonary	Blood, hydrocele fluid	Nocturnal, subperiodic	298 by 7.5–10	Pointed tail devoid of nuclei	+	Does not stain
Brugia timori	Indonesia	Mosquito	Lymphatic	Blood	Nocturnal	300 by 5–6	Nuclei in tail	+	Tends not to stain
Brugia malayi	Southeast Asia	Mosquito	Lymphatic, pulmonary	Blood	Nocturnal, subperiodic	270 by 5–6	Nuclei in tail	+	Bright pink with Giemsa stain
Onchocerca volvulus	Africa, Central and South America	Black fly	Dermal, ocular, lymphatic	Skin	None	309 by 5–9	No nuclei in tail	−	
Mansonella streptocerca	Africa	Midge	Dermal	Skin	None	210 by 5–6	"Crooked tail" in which column of nuclei extends	−	
Loa loa	Africa	Deer fly	Allergic	Blood	Diurnal	Up to 300	Irregularly arranged nuclei extend to end of tail	+	Does not stain
Mansonella perstans	Africa, South America	Midge	Probably allergic	Blood	None	203 by 4–5	Blunt tail contains nuclei	−	
Mansonella ozzardi	Central and South America	Midge	?	Blood	None	224 by 4–5	Long tail with no nuclei in it	−	

and subperiodic forms of the parasite. Nocturnally periodic forms have microfilariae present in the peripheral blood primarily at night (between 10 p.m. and 4 a.m.), whereas the subperiodic forms have microfilariae present in the blood at all times but with maximal levels in the afternoon. Generally, the subperiodic form is found only in the Pacific islands (including the Cook Islands, Tuvalu [formerly the Ellice Islands], Fiji, New Caledonia, the Marquesas, Samoa, and the Society Islands). Elsewhere, *W. bancrofti* is nocturnally periodic. The natural vectors are *Culex fatigans* mosquitoes in urban settings and usually anopheline or aedean mosquitoes in rural areas.

Brugia malayi and *Brugia timori*

The distribution of brugian filariasis is limited primarily to China, India, Indonesia, Korea, Japan, Malaysia, and the Philippines. *B. timori* has been described to occur only on two islands in Timor.

Similar to the situation with *W. bancrofti*, there are both periodic and subperiodic forms of brugian filariasis. The nocturnal periodic form is more common and is transmitted in areas where there are coastal rice fields (by mansonian and anopheline mosquitoes), while the subperiodic form is found in swamp forests (mansonian vector). Although humans are the common host, *B. malayi* also naturally infects cats.

Clinical Significance (Description of Clinical Presentation)

Lymphatic filariasis (LF) is associated with a variety of clinical manifestations. The four most common presentations are asymptomatic (or subclinical) microfilaremia, lymphedema, hydrocele, and acute attacks. Less frequently, LF can present with chyluria or tropical eosinophilia (4–6). The range of clinical disease varies somewhat across geographic locations and according to the species of nematode causing the infection (7). Additionally, the disease in previously unexposed individuals is more acute and intense than that found in natives of the region where it is endemic (2, 3). Patients with asymptomatic (or subclinical) microfilaremia rarely come to the attention of medical personnel except through the incidental finding of microfilariae in the peripheral blood. Although they may be clinically asymptomatic, virtually all persons with *W. bancrofti* or *B. malayi* microfilaremia have some degree of subclinical disease that includes dilated and tortuous lymphatics, which can be visualized by lymphoscintigraphy (8) and—in men with *W. bancrofti*—scrotal lymphangiectasia (detectable by ultrasound) (9). Despite these findings, the majority of individuals appear to remain clinically asymptomatic for years. Relatively few progress to either the acute or chronic stage of infection (10). Development of lymphedema may not occur until long after the initial infection. Lymphatic dysfunction develops as a primary event in response to adult filarial parasites and host immune responses in virtually all infected persons. This process has been shown to be progressive during infection and permanent once established (11). Lymphedema most commonly affects the lower extremities but can also affect the arms, the breasts in females, and the scrotum in males. Secondary effects such as thickening of the subcutaneous tissues, hyperkeratosis, fissuring of the skin, and hyperplastic skin changes can occur. Recurrent infections (acute dermatolymphangioadenitis [ADLA]) on the background of chronic skin changes and lymphatic dysfunction play a major role for lymphedema disease development and progression to elephantiasis (12, 13).

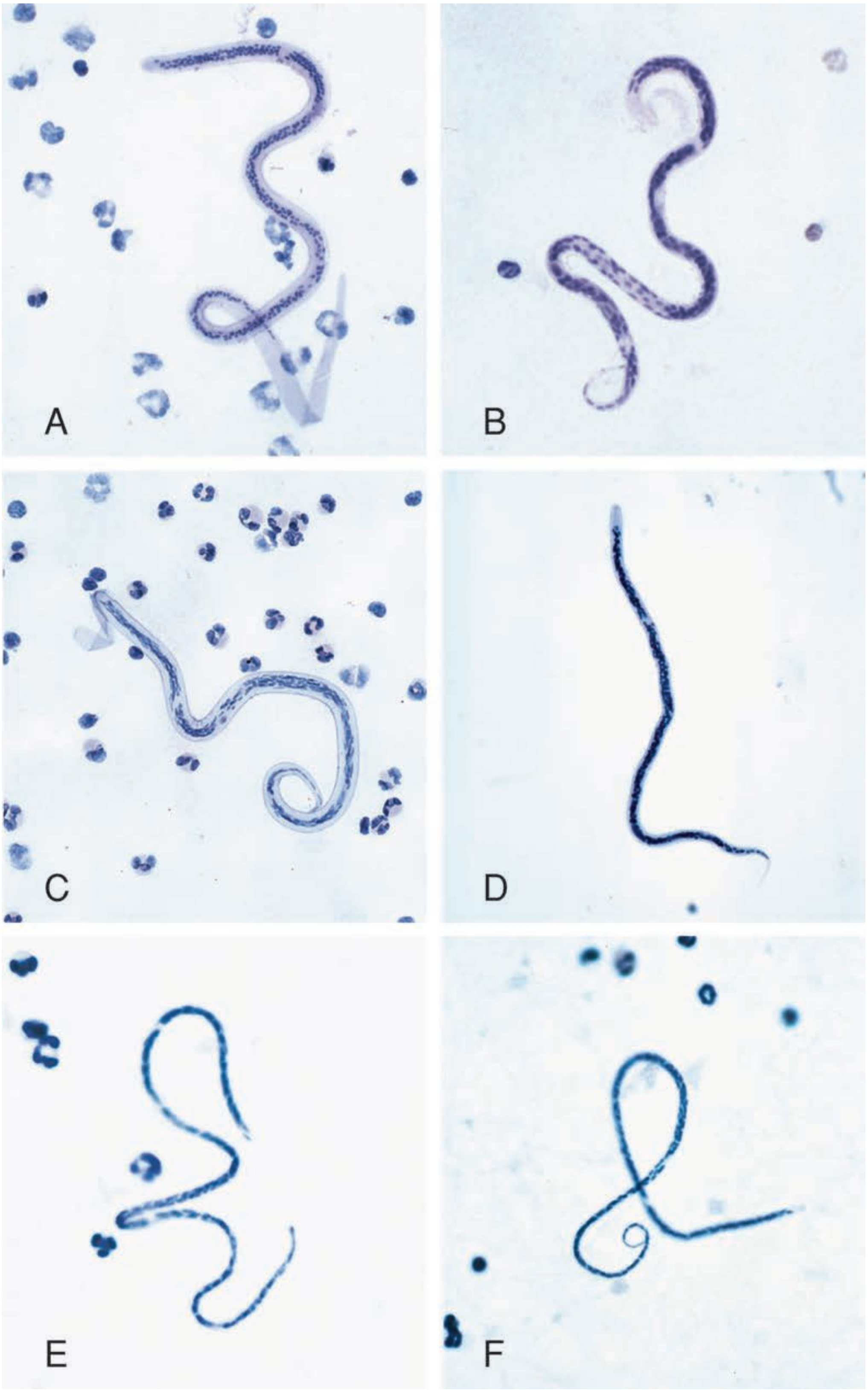

FIGURE 1 Common microfilariae found in humans. Organisms are stained with hematoxylin. Magnification, ×325. (A) *W. bancrofti*; (B) *B. malayi*; (C) *L. loa*; (D) *O. volvulus*; (E) *M. perstans*; (F) *M. ozzardi*. doi:10.1128/9781555817381.ch144.f1

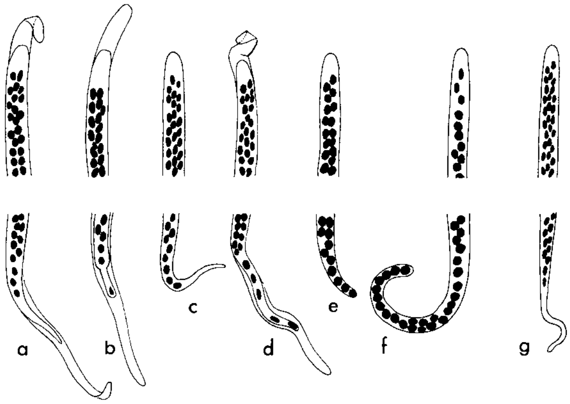

FIGURE 2 Diagrammatic representation of the anterior and posterior extremities of the common microfilariae found in humans. (a) *W. bancrofti*; (b) *B. malayi*; (c) *O. volvulus*; (d) *L. loa*; (e) *M. perstans*; (f) *M. streptocerca*; (g) *M. ozzardi*. doi:10.1128/9781555817381.ch144.f2

Acute attacks in LF cover a variety of clinical entities that present with inflammation. True filarial adenolymphangitis (ADL), considered to reflect the death of an adult worm, presents with inflammation, swelling, and retrograde lymphangitis extending peripherally from the draining nodes, where the parasites presumably reside. Regional lymph nodes are often enlarged, and the entire lymphatic channel can become indurated and inflamed. The second type of acute attack is now labeled bacterial ADL or ADLA. Skin changes cause lesions, in particular in the toe webs, that facilitate entry of bacterial skin flora (14, 15). For these reasons, limbs become susceptible to recurrent bacterial infections (16, 17). The clinical pattern of ADLA is distinctly different from that of ADL (17). The lymphangitis develops in a reticular rather than in a linear pattern, and the local and systemic symptoms, including edema, pain, fever, and chills, are frequently more severe (18). These cause considerable acute morbidity and progression of lymphedema to elephantiasis (19). ADL and ADLA occur in both the upper and the lower extremities with both bancroftian and brugian filariasis, but involvement of the genital lymphatics occurs almost exclusively with *W. bancrofti* infection.

Hydrocele formation occurs in bancroftian filariasis when adult worms block retroperitoneal and subdiaphragmatic lymphatics. In males, this causes accumulation of straw-colored lymph either unilaterally or bilaterally between the visceral and parietal layers of the tunica vaginalis. The condition presents as a translucent mass obscuring palpation of the testis and differs from a congenital hydrocele or herniation in that the tunica is sealed at the top and peritoneal fluid is not communicating. If there is obstruction of the retroperitoneal lymphatics, renal lymphatic pressure can increase to the point at which they rupture into the renal pelvis or tubules so that chyluria is seen. The chyluria is characteristically intermittent and is often prominent in the morning just after the patient arises.

Tropical pulmonary eosinophilia (TPE) is a distinct syndrome that develops in some individuals infected with LF-causing species. This syndrome affects males more often than females, most commonly during the third decade of life. The majority of cases have been reported from India, Pakistan, Sri Lanka, Brazil, Guyana, and Southeast Asia. The main features include a history of residence in regions where filaria is endemic, paroxysmal cough and wheezing that are usually nocturnal, weight loss, low-grade fever, adenopathy, and pronounced blood eosinophilia (≥3,000 eosinophils/μl). Patients are rarely found to have microfilariae in the blood. Chest radiographs may be normal but generally show increased bronchovascular markings, and diffuse miliary lesions or mottled opacities may be present in the middle and lower lung fields. Tests of pulmonary function show restrictive abnormalities in most cases and obstructive defects in half. Total serum IgE levels (10,000 to 100,000 ng/ml) and antifilarial antibody titers are characteristically elevated. TPE is now considered to be a form of occult filariasis in which rapid clearance of the microfilariae occurs, presumably on the basis of host immunologic hyperresponsiveness to the parasite (5). Although there is no single clinical or laboratory criterion that aids in distinguishing TPE from other pulmonary diseases, residence in the tropics, the presence of high levels of antifilarial antibodies, and a rapid clinical response to diethylcarbamazine (DEC) favor the diagnosis of tropical eosinophilia (20).

Diagnosis

Diagnosis of bancroftian and brugian filarial infection can be made in the right setting with clinical and historical information and noninvasively, in some cases by ultrasound. This allows adult worms to be visualized in lymphatics or dilated lymphatics to be identified. Definitive identification of parasites can be achieved with appropriate samples of blood or tissue. The timing of blood collection is critical and should be based on the periodicity of the microfilariae in the region of endemicity involved (Table 1). Recent developments in immunodiagnostic and molecular biology techniques give further options for diagnosis.

Ultrasound

In cases of suspected LF due to *W. bancrofti*, high-frequency ultrasound of the scrotum or female breast coupled with Doppler imaging may result in identification of motile adult worms ("filarial dance sign") within dilated lymphatics. Adult worms may be visualized in the lymphatics of the spermatic cord in up to 80% of infected men with microfilaremia associated with *W. bancrofti* (10). In brugian filariasis, ultrasounds have been used successfully to localize the adult worms in the female breast, the inguinal lymph nodes, and the lymphatic vessels of the thigh and calf (21).

Direct Examination

Parasites can be identified by direct examination of blood or other fluids (such as chyle, urine, or hydrocele fluid). This should take advantage of the periodicity of each organism as well as its characteristic morphologic appearance (Table 1 and Fig. 2).

Microscopy

A small volume of fluid is spread on a clean slide. The slide is then air dried, stained with Giemsa, and examined microscopically. The microfilariae of *W. bancrofti* are sheathed, lie in smooth curves in stained smears, and are 298 μm long by 7.5 to 10.0 μm in diameter. The column nuclei are dispersed; there is a short headspace, and the pointed tail is devoid of nuclei (Fig. 1A). The sheath stains faintly or not at all with Giemsa stain. The microfilariae of *W. bancrofti* must be distinguished from other sheathed microfilariae. The morphology of the *B. malayi* microfilariae is similar to that of the *W. bancrofti* microfilariae, in that they are sheathed, but they are somewhat smaller (279 μm by 5 to 6 μm). They can be differentiated from *W. bancrofti* microfilariae by the presence of subterminal and terminal nuclei in the tail (Fig. 1A and B). *B. timori* microfilariae are similar to those of *B. malayi*, with conspicuous terminal and subterminal nuclei; however, *B. timori* microfilariae are larger (more than 300 µm long) than *B. malayi*. Additionally, the *B. malayi* sheath stains bright pink with Giemsa stain, whereas the *B. timori* sheath tends not to stain and that of *W. bancrofti* never does (22).

Because microfilariae may be present in the blood in only small numbers, sensitive procedures such as Nuclepore filtration and Knott's concentration are also used routinely to detect infections.

Nuclepore Filtration

A known volume of anticoagulated blood is passed through a polycarbonate (Nuclepore) filter with a 3-µm pore. A large volume (50 ml) of distilled water is passed through (the water lyses [breaks open] the red blood cells, leaving the worms intact and more easily visible). The filter is then air dried, stained with Wright's or Giemsa stain, and examined by microscopy. For studies in the field, 1 ml of anticoagulated blood can be added to 9 ml of a solution of 2% formalin or 10% Teepol and stored for up to 9 months before performing filtration.

Knott's Concentration Technique

Anticoagulated blood (1 ml) is placed in 9 ml of 2% formalin. The tube is centrifuged at 1,500 rpm for 1 min. The sediment is spread on a slide and dried thoroughly. The slide is then stained with Wright's or Giemsa stain and examined microscopically.

Antigen Detection

Assays for circulating antigens of *W. bancrofti* permit the diagnosis of microfilaremic and cryptic (amicrofilaremic) infection. Two tests are commercially available (though not in the United States). One is an enzyme-linked immunosorbent assay (ELISA) available from TropBio Pty (Townsville, Queensland, Australia; tropbio@jcu.edu.au) and the other a rapid-format immunochromatographic card available from Alere (http://www.alere.com/ww/en/product-details/binaxnow-filariasis.html). Both assays have sensitivities that range from 96 to 100% and specificities that approach 100% (72, 73). Both tests can be used on blood drawn any time of day or night, thus avoiding the need for specific bleeding times depending on the periodicity of microfilariae. Neither of the tests is FDA approved. There are currently no tests for circulating antigens in brugian filariasis.

Nucleic Acid Detection Techniques

In appropriate laboratories, PCR can detect parasite DNA and is now the most sensitive technique for definitive diagnosis (23–25). For each of the lymphatic-dwelling parasites, primers and probes have been identified that are 100% specific and provide sensitivities that are up to 10-fold greater than parasite detection by direct examination. Recent diagnostic advances include highly sensitive real-time PCR assays capable of detecting relatively low copy numbers of target sequence in small samples of dried human blood (26, 27) and the loop-mediated isothermal application assay (28), which might potentially be used as a simple and specific test for point-of-care settings.

Serologic Tests

Immunologically based diagnosis with measured IgG or IgG4 responses against crude extracts of *Brugia* worms suffers from poor specificity. There is extensive cross-reactivity among filarial antigens and antigens of other helminths, including the common intestinal roundworms. Furthermore, serologic tests are unable to distinguish between active and past infections. However, these tests still have a role in diagnosis, as a negative test effectively excludes past or present infection. These tests are available commercially as well as from the National Institutes of Health.

Treatment and Prevention

The available chemotherapy for LF is DEC, ivermectin, and albendazole. DEC remains the treatment of choice for the individual with active LF (microfilaremia, antigen positivity, or adult worms on ultrasound), although albendazole has also been shown to have some macrofilaricidal efficacy. If the adult parasites survive, microfilaremia along with clinical symptoms can recur within months after conclusion of the therapy. Chronic low-dose DEC may also result in cure (e.g., in DEC salt). Evidence shows that the use of these drugs in combination can increase their effectiveness (29–32). The current global elimination campaign uses these three drugs in various combinations for mass treatment of communities where the parasites are endemic (33).

Most pathogenic filarial nematodes apart from *L. loa* harbor bacterial endosymbionts. These *Wolbachia* bacteria are vital for parasite larval development and adult worm fertility and viability. New approaches for treatment use antibiotics (e.g., the tetracyclines) that target *Wolbachia* and have been shown to reduce microfilarial levels (34). Once lymphedema is established, antifilarial medication is not useful if the patient does not have active infection. Management of lymphedema should concentrate on exercise, elevation, and local skin care with appropriate treatment of entry lesions (34). There has, however, been some recent evidence that treatment with doxycycline may decrease the severity in early stages of lymphedema, independent of its antimicrobial effects (35). Antifilarial medication is also not indicated in management of bacterial ADLA, which is addressed with skin care and antibiotics if indicated (15, 18). Hydroceles can be drained repeatedly or managed surgically (36).

Avoidance of mosquito bites is usually not feasible for residents of areas where the parasites are endemic, but visitors should make use of insect repellent and mosquito nets. Impregnated bednets have been shown to have a salutary effect. DEC can kill developing forms of filarial parasites and has been shown to be useful as a prophylactic agent in humans. Community-based intervention is the current approach to elimination of LF as a public health problem (33, 37). The underlying tenet of this approach is that mass annual distribution of antimicrofilarial chemotherapy (albendazole with either DEC [for all areas except where onchocercaisis is coendemic] or ivermectin) profoundly suppresses microfilaremia. If the suppression is sustained, then transmission can be interrupted. Community education and clinical care for persons already suffering from the chronic sequelae of LF are important components of filariasis control and elimination programs (38). Vaccines are not currently available but may have a role in the future.

ONCHOCERCA VOLVULUS

Taxonomy

Onchocerca volvulus belongs to the phylum Nematoda, class Secernentea, subclass Spiruria, order Spirudida, superfamily Filarioidea, and family Onchocercidae.

Description of the Agent

The adult worms of *Onchocerca volvulus* typically reside in nodules composed primarily of host tissue. These adult parasites may remain viable in the human host for decades. The morphologic appearance and other characteristics of and microfilariae and adult worms can be found in Table 1 and Fig. 1 and 2.

Epidemiology and Transmission

Onchocerciasis, sometimes called "river blindness," is caused by infection with *Onchocerca volvulus*, a subcutaneous-dwelling filarial worm. Approximately 18 million people are infected, mostly in equatorial Africa, the Sahara, Yemen, and parts of Central and South America (Guatemala, Venezuela, Mexico, Ecuador, Colombia, and Brazil). The infection is transmitted to humans through the bites of black flies of the genus *Simulium*, which breed along fast-flowing rivers in the above-mentioned tropical areas.

Clinical Significance (Description of Clinical Presentation)

The major disease manifestations of onchocerciasis are localized to the skin, lymph nodes, and eyes. Onchoceriasis is a cumulative infection. Intense infection leads to the most severe complications and is considered to reflect repeated inoculation with infective larvae.

Skin

Pruritus is the most frequent manifestation of onchocercal dermatitis. This pruritus may be accompanied by the appearance of localized areas of edema and erythema. Typically, skin disease appears as a papular, pruritic dermatitis. If the infection is prolonged, lichenification and pigment changes (either hypo- or hyperpigmentation) can occur; these often lead to atrophy, "lizard skin," and mottling of the skin.

Onchocercomata

Onchocercomata are subcutaneous nodules which can be palpable and/or visible and contain the adult worm. In African patients, they are common over bony prominences; in Latin American patients, nodules tend to develop preferentially in the upper part of the body, particularly on the head. Nodules vary in size and characteristically are firm and nontender. It has been estimated that for every palpable nodule there are four deeper nonpalpable ones (39).

Lymph Nodes

Lymphadenopathy is frequently found, particularly in the inguinal and femoral areas. The underlying pathology consists of scarring of the lymphoid areas (*O. volvulus* infection in Africa) or follicular hyperplasia (*O. volvulus* infection in Yemen). As the lymph nodes enlarge, they can come to lie within areas of loose skin (so-called "hanging groin") that predisposes to inguinal and femoral hernias.

Ocular Disease

Onchocercal eye disease can take many forms and can lead to severe visual loss or blindness. Usually seen in persons with moderate or heavy infections, ocular disease spares no part of the eye. Manifestations include conjunctivitis, anterior uveitis, iridocyclitis leading to secondary glaucoma, sclerosing keratitis, optic atrophy, and chorioretinal lesions (40).

Systemic Manifestations

Some heavily infected individuals develop cachexia with loss of adipose tissue and muscle mass. Among adults who become blind, there is a 3- to 4-fold increase in the mortality rate.

Diagnosis

Definitive diagnosis depends on finding an adult worm in an excised nodule or, more commonly, microfilariae in a skin snip. Microfilariae can occasionally be found in the blood and in urine, typically after treatment. Microfilariae may also be seen in the cornea and in the anterior chamber of the eye when viewed with a slit lamp.

For skin snip evaluation, a small piece of skin is elevated by the tip of a needle or skin hook held parallel to the surface, and a scalpel blade is used to shave off the skin area stretched across the top surface of the needle. Alternatively, a sclerocorneal punch can be used to obtain a blood-free circular skin specimen. Skin snips are generally obtained from an area of affected skin or from the scapular, gluteal, and calf areas (in the African form) and from the scapular, deltoid, and gluteal areas (in the Central American form). Once obtained, the skin snips are incubated in a physiologic solution (such as normal saline). The emergent microfilariae can be seen under a microscope typically within hours (in heavy infection) or within 24 h in light infections.

Direct Examination

Microscopy

Microfilariae lack a sheath and are approximately 309 µm long by 5 to 9 µm in diameter. The tail is tapered, usually bent or flexed, and without nuclei (Fig. 1D and 2c).

Nucleic Acid Detection Techniques

Assays using PCR to detect onchocercal DNA in skin snips are now in use in research laboratories and are highly specific and sensitive, provided that organisms are present in the skin samples obtained (41, 42).

Serologic Tests

Because direct detection of parasites in the skin or eye is invasive and insensitive, immunodiagnostic assays may be preferable. IgG antifilarial antibody assays, while positive for individuals with onchocerciasis, suffer from the same lack of specificity and positive predictive value seen in the blood-borne filarial infections; however, the combined use of three groups of recombinant antigens in conventional ELISA provides sensitivity and specificities that approach 100% for the diagnosis of onchocerciasis (43). A newer platform incorporates four recombinant antigens into a rapid, high-throughput luciferase immunoprecipitation system assay that is 100% sensitive and 80 to 90% specific in distinguishing onchocerciasis from related filarial infections (44). Although no recombinant antigen test is available commercially, an experimental rapid card test that detects IgG4 antibodies in serum or whole blood using recombinant Ov-16 protein has been shown to have >90% sensitivity and specificity (43, 45).

Treatment

The major goals of therapy are to prevent irreversible lesions and to alleviate symptoms. Surgical excision of nodules is recommended when the nodules are located on the head because of the proximity of the microfilaria-producing adult worms to the eye, but chemotherapy is the mainstay of treatment. Ivermectin, a semisynthetic macrocyclic lactone, is now considered the first-line therapy for onchocerciasis. It is characteristically given yearly or semiannually. Most patients have limited or no reaction to treatment. Pruritus, cutaneous edema, and/or a maculopapular rash occur in approximately 1 to 10% of treated individuals. Significant ocular complications are extremely rare, as is hypotension (1 in 10,000). In areas of Africa where *O. volvulus* and *L. loa* are coendemic, however, ivermectin is contraindicated because of severe posttreatment encephalopathy seen in patients who show heavy *L. loa* microfilaremia (46). There has been some recent evidence that doxycycline-based therapy targeting the *Wolbachia* endosymbiont, which is present in *Onchocerca* but not *L. loa*, might have improved efficacy in areas of coinfection with no increase in adverse events (47). Ivermectin is also contraindicated for use in pregnant or breastfeeding women, based on toxicity and teratogenicity data from animal studies. Although ivermectin treatment results in a marked drop in microfilarial density, its effect can be short-lived (<3 months in some cases). Thus, it is occasionally necessary to give ivermectin more frequently for persistent symptoms (48). A 6-week course of doxycycline that targets the *Wolbachia* endosymbiont has been demonstrated to be macrofilaristatic, rendering the female adult worms sterile for long periods (49).

Prevention

Prevention of infection is being achieved by mass treatment programs using ivermectin (50, 51). Vector control has been beneficial in areas where the infection is highly endemic in which breeding sites are vulnerable to insecticide spraying, but most areas where onchocerciasis is endemic are not suited to this type of intervention. Community-based administration of ivermectin every 6 to 12 months is now being used to interrupt transmission in areas where the infection is endemic. This measure, in conjunction with vector control, has already helped reduce the prevalence of disease in endemic foci in Africa and Latin America. No drug has proven useful for prophylaxis of *O. volvulus* infection, and no vaccine exists.

LOA LOA

Taxonomy

Loa loa belongs to the phylum Nematoda, class Chromadorea, order Spirudida, superfamily Filarioidea, and family Onchocercidae.

Description of the Agent

The adult parasite lives in the subcutaneous tissues in humans; microfilariae circulate in the bloodstream with a diurnal periodicity that peaks between noon and 2:00 p.m. The morphologic appearance and other characteristics of adult worms and microfilariae can be found in Table 1 and Fig. 1 and 2.

Epidemiology and Transmission

The distribution of *L. loa* is limited to the rain forests of West and Central Africa (52). Tabanid flies (deer flies) of the genus *Chrysops* are the vectors.

Clinical Significance (Description of Clinical Presentation)

L. loa infection may be present as asymptomatic microfilaremia, with the infection being recognized only after subconjunctival migration of an adult worm (the so-called eye worm). The classic clinical presentation is with episodic Calabar swelling (localized areas of transient angioedema) found predominantly on the extremities. If associated inflammation extends to the nearby joints or peripheral nerves, corresponding symptoms develop. Nephropathy (presumed to be immune complex mediated), encephalopathy, and cardiomyopathy due to marked eosinophilia can occur rarely.

There appear to be differences in the presentations of loiasis between those native to the area where loiasis is endemic and those who are visitors (54). The latter tend to have a greater predominance of allergic symptomatology. The episodes of Calabar swelling tend to be more frequent and debilitating, and such patients rarely have microfilaremia. In addition, those who are not native to the area of endemicity have extreme elevation of eosinophils in the blood as well as marked increases in antifilarial antibody titers.

Diagnosis

Definitive diagnosis is made parasitologically, either by finding microfilariae in the peripheral blood or by isolating the adult worm from the eye or subcutaneous biopsy material following treatment. The diagnosis must often be made on clinical grounds, however, particularly for travelers (usually amicrofilaraemic) to the region where the infection is endemic.

Direct Examination

Microscopy

The microfilariae are sheathed and are up to 300 μm long. Adult females are 50 to 70 mm long and 0.5 mm wide, whereas adult males are 25 to 35 mm long and 0.25 mm wide. In contrast to the LF parasite, the nuclei extend to the end of the tail; however, they are somewhat irregularly arranged along the length of the tail (Fig. 1C and 2d). The sheath does not stain with Giemsa stain.

Nucleic Acid Detection Techniques

PCR-based assays for the detection and quantitation of *L. loa* DNA in blood are now available in research laboratories and are highly sensitive and specific (55, 56).

Serologic Tests

Available methods using crude antigen extracts from *Brugia* or *Dirofilaria* species do not differentiate between *L. loa* and other filarial pathogens. The utility of such testing in endemic populations is limited by the presence of antifilarial antibodies in up to 95% of individuals in some regions. A *Loa*-specific recombinant protein, SXP-1, has been tested and has good specificity but only limited (50%) sensitivity in conventional ELISA (57). Incorporation of SXP-1 into a luciferase immunoprecipitation system assay increased sensitivity to near 100% while also allowing for rapid, high-throughput processing of samples (58). Antifilarial IgG and IgG4, while nonspecific, may be useful in confirming the diagnosis of loiasis in visitors to areas where the infection is endemic with suggestive clinical symptoms or unexplained eosinophilia.

Treatment and Prevention

DEC is effective against both the adult and the microfilarial forms of *L. loa*, but multiple courses are frequently necessary before the disease resolves completely (59). In cases of heavy microfilaremia, allergic or other inflammatory reactions can take place during treatment, including central nervous system involvement with coma and encephalitis (60). Heavy infections can be treated initially with apheresis to remove the microfilariae and with glucocorticoids followed by small doses of DEC. If antifilarial treatment has no adverse effects, the prednisone dose can be rapidly tapered and the dose of DEC gradually increased. Albendazole or ivermectin (although not approved for this use by the FDA) has been shown to be effective in reducing microfilarial loads, but the use of ivermectin in heavily microfilaremic individuals is contraindicated (61). DEC is an effective prophylactic regimen for loiasis (62).

MANSONELLA INFECTIONS

Taxonomy

Mansonella perstans, *Mansonella ozzardi*, and *Mansonella streptocerca* each belong to the phylum Nematoda, class Secernentea, subclass Spiruria, order Spirudida, superfamily Filarioidea, and family Onchocercidae.

Description of the Agents

The adult worms of *Mansonella perstans* reside in the body cavities (pericardial, pleural, and peritoneal) as well as in the mesentery and the perirenal and retroperitoneal tissues, whereas the location of the adult worms of *Mansonella ozzardi* is unknown. The microfilariae of both parasites circulate in the blood without periodicity. For *Mansonella streptocerca*, the adult parasites reside in the skin. *M. streptocerca* microfilariae are found predominantly in the skin. The morphologic appearance and other characteristics of adult worms and microfilariae can be found in Table 1 and Fig. 1 and 2.

Epidemiology and Transmission

Mansonella perstans is distributed across the center of Africa and in northeastern South America. The infection is transmitted to humans through the bites of midges (*Culicoides* species). *Mansonella streptocerca* is largely found in the tropical forest belt of Africa from Ghana to Zaire. It is transmitted to the human host by biting midges (*Culicoides* species). The distribution of *M. ozzardi* is restricted to Central and South America as well as certain Caribbean islands. The parasite is transmitted to the human host by biting midges (*Culicoides furens*) and black flies (*Simulium amazonicum*).

Clinical Significance (Description of Clinical Presentation)

Although most patients infected with *M. perstans* appear to be asymptomatic, clinical manifestations may include transient angioedematous swellings of the arms, face, or other body parts (similar to the Calabar swellings of *L. loa* infection); pruritus; fever; headache; arthralgias; neurologic or psychological symptoms; and right upper quadrant pain. Occasionally, pericarditis and hepatitis occur (63).

The major clinical manifestations of *M. streptocerca* infections are related to the skin: pruritus, papular rashes, and pigmentation changes. These are thought to be secondary to inflammatory reactions around microfilariae. Most infected individuals also show inguinal lymphadenopathy. Lymph nodes of affected individuals may show chronic lymphadenitis with scarring; however, many patients are completely asymptomatic (64).

The clinical details of *M. ozzardi* infection are poorly characterized. Furthermore, many consider this organism to be nonpathogenic; however, headache, articular pain, fever, pulmonary symptoms, adenopathy, hepatomegaly, and pruritus have been ascribed to infection with this organism (74). There have been reports of an association of *M. ozzardi* with keratitis (65).

Diagnosis

For *M. perstans* infections, diagnosis is made parasitologically by finding the microfilariae in the blood or in other body fluids (serosal effusions). Microfilariae are small (203 μm by 4 to 5 μm) and have a blunt tail filled with nuclei. Perstans filariasis is often associated with peripheral blood eosinophilia and antifilarial antibody elevations (63). The diagnosis of *M. ozzardi* infection is made by demonstrating the characteristic microfilariae in the peripheral blood. These are small (224 μm by 4 to 5 μm) and have long attenuated tails devoid of nuclei (Fig. 1F and 2g). PCR-based assays may provide increased sensitivity (66). The diagnosis of streptocerciasis can be made by finding the characteristic microfilariae on skin snip examination (see section above on onchocerciasis diagnosis). In areas where both *O. volvulus* and *M. streptocerca* are endemic, care must be taken to correctly identify the microfilariae. *M. streptocerca* microfilariae have no sheath, are long and slender, and measure approximately 210 μm by 5 to 6 μm. The most characteristic feature of *M. streptocerca* is its crooked tail (Fig. 2f), which contains nuclei.

Treatment and Prevention

Mansonella perstans

A number of treatment regimens have been tried, but none has been shown to be particularly effective in *M. perstans* filariasis. However, consistent with the identification of a *Wolbachia* species in *M. perstans* (67), a randomized trial in Mali has demonstrated the utility of doxycycline (200 mg daily for 6 weeks) treatment for this infection (68).

Mansonella streptocerca

DEC is particularly effective in treating infection by both the microfilarial and the adult forms of the parasite. Following treatment, as in onchocerciasis, debilitating urticaria, arthralgias, myalgias, headaches, and abdominal discomfort are often present. Nevertheless, because DEC is contraindicated in most of Africa because of concerns with posttreatment reactions in onchocerciasis, its use in this infection is limited. Consequently, ivermectin is currently the drug of choice for this infection (69).

Mansonella ozzardi

Ivermectin is the drug of choice for this infection (70, 71) but has been associated with significant posttreatment adverse events in some patients (53). There currently are no good preventative measures for any of the *Mansonella* infections beyond personal protective equipment, clothing, and insect repellents such as DEET (*N*,*N*-diethyl-meta-toluamide) or permethrin.

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Cestodes

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Cestodes have as their key characteristic a flattened body composed of the head or scolex (bearing the fixation organs—suckers, hooks, and bothria), the neck (where the cellular reproduction occurs, to form the strobila), and the strobila, formed by numerous segments or proglottids. As new proglottids develop in the neck region, existing ones mature as they become more distal. The more-distal proglottids are gravid, almost completely occupied by a uterus full of eggs which are passed with the stools of the carrier, either inside complete proglottids or free after proglottid breakage. In some species, proglottids can actively migrate out of the anus.

Tapeworms live in the lumen of the small intestine with the head or scolex as the only fixation organ, attached to the mucosa. They absorb nutrients from the host's intestine both at the head and through their tegument. Accordingly, they have developed cephalic fixation organs like hooks, suckers, or shallow grooves as longitudinal suction sulci (bothria) (Fig. 1), and a specialized tegument.

Four species of cestode tapeworms inhabit the human intestine: *Diphyllobothrium latum*, *Taenia saginata*, *Taenia solium*, and *Hymenolepis nana*. They differ widely in size, intermediate host, and other characteristics, from the 12-m *D. latum* to the 3-cm *H. nana* (Table 1). More rarely, *Dipylidium caninum* and *Hymenolepis diminuta* can also inhabit the human gut; these parasites are reviewed in chapter 147. In addition, a number of cestode larvae can produce human disease if infective tapeworm eggs are ingested, mainly cysticercosis (*Taenia solium*), cystic hydatid disease (*Echinococcus granulosus*), and alveolar hydatid disease (*Echinococccus multilocularis*). Rarer larval cestode infections affecting humans include coenurosis (*Taenia multiceps*), sparganosis (*Spirometra mansonoides*), and cysticercosis by *Taenia crassiceps*. Tapeworms and especially tapeworm larval infections still represent an important cause of morbidity and mortality, not only in most underdeveloped countries but also in industrialized countries, particularly in rural areas or among immigrants from areas of endemicity.

DIPHYLLOBOTHRIUM LATUM

Known as the fish tapeworm, *D. latum* is the longest intestinal parasite of humans. Also common in fish-eating mammals such as canids or felids (reservoir hosts), it differs from other adult tapeworms infecting humans in its morphology, biology, and epidemiology.

Taxonomy

D. latum is included in the phylum Platyhelminthes, subphylum Neodermata, class Cestoidea, subclass Eucestoda, order Pseudophyllidea, family Diphyllobothriidae, and genus *Diphyllobothrium*.

Description of the Agent

Adult Tapeworm

The adult parasite can grow to 15 m in length and apparently can live for 20 years or longer in the small intestine. It is ivory in color and has a scolex which is provided with bothria on its dorsal and ventral aspects (1, 2). *Diphyllobothrium* proglottids are much wider than they are long (~8 by 4 mm) and are easy to recognize because their genital pore is located in the center of the proglottid rather than in the lateral edges as in all other tapeworms of humans. The coiled uterus in the center of the gravid proglottids looks yellow-brown in freshly passed specimens. The uterine pore is located in the center of the proglottid near the genital pore.

Eggs

Unembryonated, operculate eggs are passed in the feces. *D. latum* eggs are oval and resemble those of trematodes but are smaller (58 to 75 μm long by 44 to 50 μm wide) and have a better-defined wall. The abopercular end usually has a small knoblike protrusion. Eggs are usually numerous, and expulsion of proglottid chains is usual.

Larvae

After the eggs embryonate in a water environment for several weeks, ciliated six-hooked embryos (coracidia) hatch. Coracidia must be ingested by appropriate species of freshwater copepods (genus, *Cyclops*) for further development. In the copepod a solid-bodied larva, the procercoid, develops as a second larval stage and becomes infective for the second intermediate host (fish). In fish, the procercoid migrates to the flesh and develops in the third larval stage, the plerocercoid or sparganum, which is the infective stage for human or animal (canids or felids) hosts.

Epidemiology, Transmission, and Prevention

The geographic distribution of *D. latum* includes lake areas in Scandinavia, other areas of northern Europe, the former

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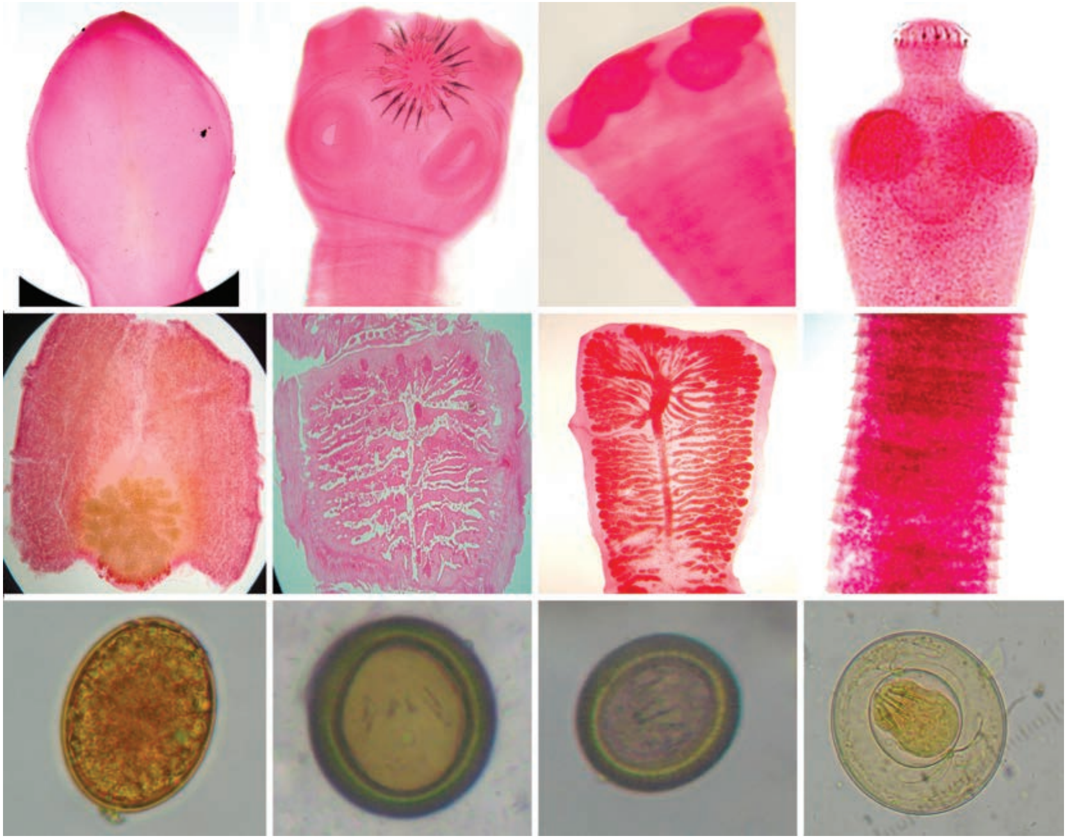

FIGURE 1 Scolices (top), gravid proglottids (middle) and eggs (bottom) of *D. pacificum*, *T. solium*, *T. saginata*, and *H. nana* (left to right). Note the coiled, central uterus in *D. pacificum*, the absence of hooks in the scolex of *T. saginata*, and the similar appearances of the eggs of *T. saginata* and *T. solium*. Morphological characteristics shown for *D. pacificum* are similar to those of *D. latum*. doi:10.1128/9781555817381.ch145.f1

Soviet Union, Finland, northern Japan, and North America, principally the upper Midwest, Alaska, and Canada, and the southwestern coast of South America. Several other *Diphyllobothrium* species (*D. pacificum*, *D. cordatum*, *D. ursi*, *D. dendriticum*, *D. lanceolatum*, *D. dalliae*, and *D. yonagoensis*) have also been reported to infect humans but less frequently (2, 3). *Diphyllobothrium pacificum*, identified by Nybellin in 1931, is a parasite of seawater found along the western coast of South America, specifically in Peru and Chile. *D. pacificum* is much smaller than *D. latum* and usually measures 50 to 200 cm long, although it can occasionally reach 3 to 4 m.

The most common sources of human *Diphyllobothrium* infection are the pike, burbot, perch, ruff, and turbot (2). Infected fish (undercooked, raw, or insufficiently treated flesh) transmit plerocercoids to humans or other fish-eating mammals. Infection with *Diphyllobothrium* is preventable by eating well-cooked fish or fish that has been deep-frozen (at least −10°C for 24 h).

Clinical Significance (Description of Clinical Presentation)

Infected individuals notice passing segment chains with their stools. The parasite may produce no clinical symptoms in some people, but when it reaches a large size it may cause mechanical bowel obstruction, diarrhea, abdominal pain, and, particularly in northern European countries, pernicious

TABLE 1 Some characteristics of main cestodes infecting humans

Organism	Length (cm)	Scolex	Gravid proglottids	Intermediate host(s)
D. latum	1,200	Spatulate, two bothria	Rosette-shaped central uterus	Copepods, fish
T. saginata	600	Squared, four suckers, no hooks	>15 main lateral uterine branches	Cattle
T. solium	300	Squared, four suckers, hooks (double crown)	<12 main lateral uterine branches	Pigs, humans
H. nana	3	Knoblike, four suckers, hooks (single crown)	Bag-shaped uterus	Insects, rodents, humans

anemia resulting from vitamin B_{12} deficiency because the tapeworm competes with the intestinal epithelium for the uptake of the vitamin. This condition is rare outside Scandinavian countries, and some authors postulate a genetic predisposition.

Collection, Transport, and Storage of Specimens

For identification purposes, eggs are well preserved in 5 to 10% formalin solutions. For DNA recovery, 95% ethanol would be a better option. Electron microscopy may require cacodylate buffer or other glutaraldehyde media. Adult tapeworm material is better defined if it is washed in saline, relaxed for better visualization of its internal structures by warming the saline at 55°C for a short period (5 min), and then placed between two glass slides and stored in a fixative solution. Fixatives could be 10% formalin, acetic acid-formaldehyde-alcohol, or sodium acetate-acetic acid-formaldehyde. Fixed pieces can be stained by injecting Semichon's carmine or India ink. The proglottids can also be sectioned and stained using hematoxylin and eosin; however, morphological characteristics can be more easily seen in whole mounts.

Direct Examination

Microscopy

Eggs can be easily seen by microscopical examination of stools. Either flotation or sedimentation techniques may be used. However, since the eggs are operculated, they generally do not float using the flotation concentration method; both the surface film and sediment need to be examined if this concentration method is used. For that reason, most laboratories routinely use the sedimentation concentration method. Low-magnification microscopy should easily permit identification of the characteristic scolex or proglottids when available (Fig. 1). Neither culture nor antigen detection is relevant for the detection and identification of *D. latum*.

Nucleic Acid Detection Techniques

Although several groups have described genus variation in *Diphyllobothrium* by using nucleic acid detection methods, the information has no clinical relevance in terms of routine tapeworm recovery and identification (4).

Serologic Tests

Serologic tests are not available.

Treatment

Both praziquantel and niclosamide are effective drugs. At recommended doses both are associated with only mild side effects, mostly gastrointestinal.

Evaluation, Interpretation, and Reporting of Results

Both stool microscopy and parasite identification are unambiguous. Eggs or tapeworm pieces should be reported as *D. latum* eggs (except in South America, where *D. pacificum* is more frequently found). Other human-infecting species are rarely found.

TAENIA SAGINATA

Known as the beef tapeworm, *Taenia saginata* is still endemic to most of the world. Humans are its only definitive host. While *T. saginata* infections do not carry major risks for the host, differential diagnosis with *Taenia solium* is important because the latter can cause neurocysticercosis.

Taxonomy

T. saginata is included in the phylum Platyhelminthes, subphylum Neodermata, class Cestoidea, subclass Eucestoda, order Cyclophyllidea, family Taeniidae, and genus *Taenia*.

Description of the Agent

Adult Tapeworm

The adult *T. saginata* tapeworm attains lengths of 4 to 8 m and has a scolex provided with four suckers and an unarmed (no hooks) rostellum. Gravid proglottids are longer than they are wide (18 to 20 mm by 5 to 7 mm). Each proglottid has a genital pore at the midlateral margin. In mature proglottids, the ovary has only two lobes and presents a vaginal sphincter. Gravid proglottids, which are highly muscular and active, break off from the strobila and actively migrate out of the anus (a pathognomic characteristic of this species).

Eggs

Eggs from *T. saginata* and *T. solium* are indistinguishable by morphological characteristics. They are spherical, measure 30 to 40 μm in diameter, and have a quite characteristic thick, yellow-brown, radiate shell (embryophore) composed of collagen subunits, which gets thicker as the eggs mature. Eggs are frequently surrounded by a thin layer of vitellum (Fig. 1). Within the egg is a six-hooked embryo, the oncosphere.

Larvae

The unarmed scolex is invaginated into a fluid-filled bladder, the cysticercus. Larval cysts are 4 to 6 mm long by 7 to 10 mm wide and have a pearl-like appearance in tissues.

Epidemiology, Transmission, and Prevention

T. saginata is distributed worldwide, although it is especially prevalent in some parts of Africa, Central and South America, eastern and western Asia, and some countries in Europe. Cattle serve as the intermediate host, and ingestion of eggs from contaminated pasturelands by grazing cattle results in development in cattle tissues of the infective cysticercus stage. After ingestion of the cysticercus in raw or inadequately cooked beef, it takes approximately 2 to 3 months for the infection to become patent in the human host.

In Southeast Asia there is a human tapeworm morphologically very similar to *Taenia saginata* (*Taenia saginata asiatica*, *Taenia asiatica*, or Taiwan taenia). In this tapeworm, the cysticercus stage occurs in the livers of pigs and, less frequently, in cattle. The adult tapeworm infects the human host, and its appearance is very similar to that of *T. saginata* (5, 6).

Clinical Significance (Description of Clinical Presentation)

Although patients may exhibit no symptoms with this infection, they usually notice passing proglottids or find them in their underwear. The mature worm can also cause abdominal discomfort, diarrhea, and occasionally intestinal obstruction as a result of its large size.

Collection, Transport, and Storage of Specimens

See instructions in "Collection, Transport, and Storage of Specimens" for *Diphyllobothrium*.

Direct Examination

Microscopy

Typical *Taenia* eggs can be found in feces. Sedimentation or the less used Kato-Katz method is apparently more sensitive for the detection of *Taenia* eggs in stools than other concentration techniques. Finding of *Taenia* eggs does not allow a species-specific diagnosis of infection; it is usually made by identification of gravid proglottids that have been passed in feces or have actively migrated out of the anus. Identification of the proglottids is based on shape and size and mainly on the morphology of the uterus, which can be demonstrated after injection with India ink or staining with carmine or hematoxylin stains. In *T. saginata* there are 15 to 20 primary lateral branches on each side of the central uterine stem (Fig. 1).

Antigens in stools (coproantigen) have been detected by enzyme-linked immunosorbent assay (ELISA) since 1990, but this assay is used mainly in research settings because of scarce availability.

Nucleic Acid Detection Techniques

Species-specific PCR techniques have been described to detect parasite DNA and differentiate *T. saginata* from *T. solium*. Most of these assays require actual parasite material, although some are apparently able to establish the difference with DNA from eggs in feces (7–10).

Serologic Tests

Serum antigen detection ELISAs for *T. saginata* cysticercosis in cattle have been developed using monoclonal antibodies to *T. saginata*. Although these assays can detect parasite burdens of <50 cysts per animal, they have not yet been routinely applied except in research settings (11–13).

Treatment

Both praziquantel and niclosamide are effective drugs. At recommended doses both are associated with only mild side effects, mostly gastrointestinal. In regions where *T. solium* is endemic, there is a possibility that latent neurocysticercosis may respond to praziquantel and cause severe headaches or seizures. Niclosamide is not absorbed from the gastrointestinal tract and thus does not carry this risk (14).

Evaluation, Interpretation, and Reporting of Results

Eggs should be reported as "*Taenia* sp." because direct observation does not confirm the species. The finding of *Taenia* sp. eggs should be notified to the attending physician to ensure prompt treatment. The presence of the scolex in the parasite material expelled (spontaneously or posttreatment) should be reported both because it allows species diagnosis and because if it is not found, the chances of treatment failure increase.

TAENIA SOLIUM

Known as the pork tapeworm, *T. solium* has an extensive geographic distribution. This infection has a huge impact on human health because of its association with seizure disorders caused by infection of the human brain with its larval stage (neurocysticercosis) (15).

Taxonomy

T. solium is included in the phylum Platyhelminthes, subphylum Neodermata, class Cestoidea, subclass Eucestoda, order Cyclophyllidea, family Taeniidae, and genus *Taenia*.

Description of the Agent

Adult Tapeworm

The adult *T. solium* tapeworm measures 2 to 4 m and has a scolex provided with four suckers and a rostellum armed with two crowns of hooks. Gravid proglottids have similar length and width (approximately 1 cm). Each proglottid has a genital pore at the midlateral margin. In mature proglottids, the ovary has two main lobes and one accessory lobe (lacking in *T. saginata*), and a vaginal sphincter muscle is lacking (present in *T. saginata*). Gravid proglottids have few (<12) lateral branches on the central uterine stem (Fig. 1). Since the eggs of *T. solium* are infective to humans and can cause cysticercosis, extreme caution in the handling of these proglottids or infective stools is recommended.

Eggs

Eggs from *T. saginata* and *T. solium* are indistinguishable by morphological characteristics. They are spherical, measure 30 to 40 μm in diameter, and have a quite characteristic thick, yellow-brown, radiate shell (embryophore) composed of collagen subunits, which gets thicker as the eggs mature. Eggs are frequently surrounded by a thin layer of vitellum (Fig. 1). Within the egg is a six-hooked embryo, the oncosphere.

Larvae

The fluid-filled bladder (cysticercus) larvae are bigger than those of *T. saginata*, measuring approximately 8 to 10 mm in diameter. They lodge in the pig's tissues, mostly in muscle and brain.

Epidemiology, Transmission, and Prevention

T. solium taeniasis and cysticercosis are highly endemic to all parts of the developing world where pigs are raised as a food source, including Latin America, most of Asia, sub-Saharan Africa, and parts of Oceania. The infection is now also increasingly diagnosed in industrialized countries due to immigration of tapeworm carriers from zones of endemicity (6, 15).

As for *T. saginata*, humans are the only definitive host. Ingestion of contaminated pork containing *T. solium* cysticerci causes human taeniasis. Conversely, *T. solium* eggs cause cysticercosis in pigs (the usual intermediate host) and humans. Pigs acquire cysticercosis by eating stools contaminated with infective eggs in places where deficient sanitation exists. Humans are infected by fecal-oral contamination from a tapeworm carrier, commonly in the household or another close environment.

Clinical Significance (Description of Clinical Presentation)

Human *T. solium* taeniasis is acquired by ingestion of infective cysticerci in inadequately cooked pork or pork products. Taeniasis seems mostly asymptomatic, and most patients do not even notice passing proglottids in stools. The clinical significance of *T. solium* infections relates to the risk of neurocysticercosis (see below), which is high for tapeworm carriers and their close contacts (15).

Collection, Transport, and Storage of Specimens

See instructions in "Collection, Transport, and Storage of Specimens" for *Diphyllobothrium*. Handling of *T. solium* proglottids or contaminated stools should be done with appropriate biosafety conditions to avoid cysticercosis.

Direct Examination

Microscopy

Typical *Taenia* eggs can be found in feces. Sedimentation or the less used Kato-Katz method is apparently more sensitive for the detection of *Taenia* eggs in stools than other concentration techniques. Finding of *Taenia* eggs does not allow a diagnosis of infection by the species, which is usually made by identification of gravid proglottids or, more rarely, the scolex passed in feces. Identification of the proglottids is based on shape and size and mainly on the morphology of the uterus, which can be demonstrated after injection with India ink or staining with carmine or hematoxylin stains. In *T. solium* there are few primary branches on each side of the central uterine stem (Fig. 1).

Antigens in stools (coproantigen) have been detected by ELISA since 1990. Coproantigen detection ELISA is much more sensitive than microscopy and thus highly recommended for the diagnosis of human taeniasis (specifically in the case of *T. solium* because of the risks of cysticercosis transmission), as well as to monitor the effectiveness of treatment, but its availability is still limited (16).

Nucleic Acid Detection Techniques

Species-specific PCR techniques that differentiate *T. saginata* from *T. solium* have been described. Most of these assays require actual parasite material, although some are apparently able to establish the difference with DNA from eggs in feces (7–10).

Serologic Tests

Recently, stage-specific serologic assays directed to the adult tapeworm have been developed, with high sensitivity and specificity. Mostly, serology is directed to the detection of *T. solium* antibodies in relation to the diagnosis of neurocysticercosis. Antibody detection by enzyme-linked immunoelectrotransfer blot assay is the method of choice, with a sensitivity of 98% in cases with more than one viable larval cyst and a specificity of 100% (17). *T. solium* antigen detection in serum or cerebrospinal fluid has been performed in cases of human cysticercosis, based on a known genus-specific cross-reaction in ELISAs for *T. saginata*. Although these assays can detect parasite burdens of <50 cysts in infected animals, they have not yet been routinely applied except in research settings. *T. solium* antigen detection is likely to be a helpful tool to monitor the evolution of patients with severe, subarachnoid neurocysticercosis, in which high antigen levels occur (18).

Treatment

Both praziquantel and niclosamide are effective drugs. At recommended doses both are associated with only mild side effects, mostly gastrointestinal. In regions where *T. solium* is endemic, there is a possibility that latent neurocysticercosis may respond to praziquantel and cause severe headaches or seizures. Niclosamide is not absorbed from the gastrointestinal tract and thus does not carry this risk (14).

Evaluation, Interpretation, and Reporting of Results

Eggs should be reported as "*Taenia* sp." because direct observation does not confirm the species. The finding of *Taenia* sp. eggs should be notified to the attending physician to ensure prompt treatment and minimize the chances of cysticercosis in the patient or his/her contacts. The presence of the scolex in the parasite material expelled (spontaneously or posttreatment) should be reported both because it allows species diagnosis and because if it is not found, the chances of treatment failure increase.

HYMENOLEPIS NANA

H. nana is the smallest of the intestinal tapeworms of humans and also the most common tapeworm infection throughout the world. It can be transmitted from person to person (an intermediate host is not necessarily required) (3).

Taxonomy

H. nana is included in the phylum Platyhelminthes, subphylum Neodermata, class Cestoidea, subclass Eucestoda, order Cyclophyllidea, family Hymenolepididae, and genus *Hymenolepis*.

Description of the Agent

Adult Tapeworm

The adult parasite measures 2 to 4 cm and seems to live for approximately 1 year. The scolex has four suckers and one crown of hooks.

Eggs

The eggs are 30 to 50 μm in diameter and thin shelled, and they contain a six-hooked oncosphere that lies in the center of the egg and is separated from the outer shell by considerable space. The oncosphere is surrounded by a membrane that has two polar thickenings from which arise four to eight filaments extending into the space between it and the outer shell (Fig. 1). These filaments are not seen in *H. diminuta*. Eggs may hatch inside the host's intestine, and the embryos (oncospheres) invade the mucosa to develop into larval stages.

Larvae

The cysticercoid larvae have an invaginated scolex but no fluid-filled bladder. They lodge in the intestinal mucosa and emerge to the intestinal lumen as young tapeworms after a few days.

Epidemiology, Transmission, and Prevention

H. nana is normally a parasite of mice, and its life cycle characteristically involves a beetle as an intermediate host. In humans, transmission is usually accomplished by direct ingestion of infective eggs containing oncospheres. When eggs are ingested, a solid-bodied larva, a cysticercoid, first develops in the wall of the small intestine. Subsequently, the larva migrates back into the intestinal lumen, where it reaches maturity as an adult tapeworm in 2 to 3 weeks. In beetles that ingest eggs of *H. nana*, the cysticercoids develop in the body cavity and have thick protective walls around them. Although humans may acquire the infection by accidental ingestion of infected beetles (often occurring in dry cereals), direct infection is far more common and is the primary reason why *H. nana* usually occurs in institutional and familial settings in which hygiene is substandard. A feature of human *H. nana* infection is the opportunity for internal autoinfection with the parasite, which may result in large worm burdens. Autoinfection occurs when eggs discharged by adult tapeworms in the lumen of the small intestine hatch rapidly and invade the wall of the intestine; here, cysticercoids are formed, and they subsequently reenter the intestine to mature as adult worms.

Clinical Significance (Description of Clinical Presentation)

Most infections cause no symptoms. However, hymenolepiasis can be associated with abdominal pain, diarrhea, headaches, or irritability, probably in infections with heavier worm burdens (19, 20).

Collection, Transport, and Storage of Specimens

See instructions in "Collection, Transport, and Storage of Specimens" for *Diphyllobothrium*.

Direct Examination

Microscopy

Diagnosis of the infection rests on finding the spherical eggs in feces by microscopy. Either flotation or sedimentation techniques are of help. Proglottids (Fig. 1) are rarely seen, since they disintegrate after breaking off from the main strobila. Neither culture, antigen detection, nor nucleic acid detection techniques are relevant for the detection and identification of *H. nana*.

Serologic Tests

Antibody detection ELISAs have been used in research settings but are of no clinical use (21).

Treatment

Both praziquantel and niclosamide are effective drugs. At recommended doses both are associated with only mild side effects, mostly gastrointestinal. A second dose of praziquantel after 10 to 15 days may decrease the likelihood of relapses. Niclosamide needs to be administered for 7 days because it is not absorbed and thus does not affect the cysticercoid larvae in the intestinal mucosa. Nitazoxanide has been reported to be useful as a therapeutic alternative (22).

Evaluation, Interpretation, and Reporting of Results

Eggs are characteristic and should be reported as *H. nana* eggs. The closest differential diagnosis is with *H. diminuta*, which rarely infects humans. The eggs of *H. diminuta* are bigger, lack polar filaments, and have a wider interior space and thus can be differentiated by microscopy.

LARVAL CESTODES INFECTING THE HUMAN HOST

The larval stages of *Taenia solium*, *Echinococcus granulosus*, *Echinococcus multilocularis*, and, less frequently, *Spirometra*, *Taenia multiceps*, and *Taenia crassiceps* can invade the human tissues. These are briefly described below.

Cysticercosis (*Taenia solium*)

In the normal life cycle of *T. solium*, humans are the definitive host and pigs act as the intermediate host, hosting the larval stage, or cysticercus. Porcine cysticercosis is a serious economic problem for pig farmers. However, the most serious consequences are associated with human cysticercosis (15). Larval vesicles located in the human central nervous system (Fig. 2) cause seizures or other neurological symptoms. Indeed, neurocysticercosis is associated with a significant proportion of seizure cases in areas of endemicity (23, 24). Clinical manifestations of neurocysticercosis are related to individual differences in the number, size, and topography of lesions and in the severity of the host's immune response to the parasites. Symptoms and signs are varied and nonspecific. Parasites in the brain parenchyma usually cause seizures and headache, whereas those located in the cerebral ventricles or in the subarachnoid space ("racemose" cysticercosis) cause intracranial hypertension and hydrocephalus. Diagnosis is made using brain imaging, either computed tomography (CT) scan or magnetic resonance imaging (MRI), and confirmed by serology. CT has a lower cost (quite important for poor areas where the disease is endemic), is more available, and has better sensitivity for the detection of calcified parasites. Conversely, MRI has better sensitivity for small lesions, those located close to the skull, and intraventricular parasites. The serologic assay of choice is an immunoblot using seven purified glycoprotein larval antigens, which has 98% sensitivity and 100% specificity except in cases with a single lesion, for which sensitivity drops to approximately 70% (17, 18). Antigen detection assays have been described, but no controlled data on sensitivity and specificity are yet available. Treatment of neurocysticercosis uses antiparasitic agents (albendazole or praziquantel) for viable parasites, usually given with steroids to ameliorate the inflammation produced by the death of the cyst. Surgery is limited to excision of single, big lesions or implantation of ventricle-peritoneal shunts. Antigen detection assays permit patient monitoring and follow-up of antiparasitic treatment (18, 25).

Cystic Hydatid Disease (*Echinococcus granulosus*)

In the normal life cycle of *Echinococcus granulosus*, the dog is the definitive host and herbivores (mainly sheep) act as the intermediate host. These become infected with the larval stage (cystic hydatid) by ingesting infective eggs dispersed from the feces of a tapeworm-infected dog. Human cystic hydatid disease is an important cause of human morbidity, requiring costly surgical and medical treatment. This cestodiasis is still endemic to most of the Old World, particularly Greece, Cyprus, Bulgaria, Lebanon, Turkey, some other European countries, South America, and Africa. Sporadic autochthonous transmission is currently recognized in Alaska and other states in the United States (26). The affected organs are most commonly the liver and lungs and, more rarely, the heart, brain, bones, or other locations. Diagnosis is made using ultrasound or CT scan for liver infection or chest X rays or CT for lung infections (Fig. 2). Antibody detection by serology is helpful, although sensitivity is lower than for other infections, reaching 80 to 85%. It is more sensitive for hepatic than for pulmonary cases. Treatment uses antiparasitic agents (albendazole or albendazole plus praziquantel) for small cysts or presurgery; PAIR (puncture, aspiration, injection, and reinjection), which is a technique of ultrasound- or CT-guided aspiration and sterilization of the cyst's contents; or either laparoscopic or open surgery (27, 28). Spillage of cyst contents could lead to acute anaphylactic reactions or dissemination of infection in the surrounding tissues. Cystic lesions may resolve without therapy in a proportion of patients (29).

Alveolar Hydatid Disease (*Echinococcus multilocularis*)

The adult stage of *E. multilocularis* lives in the small intestine of the definitive host, commonly wild predators in the northern hemisphere, occurring in parts of Europe, Asia, Japan, and North America, including Alaska (30, 31). The larval stages infect microtine rodents that usually serve as the common intermediate host. Human infections (causing alveolar hydatid disease) occur by accidental ingestion of

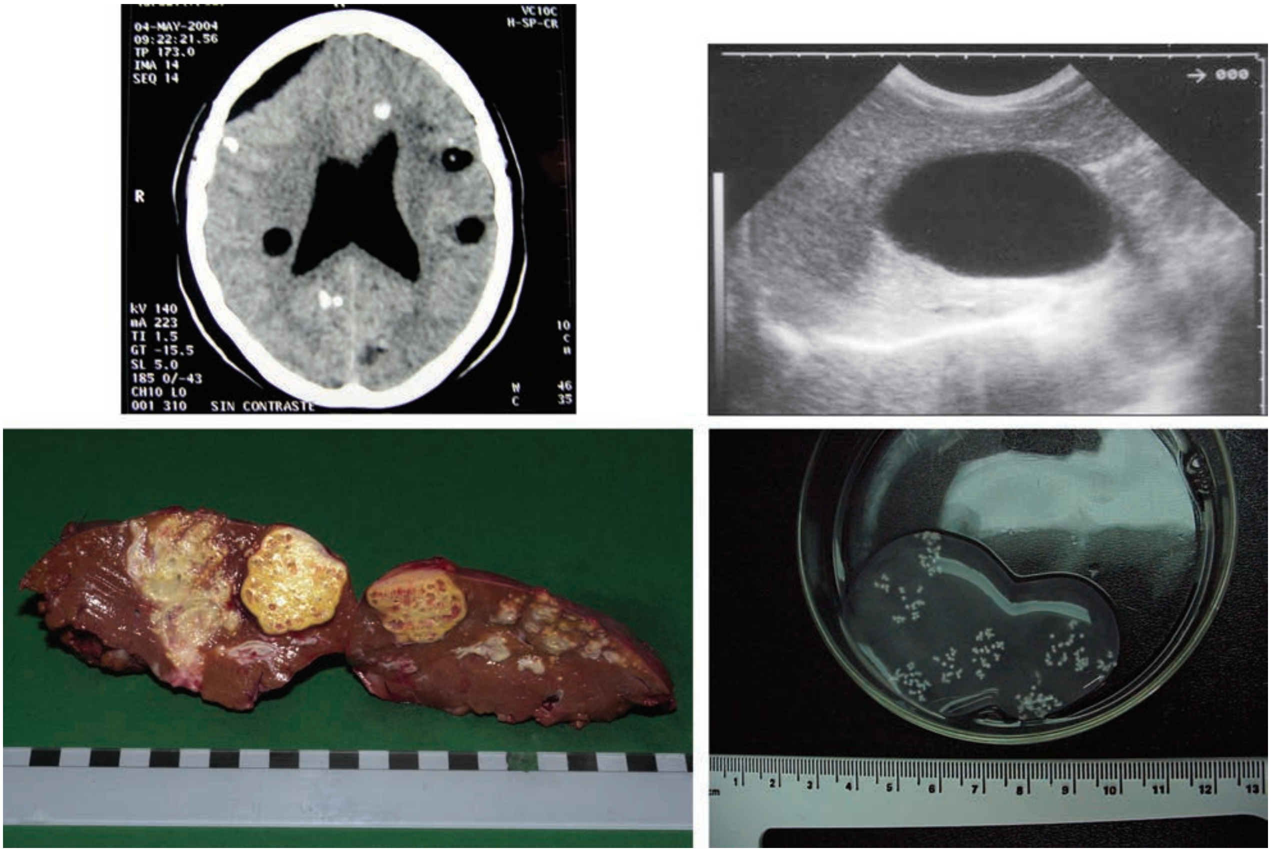

FIGURE 2 Larval cestodes infecting the human host. (Top left) *Taenia solium* cysticerci in the human brain (neurocysticercosis), shown in a noncontrasted CT scan of the brain; (top right) *Echinococcus granulosus* hydatid in the human liver (hydatid disease) as seen on liver ultrasound (kind contribution of Enrico Brunetti, Università di Pavia, Pavia, Italy); (bottom left) *Echinococcus multilocularis* alveolar hydatid disease in human liver (kind courtesy of K. Buttenschoen and P. Kern, University Hospital Ulm, Ulm, Germany); (bottom right) *Taenia multiceps* coenurus showing multiple scolices in the cystic wall. doi:10.1128/9781555817381.ch145.f2

the oncosphere by contamination with the feces of the definitive host. The manifestation of alveolar hydatid disease resembles a slowly developing "malignant tumor" of the liver, with subsequent invasion of the blood vessels and bile ducts and metastatic dissemination. The lesions vary in size and can produce minor foci up to large infiltrating structures in the host's tissue. Thus, alveolar hydatid disease differs greatly in the pathology and clinical course from cystic hydatid disease. This disease often affects persons aged over 50 years and is characterized by a chronic course lasting for months or years. Clinical manifestations relate to the extent of tumor-like lesions of the cyst. Besides physical examination, diagnosis usually is based on imaging techniques, including ultrasound, CT, and MRI, supported by serology. Treatment is mainly surgical. Chemotherapy with benzimidazole agents is restricted to residual, postsurgical, or inoperable lesions (32).

Sparganosis (*Spirometra mansonoides*)

Mainly found in Southeast Asian countries, the metacestode larvae of *Spirometra* species can invade the human tissues either by ingestion of contaminated crustaceans in drinking water or of infected meat (frog or snake) or by direct contact via a poultice. The most commonly affected sites are subcutaneous tissues and the eye. The diagnosis rests on the pathological demonstration of the larvae after excision by biopsy (33).

Coenurosis (*Taenia multiceps* or *Taenia serialis*)

Taenia multiceps and *Taenia serialis* have canids as definitive hosts and sheep as their normal intermediate host, harboring the larva, or coenurus. The coenurus is a vesicle containing a transparent fluid, with a fine membrane in which multiple (500 to 700) scolices can be seen. Infected sheep lose their balance and rotate in circles continuously, become dizzy, and fall (screw disease). It infrequently causes coenuriasis in humans. Human infections have largely been confined to the African continent, but a few cases have been described from France, England, and North and South America. The space-occupying coenurus usually invades the brain, producing lethal lesions. Diagnosis is based on pathological demonstration of the typical larval membrane and multiple scolices (34, 35).

Cysticercosis (*Taenia crassiceps*)

T. crassiceps is a common tapeworm of the red fox. Larval forms are generally found in subcutaneous tissues and body cavities of rodents. Human cases are quite rare, mostly in immunocompromised patients (subcutaneous, muscular, or ocular infections) (36, 37).

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Trematodes

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At least 70 species of digenean trematodes have been recorded as adult parasites from humans. All of these species are endoparasitic, occupying a variety of tissue sites (see Tables 1 to 3). Adult trematodes have distinctive morphology, often with a leaflike body plan. The most prominent morphological features in most species, however, are two rounded suckers. One of these, the oral sucker, surrounds the mouth, while the other, the ventral sucker, lies approximately one-third of the way along the body and serves as a primary attachment organ (1). Adult digeneans are rarely observed in clinical settings because they are endoparasitic, but they may be observed after anthelminthic purging or at autopsy. Hence, diagnosis of trematode diseases relies to a large extent on direct observations of excreted eggs. With few exceptions (2, 3), the eggs have distinct morphology and their presence is pathognomonic of specific infection. Despite the diverse range of body sites infected by adult trematodes, the eggs of most digenean flukes are voided with feces. Exceptions to this include *Schistosoma haematobium* and rarely other schistosomes, for which eggs are excreted with urine, and *Paragonimus* species, for which eggs are also observed in sputum.

Detailed descriptions of the digenean life cycles are shown in Fig. 1 and 2. There are many subtle variations in life cycle patterns, but two predominant strategies exist for these trematodes (Fig. 1 and 2). The first strategy, exemplified by the schistosomes, is one in which humans are infected by direct invasion of the skin by cercariae. The second strategy is seen among the foodborne digeneans (4), a diverse assemblage of species, which enter humans with ingested food (Fig. 2).

Two features of the digenean life cycle are noteworthy. First, digeneans often display high specificity in their choice of first intermediate host. So intimate are these host-parasite associations that the geographical distribution of a digenean is determined largely by that of its snail host. For this reason also, many digeneans show a focal distribution in countries where they are endemic. Secondly, most human parasites are zoonotic, requiring the cooccurrence of other mammalian or avian hosts in an area of endemicity to maintain human infection.

DIGENEANS OF THE CIRCULATORY SYSTEM

Schistosomes responsible for human disease are shown in Table 1, along with their distribution, snail hosts, and treatment. Currently, an estimated 779 million people are at risk and 207 million people in 76 countries and territories have schistosomiasis, with 85% of cases occurring in sub-Saharan Africa (5). Of these, some 120 million people are symptomatic and 20 million have severe illness.

Taxonomy

Schistosomes (family Schistosomatidae) are characterized by dioecious adults, cercariae with a forked tail, and a two-host life cycle (Fig. 1). Adults occupy an intravascular site in the human host, either in the mesenteric vessels or the vesical plexus. Five species commonly infect humans, of which three (*S. mansoni*, *S. japonicum*, and *S. haematobium*) are the most important (Table 1). Cross-hybridization between predominantly human- and animal-parasitic species is recognized as an increasing problem in Africa (6). Morphological descriptions of adult worms, which are not encountered in diagnostic samples, are summarized elsewhere (1).

Hepatosplenic Schistosomiasis

The most extensively studied schistosome, *S. mansoni*, occurs throughout sub-Saharan Africa, Egypt, the Middle East, Madagascar, eastern countries of South America and some islands of the Caribbean (Table 1). It is believed that the species was carried to South America with the slave trade. The distribution of *S. mansoni* overlaps that of *S. haematobium* in many parts of Africa.

Schistosoma japonicum is responsible for significant disease in foci in Asia (7) (Table 1) but has been eradicated from Japan. Sustained control efforts in China funded in part by the World Bank reduced the number of infected people from approximately 12 million to 1 to 2 million by the late 1980s and to less than 1 million in 1999 (8). These efforts, while successful in reducing morbidity to a low level, have reached a balance point and are not likely to reduce the geographic range of the parasite or eradicate the disease (8). Unlike most other human schistosomes, *S. japonicum* is a zoonosis, and in China and the Philippines, ruminants, particularly water buffalo and goats, are of primary concern in perpetuating the life cycle (9, 10).

Schistosoma mekongi has a highly focal distribution in the Mekong River Basin, with foci of endemicity in Laos and Cambodia (11). Although originally regarded as a subspecies of *S. japonicum*, the species was recognized as distinct based on morphological characteristics, pathologic effects, and life cycle patterns. Sustained large-scale chemotherapy in provinces of endemicity in Cambodia and Laos has led to

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TABLE 1 Geographical distribution, intermediate hosts, and egg morphology of the major schistosomes infecting humans

Species	Disease and geographic areas	Snail hosts	Drug regimen	Egg excretion site and egg size
Schistosoma mansoni	Intestinal schistosomiasis, infecting humans and sometimes other mammals: Angola, Benin, Botswana, Burkina Faso, Burundi, Cameroon, Central African Republic, Chad, Congo, Côte d'Ivoire, Democratic Republic of Congo, Equatorial Guinea, Eritrea, Ethiopia, Gabon, Gambia, Ghana, Guinea, Guinea-Bissau, Kenya, Liberia, Madagascar, Malawi, Mali, Mauritania, Mozambique, Namibia, Nigeria, Rwanda, Senegal, Sierra Leone, South Africa, Swaziland, Togo, Uganda, Tanzania, Zambia, Zimbabwe, Egypt, Libya, Oman, Saudi Arabia, Somalia, Sudan, Yemen, Antigua, Brazil, Dominican Republic, Guadeloupe, Martinique, Montserrat, Puerto Rico, St. Lucia, Suriname, Venezuela	*Biomphalaria* species. In Africa (Asia): many species in *B. alexandrina*, *B. choanomphala*, *B. pfeifferi*, *B. sudanica* species groups. In Americas: *B. glabrata* and 2 other species	Praziquantel, 20 mg/kg, 2 or 3 doses. Community programs usually give 40 mg/kg in single dose.	Feces (rarely urinary); 140 by 61 µm
Schistosoma japonicum	Intestinal schistosomiasis, infecting humans and bovines: China, Indonesia, Philippines	*Oncomelania hupensis*, *O. nosophora*, *O. formasona*, *O. hupensis* subsp. *quadrasi*, and *O. lindoensis*	As above, but 60 mg/kg in single dose in community programs	Feces; 85 by 60 µm
Schistosoma haematobium	Genitourinary schistosomiasis: Algeria, Angola, Benin, Botswana, Burkina Faso, Burundi, Cameroon, Central African Republic, Chad, Congo, Côte d'Ivoire, Democratic Republic of Congo, Egypt, Ethiopia, Gabon, Gambia, Ghana, Guinea, Guinea-Bissau, Iran, Iraq, Jordan, Kenya, Lebanon, Liberia, Libya, Madagascar, Malawi, Mali, Mauritania, Mauritius, Morocco, Mozambique, Namibia, Niger, Nigeria, Oman, Saudi Arabia, Senegal, Sierra Leone, Somalia, South Africa, Sudan, Swaziland, Syria, Togo, Tunisia, Uganda, United Republic of Tanzania, Yemen, Zambia, Zimbabwe	*Bulinus* species. Many species in the *Bulinus* (*Physopsis*) and *Bulinus* (*Bulinus*) subgenera of *Bulinus*	As for *S. mansoni*	Urine (rarely feces); 150 by 62 µm
Schistosoma mekongi	Intestinal schistosomiasis: Cambodia, Laos, Thailand	*Neotricula aperta*	As for *S. japonicum*	Feces; 57 by 66 µm
Schistosoma intercalatum	Rectal schistosomiasis: Cameroon, Central African Republic, Chad, Congo, Democratic Republic of Congo, Equatorial Guinea, Gabon, Mali, Nigeria, São Tomé and Principe	*Bulinus* (*Physopsis*) *africanus*, *Bulinus* (*Bulinus*) *camerunensis*		Feces; 176 by 66 µm
S. bovis, *S. curassoni*, *S. guineensis*, *S. mattheei*, *S. margrebowiei*	African schistosomes infecting bovines and wildlife; rare human infections	*Bulinus* spp.		
S. sinesium, *S. malayensis*	Asian schistosomes infecting animals; rare human infections			
Species of *Austrobilharzia*, *Bilharziella*, *Gigantobilharzia*, *Macrobilharzia*, *Ornithobilharzia*, *Trichobilharzia*	Swimmer's itch, cercarial dermatitis, worldwide distribution	*Bulinus*, *Lymnaea*, *Nassarius*, *Physa*, *Planorbis*, *Stagnicola*		Eggs not observed in humans

TABLE 2 Geographical distribution, hosts, and life cycles of lung and hepatic digeneans of humans

Family	Species	Location in human host	Disease and geographic area	Snail hosts	Source of metacercariae	Drug regimen[a]	Egg excretion site; egg size(s) and morphology
Fasciolidae	*Fasciola hepatica* and *F. gigantica*	Bile ducts	Fasciolosis of livestock: worldwide Fasciolosis of humans: Australia, Bolivia, China, Cuba, Egypt, Ecuador, France, Iran, Peru, Portugal, Turkey, Vietnam	*Galba/Fossaria* group (*F. hepatica*); *Radix* spp. (*F. gigantica*)	Vegetation (wet grass, watercress, water mint, semiaquatic vegetables); contaminated water	T, 10 mg/kg (in severe cases for 2 days)	Feces; 130–150 by 60–85 μm for *F. hepatica*; 160–190 by 70–90 μm for *F. gigantica*
Opisthorchidae	*Clonorchis sinensis*, *Opisthorchis viverrini*, and *O. felineus*	Bile ducts	Opisthorchiasis and/or clonorchiasis: China, former Soviet Union, Cambodia, Korea, Laos, Taiwan, Thailand, Vietnam	*C. sinensis*: various freshwater snails (*Alocinma*, *Bulinus*, *Parafossarulus*); *O. viverrini and O. felineus*: freshwater snails from genus *Bithynia* and related genera	Many species (>100) of freshwater fish	P, 25 mg/kg three times daily for 1–2 days	Feces; *C. sinensis*, 23–35 by 10–20 μm, *O. viverrini and O. felineus*, 30 by 12 μm
Opisthorchidae	*O. guayaquilensis*, *Metorchis albidus*, *M. conjunctus*, *Pseudamphistomum aethiopicum*, *P. truncatum*	Bile ducts	Opisthorchiasis: Asia	Not listed	Fish		
Troglotrematidae	*P. africanus*, *P. caliensis*, *P. heterotremus*, *P. hueitungensis*, *P. kellicotti*, *P. mexicanus*, *P. miyazakii*, *P. skrjabini*, *P. uterobilateralis*, *P. westermani*	Pulmonary cysts, also abdominal cavity, brain	Paragonimiasis: Cameroon, China, Colombia, Costa Rica, Côte d'Ivoire, Ecuador, Equatorial Guinea, Gabon, Guatemala, Honduras, India, Indonesia, Japan, Laos, Liberia, Malaysia, Mexico, Nepal, Nicaragua, Nigeria, North Korea, Panama, Papua New Guinea, Peru, Philippines, Poland(?), southeast Siberia, Samoa, South Korea, Sri Lanka, Taiwan, Thailand, Venezuela, Vietnam, North America	Operculate snail: *Semisulcospira*, *Thiara*, *Oncomelania*	Freshwater crabs, crayfish	T, 20 mg/kg; P, 25 mg/kg three times daily for 2 to 3 days	Feces, sputum; size varies with species
Dicrocoeliidae	*Dicrocoelium* species	Bile ducts	Dicrocoeliasis: human cases in Czech Republic, Kenya, Nigeria, Russia, Saudi Arabia, Somalia, Spain, USA	Land snails: order Stylommatophora	Insects (ants)	P?	Feces, bile; 38–45 by 22–30 μm; brown, thick walled, embryonated, operculate

[a] Abbreviations: T, triclabendazole; P, praziquantel.

TABLE 3 Digeneans of the human intestine[a]

Family	Genera	Disease and geographic area	Snail hosts	Source of metacercariae	Human infection	Drug	Egg excretion site and egg size
Echinostomatidae	*Acanthoparyphium*, *Artyfechinostomum*, *Echinochasmus*, *Echinoparyphium*, *Echinostoma*, *Episthmium*, *Euparpyhium*, *Himasthla*, *Hypoderaeum*, *Isthmiophora*, *Paryphostomum*	Echinostomiasis: China, Egypt, Hungary, India, Indonesia, Italy, Japan, Korea, Malaysia, North and South America, Philippines, Romania, Russia (including Siberia), Singapore, Taiwan, Thailand	Families Viviparidae, Planorbidae	Fish (loach), molluscs (snails, clams), amphibians (tadpoles, frogs)	Focal; prevalence, 5–44%	P, 25 mg/kg	Feces; 100 by 65–70 μm
Fasciolidae	*Fasciolopsis*	Fasciolopsiasis: Bangladesh, Cambodia, China, India, Indonesia, Korea, Laos, Pakistan, Taiwan, Thailand, Vietnam	Family Planorbidae	Water plants (water chestnut, caltrop, lotus roots, bamboo), other aquatic vegetation	Widespread but focal; prevalence rates up to 60% in children	P, 3 doses of 25 mg/kg	Feces; 130–140 by 80–85 μm
Gastrodiscidae	*Gastrodiscoides*, *Gastrodiscus*	Gastrodiscoidiasis: China, India, Myanmar, Philippines, Russia, Thailand, Vietnam	*Helicorbus*	Squid, plants, crustaceans (crayfish), amphibians (frogs, tadpoles)	Rare, focal	NR	Feces; 127–169 by 62–75 μm
Heterophyidae	*Appophalus*, *Centrocestus*, *Cryptocotyle*, *Diorchitrema*, *Haplorchis*, *Heterophyes*, *Heterophyopsis*, *Metagonimus*, *Phagicola*, *Procercovum*, *Pygidiopis*, *Stellantchasmus*, *Stichodora*	Metagonimiasis or heterophyiasis: Balkans, Brazil, China, Egypt, Greenland, Indonesia, Israel, Japan, Korea, Philippines, Russia, Spain, Sudan, Taiwan, Tunisia, Turkey, USA	Families Thiaridae, Littoriniidae	Fish (freshwater or brackish, carp, mullet, cyprinoids), crustaceans (shrimp)	Low prevalence, but common; cases with heavy infections of clinical significance	P, 10–20 mg/kg	Feces
Paramphistomidae	*Gastrodiscoides*, *Watsonia*	Malaysia, India (Assam, Bihar and Orissa), Pakistan, Philippines, Myanmar, Thailand, Vietnam, Guyana, Commonwealth of Independent States	*Helicorbis*	Aquatic vegetation	Usually asymptomatic	P, 3 doses of 25 mg/kg	Feces; 130–160 by 70 μm
Troglotrematidae	*Nanophyetus salmincola*	Nanophyetiasis: Russia, USA	*Oxytrema silicula*	Fish (salmon, trout)	Rare	P	64–97 by 34–55 μm

[a] Modified from reference 61 with permission. Abbreviations: NR, not recorded; P, praziquantel.

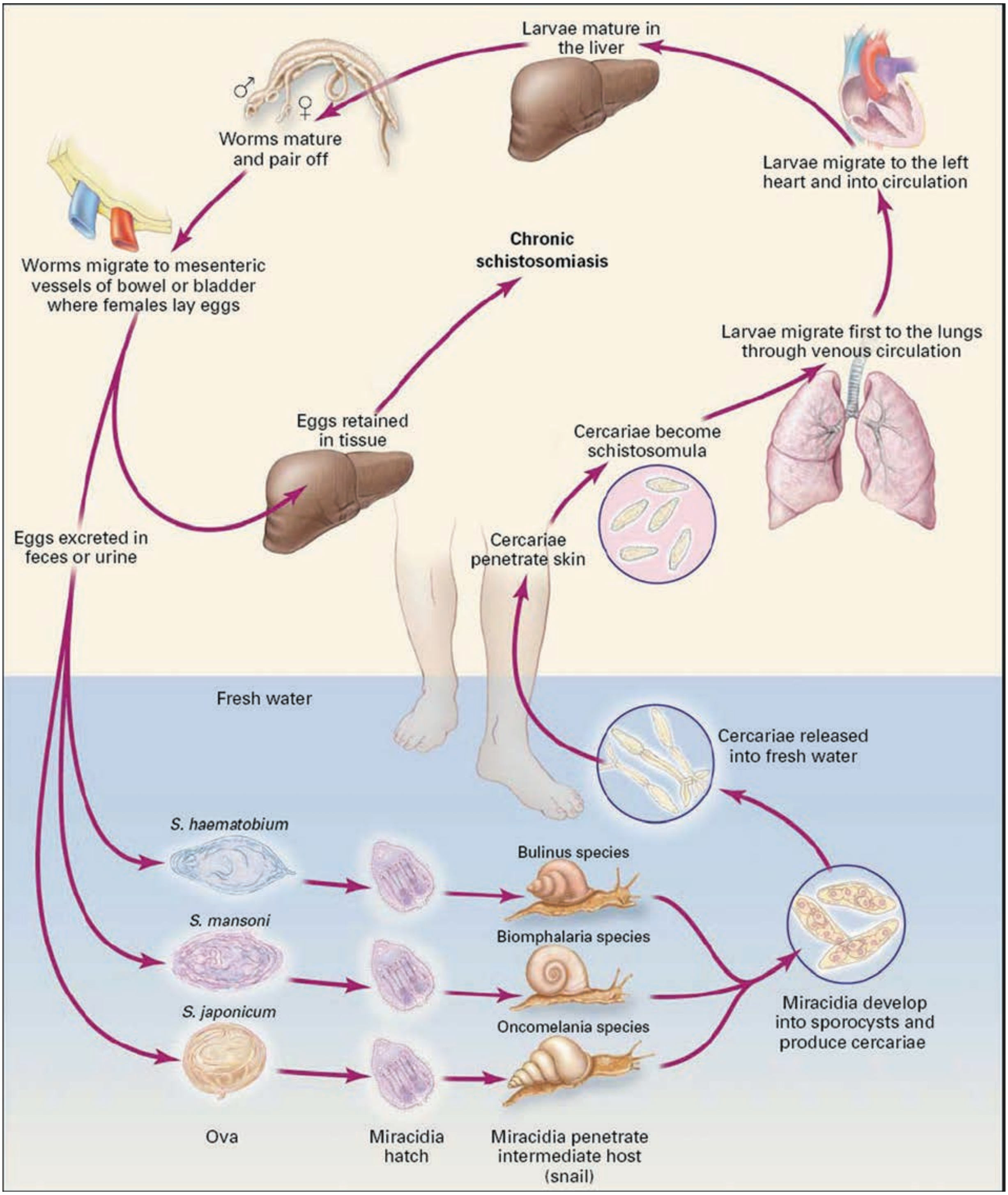
Larvae mature in the liver
♂
♀
Worms mature and pair off
Worms migrate to mesenteric vessels of bowel or bladder where females lay eggs
Larvae migrate to the left heart and into circulation
Chronic schistosomiasis
Larvae migrate first to the lungs through venous circulation
Eggs retained in tissue
Cercariae become schistosomula
Cercariae penetrate skin
Eggs excreted in feces or urine
Fresh water
Cercariae released into fresh water
S. haematobium
Bulinus species
S. mansoni
Biomphalaria species
S. japonicum
Oncomelania species
Miracidia develop into sporocysts and produce cercariae
Ova
Miracidia hatch
Miracidia penetrate intermediate host (snail)

FIGURE 1 Five species of *Schistosoma* are known to infect humans. Infection with *Schistosoma mansoni*, *S. japonicum*, *S. mekongi*, or *S. intercalatum* adults occur in mesenteric veins; *S. haematobium* adults occur in the vesicle plexus. Humans are infected after cercarial penetration of the skin. After penetration, the cercariae shed their bifurcated tails, and the resulting schistosomula enter capillaries and lymphatic vessels en route to the lungs. After several days, the worms migrate to the portal venous system, where they mature and unite. Pairs of worms then migrate to the site of patent infection. Egg production commences 4 to 6 weeks after infection. Eggs pass from the lumen of blood vessels into adjacent tissues, and many then pass through the intestinal or bladder mucosa and are shed in the feces or urine (see the text). In freshwater, the eggs hatch, releasing miracidia that, in turn, infect specific freshwater snails (Table 1). Reprinted from the *New England Journal of Medicine* (33) with permission. doi:10.1128/9781555817381.ch146.f1

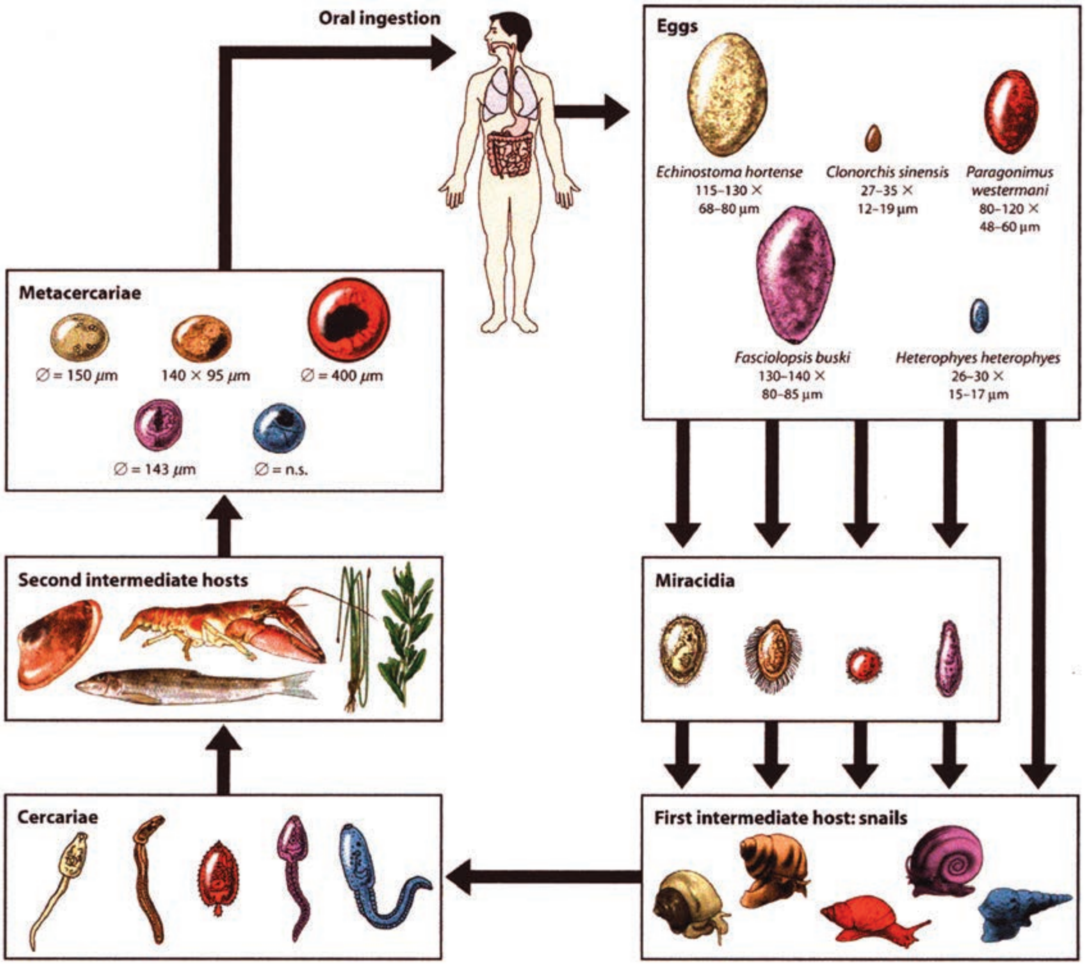
Oral ingestion
Eggs
Echinostoma hortense
115–130 ×
68–80 µm
Clonorchis sinensis
27–35 ×
12–19 µm
Paragonimus westermani
80–120 ×
48–60 µm
Fasciolopsis buski
130–140 ×
80–85 µm
Heterophyes heterophyes
26–30 ×
15–17 µm
Metacercariae
∅ = 150 µm
140 × 95 µm
∅ = 400 µm
∅ = 143 µm
∅ = n.s.
Second intermediate hosts
Miracidia
Cercariae
First intermediate host: snails

FIGURE 2 Life cycles of five different foodborne trematodes including intestinal flukes (*Echinostoma hortense*, *Fasciolopsis buski*, and *Heterophyes heterophyes*), a liver fluke (*Clonorchis sinensis*), and a lung fluke (*Paragonimus westermani*). Reprinted from *Clinical Microbiology Reviews* (38) with permission. doi:10.1128/9781555817381.ch146.f2

control of the disease, although the disease still remains at high prevalence in some pockets and new infections are occurring (12).

Schistosoma intercalatum is responsible for rectal schistosomiasis in regions of Africa. The species occurs as two geographically isolated strains (now considered distinct species [13]), the Cameroon and the Democratic Republic of the Congo strains, which display highly focal distributions (7). This parasite belongs to the *S. haematobium* group of species, characterized by, among other features, eggs with a terminal spine (Fig. 3).

Genitourinary Schistosomiasis

Schistosoma haematobium, the sole agent of urinary schistosomiasis, occurs in much of the African continent as well as Madagascar and the Middle East (1, 7) (Table 1). Adult worms live in the vesical plexus. Eggs escape the host across the bladder wall to be excreted with urine.

Epidemiology and Transmission

All schistosomes of humans use freshwater aquatic snails as the intermediate host. Humans are infected through exposure to freshwater contaminated with infective larvae, the cercariae (Fig. 1). Cercariae enter the human body by percutaneous penetration.

Within regions of endemicity, factors contributing to schistosome transmission include the distribution biology and population dynamics of the snail hosts, the extent of contamination of freshwater with feces or urine, and the degree of exposure of humans to contaminated water. The recent development of large-scale dams in some areas of endemicity has led to changes in transmission dynamics (5). The impact of the Three Gorges Dam in China on the transmission of *S. japonicum* remains uncertain (14).

Clinical Significance

Cercarial dermatitis occurs in schistosomiasis but is more commonly reported after infection with avian schistosomes (Table 1) and *Schistosoma spindale*. Acute toxemic schistosomiasis (Katayama fever) can occur with any schistosome species but is more apparent in nonimmune individuals and may be characterized by fever, headache, generalized myalgias, right-upper-quadrant pain, and bloody diarrhea (15, 16).

Eggs (Fig. 3) are laid by female worms in the vasculature and must traverse endothelia and gut or bladder mucosa to escape the host. In those schistosomes infecting mesenteric veins, eggs pass through the wall of the intestine or rectum,

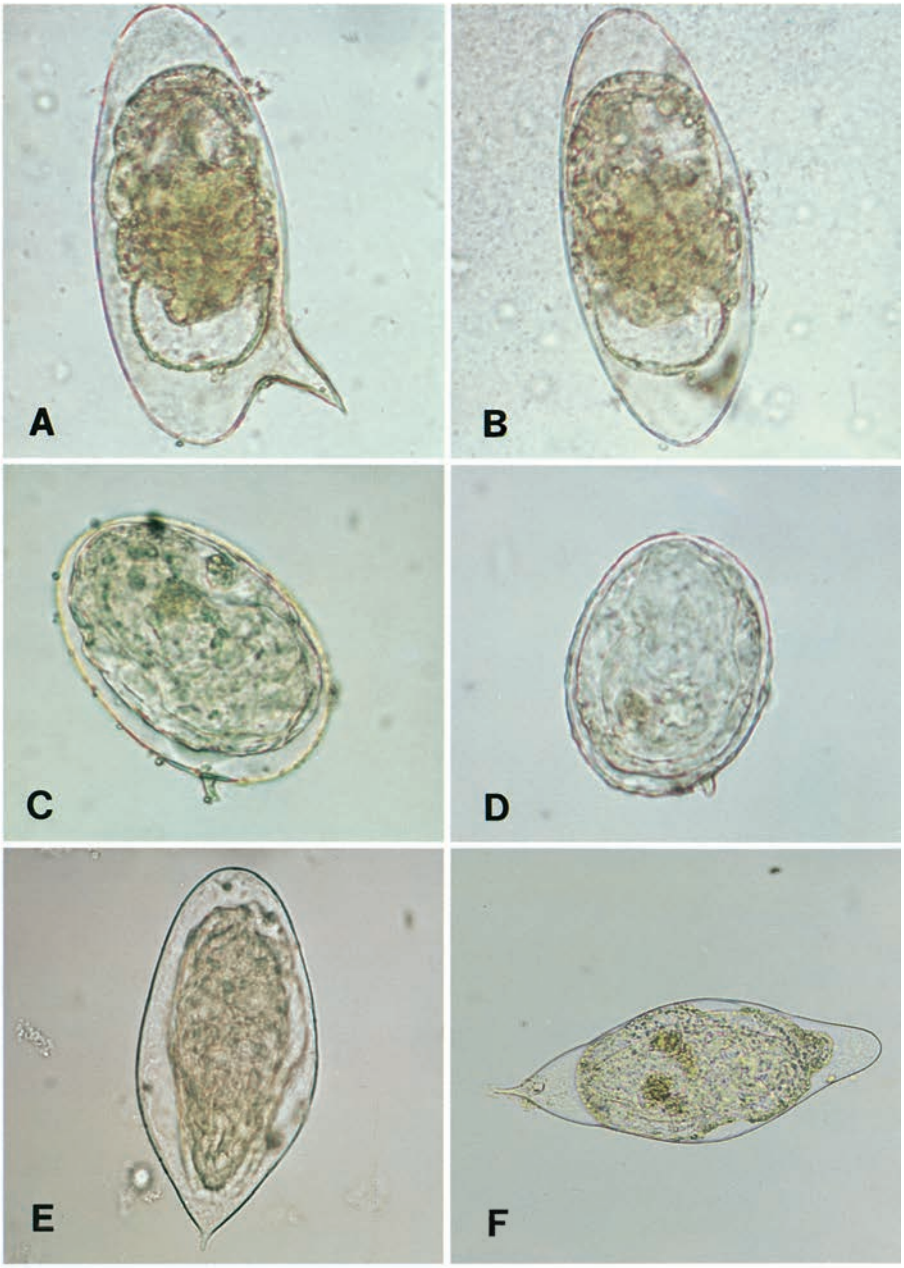

FIGURE 3 Eggs of schistosome species (magnification, ×600). (A) *S. mansoni*; (B) *S. mansoni* egg with typical lateral spine not in view; (C) *S. japonicum*; (D) *S. mekongi*; (E) *S. haematobium*; (F) *S. intercalatum*. (Panels C through E are from reference 19; used with permission.) doi:10.1128/9781555817381.ch146.f3

while for *S. haematobium*, the eggs escape across the bladder wall. For all species, however, many eggs become entrapped in tissues. The chronic effects of schistosomiasis, therefore, relate to the site of adult infections and granulomatous and fibrotic responses to entrapped parasite eggs (16). Granulomas occur in many tissues in response to entrapped eggs; however, most accumulate in tissues fed by vasculature leading from the site of adult infection. Eggs retained in the gut wall induce inflammation, hyperplasia, and ulceration, and occult blood occurs in the feces. A suggested relationship between colorectal cancer and schistosomiasis remains controversial (17). Eggs entrapped in the liver lead to portal hypertension and splenic and hepatic enlargement, potentially giving rise to the formation of fragile esophageal varices. Ascites also is common.

In urinary schistosomiasis, granulomatous inflammatory response to embolized eggs gives rise to dysuria, hematuria, and proteinuria, calcifications in the bladder, obstruction of the ureter, renal colic, hydronephrosis, and renal failure. Secondary bacterial infection of the bladder and other affected tissues may occur. There is consistent association between *S. haematobium* infection and squamous-cell carcinoma of the bladder (6). *S. haematobium* infection causes genital disease in approximately one-third of infected women, leading to vulval and perineal disease and tubal infertility, and may facilitate the transmission of HIV (18).

Collection, Transport, and Storage

Adult schistosomes are rarely encountered in clinical settings. Characteristic eggs of schistosomes (Fig. 3) voided with feces or urine are pathognomonic of infection. Detailed instructions on collection, transport, and storage of schistosome eggs in human fecal material are provided in chapter 133. The eggs of schistosomes contain fully differentiated larvae in feces or urine and hatch spontaneously upon exposure to fresh water. Although observations of viable schistosome miracidia may be advantageous for species identification, spontaneous hatching may hinder direct observations of eggs.

Detection

The presence of schistosome eggs (Fig. 3) (19) in feces or urine is diagnostic of schistosomiasis. Eggs of hepatosplenic schistosomes may be observed by light microscopy in stool specimens with or without suspension in saline. Formalin-based techniques for sedimentation and concentration are particularly useful, especially for patients releasing few eggs. Hatching tests, in which fecal matter is suspended in nonchlorinated water in darkened vessels with directed surface light, have been used to detect motile miracidia. In patients with chronic disease and with typical clinical presentation but negative urine and stool specimens, a biopsy of bladder or rectal mucosa may be helpful in diagnosis.

The Kato-Katz method of fecal smear is used in field studies for diagnosis and quantification of fecal egg burdens. Kato-Katz tests give a theoretical sensitivity cutoff of 20 eggs per g of feces (20), but the large daily variation in egg shedding and the uneven distribution of eggs in feces may lead to inconsistent counts. It has been recommended for *S. mansoni* infections that either five replicate slides, or sets of triplicate slides made from stools collected on two successive days, be used for assessment of infection status (21). A novel method, incorporating fixation in formalin-ethyl acetate coupled with sedimentation and digestion with potassium hydroxide, shows promise for quantitative assessment of eggs in bovine feces for epidemiological surveillance of *S. japonicum* (22).

Sedimentation or concentration methods are most useful for diagnosis of *S. haematobium* from urine samples. In addition, the use of tests for blood, protein, and eosinophils in urine, while not specific, may be indicative of infection (23). Portable ultrasound is useful for assessment of pathologic damage to tissues. The use of questionnaires in populations in which the infection is endemic is helpful in revealing infections for *S. japonicum* and *S. haematobium* but for *S. mansoni* may lead to underestimation of prevalence (24). An innovative test using paramagnetic beads to bind schistosome ova has been explored (25).

PCR tests for schistosomes in stools use high-repeat nuclear genomic (26) and mitochondrial sequences (27). Both tests report high sensitivity, at times exceeding that of direct coprological examination. The mitochondrial sequence probes, which amplify sequences spanning the *cox2/nad6* genes for *S. japonicum* and *S. mansoni*, *nad1* and *nad2* for *S. japonicum*, and *nad5* for *S. mansoni*, show high specificity and sensitivity (27). An interesting development is that of the use of real-time PCR to detect cell-free schistosome DNA in host plasma (28). This method utilizes a 121-bp tandem repeat sequence that represents approximately 12% of the *S. mansoni* genome as a target sequence for amplification.

An attractive method for diagnosis is a direct detection method for the schistosome antigen circulating cathodic antigen (CCA). An immunochromatographic dipstick (Rapid Medical Diagnostics, South Africa) that detects CCA in schistosomiasis mansoni is becoming increasingly valued as a diagnostic, especially in low-transmission settings, where fecal egg analyses show poor sensitivity (29). Commercial urine dipstick tests for microhematuria can serve as a rapid diagnostic proxy for *S. haematobium* infection (30).

Serology

A range of direct and indirect tests have been explored experimentally, including enzyme-linked immunosorbent assays (ELISAs) and immunoblot tests using soluble egg antigen, recombinant antigens, and detection of parasite antigen in excreta (31, 32). Host antibodies against schistosomes can persist for prolonged periods after parasitologic cure, and this, together with potential antigen cross-reactivity, can limit the value of serologic tests (31). Serology can be most valuable for diagnosis of schistosomiasis in travelers from regions of nonendemicity who visit areas that are endemic for the disease. Other indirect tests include assays for peripheral-blood eosinophilia, anemia, hypoalbuminemia, elevated urea and creatinine levels, and hypergammaglobulinemia (23, 33).

Typing

Although laboratory strains and regional variations exist among schistosomes, typing of species is of limited significance. *S. japonicum* occurs as a range of strains throughout different regions in Asia.

Anthelminthic Susceptibility and Treatment

Praziquantel is the drug of choice for treatment of schistosomiasis. The drug has been used in mass treatment campaigns in many countries, a development facilitated in part by reductions in costs associated with manufacture of the drug. Despite this, the anthelminthic has some limitations, as it is effective against only adult forms (34) and does not protect from subsequent infection.

Treatment failures for *S. mansoni* and *S. haematobium* infections with praziquantel have been observed, and the presence of resistant strains has been demonstrated experimentally (see chapter 133). Widespread resistance to praziquantel has not been observed clinically, but the application of the drug in mass treatment campaigns may result in new resistant forms emerging.

Derivatives of artemisinin, an antimalarial, have been subjected to trials against schistosomes in experimental models and clinical field settings (35). In contrast to praziquantel, artemether acts against juvenile schistosomes in the host and may be used as a chemoprophylactic agent (36). Combinations of drugs have been subjected to trials on a number of occasions. The combination artemisinin-mefloquine was effective against *S. haematobium* and *S. mansoni* infections (37).

A number of vaccines have entered clinical trials. These include Bihlvax, a vaccine targeting the glutathione *S*-transferase of *S. haematobium* and a tetraspanin (*Sm*-TSP-2) and a fatty acid binding protein (Sm14) of *S. mansoni*. There is no clear target available yet for any form of African schistosomiasis.

Schistosome Dermatitis (Swimmer's Itch)

This ailment arises from infection with a number of avian and mammalian schistosomes (Table 1). The avian schistosome cercariae cannot migrate from the dermal layers of human skin and induce local dermatitis accompanied by pruritus and papule formation.

FOODBORNE DIGENEANS

All other digeneans considered here represent a diverse assemblage of taxa, which infect humans through ingestion of uncooked or undercooked food (Fig. 2; see Tables 2 and 3). The vast majority of these species are zoonotic. The primary epidemiological features governing human infection include the distribution of suitable snail intermediate hosts, human food consumption behaviors, the presence of suitable zoonotic hosts, and the potential for water contamination with human or animal excreta (38).

Trematodes of the Respiratory System: *Paragonimus*

Taxonomy

The genus *Paragonimus*, family Troglotrematidae, contains approximately 50 species of leaf-shaped digeneans occurring in a range of mammals throughout the world. Species are distinguished on the basis of the surface spination and shape of the ovary. Human paragonimiasis is caused predominantly by 10 species distributed throughout Asia, Africa, and the Americas (Table 2) and include *Paragonimus africanus* (West Africa), *P. caliensis* (Central and South America), *P. heterotremus* (Southeast Asia, China), *P. hueitungensis* (China), *P. kellicotti* (North America), *P. mexicanus* (Central and South America), *P. miyazakii* (Japan), *P. skrjabini* (Southeast Asia, China), *P. uterobilateralis* (West Africa), and *P. westermani* (Asia) (39). The most common species infecting humans is *Paragonimus westermani*. Adult *Paragonimus* species inhabit the lungs, where they induce the formation of encapsulating cysts. The flukes are large and fleshy, measuring 8 to 16 by 4 to 8 mm. *Paragonimus* species have large, thick-shelled, and operculate eggs that are often not fully embryonated when observed in sputum or feces.

Epidemiology and Transmission

Some 23.2 million people have been estimated to have paragonimiasis (40). The global burden due to paragonimiasis is 197,000 disability-adjusted life years (DALYs) (40). The life cycle of *Paragonimus* is described in Fig. 2 and Table 2. Of the approximately 50 species, 9 have been recorded as human parasites. Eggs are passed in the lungs and are transferred up the bronchial tree with sputum. Eggs may be spat with sputum or swallowed and passed with feces. The first intermediate hosts are freshwater snails. Human infection arises after ingestion of uncooked or marinated freshwater crabs or crayfish (41), but unwashed hands of food preparers and contaminated utensils also may be a source of human infection. Wild boars can act as paratenic hosts (42). After ingestion, the immature flukes penetrate the intestinal wall and migrate to the lungs through the body cavity. All species are zoonotic, infecting carnivores, pigs, and rodents.

In the United States, autochthonous infection with *P. kellicotti* is becoming increasingly more common and native paragonimiasis is considered an emerging threat (39). Transmission is centered around the Mississippi Basin, where people are infected after eating undercooked crustaceans, especially crayfish.

Clinical Significance

Signs of infection include fever with dry cough, sometimes blood-stained sputum containing eggs, chest pain, dyspnea, and bronchitis, and symptoms sometimes resemble those of pulmonary tuberculosis. Peripheral blood eosinophilia is common. The flukes induce the formation of an epithelial cyst, measuring approximately 1 cm in diameter and which may calcify over time. Parasites may also occur in extrapulmonary locations, and serious complications occur when parasites are found in the brain. The necrosis of brain tissues and extensive granulomatous inflammatory response may induce symptoms similar to cerebral cysticercosis, caused by the cestode *Taenia solium* (23).

Collection, Transport, and Storage

Paragonimus eggs may be observed in sputum, feces, bronchoalveolar lavage (BAL) fluid, lung or pleural biopsy specimens, or surgical pleurectomy or lobectomy specimens (39) (see chapter 133). Serology is useful, as direct observations of eggs may lack sensitivity.

Detection

Diagnosis of paragonimiasis is largely dependent on observation of the eggs (19, 41) (Fig. 4) in sputum, feces, or pleural effusions. A cough with brown sputum is indicative of lung infection, and the sputum should be examined for eggs. The cysts formed around adult worms appear in X rays as characteristic rings or nodules, but direct observation of eggs is required to differentiate the disease from pulmonary tuberculosis. PCR-based methods have been developed in recent years, and though these are valuable research tools, their utility as routine diagnostic tests remains to be demonstrated (41).

Typing

Paragonimus species exist as a range of species complexes in regions of endemicity (42). While the species distinctions within a species complex has epidemiological significance, typing of species in a complex is of limited value for purposes of diagnosis or treatment and case histories are possibly most informative.

Serology

Serological tests include ELISAs using either 32- and 35-kDa antigens or parasite yolk ferritin as an antigen (23). Pleurisy with eosinophilia and dominant IgM antibody titer may be indicative of paragonimiasis (43). As *P. kellicotti* is an emerging zoonosis in North America, there is interest in improved serology testing for that species. Recently a Western blot serology test has been developed, based on whole adult worm extract of *P. kellicotti* as the antigen (44). Serology tests for paragonimiasis exist in the United States commercially and through the CDC.

Anthelminthic Susceptibility and Treatment

The current WHO recommendation for treatment is administration of praziquantel, administered at 25 mg/kg of body weight for 2 to 3 consecutive days. This treatment regime remains very effective, with the majority of patients in clinical trials being cleared of infection. Praziquantel confers no protection against subsequent infection. Triclabendazole at 10 mg/kg or 20 mg/kg in two divided doses holds promise as a therapeutic alternative (4). The only means for prevention of infection is to cook crustacean food to at least 145°F (~63°C).

Trematodes of the Liver

At least 13 species of flukes, belonging to the families Fasciolidae, Opisthorchidae, and Dicrocoeliidae (45) (Table 2), have been recorded as adult worms from the liver and bile ducts of humans. Brief information on human dicrocoeliasis is provided in Table 2, but the reader is referred to other reviews (1, 46) for more information on the lancet flukes.

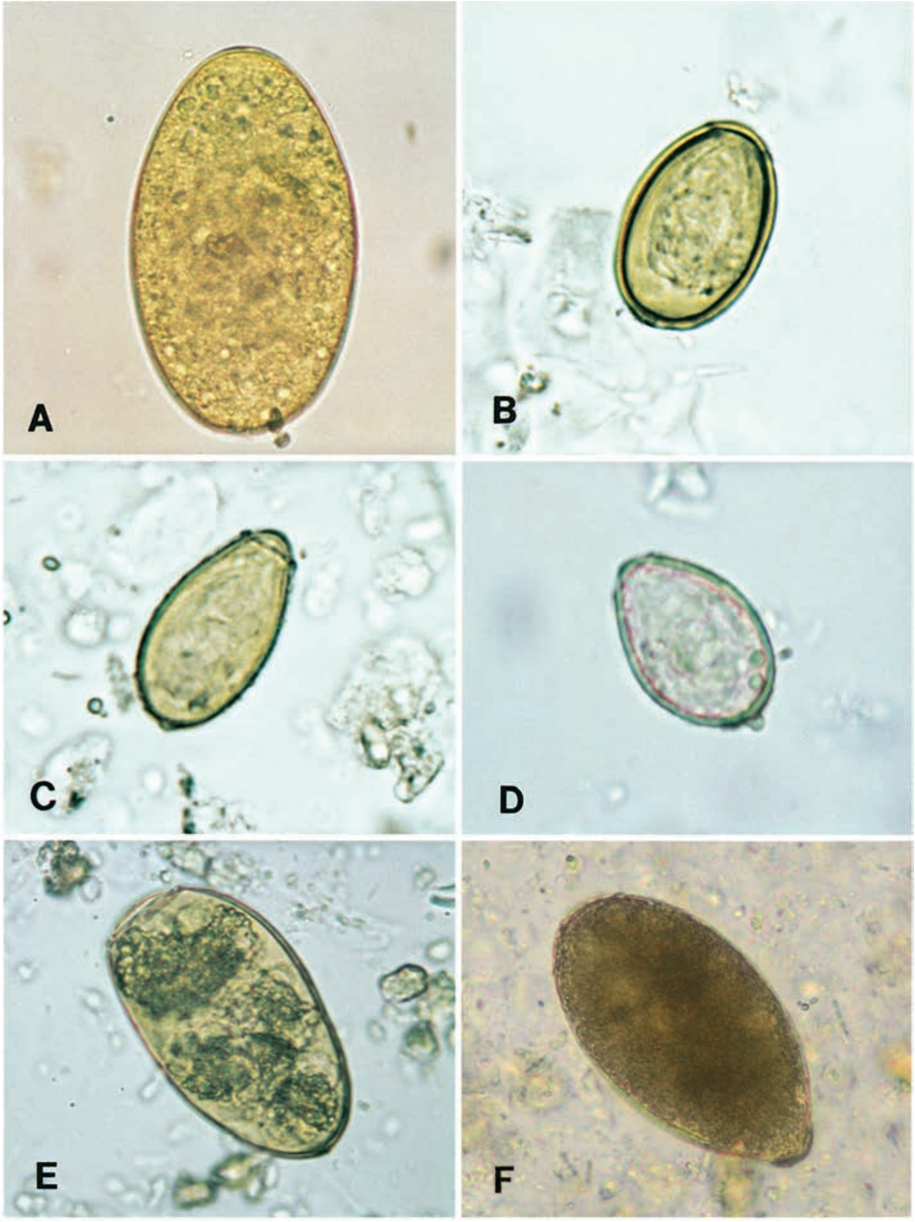

FIGURE 4 Eggs of trematode parasites. (A) *Fasciolopsis buski* (magnification, ×500); (B) *Heterophyes heterophyes* (magnification, ×1,500); (C) *Clonorchis sinensis* (magnification, ×1,500); (D) *Opisthorchis viverrini* (magnification, ×1,500); (E) *Paragonimus westermani* (magnification, ×600); (F) *Nanophyetus salmincola* (magnification, ×750). (Panels B through D are from reference 19; used with permission.) doi:10.1128/9781555817381.ch146.f4

Taxonomy

Family Opisthorchidae

Of the opisthorchid parasites infecting humans (Table 2), only three species are of major significance, namely, *Opisthorchis viverrini*, *Opisthorchis felineus*, and *Clonorchis sinensis*. Adult opisthorchids of humans are macroscopic, flattened lanceolate flukes, approximately 5 to 10 mm in length.

Family Fasciolidae

Fascioliasis, caused by flukes of the genus *Fasciola*, is primarily a disease of herbivorous mammals. Two species are responsible for hepatic disease in humans, *Fasciola hepatica* and *F. gigantica*. Both species are macroscopic flukes over 2 cm in length, with *F. gigantica* being sometimes as long as 7 cm. The diseases caused by the two flukes are similar. The species differ in geographic distribution, with *F. gigantica* occurring in Africa and Asia and *F. hepatica* occurring in these continents as well as Europe, the Americas, and Oceania.

Epidemiology and Transmission

The life cycle information for opisthorchids and fasciolids is presented in Fig 2 and Table 2. In opisthorchids, the metacercariae are found encysted in the muscles of cyprinid fish (45), and fish of other taxa are not susceptible to infection. Thus, effective transmission to humans requires the colocation of suitable snail and fish hosts. Individuals are infected by eating raw, seasoned, or undercooked fish (3).

Until recently, fascioliasis was considered a sporadic infection of humans, but it is now estimated that some 2.4 million people in 70 countries are infected, with 180 million at risk throughout the world (47). It has been calculated that fascioliasis causes 35,206 DALYs (40). The two causative species, *F. hepatica* and *F. gigantica*, have a worldwide distribution in domesticated animals; human disease is focal, and regions endemic for human fascioliasis are recognized (1, 23, 47).

Fasciolid metacercariae encyst on semiaquatic or moistened vegetation, predominantly watercress, grass, water mint, or salad vegetables (Fig. 2; Table 2). The frequency of the parasites in domesticated animals does not necessarily correlate with human disease. Areas of low endemicity in humans include regions of France (<3.1 cases per 100,000 people in Basse-Normandie) and Chile; regions of high endemicity are in Peru (15.64 to 34.2% in regions of endemicity) and Bolivia (66.7% in the Bolivian altiplano) (47). Most of the areas with a high endemicity are regions where *F. hepatica* is present. While common zoonotic hosts are cattle and sheep, other hosts, such as pigs, equines, and rats, may serve as reservoir hosts for human infection. Children and young adults are more commonly infected than adults, suggestive of the presence of age-dependent immunity (48).

Clinical Significance

Disease severity varies among the different species (3) and with intensity of infection. *Clonorchis sinensis* infects approximately 35 million people in China, Hong Kong, India, North Korea, Siberia, Taiwan, and Vietnam (38). *Opisthorchis viverrini* infects about 10 million people in Thailand, Laos, and Cambodia, and *O. felineus* is widespread throughout Northern Europe and Asia, infecting about 1.5 million people (38). An estimated 349,737 DALYs have been attributed to liver fluke infections (40). All three species live in the bile duct and are thought to feed on biliary epithelia. Light infections are usually asymptomatic, but heavy infections can induce disease. Symptoms most commonly are associated with an acute phase of infection and may include fever, abdominal pain, hepatitis-like symptoms, and eosinophilia. A number of asymptomatic hepatobiliary abnormalities are associated with infection (49). Severe infestations with these liver flukes, which are rare, might cause obstructive jaundice, cirrhosis, cholangitis, cholecystitis, bile peritonitis, biliary obstruction, intrahepatic stone formation, cholelithiasis, biliary and liver abscesses, pancreatitis, and hepatitis. The most serious complication of infections with *C. sinensis* and *O. viverrini* is cholangiocarcinoma, the malignant bile duct cancer. *C. sinensis* and *O. viverrini* have been classified by the International Agency for Research on Cancer (IARC) as definitely carcinogenic (class 1) (50).

Many infections with fasciolids remain asymptomatic. Acute disease arises because of extensive tissue damage as parasites migrate through the hepatic parenchyma to gain access to the bile ducts. Parasite activity in the bile ducts leads to proliferation of ductal epithelium, inflammation, and fibrosis. Heavy infections can lead to cholestasis and result in hepatic atrophy and periportal cirrhosis (23). Common symptoms in chronic infection include biliary colic and cholangitis. Eosinophilia is common in all stages of disease.

Collection, Transport, and Storage

Diagnosis is made by detection of characteristic embryonated eggs in feces. Eggs of opisthorchids are minute and can be confused with those of intestinal digeneans. Serology can be useful, especially in cases where chronic infection, with low egg excretion, is suspected.

Detection

Eggs of opisthorchids (Fig. 4) are embryonated when laid and are oval, yellowish brown, and operculate, with a shoulder or thickened region of eggshell surrounding the operculum. The shell surface may appear rough and may have a small knob at the abopercular pole (45). The eggs are smaller than those of many other digeneans of humans, ranging from 20 to 35 μm in length. Opisthorchid eggs may be confused with those of other taxa, especially the heterophyids (see below). Differential diagnosis may be achieved by patient history, by examination via the formalin-ether concentration technique, and by examination of purged worms in feces after anthelminthic treatment. Recently, different PCR tests that detect and discriminate between fish-borne zoonoses caused by opisthorchids and members of the Heterophyidae were developed, based on mitochondrial (51) and ribosomal sequences (52).

The eggs of fasciolids are large compared with those of other digeneans (Table 2). *Fasciola* eggs cannot be distinguished from those of the related intestinal parasite *Fasciolopsis buski* (Fig. 4) and also resemble those of *Gastrodiscoides* (Table 3). Fasciolid eggs of individual species can also vary in morphology and size in different host species, complicating species identification in regions where more than one fasciolid is endemic (53). Detection of parasite antigen in stools is useful to distinguish between present and past infections (54). Molecular diagnosis methods, notably, tests based on loop-mediated isothermal amplification (LAMP), for detection of parasite nucleic acid in feces are promising alternatives.

Typing

A number of strains of fasciolids have been isolated. These isolates generally display reduced sensitivity to the anthelminthic triclabendazole, which remains a problem in livestock industries but not yet in human treatments (55).

Serology

Many antigen tests have been subjected to trials for *O. viverrini*, but most are plagued by cross-reactivity and persistence of antibodies after parasitologic cure (56). A coproantigen ELISA using monoclonal antibodies raised against adult *O. viverrini* excretory/secretory antigen has been developed and has displayed high specificity and sensitivity, whereas ELISAs for circulating antibodies show high sensitivity but low specificity (23).

Liver fasciolids have a long (2-month) prepatent period, and because of this, fascioliasis is one disease where serological diagnosis is valuable. Immunological tests, particularly ELISAs, based on parasite excretory/secretory antigens, cysteine protease or saposin-like antigens, display high sensitivity and specificity (57, 58). Commercial and government laboratories in many countries have serological tests for human fascioliasis, including a reference laboratory at the University of Puerto Rico.

Anthelminthic Susceptibility and Treatment

Treatment for clonorchiasis or opisthorchiasis relies on oral administration of praziquantel (25 mg/kg three times per day for up to two consecutive days is a dosage commonly used in hospitals, while a single oral dose of 40 mg/kg is used for mass treatment programs). Recent proof-of-concept studies have shown that the Chinese anthelminthic tribendimidine possesses high activities against *O. viverrini* and *C. sinensis*, and hence further studies (including dose-finding and pharmacokinetic trials) have been launched (59).

Fasciola species are insensitive to praziquantel, and triclabendazole is the drug of choice (Table 2). There have been reports of triclabendazole-insensitive isolates in livestock (55). Artemether and artesunate have undergone trials for use in humans, but they have demonstrated only little effect against *Fasciola* spp. (60).

Trematodes of the Intestine

Humans can serve as host to a wide variety of intestinal flukes. Summary information on some families of intestinal digeneans is presented in Table 3. Families Brachylaimidae, Diplostomidae, Gymnophallidae, Lecithodendriidae, Microphallidae, Paramphistomatidae, Plagiorchiidae, and Strigeidae are rarely encountered in humans and are not considered further in this chapter. For further information on intestinal trematodes not provided here, the reader is referred to chapter 148 and to Fried et al. (61). Members of the families Echinostomatidae, Heterophyidae, Fasciolidae, and Troglotrematidae are commonly encountered in some countries and are discussed below.

Family Echinostomatidae

Echinostomatid flukes are small, typically 3 to 10 mm in length and 1 to 3 mm in width, with a large ventral sucker and distinctive collared spines. At least 24 species are known to parasitize humans, mostly in Asia (Table 3), for example, *Echinochasmus japonicus*, a common parasite of humans in Laos (62). All human infections by echinostomatids are zoonotic and focal in distribution, and foci are often in the vicinity of freshwater or brackish water habitats. Humans are infected by eating a range of raw or undercooked vertebrate and invertebrate foods, including snails (Table 3). In most cases, infection is asymptomatic. Heavy infections can lead to a range of symptoms including flatulence, colic, and diarrhea. Some heavy infections in children have been fatal. The eggs of echinostomatids are similar in shape to those of fasciolids but are smaller (Table 3). Interspecific variation in egg size occurs among the echinostomes, and species identification is not possible unless adult worms are obtained by purgation with anthelminthics. A single dose of praziquantel is recommended for treatment (63).

Family Fasciolidae

Fasciolopsis buski is the largest fluke parasitizing humans, measuring 8 to 10 cm in length and 1 to 3 cm in width (64). Adult worms inhabit the duodenum and jejunum of humans and a small range of other hosts that include pigs, horses, cattle, goats, and sheep. The species is distributed focally in many countries (Table 3). The snail intermediate hosts of *F. buski* are shown in Table 3. Humans are infected by eating aquatic plants on which metacercariae are encysted or by drinking metacercariae that have encysted on the water surface (61). Adults attach and feed on the intestinal wall. Human disease relates to the severity of infection, and symptoms vary from intestinal disturbance and pain, associated with eosinophilia, to severe diarrhea, gastric pain, bowel obstructions, and nausea. Feces are often profuse and yellow-green and may contain undigested food particles.

The eggs of *F. buski* are large, operculate, nonembryonated, ellipsoidal, and yellow (Table 3; Fig. 4). The eggs are very similar to those of other fasciolids from which species may be distinguished by observation of purged adults. The current drug of choice is praziquantel, but albendazole and mebendazole have also undergone trials (61, 63).

Family Heterophyidae

Many species of heterophyid trematodes are known to infect humans (61, 65). Heterophyids are mostly small parasites, less than 0.5 mm in length. Some species infect marine snails as intermediate hosts. Commonly encountered heterophyids of humans are *Heterophyes heterophyes*, *Metagonimus yokogawai*, and *Haplorchis* species. *H. heterophyes* infection in humans has been reported from many countries in Asia, North Africa, and the Middle East (61). Other species are often encountered in rural Asia (62).

The adult flukes are intestinal inhabitants of a wide range of piscivorous birds and mammals. Eggs (Fig. 4) are embryonated when passed from the host but do not hatch until ingested by a snail intermediate host (1). Cercariae encyst in a range of fish including mugilids, cyprinids, and gobiids. Humans are infected by consumption of raw, freshly salted, or undercooked fish (Table 3).

Disease symptoms in humans relate to infection intensity and arise because of parasite irritation of the intestinal mucosa. Eggs may on occasion pass into the bloodstream and lodge in tissues. These eggs have been known to cause fatal myocarditis in the Philippines (23). Diagnosis of heterophyids is facilitated by observation of eggs in feces (Table 3). Heterophyid eggs are similar in size to those of opisthorchiid liver flukes, and care must be taken to differentiate the infections. Often, this can be achieved only by examination of purged adult worms. The recommended treatment for heterophyid infection is a single dose of praziquantel (Table 3).

Family Troglotrematidae

This family of small oval flukes parasitize mammals, including humans. One species, *Nanophyetus salmincola*, found along the coastal U.S. Pacific Northwest, is found as metacercariae of salmonid fish. The species may be the most commonly encountered human trematode endemic to North America. Praziquantel is efficacious for nanophyetiasis.

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Less Common Helminths

GARY W. PROCOP AND RONALD C. NEAFIE

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This chapter covers the less common causes of helminthic parasitic infections, particularly those caused by the less common nematodes and cestodes. The trematodes are discussed thoroughly in chapter 146. This chapter is not inclusive, given the wide variety of helminthic parasites that have been reported to cause human disease, but includes some of the most interesting and challenging of parasitic diseases. The reader who desires further information about these and other helminths of medical importance is directed to *Pathology of Infectious Diseases*, volume 1, *Helminthiasis*, by the Armed Forces Institute of Pathology (AFIP) (1) and the Centers for Disease Control and Prevention's (CDC) parasitology website (http://www.cdc.gov/dpdx).

COLLECTION, TRANSPORT, AND STORAGE OF SPECIMENS

The collection, transport, and storage of specimens are similar regardless of the type of helminth present, so these guidelines are consolidated here to reduce duplication. Worms present in the lumen of the gastrointestinal tract, such as *Anisakis*, can be retrieved by endoscopy. Surgical resection specimens may contain a worm or larvae. The intact worm and surgical specimens may be preserved and transported in 10% neutral buffered formalin. Stool is an important diagnostic specimen for parasites that reproduce within the intestinal tract, e.g., *Capillaria philippinensis*, and should be collected and fixed in a standard manner similar to that used for the detection of eggs and parasites (i.e., formalin and polyvinyl alcohol), if available. Alternative, mercury-free fixatives are also acceptable (2). Blood for serology should be handled in a standard manner. Additional information about serologic tests for the diagnosis of parasites covered in this chapter is also available through the CDC at http://www.cdc.gov/dpdx/HTML/DiagnosticProcedures.htm.

LESS COMMON NEMATODES

Anisakis and Related Species

Description of the Agents

Anisakis species (for which *Anisakis simplex* is the type species), *Pseudoterranova decipiens*, and *Contracaecum* species are nematodes of the family Anisakidae, superfamily Ascaridoidea, and class Secernentea.

Epidemiology, Transmission, and Prevention

The larvae of anisakids are present in many varieties of fish for which marine mammals are the definitive host; human infections occur if raw or poorly cooked infected fish is consumed (3). Fish that have been salted or pickled may still contain viable anisakid larvae. Similarly, the smoking of fish or marinating fish in lime juice (e.g., ceviche) does not reliably kill these parasites. Disease may be prevented by consuming only thoroughly cooked fish.

Clinical Significance

The clinical features vary depending on the stage of disease. Early, within 12 h of eating infected fish, patients experience nonspecific acute abdominal pain (4). At this phase, the larvae are associated with the superficial aspects of the mucosa. Superficial larvae may migrate up the esophagus and cause coughing, potentially with expectoration of the worm. Additionally, intraluminal transit of the worm may result in mid- to lower abdominal pain, as the small intestine and colon are involved. More-severe symptoms occur if the worm penetrates through the mucosa into the submucosal and deeper tissues (Fig. 1). This evokes hypersensitivity, leukocytosis, and eosinophilia (5). Sudden, intense abdominal pain may be thought to represent appendicitis, acute gastritis, a gastric ulcer, or chronic colitis (e.g., Crohn's disease) (6). Diarrhea and constipation may occur, and the stool contains occult blood. Immunoglobulin E (IgE)-mediated hypersensitivity reactions to anisakids have been described and include acute urticaria and anaphylaxis (7).

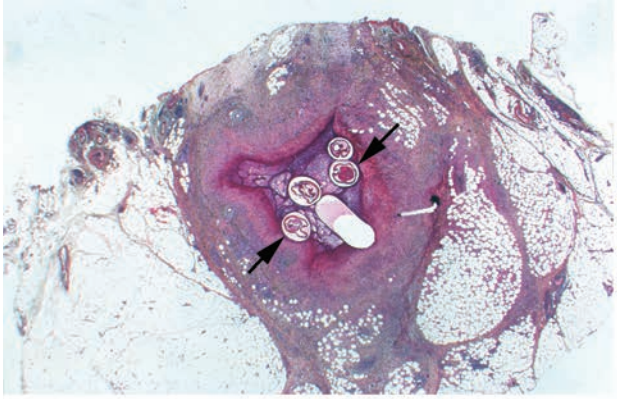
Direct Examination by Microscopy

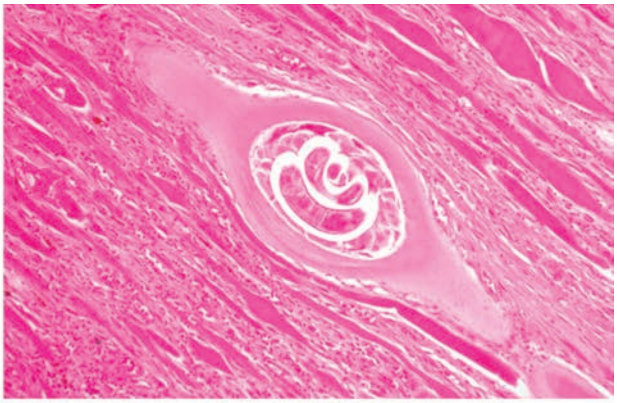
The intact worms are white to cream, nonsegmented larvae that measure 10 to 50 mm by 0.3 to 1.2 mm. One dorsal and two subventral reduced lips and a triangular boring tooth are useful for identification. The mucron or tail spine is another useful feature for the identification of *Anisakis* larvae. Histopathologic analysis of infected tissues often demonstrates cross sections of the worm and the host inflammatory response, which contains numerous eosinophils. Cross-sectional studies are useful for identifying the type of anisakid present. Species-specific PCR assays or broad-range PCR assays with DNA sequencing will afford differentiation by molecular methods once the genetic variability that exists among these parasites is determined (9).

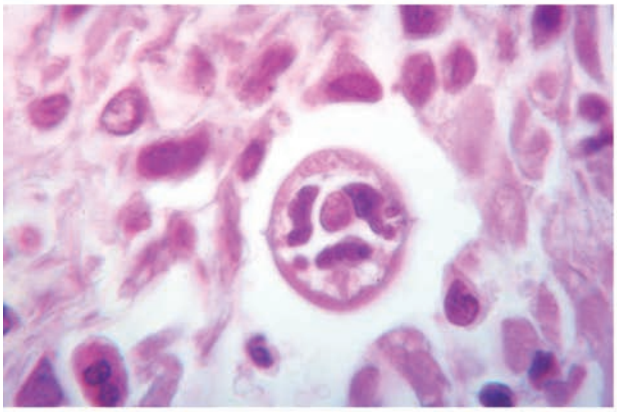
Serologic Tests

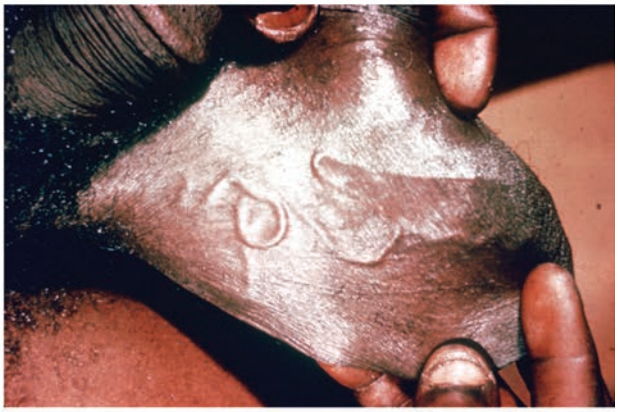
Serologic tests to detect the immunologic response to *Anisakis* and related species are available at commercial refer-

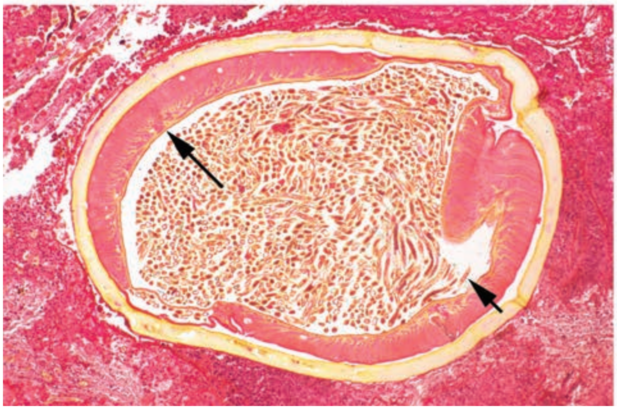
doi:10.1128/9781555817381.ch147











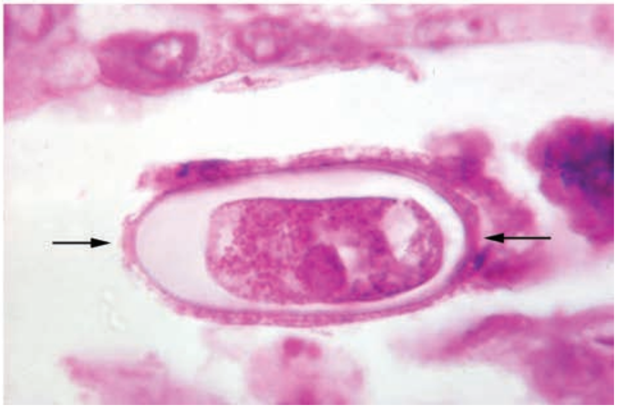


FIGURE 1 (top row, left) This *Anisakis* species (arrows) has penetrated into the deep tissues of the abdomen. Multiple cross sections of the worm, which is 300 μm in diameter, are seen in the omentum. Movat's stain; original magnification, ×2.5. doi:10.1128/9781555817381.ch147.f1

FIGURE 2 (top row, right) This coiled first-stage *T. spiralis* larva is in a "nurse cell." Note the hyaline, amorphous appearance of the external aspect of the nurse cell and the surrounding chronic inflammatory infiltrate. The worm diameter is 35 μm. Hematoxylin and eosin stain; original magnification, ×30. doi:10.1128/9781555817381.ch147.f2

FIGURE 3 (middle row, left) The minute lateral alae are useful in the identification of *Toxocara* species. The worm diameter is 18 μm. Hematoxylin and eosin stain; original magnification, ×500. doi:10.1128/9781555817381.ch147.f3

FIGURE 4 (middle row, right) The serpiginous tract of a female *D. medinensis* worm is demonstrated in the scrotum of this patient. doi:10.1128/9781555817381.ch147.f4

FIGURE 5 (bottom row, left) Rhabditiform larvae (short arrow) fill the body cavity of this gravid *D. medinensis* worm. Also note the presence of the two prominent bands of somatic muscle (long arrow). The worm diameter is 1.1 mm. Movat's stain; original magnification, ×25. doi:10.1128/9781555817381.ch147.f5

FIGURE 6 (bottom row, right) The bipolar plugs (arrows), pitted eggshell, and rectangular shape are characteristic of *Capillaria* species. This photomicrograph is from a human small intestine and demonstrates an egg that is 40 μm long. Hematoxylin and eosin stain; original magnification, ×490. doi:10.1128/9781555817381.ch147.f6 ence laboratories in the United States. Although these tests are not currently performed at the CDC, that institution may be contacted for assistance in locating a commercial laboratory that may perform this test, if necessary. A variety of serologic assays have been developed, but they vary in sensitivity and specificity. These assays identify 85 to 90% of infected individuals, with a demonstrable serologic response occurring from 10 to 35 days postinfection. The specificity of these assays suffers from cross-reactivity with other ascarids. However, this cross-reactivity may be desirable to detect related species, e.g., *Pseudoterranova*, but may limit the utility of this assay in certain populations, such as those who are likely to have or have been infected with intestinal ascarids. Conversely, more-specific assays have been devised to detect *Anisakis*, but these, unfortunately, do not detect the related species that may cause "anisakiasis" in the broadest sense (i.e., anisakidosis).

Treatment

Removal of the larva via endoscopy while it is associated only with the superficial mucosa is the most efficacious treatment. Surgical resection, however, may be needed for more deeply insinuated larvae (9). Anthelmintic drugs do not appear to be useful, but corticosteroids may be used to decrease the associated inflammation.

***Trichinella* Species**

Description of the Agents

Trichinella spiralis is the most important cause of human disease in this genus, but other species, such as *Trichinella pseudospiralis* and *Trichinella britovi*, also cause trichinosis (10). *Trichinella* species are nematodes of the family Trichinelloidea and class Adenophorea.

Epidemiology, Transmission, and Prevention

Trichinosis occurs worldwide (11). The life cycle of *T. spiralis* is different from that of other nematodes in that the infected mammal that first serves as the definitive host for the parasite also harbors infective larvae encysted within muscle (i.e., the intermediate host) (Fig. 2) (10). The domestic pig is the most important host for the transmission of this roundworm to humans, but the meat of bears, walruses, and other wild game may also contain infective larvae. The ingestion of wild game poses the greatest risk for infection in countries with strict animal husbandry regulations. The parasitic cycle is propagated in the barnyard between pigs and rodents. Disease may be prevented by eating only thoroughly cooked meat products of potential hosts and by attempting to control trichinosis in these hosts through good animal husbandry practices.

Clinical Significance

Infections vary from mild, subclinical disease to severe illness, depending upon the parasite load. The clinical manifestations vary with the stage of infection. Gastrointestinal symptoms, associated with adult worms, last only about a week and include nausea, vomiting, abdominal pain, and diarrhea. Fever, facial edema that is particularly predominant around the eyes, myalgia, and marked peripheral eosinophilia are the four cardinal features of trichinosis. The major clinical findings extracted from 5,377 well-documented cases were myalgia, diarrhea, fever, facial edema, and headaches (11). If larval migration involves the brain and meninges, then neurologic symptoms predominate, whereas involvement of the myocardium causes myocarditis and possibly arrhythmias or sudden cardiac death. Unlike skeletal muscle, the larvae do not encyst in these other tissues.

Direct Examination by Microscopy

The direct microscopic examination of a muscle biopsy sample, performed by simply compressing the fresh muscle fibers between glass slides and observing the specimen microscopically, may disclose larvae. More commonly, larvae are detected by histopathologic analysis of formalin-fixed, paraffin-embedded tissue sections (Fig. 2).

Nucleic Acid Detection Techniques

Although nucleic acid amplification assays have been developed for *Trichinella spiralis*, the clinical utility of these assays has yet to be determined. They are, however, tools for assessing the prevalence of infections in domestic pigs, as well as in wildlife (12, 13).

Serology

The antibody response begins 3 to 5 weeks after infection in 80 to 100% of patients and follows the acute phase of disease. Therefore, the conversion from a negative serologic test during acute illness is evidence of disease. The tests are highly sensitive, and although they are less than 100% specific, cross-reactivity usually results in test values in the equivocal range. These tests are available at the CDC and from several commercial laboratories.

Treatment

Therapy varies according to the stage of disease. Thiabendazole or albendazole is active against the intestinal worm but not against the encysted larvae. There is no proven method to kill the encysted larvae. Well-controlled therapeutic studies are lacking. Corticosteroids, salicylates, and antihistamines may lessen symptoms caused by the associated inflammation.

***Toxocara* Species**

Description of the Agents

Toxocara canis is the intestinal ascarid of dogs and other canids, whereas *Toxocara cati* is the intestinal ascarid of cats. Although larvae of both species can cause toxocariasis in humans, most infections are caused by *T. canis* (14). *Toxocara* species are in the family Ascarididae and the class Secernentea, within the phylum Nemathelminthes.

Epidemiology, Transmission, and Prevention

Toxocariasis has a worldwide distribution, but the prevalence of zoonotic disease varies widely by geographic area. The seroprevalence of human disease reflects helminthic control in dogs and cats. The highest infection rates occur among the poor, and there is an association with dog ownership (15). The natural cycle of *Toxocara* begins with the passage of unembryonated eggs in the feces of the definitive host. The adult female worm passes a large number of eggs per day; therefore, one instance of stool contamination of the environment, such as in a sandbox, can cause significant contamination. The L2 (i.e., second-stage) larvae develop in the eggs after 10 to 20 days of incubation in the soil; the eggs at this stage are infective. If the eggs are ingested by a suitable definitive host, then the larvae penetrate the intestinal tract and migrate through the liver, bloodstream, and lungs. The larvae then migrate up the respiratory tract, are swallowed, and mature into adult worms in the intestinal tract. Paratenic hosts (e.g., rabbits) may also participate in the life cycle by harboring infective larvae after egg ingestion; ingestion of the paratenic host by the definitive host allows the larvae to mature to adulthood. When the eggs are ingested by a nonpermissive host, such as a child, the resulting *Toxocara* larvae cannot complete their life cycle but rather wander aimlessly in the aberrant host, causing visceral larva migrans. Prevention is achieved foremost through zoonotic control by deworming dogs and cats. Deworming is most important for puppies, which are particularly permissive for infection and are often associated with children. Other preventive measures include avoidance of animal feces, thorough washing of fruits and vegetables, and thorough cooking of meats from potentially paratenic hosts (16).

Clinical Significance

Toxocara infections are common helminthic infections of humans. Fortunately, most infections are subclinical, producing only mild disease for which a definitive diagnosis is not sought. The most severe forms of disease occur with involvement of the eyes or significant involvement of the heart, brain, or other vital organs (17, 18).

Direct Examination by Microscopy

Eggs are not produced in humans in toxocariasis, so a stool evaluation is not helpful for diagnosis. However, occasionally the eggs of another geohelminth, such as *Ascaris*, hookworm, or *Trichuris*, may be present because of common risk factors. The detection of the migrating larvae in surgically excised tissues by histopathologic evaluation provides the definitive morphological diagnosis, but larvae are not always present in biopsy specimens. When present, the single minute lateral ala and the cross-sectional diameter are useful criteria for identifying these parasites in histologic sections (Fig. 3) (19).

Serologic Tests

Serologic studies are important for the establishment of the diagnosis of toxocariasis; results of these should be used in conjunction with clinical findings as well as other laboratory findings, such as increased IgE and eosinophilia (20). Positive results in the absence of other corroborative findings could represent a previous, asymptomatic infection. The sensitivity and specificity of these assays are high but less than 100%. It is difficult to determine the precise parameters of serologic assays, since there is not a better parasitological test (i.e., a gold standard) to confirm the presence of a true infection. These tests have also been used successfully in seroepidemiologic studies. Tests are available at the CDC and from commercial reference laboratories.

Treatment

There is no proven therapy. Anthelmintic therapy with albendazole or a similar therapeutic is often used. Corticosteroids may be necessary to control the inflammatory response in patients with a large parasite burden. Eye infections require a combined medical and surgical approach (21).

Dracunculus medinensis

Description of the Agent

D. medinensis, also known as the Guinea worm, is not a filarial worm but rather a member of the family Dracunculidae, which is in the class Secernentea of the phylum Nemathelminthes (23). Other *Dracunculus* species that infect a variety of other animals exist but do not cause human disease.

Epidemiology, Transmission, and Prevention

Dracunculiasis is found only in the rural parts of Africa. Disease occurs more commonly in the dry season and affects men more commonly than women. It is most common in individuals from 10 to 60 years old, which taxes the productivity of affected communities. An intensive effort by the World Health Organization, local governments, and numerous other humanitarian organizations has significantly decreased the annual incidence of disease (23). Updates and additional information are available from the WHO and CDC at their respective websites (http://www.who.int/topics/dracunculiasis/en/ and http://www.cdc.gov/parasites/guineaworm/). Significantly, there were no cases reported in January 2013, which was the first zero-case month in the history of the eradication program. At the beginning of this program (1986), an estimated 3.5 million people in 20 countries were affected. The annual incidence of worldwide disease was diminished by an astounding 98% by December 2001, and seven countries completely eradicated the disease. Substantial progress continues to be made to eradicate this disease. These substantial efforts have led to only 542 reported cases in 2012. The eradication of a devastating disease may be near at hand, but continued vigilance is necessary.

Infection follows drinking contaminated freshwater that contains copepods, which harbor the infective larvae. After ingestion, the larvae migrate to the retroperitoneum, where they mature and mate. The female worm eventually migrates to a subcutaneous location, where a blister forms, which bursts upon contact with water and releases numerous larvae. The larvae are ingested by a copepod, wherein they become infective. Preventive measures are centered on education and providing clean drinking water (26, 27). There are current challenges to provide clean drinking water in parts of South Sudan and Mali due to lack of security (23).

Clinical Significance

Patients are asymptomatic during larval penetration of the gastrointestinal tract and retroperitoneal maturation and mating of the worms. Symptoms are secondary to inflammation and tissue damage caused by worm migration. Although lesions are most common in the lower extremities, they may occur anywhere in the body. A serpiginous tract is produced, which is caused by the migrating worm under the skin (Fig. 4). The large blister that is formed may become secondarily infected. Dead worms may be absorbed, calcify, or produce symptoms secondary to location (e.g., arthritis due to location within a joint).

Direct Examination and Microscopy

The presence of the end of a worm protruding from a burst blister or ulcer in the appropriate setting is diagnostic. The microscopic analysis of these worms in cross section demonstrates a 30- to 50-μm-thick cuticle, indistinct lateral cords, prominent dorsal and ventral bands of smooth muscle, and a large uterus filled with rhabditoid larvae that fills the body cavity (Fig. 5).

Serologic Tests

Serologic tests are usually not necessary for the diagnosis, given the obvious findings in people at risk for disease. They are, however, useful for seroprevalence studies and to identify infected individuals prior to the partial emergence of the adult worm.

Treatment

The oldest and traditional treatment, as depicted in an ancient Egyptian medical text, the *Papyrus Ebers*, consists of removing the gravid worm by wrapping the exposed end of the worm around a stick and applying gentle pressure for days. Treatment with thiabendazole and metronidazole, although not lethal for the worm, facilitates removal by this process (26). Although it is often effective, risks include rupture or breaking of the worm prior to full removal. Currently, surgical excision is preferred (27). Anti-inflammatory agents and antihistamines are important for symptomatic relief. Antibiotics may be necessary to curtail secondary bacterial infections, and tetanus vaccination is important.

Capillaria philippinensis

Description of the Agent

Capillaria philippinensis is a trichurid nematode responsible for intestinal capillariasis (22, 28). *Capillaria* species are in the family Trichuridae of the class Adenophorea.

Epidemiology, Transmission, and Prevention

The natural parasitic cycle of *C. philippinensis* likely involves marine, fish-eating birds as the definitive hosts and fish as the intermediate hosts. Humans become infected by ingesting raw or undercooked fish that harbor the infective larvae. Larvae at various stages of maturation may be found in the lumen of the intestine of the definitive host or the patient. Therefore, the population of parasites within the lumen of the bowel may increase (hyperinfection) without the consumption of additional contaminated fish. Thorough cooking of fish is protective.

Clinical Significance

Infections with *C. philippinensis* are relatively rare; 82 patients with intestinal capillariasis were reported in Thailand from 1994 to 2006 (29). Rarely, because intraintestinal hyperinfection is possible, infections may be fatal. Nonspecific gastrointestinal complaints predominate early in the course of disease; these include watery diarrhea, weight loss, abdominal pain, edema, and weakness (29). As the disease progresses and the number of worms increases, there is continued diarrhea with malabsorption leading to cachexia (24). Endoscopy may be useful for establishing the diagnosis, but stool examination remains the diagnostic test of choice (25). Death occurs secondarily to malnutrition or because of secondary bacterial infections (e.g., septicemia).

Direct Examination by Microscopy

Microscopic examination of the stool may demonstrate a mixture of eggs and larvae. The eggs of *C. philippinensis* (Fig. 6), which have bipolar plugs, are superficially reminiscent of the more commonly recognized trichurid worm, *Trichuris trichiura*. The plugs of *C. philippinensis*, however, are nonprotruding, the egg is more rectangular, and there is a distinctive pitting of the eggshell (30). The adults superficially resemble *Strongyloides stercoralis* but may be differentiated by the presence of stichocytes and three bacillary bands, with the latter being recognized most easily in cross section.

Serologic Tests

Serologic tests are not available from the CDC or from commercial reference laboratories in the United States. An enzyme-linked immunosorbent assay for the screening of sera for antibodies directed against *C. philippinensis* has been developed and holds promise (31). This assay is particularly useful in infected patients who are negative by conventional stool examination. The sensitivity of the assay is excellent (100%), with a moderate specificity that is limited by cross-reactivity in patients with trichinellosis, strongyloidiasis, trichuriasis, and infections by other helminths.

Treatment

Albendazole, mebendazole, or thiabendazole may be used for therapy. Relapsing disease requires prolonged anthelmintic therapy. Aggressive electrolyte replacement and monitoring are critical, as are control of diarrhea and administration of nutrients.

Gnathostoma Species

Description of the Agents

Gnathostoma species are gastric spirurid nematode parasites for which the definitive hosts are a variety of mammals (32). Infective L3 (i.e., third-stage) larvae are known to cause disease in humans, but it is also thought that L2 (i.e., second-stage) larvae encountered in infected copepods in unclean drinking may also cause disease. Gnathostomiasis may be considered a subtype of visceral larva migrans. *Gnathostoma spinigerum* is the most common cause of human disease, but *Gnathostoma hispidum*, *Gnathostoma nipponicum*, and *Gnathostoma doloresi* may also cause human disease. *Gnathostoma* species belong in their own family, Gnathostomatidae, of the class Secernentea.

Epidemiology, Transmission, and Prevention

Gnathostoma spinigerum has essentially a worldwide distribution. Dogs and cats are the primary definitive hosts for this gnathostome, whereas *G. hispidum* and *G. doloresi* infect wild and domestic pigs and *G. nipponicum* is a parasite of weasels.

The adult male and female gnathostomes live in a tumor produced in the wall of the stomach of the definitive host, where they mate and produce eggs. The eggs are passed in the stool, and once in water, they hatch and release first-stage larvae. The larvae then infect and mature into second-stage larvae within the copepod *Cyclops*. When a variety of intermediate hosts, such as fish, snakes, eels, or frogs, eat the copepod, the larvae penetrate the gastric wall of the new host, wherein they develop into third-stage (L3) larvae, migrate to the musculature, and encyst. If an appropriate definitive host eats the intermediate host, the advanced L3 larvae excyst and penetrate the gastric wall. The L3 larvae then migrate through the liver, muscles, and connective tissues, only to return to the stomach and mature into adults. Alternatively, paratenic hosts, such as birds, may eat the second intermediate host; the L3 larvae ingested remain viable within the paratenic host, which may pass them on either to the definitive host or to humans.

Humans are accidental hosts in which the nematode larvae cannot mature to adulthood and continue to migrate aimlessly and aggressively (32). The parasite is transmitted to humans through the ingestion of raw or undercooked meat from a secondary intermediate host or a paratenic host. Disease can be prevented by thoroughly cooking potentially infected foods.

Clinical Significance

The clinical presentations of gnathostomiasis are protean, since the wandering larvae may be present in any tissue or organ system (33–36). Although the infection is not commonly fatal, significant morbidity may be associated with infection. The clinical manifestations depend on the tissues in which the larva is migrating. Panniculitis, creeping eruptions, and pseudofurunculosis are dermatologic manifestations of gnathostomiasis (34). Any visceral organ may be affected, and although *Gnathostoma* is not neurotropic, the eyes and central nervous system (CNS) may be involved (35, 36).

Direct Examination and Microscopy

Mature (adult) gnathostomes are not present in humans, but the L3 larvae are. This form is morphologically similar to the adult form but is smaller, measuring 3 to 4 mm in length by 630 μm in diameter (*G. spinigerum*). The head bulb contains four rows of cephalic hooklets, with approximately 45 hooklets per row. The body of the larva, like that of the adult, is covered with transverse rows of sharply pointed spines that diminish toward the posterior end of the worm. An entire worm may be expelled spontaneously or may require surgical excision.

Serologic Tests

Serologic assays have been developed predominantly in areas of endemicity. These are not available in the United States, but the CDC can assist in identifying a source for testing, if necessary. The possibility of cross-reactivity should be considered with these tests, as well as other serologic assays for helminthic parasites. Like any test, they should be used only in the appropriate clinical context.

Treatment

Surgical removal of the parasite is the most effective treatment but is difficult to achieve due to parasite migration; this may be aided by advanced imaging tools (37). Treatment usually involves some type of anthelmintic medication, such as albendazole or ivermectin. There remains controversy regarding the treatment of CNS gnathostomiasis, wherein there is a concern that anthelmintic therapy will kill the invading worms and increase the inflammatory reaction; treatment is supportive and may include corticosteroid use to control the inflammatory response (38).

***Parastrongylus* (*Angiostrongylus*) Species**

Description of the Agent

Parastrongylus cantonensis and *P. costaricensis* are filariform worms that are the most important causes of angiostrongyliasis (38). This parasite is also commonly referred to by the former genus name, *Angiostrongylus*. *Parastrongylus* belongs to the family Angiostrongylidae and the class Secernentea.

Epidemiology, Transmission, and Prevention

Parastrongylus spp. are widely distributed throughout the world but represent an important public health threat in Southeast Asia, the Asian Pacific Islands, Africa, and the Caribbean. These parasites persist in a wide variety of rodents, which serve as the definitive host. The adult worms *Parastrongylus cantonensis* reside in the pulmonary artery and the right side of the heart of the rodent, whereas the adult form of *P. costaricensis* resides in the ileum. Eggs released from the adult worms lodge in capillaries, where they hatch and release larvae. The larvae subsequently migrate up the trachea (*P. cantonensis*) and are swallowed and passed in the feces. These larvae infect mollusks, particularly snails and slugs that are the intermediate host. Within the snail, they mature into the infective L3 larvae. A number of animals, including shrimp, crabs, fish, and frogs, may eat the infected mollusks and serve as important paratenic hosts. Rodents that ingest either the infected mollusk or tissues from the paratenic host thereby become infected. In the definitive host, the infective larvae penetrate the intestine, become blood borne, and migrate to the CNS, where they molt twice. Thereafter, the worms reenter the systemic circulation and finally reside in either the right ventricle and pulmonary artery (*P. cantonensis*) or the ileum (*P. costaricensis*) to mature and complete the cycle. Humans become infected through the ingestion of tissue from either an infected mollusk or an infected paratenic host or through excretions from snails or slugs (i.e., slime) that contaminate other foodstuff (e.g., vegetables). Prevention of disease can be achieved through control of the local rat population, the thorough cooking of mollusks and meat from paratenic hosts, and the washing of vegetables that may be contaminated with snail or slug slime.

Clinical Significance

The clinical manifestations reflect the worm burden and the site of worm residence, the CNS for *P. cantonensis* and the ileocecal region for *P. costaricensis*. The worms of *P. cantonensis* remain juvenile, tend to remain associated with the brain and meninges in human infections (Fig. 7), and usually die in this location. Conversely, *P. costaricensis* often reaches sexual maturity and releases eggs in the intestine. Patients with CNS disease demonstrate signs and symptoms typical of meningitis or meningoencephalitis, with headache, fever, possibly eosinophilia (10 to 60%), and any of a number of neurologic disturbances, depending on the location of the worms. The cerebrospinal fluid (CSF) demonstrates pleocytosis, eosinophilia (26 to 75%), and elevated protein and occasionally contains immature worms. Alternatively, patients may have infections of the eye, with retinal detachment and blindness. Less commonly, human infections may result in pulmonary disease. Infections with *P. costaricensis* result in eosinophilic enteritis with diarrhea and abdominal pain.

Direct Examination and Microscopy

Demonstration of the worms in histologic sections or intact in clinical specimens from the CSF, eye, or other infected tissue definitively establishes the diagnosis. Eosinophilia in the CSF may be the first indicator of a possible parasitic infection of the CNS. The differential diagnosis of the causes of CSF eosinophilia, however, is broad and includes other parasitic infections, e.g., with *Gnathostoma* or *Toxocara* spp., allergic reactions, and coccidioidomycosis. The gross appearance of an adult female worm is distinctive, with the spiral winding of the uteri and ovarian branches imparting a "barber pole" appearance. Cross section of the female demonstrates a large intestine and two uteri, whereas cross section of the male reveals a large intestine and a single reproductive tube. Eggs are not produced in human tissues by *P. cantonensis*, whereas they may be produced by *P. costaricensis*. The eggs of *P. costaricensis* remain embedded in tissue in the human host and do not appear in stool. The eggs are oval, thin shelled, somewhat resembling a hookworm egg, and usually measure 60 to 65 by 40 to 45 μm.

Serologic Tests

Serologic assays are powerful tools for the diagnosis of angiostrongyliasis, particularly since *P. cantonensis* is usually located in a difficult-to-access location, the CNS. The serologic assays that have been developed vary with respect to sensitivity and specificity, so it is important that the user understand the performance characteristics and the limitations of the assay under consideration (39). Although intrathecal antibody is not always produced during infection, when detected, the presence of intrathecal antibody synthesis provides strong evidence of infection. This assay is available from some commercial laboratories in the United States.

Treatment

The optimal treatment has not been established. Fortunately, the disease is often self-limited. Removal of CSF and the administration of corticosteroids and nonsteroidal anti-inflammatory agents have been used to control pain and inflammation. A variety of anthelmintic medications have been used, with mebendazole being the current drug of choice. Careful monitoring of the patient is important, since anthelmintic therapy may sometimes exacerbate the symptoms (40). Corticosteroids may be used alone or in combination with anthelmintic therapy (40).

Dirofilaria immitis and Other *Dirofilaria* Species

Description of the Agents

Dirofilaria immitis, the filarial dog heartworm with worldwide distribution, causes human pulmonary dirofilariasis (40). There are a variety of other *Dirofilaria* species that may accidentally infect humans. For example, *Dirofilaria tenuis* is a raccoon parasite common in the southeast United States, *D. ursi* infects bears in the northern United States and Canada, and *Dirofilaria repens* is a dog parasite restricted to the Old World (41, 42). *Dirofilaria* species belong to the family Onchocercidae in the class Secernentea.

Epidemiology, Transmission, and Prevention

Although dogs are the most important host for *D. immitis*, other mammals, such as fox and bear, are also suitable hosts. The blood of infected dogs or other suitable hosts contains microfilariae that are released from the adult worm, which resides in the right ventricle of the heart. These are taken into the mosquito during a blood meal, wherein they mature into infective L3 larvae and are capable of being transferred to another mammal during a blood meal. The larvae migrate through subcutaneous tissues and eventually enter the bloodstream and the right side of the heart, wherein they mature into adults in a permissive host. Humans, however, are unsuitable or nonpermissive hosts. In humans, the worm dies before it reaches maturity and is swept into the pulmonary arterial circulation. It subsequently becomes lodged in the subsegmental pulmonary arteries and arterioles and causes thrombosis, infarction, inflammation, and eventually a granulomatous reaction surrounded by a wall of fibrous tissue (Fig. 8).

The geographic distribution of disease reflects the prevalence of canine dirofilariasis. The areas of highest prevalence in the United States are in the South, particularly along the Gulf and Atlantic coasts and along the Mississippi River (43). Interestingly, dog ownership is not a risk factor for disease. Prevention is centered on the control of zoonotic disease. The use of mosquito repellents, particularly in areas of high endemicity, is also recommended to interrupt transmission.

Clinical Significance

Slightly more than one-half of the patients with dirofilariasis are asymptomatic. The remainder have nonspecific symptoms, such as cough, chest pain, hemoptysis, low-grade fever, chills, and malaise (44). Only 5 to 10% of infected patients have peripheral blood eosinophilia. In addition to pulmonary manifestations, *D. immitis* has also rarely been identified in subcutaneous abscesses, the abdominal cavity, the eyes, and the testes. *Dirofilaria* species other than *D. immitis* are more likely to be found in a subcutaneous location (Table 1). Pampiglione et al. undertook a critical review of human pulmonary dirofilariasis in the Old World and suggested, based on traditional morphologic findings, that the cause of pulmonary dirofilariasis in these geographic regions may more likely be due to a *Dirofilaria* species other than *D. immitis*, namely *D. repens* (45). The nodules caused by *D. immitis* are often discovered by a routine chest radiograph and must be excised in most instances to exclude malignancy (43). These nodules are usually single, but occasionally two or three nodules may be present.

Direct Examination and Microscopy

The pulmonary nodules produced by *D. immitis* are characteristically small (0.8 to 4.5 cm; mean, 1.9 cm), subpleural, spherical, and well circumscribed. *Dirofilaria immitis* has a smooth, thick cuticle (5 to 25 μm) with the three distinct layers characteristic of the genus *Dirofilaria*; other *Dirofilaria* species that infect humans have a similar cuticle, but with external longitudinal ridges (Fig. 9 and 10). The thick, multilayered cuticle projects inwardly at the lateral chords, forming two prominent, opposing internal longitudinal ridges. The somatic musculature is typically prominent, but the lateral chords are usually poorly preserved. Transverse sections may reveal two large uteri and a much smaller intestine in female worms (Fig. 9); a single reproductive tube and an intestine are present in males. The definitive identification of the worm based on internal structures may be difficult or impossible, given the advanced stage of parasite degeneration in many of these specimens. The presence of a parasitic worm in a pulmonary artery and in association with a pulmonary infarct, however, is usually sufficient for a diagnosis.

Serologic Tests

Serologic tests to detect antibody to *Dirofilaria* are commercially available from several reference laboratories. Although advances have been made to increase specificity without sacrificing sensitivity, cross-reactivity may occur in patients infected with other nematodes, particularly other filarial worms. As with many serologic assays, a positive test is useful, whereas a negative test does not exclude the possibility of infection.

Treatment

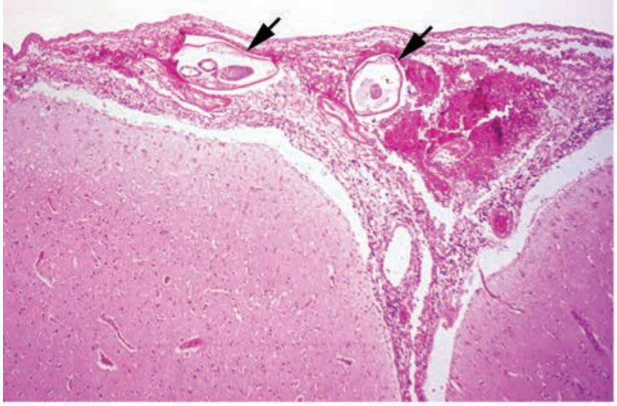
Excision of the nodule is curative. Additional antiparasitic therapy is not necessary.

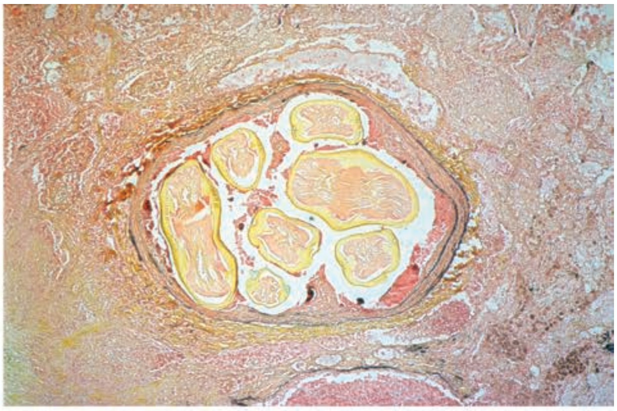
Other Less Common Nematodes

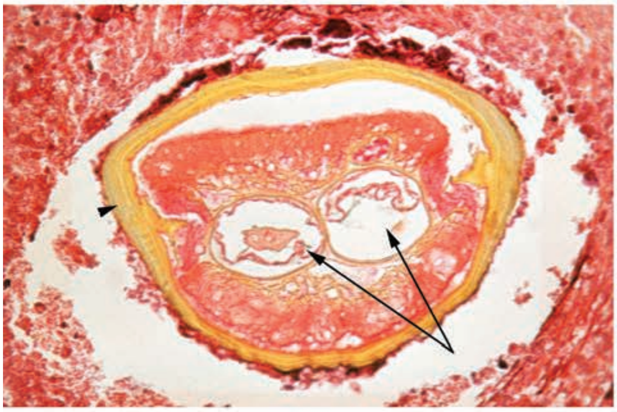
There are a number of less common nematodes other than those described here. Some of these and their important associated features are included in Table 1.

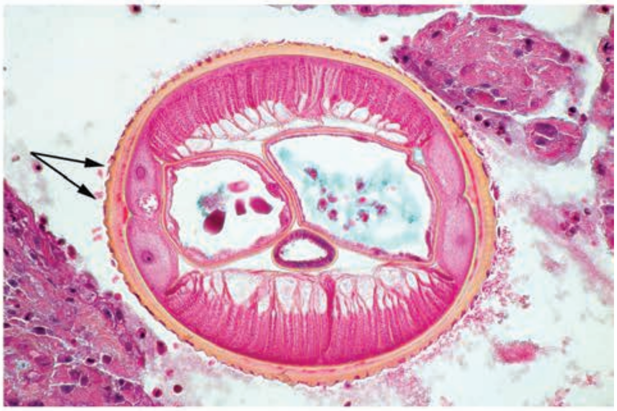
LESS COMMON CESTODES

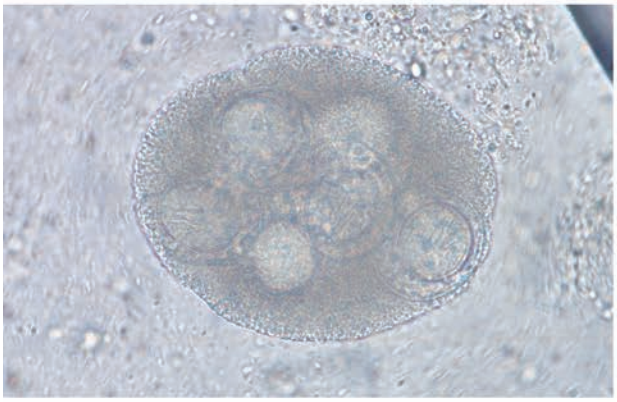
Descriptions of some less common cestodes are given below and in Table 2.

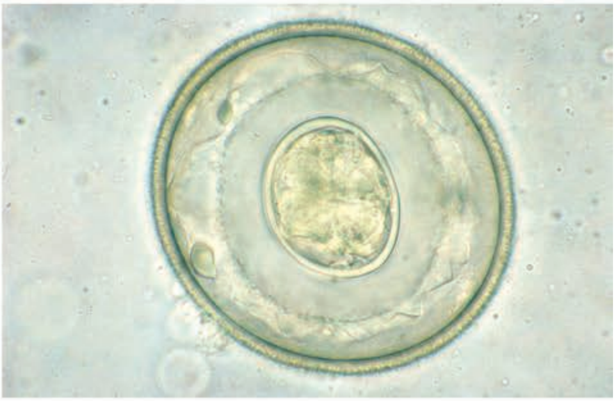












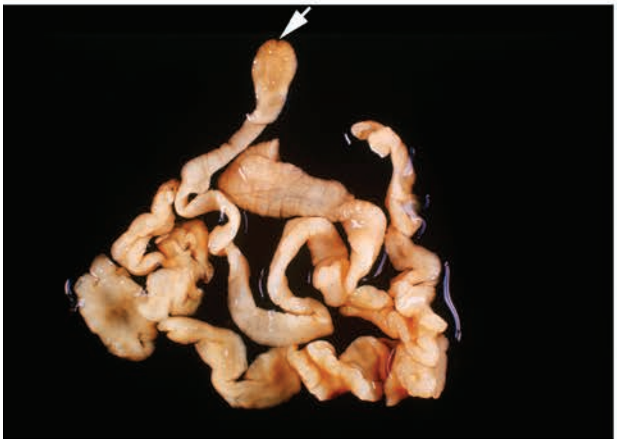


TABLE 1 Other less common nematodes

Organism	Definitive host(s)	Intermediate host(s)/vectors	Disease(s) produced	Method of diagnosis
Ancylostoma species other than *Ancylostoma duodenale*, *A. ceylanicum*, *A. caninum*, and *A. braziliense*	Cats, dogs, and hamsters	Not applicable	Ancylostomiasis, eosinophilic enteritis, cutaneous larva migrans (creeping eruption)	Detection of eggs in stool or adults in histologic sections; rarely, detection of larvae in biopsy sample of skin
Baylisascaris procyonis	Raccoon	None (geohelminth)	Larva migrans	Serology, biopsy/autopsy
Dirofilaria species other than *D. immitis*, *D. tenuis*, *D. repens*, *D. ursi*, *D. subdermata*, and *D. striata*	Raccoons (*D. tenuis*), dogs and cats (*D. repens*), bears (*D. ursi*), porcupines (*D. subdermata*), wild cats (*D. striata*)	Mosquitoes, except for *D. ursi*, which is transmitted by blackflies	Usually subcutaneous nodules that contain mature, immature, or degenerated worms	Morphological features of the worm in excised tissues (Fig. 10)

Dipylidium caninum

Description of the Agent

Dipylidium caninum, a common tapeworm of dogs and cats, also commonly infects children (46, 47). *Dipylidium* belongs to the family Dipylidiidae, the order Cyclophyllidea, and the class Cestoidea (Cestoda).

Epidemiology, Transmission, and Prevention

Dipylidiasis occurs throughout the world. This disease, like hymenolepiasis, is transmitted primarily through the ingestion of an infected flea. In the natural cycle, the dog or cat contains the intestinal adult parasite, which releases gravid proglottids filled with eggs. The eggs are ingested by fleas or lice, which are the intermediate hosts. The eggs hatch within the intermediate host, releasing larvae that penetrate the body cavity, where they mature into infective cysticercoid larvae. When an infected intermediate host is ingested, the cysticercoid metacestode larva attaches to the small intestine and matures into an adult tapeworm, completing the cycle. This maturation to the adult tapeworm occurs in the more common animal hosts, such as dogs and cats, but also occurs in humans. Prevention is achieved by deworming animals and controlling fleas and lice.

Clinical Significance

Dipylidiasis is usually an innocuous infection. Larger worm burdens may cause weight loss, abdominal pain, failure to thrive, or the appearance of colic, but such severe disease is uncommon. Disease usually comes to the attention of parents and pediatricians when motile, gravid proglottids are seen in the stool.

Direct Examination by Microscopy

The proglottids of *D. caninum* resemble a rice grain on gross examination. These differ from those of most other cestodes in that they have two genital pores (*dipylos* means two gates), which can be appreciated with the use of a dissecting microscope and by compressing the proglottid between two glass slides. The identification may also be achieved by

FIGURE 7 (row 1, left) The immature *P. cantonensis* worm (arrows) in the meninges of this patient is eliciting a marked eosinophilic response. The worm is 200 µm in diameter. Hematoxylin and eosin stain; original magnification, ×50 (AFIP negative no. 73-6862). doi:10.1128/9781555817381.ch147.f7

FIGURE 8 (row 1, right) The coiled remnants of an immature male *D. immitis* worm are present in this branch of the pulmonary artery. The maximum worm diameter is 250 µm. Movat's stain; original magnification, ×15 (AFIP negative no. 71-11563). doi:10.1128/9781555817381.ch147.f8

FIGURE 9 (row 2, left) The two uteri (arrows), muscle, and trilaminar (arrowhead), smooth cuticle are characteristic of an immature female *D. immitis* worm. The worm diameter is 250 µm. Movat's stain; original magnification, ×80 (AFIP negative no. 72-2732). doi:10.1128/9781555817381.ch147.f9

FIGURE 10 (row 2, right) The *Dirofilaria* species other than *D. immitis* have external longitudinal cuticular ridges, whereas the cuticle of *D. immitis* is smooth. *Dirofilaria tenuis* is pictured here, in cross section; it is 270 µm in diameter and has obvious cuticular ridges (arrows). *Dirofilaria* species other than *D. immitis* are often found in a subcutaneous location rather than in the pulmonary arterial vasculature. Movat's stain; original magnification, ×80 (AFIP negative no. 94-5122). doi:10.1128/9781555817381.ch147.f10

FIGURE 11 (row 3, left) An egg packet of *D. caninum*, obtained from a crushed gravid proglottid, is 150 µm in diameter. The eggs within the packet are 40 µm in diameter. Unstained (AFIP negative no. 86-7369). doi:10.1128/9781555817381.ch147.f11

FIGURE 12 (row 3, right) The thick inner membrane of the egg of *Hymenolepis diminuta* is surrounded by a gelatinous matrix and then by an outer striated shell. The eggs of *H. diminuta* are spherical, whereas those of *Hymenolepis nana* are ovoid. The egg pictured here is 80 µm in diameter. Unstained; original magnification, ×250 (AFIP negative no. 96-5119). See chapter 143 for more-detailed coverage of *Hymenolepis* spp. doi:10.1128/9781555817381.ch147.f12

FIGURE 13 (row 4, left) A sparganum superficially resembles an adult tapeworm. Close inspection, however, clarifies its immature form, with a head with only a ventral groove or bothrium (arrow) and a lack of proglottids. The maximum width is 6 mm. Unstained; original magnification, ×0.5 (AFIP negative no. 70-15303). doi:10.1128/9781555817381.ch147.f13

TABLE 2 Other less common cestodes[a]

Organism	Definitive host(s)	Intermediate host(s)	Disease produced	Diagnostic method
Hymenolepis diminuta, the rat tapeworm	Rats	Insects	Usually asymptomatic; heavy infections resemble heavy *H. nana* infections.	Eggs in the feces (Fig. 12); proglottids disintegrate before fecal passage.
Hymenolepis nana, the dwarf tapeworm[b]	Rats; human-to-human transmission possible	Fleas	This worm, the smallest adult tapeworm that infects humans, produces disease that is usually mild, and patients may be asymptomatic. Massive infections may produce abdominal pain, allergic reactions, anorexia, nausea, diarrhea or constipation, and flatulence.	Identification of characteristic eggs in the stool; proglottids disintegrate and release eggs before they are passed in the stool. The eggs are ovoid, in contrast to those of *H. diminuta*, which are spherical, and have an inner hyaline membrane, a thin egg shell, polar thickenings from which polar filaments arise, and a distinct oncosphere that contains six hooklets.
Less common *Taenia* species				
T. taeniaeformis	Cats	Rodents	Larval stage may infect liver, like cysticercosis.	Demonstration of strobilocercus in histologic sections
T. multiceps	Canids	Sheep, rabbits	Coenurosis, an infection with the coenurus in the CNS, eyes, or subcutaneous tissues (like cysticercosis)	Demonstration of the coenurus in histologic sections
Mesocestoides species	Foxes, dogs, cats, and other mammals	Unknown, possibly reptile or arthropod vectors	Asymptomatic or mild abdominal symptoms	Detection of gravid proglottid with characteristic parauterine organ. Eggs are usually not present in the stool.
Bertia species	Primates	Mites and possibly other insects	Asymptomatic or mild abdominal symptoms	Motile proglottids in the stool, similar to *D. caninum* infection. Eggs within proglottids are not within packets.
Inermicapsifer madagascariensis		Raw sugar cane ingestion has been suggested	Asymptomatic or symptoms similar to *D. caninum* infections	Motile proglottids in the stool, similar to *D. caninum* infection. Proglottid and egg morphology and number of eggs per packet are used for identification.
Raillietina species	Rodents	Insects	Asymptomatic or symptoms similar to *D. caninum* infections	Motile proglottids in the stool, similar to *D. caninum* infection. Proglottid and egg morphology and number of eggs per packet are used for identification.

[a] Praziquantel is the treatment of choice for infections with adult tapeworms; it is also effective for treatment of coenurosis. Effective preventive measures center around controlling disease in the zoonotic host (e.g., cats or dogs) or controlling the zoonotic hosts themselves (e.g., rats). Controlling intermediate hosts is also effective but may prove more difficult.

[b] This parasite is covered in detail in chapter 145 in this *Manual*.

demonstrating the characteristic egg packets (Fig. 11) and/or characteristic eggs that have distinct morphological features (i.e., four envelopes). A fecal examination is likely to be negative for eggs or egg packets, since intact proglottids are usually passed in the stool. Microdissection or histologic examination of the proglottids reveals the eggs and egg packets. In addition to the egg packets, the histologic examination of the proglottid reveals other features common to cestodes, such as a tegument, smooth muscle, and calcareous corpuscles.

Serologic Tests

Serologic tests are not usually performed, since the disease is usually subclinical and unsuspected and the diagnosis is achieved when the proglottids are discovered and examined.

Treatment

Both praziquantel and niclosamide are effective against *D. caninum*. Upon discovery, examination and treatment of household pets should proceed, as should aggressive flea control.

Spirometra Species

Description of the Agents

Sparganosis is the infection of humans by L3 plerocercoid larvae of a pseudophyllidean tapeworm (48). The plerocercoid larva in a human host does not reach maturity and is known as a sparganum. The tapeworms that cause sparganosis are *Spirometra mansoni*, *Spirometra mansonoides*, *Spirometra ranarum*, and *Spirometra erinacei*. The precise taxonomic relationship of a cestode known as *Sparganum proliferum* is unclear, but this organism may simply represent a variant of *S. mansonoides* or *S. erinacei*. *Spirometra* species belong to the family Diphyllobothriidae, the order Pseudophyllidea, and the class Cestoidea (Cestoda).

Epidemiology, Transmission, and Prevention

Adult *Spirometra* species are widely distributed tapeworms of animals, particularly dogs and cats. The parasitic cycle for these worms begins with the passage of eggs from an infected suitable host. The coracidium that emerges from the egg infects the first intermediate host, a copepod. The second intermediate host, which includes snakes, frogs, and fish, becomes infected by ingesting the infected copepod. The cycle is completed when a permissive (i.e., definitive) host ingests the second intermediate host. Humans are nonpermissive hosts and may become infected by ingesting raw or undercooked meat from a second intermediate host or by drinking contaminated water that contains the infected copepod *Cyclops*. Humans have also become infected by using infected animal flesh (e.g., frog flesh) as a poultice.

Clinical Significance

The results of ingesting a plerocercoid larva depend on both the species of the host and the species of the larva. For example, the ingestion of a plerocercoid larva of *Diphyllobothrium latum* by a human results in the development of an adult tapeworm, whereas the ingestion of a plerocercoid larva of a *Spirometra* species results only in the continued existence of the larva (i.e., the sparganum) in the new host (49). This is a situation wherein the human is behaving biologically like a second intermediate or paratenic host. The clinical features of disease are influenced by worm burden (most patients harbor only a single worm), worm location, and worm viability. Spargana migrate, but this migration usually does not cause symptoms. Migration to a subcutaneous location, however, may result in a nodule. This nodule may be excised to exclude the possibility of malignancy. Ocular sparganosis, particularly involving the conjunctiva, may result following the application of a folk medicine poultice that contains raw snake or frog tissues. Inflammation and sometimes calcification ensue following death of the sparganum, and when these occur in the brain, they may cause obstructive hydrocephalus (36).

Direct Examination and Microscopy

The spargana of *Spirometra* species are flat, ribbon-like worms that superficially resemble adult tapeworms (Fig. 13). Closer inspection demonstrates an immature anterior end without hooklets; a cleft or ventral groove, termed a bothrium, is present (Fig. 13) (50). Mature proglottids are not produced. Histopathologic examination of the sparganum demonstrates calcareous corpuscles characteristic of a cestode. Developed internal organs are not seen, but rather, irregularly scattered smooth muscle fibers and excretory ducts are seen in a loose stroma.

Serologic Tests

If the diagnosis is not suspected, it is usually made when a viable sparganum is unexpectedly discovered during surgery. The gross findings are largely diagnostic, but histopathologic examination can be used for confirmation. In such instances, serology is not useful. However, if this disease is clinically suspected, the diagnosis may be achieved through the combination of radiology and serology (51). Serologic tests for *Spirometra* are not commercially available, so one may have to send sera to specialized centers.

Treatment

Medical therapy is currently deemed unsuitable for the treatment of sparganosis; the plerocercoid larva is resistant to praziquantel. Complete surgical excision is recommended. An incomplete excision, particularly if the anterior end of the larva remains in the tissue, may result in continued growth of the organism (1).

SUMMARY

There are a wide variety of less commonly encountered helminthic parasites, which may be nematodes, cestodes, or trematodes. The diseases caused by these parasites are interesting and demonstrate their highly evolved life cycles and the complex interactions with their hosts. These diseases range from subclinical, e.g., dipylidiasis, to possibly life threatening, e.g., baylisascariasis. In many instances, the disease occurs only in a particular geographic area, which is largely determined by the biological ranges of the definitive and intermediate hosts. Dietary customs are also important in the prevalence of human disease, as many of these are associated with the ingestion of raw animal products. The treatment of these parasites varies depending on the infectious agent, but common preventive measures may significantly diminish the transmission of many of these parasitic diseases. These measures include the zoonotic control of parasitic disease in animal hosts and the vectors of transmission, washing of fruits and vegetables, access to clean drinking water, and thorough cooking of meats before consumption.

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Arthropods of Medical Importance

SAM R. TELFORD III

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Arthropods comprise a diverse group of invertebrate animals (Fig. 1), united in a common body theme (bauplan) of a jointed, chitinous exoskeleton. Four major groupings have classically been recognized: insects, arachnids, crustacea, and millipedes/centipedes; a fifth group contains only a living fossil, the horseshoe crabs, which have existed unchanged for hundreds of millions of years. All arthropod classes were extant hundreds of millions of years ago, thereby providing ample opportunity for life history traits such as parasitism to independently evolve, and evolve multiple times, in each class.

Medically important arthropods have long been considered to comprise mainly ectoparasites, parasites that limit their activities to the skin. Parasitism, however, is only one of several associations that constitute the interaction of arthropods of medical importance with humans. Arthropods may actively defend themselves against predation (crushing or swatting) by biting, stinging, piercing, or secreting noxious chemicals. Such defenses would operate regardless of the attacker, be it human or other arthropod. Passive modes of defense may inadvertently affect humans, such as irritation after brushing the urticarial hairs of certain caterpillars. Arthropods may also be medically important due to indirect effects: fear of insects, delusional parasitosis, or allergy due to dust mites. The various modes by which arthropods may affect human health thus reflect the diversity of these animals, but there are very few instances in which it may be argued that natural selection favored the reproduction of those that focused on causing misery. Accordingly, arthropods should be viewed as a normal part of the environment, which under individual circumstances may cause pathology. In addition, because of their ubiquity, spurious associations with pathology are common.

ARTHROPODS AS VECTORS

Arthropods are thought of by many in clinical settings with respect to their role as vectors, i.e., transmitters of infectious agents including viruses, bacteria, protozoa, and helminths. Infectious agents may have an obligate relationship with an arthropod (biological transmission) or may simply contaminate an arthropod (mechanical transmission). Malaria parasites undergo a complex developmental cycle within certain mosquitoes and could not perpetuate without them. In contrast, the agent of trachoma (*Chlamydia trachomatis*) is found on the external surfaces of eye gnats and flies (1) and may be transferred between hosts by the act of landing and crawling; but C. *trachomatis* more commonly perpetuates by direct contact with hosts. Mechanical transmission of an infectious agent is dependent on its stability and quantum of infection. HIV, for example, does not survive long outside the body, and the femtoliter or so of material that may contaminate the mouthparts of a mosquito would not contain enough viable lymphocytes with HIV to initiate infection; thus, mosquitoes have never been epidemiologically linked with HIV transmission, even though mosquitoes and other hematophagous arthropods obviously feed on viremic individuals.

There are five major groups of vectors: the diptera (flies and mosquitoes), the hemiptera (kissing bugs), the siphonaptera (fleas), the anoplura (lice), and the acarines (ticks and mites). The general life history strategies for each group provides the basis for understanding vectorial capacity, which is the sum of physiological and ecological attributes that allow transmission. Specific vector-pathogen relationships are discussed in detail in chapters focusing on the respective agents but are succinctly summarized here in Table 1.

Diptera

The dipteran vectors are winged insects that include mosquitoes, sandflies, blackflies, gnats, horse/deerflies, and tsetse flies. These range in size from minute (ceratopogonid midges less than 2 mm in length) to large (horseflies more than 2 cm in length). Unlike other winged insects, dipterans have only one pair of wings. Those that take blood meals as adult females may serve as vectors. Blood meals are used as nutrient to produce eggs (anautogeny); once those eggs are laid, another blood meal may be taken and more eggs produced. Thus, unless a mosquito (as an example of a dipteran) inherits infection (transovarial or vertical transmission), the first blood meal infects it and the second allows the agent to be transmitted; under favorable environmental circumstances, a mosquito may survive for several weeks and take more than two blood meals. Both male and female flies and mosquitoes also require sugar meals (usually from plant nectar), but sugar meals can result in reproduction only in certain species (autogeny).

Eggs are laid in water or within detritus. Vermiform larvae emerge from the eggs and develop through several stages (instars) in water or detritus, feeding on organic material or bacteria, culminating in pupae from which emerge new adults, thereby undergoing complete metamorphosis (holometabolous development). Depending on species and

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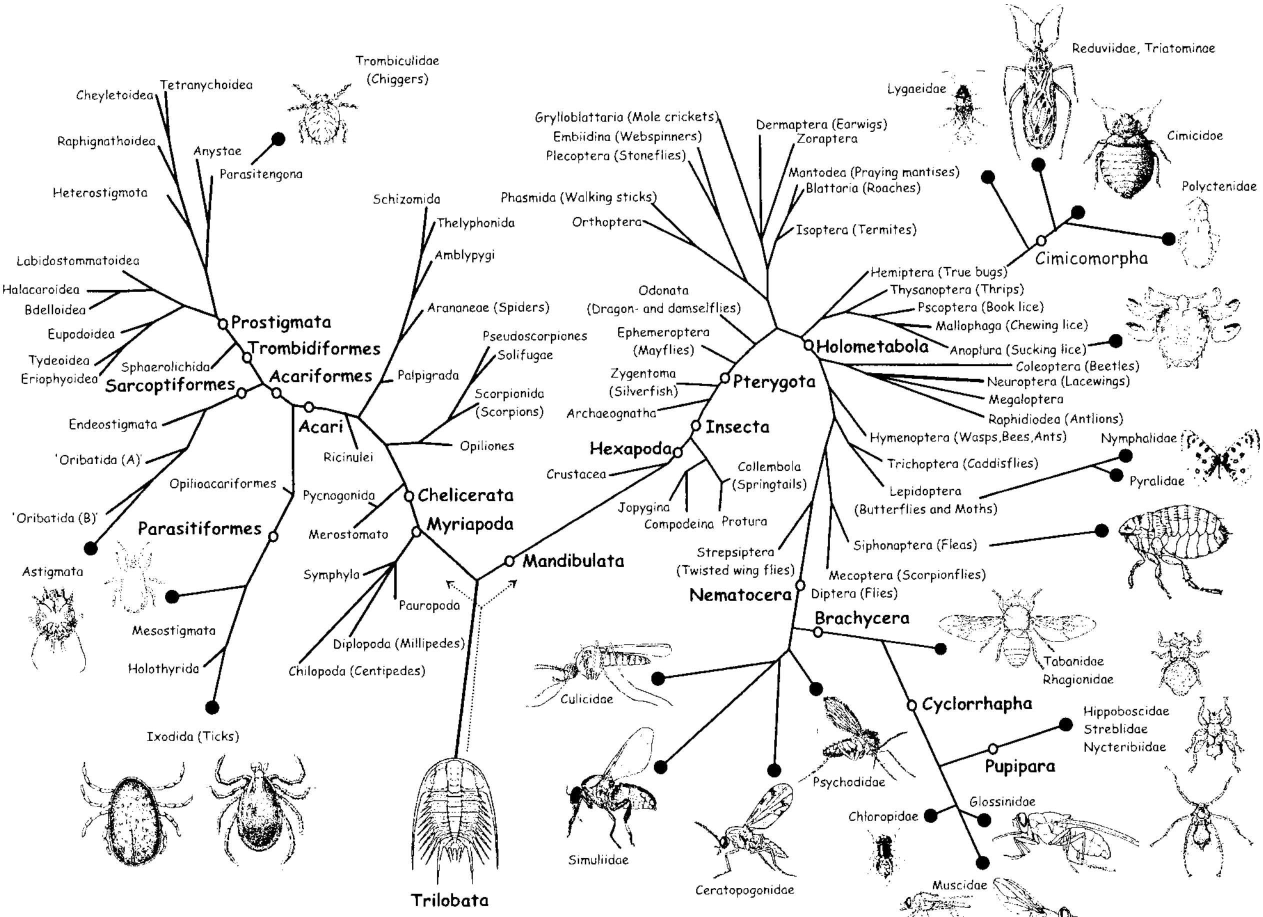
Trombiculidae
(Chiggers)
Cheyletoidea
Tetranychoidea
Raphignathoidea
Anystae
Parasitengona
Heterostigmata
Labidostommatoidea
Halacaroidea
Bdelloidea
Eupodoidea
Tydeoidea
Eriophyoidea
Sphaerolichida
Prostigmata
Trombidiformes
Sarcoptiformes
Acariformes
Endeostigmata
'Oribatida (A)'
'Oribatida (B)'
Opilioacariformes
Parasitiformes
Astigmata
Mesostigmata
Holothyrida
Ixodida (Ticks)
Acari
Ricinulei
Pycnogonida
Merostomata
Chelicerata
Myriapoda
Symphyla
Pauropoda
Diplopoda (Millipedes)
Chilopoda (Centipedes)
Mandibulata
Trilobata
Schizomida
Thelyphonida
Amblypygi
Arananeae (Spiders)
Palpigrada
Pseudoscorpiones
Solifugae
Scorpionida
(Scorpions)
Opiliones
Grylloblattaria (Mole crickets)
Embiidina (Webspinners)
Plecoptera (Stoneflies)
Phasmida (Walking sticks)
Orthoptera
Dermaptera (Earwigs)
Zoraptera
Mantodea (Praying mantises)
Blattaria (Roaches)
Isoptera (Termites)
Odonata
(Dragon- and damselflies)
Ephemeroptera
(Mayflies)
Zygentoma
(Silverfish)
Archaeognatha
Pterygota
Insecta
Hexapoda
Crustacea
Collembola
(Springtails)
Jopygina
Compodeina
Protura
Lygaeidae
Reduviidae, Triatominae
Cimicidae
Polyctenidae
Cimicomorpha
Hemiptera (True bugs)
Thysanoptera (Thrips)
Pscoptera (Book lice)
Mallophaga (Chewing lice)
Anoplura (Sucking lice)
Holometabola
Coleoptera (Beetles)
Neuroptera (Lacewings)
Megaloptera
Raphidiodea (Antlions)
Hymenoptera (Wasps,Bees,Ants)
Nymphalidae
Trichoptera (Caddisflies)
Pyralidae
Lepidoptera
(Butterflies and Moths)
Siphonaptera (Fleas)
Strepsiptera
(Twisted wing flies)
Mecoptera (Scorpionflies)
Nematocera
Diptera (Flies)
Brachycera
Tabanidae
Rhagionidae
Culicidae
Cyclorrhapha
Hippoboscidae
Streblidae
Nycteribiidae
Pupipara
Psychodidae
Chloropidae
Glossinidae
Simuliidae
Ceratopogonidae
Muscidae

FIGURE 1 Phylogeny of arthropods. (Reprinted from reference 55 with permission of Elsevier Publishing.) doi:10.1128/9781555817381.ch148.f1

ambient temperature, the duration of the dipteran life cycle may be as short as a week or so. Tsetse flies are an unusual exception to this pattern and produce only one advanced larva for each blood meal, with the larva nourished internally from milk gland analogs during a gestation of several weeks (2). This large maternal investment in offspring makes adulticiding (trapping, spraying) very effective in reducing tsetse populations and thereby reducing the transmission of the agents of sleeping sickness.

The diptera are only transiently associated with a blood meal source. Host-seeking cues include body heat, carbon dioxide (exhaled by the host), mechanical vibrations, and lactic acid or other skin-associated compounds. The flies visually seek hosts with their compound eyes, mainly larger dark-colored objects with movement, but proximal odorant cues associated with a host seem required to initiate feeding (3). The widely used repellent diethyltoluamide (DEET) works by modulating the odor-gaited ion channel formed by the dipteran odorant receptor complex on the antennae (4). Once a host has been identified, feeding is initiated and completed within minutes. Sandflies, blackflies, and mosquitoes have a diverse salivary armamentarium of pharmacologically active substances that promote finding and removing blood. During probing, antihemostatic and anti-inflammatory chemicals are secreted into the feeding channel created in the epidermis. Probing lacerates capillaries, and the ADP that is released from damaged endothelial cells would serve to promote platelet activation, but by secreting an apyrase that cleaves ADP into a monophosphate, platelet aggregation is greatly reduced during blood feeding. In addition, local inflammatory processes are temporarily diminished due to secretion of chemicals such as prostaglandin E2 (5). Thus, the first few bites from a given species remain unnoticed by the host, although factors in the saliva of blackflies may leave an egg-sized lump that is hot to the touch at the site of their bites. Such lumps may persist for several days and may be accompanied by low-grade fever. Such "blackfly fever" should not be misinterpreted as infection; it resolves on its own without treatment.

The variety of proteins that are deposited within skin during vector feeding are the basis for hypersensitivity reactions, mainly itch, in hosts that are exposed to bites more than once over the course of weeks. Repeated exposure over months may lead to tolerance and a return to failing to react to bites. Although much work remains to be done, there appears to be some cross-reactivity between salivary products from different groups of vector so that some individuals may equally react to the bites of mosquitoes, bugs, or ticks.

The blood-feeding flies have cruder salivary tools, reflecting their less elegant way of feeding, which usually involves scraping the skin with a roughened maxilla and feeding

TABLE 1 Summary of the major arthropod genera serving as vectors or scalars of infectious agents

Arthropod(s)	Etiologic agent(s)	Disease
Crustacea		
Decapods	*Paragonimus*	Paragonimiasis
Copepods	*Diphyllobothrium*	Diphyllobothriasis
	Dracunculus medinensis	Guinea worm disease
	Gnathostoma spinigerum	Gnathostomiasis
Insecta		
Anopleura		
Pediculus	*Rickettsia prowazekii*	Epidemic typhus
	Bartonella quintana	Trench fever
	Borrelia recurrentis	Epidemic relapsing fever
Siphonaptera		
Xenopsylla, Nosopsyllus	*Yersinia pestis*	Plague
	Rickettsia typhi	Murine typhus
	Hymenolepis diminuta	Rat tapeworm
Pulex, Oropsylla	*Yersinia pestis*	Plague
Ctenocephalides	*Dipylidium caninum*	Dog tapeworm disease
Hemiptera		
Panstrongylus, Rhodnius, Triatoma	*Trypanosoma cruzi*	Chagas disease
Diptera		
Aedes	*Wuchereria, Brugia*	Filariasis
	Flaviviruses	Dengue, yellow fever
	Other arboviruses	Encephalitis
Anopheles	*Plasmodium*	Malaria
	Wuchereria, Brugia	Filariasis
	Arboviruses	Encephalitis
Culex	*Wuchereria*	Filariasis
	Arboviruses	Encephalitis
Culicoides	*Mansonella*	Filariasis
Glossina	*Trypanosoma brucei*	African sleeping sickness
Chrysops	*Loa loa*	Loiasis
	Francisella tularensis	Tularemia
Simulium	*Onchocerca volvulus*	Onchocerciasis
	Mansonella ozzardi	Filariasis
Phlebotomus, Lutzomyia	*Leishmania*	Leishmaniasis
	Bartonella bacilliformis	Bartonellosis
	Phlebovirus	Sandfly fever
Arachnida		
Acari (ticks)		
Ixodes	*Borrelia burgdorferi*	Lyme disease
	Anaplasma phagocytophilum	Human granulocytic ehrlichiosis
	Rickettsia conorii	Boutonneuse fever
	Babesia	Babesiosis
Dermacentor	*Rickettsia rickettsii*	Rocky Mountain spotted fever
	Francisella tularensis	Tularemia
	Coltivirus	Colorado tick fever
	Flavivirus	Omsk hemorrhagic fever
Amblyomma	*Rickettsia rickettsii*	Rocky Mountain spotted fever
	Francisella tularensis	Tularemia
	Ehrlichia chaffeensis	Human monocytic ehrlichiosis
Hyalomma	Nairovirus	Crimean-Congo hemorrhagic fever
Rhipicephalus	*Rickettsia conorii*	Boutonneuse fever
	Rickettsia rickettsii	Rocky Mountain spotted fever
Ornithodoros	*Borrelia*	Relapsing fever
Acari (mites)		
Leptotrombidium	*Orientia tsutsugamushi*	Scrub typhus
Liponyssoides	*Rickettsia akari*	Rickettsialpox

from the pooled blood or lymph. Accordingly, fly bites are more painful regardless of prior exposure, and few successfully engorge to repletion on humans.

Freshly blood-fed diptera seek a resting place for diuresis and digestion, usually the nearest vertical surface. This behavior renders those species that bite within houses susceptible to control by indoor residual insecticide spraying.

The transient nature of infestation by blood-feeding diptera means that few specimens are submitted to clinical laboratories. Other than analyses related to confirming the diagnosis of a vector-borne infection, dipterans would come to the attention of laboratorians for issues related to hypersensitivity or for myiasis (see below).

Hemiptera

The only hemiptera that serve as vectors are the kissing or reduviid bugs, belonging to a diverse and speciose order of minute-to-large insects with compound eyes, antennae, sucking mouthparts, two pairs of wings (one delicate pair hidden under an outer pair of more robust ones), and a segmented abdomen. All are easily seen without magnification, adult bedbugs being roughly 1 cm in length, and adult triatomines ranging in size from 1 cm to more than 5 cm. Small numbers of eggs are produced by females, from which emerge miniature versions of the adults. These nymphs undergo an incomplete metamorphosis (hemimetabolous development), with development through five nymphal stages, each one requiring a blood meal to proceed. The full duration of the life cycle can be as little as 3 months or as great as 2 years. Reduviid bugs are cryptic, living within cracks of mud walls or other narrow, confined spaces. They serve as vectors of trypanosomes (*Trypanosoma cruzi*, the agent of Chagas disease; and *Trypanosoma rangeli*, an apparently nonpathogenic trypanosome that is often cotransmitted with *T. cruzi*) in the Nearctic. *Triatoma rubrofasciata* transmits a trypanosome that infects monkeys in Southeast Asia (6), but to date there is no known bug-transmitted trypanosomiasis of humans in the Palearctic. Interestingly, there are good natural cycles of *T. cruzi* transmission within the United States from California through virtually all of the southern-central states, north to Maryland, but there are only rare human cases of autochthonous Chagas disease reported. Two factors account for this paradox: people live in quality houses that are less likely to have infestations of bugs, and more importantly, the main southern U.S. vector, *Triatoma sanguisuga*, does not defecate on the host while feeding (7). Transmission of *T. cruzi* to humans requires contamination of the site of the bite or mucosa by trypanosomes that are excreted in the bug feces, although recent outbreaks that resulted from drinking sugar cane juice contaminated with reduviid excreta have been reported in Brazil (54).

As with mosquitoes, salivary products from reduviids contain a variety of pharmacologically active compounds. Unlike those of mosquitoes, repeated reduviid feedings may cause a dangerous anaphylactoid reaction in residents of houses in which the bugs are common. Scientists working with reduviid bugs often need to carry an epinephrine injector with them due to their propensity to feeding their colonies upon themselves, thereby receiving large doses of salivary antigens.

Although it is possible that a true reduviid bug may be presented by a patient for identification in clinical settings outside Latin America, it is more likely that such specimens are related heteropterans such as assassin bugs, which are insect predators, or the plant-feeding stink bugs, chinch bugs, harlequin bugs, or squash bugs. Assassin bugs may inflict extremely painful wounds by their piercing mouthparts, but these would require only typical first aid measures and perhaps a booster of tetanus toxoid given the depth of the puncture wound (probosces range in length from 3 to 8 mm). The plant feeders may issue noxious secretions, which may taste bad if accidentally ingested (from a bug that infested a fruit or vegetable that was eaten directly from the vine) but would otherwise not be a cause for concern.

Bedbugs (*Cimex lectularius*, *Cimex hemipterus*) are hemipterans but are not known to serve as vectors for any pathogen. These bugs with short broad heads, oval bodies, and four-jointed antennae undergo incomplete metamorphosis, with four nymphal stages, each taking a blood meal in order to develop. The duration of the life cycle is roughly 6 to 8 weeks but may be as long as 11 months, depending on temperature and humidity; they may survive for months without feeding. They are small, 5 mm or less in length as adults (Fig. 2). They are cryptic and require hiding places such as cracks in walls, mattress foundations, or rattan furniture. At night, bedbugs emerge and infest sleeping people, taking 10 to 20 minutes for engorgement. Feeding is often interrupted by the movements of people during their sleep, and so multiple bites may result from a single bedbug (Fig. 2). Blood meals may be taken every week or so, depending

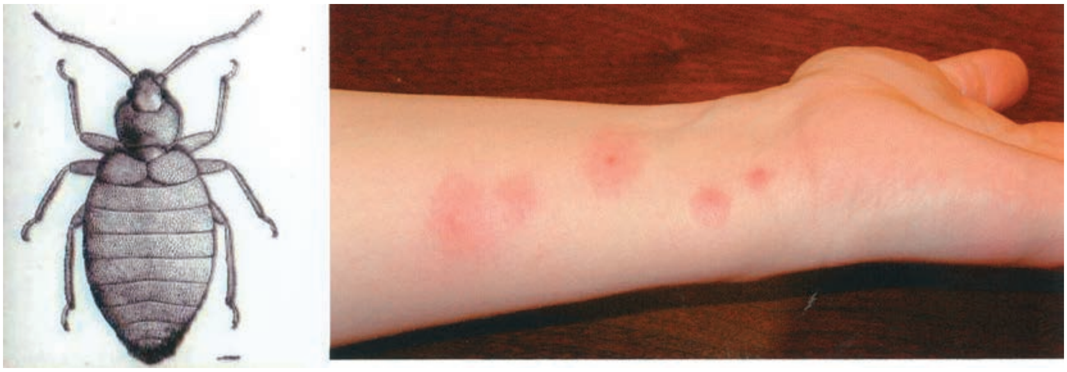

FIGURE 2 Bedbugs. (Left) *Cimex lectularius* (bedbug). Bar, 2 mm. (Source: Department of Tropical Public Health, Harvard School of Public Health). (Right) Immediate-type hypersensitivity reaction to bedbug bites acquired in a 4-star hotel in Dupont Circle, Washington, DC, in December 2005. doi:10.1128/9781555817381.ch148.f2

on the stage, with batches of 10 to 50 eggs laid; one female may lay 200 to 500 eggs in her life. Repeated exposure to bedbug bites may lead to anaphylactic reactions. Various laboratory studies have reported the survival of diverse agents such as HIV (8), hepatitis B virus, *Francisella tularensis*, or West Nile virus within artificially infected bedbugs. Epidemiologically, bedbugs have never been associated with any of these infections. Although one can never say never, the likelihood that a bedbug has served as a vector is infinitesimally small, and a physician may want to question a patient more closely on known risky practices such as intravenous drug abuse. Bedbug infestations have emerged as a major complaint of urban dwellers (http://www.cdc.gov/nceh/ehs/Publications/Bed_Bugs_CDC-EPA_Statement.htm), particularly those in multifamily homes in communities with great ethnic diversity. Bedbugs are easily transported in luggage or within discarded and repurposed furniture, particularly mattresses.

Bedbug control has become more challenging due to the development of insecticide resistance in many of their populations. A large proportion (>70%) of an infestation is associated with the bedding, mattress and box spring, and headboard and frame. The mattress and box spring should be encased in a bug-proof fabric bag. The headboard and frame need to be treated with an insecticide that penetrates all joints and cracks. All furniture within the home needs to be inspected and similarly treated, as do the baseboards of all walls and any other location where bugs and their eggs may be hiding; "bug bombs" are not appropriate for such treatments because their droplet size precludes deep penetration of such hiding places. Luggage may be treated prior to travel with the same insecticide, following the label instructions, to prevent the introduction of bedbugs into a home.

Siphonaptera

The fleas are bilaterally compressed, heavily chitinized insects with greatly modified hind legs for their characteristic jumping mode of locomotion; they lack wings. Fleas are generally small, no larger than 5 mm in length. Fleas undergo complete metamorphosis, starting from a wormlike larva feeding on organic debris and blood pellets expelled, often with remarkable force and over a long distance, from the anus of the adult flea. The larva develops through three molts (has three instars) and then secretes a silk to form a cocoon, in which it pupates. The female flea requires blood for egg production; one blood meal may serve for the production of several dozen eggs, which are laid on the fur of the host. Eggs become detached from the host's fur, coming to rest within its nest. Most fleas are known as "nest parasites." Individual fleas usually live for a couple of months, laying eggs daily, but may rarely persist for as long as a year. As with most arthropods, the duration of the life cycle is affected by temperature and relative humidity, but on average most species take 30 to 75 days to develop from egg to adult.

Of the 2,000 or so flea species that have been described, the vast majority are from rodents and have varying degrees of host specificity. Because fleas are generally nest parasites, are relatively host specific, and cannot travel long distances (crawling or jumping on the order of a few meters, implying that humans must be directly associated with their habitat for infestation to occur), only a few species are of medical importance. These include the "human" flea (*Pulex irritans*), the dog and cat fleas (*Ctenocephalides canis* and *Ctenocephalides felis*), the main plague vector (*Xenopsylla cheopis*), and the sticktight flea (*Echidnophaga gallinacea*). Of these, *C. felis* is the most notorious pest, feeding voraciously and rapidly developing dense infestations. Chronic infestations of homes are largely due to the presence of a cat or dog (despite its name, this flea does feed on either animal), their bedding, wall-to-wall carpeting, and relatively great humidity within the home. (Cold climates rarely have self-sustaining infestations because winter heating tends to dry out the carpets and molding and other places where the larval fleas tend to be hidden.) Although bites sustained over several weeks usually induce a typical delayed-type hypersensitivity reaction, with an intensely itching red spot developing at the site of the bite (usually around the ankles), note that not all members of a household react in the same manner. Some people are more attractive to arthropods than others; others react differently to bites. It is quite possible for only one person to have itchy bites and others in the same house have none, and thus a diagnosis of flea bite should not depend on the perception that if a household is infested, everyone should demonstrate similar lesions.

The human flea actually feeds on a variety of mammals, including domestic livestock. In tropical sites where homes have dirt floors and livestock share living accommodations, extremely dense infestations may develop. Although this flea is cosmopolitan in its distribution, the cat flea appears to have supplanted it as the major flea pest for humans in many countries.

An unusual flea, the chigoe or jigger (*Tunga penetrans*), attaches to a host and maintains a feeding site. Originally found in Latin America, the chigoe has been carried across to sub-Saharan Africa by humans and may be found anywhere there. This flea often penetrates under toenails or burrows into skin between toes, or in the soles of feet, an infestation known as tungiasis (Fig. 3). The female *T. penetrans* swells to 10 times its size, and the host's immune response causes skin to swell up and cover her, with only

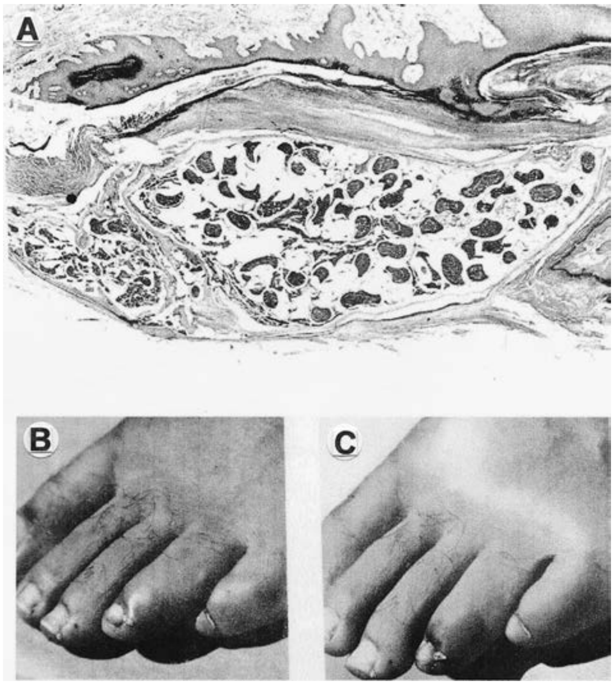

FIGURE 3 Tungiasis. (A) Low-power section of dermal lesion showing flea uterus filled with developing eggs. (B and C) Demonstration of progressive edema and secondary bacterial infection of *Tunga* lesion. (Reprinted from reference 56.) doi:10.1128/9781555817381.ch148.f3

the end of the abdomen exposed. Through this opening, she deposits eggs and feces. When the flea dies, the lesion becomes secondarily infected, causing great irritation and pain. Tourists often become infested by walking barefoot in shady spots around beaches.

Perhaps the most famous flea is the plague vector, the oriental rat flea, *Xenopsylla cheopis*. Although it prefers rats (*Rattus rattus*, *Rattus norvegicus*) as hosts, in their absence (or when they die) it feeds on humans and other animals. These fleas may be found on rats in virtually every tropical or warm temperate port city around the world, having been transported there with their hosts by trade ships. Plague is usually maintained in enzootic foci by wild rodents and their more specific fleas, but in Vietnam, Madagascar, and India, rats and *X. cheopis* appear to be important for perpetuation. Although enzootic plague usually results in sporadic cases, the great fecundity of rats under the right circumstances means that explosive outbreaks of plague may occur in urban areas, with hundreds or thousands of cases. Human exposure in the western United States, where plague is enzootic, may be due to chance contact with ground squirrels or their fleas (often *Diamanus montanus*) in sheds, disused cabins, and crawl spaces or by digging around burrows. Domestic cats often serve as an intermediary, hunting moribund rodents and subsequently exposing their owners.

Phthiraptera

Infestation by lice is called pediculosis. The lice are wingless, flat (dorsoventrally), elongate, small (0.4- to 10-mm) insects that are generally characterized by strong host specificity. Classically, two orders were recognized, the Mallophaga (chewing lice) and the Anoplura (the sucking lice); the former now comprises three suborders, and the latter remains as a suborder, all within the order Phthiraptera, which contains about 4,000 species. Of these, the sucking lice are clinically relevant, although the chewing lice may be presented as spurious ectoparasites or associated with double-pored tapeworm (*Dipylidium caninum*) infection. Most chewing lice are commensals of birds, whereas the sucking lice feed mainly on mammals.

Lice undergo incomplete (hemimetabolous) development, with nymphal forms resembling adult lice, and are often found concurrently with the adults. Thus, size may appear to greatly vary within a single collection of specimens from one host. All lice are delicate and very sensitive to temperature and humidity requirements; all die within days without the host.

Chewing lice usually are found in the feathers of birds, feeding on skin fragments. Virtually all chewing lice lack piercing mouthparts and therefore do not feed on blood. The dog-biting louse (*Trichodectes canis*) may be presented as a specimen because it is a common commensal of dogs and may be spuriously associated with "bites." Dogs serve as definitive hosts for *D. caninum*, the eggs of which are shed in feces; the feces dry on the fur, and the louse may then ingest the eggs. The tapeworm eggs hatch, and a cysticercoid becomes localized in the hemocoel of the louse. Transmission is effected when the louse is accidentally ingested, usually when the dog is grooming; humans (usually children) are incidentally infected if their hands become contaminated and then touch food or drink or are placed in their mouths.

Book lice (order Psocoptera, also known as psocids) resemble chewing lice and live on molds infesting old books. They are frequently presented as specimens in diagnostic workups for itches of unknown etiology but do not infest humans or any other animal.

The lice of greatest clinical importance (Fig. 4) are the head louse (*Pediculus humanus capitis*), the body louse (*Pediculus humanus corporis*), and the pubic louse (*Phthiris pubis*). All of these sucking lice have prominent claws attached to each of their legs, morphologically adapted for grasping the hairs of their host. They feed at least daily and deposit 1 to 10 eggs from each blood meal, gluing one egg at a time onto the shafts of hairs (or in the case of the body louse, onto threads within clothing). The eggs, or nits, are almost cylindrical in shape and have an anterior operculum. Nits hatch within 4 to 15 days, and each of the three nymphal stages lasts 3 to 8 days. Lice are transferred between hosts by close physical contact or, in the case of body lice, by sharing clothing. Schools are excellent sites for the spread of a louse infestation, not only through the sharing of hats and scarves, but by children deliberately infesting others during play. It is possible that transient circumstances, such as a subway headrest recently used by an infested head, could serve as the means for transfer. The oft-tendered excuse "I got it from a toilet seat" with reference to pubic lice should not be dismissed out of hand, but the probability

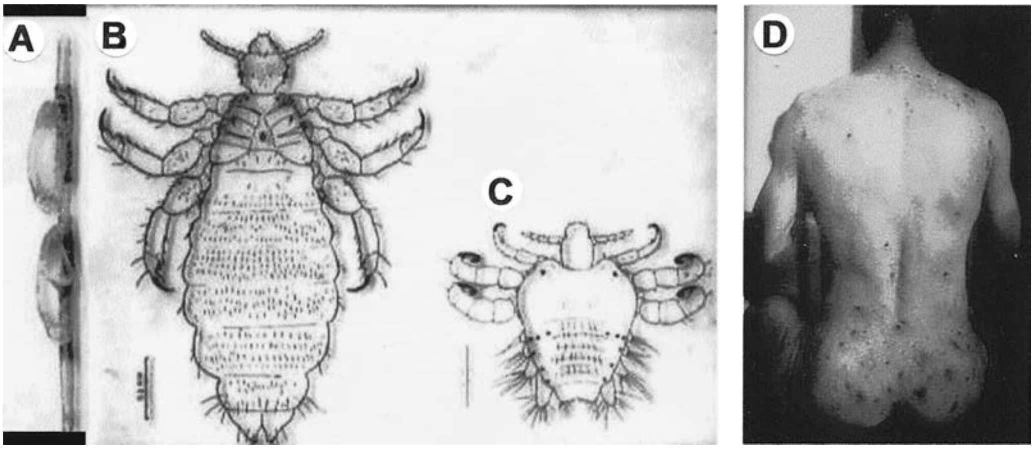

FIGURE 4 Lice. (A) Head louse nits; (B) body louse; (C) pubic louse; (D) vagabond's disease. (Source: Department of Tropical Public Health, Harvard School of Public Health).
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of acquiring a louse in this manner is far less than by skin-to-skin contact. On the other hand, the presence of pubic lice on the eyebrows usually denotes a sexually transmitted infestation, and pubic louse infestation of a child is cause for an inquiry into the possibility of child abuse.

Body or pubic louse infestation may result in intense irritation for several days, with each bite generating a red papule. Chronically infested individuals may become desensitized or may develop a nonspecific febrile illness with lymphadenopathy, edema, and arthropathy (although such signs and symptoms should prompt a search for the agent of trench fever). A very few chronically infested individuals may develop "morbus errorum" or vagabond's disease (Fig. 4), with a thickening and dark pigmentation of the skin (9).

Although body louse or pubic louse infestation may be considered evidence of poor hygiene or poor judgment, infestation by head lice should not be a stigma, as it occurs in the best of families. Nor should head louse infestation be considered to be a public health menace or even a clinical problem. Very few infestations are dense enough to cause signs or symptoms, and head lice are not vectors. Nonetheless, draconian measures are taken by many school districts, banning an infested child until treatment is thorough enough to remove all nits. Many such schools fail to discriminate between live and dead nits, insisting that all traces of them be absent. Complete removal of all nit remnants can be very difficult, relying mainly on the use of fine-toothed combs, a tool that has been found in the tombs of the Egyptian pharaoh (10).

The sucking lice are both important vectors and pests. Among the most notorious vectors in history are the body lice, which may serve as vectors for the agents of epidemic typhus, trench fever, and louse-borne relapsing fever. Napoleon's invasion of Russia in 1812 was probably thwarted by epidemic typhus decimating his troops. At the end of World War I, it is said that a typhus epidemic in Russia and Romania killed 800,000 people (11). Body lice are the product of poor hygiene, with clothes never or rarely being changed, such as in cold weather, by the homeless, or in refugee camps. Trench fever may be common in the homeless, and the most recent large outbreak of typhus was in Burundi, in Rwandan refugees. Oddly, even though the head louse is conspecific and appears vector competent in laboratory experiments, it has not been epidemiologically associated with any of these infections, nor has the pubic louse.

Body louse infestations are easily controlled by changing clothes frequently and bathing regularly. Clothes that have been removed from infested individuals should be destroyed or, at the very least, securely bagged in plastic and left for several weeks so that any lice that are present may starve. Clothing may also be washed in hot water and placed in a hot dryer for at least 30 minutes. Pubic lice may be treated by shaving the pubic hair and changing underwear regularly or by a topical pediculicide as for head lice. Head lice may be removed mechanically using specifically designed louse combs, but treatment using a pediculicide is more efficient. Although insecticide resistance is widespread, the common pediculicides containing permethrin or malathion should still be used as the first-line therapy, along with environmental hygiene (laundering bedding and clothing, particularly hats; vacuuming cushions and mattresses) and screening of family members and close friends for infestation. Retreatment a week later is required because these topical pediculicides do not act efficiently on the developing lice within nits. If infestations persist after a retreatment complemented by environmental hygiene, the new generation of prescription pediculicides such as ivermectin or spinosad should be used (12).

Acarina

The acarines are a subclass within the class Arachnida, which also contains the spiders. Acarina comprise the mites and ticks, tiny to small arthropods with eight legs as nymphs and adults (as opposed to six for insects) and with fused main body segments as opposed to three discrete ones for insects. Acarine baupläne are characterized by two functional body parts, the gnathosoma (or capitulum), which comprises the "head," and the idiosoma, which performs all the remaining functions (reproduction, motility, digestion). They undergo incomplete metamorphosis, passing through a larval and one to several nymphal stages before attaining sexual maturity. The mites are one of the most speciose groups of animals, with 45,000 recognized species. They are among the oldest terrestrial animals, dating in the fossil record to the Devonian (nearly 400 million years ago), and are found in every habitat on earth. There are two major orders of the acarines, the Acariformes and the Parasitiformes. In the former, there are two main groups, the Sarcoptiformes (astigmata) and the Trombidiformes (prostigmata), both of which contain species of clinical significance. The latter comprises three orders, the Holothyrida, the Ixodida, and the Mesostigmata. Of these, only the last two are clinically significant; the holothyrids consist of about 20 species that are found only in Australasia and some neotropical forests and are unlikely to be encountered (although they are known to secrete a toxin that may incapacitate a human that has ingested such a mite). The mesostigmata (also known as gamasida or dermanyssoidea) include some that infest birds or rodents, which under certain circumstances infest humans and cause itch. The ixodida consist of the ticks, which are essentially very large mites.

Other than the ticks and some mesostigmatids, mites are generally not considered vectors of agents that infect humans. They are, however, important for their pest potential, causing itch, dermatitis, and allergy.

The house mouse mite (*Liponyssoides sanguineus*) is the vector for rickettsialpox due to infection by *Rickettsia akari*. The relatively mild disease, characterized by fever and exanthema, was first described after a garbage strike in Kew Gardens in the Bronx, New York, NY, during the 1940s (13). Garbage piled up, and house mice populations became dense. When the strike resolved and the garbage was removed, the mice died or emigrated, leaving behind dense infestations of hungry mites. The prostigmatid trombiculid mites (chiggers) are the vectors for the agent of scrub typhus (*Orientia tsutsugamushi*), a rickettsiosis of Eurasia and northeastern Australia. Nearly 3 billion people live in countries where scrub typhus is endemic. Scrub typhus is acquired from infestation by tiny (0.2-mm) larval trombiculid mites such as *Leptotrombidium deliense* or *Leptotrombidium akamushi*; an eschar forms at the site of the chigger bite, with proximal lymphadenopathy, fever, headache, exanthema, and myalgia. Case fatality rates can range from 5 to 35% (14). Interestingly, the nymphal and adult stages of this mite feed on detritus or other arthropods and do not take vertebrate blood. Therefore, *O. tsutsugamushi* relies mainly on transovarial or vertical transmission (passage through the egg) for perpetuation, although rodent hosts that are infected may feed noninfected larvae and generate new matrilineages of infected mites. All other vector-borne agents have greater opportunities for horizontal transmission, that is, amplification by infecting a vertebrate host, and having multiple blood meals during development.

Mites are extremely difficult to identify, particularly given the likely confusion with dust mites or other ubiquitous free-living forms. All require clearing and mounting on a slide and examination under a compound microscope.

Ticks are prolific vectors, with more recognized transmitted agents than any arthropod other than mosquitoes. All of the nearly 900 known species of ticks require blood for their development and reproduction. Clinically relevant ticks belong to either the Ixodidae (the hard ticks) or the Argasidae (the soft ticks); a third family, the Nuttalliellidae, comprises a monotypic genus of soft ticks found in southern Africa and for which an association with a pathogenic agent has yet to be described. Hard ticks are so named because of the hardened sclerotized idiosomal shield or scutum. In female hard ticks, the scutum is on the anterior third of the idiosoma, with the remainder of the idiosoma consisting of pleated, leathery cuticle that allows for tremendous expansion during blood feeding. In male hard ticks, which may or may not feed at all, the scutum extends the length of the idiosoma. In contrast, soft ticks have no scutum; their entire idiosoma is leathery.

The "head," or capitulum, consists of the holdfast (hypostome), the chelicerae (which are homologs of insect mandibles), and the palps, which cover the mouthparts (hypostome and chelicerae) and serve a sensory function. Chelicerae act as cutting organs, the two sides sliding past each other, with the cutting teeth at the end gaining a purchase into a host's skin. The hypostome is thereby inserted and allows anchoring of the entire tick due to recurved, backward-facing teeth or denticles (Fig. 5). Many hard ticks also secrete a cement around the hypostome. Often, when removing an attached dog tick (*Dermacentor variabilis*) from a host, a large piece of skin comes with the hypostome, mostly cement and surrounding epidermis. Thus, by virtue of the cement and denticles, tick hypostomes rarely emerge intact when a tick is removed. "Leaving the head in" is not critical, and most times the remnant is walled off as a foreign body or works itself out, perhaps by the act of scratching. Treatment, therefore, should simply be disinfection of the site of the bite and certainly not excavation of the epidermis looking for the head. Soft ticks are transient feeders and are only rarely found attached.

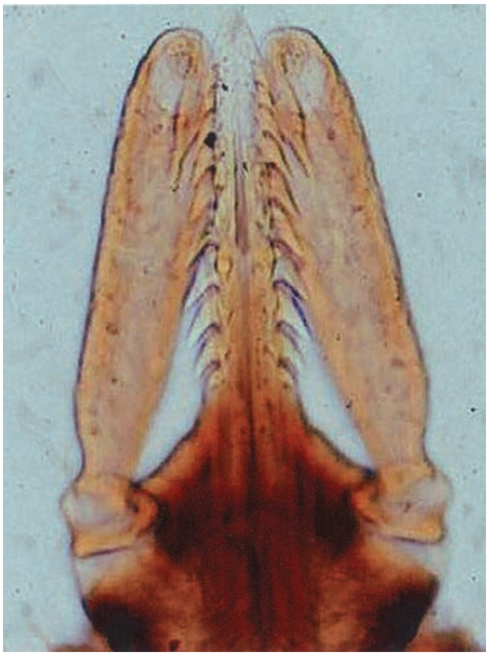


FIGURE 5 Tick hypostome, showing recurved denticles. doi:10.1128/9781555817381.ch148.f5

Hard ticks require several days to complete their blood meal; the number of days depends on the species and stage of the tick. North American deer ticks (*Ixodes dammini*, but frequently referred to as *Ixodes scapularis* [15, 16]) feed 3 days as a larva, 4 days as a nymph, and 7 days as the female. The closely related European sheep or castor bean tick, *Ixodes ricinus*, feeds 2 days as a larva, 3 days as a nymph, and 7 days as the female. The duration of feeding depends also on host immune status (previous exposure may induce immediate-type hypersensitivity, which slows down the feeding process) and temperature (*I. dammini* or *I. ricinus* feeds twice as long on cold reptiles as on those that are held at 37°C). The extended duration of feeding is required for the cuticle to soften so that the idiosoma may accommodate 10 to 100 times its weight in blood; and the site of the bite is prepared so that a pool of lymph and blood is available for removal. During the first 70% of the feeding process, very little blood or lymph appears to be present within ticks, which remain dorsoventrally flat. Hemoglobin is excreted from the anus, lipids are retained, and water from the blood is recycled back into the host as saliva (17). In the last day, usually in the last 3 or 4 hours of the blood meal, the tick takes what has been termed "the big sip," removing a large volume of whole blood and then detaching and dropping from the host.

Because they must remain attached for days, hard ticks have evolved means of temporarily disabling a host's local inflammatory response, which might inhibit its feeding. Hard tick saliva is an extremely complex mixture of anticoagulant, anti-inflammatory, and antihemostatic agents (18) that act mainly at the site of the feeding lesion. Tick saliva also neutralizes Th2 responses systemically (19). Hosts that have never been exposed to ticks do not realize that a tick is attached. Indeed, most patients with Lyme disease or spotted fever never knew that they had been "bitten" (20, 21). The most dangerous tick is not necessarily the one that a patient finds and removes, thus aborting the transmission process, but the one that he or she never knew was there and which was able to complete its feeding.

In contrast, soft ticks are more like mosquitoes in their feeding, spending tens of minutes to no more than a few hours feeding, usually as their host is sleeping. Soft tick saliva does not "need" to be as biochemically complex, and in fact some species have painful bites. The pajuello (pajaroello), *Ornithodoros coriaceus*, of California and Mexico is renowned for its "toxic bite," causing local pain and burning (9).

Tick life cycles have an extended duration, usually months or years. Deer ticks, for example, take 2 years to go from egg to egg. For this reason, there is generally no risk associated with hard ticks engorging and dropping off a companion animal within a patient's home. The engorged tick will not feed again and will take weeks to molt or lay eggs, and in the interim, usually the relative humidity within the house is too low for extended survival of the tick. On the other hand, cats as opposed to dogs appear to be a risk factor for acquiring Lyme disease, perhaps because deer ticks feed well on dogs but poorly on cats; ticks, particularly nymphs, may detach in mid-feed and readily reattach to the cat's owner (22). The exception to the lack of risk associated with ticks engorging and detaching in the household is with the brown dog tick (*Rhipicephalus sanguineus*), which is the vector of Marseille fever (Mediterranean spotted fever, boutonneuse fever) and of *Ehrlichia canis* and has also recently been documented as a vector of Rocky Mountain spotted fever. These ticks hide behind wall molding to molt and indeed are known for dense infestations covering the interior walls of dog kennels.

Ticks can be difficult to identify, depending on their state of engorgement and whether mouthparts are intact. Although tick systematists have recently altered some of the generic epithets, in general the classic Centers for Disease Control and Prevention (CDC) diagrams of the mouthparts and idiosoma (Fig. 6) can be used to classify a tick to at least the genus level. Often, simply knowing the country in which a tick may have been acquired can significantly narrow down the list of possibilities (Table 2).

ARTHROPODS AS "SCALARS"

Vectors impart directionality to a pathogen. In contrast, there are arthropod-pathogen relationships that are not characterized by directionality, and in a manner analogous to mathematical terminology, arthropods that inadvertently serve as a source of infection are called scalars (23). Helminths may use an arthropod as an intermediate host, but that arthropod does not deliver the infectious stage of the helminth during an obligate behavior such as blood feeding. Drinking water with copepods (crustacea) containing third-stage larvae of the filarial nematode *Dracunculus medinensis* initiates infection when the copepods are digested by stomach acids, thereby liberating the nematode larvae; but the copepods did not swim towards a vessel scooping water out for drinking. Accordingly, the patient's history needs to specifically address the following: the possibility of exposure via drinking from natural bodies of water (copepods and *Dracunculus*); the presence of flour beetles, fleas, or roaches (hymenolepidid cestodes, *Dipylidium caninum*; *Gongylonema* spp.); eating crabs or crayfish (*Paragonimus* spp.); or being in an environment with dense infestations of houseflies (trachoma). With the exception of houseflies and trachoma (which perpetuates in the absence of flies, spreading by direct contact with ocular exudates), all these are obligate relationships.

Muscoid Flies

The muscoid diptera include the muscids (houseflies, stable flies), the Calliphoridae (blowflies), and the Sarcophagidae (the flesh flies). Egg deposition and larval development occur in characteristic materials, *viz.*, fecal material for houseflies, decaying plant material for stable flies, live flesh for blowflies, and carrion for the flesh flies. These flies can be remarkably prolific within short periods of time: a female housefly, for example, deposits 100 to 150 eggs in moist, decaying organic material, usually excrement, at one time but may do so 20 or more times (24). Larvae emerge from the eggs within 12 hours. Three larval stages develop during a week, and a puparium is formed. The adult fly emerges from the pupa within 4 days, and copulation may occur within 1 day. Thus, a full life cycle may take as few as 12 days.

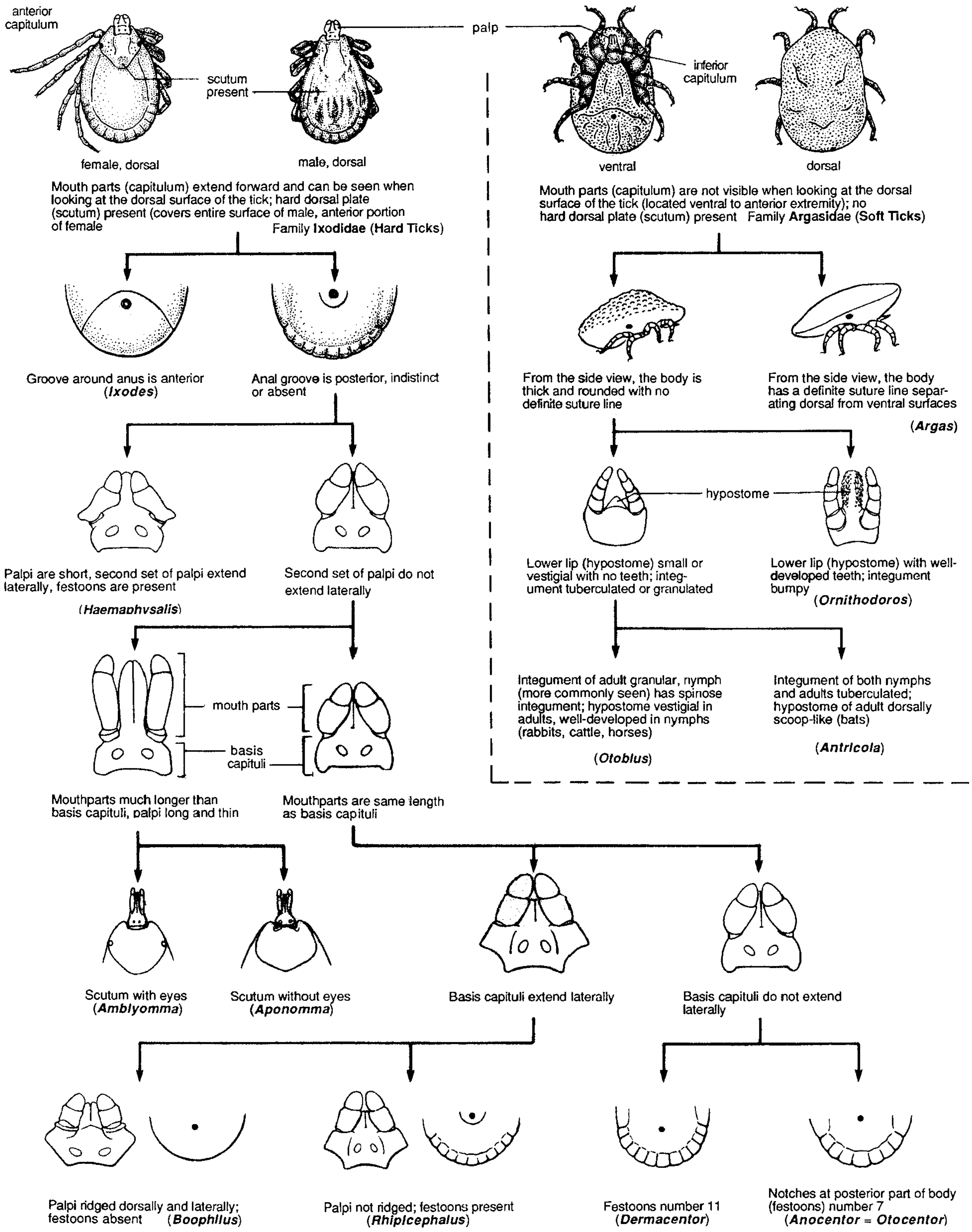
Houseflies have received much attention for their potential as scalars because of their association with poor hygiene. Flies are strong fliers and move readily from outdoors to indoors. A large fleshy structure at the apex of the proboscis provides a surface for contamination, as do the hairy body and legs of the fly. In addition, flies may regurgitate while feeding, and the vomitus may contain organisms that were acquired in a previous landing. Houseflies commonly feed on human excrement, and virtually every possible enteric pathogen (those causing amebiasis, cholera, typhoid, hepatitis A, poliomyelitis; even roundworms and *Helicobacter pylori*) has been detected within or upon them. With few exceptions, such findings are epidemiologically irrelevant inasmuch as all of the agents perpetuate in their absence. Poliovirus, for example, was recovered in flies captured from sites where polio was actively being transmitted (25), but use of dichlorodiphenyltrichloroethane (DDT) failed to curtail the epidemic. In contrast, residual DDT treatment of army camps reduced the incidence of shigellosis (26). It is likely that individual cases of enteric disease may derive from fly contamination of food, but whether the risk of such an event merits worry by patients or their health care providers remains unclear. Dense infestations of flies should be reduced (by source reduction, i.e., by preventing flies from getting access to garbage and excrement); a few flies in the house do not warrant setting off a bug bomb.

Cockroaches

Roaches are dorsoventrally flattened, smooth-bodied, winged insects with long antennae, biting-type mouthparts, and abdominal projections (cerci). The outer pair of wings is thick and leathery and the inner pair membranous. Roaches may fly but usually scuttle about on long spiny legs. They undergo incomplete metamorphosis, with the immature forms looking like miniature adults, although without wings. Eggs are laid within a hard capsule, the ootheca, which is deposited in a dark crevice. Development is slow, taking about 4 weeks between molts; many roaches have only one generation each year. Roaches can live for months without food, but water seems critical. They are omnivorous, feeding on the finest of foods to the vilest of waste, usually at night. Secretions deposited by scent glands (including trail and aggregation pheromones) give rise to a characteristic disagreeable odor that confirms an infestation even when live roaches cannot be found. Common roaches range in size from the small German cockroach (*Blattella germanica*), about half an inch in length, to the American cockroach, nearly two inches. Much work has been done attempting to incriminate roaches as scalars, even estimating nearly 14,000 CFU of bacteria (mainly *Staphylococcus* spp.) on the surface of the body of one German cockroach (9). Cholera-laden feces were fed to American cockroaches, with ingestion of as much as 200 microliters of material and recovery of live vibrios 30 to 80 hours later from roach feces (27). A roundworm (*Gongylonema* spp.) normally infecting ungulates may encyst within roaches and be transmitted to humans when ingested, usually as a contaminant of food. As with flies, the presence of roaches suggests poor environmental hygiene, but only rare instances of enteric disease might be associated with them.

The main role of cockroaches in public health appears to be as a major cause of asthma, perhaps as commonly a cause as is the dust mite, as well as a cause of wheezing, rhinitis, or atopic dermatitis. Their feces (frass) contains the allergens Bla g 1 and Bla g 2, which cross-react with known allergens such as fungi, as well as tropomyosin, a panallergen found on dust mites, crustaceans, and mollusks (28). Patients sensitized to cockroach allergens frequently (>70% of the time) have specific IgE reactivity to the 20- to 90-kDa Bla g 1 protein. Although sensitization is a main cause of frass-associated rhinitis, prior exposure is not required to trigger airway inflammation.

Dense infestations of cockroaches may develop quickly and promote respiratory illness. Hence, any detection of an



Mouth parts (capitulum) extend forward and can be seen when looking at the dorsal surface of the tick; hard dorsal plate (scutum) present (covers entire surface of male, anterior portion of female Family **Ixodidae** (**Hard Ticks**)

Groove around anus is anterior (***Ixodes***)

Anal groove is posterior, indistinct or absent

Palpi are short, second set of palpi extend laterally, festoons are present (***Haemaphysalis***)

Second set of palpi do not extend laterally

Mouthparts much longer than basis capituli, palpi long and thin

Mouthparts are same length as basis capituli

Scutum with eyes (***Amblyomma***)

Scutum without eyes (***Aponomma***)

Basis capituli extend laterally

Basis capituli do not extend laterally

Palpi ridged dorsally and laterally; festoons absent (***Boophilus***)

Palpi not ridged; festoons present (***Rhipicephalus***)

Festoons number 11 (***Dermacentor***)

Notches at posterior part of body (festoons) number 7 (***Anocentor* = *Otocentor***)

Mouth parts (capitulum) are not visible when looking at the dorsal surface of the tick (located ventral to anterior extremity); no hard dorsal plate (scutum) present Family **Argasidae** (**Soft Ticks**)

From the side view, the body is thick and rounded with no definite suture line

From the side view, the body has a definite suture line separating dorsal from ventral surfaces (***Argas***)

Lower lip (hypostome) small or vestigial with no teeth; integument tuberculated or granulated

Lower lip (hypostome) with well-developed teeth; integument bumpy (***Ornithodoros***)

Integument of adult granular, nymph (more commonly seen) has spinose integument; hypostome vestigial in adults, well-developed in nymphs (rabbits, cattle, horses) (***Otobius***)

Integument of both nymphs and adults tuberculated; hypostome of adult dorsally scoop-like (bats) (***Antricola***)

FIGURE 6 Key to major tick genera in the United States. doi:10.1128/9781555817381.ch148.f6

TABLE 2 Likely human-biting ticks and possible tick-borne infections by global region

Region	Likely tick(s) infesting humans	Possible zoonosis[a]
North America	*Ixodes dammini*, *Ixodes scapularis*, *Ixodes pacificus*, *Ixodes dentatus*, *Ixodes cookei*	Lyme disease, babesiosis, HGE, Powassan fever
	Dermacentor variabilis, *Dermacentor andersoni*, *Dermacentor occidentalis*	RMSF, CTF, tularemia
	Amblyomma americanum, *Amblyomma maculatum*	Masters' disease/STARI, RMSF, tularemia, HME
	Ornithodoros hermsi, *Ornithodoros turicata*	Relapsing fever
South America	*Amblyomma cajennense*	RMSF
	Ornithodoros spp.	Relapsing fever
Europe, Russia	*Ixodes ricinus*, *Ixodes persulcatus*	Lyme disease, babesiosis, HGE, TBE
	Dermacentor marginatus	TIBOLA
	Rhipicephalus sanguineus	Marseille fever, HME?
Japan, China, Korea	*Ixodes ovatus*, *I. persulcatus*	Lyme disease, babesiosis, HGE, TBE
	Dermacentor spp.	Tick typhus, JSF?
	Haemaphysalis spp.	JSF?
South Asia	*Haemaphysalis* spp.	KFD
	Hyalomma spp.	CCHF
	R. sanguineus	Tick typhus
Southeast Asia	*Ixodes granulatus*	Lyme disease?
	R. sanguineus	Tick typhus? HME?
Australia	*Ixodes holocyclus*	Tick typhus
North Africa	*Hyalomma* spp.	CCHF
	R. sanguineus	Marseille fever, HME?
	I. ricinus	Lyme disease
West Africa	*Hyalomma* spp.	CCHF
	Ornithodoros erraticus	Relapsing fever
	Amblyomma variegatum	ATBF
Sub-Saharan Africa	*A. variegatum*, *Amblyomma hebraeum*, *Haemaphysalis leachi*	ATBF
	Hyalomma spp.	CCHF
	Ornithodoros moubata	Relapsing fever

[a] HGE, human granulocytic ehrlichiosis (*Anaplasma phagocytophilum*); HME, human monocytic ehrlichiosis (due to either *Ehrlichia chaffeensis* or *Ehrlichia canis*); RMSF, Rocky Mountain spotted fever; STARI, southern tick-associated rash illness (etiology unknown, possibly *Borrelia*); CTF, Colorado tick fever; TIBOLA, tick-borne lymphadenopathy (*Rickettsia slovaca*); JSF, Japanese spotted fever (*Rickettsia japonica*); TBE, tick-borne encephalitis; CCHF, Crimean-Congo hemorrhagic fever; KFD, Kyasanur Forest disease; ATBF, African tick bite fever (*Rickettsia africae*).

active infestation should prompt control efforts. Control of roach infestations can be difficult. Boric acid, deposited along walls, behind moldings, and around other sites where they may hide, is effective in killing adults and nymphs by abrading cuticle between abdominal segments, rendering the roach prone to desiccation. Removing standing water (wiping up and getting rid of clutter around sinks) can also reduce infestations by preventing access to water. Roach infestations are most common in apartment buildings serving transient student or immigrant populations, who bring the insects in with their household goods.

DIRECT INJURY DUE TO ARTHROPODS

Arthropods Typically Thought of as Vectors

Vectors such as lice, ticks, bugs, fleas, mosquitoes, and blackflies may directly cause injury by their bites, either by hypersensitivity reactions or toxic effects of their salivary products. Hypersensitivity reactions manifest mainly as itch, with the concomitant potential for secondary infection due to scratching. Bedbug and flea bites may cause immediate-type hypersensitivity reactions with itchy erythema greater than 3 cm in diameter (Fig. 2). Ticks may induce a chronic local granulomatous lesion, persisting for months, perhaps due to remnants of the mouthparts (denticles) left within the epidermis. This phenomenon is particularly pronounced with "seed tick" infestation (stepping into a newly emerged batch of larval *Amblyomma* ticks, often thousands), where dozens or hundreds of ticks may attach at the belt line. Itch may be immediately relieved by calamine (or pramoxine [Caladryl] with diphenhydramine [Benadryl]) lotion or even holding the affected part under very hot running water, which will induce mast cells to degranulate. Over-the-counter hydrocortisone creams may help mild cases of itch, but severe cases may require prescription strength steroid cream (e.g., 0.05% betamethasone). Daily application of hydrocortisone should promote a resolution of itch within a week. Tick granulomas may be treated with tretinoin (Retin A) gel (0.05%), which may promote the turnover of epidermis and ejection of remaining antigenic material (personal observation).

As mentioned in the introduction to diptera, blackfly bites may produce "blackfly fever," usually as a dose-dependent reaction. Usually, the sites of the bites become edematous, with a golf ball-sized lump and an oozing punctate lesion. Fever and myalgia manifest that night and disappear within 24 hours. Such symptoms should not be construed as infection; few pathogens, if any, have such a short prepatent period. Treatment is symptomatic. Similarly, soft tick bites due to pajaroello or the African tampan (*Ornithodoros moubata*) may immediately cause pain, swelling, and irritation at the site of the bite, with raised hard wheals (29). The effects are said to last several days, with "irritability" of the affected part. Anecdotally, hunters in northern New England and the upper Great Lakes may complain of bites of *Dermacentor albipictus* larvae (a species that usually feeds only on ungulates such as deer or moose) during the early winter; apocrypha indicate that Native Americans called these larvae "bite all same as a piece of fire" (30). The condition has not been studied.

An unusual toxicosis due to tick bite is tick paralysis. The presence of certain feeding ticks induces an acute ascending paralysis. First described for sheep and cattle in Australia in 1843, a similar disease was reported for a child in Oregon in 1912. The Australian *Ixodes holocyclus* attacks cattle, sheep, and dogs but rarely humans, and thus tick paralysis is not a common clinical condition there. However, in the western United States, bites of *Dermacentor andersoni* commonly produce cases of "staggers" in cattle or sheep, which may terminate fatally. Children are the usual victims of tick paralysis, with ticks attached at the nape of the neck. The illness is characterized by fatigue, irritability, distal paresthesias, leg weakness with reduced tendon reflexes, ataxia, and lethargy. Unless the tick is removed, quadriplegia and respiratory failure may result; the case fatality rate without treatment can be 10%. Removal of the tick induces a miraculous recovery within 48 hours. (Tick biologists unromantically suggest such an etiology for the tale of Snow White, who awakens after the Prince bends over her and kisses her, probably removing a tick from behind her ear.) A 40- to 60-kDa toxin has been isolated from *I. holocyclus* and has been named holocyclotoxin (31); an antitoxin has been produced for veterinary use; others with a much smaller molecular weight have also been isolated. The toxin has not been isolated from the American tick paralysis ticks. Other ticks (*Amblyomma americanum*, *Ixodes* spp.) have also been reported to induce tick paralysis.

An enigmatic red meat allergy has been associated with the bites of Lone Star ticks in the eastern United States. A severe hypersensitivity reaction to treatment with cetuximab was found to be geographically limited and due to IgE reactivity with galactose-alpha-1,3-galactose (alpha-Gal). Such IgE reactivity was also associated with a newly recognized red meat allergy, which manifested as urticaria or anaphylaxis 3 to 6 hours after ingesting beef, pork, or lamb. Patients recalled recent multiple tick bites, and subsequently detailed studies of individual cases, epidemiological association between anti-alpha-Gal IgE and Lone Star tick bites, and correlation of IgE to tick proteins and alpha-Gal together have provided evidence for causality (32). Similar associations have also been reported for Europe and Australia, with sheep ticks (*Ixodes ricinus*) and paralysis tick (*I. holocyclus*), respectively, suspected as the culprits. Alpha-Gal is a major component of internal tick tissues, and hence the demonstrated associations are biologically plausible. Specific treatment has not been described other than avoidance of red meat and tick bites; apparently, the allergy resolves in the absence of additional tick bites. This unusual example of direct, delayed injury due to an arthropod remains to be fully understood.

Endoparasitic Arthropods

Arthropods that invade a host's body and cause disease include mites (scabies), fleas (tungiasis), fly larvae (myiasis), and pentastomes (halzoun). Tungiasis has been discussed above in "Siphoaptera." Pentastomes or tongue worms are primitive, elongate arthropods that live as adults in the lungs and air passages of hosts including fish, amphibians, reptiles, and some mammals. The body, like that of acarines, consists of a cephalothorax and indistinct abdomen, with no legs; often, the cuticle appears ringed or annulated. Chitinous hooks protrude from the head. Their size ranges from 1 to 10 cm. Although long thought to be in its own phylum, recent cladistic analyses based on morphologic and molecular characters place the tongue worm within the Crustacea and find it to be most closely related to the branchiurid fish lice (33). Pentastomes undergo a complex life cycle with incomplete development, requiring intermediate hosts in which the nymphal stages may encyst.

Human infestation by *Linguatula serrata* may be due to ingestion of eggs (from contamination by nasal discharges from the dog intermediate host) or ingestion of encysted nymphs within the raw or poorly cooked liver, lungs, or mesentery of an intermediate host such as rabbits, cattle, or sheep (34). A nasopharyngeal syndrome results, known as halzoun in the Middle East and marrara in Sudan. Facial edema, nasal discharge, coughing, and sneezing are due to the migration of nymphal forms into the nasopharynx. Removal of the offending nymphs (by visual inspection and forceps) and symptomatic treatment with antihistamines to reduce edema may be helpful. *Armillifer armillatus* and *Porocephalus crotali* of snakes also cause infections in humans, probably as a result of contaminating drinking water with eggs from their feces and not because of ingestion of poorly cooked snake meat. Usually, human infection is noted only at autopsy or by the presence of a calcified abdominal or lung object in radiographs. Treatment is symptomatic.

The most common of the ectoparasite-caused direct injury is scabies, caused by infestation with the human scabies mite (*Sarcoptes scabiei*). A number of different populations of *S. scabiei* have been treated as full species based on their tropism for other animals (including dogs, pigs, sheep, cattle, and goats), but all are morphologically identical. Infestation may occur anywhere in the world. Canine sarcoptic mange is commonly associated with scabies infestations in the owners of the dogs. In either animal or human scabies, transmission is by direct personal contact, and infestations often cluster among groups of people, particularly families. There is little evidence that environments become contaminated; fomites have not been identified.

The female scabies mite burrows beneath the stratum corneum (Fig. 7), leaving behind eggs and feces within a tracklike trail. A few dozen eggs are deposited, and these hatch within a week. Larvae form new burrows but may also emerge from the skin and move freely about. Nymphs develop from fed larvae, and they in turn develop into the adult male and female. Normal infestations consist of a dozen or two female mites. Nocturnal itching begins within a month of the first infestation but may begin within a day in previously exposed individuals. Thus, newly exposed individuals, prior to their recognizing an infestation by the presence of itching, may serve to contaminate other individuals. Erythematous papules and vesicles first appear on the webs of fingers, and spread to the arms, trunk, and buttocks. The burrows contain a granular, highly antigenic feces, which cause both delayed and immediate-type hypersensitivity reactions. Interestingly, in individuals who are immunocompromised, hundreds of female mites may be found, itching is minimal, but a hyperkeratosis is prominent. Such "crusted" or Norwegian scabies are highly infectious to other people.

Scabies infestations can be easily diagnosed by scraping a newly developed papule (not one that has been scratched) with a scalpel coated with mineral oil. The scrapings in oil may be transferred to a slide and examined at a magnification of ×100 or ×400 bright-field for 300- to 400-μm mites or the smaller black fecal granules. Scabies may be treated by topical 5% permethrin cream or 1% permethrin rinse (Nix, which is the same as used for head lice). Globally, 12.5% benzyl benzoate is the treatment of choice due to

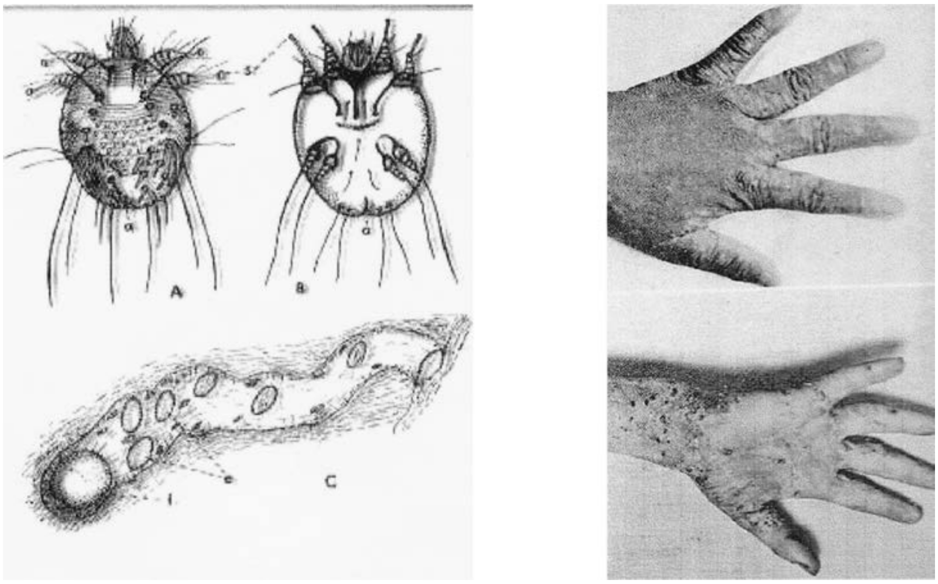


FIGURE 7 Scabies. Diagram of feeding lesion and adult female mite. Chronic scabies affecting the hands. (Reprinted from reference 56.) doi:10.1128/9781555817381.ch148.f7

cost considerations but is not Food and Drug Administration (FDA) approved as a scabicide and hence not used in the United States. Topical lindane (Kwell) is also effective, but FDA now suggests the use of permethrin first and then lindane only if permethrin fails. Lindane can be neurotoxic to children and small adults. Although oral ivermectin has recently been suggested as being more effective, at the moment topical permethrin or benzyl benzoate is still considered to be the first-line treatment when correctly applied (35).

Demodex folliculorum, the follicle mite, infests virtually everybody. This elongate 500-µm-long mite may be found within follicles on the face, the ear, and breast, and are often brought to the attention of a physician because a tweezed eyelash may have a few mites at the base of the hair shaft. Although they appear to largely be nonpathogenic, the same species causes demodectic mange in dogs and cattle. Pityriasis folliculorum, with small pustules appearing on the forehead, has been attributed to them.

Dust mite allergies (one of many causes of asthma, rhinitis, and atopic dermatitis) are due to inhalation of feces excreted by *Dermatophagoides farinae* or related pyroglyphid mites, which are human commensals that feed on flakes of skin shed from a person. The mites themselves do not infest a person but remain in the environment (usually within bedding or carpets) to feed and develop. About one-half gram of skin may be shed from a person each day; one female mite lays one egg a day, for about 2 months; thus, large accumulations of mites may readily develop. The 300- to 400-µm-long mites may be presented by patients as suspects for other nonspecific lesions or sets of signs and symptoms because they may be found in virtually all houses and can be detected if dust is allowed to settle on standing water; the mites float and move and can be seen at a magnification of ×20. Humidity less than 60% greatly reduces dust mite infestations, as do periodic vacuuming and washing of bedding and carpets.

Myiasis is the infestation of human or animal tissue by fly larvae, deposited as eggs or first-stage larvae; the larvae develop by feeding on the surrounding tissue, emerge as third-stage larvae, and pupate in the environment. There are three kinds of myiasis with respect to life cycle patterns: obligate, facultative, and accidental. Obligate myiasis reflects the need for larvae to feed well during development because adult flies do not feed or feed poorly. Botflies comprise the main examples of obligate myiasis. Most botflies normally infest animals, and thus human botfly infestation by these species is considered zoonotic. Two flies, *Dermatobia hominis* (human botfly) and tumbu fly (*Cordylobia anthropophaga*), are more adapted to humans, as illustrated by their life cycles. The former fly lays its eggs on a transport (phoretic) host such as a mosquito, and when the mosquito feeds on a human, the eggs hatch during the course of the blood meal, and the larvae penetrate the skin at the site of the mosquito bite or burrow in on their own. The latter fly is attracted to sweat, urine, or feces and oviposits on clothing that has been spread out to dry on the ground or hung up to dry, on areas of cloth that are redolent with such odors, which may remain when primitive clothes-washing practices are used. The eggs hatch when placed close to the body, and the larvae burrow into the skin. Thus, any clothing washed in tropical countries without the aid of modern soap powders and dryers should be ironed before wearing.

Facultative myiasis is usually due to infestation by blowflies (*Phormia regina*), green bottle flies (*Lucilia sericata*), and related calliphorids; by flesh flies (*Wohlfahrtia* spp.); or by common houseflies (*Musca domestica*). These flies normally deposit eggs into fecal or other rotting organic material but directly lay eggs into wounds or necrotic tissue; however, the larvae may not confine themselves to such resources and may move into healthy tissue.

Accidental myiasis includes the incidental findings of fly larvae, often housefly, under wound dressings or within unusual sites such as the gastrointestinal (GI) tract. The rat-tailed maggot (*Eristalis tenax*) is actually a hoverfly (syrphid) that breeds in sewage or dirty water. Eggs might be deposited around the anus during defecation, and the larvae may find their way into the lower GI tract or urethra. Drinking unfiltered dirty water might cause temporary infestation of the gut, with larvae surviving into the lower intestine. More often, larvae are found in containers of stool samples that are intended for the clinical laboratory and are most likely due to oviposition after the sample is taken.

A variety of clinical presentations are evident depending on the site where the larvae are present. Botflies cause furuncular lesions or migratory integumomyiasis (a serpiginous track may be produced in the skin). Wound myiasis comprises shallow or pocketlike initial lesions that become more deeply invasive. Maggots may invade the nose and accompanying structures, causing nasal or oral myiasis. Maggots may get into the ears, producing aural myiasis. Ophthalmomyiasis is due to external or internal infestation. Enteric, vaginal, or vesicomyiasis is due to invasion of the gut or genitalia. In all presentations, pathology may be due to tissue trauma or local destruction but is more often associated with secondary bacterial infection. On the other hand, many maggots do not promote bacterial infection but rather secrete bacteriolytic compounds and have been used as a surgical intervention to debride wounds (36). Indeed, sterile *L. sericata* maggots are available by prescription in the United States (www.monarchlabs.com) to help with wound debridement. Thus, the development of secondary bacterial infection in myiasis may suggest the death of the maggot.

In all cases, treatment is by removal of the maggots, laboriously picking them out using forceps. The maggots causing furuncular myiasis or migratory integumomyiasis have their posterior end visible within the lesion, exposing the spiracular plates that cover their tracheolar breathing apparatus. Although much lore on how to best remove such maggots exists, including "luring" the maggots with bacon or pork fat, the simplest method is to cover the lesion with petrolatum (Vaseline), thereby preventing the maggot from breathing. (Obstruction of the spiracles is probably the mode of action for bacon or pork rind, not a preference of maggots for tasty fat.) The maggot eventually moves out enough in an attempt to get air so that it may be grasped with forceps.

Identification of maggots can be difficult. The spiracular plates can be diagnostic (Fig. 8) and should be removed from the posterior end, mounted on slides in an appropriate mounting medium, and compared with pictorial keys. Often, a live maggot can be reared to pupation on raw meat, and the resulting adult fly can be identified. On the other hand, other than academic curiosity, most myiasis cases presenting in the developed countries are the result of tourism, and not knowing the specific identity of the fly would not influence further preventive measures; treatment would be sufficient. One important reason for accurately identifying a maggot is for forensic purposes: the developmental rates of the various obligate or facultative myiases have been described as a great aid in assigning time of death for cadavers. The American Board of Forensic Entomology (http://www.forensicentomologist.org) provides certification for experts in such tasks, and their membership may be consulted should a need for precise identification arise.

Stinging and Biting Resulting in Envenomation

Hymenoptera

Wasps, bees, and ants all belong to the order Hymenoptera, which contains well over 100,000 recognized species. Hymenoptera are minute to medium-sized insects with compound eyes, mandibles, and two pairs of transparent wings (although in the ants, wings may be seen on males or females only at certain times of the year). They undergo complete metamorphosis from a grublike larva to a pupa. Those of clinical importance are within the Aculeata, particularly the Vespidea (social and solitary wasps), Apoidea (social and solitary bees), and Formicoidea (ants). Most of the Aculeata have a characteristic constriction between the first and second abdominal segments. These insects have a complex social behavior, with males, females, and worker castes. Workers have a stinging organ, which is used for defending the colony or capturing prey.

Most stings by hymenoptera cause localized reactions, sometimes with extreme pain, and resulting in a transient induration with hyperemia. By virtue of their living in large colonies, bees and ants may swarm an intruder, and dozens if not hundreds of stings may be sustained. Airway obstruction may result should multiple stings be received on the face or neck. Although the honeybee's agricultural value in pollinating plants greatly exceeds any slight risk due to their stings, African honeybees (*Apis mellifera adansonii*, a subspecies of the regular honeybee; also known as "killer bees"), which were imported into Brazil in the hopes that they would be better pollinators, can be dangerous because they are more aggressive than the typical honeybee. Bees differ from wasps and ants in that their stinging apparatus is forcibly torn out during the act of stinging, thereby ensuring the death of individual bees as a result. Wasps and ants may sting multiple times; some fire ants may hang on by their mandibles and repeatedly insert their posteriorly located stinger.

Bee venom has been well characterized and consists of a large amount of a polypeptide (melittin), phospholipase A2, histamine, hyaluronidase, mast cell discharging peptide, and apamin. Histamine appears to be the cause of the acute pain of bee stings.

Bumblebees, paper wasps, yellow jackets, and hornets may all sting, usually as a result of intruding too near a nest. The most important clinical manifestation of bee or wasp stings is anaphylaxis. Chest tightness, nausea, vertigo, cyanosis, and urticaria may be seen even in individuals who apparently had never previously been exposed. Dozens of people die each year in the United States due to bee sting anaphylaxis.

Ants, on the other hand, rarely pose a risk for anaphylaxis but produce a reaction that may persist for a longer duration. An induration or wheal may be observed immediately after the sting, and a papule that itches or remains irritated for several days may develop. Secondary bacterial infection may ensue. Fire ants (*Solenopsis invicta*) may bite or sting, both modes accompanied by the injection of a venom, an ethyl ketone for the former and various 2,6-dialkypiperidines for the latter. Other ants (the formicinae) have a venom that is mainly formic acid.

Urticating Caterpillars

Lepidoptera (moths and butterflies) undergo complete development, with the well-known caterpillar and cocoon stages. More than 100,000 species of these familiar insects have been described. Although certain adult moths (in four superfamilies) may imbibe blood or tears, no signs or symptoms are associated with what is evidently an independent evolution of hematophagy. At least eight families of Lepidoptera, however, have caterpillars with urticating hairs, and some of the neotropical species can produce surprisingly severe reactions, including a hemorrhagic syndrome (*Lonomia achelous*, a saturniid moth of the Amazon) due to a fibrinolytic toxin exuding from poison spines (37). More typical are rashes caused by contact with hairs (erucism, or erucic rash). A common shade tree pest, the browntail moth (*Nygmia phaeorrhoea*), in Europe and northeastern North America, liberates tiny barbed hairs when the caterpillar molts. These hairs are blown about by the

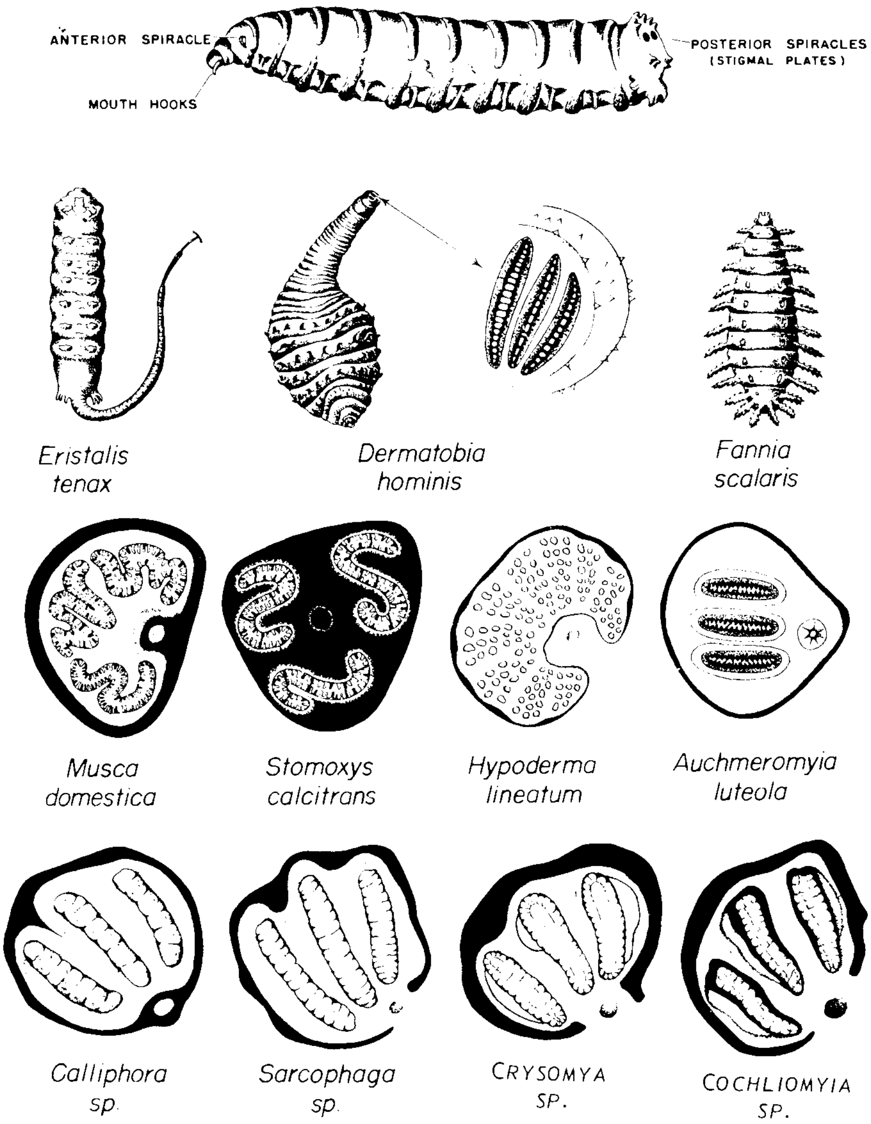

ANTERIOR SPIRACLE
MOUTH HOOKS
POSTERIOR SPIRACLES (STIGMAL PLATES)
Eristalis tenax
Dermatobia hominis
Fannia scalaris
Musca domestica
Stomoxys calcitrans
Hypoderma lineatum
Auchmeromyia luteola
Calliphora sp.
Sarcophaga sp.
CRYSOMYA SP.
COCHLIOMYIA SP.

FIGURE 8 Myiasis. Key characters of some myiasis-producing fly larvae. Row 1, mature larva of a muscoid fly. (Source: R. Hegner et al., Appleton Century Inc., New York, NY, 1938.) Row 2, larva of *Eristalis tenax*, the rat-tailed maggot; *Dermatobia hominis*, the human botfly (with an enlarged view of a posterior spiracle); and *Fannia scalaris*, the latrine fly. Rows 3 and 4, appearance of posterior spiracles of some species that produce accidental, facultative, or obligatory myiasis. (Source: Centers for Disease Control and Prevention, Atlanta, GA.)
doi:10.1128/9781555817381.ch148.f8

wind, and when skin is exposed, a severe dermatitis results; ingestion or inhalation can also cause significant irritation of the mucosa or bronchospasm. Contact with the eye may induce conjunctivitis. Dermatitis produced by urticating hairs typically comprises itchy, erythematous patches associated with small vesicles and edema. These lesions are only where the hairs have free access to the skin or where contact is made. Use of a masking tape-type lint roller can be very effective in removing urticating hairs, which may or may not be visible.

Scorpions

Scorpions are arachnids with a characteristic crablike appearance. The body consists of the cephalothorax and a segmented abdomen with a segmented tail, which terminates in a prominent stinging apparatus (aculeus). The four pairs of legs include well-defined pincers on the first pair of legs. Scorpions may range in size from 2 to 10 cm. Scorpions undergo incomplete development (immature stages look like miniature adults). Of the 1,000 or so species that are known, fewer than 50 have been reported to cause illness with their sting. Scorpions are problems mainly in the warmer climates. The bulbous end of the tail contains muscles that force venom through the stinger. All scorpions are predatory on other arthropods, immobilizing their prey with their venom. Humans are stung by walking barefoot at night, by not shaking their shoes out in the morning in an area of endemicity, by lifting rocks or logs, or in bedding that is on the floor. The stings cause local pain (probably due to the great biogenic amine content of the venom), edema, discoloration, and hypesthesia. Systemic signs can include shock, salivation, confusion or anxiety, nausea, tachycardia, and tetany. Venom characteristics differ depending on the genus of scorpion: some stimulate parasympathetic nerves and can lead to secondary stimulation of catecholamines resulting in sympathetic stimulation, which in turn may contribute to respiratory failure (38). Others affect the central nervous system, are hemolytic, or cause local necrosis. Hypersensitivity and anaphylaxis may occur in individuals who are repeatedly exposed.

Treatment is usually symptomatic, although in areas with known dangerous species, i.e., the Middle East (*Leiurus* spp., *Buthacus* spp., *Buthus* spp.), southern Africa (*Parabuthos* spp., *Buthos* spp., *Uroplectes* spp.), South and Central America (*Tityus* spp.), and the southern United States (*Centruroides* spp.), quickly applying a pressure bandage over the sting and immobilizing the limb (if that is where the sting was) would help to prevent venom from traveling via the lymphatics. Lidocaine may be injected directly into the sting to reduce pain. Medical attention should be sought as soon as possible because antivenin, when administered promptly, reduces morbidity. However, antivenin is usually species specific, and without bringing the culprit in for identification, antivenin use would be on a presumptive basis.

Centipedes and Millipedes

The centipedes (class Chilopoda, 2,800 species) and millipedes (class Diplopoda, 8,000 species) are elongate, vermiform arthropods with dozens of segments, each of which bears a pair of legs. Both undergo an incomplete development, with larvae looking like miniature adults. The word “centipedes” would suggest having 100 segments (and pairs of legs) or fewer and the word “millipedes,” more than 100 and up to 1,000. This simplification is not quite correct, but in general, possession of many legs indicates a millipede and fewer legs (but more than insects or ticks) indicates a centipede. Other differences are apparent: millipedes are rounded in cross section, but centipedes are dorsoventrally flattened; millipedes have mouthparts that are ventral and nonpiercing, whereas centipedes have mouthparts that protrude anteriorly and are clearly capable of piercing. Millipedes move slowly, reflecting their mode of life as feeders on detritus. Centipedes are very rapid predators on other arthropods.

The diversity of both millipedes and centipedes is greatest in the tropics, and virtually all those of medical importance are found in warm climates. Millipedes may squirt a noxious, corrosive fluid from pores on their segments. Such fluid may contain benzoquinone, aldehydes, and hydrocyanic acid and cause an immediate burning sensation followed by erythema and edema, even progressing to blistering (39). Most millipedes also have a repugnant smell; both the corrosive fluid and the smell tend to protect them from predation. People become exposed when they step on or sleep on millipedes or provoke them (children playing with them are often victims). Treatment consists of washing the affected site as soon as possible to dilute and remove the corrosive fluids and is symptomatic for the skin lesions and pain. Centipedes have powerful biting mandibles and small fanglike structures (forcipules) situated between them and derived from the first pair of legs that may inject a venom. This venom is used to immobilize prey but is also used in defense. Centipede bites occur when people step on or sleep on them or play with them. Envenomation is manifested by local pain and swelling, with proximal lymphadenopathy. Headache, nausea, and anxiety are common. Skin lesions may ulcerate and become necrotic. Death due to centipede bite has been confirmed for only one case, a Filipino child who was bitten on the head by a large *Scolopendra* sp. The common house centipede, *Scutigera coleoptrata* of the eastern United States, is commonly found in bathtubs. This hairy-looking, small (5- to 8-cm-long) centipede is actually beneficial, preying on roaches and other potential pests within houses. Its strong mandibles can, however, inflict a painful pinch, and it does have a mild venom that produces a bite similar in quality to a bee sting.

Spiders

The order Araneae comprises nearly 30,000 species, but only a fraction of them have any medical importance. Most are very small (0.5 cm or smaller in length), but a few may be as large as a man’s hand. Two major suborders are recognized: the Mygalomorphae, in which the fang-tipped chelicerae operate in parallel using an up-and-down strike, and the Aranaeomorphae, in which the chelicerae operate like insect mandibles, with a side-to-side motion. Spiders have an unsegmented cephalothorax, an abdomen, four pairs of legs, the prominent chelicerae, and spinnerets, specialized organs that secrete the silk for making webs. All spiders are predatory on other arthropods and use their venom to immobilize prey, which are stored live. Spiders rend their prey with their chelicerae and bathe them with a digestion fluid for hours prior to ingestion. Thus, although all spiders have stout chelicerae and can bite and all spiders have a venom with which to immobilize their prey, most are too small to be noticed by humans even if they were to be bitten. The spiders of main medical interest belong to four groups (40, 41): the funnel web or trapdoor spiders of Australia (*Atrax* spp., *Hydronyche* spp.), New World and southern African recluse spiders (*Loxosceles* spp.), South American armed spiders (*Phoneutria* spp.), and the cosmopolitan widow spiders (*Latrodectus* spp.). The recluse and widows have been widely transported by humans.

The clinical manifestations and complications of envenomation may differ between the four main spiders, and the syndromes caused by each have been given names that reflect the identity of the spider. With spider bites in general, there are local pain and erythema at the site of the bite, and this may be accompanied by fever, chills, nausea, and joint pains. In loxoscelism, the site of the bite ulcerates and becomes necrotic. Skin may slough, and there may be destruction of the adjacent tissues. Hemolysis, thrombocytopenia, and renal failure may ensue. With latrodectism, phoneutrism, and funnel web neurotoxicity, the venoms have a strong neurotoxic action. Muscle rigidity and cramping (similar to acute abdomen) are seen with latrodectism; complications include electrocardiogram (EKG) abnormalities and hypotension. With phoneutrism, visual disturbances, vertigo, and prostration may occur; complications include hypotension and respiratory paralysis (40). Funnel web spiders induce autonomic nervous system excitation, with muscular twitching, salivation and lachrymation, nausea and vomiting, and diarrhea; fatal respiratory arrest may result from apnea or laryngospasm (42).

Funnel web spider bites require prompt first aid, and the same recommendations could be used for latrodectism or phoneutrism. A compression bandage should be applied over the site of the bite, and the affected limb should be immobilized by splinting with a compression bandage if bitten on an extremity (standard procedures for snakebite). This may help prevent the venom from moving from the local lymphatics. The patient should seek medical attention as soon as possible for antivenin treatment. Otherwise, treatment is symptomatic with analgesics and antipyretics.

Tarantulas are popular pets and appear fearsome. Fortunately, they are docile, and most injuries associated with them are due to their urticating hairs, particularly with respect to conjunctivitis. They do, however, have robust mandibles and venom and should not be provoked; bites are similar in quality to a bee sting.

Necrotic dermal lesions are often classified as loxoscelism even if the appropriate spiders are not known to be present in the area: only 35 of 216 diagnoses of "brown recluse spider bites" proved to be supported by the minimum evidence for assigning such a specific etiology, *viz.*, the known presence of *Loxosceles reclusa* within the geographic area of exposure (43). In the eastern United States, some necrotic dermal lesions may actually be due to the bite of the yellow sac spider, *Cheiracanthium mildei* (44), or the related *Cheiracanthium inclusum*, causing a painful eschar that may last for weeks (but see reference 45). Severe reactions to tick bite or even the erythema migrans of Lyme disease may be confused with recluse or other spider bites (46).

Miscellaneous Injury Due to Arthropods

Beetles (coleoptera) comprise 40% of all insect species, but even with this great diversity, very few may be harmful. Most injury is related to vesication by secretions containing cantharidin. The hemolymph (blood) of some of these beetles, generally called the blister beetles, also contains this irritant, and thus exposure may be by direct contact with live or dead (crushed) beetles. Cantharidin is the active ingredient of the Spanish fly, the alleged aphrodisiac made from pulverized *Lytta vesicatoria*. Ingestion of Spanish fly would irritate the ureter and urethra and cause painful priapism. Overdose or chronic use causes renal tubular necrosis. It is likely that Spanish fly is no longer in demand given the availability of nitric oxide inhibitors.

An unusual group of carabids, the bombardier beetles, spray a boiling-hot (100°C) jet of benzoquinone as a defense (47), causing burns and blistering. Blistering is also produced by crushing the staphylinid beetle *Paederus fusca* of Southeast Asia, which contains a toxic alkaloid, pederin.

More commonly, carpet beetles (dermestids), which feed on wool rugs and other animal fur products, are associated with a papulovesicular eruption. In particular, larval dermestids and their hairs or shed skins (exuviae) cause a contact dermatitis.

Water beetles (actually members of the Hemiptera), also known as water skimmers, can inflict strong pinches on unsuspecting toes wading through water.

OTHER INJURY

Delusional or Illusion of Parasitosis

Illusory parasitosis is a condition in which a patient who has a real itch has the mistaken belief that the current itch is due to active infestation by irrelevant arthropods. Often, the itch is due to a drug reaction, sunburn, detergents, irritating dusts, poison ivy, or even *bona fide* arthropod bites (among many other potential sources of itch) that were sustained months in the past. Once a patient has been helped to identify the actual cause of the itch, he or she will not persist in blaming an arthropod. In contrast, in delusory parasitosis (also known as Ekbom syndrome), the patient cannot be dissuaded that the source of discomfort is probably not an arthropod. In some patients, there are no objective lesions but simply an insistence that they are infested. Many such cases are highly educated people and can describe their infestation with great detail. However, they rarely produce a specimen, and when they do, it is usually not an arthropod or part thereof. Delusory parasitosis can be a serious illness, which can be treated by experienced mental health professionals.

IDENTIFYING SUBMITTED SPECIMENS

For most purposes, identification to the species level ("this is *Eutrombicula alfreddugesi*") may not provide any more clinically relevant information than identification to a higher taxonomic level ("this is a chigger"). In some cases, simply being able to say "this is definitely an arthropod part, perhaps beetle" may be all the information that is required (in this example, a beetle part may suggest dwarf tapeworm infection or be consistent with urticaria due to the hairs of dermestid beetle larvae or with vesication due to the hot spray of the bombardier beetle). A whole arthropod may be compared with the bauplan diagram to place it within a general group. A classic pictorial key published by the CDC (available from http://www.dpd.cdc.gov/dpdx/HTML/CDProducts.htm) or a simple dichotomous key (Table 3) may be used to narrow the identification down to a known genus of medically important arthropod. Anything more specific usually requires consulting a taxonomic reference or finding an entomologist who can help. Agricultural extension services, university entomology departments, local mosquito or pest control organizations, parasitologists, or local bug-collecting enthusiast clubs may all be of help in identifying a specimen and usually do not charge out of professional courtesy. (Note that few such entities may be Clinical Laboratory Improvement Amendments [CLIA] certified, and thus fee for service is problematic.)

In some cases, a patient may insist that an arthropod specimen be tested for the presence of an infectious agent. This is particularly common in areas where Lyme disease is endemic. The value of such a practice is dubious, other than for psychologically satisfying the patient's demand. Some commercial laboratories test for the presence of Lyme disease spirochetes using a PCR assay, but given that the prevalence of infection in the vector may range from 15 to 70%, a positive test is likely. Furthermore, at least with deer ticks, other agents, such as those causing human granulocytic ehrlichiosis (HGE) and babesiosis, may be present but not revealed by tick testing for Lyme disease spirochetes. Other tick species also are likely to have guilds of microbes as opposed to a sole agent (48). In any event, by the time a PCR result is rendered, the patient may already be experiencing signs and symptoms. For Lyme disease, and likely for ehrlichiosis, postexposure prophylaxis may be provided in the form of two doses of 100 mg doxycycline, if the patient remains unconvinced.

The most important variable in determining the riskiness of a tick bite is how long the tick has fed (Fig. 9). If a deer tick is attached for no more than 24 hours, regardless of whether it is demonstrated to be infected with Lyme disease spirochetes, *Babesia microti*, or the agent of granulocytic ehrlichiosis, the likelihood of an infectious dose of organisms being transmitted is very small. The biological basis for this "grace period" is a phenomenon known as reactivation, wherein pathogens within ticks require a period of replication after emerging from a period of dormancy that they enter during the long interstadial period between blood meals (49). Reactivation was first described for the agent of Rocky Mountain spotted fever, which attains infectivity

TABLE 3 Key to the common arthropod classes, subclasses, and orders of medical importance, adult stages only[a]

1. Three or four pairs of legs [2]
   Five or more pairs of legs [22]
2. Three pairs of legs with antennae **(insects: class Insecta)** [3]
   Four pairs of legs without antennae **(spiders, ticks, mites, scorpions: class Arachnida)** [20]
3. Wings present, well developed [4]
   Wings absent or rudimentary [12]
4. One pair of wings **(flies, mosquitoes, midges: order Diptera)** [5]
   Two pairs of wings [6]
5. Wings with scales **(mosquitoes: order Diptera)**
   Wings without scales (**other flies: order Diptera**)
6. Mouthparts adapted for sucking, with an elongate proboscis [7]
   Mouthparts adapted for chewing, without an elongate proboscis [8]
7. Wings densely covered with scales, proboscis coiled **(butterflies and moths: order Lepidoptera)**
   Wings not covered with scales, proboscis not coiled but directed backward **(bedbugs and kissing bugs: order Hemiptera)**
8. Both pairs of wings membranous, similar in structure, although size may vary [9]
   Front pair of wings leathery or shell-like, serving as covers for the second pair [10]
9. Both pairs of wings similar in size **(termites: order Isoptera)**
   Hind wings much smaller than front wings **(wasps, hornets, and bees: order Hymenoptera)**
10. Front wings horny or leathery, without distinct veins, meeting in a straight line down the middle [11]
    Front wings leathery or paperlike, with distinct veins, usually overlapping in the middle **(cockroaches: order Dictyoptera)**
11. Abdomen with prominent cerci or forceps; wings shorter than abdomen **(earwigs: order Dermaptera)**
    Abdomen without prominent cerci or forceps; wings covering abdomen **(beetles: order Coleoptera)**
12. Abdomen with three long terminal tails **(silverfish and firebrats: order Thysanura)**
    Abdomen without three long terminal tails [13]
13. Abdomen with narrow waist **(ants: order Hymenoptera)**
    Abdomen without narrow waist [14]
14. Abdomen with prominent pair of cerci or forceps **(earwigs: order Dermaptera)**
    Abdomen without cerci or forceps [15]
15. Body flattened laterally, antennae small, fitting into grooves in side of head **(fleas: order Siphonaptera)**
    Body flattened dorsoventrally, antennae projecting from side of head, not fitting into grooves [16]
16. Antennae with nine or more segments [17]
    Antennae with three to five segments [18]
17. Pronotum covering head **(cockroaches: order Dictyoptera)**
    Pronotum not covering head (**termites: order Isoptera**)
18. Mouthparts consisting of tubular joined beak; three- to five-segmented tarsi **(bedbugs: order Hemiptera)**
    Mouthparts retracted into head or of the chewing type; one- or two-segmented tarsi [19]
19. Mouthparts retracted into the head; adapted for sucking blood (**sucking lice: order Anopleura)**
    Mouthparts of the chewing type **(chewing lice: order Mallophaga)**
20. Body oval, consisting of a single saclike region **(ticks and mites: subclass Acari)**
    Body divided into two distinct regions, a cephalothorax and an abdomen [21]
21. Abdomen joined to the cephalothorax by a slender waist; abdomen with segmentation indistinct or absent; stinger absent **(spiders: subclass Araneae)**
    Abdomen broadly joined to the cephalothorax; abdomen distinctly segmented, ending with a stinger **(scorpions: subclass Scorpiones)**
22. Five to nine pairs of legs or swimmerets; one or two pairs of antennae; principally aquatic organisms **(copepods, crabs, and crayfish: class Crustacea)**
    Ten or more pairs of legs or swimmerets absent; one pair of antennae present; terrestrial organisms [23]
23. Body segments each with only one pair of legs **(centipedes: class Chilopoda)**
    Body segments each with two pairs of legs **(millipedes: class Diplopoda)**

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[a] Data from references 9 and 58.

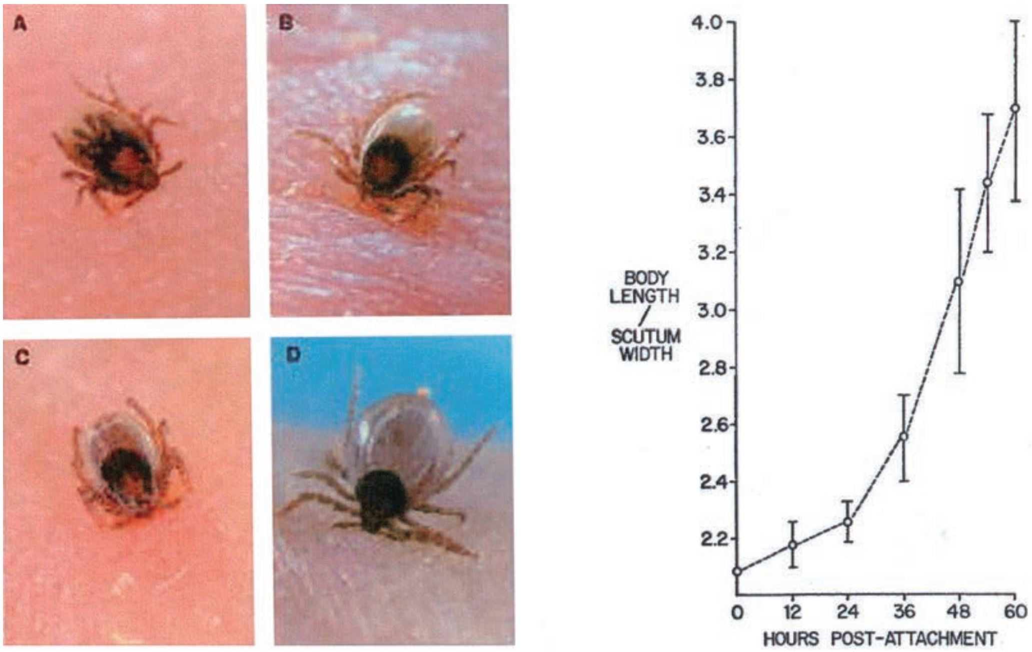

FIGURE 9 Gradual engorgement of feeding ixodid ticks. (Left) Four-day feeding sequence of nymphal *Ixodes ricinus*. (Reprinted from reference 57, with permission from Elsevier.) (Right) Scutal index of engorgement for *Ixodes dammini*. (Reprinted from reference 50 with permission of the American Society of Tropical Medicine and Hygiene.) doi:10.1128/9781555817381.ch148.f9

within tick salivary glands only after 12 to 18 hours of exposure to a host. Reactivation is probably a general phenomenon with most ixodid (hard) tick-transmitted agents, the only known exceptions being the viruses causing tick-borne encephalitis and the related Powassan fever, in which transmission is thought to be instantaneous with attachment.

Estimating the degree of engorgement may provide more of an index of individual risk than the actual presence or absence of infection. A simple measurement may be made with deer ticks and might be tried for other species of ticks. The scutal index is the ratio of the length of the tick from the tip of the mouthparts to the caudal edge of the tick to the width of the scutum, the dark shield on the dorsum of the tick. A scutal index of >2.5 suggests that a deer tick has been attached for more than 24 hours, and therefore transmission is likely (50). Such a finding might prompt a physician to provide prophylactic antibiotics, which would effectively abort Lyme disease or HGE. Although such prophylaxis would not prevent babesiosis, note that of the agents transmitted by deer ticks, *B. microti* requires the greatest duration of attachment for infectivity, probably relating to the requirement for sporogony during attachment.

Much progress has been made in a universal barcoding system for identifying animals by means of DNA sequences (51). In particular, the 5′ end of the mitochondrial cytochrome C oxidase subunit I (COI), usually a 648-base pair portion, is used as a PCR target, and the nucleotide sequence that is obtained from the amplification product can be compared to a large online database (http://www.boldsystems.org/views/login.php). Most species of animals contain unique sequences in their COI gene and thus can be discriminated from other related species. Nearly 750,000 sequences have been accessioned in the BOLD database. For the phylum Arthropoda, barcode sequences exist for all 16 classes; for Insecta, all 30 orders are represented. Accordingly, virtually any putative arthropod sample may be identified to the order level and possibly to family or genus level by extracting its DNA, amplifying the COI gene, sequencing, and then submitting the data in FASTA format to the BOLD server. Assuming that the equipment is available (thermal cycler, gel electrophoresis, pipettors, and other materials common to molecular biology laboratories), such an identification might cost on the order of $150 to $200 (5 hours of technician time, PCR reagents, commercial rate for sequencing 300 base pairs) with a few days' turnaround (usually, a minimum of 24 to 48 hours from submission of the DNA to receipt of the sequencing chromatogram is required for DNA sequencing). A simple DNA extraction technique ("HotSHOT," which uses hot sodium hydroxide and Tris neutralization) may be used on most samples to prepare them for PCR analysis (52). The assay conditions and PCR primers for amplifying an informative portion of the COI gene may be adopted from published analyses of preserved museum specimens, which often contain degraded DNA similar to that of many forensic or clinical samples (53). The advantage to the barcoding approach is that the method is not subjective and does not require the extensive training and expertise for morphological identification; anyone with general expertise in molecular biology may be engaged. Classically based methods, however, require little more than a microscope, can be virtually instantaneous, and may be rendered as a professional courtesy without fee. Some commercial websites (e.g., www.identifyus.com) offer virtual morphologic identification in real time, with clients uploading a photograph of the offending arthropod to be identified quickly by an expert. Alternatively, amateur entomologist websites (e.g., www.bugguide.net) can be extremely helpful in identifying samples.

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section IX ANTIPARASITIC AGENTS AND SUSCEPTIBILITY TEST METHODS

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Antiparasitic Agents

KARIN LEDER AND PETER F. WELLER

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A number of effective antiprotozoal and anthelmintic drugs are currently available. Antiparasitic agents are important both for therapy of infected individual patients and for control of parasitic infections at the community level. Large-scale chemotherapy is reducing transmission, morbidity, and mortality of infections including lymphatic filariasis, onchocerciasis, schistosomiasis, and infection with intestinal nematodes. However, the lack of financial incentives to develop new agents is a major limitation to the future of antiparasitic chemotherapy. Emerging resistance among parasites, a lack of effective antiparasitic vaccines, and the enormous burden of disease worldwide also pose challenges to the effective management of parasitic infections.

This chapter focuses on the mechanisms of action, pharmacology, clinical utility, and adverse effects of common first-line antiparasitic therapies and newer drug alternatives. Most helminth infections in humans can be treated with one of five drugs, namely, albendazole, mebendazole, praziquantel, ivermectin, and diethylcarbamazine (DEC), so these five drugs are reviewed in detail. Another agent, nitazoxanide, has both anthelmintic and antiprotozoal activity and is also discussed. Other major antiprotozoal drugs, including those used for malaria, gastrointestinal protozoal infection, leishmaniasis, and trypanosomiasis, are also reviewed, but an exhaustive list of all antiparasitic drugs is not included. Specifically, we have excluded discussion of agents without a first-line indication or that are recommended only in special situations, such as furazolidone in children. Tribendimidine is a promising new agent for treatment of liver flukes (clonorchiasis and opisthorchiasis), but trials are still under way and it has not yet been approved for use so is not discussed. Additionally, antibacterial and antifungal agents that can also be used for treatment of protozoal infections, such as the 5-nitroimidazoles, trimethoprim-sulfamethoxazole, azithromycin, and amphotericin, are not discussed in detail here, but their general indications for parasitic infections are shown in Tables 1 and 2. Resistance to antiparasitic agents and drug susceptibility testing are dealt with in separate chapters.

ANTHELMINTIC AGENTS

Benzimidazoles

The benzimidazoles are antiparasitic agents with a broad spectrum of activity against many helminthic and certain protozoal infections. All members of the benzimidazole class have in common a bicyclic ring system in which benzene has been inserted. Mebendazole and albendazole, both of which are synthetic agents, are the most widely used drugs of this class. Mebendazole is 5-benzoyl-2-benzimidazole carbamic acid, and albendazole is methyl 5-(propylthio)-2-benzimidazole carbamate. The low cost, high efficacy, and ease of administration of these two agents have led to their widespread use for many human parasitic infections. Major indications for their use are shown in Tables 3 and 4. Whereas mebendazole has been approved for treatment of multiple nematode infections by the U.S. Food and Drug Administration (FDA), albendazole is used preferentially as first-line treatment for many parasite infections but is nevertheless considered investigational and given as a "non-approved indication" in all cases except when used as treatment for hydatid infections and neurocysticercosis (1).

Other members of this drug class include flubendazole, thiabendazole, and triclabendazole. Flubendazole, a parafluoro analogue of mebendazole, has the same mechanism of action as mebendazole and albendazole. It is licensed in Europe for the treatment of intestinal nematodes but is not licensed in the United States. It has shown good activity against adult filarial parasites in animal models if given parenterally (2). It also exhibits activity against protoscoleces of *Echinococcus granulosus*, but currently there are no data on its efficacy for treatment of hydatid disease in humans (3). Thiabendazole, 2-(4-thiazolyl)-1H-benzimidazole, has similar mechanisms of action to those of the other benzimidazoles, but it is frequently associated with side effects. It has now been replaced by other anthelmintic agents (4) but is sometimes still used topically for treatment of cutaneous larva migrans. Triclabendazole is a newer imidazole derivative that has been used as a veterinary agent for many years. It is thought to act on microtubules, causing decreased parasite motility. It is the drug of choice for fascioliasis (5, 6), although resistance is emerging in animals and this may pose a threat to treatment of human patients in the future (7–9). It is the preferred therapy for paragonimiasis (10). Triclabendazole is well tolerated, and few significant adverse effects have been described, but it is not recommended for use during pregnancy because of insufficient safety data. It is considered investigational by the U.S. FDA and may be obtained from the Centers for Disease Control and Prevention (CDC) in the United States for paragonimiasis, but it is not widely available. These three agents are not discussed further.

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TABLE 1 Treatment of intestinal, blood/tissue (nonplasmodial), and urogenital protozoal infections[a]

Organism or disease	Primary agent used for treatment	Alternative agents
Intestinal protozoa		
Entamoeba histolytica	Invasive trophozoites: metronidazole	Tinidazole
	Luminal agent: iodoquinol	Alternative luminal agents: diloxanide furoate, paromomycin
	Tetracycline	Metronidazole, iodoquinol
Balantidium coli	Nitazoxanide	
Cryptosporidium parvum	Trimethoprim-sulfamethoxazole	Ciprofloxacin
Cyclospora cayetanensis	Trimethoprim-sulfamethoxazole	
Cystoisospora belli	Metronidazole	Iodoquinol, paromomycin
Dientamoeba fragilis	Metronidazole	Tinidazole, nitazoxanide, quinacrine, albendazole, furazolidone, paromomycin
Giardia duodenalis		
Blood and tissue protozoa		
Amebic meningoencephalitis	Amphotericin B	Miltefosine, variety of combinations
		Clindamycin plus quinine
Babesia species	Atovaquone plus azithromycin	Miltefosine, pentamidine, paromomycin
Leishmania species	Liposomal amphotericin B or pentavalent antimonial	Pyrimethamine plus clindamycin
Toxoplasma gondii	Pyrimethamine plus sulfadiazine	
Trypanosoma brucei species (African trypanosomiasis)	Early: suramin (*T. b. rhodesiense*) or pentamidine (*T. b. gambiense*)	
	Late: melarsoprol (*T. b. rhodesiense*) or eflornithine plus nifurtimox (*T. b. gambiense*)	Benznidazole
Trypanosoma cruzi (American trypanosomiasis)	Nifurtimox	
Urogenital protozoa		
Trichomonas vaginalis	Metronidazole	Tinidazole

[a] Adapted from reference 1.

Mechanism of action. The antiparasitic activity of albendazole and mebendazole results mainly from their ability to bind to a cytoskeletal protein of parasites called β-tubulin, thereby inhibiting the polymerization of tubulin into microtubules (11). The disruption of microtubule synthesis within parasitic intestinal cells results in decreased absorptive function. In addition, mebendazole and albendazole directly inhibit glucose absorption by parasites, leading to a depletion of parasite glycogen stores, insufficient energy sources for formation of ATP, and inability to reproduce or survive (12, 13). Although tubulin is also present in mammalian hosts, the benzimidazoles bind to parasite tubulin with an affinity that is hundreds of times greater than that for mammalian tubulin, thereby causing minimal mammalian toxicity (4).

Pharmacokinetics. Benzimidazoles are poorly soluble in water and therefore are not well absorbed following oral administration. Although this limits their activity against tissue-dwelling parasites, it contributes to their minimal toxicity and to their efficacy in the treatment of many intestinal helminthic infections (14). Less than 20% of mebendazole is absorbed after oral administration, with peak plasma concentrations seen at 2 to 4 h. It is metabolized in the liver to inactive compounds, eliminated in the bile, and excreted predominantly in the feces. It is 95% protein bound in plasma, and its serum half-life is 2.5 to 5.5 h. Levels in serum are markedly variable between individuals, but concentrations in tissue and in echinococcal cysts tend to be low (15).

The oral bioavailability of albendazole is also poor, with <10% absorption following an oral dose (16). Administration of albendazole with a fatty meal markedly improves bioavailability, by up to 5-fold. It is rapidly metabolized in the liver, and concentrations of the parent drug in plasma are negligible. However, its primary metabolite, albendazole sulfoxide, also has anthelmintic activity (17). This results in a higher efficacy of albendazole than that of mebendazole for most indications. Albendazole sulfoxide is 70% protein bound and is widely distributed throughout the body. Peak concentrations of albendazole sulfoxide in plasma are seen after 2 to 5 h but show great intersubject variability, ranging from 0.45 to 2.96 mg/liter following a single dose of 15 mg/kg of body weight (18). Albendazole induces enzymes of the cytochrome P450 system responsible for its metabolism. Cimetidine and dexamethasone both raise drug levels (19), and coadministration with praziquantel also increases the levels of both active drugs (20, 21). Albendazole sulfoxide has been detected in urine, bile, liver, cyst fluid, and cerebrospinal fluid (CSF). Levels in plasma are 3- to 10-fold and 2- to 4-fold higher than those in cyst fluid and CSF, respectively (22, 23). Albendazole sulfoxide has a half-life of ~9 h. It is oxidized further to inactive compounds such as albendazole sulfone and is mainly excreted in the urine.

Neither mebendazole nor albendazole is dialyzable. No dosage adjustment is required for individuals with renal impairment, but a reduction in dose should be considered if there is significant hepatic insufficiency. Benzimidazoles are not available as intravenous formulations.

Spectrum of activity. The benzimidazoles are effective against adult worms and developing helminthic embryos. Albendazole and mebendazole have similar and broad ranges of activity (Tables 3 and 4). Both drugs have good efficacy against many common intestinal nematode infections, including ascariasis, trichuriasis, enterobiasis, and hookworm infections. Three-day regimens of mebendazole and 1 to 3 days of albendazole are generally recommended for therapy

TABLE 2 Treatment of major helminthic infections[a]

Organism or disease[b]	Primary agent used for treatment	Alternative agents
Nematodes		
Ancylostoma caninum (eosinophilic enterocolitis)	Albendazole	Mebendazole
Angiostrongylus cantonensis	Supportive	Albendazole plus steroids
Ascaris lumbricoides	Albendazole	Mebendazole, ivermectin
Capillaria species	Mebendazole	Albendazole
Cutaneous larva migrans	Albendazole	Ivermectin
Enterobius vermicularis	Albendazole	Mebendazole
Filariasis (*Wuchereria bancrofti*, *Brugia malayi*)	Diethylcarbamazine with or without albendazole or ivermectin	
Gnathostoma species	Albendazole	Ivermectin, surgical removal
Hookworm	Albendazole	Mebendazole
Loa loa (only treat without high microfilaremia)	Diethylcarbamazine with or without albendazole or ivermectin	
Onchocerca volvulus	Ivermectin	
Strongyloides stercoralis	Ivermectin	Albendazole
Toxocara species (visceral larva migrans)	Albendazole	Mebendazole
Trichinella spiralis	Albendazole plus steroids	Mebendazole
Trichostrongylus species	Albendazole	Mebendazole
Trichuris trichiura	Mebendazole	Albendazole, ivermectin
Cestodes		
Cysticercosis	Albendazole	Praziquantel
Diphyllobothrium latum	Praziquantel	
Dipylidium caninum	Praziquantel	
Echinococcus species	Albendazole	Praziquantel
Hymenolepis nana	Praziquantel	
Taenia saginata	Praziquantel	
Taenia solium	Praziquantel	
Trematodes		
Clonorchis sinensis	Praziquantel	Albendazole
Fasciola hepatica	Triclabendazole	
Intestinal flukes	Praziquantel	
Metorchis conjunctus	Praziquantel	
Opisthorchis viverrini	Praziquantel	
Paragonimus species	Triclabendazole	Praziquantel
Schistosoma species	Praziquantel	

[a] Adapted from reference 1.
[b] This is not an exhaustive list of all possible parasitic infections, but commonly encountered parasites are included.

of individual patients (with the longer duration being particularly preferable for trichuriasis and hookworm infection therapy) (24). A single dose of either drug is often used for mass or targeted community treatment of intestinal nematodes in areas of endemicity. Albendazole is preferred in most instances, but the efficacy of albendazole against very heavy infections with *Trichuris trichiura* remains questionable, and mebendazole may be preferable in this circumstance. The reported curative efficacies of both drugs for different parasitic infections vary according to the baseline intensity of infection in the patient, geographic location, diagnostic tests employed, and duration of follow-up posttreatment.

Mebendazole and albendazole also have activity against other nematode infections, including angiostrongyliasis, trichostrongyliasis, capillariasis, trichinellosis, gnathostomiasis, toxocariasis, and cutaneous larva migrans. Additionally, albendazole is increasingly being used in combination with either DEC or ivermectin for treatment of Bancroftian or Brugian filariasis and loiasis; it is particularly useful for these infections as part of a single-dose regimen for mass chemotherapy programs. Although albendazole displays some efficacy against *Strongyloides stercoralis*, ivermectin consistently yields higher cure rates and is the recommended treatment of choice.

Both mebendazole and albendazole also show activity against certain cestode infections. Albendazole is again preferred because of its more favorable pharmacokinetics, and it is now considered the drug of choice for medical management or adjunctive treatment of hydatid disease due to *E. granulosis*. Prolonged albendazole therapy (minimum of 10 years) can also be used for inoperable alveolar echinococcosis or as adjuvant therapy for patients with *Echinococcus multilocularis* infection. Additionally, albendazole is used for treatment of parenchymal neurocysticercosis. Although considered primarily an anthelmintic agent, albendazole is also an alternative agent for giardiasis.

Adverse effects. Adverse effects following short courses of the benzimidazoles mebendazole and albendazole are infrequent and generally mild. Transient abdominal pain, nausea, and diarrhea may develop. Headache, dizziness, insomnia, and allergic phenomena are also reported. With prolonged high-dose therapy, such as for echinococcosis,

TABLE 3 Major indications for albendazole[a]

Indication	Usual dose	Reported efficacy
Echinococcus granulosis	400 mg twice daily, usually a minimum of 1–6 mo	Clinical cure, as evidenced by cyst disappearance in one-third of recipients and improvement in radiological appearance in an additional 30–50%
Cysticercosis	400 mg twice daily, usually for 8 days (8–30 days)	75–95% of parenchymal cysts destroyed and 40–70% of patients show resolution of all active cysts
Ascaris lumbricoides	Single 400-mg dose	Median cure rate of 95–98% and egg reduction rate of 99–100%
Cutaneous larva migrans	400 mg daily for 3 days	No large clinical trials; generally reserved for those with severe or disseminated infection
Enterobius vermicularis	400-mg dose, repeat in 2 wk	Cure rate close to 100%
Hookworm	Single 400-mg dose	70–90% cure rate and 85–100% egg reduction rate
Trichuris trichiura	400-mg dose for 3 days	Cure rate of 35–70% and egg reduction rate of 50–90%
Toxocariasis	400 mg twice daily for 5 days	No large clinical trials
Lymphatic filariasis	400-mg dose for 1–7 days	Microfilaremia reduced by 98–99% for prolonged periods when administered in combination with doxycycline, DEC, or ivermectin; prolonged high doses also have a macrofilaricidal effect
Loa loa	Single 400-mg dose	No microfilaricidal effect, but partial macrofilaricidal effect with sterilization and/or death of adult worms
Giardia duodenalis	400 mg daily for 5 days	Cure rate of 80–97%
Gnathostoma spinigerum	400 mg twice daily for 21 days	No large clinical trials

[a] Adapted from reference 1.

transient and reversible elevations of serum transaminases occur in 1 to 5% of recipients. Occasionally, alopecia (<1%) and reversible leukopenia (<1%) are seen. Discontinuation of therapy is infrequently needed, but a death related to albendazole-induced pancytopenia has been described (25). There are rare reports of albendazole therapy triggering seizures in people with undiagnosed neurocysticercosis (26, 27).

When mebendazole (pregnancy category C) has been used by pregnant women, the incidence of spontaneous abortions or fetal malformations has not been greater than the background population rate. There are also no reports of teratogenicity from albendazole (pregnancy category C), and women who have received it inadvertently during pregnancy have not experienced adverse fetal outcomes (28). However, studies of benzimidazole drugs performed with animals suggest a possible teratogenic and embryotoxic effect. Since there are no good prospective safety data on either of these agents for humans, it is recommended that neither albendazole nor mebendazole be administered during pregnancy, particularly during the first trimester, unless the potential benefits justify the possible risk to the fetus. Albendazole and mebendazole are excreted in low concentrations in breast milk, and although they are unlikely to be harmful to infants, these drugs should be used with caution in lactating women (29).

Praziquantel

Praziquantel is a synthetic heterocyclic isoquinolone-pyrazine derivative. Major indications for its use are shown in Table 5. It has the unique characteristic of being active against almost all trematodes and cestodes, but it is not useful for treatment of nematode infections (4). It has been approved for use by the U.S. FDA (1).

Mechanism of action. Praziquantel induces ultrastructural changes in the teguments of parasites, resulting in increased permeability to calcium ions. Calcium ions accumulate in the parasite cytosol, leading to muscular contractions and ultimately to paralysis of adult worms. By damaging the tegument membrane, praziquantel also exposes parasite antigens to host immune responses (30, 31). These effects lead to dislodgment of worms from their intestinal sites and subsequent expulsion by peristalsis.

Pharmacokinetics. Praziquantel is available for oral administration, and >80% of the drug is absorbed. Coadministration with a high-carbohydrate meal increases drug concentrations in serum (32). The drug is biotransformed in the liver, and metabolites are excreted mainly in the urine. The cytochrome P450 hepatic metabolism of praziquantel is induced by corticosteroids, phenytoin, and phenobarbital.

TABLE 4 Major indications for mebendazole[a]

Indication	Usual dose	Reported efficacy
Ascaris lumbricoides	Single 500-mg dose or 100 mg twice daily for 3 days	Median cure rate of 95–98% and egg reduction rate of 99–100%
Enterobius vermicularis	Single 100-mg dose, repeated after 2–3 wk	Mean cure rate of 95%
Hookworm	Single 500-mg dose or 100 mg twice daily for 3 days	Cure rate of 15–30% and egg reduction rate of 60–95% (~70% cure rate achieved with 3 days of mebendazole 500 mg daily)
Trichuris trichiura	100 mg twice daily for 3 days	Cure rate of 70–90% and egg reduction rate of 90–95%
Toxocariasis	100–200 mg twice daily for 5 days	No large clinical trials

[a] Adapted from reference 1.

TABLE 5 Major indications for praziquantel[a]

Indication	Usual dose	Reported efficacy
Clonorchiasis	75 mg/kg/day in 3 doses for 2 days	Cure rate of 85–100%
Cysticercosis	50–100 mg/kg/day in 3 doses for 1–30 days	Variable
Opisthorchiasis	75 mg/kg/day in 3 doses for 2 days	Cure rate of >95%
Intestinal flukes	75 mg/kg/day in 3 doses for 1 day	No large clinical trials
Paragonimiasis	75 mg/kg/day in 3 doses for 2 days	>95% cure rate for pulmonary infections (may be lower in ectopic sites)
Schistosomiasis	40–60 mg/kg/day in 2–3 doses for 1 day	Cure rate of 75–100% and egg reduction rate of 90–95% in those not cured
Tapeworms	Single dose of 5–25 mg/kg	Cure rates of >95% for *Taenia*, *Diphyllobothrium*, and *Hymenolepis* species infections

[a] Adapted from reference 1.

Praziquantel levels in serum are therefore lowered when any of these drugs are coadministered. Cimetidine, which inhibits P450-mediated metabolism, can be given concurrently to increase levels of praziquantel in plasma. Levels in plasma peak after 1.5 to 2 h, and after a single 40-mg/kg dose, peak levels have been reported as 1.007 to 1.625 mg/liter (33). Praziquantel does not cross the blood-brain barrier well, so levels in CSF are only approximately 20 to 25% of those in plasma (14, 32). It is 80% protein bound, and its half-life in serum is 1 to 3 h. It is not dialyzable, and no adjustment in dose is recommended in either renal or hepatic insufficiency.

Spectrum of activity. Praziquantel is active against the larval and adult stages of many trematodes. It is the drug of choice for schistosomiasis and is effective for all *Schistosoma* species that infect humans. It is used both for treatment of individuals and in mass community chemotherapy programs and leads to decreased transmission and prevalence of infection. Praziquantel is also used for treatment of opisthorchiasis, clonorchiasis, paragonimiasis, and intestinal fluke infections, including fasciolopsiasis, heterophyiasis, and metagonimiasis. In contrast to other human trematode infections, praziquantel has not proven to be effective in the treatment of *Fasciola hepatica* infection.

Many cestode infections can also be treated with praziquantel. Most tapeworm infections respond, including those caused by *Taenia*, *Diphyllobothrium*, and *Hymenolepis* species. Because praziquantel does not kill eggs, precautions should be taken to prevent autoinfection and laboratory-acquired infection, particularly for *Taenia solium*. Praziquantel is also used in the treatment of neurocysticercosis as an alternative or adjunct to albendazole, although its overall benefit remains unclear.

Praziquantel has also been used in combination with albendazole for treatment of echinococcal infections. Praziquantel has high protoscolicidal activity *in vitro*, and some reports have suggested superior efficacy of the combination to that of either drug alone (34–36).

Adverse effects. Adverse effects of praziquantel are generally mild, but many studies report that some side effects occur in >30% of patients. Common reactions include dizziness, lethargy, headache, nausea, and abdominal pain. Hypersensitivity reactions occur rarely (37, 38). Severe adverse reactions are uncommon, although administration to individuals with neurocysticercosis can result in seizures and neurological sequelae related to precipitation of an inflammatory response (39).

Animal studies do not suggest a teratogenic effect of praziquantel (pregnancy category B), but an increased abortion rate has been seen in rats. There are minimal data on its safety in humans, but there is a very low potential for adverse effects on either the mother or her unborn child (40), and when praziquantel has been used during pregnancy, no increase in abortion rates, preterm deliveries, or congenital abnormalities has been noted (41). Consequently, praziquantel can be given after the first trimester. No adverse effects of praziquantel administration during lactation have been reported, but it is excreted in human breast milk and discontinuation of breast feeding on the day of therapy and for the following 72 h is sometimes suggested. However, owing to available data regarding its safety profile, in 2002 the World Health Organization recommended that it can be considered for use in pregnant and lactating women (42). It is FDA approved for children aged 4 years and over.

Ivermectin

Ivermectin is a semisynthetic macrocyclic lactone derivative of avermectins, which are natural substances derived from the actinomycete *Streptomyces avermitilis*. Major indications for its use are shown in Table 6. It was initially developed as an agent for veterinary use but is now used widely in humans. Ivermectin is a potent oral agent with relatively broad-spectrum anthelmintic activity. It has been approved by the U.S. FDA as an oral therapy for onchocerciasis and uncomplicated strongyloidiasis (1) and as a topical treatment for head lice.

Mechanism of action. Ivermectin causes an influx of chloride ions across glutamate-gated chloride channels in nerve and muscle cell membranes, resulting in hyperpolarization of the affected cells and consequent paralysis and death of parasites (4, 43–46). It has also been postulated that ivermectin may act as an antagonist of the neurotransmitter γ-aminobutyric acid (47). Although specific ivermectin binding sites have been identified in mammalian brain tissue, the affinity of ivermectin for sites within parasites is ~100 times greater than that for mammalian tissue.

Pharmacokinetics. Ivermectin is available as an oral preparation. There is no parenteral preparation of ivermectin approved by the FDA, but it has been given via the rectal and subcutaneous routes to critically ill patients with disseminated strongyloidiasis who are unable to tolerate oral therapy (48, 49). It is rapidly absorbed following oral administration. Bioavailability is thought to increase ~2.5-fold if it is taken with a high-fat meal; recommendations are to administer the drug on an empty stomach with water. Ivermectin is metabolized in the liver and excreted almost

TABLE 6 Major indications for ivermectin[a]

Indication	Usual dose	Reported efficacy
Ascaris lumbricoides	Single dose of 150–200 μg/kg	Cure rate of 78–99%
Cutaneous larva migrans	200 μg/kg daily (usually 12 mg) for 1–2 days	Cure rate of 77–100%
Gnathostoma spinigerum	200 μg/kg daily for 2 days	Cure rate of 76–95%
Onchocerca volvulus	Single dose of 150 μg/kg, repeat every 6–12 mo until asymptomatic	Skin microfilarial counts reduced by 85–95%, and levels remain suppressed by >90% at 1 yr
Strongyloides stercoralis	200 μg/kg daily for 2 doses (doses given either on consecutive days or 2 weeks apart)	Cure rate of 85–97% in uncomplicated infection (normal or immunocompromised hosts)
Trichuris trichiura	200 μg/kg daily for 3 days	Cure rate of 35–84%
Ectoparasites: scabies and lice	200-μg/kg dose, repeat after 2 wk for scabies	Almost 100% efficacy

[a] Adapted from reference 1.

entirely in the feces. Peak levels in serum occur at 4 to 5 h, and levels of ~46 μg/liter have been reported after a single 12-mg dose (15). It is highly protein bound in plasma, and it has a half-life of 10 to 18 h. The drug accumulates in fat tissue and does not readily cross the blood-brain barrier (50).

Spectrum of activity. Ivermectin is the drug of choice for onchocerciasis and strongyloidiasis. In onchocercal infections, ivermectin does not significantly affect the viability of adult worms, but it impairs release of microfilariae, is a potent microfilaricide, and leads to a sustained reduction in microfilaremia for many months (44). It can be used both for the treatment of individual patients and in mass chemotherapy programs in areas where onchocerciasis is endemic. The role of combination therapy with ivermectin is being explored. In uncomplicated strongyloidiasis, ivermectin has excellent efficacy in immunocompetent patients (47). In disseminated strongyloidiasis, ivermectin is administered daily until stool and sputum exams are negative for larvae.

Ivermectin also has activity against microfilariae of *Wuchereria bancrofti*, *Brugia malayi*, and *Loa loa*. It does not have a significant effect on adult worm viability in these infections, so reduced microfilaremia is sustained only with repeated doses, and it has not replaced DEC as first-line therapy for these infections. Ivermectin also has activity against *Mansonella ozzardi* and *Mansonella streptocerca* microfilariae.

Ivermectin also has efficacy against many intestinal helminths, including *Ascaris lumbricoides*, *T. trichiura*, *Enterobius vermicularis*, and cutaneous larva migrans (45, 51–53). However, it is not generally used as first-line treatment for these indications due to the widespread availability and excellent efficacy of albendazole. It also can be used for treatment of gnathostomiasis (54, 55). It is not active against trematodes or cestodes. In addition to its anthelmintic activity, ivermectin is the drug of choice for ectoparasitic infestations, including those of scabies and lice.

Adverse effects. Ivermectin is generally well tolerated. Most of the adverse effects that occur following its administration are a result of the host's immune response to destruction of parasites rather than to toxic effects of the drug per se. Adverse effects include fever, rash, dizziness, pruritis, myalgia, arthralgia, and tender lymphadenopathy; the severity of these symptoms relates to the pretreatment intensity of infection rather than to ivermectin concentrations in serum (56). Transaminitis is occasionally reported. Severe reactions occasionally occur, including a hypersensitivity response to dying microfilarial parasites known as the Mazzotti reaction. This anaphylactoid response is characterized by allergic manifestations including pruritis, edema, fever, and systemic hypotension. However, these reactions are primarily restricted to individuals with high parasite loads. In patients infected with *L. loa* who have elevated levels of microfilaremia, ivermectin has been associated with the development of fatal encephalopathy (57) and so should be avoided.

Ivermectin (pregnancy category C) has been shown to be teratogenic in mice, rats, and rabbits when given in repeated doses of 0.2, 8.1, and 4.5 times the maximum recommended human dose, respectively. Teratogenicity was characterized in the three species by cleft palate; clubbed forepaws were additionally observed in rabbits. These developmental effects were found only at or near doses that were maternotoxic to the pregnant female (14, 44). There are insufficient data to recommend its use in pregnant women, although the risk of fetal damage in 203 pregnant women inadvertently treated with ivermectin was no greater than that in controls, and it has been suggested that ivermectin can be given safely after the first trimester (58–60). It is excreted in breast milk in low concentrations, so it should be avoided in lactating women when possible. It is not recommended in children weighing <15 kg.

Diethylcarbamazine

DEC is a piperazine derivative. Its main use is in filarial infections. Major indications for its use are shown in Table 7. DEC is not licensed for use in the United States, but it can be obtained from the CDC under an Investigational New Drug (IND) protocol.

Mechanism of action. The mode of action of DEC is uncertain. It is predominantly a microfilaricidal agent, and it is thought that its main effect is to inhibit arachidonic acid metabolism and alter the surface membranes of microfilariae, thereby enhancing destruction via host immune responses (12, 13). It also has some macrofilaricidal activity under certain conditions, likely via hyperpolarization and immobilization of adult worms (61).

Pharmacokinetics. DEC is available only in tablet form. It is freely soluble in water and is almost completely absorbed after oral administration. The drug is metabolized in the liver, although >50% is excreted unchanged in the urine. There is negligible protein binding, and it is widely distrib-

TABLE 7 Major indications for DEC[a]

Indication	Usual dose	Reported efficacy
Loa loa	Up to 9 mg/kg/day in 3 doses for 12 days	Few large trials, but single course is curative in <50% of patients
Wuchereria bancrofti and *Brugia* spp. infections	Up to 6 mg/kg/day in 3 doses for 12 days (or repeated single doses)	>90–99% reduction in microfilaremia, but often need additional courses to eradicate adult worms

[a] Adapted from reference 1.

uted in tissues. It readily crosses the blood-brain barrier. Peak levels in plasma of 100 to 150 μg/liter at 1 to 2 h have been reported after a single 0.5-mg/kg dose (62). The half-life of DEC is 2 to 10 h. Renal excretion is reduced in the presence of an alkaline urinary pH, and dose reductions are required in patients with renal impairment.

Spectrum of activity. DEC is an effective microfilaricidal drug against *W. bancrofti*, *B. malayi*, *Brugia timori*, *Onchocerca volvulus*, *L. loa*, and *M. streptocerca*, but it has little or no effect on *Mansonella perstans* or *M. ozzardi* microfilariae (4). It has been the drug of choice for lymphatic filariasis for the last 50 years. It is used both for individual therapy for filarial infections and in mass community chemotherapy programs, either alone or in combination with ivermectin, albendazole, or doxycycline. It is predominantly a microfilaricidal agent, although it has some macrofilaricidal activity in *W. bancrofti*, *B. malayi*, *B. timori*, and *L. loa* infections (63–65). DEC has also been used in the treatment of toxocaral visceral larva migrans, but albendazole is now the preferred agent because of its better safety profile.

Adverse effects. The side effects of DEC include mild headache, dizziness, anorexia, nausea, and arthralgias. Administration of the drug to individuals with filarial infections can induce adverse results that are not due to the drug itself but instead are related to host responses, in part to release of *Wolbachia* endosymbionts from filariae, following damage to adult worms (local reactions) and death of microfilariae (systemic reactions). These reactions tend to be relatively mild with lymphatic filariasis and infrequent with loiasis but can be severe with onchocerciasis or with heavy *L. loa* infections and result in intense pruritis, rash, fever, hypotension, and encephalopathy. The potentially fatal Mazzotti reaction and serious ophthalmic adverse effects can occur following treatment of *O. volvulus* with DEC, so DEC is contraindicated for onchocercal infections. For heavy *L. loa* infections, DEC is contraindicated to avoid potentially fatal encephalopathy. Animal studies have not shown DEC to be teratogenic, but it may increase the risk of abortion and so should be avoided in pregnancy when possible (14, 66). It is not excreted in breast milk and is safe during lactation.

Pyrantel Pamoate

Pyrantel, a tetrahydropyrimidine, is a relatively broad-spectrum agent against nematodes. It is associated with more side effects and lower efficacy than the benzimidazoles, so it has now largely been replaced. It is approved by the U.S. FDA but is considered investigational for most indications except enterobiasis.

Mechanism of action. Pyrantel is a depolarizing neuromuscular blocking agent. It exerts its anthelmintic effect via release of acetylcholine and inhibition of helminthic acetylcholinesterase. This results in stimulation of ganglionic receptors and spastic paralysis of adult worms. The worms become dislodged from the intestinal wall and are expelled in the feces by normal peristalsis.

Pharmacokinetics. Pyrantel is administered orally but is almost insoluble in water and is therefore poorly absorbed from the gastrointestinal tract. Peak levels in serum occur after 1 to 3 h. More than 50% is excreted unchanged in the feces. The absorbed drug is partially metabolized in the liver. There is no significant interaction with food.

Spectrum of activity. Pyrantel has excellent efficacy in the treatment of ascariasis and hookworm and pinworm infections. It also has some activity against *Trichostrongylus*. It is not active against *T. trichiura*.

Adverse effects. Although pyrantel is generally well tolerated, it can lead to adverse reactions, including anorexia, nausea, vomiting, abdominal cramps, and diarrhea. It has also been associated with neurotoxic effects, including headache, dizziness, drowsiness, and insomnia. Transient increases in hepatic enzymes have also been reported, and one study reported nephrotic syndrome temporally related to its use (67). Animal studies have not shown adverse effects in the fetus, and it has been used during pregnancy in humans without harmful fetal effects (pregnancy category C) (68). It is not recommended for children <2 years of age.

AN AGENT WITH ANTHELMINTIC AND ANTIPROTOZOAL ACTIVITIES: NITAZOXANIDE

Nitazoxanide is a 5-nitrothiazole derivative with broad-spectrum activity against numerous intestinal protozoa, helminths, and anaerobic bacteria. Major indications for its use are shown in Table 8. It was initially developed as a veterinary anthelmintic with activity against intestinal nematodes, cestodes, and liver trematodes (69). The U.S. FDA has approved it for use for the treatment of diarrhea caused by *Cryptosporidium* species and *Giardia duodenalis* in pediatric and adult patients (69, 70).

Mechanism of action. Nitazoxanide inhibits pyruvate ferredoxin oxidoreductase, an enzyme essential to anaerobic energy metabolism. This is its mechanism of action against anaerobic protozoa and bacteria (e.g., *Trichomonas vaginalis*, *Entamoeba histolytica*, and *Clostridium perfringens*), although for protozoa additional mechanisms may also be involved (71). The exact mechanism of nitazoxanide's activity against helminths has not yet been determined.

Pharmacokinetics. Nitazoxanide is given by the oral route and is available as a suspension or in tablet formulation. Bioavailability is nearly doubled by administration with food (72). It is absorbed from the gastrointestinal tract, with approximately one-third of the oral dose excreted in

TABLE 8 Major indications for nitazoxanide[a]

Indication	Usual dose[b]	Reported efficacy
Cryptosporidium parvum	Immunocompetent children 1–11 yr old[c] and adults[c]: b.i.d. treatment for 3 days	Cure rate of 52–80%
	Immunocompromised patients: 1,000 mg b.i.d. for 2–8 wk	Cure rate of 18–67% (efficacy varies with CD4 count in HIV-positive patients)
Giardia duodenalis	b.i.d. treatment for 3 days[c]	Cure rate of 71–94%

[a] Adapted from reference 69.

[b] The dosing schedule of nitazoxanide given as a twice-daily (b.i.d.) treatment course for 3 days consists of the following: for children 1–3 yr of age, 100 mg b.i.d. for 3 days; for children 4–11 yr of age, 200 mg b.i.d. for 3 days; and for adults, 500 mg b.i.d. for 3 days.

[c] Licensed for this use by the U.S. FDA.

urine and two-thirds excreted in feces (73, 74). In blood, nitazoxanide is rapidly hydrolyzed to form an active metabolite, tizoxanide. The maximum concentration of tizoxanide in plasma following an oral dose of 500 mg nitazoxanide is 2 mg/liter (6.5 μM), and its half-life in plasma is 1 to 2 h (75). Tizoxanide is extensively bound to plasma proteins (>99%), and its urinary elimination half-life is 7.3 h (76). Tizoxanide then undergoes glucuronidation to form tizoxanide glucuronide. Nitazoxanide is not detected in plasma, urine, bile, or feces, but tizoxanide is found in plasma, urine, bile, and feces and tizoxanide glucuronide is found in plasma, bile, and urine (74). The pharmacokinetics of nitazoxanide in patients with impaired liver or renal function has not been studied, and it must be administered with caution to these patients (69).

Spectrum of activity. While nitazoxanide has been reported to have activity against a broad range of parasites, it is FDA approved only for treatment of *Cryptosporidium* species and *G. duodenalis*, and its role in treating other infections is ill defined.

Adverse effects. Nitazoxanide is well tolerated, with adverse effects similar to those of placebo. Mild and transient side effects have been seen in only 3 to 4% of patients, principally related to the gastrointestinal tract (abdominal pain, diarrhea, and nausea) (77). No significant adverse effects on electrocardiography, hematology, clinical chemistry, or urinalysis parameters in humans have been noted (71, 78). There are minimal data on the safety of nitazoxanide in pregnant (pregnancy category B) or lactating women (69).

ANTIMALARIALS

A number of agents are approved for use for treatment of malaria. The most commonly recommended agents are shown in Table 9.

Quinoline Derivatives

The quinoline derivatives can be divided into four groups: the 4-aminoquinolines; the cinchona alkaloids; synthetic compounds, such as mefloquine and halofantrine; and the 8-aminoquinolines. A related drug, piperaquine, which is a bisquinoline, is now often used in combination with artemisinin derivatives for treatment of malaria. Although it is used in Europe, it is not approved by the U.S. FDA.

4-Aminoquinolines

The 4-aminoquinolines include chloroquine, hydroxychloroquine, and amodiaquine. Chloroquine is the most widely used of these agents. It is an inexpensive, safe drug that has been used extensively for treatment and prophylaxis of all *Plasmodium* species that infect humans, although resistance to chloroquine in *Plasmodium falciparum* is prevalent globally in most malarious regions. Hydroxychloroquine is a related synthetic compound with an identical clinical spectrum, similar pharmacokinetics, and similar adverse effects. Amodiaquine is another related agent with the same mechanism of action and spectrum of activity. It is reported to be more effective than chloroquine for parasite clearance, but its use has been restricted due to uncommon serious side effects, as noted below. It has not been approved by the U.S. FDA (1).

Mechanism of action. The main mechanism of action of the 4-aminoquinolines is thought to be via nonenzymatic inhibition of heme polymerization. Asexual intraerythrocytic malaria parasites actively concentrate quinoline ring compounds within hemoglobin-containing vesicles. In the absence of drug, plasmodia degrade host erythrocyte hemoglobin to provide amino acid nutrients essential for parasite growth. The degradation of hemoglobin produces free heme, which is stored as ferriprotoporphyrin IX within the red blood cell. Ferriprotoporphyrin IX is toxic to the parasite and is usually polymerized into nontoxic malaria pigment (hemozoin). In the presence of drug, there is inhibition of the conversion of heme into hemozoin, leading to the accumulation of products toxic to the parasite and resulting in parasite death (79). These agents also inhibit protein synthesis by inhibiting incorporation of phosphate into DNA and RNA and by inhibiting DNA and RNA polymerases (13). Chloroquine also raises the pH of the vesicle (80).

TABLE 9 Treatment regimens for malaria

Organism	Primary agent used for treatment	Alternative agents
Plasmodium vivax and *Plasmodium ovale*	Artemisinin derivative or Malarone or chloroquine, followed by primaquine	Quinine sulfate plus doxycycline or plus clindamycin
Plasmodium falciparum	Artemisinin derivative or Malarone	
Plasmodium malariae	Chloroquine	
Plasmodium knowlesi	Artemisinin derivative or Malarone or chloroquine	

Pharmacokinetics. The 4-aminoquinolines are extensively distributed in tissues and are characterized by a long elimination half-life. Despite similarities in their chemical structures, these drugs show differences in their biotransformation and routes of elimination (81).

Chloroquine is available in oral and parenteral forms. Many different formulations are manufactured worldwide. Chloroquine is rapidly absorbed from the gastrointestinal tract after oral administration and has oral bioavailability exceeding 75%. Food has variable effects on absorption. The drug is distributed extensively in body tissues and reaches high levels within the brain (82). Chloroquine binds to melanin-containing cells in the skin and eye, so it can also reach high levels at these sites. There is marked variability in peak concentrations in plasma between individuals, but within 3 h of initiating standard oral treatment doses (10 mg chloroquine base/kg, followed by three doses of 5 mg/kg at 6, 24, and 48 h), concentrations in blood remain above 1 mmol/liter for at least 4 days (83). It is ~60% protein bound and has a half-life of 3 to 6 days. Approximately 30 to 50% of the drug is metabolized to inactive compounds in the liver, and the remainder is excreted in the urine. Treatment reduction (usually 50% of the normal dose) is required in patients with severe renal or hepatic failure. It is not dialyzable.

In contrast, amodiaquine is a prodrug and is almost entirely metabolized to a biologically active metabolite, desethylamodiaquine, following oral administration. Otherwise, it has pharmacokinetic properties similar to those of chloroquine but has a smaller volume of distribution.

Spectrum of activity. The 4-aminoquinolines are efficient and rapidly acting blood schizonticides. They can be used in both the treatment and prophylaxis of infection with susceptible strains of all *Plasmodium* species. They have no effect on tissue schizonts or exoerythrocytic stages. They are gametocytocidal for *Plasmodium vivax* and *Plasmodium malariae* but have minimal effect on *P. falciparum* gametocytes. Following infections with *P. vivax* or *Plasmodium ovale*, primaquine is also needed to eradicate liver hypnozoites and prevent relapses of infection.

Chloroquine currently is still a first-line option for therapy for some *P. vivax*, *P. malariae*, and *P. ovale* infections, but increasing resistance among *P. vivax* isolates globally, and especially among *P. vivax* infections acquired in Indonesia, East Timor, Papua New Guinea, and the Solomon Islands, is emerging. It also remains the treatment of choice for susceptible *P. falciparum* strains, although *P. falciparum* strains from almost all areas of the world have developed resistance to it and so it is now rarely used for *P. falciparum*. It is also effective against *Plasmodium knowlesi* infections (84). Because of its potential toxicity, amodiaquine is not recommended for prophylaxis of malaria and is generally not used as first-line treatment. However, it results in faster parasite clearance and more rapid resolution of symptoms than those with chloroquine, and it may be effective in some cases of chloroquine-resistant malaria, so it is used as an alternative treatment regimen in some areas.

Chloroquine is also active against *E. histolytica* trophozoites but is rarely used for this indication, as the nitro-5-imidazoles are the drugs of choice.

Adverse effects. Chloroquine has a bitter taste. It is generally well tolerated at the doses required for malaria prevention or treatment, even when taken for prolonged periods. However, it can lead to nausea, abdominal discomfort, dizziness, retinal pigmentation, blurred vision, electrocardiographic changes, muscular weakness, and, rarely, transient psychiatric symptoms. It can also cause severe pruritis, particularly in African blacks. Irreversible neuroretinitis can result if it is taken at high doses for prolonged periods. If taken as an overdose, it can cause shock, arrhythmia, and death.

At the doses used for malaria treatment or prophylaxis, chloroquine has rarely been reported to cause adverse congenital effects (pregnancy category C) (85). However, affinity for melanin-containing tissues, such as the retina, iris, and choroid of the eye, has been reported, and definitive delineation of fetal risk remains undefined. It is used commonly for treatment and prophylaxis of malaria in pregnant women, without evidence of teratogenicity, and it is generally agreed that the benefits of preventing and treating malaria in pregnant women outweigh the potential fetal risks. Chloroquine is excreted in small amounts in breast milk.

Amodiaquine is more palatable than chloroquine and seems to cause less itching. However, serious adverse events, including agranulocytosis, aplastic anemia, and drug-induced hepatitis, have been reported. These have occurred predominantly following long-term amodiaquine use (mean, 7 to 8 weeks) for malaria prophylaxis. While short-term treatment regimens are thought to be safe (86), this drug is now used uncommonly.

Cinchona Alkaloids

The cinchona alkaloids, quinine and quinidine, contain a quinoline ring. Quinidine is the diastereoisomer of quinine. Quinine was originally extracted from the bark of the South American cinchona tree, but a synthetic form is now available, usually as a quinine sulfate salt. Quinidine is more active than quinine, but it is also more cardiotoxic.

Mechanism of action. The exact target of cinchona alkaloids is unknown. They are thought to act by forming complexes with ferriprotoporphyrin IX, thereby interfering with hemoglobin digestion and resulting in cell lysis and death of schizonts (87). They also interfere with the function of plasmodial DNA and inhibit the synthesis of parasite nucleic acids and proteins. Quinine also interacts with certain fatty acids present in parasitized erythrocytes, preventing red blood cell lysis and interrupting schizont maturation (13). Additionally, it increases intracellular pH, resulting in lethal effects on the parasite.

Pharmacokinetics. Quinine is available for oral administration as a sulfate salt and for parenteral administration as quinine dihydrochloride. It is >80% absorbed from the gastrointestinal tract following oral doses. It is widely distributed in body tissues, but concentrations in CSF are <10% of concurrent levels in plasma. It is >90% protein bound. Quinine is metabolized in the liver, and the native drug and its metabolites are excreted in the urine (82). It has a short half-life of 8 to 12 h, necessitating multiple daily doses. After a single dose of 650 mg of quinine sulfate, peak concentrations in serum are ~3.2 mg/liter in healthy individuals but are higher (8.4 mg/liter) in patients with malaria. Intravenous quinine is used in many countries when oral therapy cannot be tolerated, but quinidine gluconate is considered the parenteral drug of choice in the United States (1). Both agents have similar pharmacokinetic properties.

The pharmacokinetic properties of the cinchona alkaloids are considerably altered in patients with malaria, with a reduction in clearance that is proportional to the severity of disease. Consequently, doses should be decreased by 30 to 50% after the third day of treatment to avoid accumulation of drug in seriously ill patients (88). Drug levels may also be increased by administration with foods that alkalinize the urine, because increased tubular reabsorption results. Caution is recommended for patients with significant liver impairment, and dose reduction is required if there is renal impairment. Both agents are partially dialyzable.

Spectrum of activity. The cinchona alkaloids can be used in the treatment of all *Plasmodium* species that infect humans. Their main indication is for chloroquine-resistant *P. falciparum*. Oral therapy is indicated for uncomplicated malaria, but intravenous formulations of quinine dihydrochloride or quinidine gluconate are used in severe infections.

Quinine and quinidine are blood schizonticides but have little effect on sporozoites or preerythrocytic forms of the parasite. Consequently, they do not eradicate *P. vivax* or *P. ovale* hypnozoites in the liver. They also are not gametocytocidal against *P. falciparum*. Although resistance to these agents has emerged in Southeast Asia and Africa, they remain useful drugs for the treatment of malaria worldwide; however, over recent years they have been replaced by artemisinin derivatives for first-line malaria treatment.

Quinine is also used for the treatment of babesiosis. It is ineffective when used as a single agent but can be given together with clindamycin or azithromycin (1).

Adverse effects. Quinine has an extremely bitter taste and can be associated with nausea, vomiting, and epigastric pain. It also often leads to the symptom complex of cinchonism (nausea, tinnitus, dysphoria, and reversible high-tone deafness). Quinine can also cause hyperinsulinemic hypoglycemia, especially in children and pregnant women with severe malaria, as it increases release of insulin from the pancreas. It has also been associated with massive hemolysis in patients with heavy *P. falciparum* infections. Agranulocytosis, thrombocytopenia, retinopathy, and tongue discoloration are additional adverse effects that have been described. Overdose of quinine can lead to ataxia, convulsions, and coma.

When used as treatment for severe malaria, intravenous quinidine is associated with cardiac arrhythmias. It prolongs the QT interval, widens the QRS complex, and prolongs the PR interval. It can therefore lead to hypotension and ventricular arrhythmias, including torsades de pointes. Consequently, it should be administered only in an intensive care setting with cardiac monitoring. As with quinine, administration can also result in blood dyscrasias and cinchonism.

Despite reports of congenital defects following administration of quinine during pregnancy, it can be administered during pregnancy when the benefits of maternal treatment outweigh the potential fetal risks (pregnancy category C) (89, 90). Quinidine has not been reported to be teratogenic. Quinine can have an abortificient effect and can lead to induction of labor. It is excreted in small amounts in breast milk but can be administered during breast feeding when necessary.

Synthetic Quinoline Compounds

Mefloquine

Mefloquine is a synthetic 4-quinoline methanol compound structurally related to quinine.

Mechanism of action. Mefloquine interacts both with host cell phospholipids and with ferriprotoporphyrin IX of the parasitized erythrocyte (13). Its action is thought to rely on interference with the digestion of hemoglobin during the blood stages of the malaria life cycle, likely via a mechanism similar to that of quinine (87). It does not inhibit protein synthesis.

Pharmacokinetics. Mefloquine is available for oral administration only. Food enhances bioavailability, and it should not be taken on an empty stomach. It is >85% absorbed following oral administration and is concentrated within red blood cells (91). It is >95% protein bound and has a half-life of 2 to 4 weeks. Because of its long half-life, mefloquine is frequently used for prophylaxis of malaria, as a once-weekly dose. However, when mefloquine is administered weekly, it requires about 8 weeks before steady-state drug levels are reached, so a loading dose is often recommended. Peak concentrations in plasma occur at 6 to 24 h, and following a single dose of 500 mg or 1,000 mg orally, they are 430 and 800 μg/liter, respectively. In healthy volunteers, a dose of 250 mg once weekly produces maximum steady-state plasma concentrations of 1,000 to 2,000 μg/liter, which are reached after 7 to 10 weeks. It is highly lipophilic, is widely distributed throughout the body, and can cross the blood-brain barrier. Mefloquine is metabolized in the liver and excreted through the bile and feces. There are no specific recommendations regarding the need for dosage adjustment in patients with renal or hepatic failure. It is not dialyzable.

Spectrum of activity. Mefloquine is active against the erythrocytic schizonts of all *Plasmodium* species causing human malaria, and it has been used for both chemoprophylaxis and therapy. Since weekly administration is sufficient for chemoprophylaxis, it is convenient for use in travelers to areas where malaria is endemic. Its main utility in malaria treatment results from its activity against most chloroquine-resistant *P. falciparum* strains, although resistance has been recognized, particularly in some areas of Southeast Asia. When used for therapy, it should be combined with another agent, usually an artemisinin derivative. Mefloquine does not kill tissue schizonts, so patients infected with *P. vivax* should subsequently be treated with an 8-aminoquinoline. It also has no effect on gametocytes.

Adverse effects. Adverse reactions to mefloquine include nausea and vomiting, agranulocytosis, and aplastic anemia, as well as central nervous system (CNS) effects such as dysphoria, dizziness, disturbed sleep, nightmares, and ataxia. Severe neuropsychiatric reactions, including delirium and seizures, have been reported occasionally and are thought to occur in approximately 1:200 to 1:1,300 patients treated for acute falciparum malaria (92, 93). Mefloquine can also potentiate dysrhythmias in individuals on β-blockers. Mefloquine is teratogenic in high doses in animals, but reports on humans do not support teratogenic effects (pregnancy category B) (94). A possible higher rate of spontaneous abortion has been suggested, so it is generally avoided in the first trimester of pregnancy if possible. However, limited data suggest that it is probably safe to use even during the first trimester, and it can be used in later stages of pregnancy if the benefits outweigh the potential risks (95). It is excreted in low concentrations in breast milk but can be used in lactating women when necessary.

Halofantrine

Halofantrine is a synthetic phenanthrene-methanol compound. It has not been approved by the U.S. FDA (1), and because of its potential cardiac side effects, it has limited indications for use.

Mechanism of action. Halofantrine has activity against the asexual erythrocytic stages of malaria parasites, although the exact mechanism of action is unclear.

Pharmacokinetics. Halofantrine is available only for oral administration but has variable bioavailability. Absorption is enhanced by administration with fatty food, but because high levels in blood enhance toxicity, it is recommended for administration on an empty stomach. After three doses of 500 mg of halofantrine hydrochloride (at 0, 6, and 12 h), a maximum concentration in plasma of 896 μg/liter was reported (96). Halofantrine is metabolized in the liver to an active metabolite, *N*-desbutylhalofantrine. The half-lives of halofantrine and its metabolite are 6 to 10 days and 3 to 4 days, respectively. It is excreted mainly in the feces.

Spectrum of activity. Halofantrine is efficacious in the treatment of *P. vivax* and *P. falciparum* malaria, but data concerning *P. ovale* and *P. malariae* are limited (97). It is not recommended for prophylaxis of malaria because of toxicity. It is active against blood-stage schizonts only and appears to have no effect against sporozoites, gametocytes, or tissue-stage parasites. Halofantrine is more active than mefloquine, but cross-resistance between these drugs occur. Its expense and potential toxicity also limit its use.

Adverse effects. Halofantrine leads to gastrointestinal adverse effects, including nausea, vomiting, diarrhea, and abdominal pain. It also has potential cardiovascular toxicity and causes concentration-dependent prolongation of the QT interval. It is therefore contraindicated in patients with long QT syndrome, as it can lead to cardiac arrest (98). It can also lead to pruritis and hepatic enzyme elevations. Halofantrine is contraindicated in pregnancy. The degree of excretion in breast milk is unknown, and it is not advised for use in lactating women.

Lumefantrine

Lumefantrine is a drug with a similar structure to that of halofantrine. It is widely used as a long-acting partner drug to artemether in a fixed-dose combination for malaria.

Mechanism of action. The exact mechanism by which lumefantrine exerts its antimalarial effect is unknown. However, it seems to inhibit the formation of β-hematin by forming a complex with hemin and inhibits nucleic acid and protein synthesis.

Pharmacokinetics. The oral bioavailability of lumefantrine is highly variable and increases up to 3- to 4-fold when it is taken with a high-fat meal. Peak levels in plasma are seen after 6 to 8 h. Lumefantrine is 99.7% protein bound, and its half-life is 3 to 6 days. It is highly lipophilic and has an apparent large volume of distribution. Peak levels in plasma vary considerably, but after administration of six tablets containing 2,780 mg of lumefantrine, median levels in plasma of 8 to 9 μg/ml were reported (99). Lumefantrine is extensively metabolized in the liver, and the major metabolite found in plasma is desbutyl-lumefantrine. It should be used with caution in patients with severe renal or hepatic failure.

Spectrum of activity. In its fixed-dose combination with artemether, lumefantrine has efficacy against all *Plasmodium* species.

Adverse effects. Lumefantrine is well tolerated, with rare mild adverse reactions such as diarrhea, nausea, abdominal pain, and vomiting. There is no evidence of significant cardiotoxicity associated with lumefantrine use, but it is recommended that it be avoided in patients at risk for QT prolongation. Artemether-lumefantrine has been assigned to pregnancy category C by the FDA. There are no human data on the excretion of lumefantrine into breast milk, but animal data suggest some excretion. The effects in the nursing infant are unknown, and it should be used with caution in lactating women.

8-Aminoquinolines

The 8-aminoquinolines are primaquine and tafenoquine (WR 238,605). Tafenoquine is not yet commercially available and has not been approved by the U.S. FDA (1).

Mechanism of action. The 8-aminoquinolines interfere with parasite mitochondrial enzymes involved in energy production. They also have an inhibitory action against DNA, although the exact mechanism by which this occurs in unclear (13). An active metabolite of the drug is thought to interrupt the mitochondrial transport system and pyrimidine synthesis in hypnozoites (100).

Pharmacokinetics. The 8-aminoquinolines are well absorbed after oral administration, with >90% bioavailability. Primaquine is rapidly metabolized in the liver, and its half-life is 4 to 9 h, so it needs to be administered daily. Following a single 45-mg oral dose, a mean peak level in serum of 153.3 μg/liter was observed after 2 to 3 h (101). It is found at relatively low concentrations in most body sites. Tafenoquine has a longer half-life of 2 to 4 weeks. Weekly or possibly monthly doses seem to be sufficient for prophylaxis, thus making it better tolerated than primaquine (102). A single dose of tafenoquine may be sufficient for prevention of relapse following *P. vivax* infections (103). Optimal dose-finding studies are being performed.

Spectrum of activity. Primaquine is less active against blood-stage malarial forms than most other antimalarial agents are, but it is very active against preerythrocytic sporozoites and exoerythrocytic tissue schizonts of all malarial species. Its main use is to prevent relapse of *P. vivax* and *P. ovale* infections from latent hypnozoites following treatment with chloroquine. Additionally, it is gametocytocidal against *Plasmodium*, especially *P. falciparum*, and can interrupt transmission of malaria. It is also an effective causal prophylactic agent but traditionally has been used infrequently in this way for travelers. Tafenoquine is reported to be more active than primaquine and has higher schizonticidal activity (104).

Adverse effects. Mild gastrointestinal side effects, including nausea and abdominal pain, are common following 8-aminoquinoline administration. They should not be used in people with glucose-6-phosphate dehydrogenase deficiency, as they can induce hemolysis. Patients with NADH methemoglobin reductase deficiency are at risk of developing methemoglobinemia. Primaquine also occasionally causes arrhythmias. Interference with visual accommodation has also been reported. These agents should also not be used during pregnancy or lactation because of the potential risk of hemolytic effects in the fetus.

Artemisinin (Qinghasou) Derivatives

Artemisinin is an extract from the Chinese herbal plant *Artemisia annua*, also known as qinghasou. It is a sesquiterpene lactone peroxide. Synthetic derivatives include artemether, dihydroartemisinin, arteether, and artesunate. Although not officially approved for use by the U.S. FDA, the intravenous formulation of artesunate is available via the CDC under an IND for patients with severe malaria.

Mechanism of action. Artemisinin and its derivatives act mainly against the asexual erythrocytic stages of malaria parasites. They have an antiparasitic effect, particularly on young, ring-form parasites, leading to their clearance and preventing development of more mature pathogenic forms. They bind to the parasite membrane and to ferriprotoporphyrin IX, so they are highly concentrated within parasites and reach 100 to 300 times higher concentrations in *P. falciparum*-infected red cells than in uninfected cells (105). By binding iron in the malarial pigment, they lead to the production of toxic oxidative free radicals that damage parasite organelles and alkylate parasitic proteins, leading to inhibition of protein synthesis and ultimately to parasite death.

Pharmacokinetics. The derivatives of artemisinin have greater solubility than artemisinin and consequently have been developed for easier administration by a variety of routes. Artesunate is water-soluble and can be given intravenously, intramuscularly, orally, or by suppository. Artemether and arteether are oil-soluble and are available in tablet, capsule, and intramuscular injection forms. Although artesunate is the most potent *in vitro*, there is no apparent clinical difference in efficacy between the formulations. The artemisinin derivatives have a short half-life of <1 to 2 h. They are usually administered once daily for a minimum of 3 days. All are metabolized to the active compound, dihydroartemisinin. After single oral doses of 2 and 4 mg of dihydroartemisinin/kg of body weight in healthy volunteers, median peak values in plasma of 181 and 360 µg/liter, respectively, were observed (106). Inhibitors of cytochrome P450, such as grapefruit juice, increase the levels of artemether in plasma. Artemisinin is eliminated by glucuronidation to inactive metabolites (4, 107). The pharmacokinetics of artesunate may be altered by pregnancy and by acute malaria infection. Artemisinin derivatives should be used with caution in individuals with hepatic or renal impairment.

Spectrum of activity. The artemisinin derivatives are effective against *P. falciparum* and *P. vivax* and are the most potent and rapidly acting parasiticidal drugs. They act specifically against the erythrocytic stages of *Plasmodium*. They are effective against multidrug-resistant *P. falciparum* and are the drugs of choice against mefloquine- and/or quinine-resistant *P. falciparum* isolates and against chloroquine-resistant *P. vivax*. The artemisinin derivatives are also active against gametocytes, reducing gametocyte carriage by ~90% and therefore decreasing malaria transmission in areas where they are widely used (108, 109). They are not effective against the intrahepatic hypnozoite stage of *P. vivax* or *P. ovale* infections. Late recrudescence is common unless these agents are combined with another drug, so they should be administered with a second agent such as mefloquine or doxycycline or in fixed combinations such as artemether-lumafantrine, artesunate-amodiaquine, or dihydroartemisinin-piperaquine. The artemisinin derivatives are usually associated with quick clearance of parasitemia, and recent randomized trials comparing them with quinine have shown a benefit in terms of mortality in adults treated for severe *P. falciparum* malaria (110–112). However, artemisinin-resistant *P. falciparum* malaria is now also emerging, particularly along the Thai-Cambodian border, characterized clinically by a substantial delay in parasite clearance (113). Despite limited data, the oral artemisinin derivative combinations seem effective against *P. knowlesi*, *P. ovale*, and *P. malariae* infections. Intravenous artesunate is used for those affected by severe malaria due to *P. knowlesi* (114). The artemisinin derivatives also have antitrematode activity and have been studied in schistosomiasis. They are less effective than praziquantel but may have a role as part of combination therapy in the future (115–117).

Adverse effects. The artemisinin derivatives are very well tolerated, with no serious toxicity or subjective adverse effects, although hematopoietic suppression has been described. They have been associated with adverse neurological effects in animal models, but there is no evidence that this occurs in humans. Fetal deaths and congenital malformations seen in rodent studies have not been observed in clinical trials involving 1,837 pregnant women, including 176 patients in the first trimester, exposed to an artemisinin agent or artemisinin-based combination therapy (118, 119). Although it is generally recommended that they be avoided in pregnancy because of insufficient safety data, they can be used, particularly after the first trimester, if the benefits outweigh the risks. The amount excreted in breast milk is unknown.

Antifolates

Pyrimethamine-sulfadoxine, also known as Fansidar, is still used for treatment of malaria in some countries despite widespread resistance. Pyrimethamine is a synthetic aminopyrimidine antimalarial agent, and sulfadoxine is a long-acting sulfonamide agent.

Mechanism of action. Pyrimethamine acts against the asexual erythrocytic stage of *Plasmodium* by inhibiting the plasmodial enzyme dihydrofolate reductase. Although it is active against *P. falciparum*, rapid development of resistance occurs and is a major factor limiting its use. Combining pyrimethamine with a sulfonamide or sulfone provides sequential, synergistic inhibition of the folate biosynthesis pathway. Malaria parasites are unable to utilize host-derived folic acid, so inhibition of folic acid biosynthesis prevents malarial DNA replication, ultimately leading to cell death.

Pharmacokinetics. Fansidar tablets comprise 25 mg of pyrimethamine and 500 mg of sulfadoxine. Both drugs are well absorbed orally. After oral administration of a single tablet, peak concentrations of pyrimethamine and sulfadoxine in plasma are 0.13 to 0.4 mg/liter and 51 to 76 mg/liter, respectively. The half-life of pyrimethamine is 80 to 95 h, and the half-life of sulfadoxine is 150 to 200 h (88). Both components are ~90% protein bound. Sulfadoxine is metabolized in the liver, and both agents are excreted mainly in the urine.

Spectrum of activity. Pyrimethamine-sulfadoxine is effective for both treatment and chemoprophylaxis of *P. falciparum* malaria. It is no longer recommended for routine prophylaxis because of the potential for severe adverse effects. It acts mainly against blood schizonts and does not have significant gametocytocidal activity. Pyrimethamine-sulfadoxine also has some efficacy in the treatment of *P. vivax* infection, but it has longer parasite and fever clearance times and higher failure rates (30 to 40%) than chloroquine and thus is not recommended. The efficacy of pyrimethamine-sulfadoxine against *P. ovale* and *P. malariae* has not been adequately evaluated.

Pyrimethamine is also used in combination with sulfadiazine for the treatment of toxoplasmosis.

Adverse effects. Pyrimethamine-sulfadoxine can result in adverse effects, including rash, nausea, vomiting, headache, and peripheral neuritis. It can also be associated with more serious and occasionally fatal reactions, including Stevens-Johnson syndrome and blood dyscrasias (particularly agranulocytosis and megaloblastic anemia). Other reported adverse effects include hepatitis, toxic nephrosis, exfoliative dermatitis, and erythema multiforme. The long half-life of the sulfa component means that sensitivity reactions can be sustained for prolonged periods even after the drug is discontinued.

Pyrimethamine has been associated with teratogenic effects in animals. There are no controlled human studies, but it has been used frequently during pregnancy, particularly in African countries, and has been associated with good fetal outcomes. It is officially recommended for use during pregnancy only when potential benefits outweigh the possible risks to the fetus. Pyrimethamine-sulfadoxine can cause kernicterus in infants, so it should be used with caution in pregnant women late in the third trimester. Both pyrimethamine and sulfadoxine are excreted into breast milk and are preferably avoided during lactation.

Atovaquone-Proguanil (Malarone)

Malarone is a tablet combination of 250 mg of atovaquone and 100 mg of proguanil. Atovaquone is a hydroxynaphthoquinone, and proguanil is an antifolate. Malarone has been approved for use by the U.S. FDA for prophylaxis and treatment of malaria.

Mechanism of action. *Plasmodium* species are dependent on *de novo* pyrimidine biosynthesis, which is selectively coupled with electron transport. Atovaquone inhibits the electron transport system in the mitochondria of parasites, thereby blocking nucleic acid synthesis and inhibiting replication (120). When used as monotherapy, atovaquone is associated with high recrudescence rates. Proguanil also acts against the asexual erythrocytic stage of the parasite by selectively inhibiting plasmodial dihydrofolate reductase. However, in combination with atovaquone, it acts via a different mechanism and directly lowers the effective concentration at which atovaquone causes collapse of the mitochondrial membrane potential (121).

Pharmacokinetics. Atovaquone is a highly lipophilic compound with low aqueous solubility and poor and variable oral availability. Its absorption is increased if administered with fatty foods or a milky drink. It is not metabolized and is excreted almost exclusively in the feces. It is 99% plasma protein bound, and its half-life is 2 to 4 days. Atovaquone levels vary widely between individuals (122). Proguanil is rapidly and extensively absorbed after oral administration. It is metabolized by cytochrome P450 in the liver to the active cyclic triazine metabolite, cycloguanil. It is 75% protein bound and is excreted mainly in the urine. Its half-life is 12 to 21 h. After two Malarone tablets twice daily for 3 days, mean levels of proguanil in plasma of 170 μg/liter have been reported. The pharmacokinetics of Malarone are altered during pregnancy. No dosing adjustments are required in the setting of mild to moderate hepatic or renal insufficiency. However, with severe renal impairment (creatinine clearance of <30 ml/min), use of Malarone is contraindicated for prophylaxis, but it can be used for treatment if the benefits outweigh the risks.

Spectrum of activity. Atovaquone-proguanil is effective against asexual and sexual forms of the *P. falciparum* parasite and is recommended for treatment and prophylaxis of falciparum malaria. It is used frequently for chemoprophylaxis in travelers to areas where malaria is endemic. Atovaquone-proguanil is also effective for treating *P. vivax* and *P. ovale* infections, but neither drug is effective against hypnozoites, so primaquine is additionally required to prevent relapses after drug discontinuation. Atovaquone-proguanil also showed good efficacy against *P. malariae* and *P. knowlesi* in limited studies (114, 123). Reports of clinical failures and resistance of *P. falciparum* isolates to atovaquone-proguanil via single mutations to the cytochrome *b* gene are emerging.

Adverse effects. Atovaquone-proguanil is generally very well tolerated. Side effects are mild and include anorexia, nausea, vomiting, abdominal pain, diarrhea, pruritis, and headache. Between 5 and 10% of recipients develop transient asymptomatic elevations in transaminases and amylase. Because of inadequate safety data, it is not recommended for prophylaxis during pregnancy (pregnancy category C) or lactation. It is also not recommended for treatment during pregnancy but can be considered if warranted. There are insufficient safety data to recommend its use in children weighing <5 kg.

OTHER ANTIPROTOZOAL AGENTS

Diloxanide Furoate

Diloxanide furoate, also known as Furamide, is a substituted acetanilide. Its main use is as a luminal amebicidal agent. It is not widely available in the United States and can be obtained only from specific pharmacies (1).

Mechanism of action. The mechanism of action of diloxanide furoate is unknown.

Pharmacokinetics. Diloxanide furoate is available in tablet form. The parent drug is poorly absorbed following oral administration, but it is hydrolyzed in the bowel lumen to an active compound, diloxanide. This is >90% absorbed and is glucuronidated in the liver, reaching peak levels in serum within 1 to 2 h. Metabolites are excreted primarily in the urine.

Spectrum of activity. Diloxanide furoate acts primarily as a luminal agent and helps to clear the bowel of *E. histolytica* cysts, thereby preventing relapse in cyst carriers (124). It is not effective for amebae in the bowel wall or in other tissues such as the liver, so it is generally given with a 5-nitroimidazole.

Adverse effects. Side effects are generally not severe but include rash, nausea, abdominal pain, diarrhea, and flatulence. It is not recommended in pregnancy or during lactation.

Iodoquinol

Iodoquinol is a halogenated 8-hydroxyquinoline derivative, diiodohydroxyquin.

Mechanism of action. Iodoquinol is thought to act by inactivating essential parasitic enzymes and inhibiting parasite multiplication (13).

Pharmacokinetics. Iodoquinol is available for oral administration but is very slowly absorbed, with <8% reaching the systemic circulation, so it is excreted primarily in the feces. Small amounts of absorbed drug are glucuronidated in the liver, and small quantities of glucuronic acid metabolites are excreted in the urine. It should not be used in individuals with renal or hepatic insufficiency and should be used with caution in patients with thyroid or neurologic disease.

Spectrum of activity. Iodoquinol is a potent amebicidal drug. It is effective against trophozoites and cysts of *E. histolytica* located within the lumen of the intestine and is used to eradicate amebic cysts to help prevent relapse of infection (124). Because it is poorly absorbed systemically, it is not effective for invasive intestinal or extraintestinal *E. histolytica* infections and is therefore frequently combined with a 5-nitroimidazole.

Iodoquinol also has activity against *Balantidium coli*, *Dientamoeba fragilis*, and *G. duodenalis*. It has also been used for *Blastocystis hominis*, although the significance of this agent as a true pathogen remains controversial (125).

Adverse effects. The main side effects of iodoquinol include nausea, abdominal cramps, diarrhea, headache, pruritis, and rash. Skin and hair may temporarily be stained yellow-brown following exposure. At high doses or with prolonged use, it can cause optic neuritis, optic atrophy, peripheral neuropathy, ataxia, and seizures. It is also associated with nephrotoxicity. It should be avoided in individuals who are sensitive to iodine. The degree of safety associated with its use in pregnancy or lactation is uncertain, so it is recommended that it be avoided.

Pentavalent Antimonial Compounds

The pentavalent antimony derivatives are used for treatment of leishmaniasis. They include sodium antimonylgluconate (or stibogluconate), also known as Pentostam, and *N*-methylglucamine antimoniate (or meglumine antimoniate), also known as Glucantime. Selection of one drug over the other is based primarily on cost and availability. Neither drug is licensed for use in the United States, but sodium stibogluconate is available from the CDC for individual patient use (1).

Mechanism of action. The precise mechanism of action of the pentavalent antimony derivatives remains unclear. They are thought to inhibit enzymes of glycolysis within parasites. Because glycolysis is the major source of parasitic ATP, the blockade of this source of energy is fatal to parasites (13).

Pharmacokinetics. The pentavalent antimonials are administered parenterally (intramuscularly or intravenously) or via intralesional injection. New formulations and drug delivery approaches are being investigated. The drugs remain in plasma and are excreted predominantly by the kidneys. Small amounts are metabolized in the liver to trivalent antimony, which contributes to the toxicity associated with their use. Following intramuscular administration of an initial dose of 10 mg of antimony per kg of body weight, mean peak antimony concentrations in blood of 9 to 12 mg/liter at 2 h have been reported (126). There are no specific guidelines regarding dose adjustment for renal impairment.

Spectrum of activity. Antimony preparations are efficient in killing many protozoan and helminthic parasites but are no longer recommended for most parasitic infections because of their toxicity. They are still used for the treatment of visceral, mucocutaneous, and cutaneous leishmaniasis, as few effective alternatives exist, although increasing drug resistance is emerging. Various treatment regimens are used, often involving 28 days of therapy, but the exact duration and efficacy vary depending on the type of leishmaniasis, the severity of the lesion, and the area of endemicity.

Adverse effects. Minor adverse effects from the pentavalent antimonials are common and include nausea, vomiting, headache, and malaise (127). More severe reactions, such as leukopenia, agranulocytosis, and electrocardiographic changes (prolongation of the QT interval and ventricular arrhythmias), can also occur. Renal insufficiency, proteinuria, and elevation of hepatic and pancreatic enzymes have also been described.

Miltefosine

Miltefosine is a phosphocholine analogue that was originally developed as an anticancer compound. It is the first effective oral agent for visceral leishmaniasis and is becoming increasingly important because of growing resistance of *Leishmania* strains to pentavalent antimonials. Miltefosine was registered in 2002 for the oral treatment of visceral leishmaniasis in India and has been approved for use in Germany. It is not approved by the U.S. FDA (128) and requires an emergency IND request to the FDA (1). Miltefosine has shown ameba-killing activity *in vitro* against the free-living ameba *Naegleria fowleri* and has been used successfully to treat patients infected with *Balamuthia* and disseminated *Acanthamoeba* infections.

Mechanism of action. The mechanism of action of miltefosine is not well understood. The drug interferes with cell signaling pathways and appears to act on key enzymes involved in the metabolism of ether lipids present on the surfaces of parasites (129, 130). Miltefosine does not appear to have a direct immunostimulatory effect, but it does induce apoptotic cell death (131, 132). It has been shown to be active against both the extracellular promastigote form and the intracellular amastigote form of *Leishmania* parasites both *in vitro* and *in vivo* (133).

Pharmacokinetics. Miltefosine is well absorbed after oral administration and is widely distributed. Minimal pharmacokinetic data are available for humans, but in rats the drug is rapidly taken up and accumulates in the kidney, liver, lung, spleen, and adrenal glands (130). Upon oral administration of miltefosine at 30 mg/kg of body weight twice per day, concentrations of 155 to 189 nmol/g of tissue are achieved (129). Miltefosine has a long half-life of about 8 days and is slowly metabolized by phospholipase.

Spectrum of activity. Miltefosine has activity against *Leishmania* spp. and *Trypanosoma cruzi* both *in vitro* and *in vivo*, but clinical studies to date have been limited to leishmaniasis. *In vitro* activity of miltefosine against *Trypanosoma brucei* spp., *E. histolytica*, and *Acanthamoeba* spp. has also been demonstrated (134). The majority of studies using miltefosine have examined its role against visceral leishmaniasis in India. In clinical trials, miltefosine has been found to have a cure rate of >90% at 6 months in both adults and children (135, 136), although emerging resistance is reducing its efficacy. Different combination therapy strategies involving miltefosine are being used in various geographic regions. Recent studies have also examined its efficacy in New World cutaneous leishmaniasis and have generally found 70 to 90% efficacy for most species (137). It is also used for post-kala-azar dermal leishmaniasis. There are also recent case reports of clinical success using miltefosine in combination with other agents for granulomatous amebic encephalitis (138, 139).

Adverse effects. In various clinical trials, toxic effects associated with miltefosine have usually been found to be tolerable and reversible, although the therapeutic window appears to be narrow (131). Mild to moderate gastrointestinal side effects, including nausea, vomiting, and diarrhea, occur in up to 60% of patients. Dose-related motion sickness is also reported in up to 40% of patients (140). A mild increase in aspartate aminotransferase and creatinine and/or blood urea nitrogen levels has been reported, with reversible hepatotoxicity and renal damage in a few cases (130, 135). Miltefosine is abortifacient and teratogenic in animals and should not be used in pregnancy or breast feeding. Contraception must be used in women of childbearing age during and for 3 months after treatment.

Pentamidine

Pentamidine is an aromatic diamidine compound that is used as an antiprotozoal agent in the treatment of leishmaniasis and African trypanosomiasis. Its use has been approved by the U.S. FDA (1).

Mechanism of action. Pentamidine is chemically related to guanidine. Its mechanism of action has not been defined clearly and may not be uniform against different organisms. It is possible it inhibits dihydrofolate reductase and interferes with aerobic glycolysis in protozoa. It may also interfere with amino acid transport; precipitate nucleotides and nucleotide-containing coenzymes; and inhibit DNA, RNA, and protein synthesis (141).

Pharmacokinetics. Pentamidine is currently available as an isothionate salt. It is poorly absorbed from the gastrointestinal tract. When used for protozoal infections, it must therefore be administered intramuscularly or intravenously, but it can be given via inhalation for prevention of *Pneumocystis jirovecii* pneumonia. Following intravenous administration of a 4-mg/kg dose, concentrations in plasma of 0.3 to 1.4 mg/liter have been reported (142). The highest concentrations of the drug are found in the kidney, liver, and spleen, and pentamidine penetrates poorly into the CNS. It has a short half-life in serum of 6.5 to 9 h because it is rapidly and extensively taken up by tissues. Its extensive tissue binding results in prolonged excretion over a period of 6 to 8 weeks, and it is eliminated unchanged via the kidneys. Pentamidine should be used cautiously in the presence of renal or hepatic failure, but no dosage adjustment is recommended.

Spectrum of activity. The antiparasitic indications for pentamidine include leishmaniasis and African trypanosomiasis, but its main use is as an antifungal agent for treatment and prophylaxis of *P. jirovecii* pneumonia. Despite activity against *Leishmania* spp., pentamidine's toxicity means that it is used predominantly in individuals intolerant of antimonial compounds, in disease that is refractory to other treatment, or in combination therapy. Pentamidine is also active against African trypanosomes, and it is first-line treatment in early disease, but its utility is restricted to trypanosomiasis without CNS involvement. Since CNS involvement occurs early with *T. brucei rhodesiense*, it is used more frequently for *T. brucei gambiense* infections. Pentamidine is also sometimes used in combination therapy for granulomatous amebic encephalitis caused by *Acanthamoeba* and *Balamuthia* species.

Adverse effects. More than half of parenteral pentamidine recipients experience some adverse effect from therapy. Administration is associated with a variety of reactions that appear to be unrelated to drug concentrations in plasma (141). Immediate reactions include nausea, anorexia, dizziness, pruritis, and hypotension. Pentamidine can also produce local effects, including pain and necrosis at the site of injection. In addition, pentamidine administration is associated with hematologic effects, particularly leukopenia (up to 10% of recipients) and thrombocytopenia (~5% of recipients), as well as with electrolyte abnormalities, including hyperkalemia, hypomagnesemia, and hypocalcemia. Other severe adverse effects include ventricular arrhythmias, pancreatitis, hypo- or hyperglycemia, hepatotoxicity, and acute renal failure. Pentamidine has also been associated with Stevens-Johnson syndrome. Finally, occasional seizures and hallucinations have been reported. There are minimal available safety data regarding the use of pentamidine in pregnancy (category C) or lactation, and its use is therefore not recommended.

Paromomycin

Paromomycin, which was initially named aminosidine, is an oral, poorly absorbed, broad-spectrum aminoglycoside. It is active against Gram-negative and many Gram-positive bacteria as well as some protozoa and cestodes. It is approved by the U.S. FDA for use in noninvasive intestinal amebiasis and is sometimes used off-label for cryptosporidiosis, *D. fragilis* infection, and giardiasis. Paromomycin is used in parenteral intramuscular (visceral and cutaneous leishmaniasis), topical (Old World and New World cutaneous leishmaniasis), and oral (protozoal infections) formulations.

Mechanism of action. Paromomycin is a protein synthesis inhibitor that exerts its function by binding to 16S rRNA, thus inhibiting protein synthesis. However, when used for treatment of leishmaniasis, it has additional mechanisms of action that are incompletely understood but seem to involve inhibition of parasite metabolism and mitochondrial respiration (143).

Pharmacokinetics. Paromomycin is not systemically absorbed after oral administration and is passed in feces without being metabolized. Following an intramuscular injection, peak concentrations in plasma are achieved within 0.5 to 1.5 h and the half-life is 2 to 3 h. Plasma protein binding is negligible. Mean concentrations in plasma 1 h after a dose of 12 to 15 mg/kg are 18.3 to 20.5 μg/ml. Paromomycin is widely distributed in the body after parenteral administration, with measurable concentrations achieved in bone, synovial fluid, and peritoneal fluid but with negligible CNS penetration. It should be used with caution in patients with renal impairment.

Spectrum of activity. Oral paromomycin has activity against the intestinal protozoa *G. duodenalis*, *Cryptosporidium parvum*, and *D. fragilis*. It is the drug of choice for giardiasis in pregnancy because of its safety and is also used in cases that have not responded to other agents (144). In addition, it has activity as a luminal agent to clear intestinal infections with *E. histolytica*, but it is not effective in extraintestinal amebiasis. It has also been used for treatment of *B.*

hominis infections, and it has activity against *T. vaginalis*. Paromomycin also is used parenterally or topically in visceral and cutaneous leishmaniasis, sometimes in combination with other agents. Although it has activity against most tapeworm infections, it is not used for this indication due to the availability of alternate agents (145, 146).

Adverse effects. The most common side effects of paromomycin are nausea, increased gastrointestinal motility, abdominal pain, and diarrhea. As with other aminoglycosides, systemic absorption of paromomycin following intramuscular injection may cause reversible ototoxicity and nephrotoxicity. It can also cause an increase in hepatic transaminases. With parenteral administration, ~50% of patients experience mild pain at the injection site.

Because oral paromomycin is not systemically absorbed, it does not adversely affect the fetus or infant so can be used during pregnancy and lactation. However, parenteral aminoglycosides cross the placenta and may accumulate in fetal plasma and amniotic fluid. No reproductive toxicity has been observed in animals, but insufficient data are available regarding the use of paromomycin in pregnant women to recommend its use. All results to date indicate that parenteral paromomycin is safe during lactation provided that the mother and infant have normal renal function (143).

Suramin

Suramin is a polysulfonated naphthylamine derivative of urea. Its main indication is in the treatment of African trypanosomiasis. It is not licensed for use in the United States but is available from the CDC via a compassionate drug protocol (1).

Mechanism of action. The mechanism of action of suramin is not fully understood but is thought to be via inhibition of enzymes associated with DNA metabolism and protein synthesis of the trypanosomal parasites (147).

Pharmacokinetics. Suramin is dispensed as a sodium salt and is soluble in water. It is not absorbed when given orally and is usually administered as a 10% solution by slow intravenous infusion. Following intravenous injection, it is rapidly distributed, and >99% becomes bound to plasma proteins. It does not cross the blood-brain barrier. It undergoes little or no metabolism and has a half-life of 41 to 78 days. It is excreted in the urine. It should not be used in the presence of renal failure or significant hepatic dysfunction.

Spectrum of activity. Suramin is an effective drug for early hemolymphatic stages of *T. b. gambiense* infections. It also has some effect in early *T. b. rhodesiense* infection, provided that there is no CNS involvement (148). Suramin was used to treat onchocerciasis prior to the development of ivermectin. An advantage of suramin is that it has macrofilaricidal activity, damaging the intestinal epithelium of adult *O. volvulus* worms and resulting in their death, but it is associated with frequent toxic effects at required doses (149). Suramin also has activity against adult *W. bancrofti* worms but is not recommended for this indication (13).

Adverse effects. Potential side effects include an immediate reaction, with nausea, vomiting, shock, and loss of consciousness, following suramin injection. Other adverse effects include renal impairment, exfoliative dermatitis, and neurological toxicity. It has also been associated with pancytopenia. Suramin has been reported to be teratogenic in rodents (150). No case of infant malformation has been described in humans, but it is not recommended for use during pregnancy except in circumstances where there is no suitable alternative.

Melarsoprol

Melarsoprol is a trivalent arsenical compound. Its main use is in the treatment of African trypanosomiasis. It is not licensed for use in the United States but is available from the CDC via a compassionate drug protocol (1).

Mechanism of action. Melarsoprol appears to act by binding to essential thiol groups of trypanosomes and has a particularly high affinity for the active site for pyruvate kinase. This results in interference with energy generation within parasites, thereby preventing trophozoite multiplication.

Pharmacokinetics. Melarsoprol is absorbed if given orally but is generally administered by the intravenous route. It is prepared as a solution in propylene glycol and is given by slow intravenous infusion. It is estimated that <1% penetrates the CNS, and concentrations in CSF are up to 50-fold lower than concentrations in serum (12, 151). However, it is an efficacious agent that can be used for late stages of trypanosomal disease, although resistance has been reported. Melarsoprol has a half-life of ~35 h. Its metabolism has not been well studied, but it is excreted predominantly in the urine.

Spectrum of activity. Melarsoprol is active in the treatment of all stages of African trypanosomiasis due to *T. b. gambiense* and *T. b. rhodesiense* and is the only available treatment for late-stage *T. b. rhodesiense* infection. Treatment courses have been reduced from 1 month to 10 days (152). However, because of its toxicity, it is generally reserved for use in late stages of disease involving the CNS.

Adverse effects. Melarsoprol is commonly associated with significant toxicity, including vomiting, abdominal pain, hepatotoxicity, peripheral neuropathy, hypersensitivity reactions, myocarditis, cardiac arrhythmias, and albuminuria. Administration can also lead to a reactive encephalopathy in up to 10% of patients, and this is associated with significant mortality. Hypersensitivity reactions are also relatively frequent. The injection is very irritating, and extravasation during intravenous administration should be avoided. There are minimal data available regarding potential teratogenic effects.

Eflornithine

The main indication for use of eflornithine (also known as difluoromethylornithine) is for treatment of African trypanosomiasis. Drug availability is limited, and it is not available in the United States for systemic use.

Mechanism of action. Eflornithine selectively and irreversibly inhibits ornithine decarboxylase, an enzyme required for the formation of polyamines needed for cellular proliferation and differentiation in parasites. It therefore leads to inhibition of parasite growth (153).

Pharmacokinetics. Eflornithine can be administered by mouth and has >50% oral bioavailability, but significant diarrhea frequently results, so it is usually given intravenously. No protein binding of the drug occurs following intravenous administration. It crosses the blood-brain barrier and produces CSF-to-blood ratios between 0.13 and 0.5 (154, 155). The half-life is 3 to 3.5 h, and ~80% of the dose is excreted unchanged by the kidneys. Dose reduction is required in patients with significant renal impairment.

Spectrum of activity. Eflornithine is used for treatment of *T. b. gambiense* when there is CNS involvement. Nifurtimox-eflornithine combination therapy is now standard first-line treatment for CNS-stage *T. b. gambiense* infection (152, 156). Eflornithine is ineffective as monotherapy in *T. b. rhodesiense* infection (157). It displays some activity against other parasites, including *Plasmodium* species, *C. parvum*, and *T. vaginalis*, but is not used for these indications because of toxicity.

Adverse effects. Side effects of eflornithine occur in up to 40% of patients. Common adverse reactions include vomiting, abdominal pain, diarrhea, dizziness, arthralgias, hearing loss, and rash. Bone marrow toxicity resulting in anemia, thrombocytopenia, and leukopenia has also been described. Eflornithine has been shown to arrest embryonic development in animals (158). There are no good studies of its safety in pregnancy or lactation (pregnancy category C), so it should only be used when the potential maternal benefits outweigh the possible risks to the fetus.

Nifurtimox and Benznidazole

The two agents used for treatment of American trypanosomiasis are nifurtimox and benznidazole. Nifurtimox is a synthetic nitrofuran, and benznidazole is a 2-nitroimidazole derivative. Neither is approved for use by the U.S. FDA, but nifurtimox is available from the CDC for compassionate use (1).

Mechanism of action. Benznidazole has an inhibitory effect on protein and RNA synthesis in *T. cruzi* cells (159). It is thought to cause increased phagocytosis, cytokine release, and production of reactive mitogen intermediates that result in destruction of intracellular parasites (160).

The mechanism of action of nifurtimox seems to be related to its metabolism to chemically reactive radicals that cause production of toxic reduced products of oxygen, such as superoxide, hydrogen peroxide, and hydroxyl radicals (12, 161). These compounds accumulate within trypanosomes, leading to toxic effects, including membrane damage and enzyme inactivation. It may also cause direct inhibition of protein synthesis via damage to parasite DNA (162).

Pharmacokinetics. Benznidazole is available for oral administration and has a bioavailability of >90%. It is ~40% protein bound. It has a half-life of ~12 h and has good tissue penetration (163). Nifurtimox is administered orally but has poor oral bioavailability. It is metabolized in the liver and has a half-life of ~3 h. Dose reduction is advised for patients with significant hepatic or renal impairment, but no specific guidelines exist.

Spectrum of activity. Both benznidazole and nifurtimox are used for the treatment of acute *T. cruzi* infection (Chagas' disease). Neither agent has demonstrated efficacy in late stages of disease, and indications for treatment of chronic infection remain controversial but are expanding (164–166). No randomized trial has evaluated the comparative safety and efficacy of nifurtimox and benznidazole in adults (167). Nifurtimox is now also increasingly being used in combination with eflornithine for first-line treatment of *T. b. gambiense* infection. It also has been shown to have some activity in leishmaniasis but is not routinely used for this indication.

Adverse effects. Side effects are common with benznidazole and are seen in up to 40% of treated individuals, commonly including vomiting, abdominal pain, peripheral neuropathy, rash, and pruritis. Bone marrow suppression and neuropsychiatric reactions have also been reported. Nifurtimox has significant side effects that preclude the completion of therapy in many patients. Adverse effects include anorexia, nausea, rash, headache, sleep disturbance, peripheral neuropathy, and myalgias. Less-frequent but more severe toxicities include psychosis and convulsions. Benznidazole crosses the placenta, but there are minimal data regarding teratogenic effects for either agent in either animals or humans (168). Nifurtimox is detected in breast milk, so caution is recommended during breast feeding (169). Similarly, safety data for benznidazole and lactation are lacking, so withholding treatment during breast feeding is again recommended.

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Mechanisms of Resistance to Antiparasitic Agents

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Parasitic diseases rank among the most prevalent and severe diseases worldwide and yet their control relies heavily on a single tool: the drugs used for chemotherapy or prophylaxis. This situation exists because no effective antiparasitic vaccines are available and implementation of other control measures often proves to be difficult in countries where parasitic diseases are endemic. This dependence on drugs is compounded by the relative paucity of the current armamentarium of antiparasitic products, a situation attributable largely to a lack of economic incentives for research and development. Furthermore, those drugs that are available are too often used incorrectly in communities and in control programs, a practice that encourages the selection of drug-resistant parasites.

The complex biologic interactions between parasites and their hosts (and at times vectors) significantly influence the emergence and expression of drug resistance. In many cases the observed resistance is true resistance, attributable to biologic characteristics of the parasites that enable them to survive drug concentrations that are lethal to susceptible members of the species. Mechanisms for such true resistance are varied and include a decrease in drug accumulation within the parasite or modifications in parasite enzyme structure or metabolic pathways. However, various host factors modulate the clinical and parasitologic responses to drug treatment, and the observed responses do not necessarily reflect true parasite resistance or susceptibility. For example, in populations with high rates of exposure to parasitic infections, the resulting high rates of immunity might suffice to eliminate an infection with drug-resistant parasites or treated with an ineffective drug. Conversely, treatment with a drug to which the parasite is biologically susceptible will not necessarily result in therapeutic success if the host takes an inadequate dose of the drug, absorbs it poorly, or lacks the immune response that might be needed for a successful antiparasitic synergism with the drug. Such host factors may be especially important in the areas where most parasitic diseases prevail, where high rates of parasite transmission result in high rates of immunity in most of the population, or where, conversely, malnutrition and human immunodeficiency virus (HIV) infection frequently decrease the patient's immune status.

Greater understanding of the epidemiology and mechanisms of drug resistance can provide valuable guidance for a better use of existing compounds and for the development of novel products. A selective review of drug resistance in five diseases will illustrate the existing problems and their potential solutions. A summary of the proposed mechanisms of resistance is provided in Table 1.

MALARIA

Overview

Malaria remains the most visible indicator of the adequacy of health control in all regions of the world with a hot and humid season. By 1955, the World Health Organization (WHO) had established projects for malaria eradication by using indoor residual spraying of insecticides to limit contact between humans and the anophele vector and by mass administration of pyrimethamine and chloroquine to kill erythrocytic-replicating forms of the parasites (1). As these programs faced infrastructure deficiencies and resistance to insecticides, the vector control was often neglected and the bulk of expenditure was devoted to the use of antimalarial drugs against fever, mainly chloroquine, with sulfadoxine-pyrimethamine as a secondary drug. *Plasmodium falciparum*, the most virulent species, has become the dominant species and its resistance to chloroquine and sulfadoxine-pyrimethamine, and further, to all known drugs, has developed to various degrees. Sub-Saharan Africa alone contributes about 80% of the annual 207 million patients worldwide suffering from malaria, leading to an estimated 627,000 deaths (2). Severe malaria affects mainly those without acquired adequate clinical immunity, such as young children or particular groups, such as pregnant women. Since the beginning of the 21st century, a specific effort of integrated control has been made and a reduction in transmission is starting to be observed in parts of Africa (3). The most spectacular action (together with the widespread use of long-lasting insecticidal nets) was the global establishment of malaria treatment with artemisinin-based combination therapies (ACTs), associating a curative dose of a drug with long elimination half-life and a dose of a rapidly active drug able to destroy a large parasite load in few hours. This bitherapy is based on a derivative of artemisinin, a substance extracted from sagebrush grown mainly in China, which generates oxidative stress in the parasite. The combination of multiple drugs enhances clinical efficacy and may delay the acquisition of parasite resistance. We need to consider the history of the emergence and spread of resistance to chloroquine and sulfadoxine-pyrimethamine as premonitory of the risk of losing the effectiveness of ACTs if their use is not controlled.

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TABLE 1 Summary of proposed mechanisms of resistance to selected antiparasitic drugs

Disease	Drug(s)	Mechanism(s) of resistance
Malaria	Chloroquine	Decreased accumulation of the drug by the parasite, resulting from altered transport properties of mutant PfCRT and PfPGH-1
	Pyrimethamine	Alteration in binding affinities between the drug and the parasite dihydrofolate reductase-thymidylate synthase, resulting from mutations on the corresponding gene
	Sulfadoxine	Alteration in binding affinities between the drug and the parasite dihydropteroate synthase, resulting from mutations on the corresponding gene
	Atovaquone	Alteration in binding affinities between the drug and the parasite cytochrome b, resulting from mutations on the corresponding gene
	Artemisinins	Mutated Kelch13 protein; mechanism unknown
Trichomoniasis	Metronidazole, tinidazole	Reduced concentration of enzymes or coenzymes necessary to activate nitro group
Leishmaniasis	Pentavalent antimonials	Decreased active intracellular drug concentration through decreased uptake, increased efflux, or decreased conversion to active trivalent form, glycans that increase host IL-10 production
	Miltefosine	Replaced pentavalent antimonials as primary treatment in some areas, resistance by increased drug efflux or increases in strain infectivity
	Amphotericin B	Increased drug efflux, altered thiol metabolism
African trypanosomiasis	Pentamidine (1st stage)	Mutation/loss of P2 adenosine and/or aquaglyceroporin 2 transporters that uptake drug
	Suramin (1st stage)	Not useful in west Africa where *T. b. gambiense* is the primary infection because of severe allergic reactions in onchocerciasis patients
	Melarsoprol (2nd stage)	Mutation/loss of P2 adenosine and/or aquaglyceroporin 2 transporters that uptake drug
	Eflornithine or combination eflornithine/nifurtimox	*T. b. rhodesiense* naturally tolerant, thus only useful against *T. b. gambiense*
Schistosomiasis	Praziquantel	Widespread clinical resistance not currently recognized as important problem for public health, although genetic bottlenecking observed in some treatment areas and resistance demonstrated in laboratory strains

The 4-amino quinoline drug chloroquine, a cornerstone of antimalarial chemotherapy since the 1940s due to its low cost, its safety, and its rapid action, lost most of its usefulness as the frequency of *P. falciparum* chloroquine-resistant strains increased and peaked in the 1980s. From limited original foci in Southeast Asia, South America, and Papua New Guinea, resistance has spread inescapably and is now found in most areas of endemicity, including Africa, the continent with the heaviest malaria burden. Chloroquine no longer constitutes an appropriate option for prompt and effective treatment (or prophylaxis) in most countries where *P. falciparum* malaria is the dominant endemic species. With increased use of sulfadoxine-pyrimethamine, the second affordable, relatively safe, and easily administered drug after chloroquine, parasite resistance to sulfadoxine-pyrimethamine has developed very quickly following the same itineraries as the spread of chloroquine resistance a few years before. Nevertheless, specific groups of people in areas of endemicity still rely on sulfadoxine-pyrimethamine for presumptive treatment of fever or for intermittent preventive treatment as the most effective way to prevent severe consequences of malaria. Losing the two low-cost antimalarials is often seen by experts as a public health disaster. Reducing transmission intensity could slow the spread of resistance but, paradoxically, below a critical level, it may accelerate the selection of multigenic resistance (3). This critical situation has prompted international initiatives to help face the high cost of ACTs, to make affordable rapid-diagnostic tests available everywhere, and to renew mosquito-control programs by an extensive distribution of insecticide-impregnated nets. Learning from the past that delay in detection and control of resistance to ACTs may ruin all programs without alternatives for decades, forced real-time drug-resistance surveillance to be set up and drug development to be reinforced.

Mechanisms of resistance to selected antimalarials

Chloroquine concentrates from nanomolar levels outside the parasite to millimolar levels within the digestive vacuole of the intraerythrocytic trophozoite, where it inhibits hemoglobin degradation (4). Chloroquine forms complexes with hematin, a by-product of host-cell hemoglobin digestion by the parasite, which accumulates in large quantities and eventually kills the parasite. The resistant isolates have in common a defect in chloroquine accumulation in the digestive vacuole (5). Several mechanisms have been proposed to explain the altered chloroquine accumulation, such as changes in the pH gradient or altered membrane permeability leading to a decreased drug uptake or increased drug efflux (6). Chloroquine accumulation has high structural specificity; this suggests the involvement of either a specific transporter/permease or a molecule associated with hematin in the digestive vacuole (7). Following demonstration that chloroquine resistance is reversible by verapamil, earlier studies focused on the orthologue of the mammalian multidrug resistance (*mdr*) gene, whose products are overexpressed in cancer cells where they expel cytotoxic drugs (8). This strategy led to the identification of the *pfmdr1* gene product (protein PfPGH-1), a transporter located in the membrane of the parasite digestive vacuole (9). Chloroquine susceptibility was found to be altered by PfPGH-1 mutated at specific amino acid residues and in specific parasite genetic backgrounds (10). However, current evidence indicates that *pfmdr1* does play a secondary role in chloroquine resistance, except in Madagascar where it plays a major role in chloroquine-treatment failures (11). In following years, using a genetic cross between chloroquine-sensitive and chloroquine-resistant parasites, inheritance data led to the key discovery of the *P. falciparum* chloroquine resistance transporter (*pfcrt*) gene (12–13). The *pfcrt* gene encodes a transmembrane protein (PfCRT), as does PfPGH-1, located in the membrane of the digestive vacuole (13). A complex set of mutations (or haplotype) of this gene is found in most natural isolates from chloroquine-treatment failures and in isolates with an *in vitro* chloroquine-resistant phenotype (13–15). Finally, transfection of chloroquine-sensitive parasites with the mutant *pfcrt* genotypes found in resistant isolates suffices to confer chloroquine resistance (16). Mutant PfCRT has acquired the property to expel chloroquine outside of the digestive vacuole (17) which is dependent on a charge loss at codon 76 (usually the K76T mutation) in the first transmembrane helix of the transporter (17–18). The role of the other *pfcrt* mutations which systematically accompany K76T remains elusive (6). At least four independent mutant *pfcrt* haplotypes carrying the K76T mutation are seen, varying geographically: Asia-Africa, Papua, and South America 1 and 2 (15). A major event in chloroquine resistance was the emergence on the Thai-Cambodian border in the 1950s of CVIET haplotype in codons 72–76 of the *pfcrt* gene under drug-pressure selection; this haplotype has now spread to most of the *P. falciparum* Asian and African territories (15, 19). Nonetheless, the wild *pfcrt* parasites have not been totally replaced, and their prevalence increases when drug pressure is removed (20). The *pfcrt* K76T mutation is now a valuable molecular marker used in epidemiological surveys of chloroquine resistance. Its estimated prevalence may offer a useful predictor of the clinical efficacy of chloroquine in a given area, provided that appropriate adjustments are made for host factors, particularly immunity, that may result in parasite clearance in spite of treatment with an ineffective drug (21).

Amodiaquine, piperaquine, and pyronaridine, which are in the first line of therapy as partners of artemisinins, share with chloroquine a quinoline scaffold, whereas mefloquine and lumefantrine, the other partners of ACTs, belong to amino-alcohols. Neither the mechanism of action of these drugs nor resistances to them are as clearly understood, as for chloroquine. However, the *pfmdr1* gene appears to be involved in parasite response to diverse antimalarials as mutations or amplification of the gene alters parasite susceptibility to desethyl amodiaquine, the active metabolite of amodiaquine (22), quinine, mefloquine, lumefantrine, and artemisinins (10, 23–26). Similarly, wild-type and various mutant *pfcrt* alleles associate with altered susceptibility to various antimalarials (lumefantrine, amodiaquine, artemisinins) in addition to choloroquine (6, 16, 27). Transporters other than PfCRT and PfPGH-1, such as PfMRP-1 and PfNHE-1, may be involved in resistance to mefloquine, lumefantrine, pyrimethamine (28, 29), and quinine, respectively.

Resistance to artemisinins recently emerged in western Cambodia and has now spread across mainland Southeast Asia. It manifests as a delayed parasite-clearance time following treatment initiation and as higher failure rate (30–31). Extensive genetic studies have shown that artemisinin resistance is a genetically determined trait (32) and pointed out specific regions of the parasite genome that are associated with the *in vivo* delayed parasite-clearance time (33–35). Comparative genomics of laboratory-selected and Cambodian field-derived artemisinin-resistant parasites led to the identification of missense mutations in the *kelch13* gene as an *in vitro* and *in vivo* marker of artemisinin resistance (36). How this mutant protein contributes to artemisinin resistance remains to be clarified.

Regarding drugs that antagonize a single enzyme, such as antifolates or inhibitors of the mitochondrial respiratory chain, a single gene modification is often sufficient to generate high-grade resistance. Malaria parasites mostly rely on *de novo* synthesis for folate supply. Cycloguanil (produced by the pro-drug proguanil), and its analogue, pyrimethamine, were the first satisfactory synthetic antifolate antimalarials to be on the market in the 1940s. Both inhibit the plasmodial dihydrofolate reductase (PfDHFR), a key enzyme in the folate synthesis pathway of the parasite. Unfortunately, resistance emerged almost instantaneously and independently from several areas where the drugs had been introduced and these antimalarials were soon supplanted by chloroquine (37). A resurgence in the use of PfDHFR inhibitors took place with the demonstration in 1967 that potentiation with other antifolates from the sulfone/sulfonamide group (such as sulfadoxine or dapsone), which inhibit the plasmodial dihydropteroate synthase (PfDHPS), bypassed resistance and sulfadoxine-pyrimethamine became the new spearhead to face chloroquine resistance in Southeast Asia (37). However, resistance to sulfadoxine-pyrimethamine emerged soon after the increased use of sulfadoxine-pyrimethamine in Thailand. Resistance to PfDHFR inhibitors is conferred by mutant PfDHFR enzymes to which antifolate drugs bind less efficiently compared to the wild-type version (38–39). Stepwise acquisitions of pyrimethamine and then sulfadoxine-pyrimethamine resistance in *P. falciparum* are mirrored by the stepwise acquisitions of PfDHFR mutations: first the key mutation S108N, then the additive mutations N51I and C59R, and finally the additive I164L mutation (39, 40). Other additive mutations can be found in specific areas such as in South America (41). The quadruple-mutant N51I-C59R-S108N-I164L which associates with the highest level of sulfadoxine-pyrimethamine resistance as well as to decreased sensitivity to chlorproguanil-dapsone (an attempt to develop a new antifolate combination similar to sulfadoxine-pyrimethamine) has been reported mostly in Southeast Asia. However, the triple mutant N51I-C59R-S108N, which is widespread in Africa, also associates with treatment failure to sulfadoxine-pyrimethamine (42). This triple mutant N51I-C59R-S108N *pfdhfr* gene emerged in Southeast Asia, and spread all over Asia and Africa in the following years (43, 44).

Resistance to sulfone/sulfonamide group (sulfadoxine being the major antimalarial compound) is conferred by mutant PfDHPS enzymes to which antifolate drugs bind less efficiently compared to the wild-type version (45, 46). Resistance to sulfadoxine has been traced to a set of sequential mutations in the *pfdhps* gene. The likely initial event consisted of an A437G mutation, with subsequent additional mutations conferring increasing degrees of resistance (37). These resistance mutations have appeared independently multiple times and in multiple endemic sites (47). Altogether, resistance to the sulfadoxine-pyrimethamine combination appears to require three mutations in the *pfdhfr* gene, and the probability of sulfadoxine-pyrimethamine treatment failure increases with additional mutations in *pfdhps*. The clinical outcome of a sulfadoxine-pyrimethamine treatment is subjected to additional host factors such as the level of folates and of acquired immunity and drug absorption and metabolism (42, 48). Cross-resistance has been demonstrated between cycloguanil and pyrimethamine (38, 39, 49). Consequently, interest in other antifolates, such as chlorproguanil plus dapsone (LapDap), to treat parasites resistant to sulfadoxine-pyrimethamine has been limited.

The combination of atovaquone and proguanil was registered in 1996 in North America and Europe where, within 10 years, it became the most used antimalarial for prophylaxis and first-line treatment of non-severe *P. falciparum* malaria. Atovaquone is a ubiquinone analogue and binds to cytochrome *b* (PfCytB), a component of the complex III in the mitochondrial-respiratory chain (50). In association with proguanil, the effective concentration at which it collapses the mitochondrial membrane potential is diminished (51). In addition, proguanil is partially metabolized to the antifolate cycloguanil by human P450 cytochromes. The contribution of the resulting low cycloguanil blood concentrations to the therapeutic efficacy of atovaquone-proguanil remains yet to be substantiated. As with the antifolates, atovaquone resistance emerged almost instantaneously (52). Resistance to atovaquone is conferred by mutant PfCytB to which atovaquone binds less efficiently compared to the wild-type version (53). The substitution of Y for S or N or C in codon 268 of PfCytB is found associated with treatment failures and confers a high level of atovaquone resistance (54) that proguanil could not thwart (55). Remarkably, the resistance mutation is not detected in areas of endemicity (56), and it seems to evolve repeatedly during primary infections (57). Due to the risk of rapid extension of resistance and high cost of the drug, the deployment of atovaquone in regions where malaria is endemic has not been considered as a priority.

Chloroquine resistance in *Plasmodium vivax*, the second most common malaria parasite, has been very limited despite widespread chloroquine use. *P. vivax,* which relapses from dormant parasites in the liver, developed partial resistance to primaquine, the only drug active against liver forms (58). Primaquine's diminished efficacy is also associated with polymorphisms in the host cytochrome P-450 2D6, resulting in altered concentrations of the active metabolites (59). While drug resistance in *P. vivax* remains at low magnitude, increasing attention has recently focused to tackle this threat. Investigations on mechanisms of resistance in this species currently examine the potential role of *P. vivax* homologues of *pfcrt* (60), *pfmdr1* (61), *pfdhfr* (62), and *pfdhps* (63).

TRICHOMONIASIS

Infection with *Trichomonas vaginalis* is one of the most common causes of human vaginitis as well as the most prevalent nonviral sexually transmitted disease (64). *T. vaginalis* infections are associated with preterm delivery, low birth weight, and greater susceptibility to infection with HIV as well as increased shedding of virus in HIV-infected individuals (65–67). As a result, expedient treatment of this infection has become an important public health concern (68, 69).

T. vaginalis is a facultative anaerobe and trichomoniasis is most commonly treated with the 5-nitroimidazole class of drugs. Two members of this group, metronidazole and tinidazole, are the only drugs licensed for treatment of trichomoniasis in the United States. Tinidazole is more active at equimolar concentrations than metronidazole and is recommended if treatment with metronidazole fails (70–72). However, strains clinically resistant to metronidazole can have cross-resistance to tinidazole. The molecular epidemiology of *T. vaginalis* suggests that clinically resistant isolates are genetically related and are concentrated within one of two major subpopulations (73–75). In a survey of women attending sexually transmitted disease clinics in six U.S. cities, 4.3% of isolates exhibited drug resistance (76), suggesting that almost 160,000 residents in the U.S. alone may be affected (171).

The 5-nitroimidazoles enter parasites in an inactive form by passive diffusion. Once inside, the drug is reduced to the active nitro-radical anion that is thought to cause parasite death by breaking or disrupting DNA. A number of electron donors have been proposed for drug activation, including ferredoxin, pyruvate-ferredoxin oxidoreductase (PFOR), and malic enzyme in the hydrogenosome, which is the source of ATP generation in these amitochondriate parasites (77–79). Drug resistance occurs when transcription of one or more of these enzymes is decreased, and laboratory-generated resistant isolates have smaller hydrogenosomes (80). However, clinical resistance does not correlate with lower transcript levels of these enzymes or smaller hydrogenesome size (80, 81).

The nitroimidazole drugs are also reduced by the flavin enzyme thioredoxin reductase and covalently bind and inhibit proteins associated with thioredoxin-mediated redox regulation (82). An *in vitro*-induced nitroimidazole-resistant strain demonstrated reduced thioredoxin reductase activity, not as a result of decreased enzyme concentration but because of a deficiency in the necessary FAD cofactor. Trichomonads with minimal PFOR and malate-dehydrogenase activity remained susceptible to metronidazole (82). Furthermore, use of a flavin inhibitor rendered a normally susceptible isolate resistant to high concentrations of metronidazole. Flavin inhibition also reduced levels of PFOR and malate dehydrogenase, suggesting that the decreased hydrogenosomal redox enzymes observed in resistant isolates may be a consequence rather than a cause of metronidazole resistance (83). A third possible mechanism for nitroimidazole resistance was suggested by the observation that trichomonas isolates that harbored *Mycoplasma hominis* symbionts had a mean *in vitro* resistance level 10-fold higher than noninfected trichomonads (84). However, the increased mean resistance of the *M. hominis*-infected isolates was still lower than typically observed for resistant isolates. In a separate study, there was no association of mycoplasma infection with clinical resistance (85). It remains unclear which, if any, of these mechanisms is responsible for the clinical nitroimidazole resistance observed in some *T. vaginalis* infections.

Treatment of patients who have metronidazole-resistant trichomoniasis often results in an immediate resolution of symptoms and a negative wet mount. However, within 3 to 4 weeks, in the absence of further exposure, symptoms may recur as the number of organisms rises. Thus, it is important to monitor efficacy of treatment for up to a month and to encourage patients to avoid unprotected intercourse during this time. When nitroimidazole resistance is encountered, patients are often successfully treated with increased doses of drug for a longer time (71, 72); however, many patients cannot tolerate high doses of metronidazole and such practices may only exacerbate the development of drug resistance. In addition, some patients experience hypersensitivity reactions in response to metronidazole and tinidazole.

Clearly, alternatives to the nitroimidazoles for treatment of *T. vaginalis* infections are needed (86). A library of 1,040 FDA-approved drugs was recently tested against metronidazole-sensitive and -resistant isolates *in vitro* in an attempt to find alternate oral therapies for trichomoniasis. While no drugs were as effective as the 5-nitroimidazoles, some enhanced the activity of metronidazole when used in combination (87). Intravaginal treatment with drugs such as furazolidone and paromomycin sulfate that are not absorbed well from the intestine or cannot be ingested has been successful to cure some patients but, in general, has limited efficacy (86, 88). Povidone-iodine and boric acid have also shown efficacy for some patients but additional clinical testing is needed (88, 89).

LEISHMANIASIS

Leishmaniasis is transmitted to humans by phlebotomine sandfly vectors and manifests itself in a variety of syndromes. Depending in part on which of the possible 20 species of *Leishmania* that infects humans is present, pathology can range from a cutaneous lesion that is self-limiting to the more severe mucosal or visceral forms. The identification of leishmaniasis as an important opportunistic infection in patients with AIDS has presented new challenges for treatment of this disease, with increased treatment failures and drug toxicity in HIV-1 positive individuals (90, 91). Use of antileishmanial drugs is limited by their high cost, the difficulty of their administration (injections for several weeks), and/or their associated toxicity. These factors are even more consequential in the developing countries where leishmaniasis is endemic and can lead to premature self-termination of therapy, which in turn may promote increased levels of resistance (92). While true drug resistance has been described for isolates of some *Leishmania* spp., other species or isolates may just differ in their intrinsic sensitivity to certain compounds (93–96). True resistance is more likely in anthroponotic forms of leishmaniaisis, such as *Leishmania donovani* and *Leishmania tropica*, because the zoonotic species that primarily infect animals, with humans as an occasional host, rarely encounter drugs and serve as a reservoir for drug-sensitive parasites (94, 97).

The frontline drugs for treating *Leishmania* infections caused by all species and all clinical forms have long been the pentavalent-antimonial compounds such as sodium stibogluconate and meglumine antimoniate. These compounds are inexpensive compared to other anti-leishmanial drugs but their extensive use has led to widespread treatment failure. For example, in some areas of endemicity in India, treatment failure of visceral leishmaniasis caused by *L. donovani* is as high as 65% (98). Evidence for true drug resistance in this setting comes from observations that isolates from clinically resistant patients require higher *in vitro* concentrations of drug to kill the parasites than do isolates from patients who respond to treatment (99). However, the correlation between clinical outcome of treatment for leishmaniasis and the *in vitro* susceptibility of the causative isolate is not always clear (93–95, 100).

Pentavalent antimonials are prodrugs that become reduced within the mammalian host cell or parasite to an active trivalent form that boosts the intracellular cytotoxic potential of macrophages, as well as disrupts the parasite's redox metabolism; many of the proposed mechanisms of resistance across the various *Leishmania* spp. involve a lower intracellular concentration of active drug (101, 102). One mechanism by which this occurs is a decrease in aquaglyceroporins on the parasite's surface, resulting in less uptake of drug (103, 104). Resistance has also been associated with increased production of trypanothione or glutathione that binds with the trivalent antimonials and sequestration of the resulting thiol-drug conjugates into intracellular organelles by ATP-binding cassette (ABC) transporters (101, 105, 106). Increased expression of the enzymes involved in thiol synthesis or of the ABC transporter promotes resistance to pentavalent antimonials; inhibition of these pathways in resistant strains increases susceptibility to the drug (102, 103, 105–109). Adding to the difficulty of defining resistance mechanisms, more than one of these mechanisms may arise, even within closely related parasite strains (101). Genomic, proteomic, and metabolomic approaches have further revealed the complex, multifactorial nature of antimonial resistance in *Leishmania* spp. as well as indicate other mechanisms that could be in play, such as protection from reactive-oxygen species and stress response (95, 96, 100, 101, 104, 110, 111).

Because of the high level of pentavalent-antimonial resistance, this treatment is no longer considered useful in parts of India and, in these places, miltefosine is now the primary treatment for visceral leishmaniasis (96, 112). Miltefosine was originally developed as an anti-cancer drug and is particularly promising because it can be taken orally and has fewer side effects than most of the parenteral treatments for leishmaniasis (113). As a result, although the drug is more expensive than other therapies, it can be administered on an outpatient basis, reducing the overall cost for treatment in comparison to less costly drugs that require hospitalization. Enthusiasm is tempered, however, both because it is teratogenic, thereby limiting its unregulated use in women of child-bearing age, as well as the observation that leishmanial resistance to miltefosine develops easily *in vitro* (113). Relapse rates of 20% within 1 year after miltefosine treatment have already been reported (114). As with the antimonials, resistance to miltefosine has been associated with mechanisms that decrease intracellular concentrations, and inhibitors of ABC transporters can restore drug susceptibility *in vitro* (115–117). However, recent studies suggest that these mechanisms do not explain all of the clinical resistance to miltefosine and other mechanisms, such as infectivity of the parasite strain, may be involved (112, 114, 118).

Another drug now being used for primary treatment of visceral leishmaniasis is amphotericin B, which interacts with parasite-specific 24-alkyl sterols and induces pore formation in the parasite plasma membranes (92). The use of amphotericin B has been limited in the past due to its high cost and toxicity; however, new lipid-associated formulations of amphotericin B have greatly reduced toxicities and retain good efficacy even when administered in lower doses (119). Like the pentavalent antimonials, optimal activity of amphotericin B may require competent host immune responses (120, 121). Nevertheless, amphotericin B seems to be superior to antimonials for treatment of leishmaniasis in HIV-infected individuals (90, 91). Lipid-associated formulations of amphotericin B are phagocytized by host monocytes and accumulate in the phagocytic lysosomes where *Leishmania* amastigotes reside. Although parasite isolates from HIV-positive patients who relapsed with *L. infantum* demonstrated no decrease in their *in vitro* susceptibility to amphotericin B (121), clinically resistant isolates have been obtained from patients infected with *L. donovani*. Interestingly, although the mode of action of amphotericin B is thought to differ greatly from that of the antimonials, field isolates indicated that those with greater sodium-antimony-gluconate resistance had greater *in vitro* resistance to amphotericin B (115). As with the other anti-lesihmania drugs, amphotericin B resistance is associated with greater drug efflux and altered thiol metabolism (122).

Part of the challenge for understanding drug activity and treatment failure during leishmaniasis relates to the intracellular location of the amastigotes within host macrophages. As a result, treatment efficacy involves host factors in addition to the parasites and therapeutic compounds (123). For example, intracellular killing of amastigotes is dependent on macrophage production of nitric oxide, which is inhibited by the cytokine interleukin-10 (IL-10). Interestingly, some antimony-resistant isolates of *L. donovani* express a unique glycan that upregulates the ABC transporter in the parasite as well as IL-10 production by the host (108, 124). As a result, neutralization of IL-10 has been suggested as an adjunct therapy for visceral leishmaniasis (125).

AFRICAN TRYPANOSOMIASIS

Trypanosoma brucei rhodesiense and *Trypanosoma brucei gambiense* are the etiologic agents of human African trypanosomiasis (HAT). The two subspecies are endemic in east- and west-central Africa, respectively, with *T. b. gambiense* causing the vast majority of infections (126, 127). Because these parasites possess antigenic-switching mechanisms, host immune responses are ineffective and the prospects for the development of vaccines against these organisms are meager. Control efforts have largely been focused on prevention through reduction of the tsetse fly vector, usually through traps or insecticides (126). However, recent political unrest with subsequent loss of an effective public health infrastructure in parts of Africa has caused a resurgence of this disease. As a result, drug treatment is the only medical intervention available to combat sleeping sickness for the foreseeable future.

HAT has two stages; an initial bloodstream stage followed by invasion of the central nervous system in the second stage, which causes the meningoencephalitic systems associated with "sleeping sickness," the more familiar name for HAT (126). Pentamidine and suramin are drugs used for treatment of the first stage but not advanced disease because they are highly ionic and do not cross the blood-brain barrier. Furthermore, use of suramin is avoided for treatment of *T. b. gambiense* because of the risk of severe allergic reactions that it can cause in onchocerciasis patients (126, 127). While clinical resistance to these drugs does not seem to be a problem, failures can occur when infections are diagnosed and treated after disease has progressed past the hemolymphatic stage. Late-stage, central nervous system disease is treated with melarsoprol, eflornithine, or nifurtimox-eflornitihine combination therapy (126, 128). Eflornithine is effective against late-stage *T. b. gambiense* infections that are resistant to melarsoprol, but *T. b. rhodesiense* parasites are naturally tolerant to this drug (129). The drugs for HAT are difficult to administer or are relatively toxic, which can contribute to premature cessation of treatment that can in turn contribute to the development of drug resistance.

Pentamidine and melarsoprol share an amidinium-like moiety with amino purines that is recognized and actively taken up by nucleoside transporters in the trypanosome membrane, resulting in concentration of these compounds within the parasite (127). One of these receptors, the *T. brucei* P2 adenosine transporter, or TbAT1, has been extensively studied and resistance to pentamidine and melarsoprol is associated with loss of functional *tbat1* expression, including in field isolates (127, 130, 131). However, *tbat1* knockout parasites were only partially resistant, leading to the discovery of a high-affinity pentamidine transporter (HAPT) that also concentrates pentamidine within the parasites (132). Recently, HAPT has been identified as aquaglyceroporin 2, and loss of this receptor is shown to confer melarsoprol/pentamidine cross-resistance (133, 134). *T. brucei* parasites also express an ABC transporter that, similar to *Leishmania*, functions as an efflux pump and may contribute to the melarsoprol resistance of certain isolates (127, 128, 135).

While melarsoprol treatment failure may be as high as 20% in some settings (136), field resistance to eflornithine and nifurtimox has not been documented. Nevertheless, resistance in laboratory strains has been readily induced for both drugs (137, 138). Eflornithine resistance is associated with the loss of a nonessential amino acid transporter that is responsible for drug uptake, raising the concern that field resistance could easily develop (137).

SCHISTOSOMIASIS

Drug resistance in human helminths is rare, a fact attributed to their long reproduction cycles and to their lack of multiplication inside the human host (the exception being *Strongyloides stercoralis*). Thus, when considering treatment failures in schistosomiasis, it is important to distinguish characteristics leading to reduced drug efficacy from true drug resistance. For example, persons with very high levels of infection are less likely to be cured with single-dose therapy than individuals with lower worm burdens (139, 140). This is in part related to the fact that the standard drug used to treat schistosomiasis, praziquantel, is only effective against the adult stage of the parasite and, even under the best conditions, a single dose of praziquantel does not demonstrate complete efficacy (141, 142). Immature worms that may be present at the time of drug treatment, especially in areas of high transmission, are not susceptible to the initial treatment and subsequently develop into patent infections that give the impression of drug resistance. As a result, two treatments spaced 4 to 6 weeks apart are more effective than a single treatment and should be attempted when drug resistance is suspected (139, 143, 144). Rapid reinfection in areas of high transmission should also be considered as a possible explanation for apparent praziquantel-treatment failure (145, 146).

Suspected true praziquantel resistance has been described for *Schistosoma mansoni* infections in Egypt and Kenya (147, 148). Eggs obtained from the feces of individuals who could not be successfully treated have been used to establish infections in mice, confirming the drug-resistance phenotype (147–149). However, widespread clinical resistance has not developed, even in areas with high-intensity treatment pressure for a prolonged time (150–152). Laboratory and field studies have shown decreased diversity of schistosomes following praziquantel treatment suggestive of a genetic bottleneck, a warning sign for development of drug resistance (153, 154). However, this observation has not been consistent in all field studies (155, 156).

The exact mechanism of praziquantel, and as a result the mechanism of drug resistance, is not definitively understood. In drug-susceptible parasites, praziquantel-induced damage to the tegument of adult schistosomes renders the worms susceptible to attack and killing by the host's immune response; the effect of drug on resistant parasites is reduced (157). The unique beta subunit of the schistosome calcium-ion channel is a molecular target for praziquantel with treatment rapidly inducing a calcium-dependent, sustained muscle contraction in the worm's tegument (158, 159). However, no differences in this gene's sequence or expression were observed among a limited number of praziquantel-resistant and -susceptible parasite strains (160). Furthermore, cytochalasin D reverses the effects of praziquantel without altering the calcium influx, thus raising the possibility that this is not the effector of parasite death (161). Other proposed mechanisms of praziquantel action include inhibition of the worm's nucleoside uptake and binding to, and altering the function of, schistosome myosin-light chain (162, 163). One potential mechanism of drug resistance includes increased expression of a P-glycoprotein ATP-dependent efflux-pump homologue in parasite strains that have reduced susceptibility to praziquantel (164, 165). Cross-breeding of adult worms with different levels of sensitivity to drug results in offspring with an intermediate phenotype, suggesting that at least artificially induced resistance displays partial dominance (166). Differential gene transcription between susceptible and resisitant parasites and between adult and juvenile worms in response to praziquantel may also play a role in drug susceptibility (167, 168).

Although widespread praziquantel resistance has not been observed, as mass drug-administration programs are increasingly employed, there is a fear that resistance may emerge (169). This risk reiterates the need for ongoing monitoring of praziquantel resistance and efforts for the discovery of new drugs or repositioning of existing compounds to treat for schistosomiasis (170).

FUTURE PERSPECTIVES

Several factors contribute to the emergence of drug-resistant parasites. Those parasite species with short life cycles and high multiplication rates that occur in areas of intense transmission are most likely to develop resistant subpopulations. The selection of such populations is encouraged when the parasites are repeatedly exposed to suboptimal drug concentrations. This pattern can result from the use of drugs with long half-lives, or more typically, from the frequent, often unjustified use, of inadequate doses of drugs, a common occurrence in countries where parasite infections are endemic. Public health interventions to correct these factors have not always been successful and would benefit from a better understanding of the drug-resistance mechanisms used by parasites. These mechanisms are very diverse and have been difficult to study, but recent technological advances now provide long-awaited tools that will facilitate the task. The genome sequences for several *Plasmodium* species, *T. vaginalis*, *Leishmania major*, *T. brucei*, *S. mansoni*, and *Schistosoma haematobium*, have been compiled. When these data are used, for example, in combination with microarray technology or whole-genome sequencing, where the DNA of drug-resistant parasite strains is compared to drug-susceptible strains, identification of the genes that confer resistance should proceed even more rapidly than in the last few years. In addition, genomic, transcriptomic, and proteomic data may also be useful in the design of new chemotherapeutic agents as they help researchers identify metabolic processes of parasites that are sufficiently different from those of their human hosts to allow specific attack.

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Susceptibility Test Methods: Parasites

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Accurate methods for ascertaining responses of parasites to antiparasitic drugs can prove useful at several levels. They can assist in the clinical management of individual patients, they can yield epidemiologic information that may guide drug use policies and public health interventions, and they offer crucial research tools for the development of new and better drugs. Drug susceptibility tests fall into four broad categories: *in vivo* tests, *in vitro* tests, tests with experimental animals, and molecular tests.

In vivo tests with patients directly assess the clinical efficacies of existing compounds. These tests are performed in actual epidemiologic investigations, and their modest technical requirements make them suitable for use under field conditions in developing countries. The interpretation of *in vivo* tests is limited by potential interference by factors related to the host (e.g., immunity or variations in drug intake or metabolism) or to the environment (e.g., reinfections). However, such tests have proven instrumental in guiding drug use policies, particularly for malaria.

In vitro tests circumvent these interferences by isolating the parasites from their hosts and investigating them in culture under controlled laboratory conditions, which provides opportunities for repeated assessments against multiple compounds, including experimental compounds. *In vitro* tests, however, are technically more demanding and therefore less amenable to performance under field conditions. They are most informative if they are used to investigate parasites that multiply rapidly in culture, a select group consisting mostly of protozoa. They are of limited use for assessing inactive drug precursors that must be activated by the host or drugs whose antiparasitic activities necessitate the synergistic effect of the host's immune defenses.

Tests with experimental animal models permit investigations of parasites that cannot be grown in culture or of drugs not yet approved for use in humans. This approach accommodates the advantages of human *in vivo* drug testing without the potential of exposing persons to toxic drugs that may be ineffective. However, its use is predicated on a suitable animal model for the infection and on the availability of appropriate facilities for maintaining the test animals. Furthermore, for animal models to be relevant, the pharmacokinetics of the drug under investigation should be similar in the particular model used and in humans.

Molecular tests detect genetic variations that are potentially linked with resistance. Such tests offer unique advantages. PCRs can be performed with minute amounts of nonviable parasite genetic material. They can be run in batches, allowing large-scale epidemiologic studies. Molecular analysis can circumvent potential ambiguities associated with the polyclonality of parasites infecting a single host occasionally encountered in *in vivo* or *in vitro* tests and allows the dissection of such within-host parasite populations. Because of their short duration (hours), molecular diagnostic procedures can potentially be used to guide patient management. *In vivo* and *in vitro* tests usually require more time (days to weeks) and yield results that are used mainly for epidemiologic surveillance and experimental chemotherapy studies. Drawbacks of molecular tests reside in their technical requirements and in the need to be certain that the genes evaluated correlate with functional resistance. However, thanks to the development of more-practical protocols and more-robust automated equipment, as well as a better understanding of the genes that confer resistance, molecular techniques are being used in an increasing number of laboratories, including field facilities.

These different categories of tests provide complementary information. At one end of the spectrum, molecular tests analyze parasites at their most basic biologic level, without any outside interference. At the other end, *in vivo* tests in patients reflect complex interactions between host and parasite yet are most relevant for clinicians and public health practitioners. While a good correlation between results of various test methods is desirable, some degree of discrepancy should be expected to result from factors linked to the host or the culture conditions. Indeed, a judicious analysis of such discrepancies might provide valuable insights into the mechanisms of drug action and resistance.

These points are illustrated in the following discussions of five parasitic diseases, selected for their particular chemotherapeutic challenges. A summary is provided in Table 1.

MALARIA

Most drug resistance tests in malaria concern *Plasmodium falciparum*, the most prevalent and virulent species. Initial observations of drug-resistant malaria occur most often in a clinical context, and their confirmation is frequently sought by *in vivo* tests. These aim to document the parasitological and clinical response of a malaria infection in a patient treated with a standard dose of the test drug and monitored under controlled conditions. Initially standardized by the WHO for the response of *P. falciparum* to chloroquine, *in vivo* tests have been modified several times for increased performance and assessments of other drugs (1). An increasing number of

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TABLE 1 Selected antiparasitic agents and susceptibility testing methods[a]

Disease and drugs	Testing method(s)	Remarks
Malaria[b]		
Chloroquine, amodiaquine, quinine, mefloquine, lumefantrine, piperaquine, pyronaridine, artemisinin, sulfadoxine-pyrimethamine, atovaquone-proguanil, tetracycline, primaquine, and others	*In vivo* tests in patients with *Plasmodium falciparum* or *P. vivax* malaria. Culture of erythrocytic stages of *P. falciparum*. Criteria for assessment are (i) microscopic examination (maturation from rings to schizonts; parasite multiplication), (ii) metabolic activity (incorporation of [^{3}H]hypoxanthine; production of pLDH, HRP2), and (iii) DNA quantitation. PCR-based genetic analysis (mostly of *Plasmodium falciparum*) of mutations in and amplification of genes putatively involved in resistance to chloroquine (*pfcrt*, *pfmdr1*), mefloquine (*pfmdr1*), antifolates (*dhfr*, *dhps* genes), atovaquone (*pfcytb* gene), artemisinins (*kelch13* gene), and others.	Drug resistance is a major problem, especially in *P. falciparum*; it also occurs in *P. vivax*. Tests are used for epidemiologic assessment as well as for laboratory investigations. Short-term culture tests are also described for erythrocytic stages of *P. vivax*. On an experimental basis, there are *in vitro* tests to determine a drug's effect on liver stages and sexual stages (gametocytes).
Trichomoniasis[c]		
Metronidazole and tinidazole	Culture under aerobic and anaerobic conditions. The criterion for assessment is parasite mobility.	Resistance to metronidazole is relative; testing is performed over a wide range of concentrations.
Leishmaniasis[d]		
Sodium stibogluconate, meglumine antimoniate, pentamidine, amphotericin B, paromomycin, and miltefosine	Culture of promastigotes. Criteria for assessment are microscopic examination with parasite counting and metabolic activity (incorporation of [^{3}H]thymidine, hydrolysis of *p*-nitrophenyl phosphate, conversion of MTT or resazurin). Culture of amastigotes (intracellular or axenic). Criteria for assessment are microscopic examination with counting of stained intracellular parasites, luciferase activity of transfected parasites, flow cytometry using GFP-transfected parasites, lysis of host cells, transformation of amastigotes back to promastigotes, and promastigote assay.	Problems with most drugs are their high cost, difficulty in administration, and toxicity. There is a high level of failure of pentavalent antimonials in some areas. The choices of promastigote or amastigote assay differ with the drug being tested. Tests with intracellular or axenic amastigotes show better correlation with clinical drug efficacy but are not absolute.
African trypanosomiasis[e]		
Pentamidine, suramin, melarsoprol, and eflornithine	Culture of trypomastigotes and assessment by microscopic examination with parasite counting, metabolic activity (hydrolysis of *p*-nitrophenyl phosphate, reduction of resazurin), and uptake of radiolabeled drug.	*In vitro* tests are used mainly for laboratory investigations. The ability to detect mutations in the transporter responsible for drug uptake may soon allow field-applicable tests.
Schistosomiasis[f]		
Praziquantel	Examination of damage to adult worms from experimental infections with suspected resistant strains, egg-hatching efficiency, miracidial morphology, and cercarial tail shedding.	*In vivo* animal tests are needed for confirmation due to the dependence of drug effect on the host immune response.

[a] See the text for details.

[b] Disease caused predominantly by *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium knowlesi*.

[c] Disease caused by *Trichomonas vaginalis*.

[d] Disease caused by one or several *Leishmania* species.

[e] Disease caused by *Trypanosoma brucei rhodesiense* and *T. brucei gambiense*.

[f] Disease caused predominantly by *Schistosoma mansoni*, *Schistosoma japonicum*, and *Schistosoma haematobium*.

in vivo tests comparing drug schemes have been performed since 1996, as it was necessary to assess the response of *P. falciparum* to artemisinin-based combined therapy (2). With regard to the fast-acting artemisinin derivatives, measurement of parasite clearance times, in addition to determination of standard clinical outcomes, has been introduced (3). Nevertheless, the diversity of study designs and analytical methods undermines the possibility of monitoring antimalarial drug efficacy over time from diverse regions of endemicity (4). Among key variables considered to define the therapeutic response are *Plasmodium* species, outcome, level of immunity, and PCR adjustment to discriminate recrudescence of the initial infestation and recurrence (*Plasmodium vivax*) or reinfestation. Incorporating pharmacokinetic parameters is now considered for true resistance identification within partners of artemisinins, which are frequently poorly absorbed or slowly eliminated drugs (5, 6).

The standard *in vitro* antimalarial drug susceptibility assay determines the *ex vivo* growth of replicating intraerythrocytic parasites from the ring stage (the only asexual *P. falciparum* stage found in patient peripheral blood) to the schizont stage in 24 to 72 h in the presence of serial drug concentrations under conditions close to *in vivo* conditions. With regard to the standard assay, a well-suited 96-well microtiter plate format was designed using the Trager and Jensen cultivation parameters (hypoxia and buffered RPMI medium with human serum), which is the basis of the most-used tests (7, 8). The simplest test format has been adapted to field work and uses 100 µl of fingerpick capillary blood mixed with medium, a 24- to 30-h candle jar incubation, a microscopic count of multinucleated schizonts, and calculation of the 50% inhibitory concentration (IC_{50}) and IC_{99} of the drug from a dose-response curve (9). Beyond this simplest format, whose reagents were prepared and commercialized through the WHO in the years 1980 to 1990, other *in vitro* tests that offer valuable advantages have been developed. All tests are applicable both to field-collected parasites and to the parasites growing asynchronously in long-term laboratory culture and can also be used for screening potential new antimalarials and for investigations on drug modes of action or resistance. The activities of some antimalarials, such as quinine, address only a part of the asexual erythrocytic cycle. This implies that when long-term laboratory culture of patient isolates or reference clones is used, synchronization at ring stage can improve the reproducibility of susceptibility results. Parasite growth and inhibition can be assessed using different methods. A parasite count by microscopic examination of culture smears is cumbersome and often poorly reproducible. Measurement of uptake of $[^3H]$hypoxanthine offers a semiautomated, quantitative, high-output approach but necessitates use of radioactive material and specialized equipment in authorized laboratories, leading to high costs because of the handling of radioactive waste (10). Measurement of highly produced *Plasmodium* proteins, such as parasite lactate dehydrogenase (11) or histidine-rich protein 2 (12), by double-site enzyme-linked immunosorbent assay demonstrated a higher sensitivity than use of radioisotopes, although these tests are time-consuming and commercial kits are costly (13). Other tests measure the production of DNA during the maturation of parasites using SYBR green I fluorescent dye (14–16). The successful completion and interpretation of all *in vitro* tests depends on several factors that in turn depend on samples, materials, culture, and statistical methods to generate inhibitory constants. Sample factors to be considered include a recent intake of antimalarial drugs by the patient, which may decrease the test success rate; high parasite inocula, which can lead to an overestimation of resistance to some drugs (17); and folate and *para*-aminobenzoic acid in the culture medium, which antagonize the *in vitro* effect of antifolate drugs (18). Another sample factor is the short life of erythrocyte *P. falciparum* parasites outside the host; each day that passes at 4°C halves their capacity to survive and grow *in vitro* (19). Processes include the necessity of preparing and distributing dilutions of drugs in wells of plates, which results in particular difficulties for drugs other than chloroquine, as most are poorly soluble or have limited shelf lives. Culture necessitates supplementation of RPMI medium with human serum or AlbuMAX, although this can affect IC_{50}s, as they interact with drugs differently (20). All these fundamental methodological issues undermine accurate comparisons of *in vitro* susceptibilities either between laboratories or within a single laboratory over time. Consensus exists on parameters of culture, and suggested improvements include measures of quality control of key parameters, such as predosed plates (titrated drug solutions) and reference of endpoints of isolate susceptibilities to those of reference clones with known susceptibilities (21). Finally, standardized mathematical analysis of concentration inhibition assays is possible through free Web-based tools (22, 23). Drug concentrations associated with the IC_{50} are determined by a modified sigmoid maximum-effect (E_{max}) model-fitting algorithm and display the precision of IC_{50} estimation. The standard *in vitro* antimalarial drug susceptibility assay described above, however, does not permit accurate potency measurement of the fast-acting and stage-specific artemisinin derivatives for which specific protocols have been developed recently (24–26). These new assays test the capacity of early-ring-stage parasites to survive a brief pulse exposure to high artemisinin concentrations (the so-called ring-stage survival assay [RSA]), better mimicking the situation faced by parasites *in vivo*. In a study with Cambodian parasite isolates, the survival rate estimated with the RSA correlates with *in vivo* parasite clearance half-lives (25).

Tests exploring antimalarial efficacy at various stages of the *Plasmodium* life cycle have been designed but are not adapted for routine laboratory use (27). However, the microtechnique has been adapted for testing of erythrocytic stages of *P. vivax* (28), and cultures of the liver and sexual blood stages of *P. falciparum* can also be used, although they are substantially more cumbersome (29, 30).

Genetic markers that now offer tools for assessing parasite drug resistance have been identified for several major antimalarials. Chloroquine resistance has been linked to a *P. falciparum* chloroquine resistance transporter (*Pf*CRT) K76T change on a transmembrane channel in the digestive vacuole of *P. falciparum*, following a mutation of the *pfcrt* gene (31, 32). *P. falciparum* multidrug resistance gene 1 (*pfmdr1*) encodes a P glycoprotein homologue of a human ABC transporter that transports toxic compounds across the digestive vacuole membrane. Point mutations and gene amplification of *pfmdr1* have been linked, to various extents, to altered susceptibilities to various antimalarials (chloroquine and other 4-aminoquinolines, quinine and other amino-alcohols, and artemisinin derivatives) (33–35). Regarding the artemisinins, missense mutations in the *kelch13* gene have been proposed as *in vitro* and *in vivo* markers of artemisinin resistance (36). Resistance to antifolates, such as sulfadoxine-pyrimethamine, has been associated with S108N, C59R, and N51I changes in *P. falciparum* dihydrofolate reductase (37–39) or its homologues in *P. vivax* (40). High levels of resistance to antifolate drugs are associated with the *P. falciparum* dihydrofolate reductase I164L change (37, 41) or changes in the *P. falciparum* dihydropteroate synthase target of sulfa drugs (42). Resistance to atovaquone-proguanil has been linked to substitutions in codon 268 of the *P. falciparum* cytochrome b (*pfcytb*) gene, resulting in the amino acid change S, N, or C, leading to high-level resistance (43). Such genetic polymorphisms can be identified in individual parasite isolates by various standard techniques, which include mutation-specific nested PCR (44) or PCR followed by sequencing (45), restriction fragment length polymorphism analysis (46), and single-nucleotide primer extension with detection of fluorescent products on a capillary sequencer (47), or by using a DNA microarray-based method (48). Next-generation sequencing also allows accurate detection of drug resistance genotypes from pooled parasite isolates (49). Possibilities for analyzing haplotypes in mixed parasite isolates may improve our ability to find the clinical relevance of combined mutations (50).

As long as validated molecular markers are lacking for resistance to artemisinin's partner drugs, *in vivo* and *in vitro* surveillance will remain critical surveillance tools for the emergence of resistance to artemisinin-based combined therapy (51, 52). The judicious use of *in vivo*, *in vitro*, and molecular tests can yield valuable information that will help us to adapt drug policies before extension of resistance brings about elevated morbidity consequences (53, 54).

TRICHOMONIASIS

Trichomonas vaginalis is the most common nonviral sexually transmitted infection in the United States and throughout the world; however, metronidazole and tinidazole are the only drugs currently approved by the U.S. Food and Drug Administration to treat trichomoniasis. With 4.3% of *T. vaginalis*-infected sexually transmitted disease clinic patients in the United States demonstrating some degree of resistance to metronidazole, standard treatment regimens may not be effective for approximately 159,000 individuals (55, 56). Fortunately, drug susceptibility testing has proven useful in identifying alternative treatment protocols that are usually successful in effecting patient cure (57). *In vitro* testing for resistance to 5-nitroimidazoles is indicated following failure of two standard treatments to cure the patient's infection.

Susceptibility testing for *T. vaginalis* is a simple assay of parasite motility in the presence of drug (58). Axenic trichomonads are cultured in Diamond's Trypticase-yeast-maltose medium with serial dilutions (400 to 0.2 μg/ml) of metronidazole or tinidazole dissolved in dimethyl sulfoxide (DMSO) and the appropriate parallel concentrations of DMSO in U-bottom microtiter plates. Plates are incubated at 37°C for 48 h and are then examined microscopically with an inverted phase-contrast microscope. The lowest concentration of drug in which no motile organisms are observed is defined as the minimum lethal concentration. Minimal lethal concentrations greater than 100 μg of metronidazole per ml have been associated with clinical resistance (59). This assay has been adapted to determine IC_{50}s and screen novel compounds for antitrichomonad activity by measuring incorporation of [^{3}H]thymidine (60, 61) or acid phosphatase activity (62). These modifications have not as yet been adapted to monitor clinically resistant isolates, but theoretically, this could be accomplished rather easily.

One difficulty of the *in vitro* culture method for assessing the resistance of *T. vaginalis* isolates is the need to derive axenic cultures from clinical specimens. As individuals with trichomoniasis are often infected with other sexually transmitted disease organisms that also grow in Diamond's medium, this may require an extended time. Molecular comparisons of *T. vaginalis* isolates suggest that genetic markers for metronidazole resistance could be identified (63–66). The prospect of a molecular marker for resistant *T. vaginalis* is exciting, as it may make possible a PCR-based assay for resistance. This would obviate the need for establishing axenic cultures and may allow direct testing of specimens from patients suspected of harboring a resistant isolate.

LEISHMANIASIS

Treatment failure in leishmaniasis may result from either true drug resistance or patient factors, such as poor drug absorption, treatment compliance, or immunodeficiencies, that preclude effective chemotherapeutic action. Differentiation of these two possibilities is important for both individual patient care and general public health. Traditional testing for drug resistance in *Leishmania* is performed by culturing the promastigote form of the parasite with drug in standard cell culture media (Schneider's medium, RPMI medium, or M199 medium) supplemented with bovine sera for 42 to 72 h at 26 to 37°C and by assessing viability by direct counting (67), [^{3}H]thymidine incorporation (68), enzymatic hydrolysis of *p*-nitrophenyl phosphate (69), conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (70), or reduction of resazurin (71). [^{3}H]thymidine incorporation is perhaps the most sensitive method, but it requires access to radioactive materials and a scintillation counter. Hydrolysis of *p*-nitrophenyl phosphate and conversion of MTT are colorimetric assays, but *p*-nitrophenyl phosphate hydrolysis has high backgrounds caused by medium components and MTT may interact with certain drugs (e.g., meglumine antimoniate). An advantage of resazurin (also known as Alamar Blue) is that it is not necessary to further manipulate the parasites in order to read the assay. Thus, drug activities can be monitored at different time points after initiation of cultures.

Although promastigote assays are the easiest to use, they yield clinically reliable results only for drugs (such as miltefosine and amphotericin B) that do not require cellular mechanisms for activation (72, 73). In contrast, the more widely used pentavalent antimonials require reduction by host cells to the active trivalent form. As a result, promastigote assays correlate poorly with clinical resistance, and assays that test drug susceptibility of the intracellular amastigote form of leishmania parasites are recommended (74, 75). Unfortunately, intracellular assays are very labor-intensive. Macrophages are infected with promastigotes that must then transform into amastigotes prior to exposure to drug. Each test condition necessitates preparation of slides, which are then stained for microscopic counting of the proportion of infected macrophages and the number of amastigotes per macrophage in drug-treated and control cultures. Furthermore, promastigotes from field isolates are not uniformly infective for macrophages, leading to selection bias that may reduce the correlation between drug susceptibility assays and clinical treatment outcome (76, 77).

Intracellular assays have been facilitated by stable transformation of isolates with firefly luciferase. Cells are simply lysed, the substrate luciferin is added, and activity is measured on a luminometer (78). Similarly, transfection of isolates with the gene encoding green fluorescent protein (GFP) can be used (79). Following incubation with the drug, parasites are enumerated on a flow cytometer, with no need for addition of substrates because GFP is intrinsically fluorescent. An advantage of transfection assays is the ability to compare drug activities against promastigotes, axenic amastigotes, and intracellular amastigotes. However, these assays are more useful for screening experimental compounds rather than testing field isolates because of the challenges of standardized transfection between isolates and the expensive equipment needed to measure the results.

If transfection with exogenous genes is undesirable, mammalian cells that have been infected with parasites and treated with drug can be fixed and permeabilized. Intracellular amastigotes are then detected with anti-*Leishmania* lipophosphoglycan monoclonal antibody followed by a fluorescent-marker-labeled anti-mouse immunoglobulin antibody (80). This assay can be used for assessing the drug sensitivities of a variety of *Leishmania* species, and it is adaptable to use with individual patient isolates. Alternatively, following incubation of infected cells with a drug, controlled lysis of macrophages can be performed to release amastigotes that are then transformed back into promastigotes and enumerated using one of the microscopic, [^{3}H]thymidine incorporation, colorimetric, or fluorometric assays described above (81).

As molecular mechanisms of drug resistance in *Leishmania* spp. become better defined, it may be possible to utilize PCR and DNA sequencing of single nucleotide polymorphisms (SNPs) for epidemiologic studies of resistant isolates. Resistance-associated SNPs from both visceral and cutaneous *Leishmania* species have been identified in genes for drug transporters, the stress response, and thiol and redox metabolism (82, 83). These assays are of interest, as no manipulation beyond growing parasites is needed. However, it is likely that applicability will be species and drug restricted.

AFRICAN TRYPANOSOMIASIS

As in leishmania drug sensitivity testing, trypanosomes can be cultured *in vitro* with drug and monitored for viability by enzymatic hydrolysis of *p*-nitrophenyl phosphate (69) or by direct counting (84). Parasites are cultured at 37°C for 24 h in 4 to 5% CO_2 in phenol red-free Iscove's medium containing hypoxanthine, thymidine, glutamine, L-cysteine, pyruvate, β-mercaptoethanol, and heat-inactivated bovine or horse sera. An *in vitro* lysis assay has been developed for testing the sensitivities of *Trypanosoma brucei* subspecies to melarsoprol (85). It requires the ability to culture trypanosomes and a thermostatically controlled recording spectrophotometer. As susceptible organisms die, their absorbance at 500 nm is reduced over the course of 30 min. If drug-susceptible and drug-resistant strains are available to use as controls, this technique may perhaps be adapted to field use because differences in intensity at this wavelength can be distinguished by the unaided eye. Visible color change can also be observed when trypanosomes are incubated with resazurin (86). However, unlike in assays for *Leishmania* drug sensitivity, the dye is added after parasites are incubated with drugs for 66 to 72 h, depending on the parasite subspecies.

Because drug resistance in African trypanosomes is primarily a function of reduced drug uptake, radiolabeled drugs can be incubated with parasites to identify resistant isolates, with reduced cellular incorporation of radioactivity suggesting resistance (87). Similarly, because drug uptake is reduced in isolates with point mutations or deletions of aminopurine transporters, it may soon be possible to utilize molecular tools to identify trypanosome isolates that are drug resistant (88). One of the genetic mutations leading to an amino acid change in the transporter responsible for melarsoprol uptake abrogates a restriction endonuclease site, while a different mutation creates one (89). Thus, PCR amplification of the appropriate gene followed by enzymatic digestion and gel electrophoresis results in different DNA banding patterns that might be used to distinguish melarsoprol-susceptible and -resistant isolates. While more clinical drug-resistant isolates must be evaluated to confirm the utility of this test, a similar banding pattern between melarsoprol-resistant laboratory-derived and field isolates (89) suggests that this tool may be useful for both management of individual patients' infections and tracking the spread of resistance in a population (90).

SCHISTOSOMIASIS

Drug resistance testing of schistosomes differs greatly from that of protozoan parasites in both purpose and methods. Adult worms do not replicate within the definitive host, thus circumventing one of the mechanisms associated with rapid development of drug resistance. As a result, widespread resistance to praziquantel, the drug of choice for schistosomiasis, has not emerged even under heavy drug pressure (91–93). Thus, the need to evaluate drug resistance for individual infections is rare. However, because reliance on a sole drug is risky and schistosomes have developed resistance to other drugs, techniques to monitor praziquantel resistance are needed. The failure of adult worms to replicate makes drug susceptibility testing methods more challenging. Investigation of drug resistance has typically been performed by (i) obtaining eggs from an unsuccessfully treated mammalian host, (ii) infecting the appropriate intermediate snail host, (iii) isolating cercariae from the infected snail, and (iv) infecting and treating experimental animals. A protocol to determine the 50% effective dose in mice has been developed and demonstrates good reproducibility among different laboratories (94). This approach has the drawbacks of being very time-consuming and technically challenging, especially in areas of endemicity that may not have well-developed experimental snail and rodent colonies. Additionally, resistance phenotypes may not persist in the absence of drug pressure, clouding our ability to interpret results (95). Fifty-percent effective doses can also be estimated in an *in vitro* assay by assessing contraction of worms following perfusion and incubation with praziquantel and correspond well with *in vivo* results (96, 97). Although this approach is less complicated than infecting, treating, and perfusing additional mice, it still requires the availability of naive snails and some mice to obtain the adult worms, along with the time and expertise needed to perpetuate the life cycle. In addition, the parasites that successfully infect mice may not accurately represent the diversity of field isolates. Recently, an *in vitro* method that circumvents these problems has been adapted to field use (98, 99). Eggs from stools of infected individuals are isolated and hatched to release miracidia, which are then exposed to praziquantel, and effects on morphology are monitored. The miracidia from persons who then cleared their infection following treatment were more affected by the drug than the miracidia from stools whose donors did not clear their infections. Evaluating changes in the parasite's population structure using miracidia obtained from eggs in patients' stools is another approach to monitor development of potential drug resistance. Changes in the genetic composition of schistosomes following treatment suggest praziquantel efficacy, while maintenance of the same microsatellite distribution may indicate treatment failure (100, 101). Ongoing surveillance for development of drug resistance will be an important component for schistosomiasis control programs that are dependent on mass drug administration.

FUTURE PERSPECTIVES

Most susceptibility tests for antiparasitic drugs are not routinely available in clinical diagnostic laboratories, because these procedures are not in frequent demand and present special technical requirements. Such tests are performed mainly in reference or research laboratories or during epidemiologic investigations in areas of endemicity. Thanks to recent advances in laboratory technology and genetic analysis of parasites, available tests are increasingly sophisticated and informative. The development of tests that are robust and simple to use will facilitate field studies that aim at optimizing the deployment of currently available drugs.

These field tests usefully complement the more sophisticated procedures used in research laboratories whose main orientation is toward the development of novel antiparasitic compounds and deciphering drug resistance mechanisms.

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